From adaptation to motion: In-depth characterization of γ-proteobacteria under acid or hormone stress

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Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbstständig und ohne unerlaubte Hilfe angefertigt wurde. Des Weiteren erkläre ich, dass ich nicht anderweitig ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen. Die folgende Dissertation liegt weder ganz, noch in wesentlichen Teilen einer anderen Prüfungskommission vor.

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I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources. As well I declare, that I have not submitted a dissertation without success and not passed the oral exam. The present dissertation (neither the entire dissertation nor parts) has not been presented to another examination board.

Kilian Schumacher, Munich 04.07.2024

Contents

Nomenclature

Proteins are numbered according to the first methionine/valine in the wild-type amino acid sequence designated as '1'. N- or C-terminal affinity tags are indicated corresponding to their position (e.g. sORF15:3xFLAG).

Gene deletions are indicated by the 'Δ' symbol, e.g., Δ*mhpR*.

'P' indicates a promoter, e.g., P*mhpR*.

Abbreviations

ARs	acid resistance systems
ATP	adenosine triphosphate
bp	base pair
cDNA	complementary DNA
CFAs	cyclopropane fatty acids
Co-IP	co-immunoprecipitation
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EHEC	enterohemorrhagic *Escherichia coli*
EPI	epinephrine
EtOH	ethanol
G3P	glycerol 3-phosphate
GABA	gamma-aminobutyric acid
GO	gene ontology
GSEA	gene set enrichment analysis
HK	histidine kinase
IS	insertion sequence
LAB	labetalol
LB	lysogeny broth
LC-MS	liquid chromatography-mass spectrometry
MALDI-TOF	matrix-assisted laser desorption/ionization - time of flight
MCPs	methyl-accepting chemotaxis proteins
MNase	micrococcal nuclease
mRNA	messenger RNA
MST	microscale thermophoresis

NE	norepinephrine
NGS	next generation sequencing
nt	nucleotide
OD_{600}	optical density at 600 nanometer
OMP	outer membrane protein
PE	phenylephrine
qRT-PCR	quantitative real-time PCR
RNA	ribonucleic acid
RNA-Seq	RNA sequencing
ROS	reactive oxygen species
RPFs	ribosome-protected fragments
RR	response regulator
rRNA	ribosomal RNA
SCFAs	short-chain fatty acids
scRNA-Seq	single-cell RNA sequencing
sORF	small open reading frame
sRNA	small RNA
UTR	untranslated region
UV light	ultraviolet light
w/v	weight per volume

List of publications:

Chapter 2.1:

<u>Schumacher, K.</u>, Gelhausen, R., Kion-Crosby, W., Barquist, L., Backofen, R., and Jung, K. (2023). Ribosome profiling reveals the fine-tuned response of *Escherichia coli* to mild and severe acid stress. mSystems.10.1128/msystems.01037-23.

Chapter 2.2:

<u>Schumacher, K.</u>, Braun, D., Kleigrewe, K. and Jung, K. (2024). Motility-activating mutations upstream of *flhDC* reduce acid shock survival of *Escherichia coli*. Microbiol Spectr. 12:e0054424.

Chapter 2.3:

Muñoz, A.W., Hoyer, E., <u>Schumacher, K.</u>, Grognot, M., Taute, K.M., Hacker, S.M., Sieber, S.A., and Jung, K. (2022). Eukaryotic catecholamine hormones influence the chemotactic control of *Vibrio campbellii* by binding to the coupling protein CheW. Proc. Natl. Acad. Sci. U. S. A. *119*. 10.1073/pnas.2118227119.

Chapter 2.4:

Brameyer, S., <u>Schumacher, K.</u>, Kuppermann, S., and Jung, K. (2022). Division of labor and collective functionality in *Escherichia coli* under acid stress. Commun. Biol. *5*, 1–14. 10.1038/s42003-022-03281-4.

Chapter 2.5:

Schwarz, J.*, <u>Schumacher, K.</u>*, Brameyer, S., and Jung K. (2022). Bacterial battle against acidity. FEMS Microbiol. Rev. *46*. 10.1093/femsre/fuac037.

* These authors contributed equally

Chapter 2.6:

<u>Schumacher, K.</u>, Brameyer, S., and Jung, K. (2023). Bacterial acid stress response: from cellular changes to antibiotic tolerance and phenotypic heterogeneity. Curr. Opin. Microbiol. *75*:102367.10.1016/j.mib.2023.102367

Declaration of contributions

Chapter 2.1:

KS and KJ initiated and designed the study. KS performed all *in vivo* experiments and created all strains and plasmids. Ribo-Seq and RNA-Seq data analysis using the HRIBO pipeline was performed by RG with input from KS. Gene set enrichment analysis and novel sORF detection were performed by KS and RG. The identification of novel transcription factors involved in the acid stress response of *E. coli* was performed by KS. The autoencoder analysis to differentiate the acid stress and general stress responses was conducted by WKC with input from KS. KS designed the figures, and KS and KJ wrote the manuscript with input from RG, WKC, LB, and RB. KS was assisted by the research students Pol Bannasch and Annika Krimmel.

Chapter 2.2:

KS and KJ initiated and designed the study. KS performed all *in vivo* experiments and created all strains and plasmids. DB assisted KS with strain- and plasmid construction, promoter activity assays, and acid shock experiments. KG conducted LC-MS and analyzed the respective data. KS and KJ wrote the manuscript with input from all authors.

Chapter 2.3:

SAS and KJ initiated and designed the study. AWM conducted the synthesis of tailored probes for chemical proteomic experiments, photoaffinity labeling, and Co-IP. EH performed soft agar colony expansion assays. EH and KS performed protein purification, MST experiments, and growth assays. KS conducted chemotaxis capillary assays. MG and KT performed 3D chemotaxis assays. AWM, EH, KS, MG, and SMH analyzed data. AWM, SAS, and KJ wrote the manuscript with input from all authors.

Chapter 2.4:

SB and KJ initiated and designed the study. SB and KS performed fluorescence microscopy and data analysis. SB and KS constructed all strains and plasmids. KS verified the functionality of fluorescent antiporter hybrids by western blot. SK performed phylogenetic analysis. SB and KJ wrote the manuscript with input from KS and SK.

Chapter 2.5:

KJ initiated and designed the study. JS summarized the literature and wrote the text for the following chapters: 'Enzyme-based H$^+$-consuming acid stress resistance systems', 'Acid-resistance system 1 (AR1)', Arginine-dependent AR (Adi) system (AR3)', 'Lysine-dependent AR (Cad) system (AR4)', 'Ornithine-dependent AR (Orn) system (AR5)', 'Deiminase and deaminase systems', 'ToxR-like receptor CadC', and 'Transcriptional regulator AphB'. KS summarized the literature and wrote the text for the following chapters: 'pH homeostasis and ion transport', 'Acid-dependent chemotaxis', 'Glutamate-dependent AR (Gad) system (AR2)', 'Synthesis of acid-shock proteins', 'Urease', 'Hybrid HK EvgS', 'HK PhoQ', 'Chemoreceptors Tar and Tsr', and 'Chemoreceptor TlpB'. SB summarized the literature and wrote the text for the following chapters: 'Phylogenetic occurrence of the Gad, Adi, Cad, and Orn systems in the bacterial kingdom', 'HK SsrA', 'HK ArsS', and 'Functional diversification of bacterial populations under acid stress'. KJ summarized the literature and wrote the text for the following chapters: 'Introduction', 'Synthesis of acid-shock proteins', 'Synthesis of chaperones', 'Modifications of the cytoplasmic membrane', 'HK PmrB', 'Functional diversification of bacterial populations under acid stress', and 'Concluding remarks'. Above mentioned chapters were corrected and modified with input from all authors. SB and KJ designed the figures with input from all authors.

Chapter 2.6:

KJ initiated and designed the study. KS summarized the literature and wrote the text for the chapter 'Acid stress-dependent alterations of the transcriptome and proteome of various bacteria'. SB summarized the literature and wrote the text for the chapter 'Acid stress and antibiotic tolerance'. KJ summarized the literature and wrote the text for the chapters 'Introduction', 'The heterogeneous acid stress response', and 'Conclusions and perspectives'. Above mentioned chapters were corrected and modified with input from all authors. KS and SB designed the figures with input from KJ.

We hereby confirm the above statements:

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Kilian Schumacher   Kirsten Jung

Summary

Sensing and responding to acidity is crucial for bacteria to survive in a variety of habitats, such as the gastrointestinal tracts of vertebrates. However, previous approaches to systematically study the response of bacteria to acid stress have been restricted to methods with significant drawbacks involving proteomics and microarrays or were limited to mildly acidic conditions. In this thesis, the advantages of ribosome profiling (Ribo-Seq) were leveraged and combined with RNA-Seq to study genome-wide adaptations of *Escherichia coli* to mild (pH 5.8) and severe near-lethal acidic conditions (pH 4.4). The obtained findings included novel strategies of *E. coli* to gain acid tolerance, such as reduction of membrane protein synthesis and global metabolism, as well as the induction of iron uptake and siderophore synthesis. Moreover, Ribo-Seq uncovered novel small proteins that were exclusively detectable under acid stress, and acid-specific adaptations were distinguished from other stressors using autoencoder-based machine learning.

Ribo-Seq and RNA-Seq also revealed an induction of motility genes at pH 5.8 but not at pH 4.4, indicating that flagella biosynthesis is dependent on the degree of acid stress. Follow-up studies were conducted focusing on stress-dependent alterations of chemotaxis and motility. These revealed that motility-activating mutations upstream of the master regulatory genes *flhDC* compromised the survival of *E. coli* under acid shock. Accordingly, an inverse correlation between motility and shock survival was demonstrated, suggesting a differentiation of *E. coli* into a motile and an acid-tolerant subpopulation. This trade-off between motility and the ability to tolerate acid shock periods was found to depend on the differential integration of insertion sequence elements in the promoter region of *flhDC*. In another study, stress-dependent adaptations of chemotaxis in the marine pathogen *Vibrio campbellii* were highlighted. Stress hormones such as eukaryotic epinephrine bound to the chemotaxis coupling protein CheW and affected the sensing of chemoattractants. Finally, the use of a triple-fluorescent reporter strain demonstrated that the three major acid-resistance systems are heterogeneously activated in an *E. coli* population. These results indicated a 'bet-hedging' strategy and division of labor under acid stress conditions.

Overall, the results obtained in this thesis shed new light on bacterial stress adaptations and provide new starting points for future research, such as an in-depth characterization of novel acid-induced small proteins, the analysis of subpopulations with different acid tolerance, and the search for further stress-dependent mechanisms of chemotaxis and motility.

Zusammenfassung

Das Wahrnehmen und Reagieren auf Säurestress ist essentiell für das Überleben von Bakterien in Lebensräumen wie dem Verdauungstrakt von Wirbeltieren. Frühere Versuche die Reaktion von Bakterien auf Säurestress systematisch zu analysieren beschränkten sich jedoch auf Methoden wie Proteomics und Microarrays, oder auf milden Säurestress. In dieser Arbeit wurden die Vorteile von Ribo-Seq genutzt und mit RNA-Seq kombiniert um die Anpassungen von *Escherichia coli* an milden (pH 5.8) und starken, fast tödlichen, Säurestress (pH 4.4) aufzuzeigen. Die Ergebnisse beinhalteten neue Säuretoleranzstrategien wie die reduzierte Synthese von Membranproteinen, einen minimierten Stoffwechsel und die Induktion der Eisenaufnahme und Siderophorsynthese. Zudem wurden neue kleine Proteine durch Ribo-Seq entdeckt, welche nur unter Säurestress detektierbar waren. Außerdem wurden durch den Einsatz von Autoencoder-basiertem maschinellen Lernen säurestressspezifische Anpassungen von anderen Stressoren abgegrenzt.

Die Ribo-Seq und RNA-Seq Ergebnisse zeigten auch eine erhöhte Expression von Motilitätsgenen bei pH 5.8, nicht aber bei pH 4.4, was auf eine Regulation der Flagellensynthese in Abhängigkeit der Stressintensität hindeutete. Daher wurden weitere Studien zu säurestressabhängigen Änderungen der Chemotaxis und Beweglichkeit durchgeführt. Dabei wurde gezeigt, dass Mutationen nahe der Masterregulatorengene *flhDC*, welche die Beweglichkeit erhöhen, das Überleben von *E. coli* unter Säureschockbedingungen verringern. Gleichzeitig wurde eine inverse Korrelation zwischen der Motilität und dem Überleben unter Säureschock festgestellt, was eine Differenzierung von *E. coli* in eine bewegliche und eine säuretolerante Subpopulation unter Säurestress nahelegte. Es konnte schließlich gezeigt werden, dass der Kompromiss zwischen Beweglichkeit und dem Überleben unter Säureschock von der unterschiedlichen Integration von Insertionssequenzelementen in der Promotorregion von *flhDC* abhängig ist. In einer weiteren Studie wurde die stressabhängige Anpassung der Chemotaxis im marinen Pathogen *Vibrio campbellii* demonstriert. Stresshormone wie das eukaryotische Adrenalin banden an das chemotaktische Kopplungsprotein CheW und beeinflussten das Wahrnehmen von Lockstoffen. Schließlich konnte mit Hilfe eines fluoreszierenden Reporterstamms die heterogene Aktivierung der drei hauptsächlichen Säureresistenzsysteme innerhalb einer *E. coli* Population gezeigt werden. Diese Ergebnisse weisen auf eine funktionelle Arbeitsteilung unter Stressbedingungen hin.

Zusammenfassend werfen die Ergebnisse dieser Doktorarbeit neues Licht auf bakterielle Stressanpassungen und bieten viele neue Ansatzpunkte für zukünftige Forschungsprojekte, wie z.B. die Charakterisierung von neuen säureinduzierten kleinen Proteinen, die Analyse von Subpopulationen mit unterschiedlicher Säuretoleranz, sowie die Suche nach weiteren stressabhängigen Anpassungen der Beweglichkeit und Chemotaxis.

1 Introduction

1.1 Overview of acidic environments

pH is defined as the 'power of hydrogen' and measures the acidity or basicity of an aqueous solution (1). It is calculated by taking the negative logarithm of hydrogen ion concentration and ranges from 1 to 14. By definition, solutions with pH < 7 are acidic, pH > 7 are basic, and solutions with a pH of exactly 7 are neutral at 25 °C (2). The pH varies significantly in different environmental habitats and can range from less than 2 in acid mine drainage to a pH of 10 to 11 in soda lakes (3, 4).

This thesis focuses on γ-proteobacteria exposed to acidic environments such as gastrointestinal tracts, macrophage phagosomes, acidic soils, peat bogs, industrial fermentation tanks, or biomining sites (Figure 1). Bacteria within the gastrointestinal tract of humans and animals encounter varying levels of acidity which is crucial for digestion and immune defense (5). The stomach possesses an exceptionally low pH ranging from 1.5 to 3.5 due to the presence of hydrochloric acid secreted by gastric glands (6). Correspondingly, the stomach constitutes the major bactericidal barrier of the gastrointestinal tract for pathogens (7). It is important to note that the capacity of microbes for tolerating the acidity of the stomach varies significantly and results in infective doses that differ by several orders of magnitude (Chapter 2.5). For instance, *Escherichia* and *Shigella* spp. are highly tolerant towards acidity and have low infective doses whereas *Vibrio cholerae* requires $\sim 10^8$ ingested cells to conduct an infection (8–10). Mildly acidic conditions are present in the duodenum which is in direct contact with the stomach and exhibits a pH of ~6 (11, 12). Moving further along the tract, bacteria that survived the stomach acidity are also confronted with mild acidity in the proximal colon including the cecum (12, 13). In this environment, short-chain fatty acids (SCFAs) are produced by anaerobic fermenting bacteria resulting in a pH of 6.4 or lower (14). In a medical context, acidic conditions can be faced in macrophage phagosomes (Figure 1) (15), where intracellular pathogens including *Salmonella*, *Legionella*, and *Mycobacterium* are exposed to acidity as a consequence of the activity of vacuolar-type proton ATPases (V-ATPases) and Na^+/H^+ antiporters (16).

Acidic environments are also found to large extents in soil (Figure 1). In these surroundings, acidification can be triggered by carbonic acid (H_2CO_3) formation as a consequence of the combination of carbon dioxide (CO_2) emissions with atmospheric water (17). Additionally, burning fossil fuels releases sulfur and nitrogen oxides, which, upon reacting with water, form sulfuric and nitric acids. These acidic compounds accumulate in the soil through acidic rain (18). Furthermore, root respiration and the breakdown of organic matter by microorganisms release CO_2, elevating H_2CO_3 levels in the soil (19). This process triggers cation

gastrointestinal tracts
pH 1.5 - 6.0

macrophage phagosomes
pH 4.0 - 5.0

acidic soils
pH 3.0 - 5.5

peat bogs
pH 3.0 - 5.0

industrial fermentation tanks
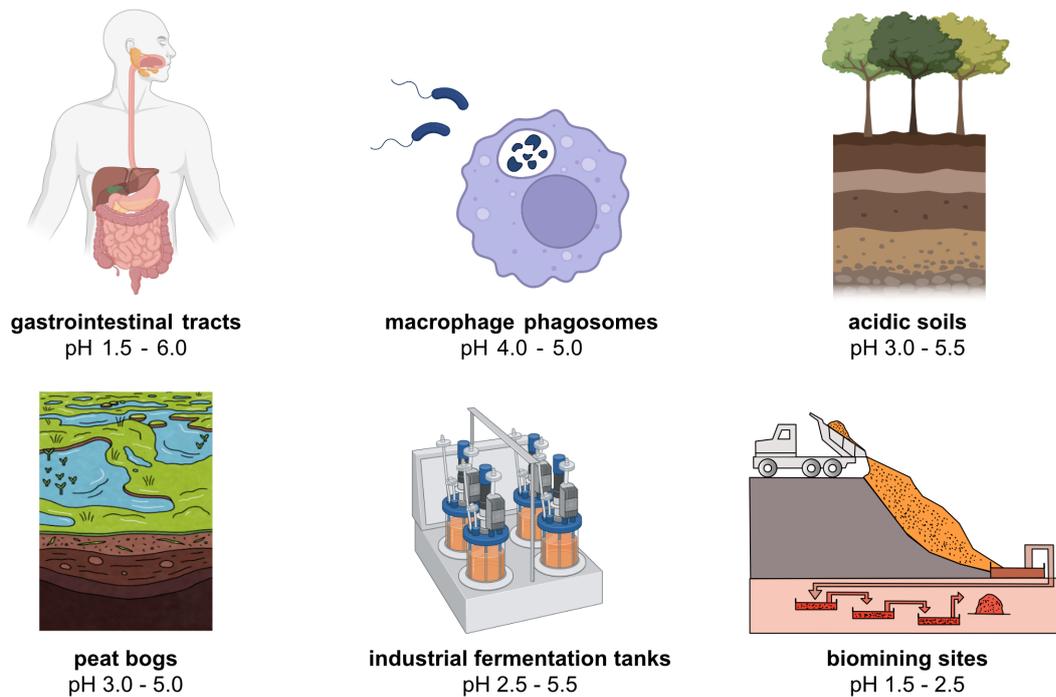pH 2.5 - 5.5

biomining sites
pH 1.5 - 2.5

Figure 1: Schematic illustration of habitats where acidophilic and neutralophilic bacteria can be exposed to acidity.

exchange, leading to the leaching of base cations such as Ca^{2+} and Mg^{2+} from the soil, consequently increasing the proportion of Al^{3+} and H^+ ions (20). Overall, acidic soils account for 30% of the global ice-free land with pH values of as low as < 3.5 (21). Additionally, low pH can be encountered by bacteria in peat bogs (Figure 1). *Sphagnum*-dominated acidic peat bogs account for ~3% of the terrestrial surface on earth and are characterized by a pH between 3 and 5 (22). In these habitats, which are usually mineral nutrient deprived, methanotrophic communities are commonly encountered (22–24).

Acidity also holds significant importance for the food industry (Figure 1). Predominantly found in dairy products, homofermentative and heterofermentative lactic acid bacteria along with propionic bacteria prompt the generation of lactic acid, propionic acid, EtOH, and CO_2. These compounds contribute to improved flavor, digestibility, nutritional qualities, and preservation of the products (25, 26). Consequently, bacteria encounter the acidity resulting from their own fermentation activities and are commonly exposed to a pH between 4.0 and 5.5 (27). Another industry relying on acid-tolerant microbes is the biomining industry (Figure 1), which is specialized in recovering valuable metals from reduced sulfides present in ores and concentrates (28). In this process, metal ions undergo oxidation which leads to the production of acid mine drainage, consisting mainly of sulfuric acid and causing ambient pH values below 1.5 (29).

In summary, the significance of acid tolerance spans several domains impacting public health, industrial sectors, and ecological landscapes which are characterized by different levels of acidity.

1.2 Bacterial acid stress response mechanisms

Bacteria evolved a magnitude of sophisticated stress response mechanisms to survive in environments with fluctuating pH. For instance, the intrinsic buffering capacity of the cytoplasm is essential in habitats with high proton concentrations. In particular, biological macromolecules including amino-acid side chains, polyamines, polyphosphates, and inorganic phosphate, can be protonated (30). Thus, fluctuating H^+ concentrations can be balanced through the sequestration of pH-titratable cellular components.

Upon exposure to acidity, the cytoplasmic membrane serves as the primary barrier for protons and limits intrusion into the cell. However, H^+ can still permeate into the cytoplasm via ion channels, water chains, or damaged membranes (31). To prevent the latter, modifications of the cytoplasmic membrane which alter the lipid composition, integrity, or fluidity, occur in bacteria. One common adaptation of this kind is incorporating cyclopropane fatty acids (CFAs) into the membrane (Figure 2). CFAs are synthesized from unsaturated fatty acids by adding a methyl group from S-adenosine-methionine to a double bond and CFA-enriched membranes were demonstrated to be less permeable for protons (32). Notably, an *E. coli* mutant lacking the CFA synthase gene was more sensitive to acid stress (32, 33). Furthermore, the proportion of saturated to unsaturated fatty acids in the membrane can increase in low-pH environments (34). Besides fatty acids also lipid types are modified upon acidification. Such adaptations involve the presence of aminoacylated derivates of phosphatidylglycerol, the formation of hopanoids and sphingolipids, and the increase of ornithine lipids (35).

In addition to the restriction of H^+ uptake, proton export constitutively occurs in bacteria via proton-cation antiporters, or proton pumps associated with the respiratory chain (Figure 2). Nevertheless, the activity of these pumps depends on the generation of the proton motive force (36). Under mildly acidic conditions, *E. coli* restricts the production of the F_OF_1 ATPase to limit proton re-entry (37, 38). Conversely, the F_OF_1-ATPase synthase was also shown to actively pump protons out of the cell in an ATP-dependent manner in several species under severe acid stress (38–41).

Alongside constitutive response mechanisms, inducible defense strategies against acidity exist in γ-proteobacteria. Such responses are commonly initiated by acid sensors which are responsible for the detection of cytoplasmic and external proton concentrations. Acid sensors can be implemented in bacterial cells in different forms such as HKs of two-component systems, one-component-systems, or cytoplasmic transcription factors (Chapter 2.5). In many cases, H^+ are sensed via the protonation of single amino acid residues or patches of amino acids, and conformational changes are triggered which propagate the signal (42–44). Examples of bacterial acid stress sensors include ArsS, EvgS, PhoQ, PmrB, SsrA, CadC, and AphB, which are reviewed in Chapter 2.5.
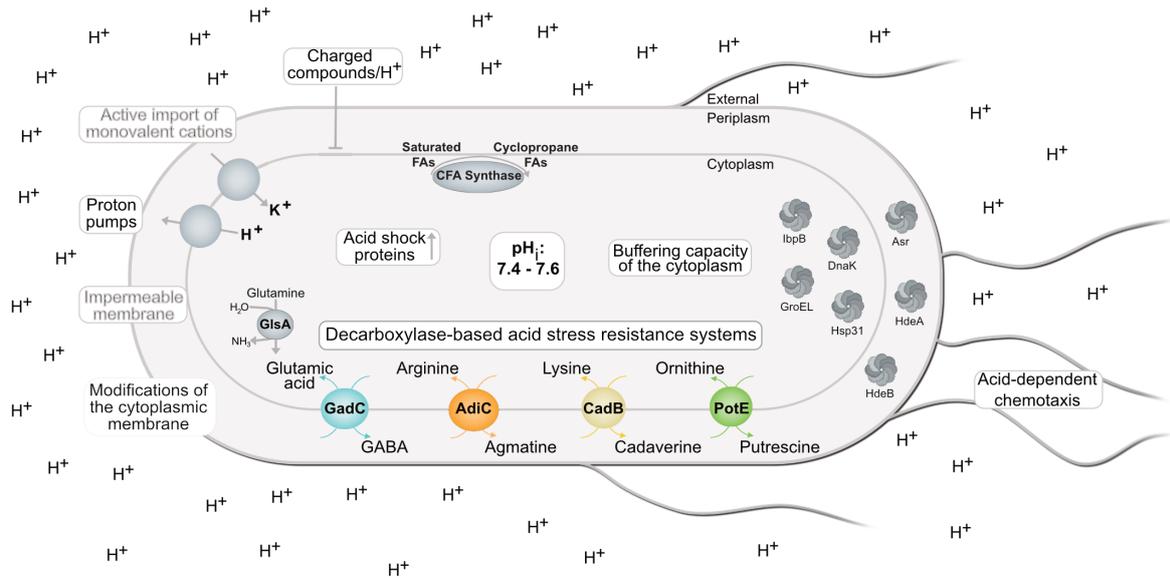
Figure 2: Summary of acid stress response mechanisms in γ-proteobacteria. This figure was adapted from Schwarz and Schumacher *et al.* 2022 (Chapter 2.5).

A prime example of inducible acid defense mechanisms are so-called enzyme-based H^+-consuming acid resistance systems (ARs). These systems elevate the pH via amino acid decarboxylases and the more alkaline reaction products, which are exported by antiporters in exchange for amino acid substrates (Figure 2). ARs were mostly studied in *E. coli*, which is equipped with four main ARs including the glutamate decarboxylase (Gad, AR2), arginine decarboxylase (Adi, AR3), lysine decarboxylase (Cad, AR4), and ornithine decarboxylase (Orn, AR5) systems. Each system is comprised of an H^+-consuming decarboxylase (GadA/GadB, AdiA, CadA, SpeF) and a corresponding antiporter (GadC, AdiC, CadB, PotE) (43–45). Gene regulation of AR components and the exact mechanisms of acid stress relief have been reviewed by Foster 2004, de Biase and Pennacchietti 2012, Kanjee and Houry 2013, Lund *et al.* 2014, Guan and Liu 2020, and Arcari *et al.* 2020 (see also Chapter 2.5) (42–47).

In brief, AR systems simultaneously increase the internal and external pH by proton consumption via decarboxylation of the respective amino acids (glutamate, arginine, lysine, or ornithine) and export of the more alkaline reaction products (GABA, agmatine, cadaverine, or putrescine) (Figure 2). Importantly, each AR is activated at a distinct pH range and growth phase (43–45). While the Cad, Adi, and Orn systems entirely depend on the presence of the respective amino acids (48–50), the Gad system is activated in the stationary phase irrespective of the presence of glutamate (51, 52). Moreover, transcriptional regulation of *gadA* and *gadBC* constitutes one of the most complex mechanisms known in prokaryotes (46). On the other hand, *cadBA*, *adiA*, and *adiC* expression is controlled by a single regulator, AdiY or CadC respectively (50, 53). Regulators involved in transcriptional regulation of *speF* and *potE*

of the Orn system are currently unknown. In addition to the four systems mentioned above, *E. coli* is equipped with another unexplored system referred to as AR1. In contrast to AR2-5, AR1 is not dependent on the uptake of external amino acids but relies on the consumption of internally derived glutamate using the decarboxylating enzymes of the Gad system (54).

Inducible chaperones and acid shock proteins also confer acid resistance to bacteria. Periplasmic chaperones such as HdeA and HdeB restrict protein aggregation in the periplasm and are essential for survival considering that acidification triggers protein damage, denaturation, and unfolding (55). HdeA and HdeB show maximal activity at pH < 4 and bind proteins unfolded at low pH to prevent them from aggregation (56, 57). Another protective periplasmic chaperone is Asr, which is annotated as an 'acid shock protein'. Asr is induced in a pH range of 3-4 and knockout mutants are highly vulnerable to acid shock (58). Recently, the molecular mechanism of Asr-mediated acid protection was unraveled. Asr acts as an intrinsically disordered chaperone which guarantees outer membrane integrity by preventing aggregation of proteins carrying positive net charges (59). Besides the periplasmic examples mentioned above, other protective chaperones act in the cytoplasm. These involve GroEL, DnaK, IbpB, and Hsp31 (*hchA*) (Figure 2).

Moreover, multidrug efflux pumps such as MdtEF are induced by acidity (60, 61). In addition to their role in drug extrusion, efflux pumps can export protons and consequently relieve acid stress (62). Importantly, several lines of evidence for facilitated development of antibiotic resistance as a consequence of induced acid defense mechanisms exist (63–65). This overlap between acid stress and antibiotic stress responses is summarized in Chapter 2.6.

1.3 pH-induced chemotaxis and motility

Bacteria can detect chemical gradients, enabling them to move toward or away from specific stimuli. This process involves specialized structures such as flagella, allowing the bacteria to swim and navigate to favorable environments or away from harmful substances (66, 67).

Considering acidity, the chemoreceptors Tar and Tsr are essential for navigating *E. coli* to habitats with desired pH levels. These receptors are methyl-accepting chemotaxis proteins (MCPs) and form clusters in the cell membrane which associate with the HK CheA and the adaptor protein CheW (Figure 3) (68–70). Both MCPs can sense the external and cytoplasmic pH and induce a signal transduction cascade, ultimately resulting in control of the flagellar motor (Figure 3) (71, 72). Adaptive receptor methylation in both Tar and Tsr fine-tunes the pH response and a 'push-pull' mode of action has been noted (73). Another crucial receptor for pH taxis is TlpB of *Helicobacter pylori* (Figure 3). Notably, *tlpB* knockout mutants have been characterized as pH non-responsive (74, 75).
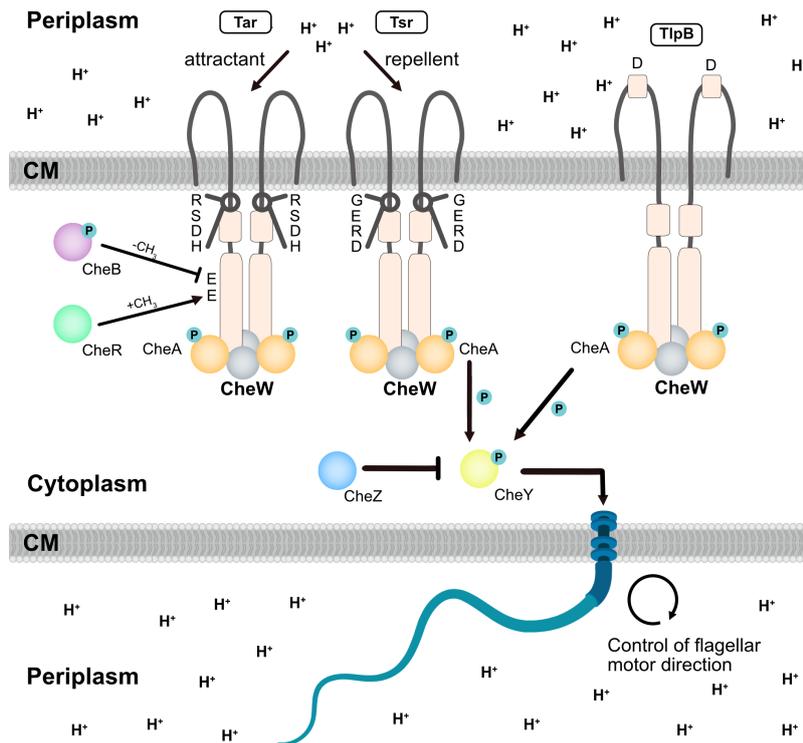
Figure 3: Control of bacterial chemotaxis and flagellar movement in response to acid stress. Amino acid residues are marked in single letter code when described to be important for pH-sensing. CM, cytoplasmic membrane.

Upon signal propagation from chemoreceptors, the kinase CheA is phosphorylated while CheW serves as an adaptor protein. Subsequently, activated CheA transfers its phosphoryl group to the RR CheY, converting it into its active, phosphorylated form (CheY-P). CheY-P is essential for controlling the flagellar motor rotation as it binds and interacts with the motor switch complex (Figure 3). When CheY-P dissociates, the flagellar motor resumes its normal spinning motion, propelling the bacterium forward in a straight run. CheY-P can also be dephosphorylated by the phosphatase CheZ, and CheB as well as CheR proteins are involved in adaptation (Figure 3). CheR methylates the receptors, while CheB demethylates them (66, 67).

Besides alterations in chemotactic behaviors, differential expression of flagellar components has been observed under acidity. For instance, flagellar genes were differentially expressed in *E. coli* at pH 5.0 (37) and similar observations were made in *Brucella suis*, *Listeria monocytogenes,* and *Campylobacter jejuni* (see Chapter 2.6), suggesting a universal phenomenon in motile bacteria. It is worth noting, that motility and chemotaxis appear to be strictly dependent on the degree of acid stress, considering that taxis towards aspartate was reported to be abolished at pH <5 and bacteria were demonstrated to be non-motile at pH 4 (76). Moreover, flagellar excision and rapid shedding of flagella upon sudden drops to pH 4 have been noted (77, 78). This suggests, that bacteria when exposed to acidity aim to migrate to habitats with lower H^+ concentrations only under mildly acidic conditions.

1.4 Bacterial exposure to eukaryotic stress hormones

Besides acid stress, bacteria can encounter a variety of other stressors in their environments such as temperature fluctuations, nutrient scarcity, toxic chemicals, oxidative stress, or antibiotic stress (79). Another stressor which gained increasing attention over the last years in the field of bacteriology are eukaryotic stress hormones. For instance, catecholamine hormones such as epinephrine (EPI), phenylephrine (PE), norepinephrine (NE), labetalol (LAB), or dopamine, are present in a wide range of hosts such as animals and humans where they function as neurotransmitters and stress hormones (80). These compounds harbor side-chain amines and a catechol motif (Figure 4).
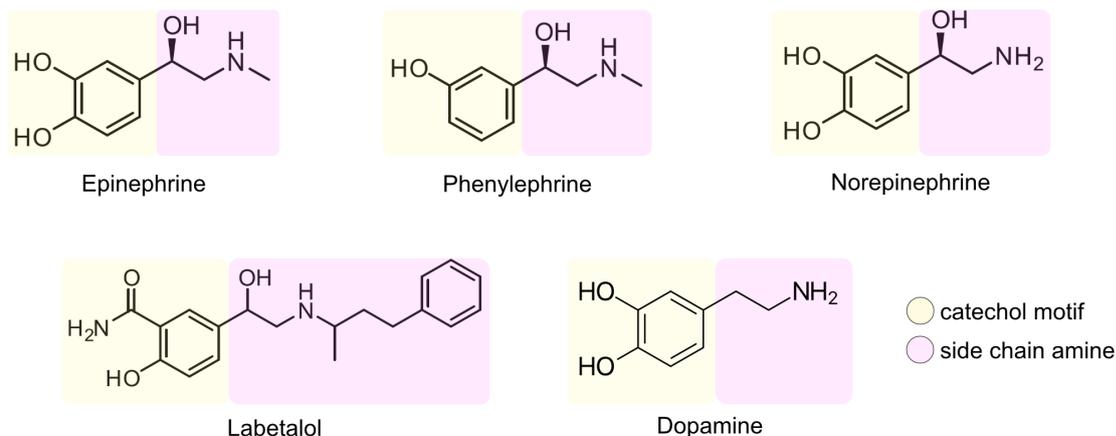
Figure 4: Chemical structures of a selection of eukaryotic catecholamine hormones. Catechol motifs and side chain amines are colored yellow and purple.

It was previously indicated that pathogenic infections of mammalian hosts were facilitated when the host organism was stressed (81, 82). Along this line, catecholamine hormones induced the growth of several bacterial species due to the iron-sequestering properties of the catechol siderophore (83). Importantly, catecholamine hormones can also affect biofilm formation, virulence, siderophore production, and the invasion of epithelial cells (82, 84–87). Hence, certain pathogens may use these compounds produced under stress as cues for recognition of the eukaryotic environment with the ultimate purpose of occupying a specific niche within the host (88). This hypothesis is further supported as bacterial chemotaxis was impacted by stress hormones. For example, NE was reported to act either as an attractant or a repellent for *E. coli*, depending on the concentration (89). Moreover, stress hormones can increase bacterial horizontal gene transfer efficiencies, enhance the expression of pili as well as the attachment to gut tissue, and activate type II secretion systems (90–92).

In eukaryotes, the binding of catecholamines to G protein-coupled receptors is well known (93, 94). Based on the effects in bacteria described above, it appears plausible that catecholamine receptors may have also evolved in prokaryotes. Indeed, a former study in EHEC indicated the

binding of NE and EPI to the HKs QseE and QseC (95, 96). Nevertheless, knockout mutants lacking the above mentioned genes still responded to eukaryotic stress hormones in *V. cholerae* and *Salmonella* (87, 97, 98). This suggests, that further yet unknown receptors exist in bacteria. For the marine pathogen *Vibrio campbellii*, NE and dopamine simultaneously increased growth, siderophore production, motility, as well as the expression of virulence genes (86). Notably, while α-adrenoreceptor antagonists like LAB inhibited the effect of NE on motility, β-adrenoreceptor antagonists such as propranolol (PRO) did not exhibit any effect. This indicates, that catecholamines likely act through a specific receptor in *V. campbellii* (86).

V. campbellii, formerly known as *V. harveyi*, is an important model organism for the study of quorum sensing (99, 100). This marine bacterium belongs to the γ-proteobacteria class, exhibits motility and bioluminescence, and is an opportunistic pathogen for marine species such as fish, shrimp, squid, and other marine invertebrates (101). To detect the potential receptor of catecholamine hormones in *V. campbellii*, or other target proteins, chemical proteomics shall be applied (see Chapter 1.7). Chemical proteomics involves synthesizing chemical probes containing an alkyne handle suitable for target protein enrichment via affinity-based protein profiling, followed by mass spectrometry (MS)-based proteome profiling (102, 103). This approach successfully identified target proteins of diverse chemicals in various bacteria (104–106). Thus, it represents a promising approach for gaining further insights into the interplay of eukaryotic catecholamine hormones and target proteins in prokaryotes.

1.5 Heterogeneous stress responses

Heterogeneous stress responses in bacteria refer to the varied and non-uniform reactions and gene expression levels of individual cells within a population to environmental or physiological stressors (107, 108). Unlike multicellular organisms, where heterogeneity can occur at different levels (cellular, tissue, organismal), the variability in bacteria is observed at the level of individual cells (109). Heterogeneity arises from internal factors including the cellular physiological state and stochastic gene expression, often referred to as 'noise', but can be influenced by external factors such as stressors, nutrient availability, and cell density (110, 111). Thereby, risks associated with unpredictable environments can be mitigated due to diversification, a strategy referred to as 'bet-hedging' (112, 113). It is important to note that phenotypic heterogeneity refers to the presence of diverse phenotypes within a genetically identical population, whereas homogeneous populations are characterized by uniform gene expression levels (Figure 5). In the field of bacteriology, both fluorescence microscopy (Chapter 2.4) and single-cell RNA sequencing (scRNA-Seq) are commonly used to study heterogeneous expression levels of genes and pathways (114).
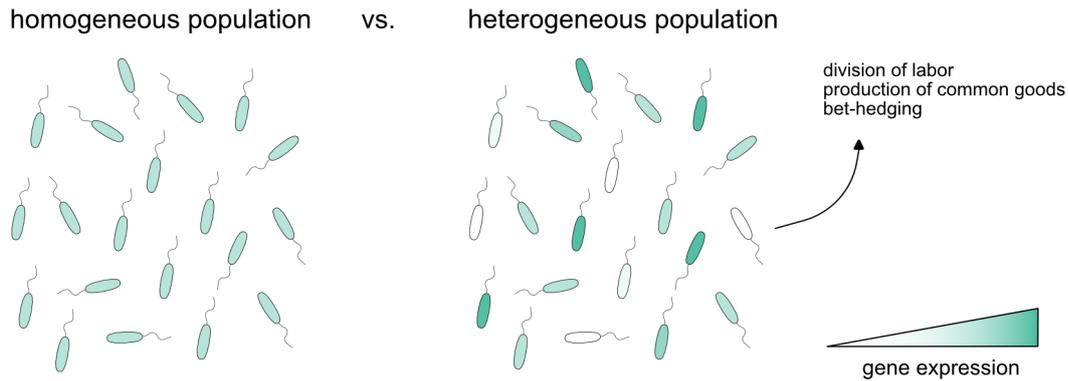
Figure 5: Schematic illustration of homogeneous and heterogeneous bacterial populations. Differential gene expression levels among individual cells are indicated using green and white colors.

This work focuses on heterogeneous responses under acid stress. Nevertheless, division of labor and bet-hedging have previously been observed upon exposure of bacteria to other stressors. For instance, cell-to-cell variability was noted upon exposure of *E. coli* to oxidative stress. In this case, supplementation of H_2O_2 triggered heterogeneity which was dependent on the ROS levels within the immediate environment of single cells and was affected by cell-cell interactions (115). Moreover, *Bacillus cereus* exhibited high-level heterogeneity during the adaptive salt stress response (116). Phenotypic heterogeneity under antibiotic stress is also a well-known phenomenon and was summarized in several literature reviews (see also Chapter 2.6) (117–119). In terms of acid stress, cell-to-cell variability was indicated in *Salmonella* which is confronted with acidity in vacuoles of macrophages (120). In *E. coli*, GadE, the master regulator of the Gad system, is heterogeneously expressed irrespective of the stress intensity. This results in a preemptive split into subpopulations with different acid tolerance among an isogenic population (121).

Further, the Cad system is heterogeneously activated among single cells in *E. coli*, but not in *V. campbellii*. Fluorescence microscopy revealed around 70% ON cells and 30% OFF cells in terms of CadB-eGFP expression. The underlying reason for this was reported to be a ribosome stalling motif which is present in the transcriptional activator CadC in *E. coli*, but missing in the *Vibrionaceae* family. This polyproline motif causes low CadC copy numbers (≤ 4 molecules per cell) which in turn lead to noisy gene expression of the target *cadBA* operon. This design of the signaling system represents an example of heterogeneity caused by the stochastic distribution of regulators among the population (122).

The ON/OFF behavior of the *E. coli* population in activating the Cad system raises the question of whether cells that do not express the Cad system induce the Adi and/or Gad system, which would indicate a division of labor under acid stress. To address this question, a comprehensive analysis of the distribution of the three major ARs in *E. coli* under consecutively increasing acid stress is provided in Chapter 2.4.

1.6 Previous and current approaches to systematically study acid stress

Next generation sequencing (NGS), also known as high-throughput sequencing, refers to a set of advanced sequencing technologies that enable rapid and parallel sequencing of millions of nucleic acid fragments. These advances revolutionized the field of genomics by providing the possibility to quickly and cost-effectively analyze large amounts of genetic information (123, 124). Before the availability of NGS, bacterial transcriptomes were mainly studied using microarrays (Figure 6) (125). A microarray constitutes a solid surface onto which DNA or RNA probes can be attached in a spatially defined manner. Probes are designed as complementary to specific genes or transcripts of interest and abundance is measured based on the emission of fluorescently-labeled cDNA binding to immobilized probes (126, 127). While microarrays are a cost-effective option, they are less sensitive compared to NGS-based methods, have limited genome coverage, and have a fixed content due to probe design which limits the detection of novel transcripts (128). In the context of bacterial acid stress resistance, a selected part of the *E. coli* transcriptome was studied via a microarray at pH 7.0 and 5.0 (37).

Beforehand, global adaptations of *E. coli* to acidity were also captured on the protein level by two-dimensional gel electrophoresis (Figure 6), separating proteins based on their isoelectric point and molecular weight (129, 130). With this approach, abundant proteins at pH 4.4, pH 4.9, and pH 6.0 were identified via excision from gels, followed by N-terminal sequence analysis (129), or MALDI-TOF (130). In recent years, the adaptations to acidity of a wide range of bacterial species have been studied using RNA-Seq and/or proteomics (summarized in Chapter 2.6).

Proteomics is defined as the large-scale study of proteins to characterize the entire protein content of a cell, which is usually based on liquid chromatography-mass spectrometry (LC-MS) (Figure 6) (131, 132). Nevertheless, the percentage of detected proteins in proteomics relative to total protein abundance remains in many cases limited due to issues based on aggregation or precipitation of membrane proteins. On average, membrane proteins constitute 30% of a proteome and target residues for tryptic cleavage are to a large extent absent in transmembrane helices and rather found in hydrophilic areas (133).

A powerful strategy to overcome the above mentioned limitations is to combine RNA-Seq with ribosome profiling (Ribo-Seq). This approach enables simultaneous detection of genome-wide mRNA levels and protein synthesis rates.
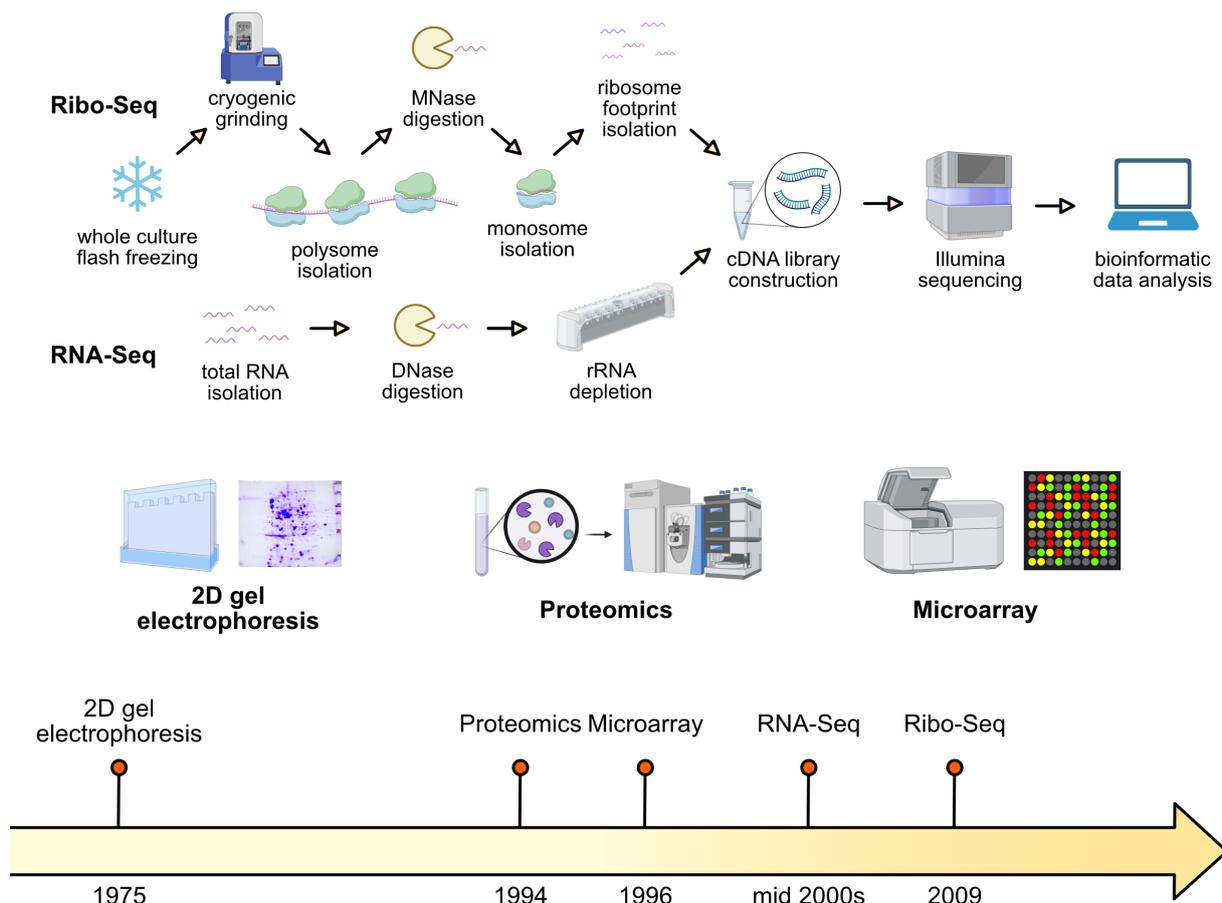
Figure 6: Schematic overview of methods for detection of genome-wide alterations in RNA and protein levels. The timeline indicates the year in which the respective methods were first described in the literature (134–139). Parts of this figure were created using BioRender.com.

1.6.1 Detection of transcriptional regulation using RNA-Seq

RNA-seq is a high-throughput sequencing technique that enables the genome-wide quantification and profiling of RNA and the comparison of transcriptomic responses in different experimental conditions. RNA-Seq workflows involve RNA extraction, DNase digestion, rRNA depletion, cDNA library construction, high-throughput sequencing, and bioinformatic analysis (Figure 6) (140, 141). RNA isolation is frequently conducted by commercially available kits or phenol-chloroform extraction (142). Subsequently, genomic DNA contaminations are removed by DNase digestion. Considering that rRNA accounts for the vast majority of bacterial transcripts, rRNA is depleted to enrich mRNA transcripts, for instance via hybridization to magnetic bead-linked complementary oligonucleotides (143). Finally, mRNA is converted to cDNA via random hexamer-primed reverse transcription, and adaptors, as well as unique indices, are ligated to cDNA fragments (140). Overall, the introduction of RNA-Seq has enabled the detection of numerous novel coding sequences, non-coding RNAs, and transcriptional start sites in bacteria (140, 141).

1.6.2 Assessment of protein synthesis rates using Ribo-Seq

RNA-Seq combined with Ribo-Seq enables simultaneous assessment of genome-wide mRNA levels and protein synthesis rates and allows the calculation of global translation efficiencies (139, 144). Protein synthesis is measured via Ribo-Seq by detection of transcripts which are protected from nuclease digestion by ribosome coverage. Therefore, in contrast to RNA-Seq, not the whole mRNA fraction is converted to cDNA but only RNA fragments shielded by ribosomes at the moment of sample collection. This allows genome-wide mapping of ribosome positions with single-nucleotide resolution. Ribosome-protected fragments (RPFs) are also referred to as ribosome footprints and are obtained by nuclease digestion of non-ribosome-covered RNA (139, 144–146). In eukaryotes, RNaseI is commonly used for digestion, which is however inactivated by bacterial ribosomes (147). To this end, MNase obtained from *Staphylococcus aureus* is commonly used for bacterial Ribo-Seq (148–151). In contrast to eukaryotes where RPFs display a uniform length of 28 nt, bacterial ribosome footprints range from 15 to 45 nt (139, 150, 152). Before MNase digestion, cells are commonly harvested by flash-freezing and lysed through cryogenic grinding. Bacterial polysomes are then isolated and converted to monosomes upon nuclease addition (Figure 6). Subsequently, RNA is isolated and enriched for RPFs via size selection and the remaining ribosome footprints are converted to cDNA (145, 150, 151, 153). In terms of stress conditions, Ribo-Seq combined with RNA-Seq has so far only been conducted in *E. coli* under heat stress (45 °C) (154).

1.6.3 Unravelling novel sORFs through Ribo-Seq

sORFs are open reading frames that are defined by their short length. However, the definition is inconsistent across literature and ranges from < 50 aa, to <70 aa, and < 100 aa (155–158). To a large extent, sORFs were previously overlooked due to technical limitations and were thus lacking in bacterial genome annotations. Beyond their size, small proteins are characterized by hydrophobicity and sometimes nonexistent charge which complicates their detection and the characterization of molecular functions (155–160). Furthermore, small proteins oftentimes harbor alpha-helical structures and are incorporated, or associated with the bacterial membrane (161, 162). Considering genomic locations, sORFs can be encoded independently in intergenic regions, antisense, nested (in the same or alternative frame) relative to known ORFs, or in the 5'- or 3' UTR of annotated genes. Moreover, sRNAs that encode a small peptide are considered dual-function RNAs, and small proteins can also be obtained by protease cleavage of a larger peptide (155, 157).

In recent years, Ribo-Seq and mass spectrometry-based approaches optimized for small proteins (peptidomics) have revealed a large reservoir of sORFs in a variety of bacterial species. Detection of sORFs through Ribo-Seq is commonly assisted by the supplementation of translation-arresting drugs before sample collection. Trapping ribosomes at the translation

initiation site (TIS) or translation termination site (TTS) is especially important for detecting sORFs encoded nested on the same strand within known sORFs (156, 163–165). The immense potential of novel sORF detection by Ribo-Seq was illustrated in various bacterial and archaeal species (157). Specifically, using mainly TIS- and TTS-Seq,139 novel candidate sORFs have been identified in *Salmonella* (166), 47 in *H. volcanii* (167), and 37 in *S.meliloti* (168). Nevertheless, experiments for the detection of novel sORFs in various organisms have so far been restricted to standard growth conditions. Thus, it will be of intriguing interest to conduct Ribo-Seq under various experimental setups, such as under stress conditions (see Chapter 2.1).

1.7 Scope of this thesis

Adaptations to acid stress are crucial for the survival of γ-proteobacteria throughout the passage of gastrointestinal tracts. In recent decades, various studies have been conducted to describe global systematic acid stress responses. Nevertheless, these investigations were mainly limited due to technical constraints and/or were conducted only under mildly acidic conditions. Within this thesis, the advantages of NGS, in particular Ribo-Seq, shall be leveraged to discover genome-wide adaptations of *E. coli* to mild (pH 5.8) and near-lethal (pH 4.4) acid stress, as well as to find novel differentially expressed biological pathways and key transcriptional regulators. These large-scale datasets can subsequently be mined to reveal novel sORFs exclusively expressed under acidity, and used to highlight acid-specific adaptations guided by autoencoder-based machine learning (see Chapter 2.1).

Moreover, alterations in flagella and chemotaxis gene expression were previously indicated to be crucial for bacterial acid stress responses. To further explore this relationship, a strain from the single-gene knockout Keio collection (BW25113 *mhpR::km*) shall be analyzed in terms of acid shock survival rates and motility. This mutant is characterized by an IS insertion in the promoter region of the chemotaxis and motility master regulatory genes *flhDC* and is thus hypermotile and suitable for exploring a potential correlation between motility and the survival of acid shock periods (see Chapter 2.2). Potential adaptations of γ-proteobacterial chemotactic signaling are further investigated in Chapter 2.3 by studying the response of the marine pathogen *Vibrio campbellii* to eukaryotic stress hormones. This study aims to discover protein targets of catecholamine hormones through chemical proteomics including photoaffinity labeling.

Finally, the three main inducible ARs of *E. coli* shall be analyzed in-depth concerning potential heterogeneous activation among single cells. By constructing a triple reporter strain and conducting fluorescence microscopy under consecutively increasing acid stress, potential bet-hedging strategies and division of labor in *E. coli* are investigated (Chapter 2.4).

2 Results

2.1 Ribosome profiling reveals the fine-tuned response of *Escherichia coli* to mild and severe acid stress

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🔓 | Systems Biology | Research Article

Ribosome profiling reveals the fine-tuned response of *Escherichia coli* to mild and severe acid stress

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ABSTRACT The ability to respond to acidic environments is crucial for neutralophilic bacteria. *Escherichia coli* has a well-characterized regulatory network that triggers a multitude of defense mechanisms to counteract excess protons. Nevertheless, systemic studies of the transcriptional and translational reprogramming of *E. coli* to different degrees of acid stress have not yet been performed. Here, we used ribosome profiling and RNA sequencing to compare the response of *E. coli* (pH 7.6) to sudden mild (pH 5.8) and severe near-lethal acid stress (pH 4.4) conditions that mimic passage through the gastrointestinal tract. We uncovered new differentially regulated genes and pathways, key transcriptional regulators, and 18 novel acid-induced candidate small open reading frames. By using machine learning and leveraging large compendia of publicly available *E. coli* expression data, we were able to distinguish between the response to acid stress and general stress. These results expand the acid resistance network and provide new insights into the fine-tuned response of *E. coli* to mild and severe acid stress.

IMPORTANCE Bacteria react very differently to survive in acidic environments, such as the human gastrointestinal tract. *Escherichia coli* is one of the extremely acid-resistant bacteria and has a variety of acid-defense mechanisms. Here, we provide the first genome-wide overview of the adaptations of *E. coli* K-12 to mild and severe acid stress at both the transcriptional and translational levels. Using ribosome profiling and RNA sequencing, we uncover novel adaptations to different degrees of acidity, including previously hidden stress-induced small proteins and novel key transcription factors for acid defense, and report mRNAs with pH-dependent differential translation efficiency. In addition, we distinguish between acid-specific adaptations and general stress response mechanisms using denoising autoencoders. This workflow represents a powerful approach that takes advantage of next-generation sequencing techniques and machine learning to systematically analyze bacterial stress responses.

KEYWORDS acid resistance, small proteins, transcription factor, ribosome profiling, RNA-Seq, machine learning

T he infective dose of enteropathogens varies significantly among bacterial genera and is dependent on the number and complexity of acid resistance mechanisms (1). *Escherichia coli* is equipped with a high number of defense mechanisms to survive the acidity of the stomach and, correspondingly, can have an infective dose as low as less than 50 cells (2). Enterobacteria that survive the stomach also confront mild acid stress in the colon due to the presence of short-chain fatty acids produced by obligate anaerobes (3). Other neutralophilic bacteria encounter low pH environments in a variety of settings, such as acidic soils, fermented food, or phagosomes within macrophages (1).

The cytoplasmic membrane represents a primary barrier for protons (H^+). Nevertheless, at low pH, H^+ can permeate into the cytoplasm via protonated water chains, ion channels, or damaged membranes (4). Upon acidification, the cytoplasmic pH transiently

decreases but returns to neutral within short time intervals. For example, a reduction in external pH to 5.5 causes a temporary decline in internal pH to ~6.0 (5). Intracellular acidification leads to the protonation of biological molecules, ultimately altering their charge and structure. This, in turn, may lead to protein unfolding, denaturation, and reduced enzymatic activities (6). Furthermore, acidification causes membrane and DNA damage (7, 8).

To maintain a constant internal pH and balance fluctuations in H^+ concentrations, the intrinsic buffering capacity of the cytoplasm is crucial, as protons can be sequestered by side-chains of proteins, inorganic phosphates, polyphosphates, or polyamines (9). Additional protective mechanisms that counteract acid stress and ensure survival in low pH habitats involve proton pumps, membrane remodeling, acid-dependent chemotaxis, chaperones, acid shock proteins, and the induction of enzyme-based H^+-consuming acid resistance (AR) systems (10, 11).

E. coli is equipped with four different AR systems, namely, the glutamate decarboxylase (Gad, AR2), arginine decarboxylase (Adi, AR3), lysine decarboxylase (Cad, AR4), and ornithine decarboxylase (Orn, AR5) systems (12, 13). Each AR system consists of an H^+-consuming amino acid decarboxylase (GadA/GadB, AdiA, CadA, SpeF) and a corresponding antiporter (GadC, AdiC, CadB, PotE), which serves to uptake the amino acid and export the more alkaline reaction product into the surrounding medium. This strategy ensures a simultaneous increase in intracellular and extracellular pH (7, 13, 14). Notably, each AR system is activated at different external pH values and growth phases (7, 15). We have previously found that individual *E. coli* cells exposed to consecutively increasing acid stress activate the Gad, Adi, and Cad systems heterogeneously, resulting in functional diversification and enhanced population fitness (15, 16). The regulatory network of the Gad system is highly complex and involves more than 10 regulatory elements (17), whereas the Adi and Cad systems are each regulated by a single transcriptional regulator, AdiY and CadC, respectively (15). Nevertheless, additional regulatory elements seem to exist that not only link these three ARs in individual cells but also contribute to a fine-tuned response of *E. coli* to different levels of acid stress (15).

Previous systematic studies to determine genome-wide adaptations within the acid stress response of *E. coli* have either been restricted to microarrays (18), two-dimensional gel electrophoresis (19, 20), or have only been performed under mild acidic conditions (pH 5.0–6.0) (21–23). Here, we present the first systemic study of global adaptations to different intensities of acid stress in *E. coli* at both the transcriptional and translational levels. Specifically, we compare gene expression and translation at pH 5.8 and pH 4.4 with pH 7.6, using RNA sequencing (RNA-Seq) and ribosome profiling (Ribo-Seq). Ribo-Seq allows deep-sequencing of ribosome-protected mRNA fragments (RPFs) (24, 25), which are obtained by nuclease digestion of non-ribosome-covered RNA, and has significantly advanced the current understanding of translational regulation (26). The applications of Ribo-Seq are manifold and include monitoring protein synthesis rates across the proteome, the identification of novel small open reading frames (sORFs), as well as determining protein copy numbers per cell during steady-state growth (27, 28). In contrast to mass spectrometry-based approaches, ribosome profiling is independent of protein biochemistry and allows detection of small proteins with less than 50 amino acids in length (29). In recent years, substantial advances in understanding translational events have been made through the utilization of Ribo-Seq, not only in various bacterial species but also in archaea, bacteriophages, and microbiomes (28, 30–35).

Our results reveal hundreds of differentially transcribed and translated genes of *E. coli* K-12 at pH 5.8 and 4.4, as well as examples of pH-dependent changes in translation efficiency. We identified previously undiscovered biological processes in response to acidity, including increased siderophore synthesis, glycerol catabolism, copper efflux, nucleotide biosynthesis, and spermidine/multidrug export, as well as decreased membrane transport and metabolic activities. In addition, we have identified new transcription factors (TFs) as key players during low pH exposure and 18 novel candidate sORFs involved in the response to acid stress. Finally, we differentiated

acid-specific transcriptional adaptations by using machine learning to compare the low pH response to that of other stressors.

RESULTS AND DISCUSSION

Examination of alterations in the translatome and transcriptome of *E. coli* in response to varying degrees of acid stress

To mimic natural stress conditions, such as the passage of *E. coli* through the gastrointestinal tract, we established the following protocol involving a sudden change to low pH, detection of a rapid response, and severe, near-lethal acid stress. Specifically, *E. coli* K-12 MG1655 was cultivated in unbuffered lysogeny broth (LB) medium at pH 7.6 until the exponential growth phase ($OD_{600} = 0.5$). Then, 5 M hydrochloric acid was added directly to expose the cells to a pH of 5.8 or stepwise to a pH of 4.4, corresponding to mild and severe acid stress (Fig. 1A). The final optical densities were comparable (pH 7.6: $OD_{600} =$
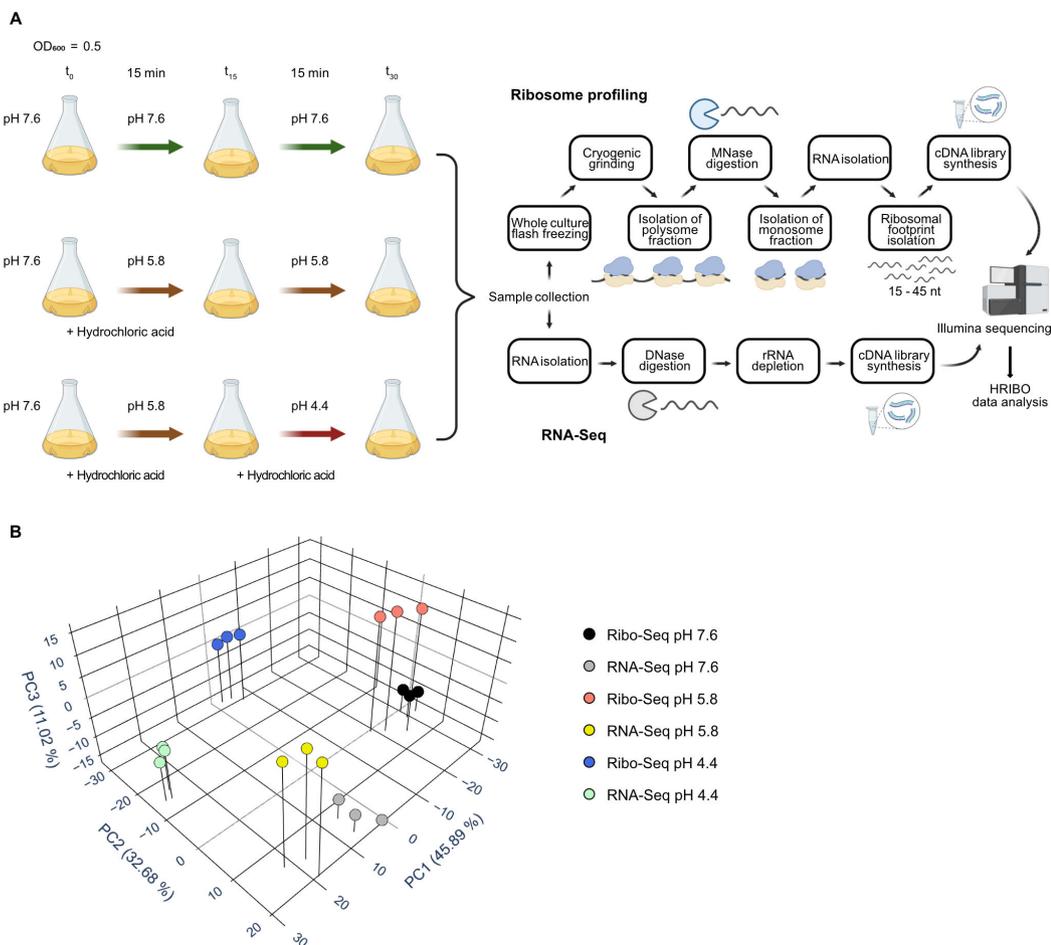
FIG 1   A schematic overview of culture conditions, pH-shift procedures, and the Ribo-Seq and RNA-Seq workflows. (A) Biological triplicates of *E. coli* MG1655 cells were grown in unbuffered LB medium (pH 7.6) to an OD_{600} of 0.5 (t_0). Subsequently, the cultures were either grown for an additional 30 min at pH 7.6, shifted for 30 min to pH 5.8, or shifted first for 15 min to pH 5.8 (t_{15}) and then to pH 4.4 (t_{30}). pH shifts were initiated by the direct addition of 5 M HCl to the cultures. At t_{30}, samples were collected. For RNA-Seq, total RNA was isolated, DNase digested, and ribosomal RNA was depleted prior to cDNA library preparation. For Ribo-Seq, whole cultures were flash-frozen in liquid nitrogen and cryogenically grinded using a freezer mill. Polysome fractions were isolated, and non-ribosome-protected RNA was digested using MNase. Ribosomal footprints were purified from the monosome fraction using a size selection gel and converted to cDNA libraries. Upon Illumina sequencing, the data were analyzed using the HRIBO pipeline (37). (B) Principal component analysis of median-of-ratios normalized and regularized log-transformed (rlog) read count values for *E. coli* Ribo-Seq and RNA-Seq triplicate data at pH 4.4, 5.8, and 7.6. Panel (A) was created with BioRender.com.

~1.1; pH 4.4: OD_{600} = ~0.7) (Table S1), and the pH values hardly changed compared to t_0 (Fig. 1A; Table S2). To investigate whether a 15-min exposure to pH 4.4 was sufficient to induce cellular adaptation to severe acid stress, we examined the temporal dynamics of *adiA* expression by quantitative reverse transcription PCR (RT-qPCR). We detected an increase in *adiA* mRNA levels as early as 15 min after the shift to pH 4.4 and no substantial further increase after 30 or 60 min (Fig. S1). Additionally, we evaluated cell viability at the final experimental time points using propidium iodide (PI) staining (36) and examined colony-forming units (CFUs). The average percentage of dead cells detected by PI staining was less than 1% at pH 7.6, 5% at pH 5.8, and 18% at pH 4.4 (Fig. S2A). A positive control (5-min heat treatment at 80°C) resulted in an average of 97.2% non-viable cells, as determined by PI staining. The CFU count results underline that at least 2×10^8 viable cells were collected irrespective of pH at the moment of sample collection for Ribo- and RNA-Seq (Fig. S2B).

Cells were harvested and lysed as previously described by whole-culture flash freezing and cryogenic grinding in a freezer mill to avoid bias from translation-arresting drugs and filtering (38). The subsequent steps of our ribosome profiling protocol were a combination of methodologies reported by Latif and colleagues (39) and Mohammad and Buskirk (40) (see Materials and Methods for details). Strand-specific Illumina sequencing yielded an average of approximately 30 million cDNA reads per sample for Ribo-Seq and 5–10 million for RNA-Seq. The next-generation sequencing data were analyzed using an extended version of the high-throughput HRIBO data analysis pipeline (37). All samples achieved sufficient coverage with over two million reads, each mapping uniquely to the coding regions. The rRNA contamination was higher in the pH 4.4 Ribo-Seq samples than in other conditions but accounted for less than 15% in all cDNA libraries (Fig. S3). The length distribution of the generated RPFs was broad, ranging from 15 to 45 nucleotides (Fig. S4), consistent with previous observations in other prokaryotic ribosome profiling analyses (38, 39). We did not detect stress-induced increased relative ribosome occupancy in the initiation region of ORFs under acid stress compared to neutral pH. This is in contrast to previous observations in *E. coli* under heat stress (41) and in yeast under oxidative stress (42), which reported increased relative ribosome accumulation at start codons in stressed cells. In fact, ribosome occupancy in the translation initiation regions was slightly reduced at pH 4.4 and 5.8 compared with physiological pH (Fig. S5). This could be explained due to diminished ribosome-RNA complex stability and increased ribosome drop-off under acidic conditions. The biological triplicates for each experimental condition clustered on the first three principal components in a principle component analysis (PCA) plot (Fig. 1B). Notably, the global gene expression profiles were highly distinct at pH 4.4 compared with both pH 5.8 and 7.6.

Coordinated regulation of transcription and translation in response to acid stress

The tool *deltaTE* (43) was used to assess transcriptional and translational changes (i.e., differential expression and differential translation efficiency) in response to mild and severe acid stress. Low-expression transcripts were filtered out, and we focused our analysis on 3,654 genes with mean reads per kilobase per million reads mapped (rpkm) values ≥5 across all investigated conditions. Our findings reveal that 702 transcripts were significantly altered at pH 5.8 compared with physiological pH [absolute mRNA log_2 fold change (FC) ≥1 and false discovery rate (FDR) adjusted $P \leq 0.05$], and 1,030 genes showed significant differences in mRNA levels at pH 4.4 (Fig. 2A). These results suggest that extensive transcriptional reprogramming occurred, which was influenced by the degree of acid stress. As illustrated by the Venn diagram overlaps (Fig. 2), a large number of adaptations occurred regardless of the degree of acid stress. Nonetheless, several hundred genes were differentially expressed exclusively at pH 5.8 or 4.4 (Fig. 2A). This suggests that in addition to universal adaptations at low pH, specific adaptations for mild and severe acid stress occur. We further determined the number of genes with
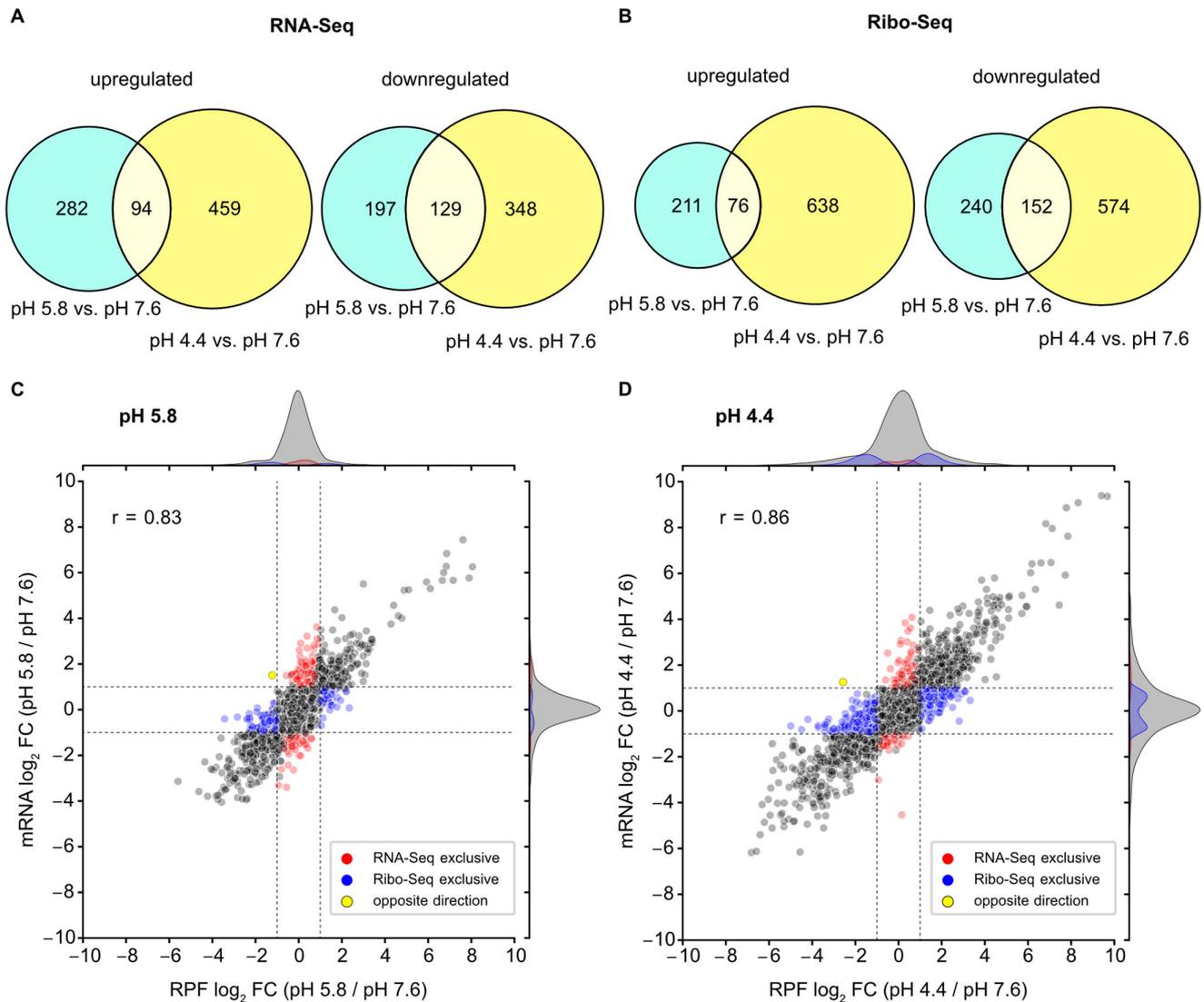
FIG 2 Genome-wide adaptations correlate at the transcriptional and translational levels in *E. coli* under acid stress. Weighted Venn diagrams show the total number and overlap of genes with significant FCs (absolute log$_2$ FC ≥1 and *P*-adjust ≤0.05) determined by (A) RNA-Seq or (B) Ribo-Seq for cells exposed to pH 5.8 or pH 4.4, compared to the control (pH 7.6). (C) Comparison of global RPF and mRNA log$_2$ FC values for pH 5.8, or (D) pH 4.4 vs pH 7.6. Dashed lines indicate log$_2$ fold change values of +1 or −1. Hundreds of genes exhibited differential expression (absolute log$_2$ FC ≥1 and *P*-adjust ≤0.05) at both the transcriptional and translational levels, whereas others were exclusively detected by either RNA-Seq (red dots) or Ribo-Seq (blue dots) or had significant changes in opposite directions (yellow dots). Values of the Pearson correlation coefficient (*r*) are indicated.

stress-dependent alterations in RPF counts to be 679 at pH 5.8 and 1,440 at pH 4.4 (absolute RPF log$_2$ FC ≥1 and FDR adjusted *P* ≤ 0.05), which was in a similar range compared with the RNA-Seq data (Fig. 2A and B). Accordingly, the global FC values for mRNA and RPF levels showed a high Pearson correlation coefficient (*r*) under both conditions (Fig. 2C and D, gray dots). This indicates that transcriptional regulation of these genes is the predominant response to acid stress. However, a subset of genes exhibited exclusive and significant regulation at either the transcriptional (red dots) or translational (blue dots) level.

Specifically, at pH 5.8, 193 genes were detected to be significantly regulated exclusively by RNA-Seq, while 216 genes were exclusively affected in the Ribo-Seq data (Fig. 2C; Table S3). At pH 4.4, 127 differentially regulated genes were found exclusively by RNA-Seq and 570 genes by Ribo-Seq (Fig. 2D; Table S3). Notably, for *fruA* at pH 5.8 and *yecH* at pH 4.4, opposite changes were observed at the transcriptional and translational

levels (Fig. 2C and D, yellow dots). FruA is the fructose permease of the phosphoenolpyruvate-dependent sugar phosphotransferase system (44), whereas the function of YecH remains unknown.

Next, we investigated translation efficiency (TE) to identify genes that undergo translational regulation in response to acidic conditions. TE provides information regarding ribosome counts per mRNA and is calculated as the ratio of RPFs over transcript counts within a gene's coding sequence normalized to mRNA abundance (43). We identified 22 genes at pH 5.8 and 89 genes at pH 4.4, which displayed significantly altered TEs (absolute $\log_2$ TE fold change ≥1 and P-adjust ≤0.05) (Table S4). The highest increase in TE at pH 4.4 was found for the KpLE2 phage-like element (*topAI*), a hydroxyethylthiazole kinase (*thiM*), and a palmitoleoyl acyltransferase (*lpxP*). In contrast, *yecH* and *yjbE*, both encoding uncharacterized proteins, and *malM* of the maltose regulon showed the most prominent decrease in TE at pH 4.4 (Table S4). At pH 5.8, we noted the largest increase in TE for a ferredoxin-type protein encoded by *napF*, an iron transport protein (*feoA*), and a tagaturonate reductase (*uxaB*). Conversely, the largest decrease was observed for a protein of the fructose-specific phosphotransferase system (*fruA*), a tripartite efflux pump membrane fusion protein (*emrK*), and an HTH-type transcriptional regulator (*ydeO*) (Table S4).

In summary, besides extensive transcriptional reprogramming, dozens of genes exhibit significant FCs either at the transcriptional or translational level in response to acid stress. This underlines that transcription and translation are not always coupled in bacteria. Similar findings were reported by Zhang and colleagues (41), who conducted Ribo-Seq and RNA-Seq analyses for *E. coli* under heat stress (41). Overall, such differential regulation can be explained, for example, by delayed translation relative to transcript synthesis, selective recruitment or release of ribosomes, or regulation during translation initiation, elongation, or ribosome biogenesis (45–49), which could be beneficial under stress conditions.

Functional implications of genes with differential mRNA and RPF levels under mild acid stress

To obtain a more profound understanding of the fine-tuned response of *E. coli* to different degrees of acid stress, we first analyzed all genes with differential mRNA and ribosome coverage levels during mild acid stress (pH 5.8). Under this condition, the top candidates with the highest FC values for mRNA and RPF are as follows: (i) the *cad* operon, encoding the core components of the Cad AR system (see also below); (ii) the *glp* regulon, responsible for glycerol and *sn*-glycerol 3-phosphate uptake and catabolism (50); (iii) the *mdtJI* operon, encoding a heterodimeric multidrug/spermidine exporter (51); and (iv) genes encoding proteins involved in motility and flagella biosynthesis (Table 1). A comprehensive list of normalized read counts, mRNA and RPF FCs, and TEs for all *E. coli* genes is provided in Table S5. We tested a representative selection of differentially expressed genes by RT-qPCR. In all cases, the detected changes in mRNA levels were consistent with the data gathered by RNA-Seq (Fig. S6A). Both *recA* and *secA* were chosen as reference genes for RT-qPCR because their rpkm counts were relatively constant under the conditions tested (Table S5; Fig. S6B).

Next, we performed gene set enrichment analysis (GSEA) using *clusterProfiler* (52) to identify biological processes associated with differentially expressed genes at pH 5.8. Among the most enriched Gene Ontology (GO) terms for biological processes at pH 5.8 was "spermidine transmembrane transport" (Fig. 3), which corresponds to the induction of *mdtJI* (Table 1) and a polyamine ABC transporter encoded by *potABCD* (Table S5). Polyamines are crucial for survival under acid stress, as they reduce membrane permeability by blocking OmpF and OmpC porins (53–55). External spermidine supplementation also improved acid resistance in *Streptococcus pyogenes* (56). On the other hand, overaccumulation of polyamines can be toxic and potentially lethal for *E. coli* (51, 57). Therefore, precise transmembrane transport of polyamines in acidic environments is critical and contributes to survival in acidic conditions.

TABLE 1 Top 20 genes with increased RPF levels at pH 5.8 compared to pH 7.6, sorted in descending order by Ribo-Seq log$_2$ FC values[a]

Gene name	Log$_2$ FC (Ribo)	P-adjust (Ribo)	Log$_2$ FC (RNA)	P-adjust (RNA)	Cellular location	Annotation
glpA	8.05	1×10^{-159}	6.26	2×10^{-38}	C	Anaerobic glycerol-3-phosphate dehydrogenase subunit A
glpB	7.89	2×10^{-141}	5.77	6×10^{-29}	C	Anaerobic glycerol-3-phosphate dehydrogenase subunit B
cadA	7.61	2×10^{-144}	7.44	3×10^{-57}	C	Lysine decarboxylase 1
glpC	7.16	5×10^{-89}	5.67	4×10^{-35}	C, IM	Anaerobic glycerol-3-phosphate dehydrogenase subunit C
cadB	6.86	8×10^{-96}	6.85	5×10^{-35}	IM	Lysine:cadaverine antiporter
glpT	6.84	2×10^{-105}	6.28	2×10^{-39}	IM	sn-Glycerol 3-phosphate:phosphate antiporter
glpD	6.72	4×10^{-126}	6.00	2×10^{-52}	C, IM	Aerobic glycerol 3-phosphate dehydrogenase
mdtJ	6.65	2×10^{-67}	5.67	6×10^{-36}	IM	Multidrug/spermidine efflux pump membrane subunit MdtJ
glpQ	6.11	2×10^{-89}	5.31	2×10^{-29}	P	Glycerophosphoryl diester phosphodiesterase
mdtI	5.93	3×10^{-49}	5.60	9×10^{-34}	IM	Multidrug/spermidine efflux pump membrane subunit MdtI
glpF	5.10	7×10^{-64}	5.26	1×10^{-19}	IM	Glycerol facilitator
yfdX	4.89	2×10^{-24}	5.23	4×10^{-25}	P	Protein YfdX
frc	4.78	1×10^{-36}	4.01	2×10^{-15}	C	Formyl-CoA transferase
glpK	4.62	9×10^{-54}	4.10	1×10^{-25}	C	Glycerol kinase
yfdV	4.40	3×10^{-18}	4.58	1×10^{-12}	IM	Putative transport protein YfdV
oxc	4.28	2×10^{-28}	3.75	1×10^{-14}	C	Xalyl-CoA decarboxylase
flgC	3.41	3×10^{-22}	2.95	3×10^{-9}	P	Flagellar basal-body rod protein FlgC
flgB	3.40	1×10^{-24}	3.09	1×10^{-10}	P	Flagellar basal-body rod protein FlgB
dhaL	3.39	9×10^{-13}	3.08	1×10^{-10}	C	Dihydroxyacetone kinase subunit L
flgD	3.33	7×10^{-25}	2.90	5×10^{-9}	C	Flagellar biosynthesis, initiation of hook assembly

[a]C, cytosol; P, periplasm; IM, inner membrane.
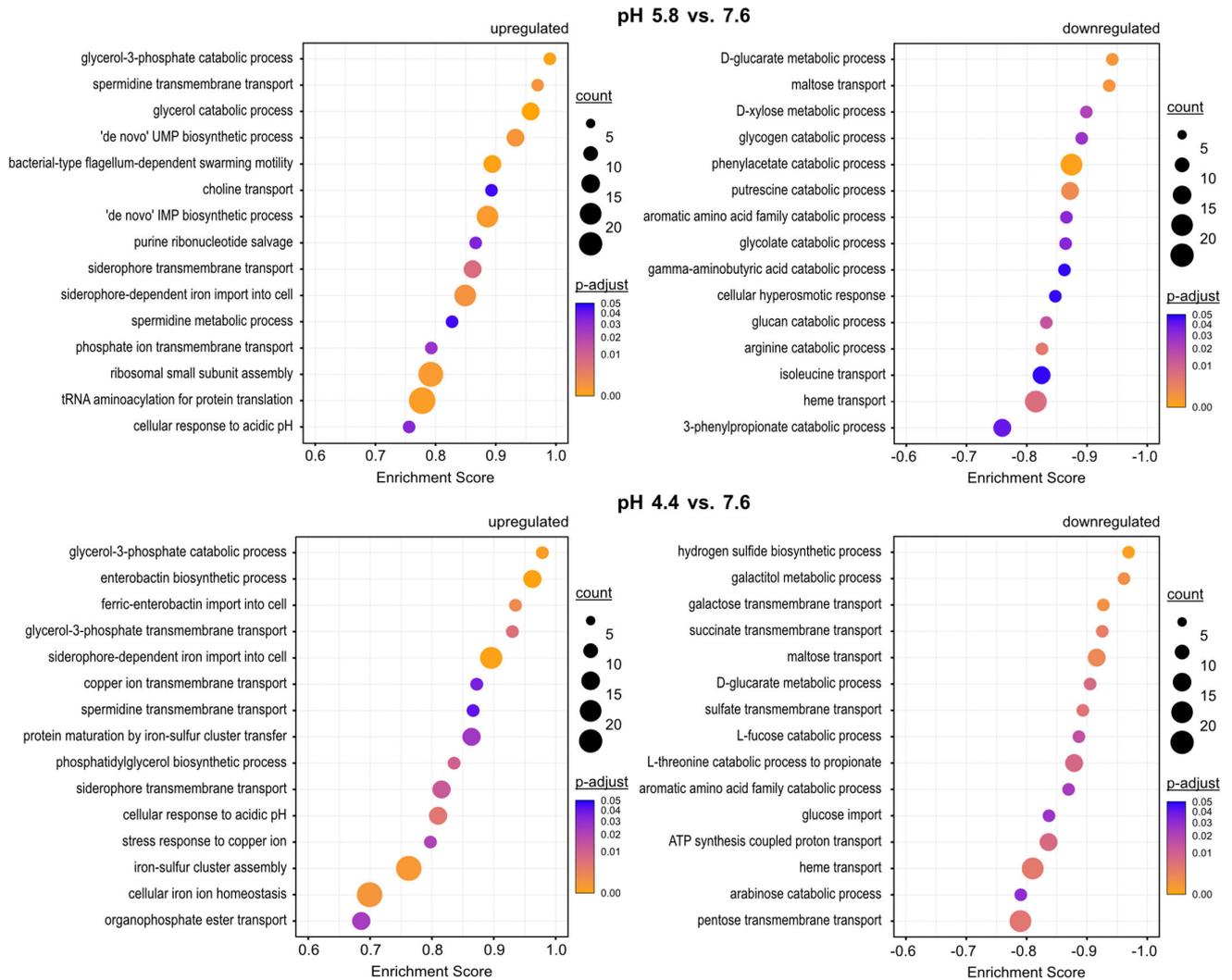
FIG 3 Up- and downregulated biological processes in *E. coli* at pH 5.8 and 4.4. GSEA was conducted using the *gseGO* function in the *clusterProfiler* package (52) with the ribosome profiling differential expression data sorted by log$_2$ fold change values as input. GO terms were considered up- or downregulated if *P*-adjust values were ≤0.05. The top 15 non-redundant GO terms were sorted in descending order by the *clusterProfiler* enrichment score and are shown for pH 5.8 vs 7.6 and pH 4.4 vs 7.6. The dot size represents the number of genes associated with each GO term, and the dot color represents adjusted *P*-values corrected for the false discovery rate.

The enrichment of the GO terms "glycerol-3-phosphate catabolic process" and "glycerol catabolic process" at pH 5.8 (Fig. 3) has not yet been associated with acid stress to our knowledge. Notably, of the 14 genes with the largest increase in RPF counts at pH 5.8, 7 belong to the *glp* regulon (Table 1). This regulon is required for the uptake and catabolism of glycerol and *sn*-glycerol 3-phosphate (G3P) (50). In this pathway, G3P is converted to dihydroxyacetone phosphate by membrane-bound dehydrogenases, either aerobically via GlpD or anaerobically by the GlpABC complex (58, 59). Alternatively, dihydroxyacetone phosphate can be produced directly from glycerol by GldA and the protein products of the *dhaKLM* operon (60). The *dhaKLM* operon was also induced at pH 5.8 (Table S5). It remains unclear whether glycerol and G3P catabolism directly contribute to acid tolerance or whether the *glp* regulon is activated as a consequence of other low pH adjustments. Expression of *glp* genes is regulated by the repressor GlpR, which is inactivated upon binding of glycerol or G3P (61). We hypothesize that changes in phospholipid composition under acid stress conditions (62) may release G3P, which in

turn induces the *glp* regulon. Accordingly, the GO term "phosphatidylglycerol biosynthetic process" was enriched under acid stress (Fig. 3).

Another observation is the upregulation of *de novo* biosynthesis pathways for pyrimidine and purine nucleotides at pH 5.8 (Fig. 3). The induction of a large proportion of the PurR-dependent regulon involved in *de novo* nucleotide synthesis (Fig. 3; Table S5) suggests that *E. coli* requires additional nucleotides to cope with the extensive transcriptional reprogramming. Besides, intracellular acidification can lead to DNA damage, such as depurination (63), making enhanced nucleotide biosynthesis a critical compensatory mechanism. Recently, *Oenococcus oeni* was reported to experience a decrease in the abundance of both purines and pyrimidines under acid stress, while nucleotide metabolism and transport increased (64), suggesting a similar phenomenon in this species. Other enriched GO terms under mild acid stress include "choline transport," "siderophore transmembrane transport," "phosphate ion transmembrane transport," "ribosomal small subunit assembly," "tRNA aminoacylation for protein translation," and "bacterial-type flagellum-dependent swarming motility" (Fig. 3). pH-dependent motility has previously been observed in *E. coli*, *Salmonella*, and *Helicobacter* (1). These observations suggest that bacterial cells use an escape strategy to migrate to more favorable pH environments when challenged with acidic conditions.

On the contrary, our findings reveal that many membrane and periplasmic proteins (18 of the 20 genes with the most diminished RPF counts, Table 2) were among the top candidates with decreased mRNA and RPF levels under mild acid stress. This affected, for example, genes encoding ABC transporters (*mal* regulon, *dpp* operon) and symporters (*actP*, *melB*, *gabP*), highlighting the superiority of Ribo-Seq over mass spectrometry-based approaches, namely, its independence of protein biochemistry and higher sensitivity (29). Furthermore, GSEA identified membrane transport and metabolic activities as the most downregulated biological processes in response to mild acid stress. For example, "maltose transport," "isoleucine transport," "heme transport," "putrescine catabolic process," "glycolate catabolic process," and "aromatic amino acid family catabolic process" were among the most downregulated GO terms at pH 5.8 (Fig. 3). Downregulation of H^+-coupled transport processes represents a key mechanism by which *E. coli* restricts proton influx into cells. In addition, the downregulated metabolic processes are in many cases associated with the synthesis and conversion of amino acids and carbon sources. For example, the catabolism of aromatic amino acids and arginine was also reduced at pH 5.8 (Fig. 3). Particularly noteworthy is the downregulation of the arginine catabolic pathway, which involves the protein products of the *astEBDAC* operon. At pH 5.8, hardly any reads were mapped in the *astEBDAC* region, despite detectable expression at pH 7.6 and pH 4.4 (Table S5). Presumably, *E. coli* preserves the intracellular arginine pool at pH 5.8, as this amino acid serves as a substrate for the Adi system during severe acid stress (15, 65).

In summary, the response of *E. coli* to mild acid stress is characterized by the activation of the motility machinery to escape to less acidic habitats, by induction of the *cad* operon, and by genes involved in polyamine transport and glycerol-3-phosphate conversion (Tables 1 and 2; Fig. 3). In addition, *E. coli* restricts the influx of protons and conserves energy by reducing its metabolic activities.

Functional implications of genes with differential mRNA and RPF levels under severe acid stress

Next, we analyzed genes with differential mRNA and ribosome coverage levels in response to severe acid stress (pH 4.4) compared with non-stress (pH 7.6). Genes with the highest number of increased read counts, which were not already upregulated at pH 5.8, were *asr*, encoding an acid shock protein, followed by *bdm*, encoding a biofilm-modulation protein, and *bhsA*, encoding a multiple stress resistance outer membrane protein (Table 3). Originally, Asr was classified as a periplasmic acid shock protein, although its role in acid adaptation remained unclear (66). Recently, Asr was shown to be an intrinsically disordered chaperone that contributes to outer membrane integrity and to

TABLE 2 Top 20 genes with decreased RPF levels at pH 5.8 compared to pH 7.6, sorted in ascending order by Ribo-Seq log$_2$ FC values[a]

Gene name	Log$_2$ FC (Ribo)	P-adjust (Ribo)	Log$_2$ FC (RNA)	P-adjust (RNA)	Cellular location	Annotation
yjcH	-5.59	3×10^{-27}	-3.14	1×10^{-8}	IM	Conserved inner membrane protein YjcH
actP	-4.63	2×10^{-47}	-3.58	1×10^{-11}	IM	Acetate/glycolate:cation symporter
lamB	-4.32	3×10^{-6}	-2.27	6×10^{-6}	OM	Maltose outer membrane channel/phage lambda receptor protein
dppD	-4.23	5×10^{-33}	-3.79	9×10^{-15}	IM	Dipeptide ABC transporter ATP-binding subunit DppD
acs	-4.06	3×10^{-20}	-2.60	3×10^{-6}	C	Acetyl-CoA synthetase (AMP-forming)
malK	-3.96	2×10^{-18}	-2.64	2×10^{-10}	IM	Maltose ABC transporter ATP-binding subunit
dppC	-3.83	1×10^{-28}	-3.78	5×10^{-13}	IM	Dipeptide ABC transporter membrane subunit DppC
malG	-3.82	5×10^{-20}	-2.52	4×10^{-7}	IM	Maltose ABC transporter membrane subunit MalG
dppF	-3.75	4×10^{-34}	-3.93	4×10^{-18}	IM	Dipeptide ABC transporter ATP-binding subunit DppF
dppB	-3.74	1×10^{-31}	-3.74	1×10^{-16}	IM	Dipeptide ABC transporter membrane subunit DppB
malM	-3.69	7×10^{-5}	-2.06	2×10^{-6}	P	Maltose regulon periplasmic protein
melB	-3.60	6×10^{-28}	-1.24	0.03	IM	Melibiose:H$^+$/Na$^+$/Li$^+$symporter
ydcT	-3.59	2×10^{-27}	-3.61	1×10^{-10}	IM	Putative ABC transporter ATP-binding protein YdcT
ydcS	-3.57	1×10^{-31}	-4.05	9×10^{-15}	IM, P	Putative ABC transporter periplasmic–binding protein/polyhydroxybutyrate synthase
gabP	-3.56	5×10^{-19}	-2.76	9×10^{-8}	IM	4-Aminobutyrate:H$^+$ symporter
ydcV	-3.48	1×10^{-23}	-3.39	1×10^{-8}	IM	Putative ABC transporter membrane subunit YdcV
ydhY	-3.44	5×10^{-16}	-0.40	0.23	IM	Putative 4Fe-4S ferredoxin-type protein
gudP	-3.42	5×10^{-21}	-3.50	4×10^{-7}	IM	Galactarate/glucarate/glycerate transporter GudP
astE	-3.34	2×10^{-15}	-3.47	1×10^{-5}	C	Succinylglutamate desuccinylase
malE	-3.32	1×10^{-12}	-2.52	1×10^{-9}	IM, P	Maltose ABC transporter periplasmic–binding protein

[a]C, cytosol; P, periplasm; IM, inner membrane; OM, outer membrane.

TABLE 3 Top 20 genes with increased RPF levels at pH 4.4 compared to pH 7.6, sorted in descending order by Ribo-Seq $\log_2$ FC values[a]

Gene name	$\log_2$ FC (Ribo)	P-adjust (Ribo)	$\log_2$ FC (RNA)	P-adjust (RNA)	Cellular location	Annotation
asr	9.68	1×10^{-214}	9.37	2×10^{-52}	P	Acid shock protein
cadA	9.40	8×10^{-186}	9.39	5×10^{-92}	C	Lysine decarboxylase 1
ydgU	9.22	5×10^{-35}	12.39	3×10^{-16}	IM	Uncharacterized protein YdgU
yhcN	8.32	5×10^{-125}	9.08	3×10^{-94}	P	DUF1471 domain-containing stress-induced protein YhcN
bdm	7.85	4×10^{-71}	7.62	1×10^{-47}	C	Biofilm-dependent modulation protein
cadB	7.78	2×10^{-108}	8.90	2×10^{-51}	IM	Lysine:cadaverine antiporter
yjcB	7.73	4×10^{-45}	5.92	6×10^{-23}	IM	Uncharacterized protein YjcB
mdtJ	7.45	2×10^{-58}	4.62	1×10^{-23}	IM	Multidrug/spermidine efflux pump membrane subunit MdtJ
bhsA	7.12	3×10^{-110}	7.96	2×10^{-54}	OM	DUF1471 domain-containing multiple stress resistance outer membrane protein BhsA
glpD	7.06	2×10^{-122}	6.48	8×10^{-48}	C, IM	Aerobic glycerol 3-phosphate dehydrogenase
azuC	6.83	5×10^{-24}	8.17	1×10^{-26}	IM	Uncharacterized protein AzuC
nrdH	6.60	2×10^{-30}	6.46	1×10^{-29}	C	Glutaredoxin-like protein
yedR	6.35	2×10^{-21}	5.30	8×10^{-20}	IM	Putative inner membrane protein
yhdV	6.18	4×10^{-35}	6.43	3×10^{-18}	IM	Lipoprotein YhdV
nrdI	6.12	3×10^{-42}	6.03	3×10^{-28}	C	Dimanganese-tyrosyl radical cofactor maintenance flavodoxin NrdI
ybiJ	5.94	6×10^{-54}	4.56	2×10^{-21}	P	DUF1471 domain-containing protein YbiJ
fhuF	5.92	9×10^{-38}	4.55	8×10^{-21}	C	Hydroxamate siderophore iron reductase
ycgZ	5.73	3×10^{-50}	5.04	6×10^{-19}	C	Putative two-component system connector protein YcgZ
mdtI	5.72	7×10^{-31}	4.25	7×10^{-18}	IM	Multidrug/spermidine efflux pump membrane subunit MdtI
ycfJ	5.15	7×10^{-48}	5.03	1×10^{-17}	IM	PF05433 family protein YcfJ

[a]C, cytosol; P, periplasm; IM, inner membrane; OM, outer membrane.

act as an aggregase in order to prevent aggregation of proteins with positive charges (67). Our Ribo-seq data clearly illustrate the enormous importance of Asr under severe acid stress in *E. coli*, as it is one of the most abundant proteins in the cell, with approximately 2% of all reads mapping in the *asr* coding region at pH 4.4 (corresponding to an ~1,000-fold upregulation compared to pH 7.6). Strikingly, almost half of the top 20 genes with increased ribosome coverage of transcripts (*ydgU, yhcN, yjcB, yedR, yhdV, ybiJ, ycgZ,* and *ycfJ*) are poorly characterized (Table 3). So far, only YhcN from the above list has been shown to be involved in the response to acid stress (68).

GSEA for biological processes identified the GO terms "enterobactin biosynthetic process," "ferric-enterobactin import into cell," "siderophore-dependent iron import into cell," and "siderophore transmembrane transport" as significantly enriched at pH 4.4 (Fig. 3). Specifically, the complete enterobactin biosynthesis pathway, comprising the *entCEBAH* operon, *entF, entH,* and *ybdZ,* revealed significant enrichment under severe acidic conditions (Table S5). Furthermore, all subunits of the Ton complex (*tonB, exbB, exbD*) and its putative outer membrane receptor encoded by *yncD* exhibited significantly higher RPF and mRNA levels at pH 5.8 and pH 4.4 (Table S5). The Ton complex functions as a proton motive force-dependent molecular motor that facilitates the import of iron-bound siderophores (69, 70). Several other iron uptake systems, including a ferric dicitrate ABC transport system (*fecABCDE*), an iron (III) hydroxamate ABC transport system (*fhuACDB*), a ferric enterobactin ABC transport system (*fepA, fepB, fepCGD*), and a TonB-dependent iron-catecholate outer membrane transporter (*cirA*), were also induced under acidic conditions (Table S5). Moreover, the GO terms "protein maturation by iron-sulfur cluster assembly" and "iron-sulfur cluster assembly" were enriched at pH 4.4

(Fig. 3). Specifically, we detected a fivefold upregulation of all genes of the *isc* and *suf* operons (Table S5), which encode components of the complex machinery responsible for iron-sulfur cluster assembly in *E. coli* (71). In contrast, heme transport was among the most downregulated biological processes at both pH 5.8 and 4.4 (Fig. 3), which could potentially be the cause of iron limitation. Moreover, at low pH, the solubility of iron ions increases, which can destabilize iron-sulfur clusters (72). The iron limitation would be consistent with our data that *E. coli* upregulates the synthesis of iron-chelating sidero-phores and their transporters, as well as the components of the iron-sulfur assembly machinery. Given the better solubility of iron in a low pH environment, the question arises whether *E. coli* synthesizes siderophores to respond to iron limitation, or rather, protects itself against an iron excess. The latter function has been demonstrated for *Pseudomonas aeruginosa*, where siderophores protected cells from the harmful effects of reactive oxygen species. In this case, *P. aeruginosa* no longer secreted siderophores into the extracellular environment but instead stored them intracellularly (73). In conclusion, these results prompt the question of whether the upregulation of the iron uptake machinery counteracts iron limitation or rather provides protection against iron excess under severe acid stress.

We also detected a significant enrichment for the GO terms "cellular response to acidic pH," "stress response to copper ion," and "copper ion transmembrane transport" at pH 4.4 (Fig. 3). These results are in line with previous studies that have suggested an interplay between resistance to copper and acid stress in *Escherichia coli* (74, 75). This overlap between the two stress responses is further emphasized by our findings because at pH 4.4, substantial upregulation of the Cu^+-exporting ATPase CopA and CusA, a component of the copper efflux system, was detected (Table S5). These results are of important physiological relevance, given that copper is an important antibacterial component in the innate immune system (76, 77).

Among the downregulated genes at pH 4.4, the *tnaAB* operon and its leader peptide (*tnaC*) showed the most significant decrease in terms of RPF counts (Table 4). *tnaA* encodes a tryptophanase, which cleaves L-tryptophan into indole, pyruvate, and NH_4^+, whereas *tnaB* encodes a tryptophan:H^+ symporter (78). This finding is particularly intriguing because, in a previous study, persister cell formation in *E. coli* was related to a lower cytoplasmic pH associated with tryptophan metabolism (79). It is important to note that we also detected a substantial upregulation in RPFs for *hipA* (Table S5), which encodes a serine/threonine kinase that plays a role in persistence in *E. coli* (80). Therefore, our data provide further evidence for the link between internal pH and persistence.

The expression of several outer membrane proteins and porins (*ompW*, *ompF*, *nmpC*, *lamB*) was also downregulated at pH 4.4 (Table 4). This observation is consistent with the extensive restructuring of the *E. coli* lipid bilayers to reduce membrane permeability and limit proton entry. Similar to pH 5.8, the majority of the 20 proteins with the most reduced RPF levels compared with physiological pH are membrane proteins (Table 4). Moreover, the GO term "ATP synthesis coupled proton transport" was significantly reduced at pH 4.4 (Fig. 3). This is explained by the reduction in RPF levels of genes encoding subunits of the F_OF_1-ATPase (Table S5). F_OF_1-ATPase uses the electrochemical gradient of protons to synthesize adenosine 5′-triphosphate (ATP) from ADP and inorganic phosphate but can also hydrolyze ATP to pump protons out of the cytoplasm (81, 82). As at pH 5.8, the most downregulated biological processes at pH 4.4 were almost exclusively GO terms related to transport and cellular metabolism (Fig. 3).

In summary, the response of *E. coli* to severe acid stress is dominated by the activation of survival strategies that limit the entry of protons into the cell, prevent protein aggregation, and maintain iron homeostasis. Severe acid stress leads to a reduction in metabolic, transcriptional, and translational activity, thereby preparing *E. coli* for a dormant state. Eventually, these dormant cells may be able to withstand antibiotic attack (i.e., persister cells).

TABLE 4 Top 20 genes with decreased RPF levels at pH 4.4 compared to pH 7.6, sorted in ascending order by Ribo-Seq $\log_2$ FC values[a]

Gene name	$\log_2$ FC (Ribo)	P-adjust (Ribo)	$\log_2$ FC (RNA)	P-adjust (RNA)	Cellular location	Annotation
tnaC	-6.82	4×10^{-39}	-6.18	5×10^{-27}	C	tnaAB operon leader peptide
tnaA	-6.53	6×10^{-7}	-6.15	1×10^{-34}	C	Tryptophanase
tnaB	-6.40	6×10^{-47}	-5.40	6×10^{-26}	IM	Tryptophan:H$^+$ symporter TnaB
mglB	-6.31	7×10^{-51}	-4.08	1×10^{-17}	IM, P	D-galactose/methyl-galactoside ABC transporter periplasmic–binding protein
ompW	-6.21	1×10^{-39}	-4.83	2×10^{-13}	OM	Outer membrane protein W
nmpC	-6.05	3×10^{-62}	-3.87	1×10^{-17}	OM	DLP12 prophage; putative outer membrane porin NmpC
treB	-5.94	3×10^{-62}	-4.57	3×10^{-26}	IM	Trehalose-specific PTS enzyme IIBC component
borD	-5.92	1×10^{-25}	-3.36	5×10^{-9}	IM	DLP12 prophage; prophage lipoprotein BorD
garP	-5.86	4×10^{-47}	-5.10	1×10^{-16}	IM	Galactarate/glucarate/glycerate transporter GarP
tdcA	-5.84	9×10^{-32}	-1.27	4×10^{-3}	C	DNA-binding transcriptional activator TdcA
lamB	-5.79	2×10^{-54}	-2.60	4×10^{-8}	OM	Maltose outer membrane channel/phage lambda receptor protein
malK	-5.65	2×10^{-70}	-2.14	7×10^{-5}	IM	Maltose ABC transporter ATP-binding subunit
ompF	-5.59	1×10^{-63}	-4.03	6×10^{-20}	OM	Outer membrane porin F
garD	-5.54	3×10^{-42}	-3.12	2×10^{-6}	C	Galactarate dehydratase
nrfB	-5.47	4×10^{-24}	-2.36	1×10^{-3}	P	Periplasmic nitrite reductase penta-heme c-type cytochrome
xylF	-5.46	3×10^{-22}	-4.54	3×10^{-16}	IM, P	Xylose ABC transporter periplasmic–binding protein
malM	-5.38	3×10^{-65}	-1.93	7×10^{-5}	P	Maltose regulon periplasmic protein
treC	-5.38	1×10^{-44}	-4.47	3×10^{-23}	C	Trehalose-6-phosphate hydrolase
malE	-5.31	8×10^{-35}	-2.53	2×10^{-8}	IM, P	Maltose ABC transporter periplasmic–binding protein
ydeN	-5.31	2×10^{-49}	-5.05	5×10^{-30}	P	Putative sulfatase

[a]C, cytosol; P, periplasm; IM, inner membrane; OM, outer membrane.

Expanding the regulatory network of enzyme-based H⁺-consuming acid resistance systems

Recently, we have shown that the Adi and Cad AR systems are mutually exclusively activated in individual *E. coli* cells, indicating functional diversification and division of labor under acid stress (15). To gain further insights into the fine-tuned regulation of the three major AR systems, we first studied the mRNA and RPF levels of known enzyme-based H⁺-consuming AR components. The core components of the Gad system (AR2) (*gadA*, *gadB*, and *gadC*) and several transcriptional components (*gadW*, *gadX*, *gadY*, *phoP*, *phoQ*) showed an increase in mRNA and RPF levels by approximately two- to sixfold at pH 4.4, but not at pH 5.8, whereas the expression of *ydeO* was massively induced at pH 5.8 (particularly at the mRNA level), and RPF levels were decreased at pH 4.4 (Fig. S7). Expression of the core components of the Adi system (AR3), *adiA* and *adiC*, was induced at severe acid stress but not at pH 5.8, consistent with our previous study (15). Upregulation was not detected for regulatory components of the Adi system. A novel finding was that the levels of *adiA* but not *adiC* were significantly higher in the Ribo-Seq data than in the RNA-Seq data (Fig. S7). In fact, *adiA* had the sixth highest increase in TE among all *E. coli* genes at pH 4.4 (Table S4), indicating translational regulation by a thus far unknown mechanism. The only other component of an AR system in *E. coli*, known to be subject to translational regulation, is the major regulator CadC of the Cad (AR4) system. CadC contains a polyproline motif, and its translation therefore depends on the elongation factor P, a process that keeps the copy number of CadC extremely low (83). As expected, expression of the core components of the Cad system (AR4), *cadA* and *cadB*, was tremendously increased at both pH 5.8 and 4.4. Genes of the Orn system (AR5) were not induced in our experimental setup (Fig. S7).

Next, we analyzed the mRNA and RPF levels of all annotated TFs to search for other potential TFs involved in the acid stress response of *E. coli* (Fig. 4A and B). At pH 5.8, YdeO showed by far the strongest induction at the transcriptional and translational levels, but for all other TFs, the expression levels hardly changed (Fig. 4A). At pH 4.4, the expression of numerous TFs was induced, including GadW, YdcI, and the antibiotic resistance-controlling regulator MarR. The strongest upregulation was found for the IclR-type regulator MhpR and the iron-sulfur cluster-containing regulator IscR (Fig. 4B). Notably, while most acid-induced TFs were differentially expressed and displayed constant TE, YdcI exhibited constant mRNA levels but was differentially translated in response to acid stress (Fig. 4B). The contribution of all TFs with high FC values to survival under acid stress (Table S6) was tested in an acid shock assay. Cells of the corresponding knockout mutants (84) and, for comparison, the *rcsB* and *gadE* mutants (each lacking a TF important for acid resistance) were exposed to pH 3 for 1 h. All mutants except *marR* and *ydeO* showed significantly reduced survival compared to the parental strain (Fig. 4C). For *ydeO*, this result was consistent with our finding that transcript abundance and occupancy with ribosomes were upregulated at mild but not severe acid stress (Fig. 4; Fig. S7). Thus, YdeO appears to be only crucial under mild acid stress (Fig. 4A). In contrast, the *mhpR* mutant had a low survival rate comparable to that of *rcsB* and *gadE*, and the survival rates of the *iscR*, *ydcI*, and *gadW* mutants were only slightly higher (Fig. 4C). These results confirm the physiological relevance of these TFs for acid resistance. As controls, we re-introduced the corresponding genes *in trans* using isopropyl-β-D-thiogalactopyranosid (IPTG)-inducible pCA24N plasmids from the ASKA collection (85). Complementation of the *mhpR*, *iscR*, *ydcI*, and *gadW* mutants, as well as *rcsB* and *gadE* controls, resulted in strains with survival rates comparable to the wild-type (WT) strain carrying the pCA24N control vector (Fig. S8).

Subsequently, we tested whether these TFs are involved in the regulation and interconnectivity of the Gad, Adi, and Cad systems. Therefore, we examined the promoter activities of *gadBC*, *adiA*, and *cadBA* in the corresponding knockout mutants (84) using transcriptional reporter plasmids (promoter-*lux* fusions). The cultivation conditions were the same as those used for Ribo-Seq and RNA-Seq (Fig. 1A), and luciferase activity was monitored during growth in microtiter plates. We found that YdcI significantly
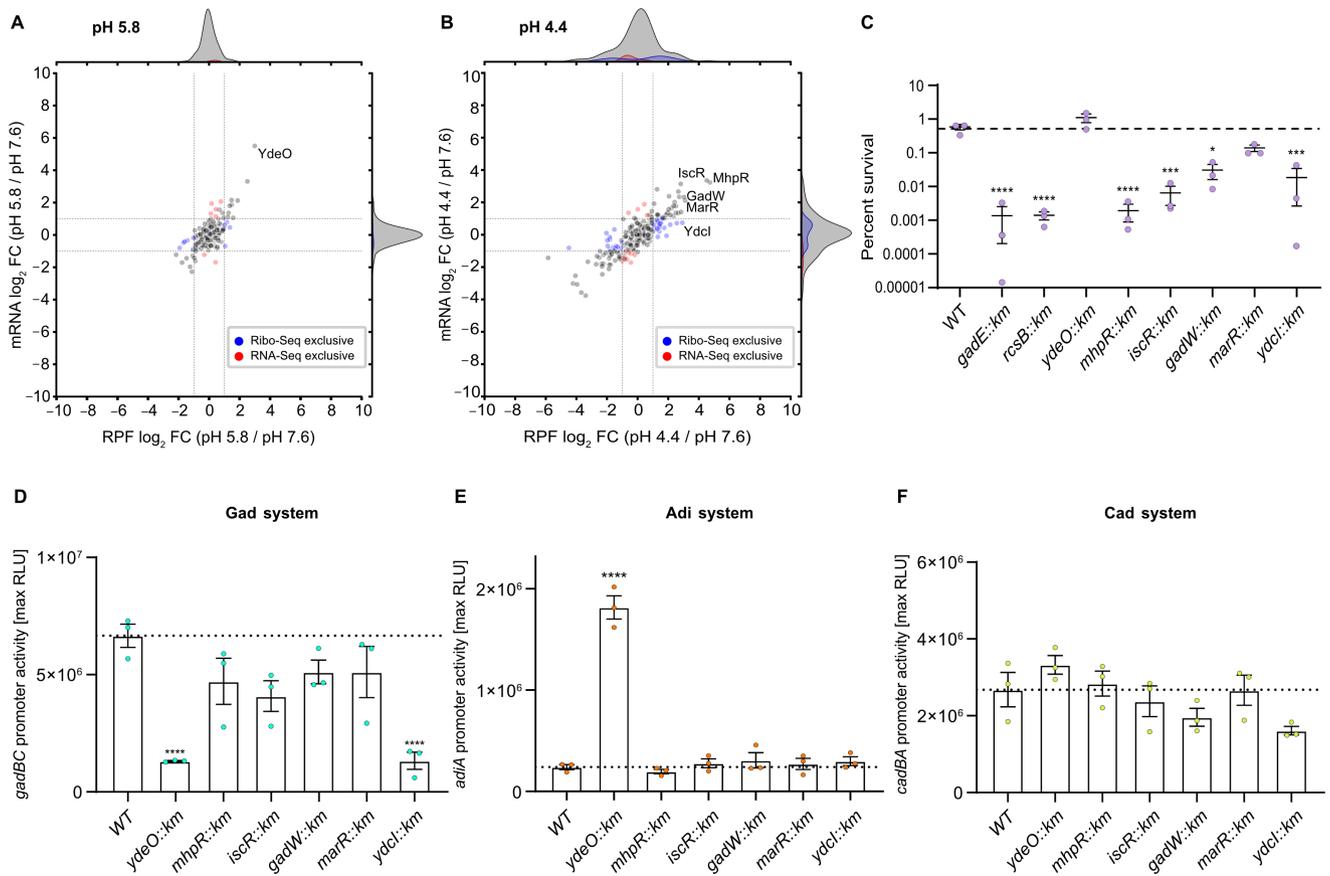
FIG 4 Contribution of TFs to survival and AR induction under acid stress. Comparison of global RPF and mRNA log$_2$ FC values of transcriptional regulators for (A) pH 5.8 vs pH 7.6 and (B) pH 4.4 vs pH 7.6. Dashed lines indicate log$_2$ fold changes of +1 or −1. Changes detected exclusively by either RNA-Seq (red dots) or Ribo-Seq (blue dots) are colored. TFs described in panels C-F are highlighted. (C) Acid shock assay to test the survival of *E. coli* BW25113 (WT) and the indicated mutants (84). Cells were grown in LB pH 7.6 to OD$_{600}$ = 0.5. The cultures were split and then either grown at pH 7.6 or stepwise stressed (15 min pH 5.8, 15 min pH 4.4) before being exposed to LB pH 3 for 1 h. Colony-forming units were counted, and the ratio of surviving cells was calculated. The dashed line indicates the average percentage of surviving WT cells. (D through F) Luciferase-based promoter assays. WT and the indicated mutants were transformed either with plasmid pBBR1-P*gadBC:lux* (D), pBBR1-P*adiA:lux* (E), or pBBR1-P*cadBA:lux* (F) and grown in LB medium (pH 7.6) until OD$_{600}$ = 0.5. The medium pH was then adjusted to 5.8 to induce the Cad system or to pH 4.4 to induce the Adi and Gad systems. Luminescence and growth were determined every 10 min in microtiter plates using a CLARIOstar plus plate reader (BMG Labtech). Data are reported as relative light units (RLUs) in counts per second per OD$_{600}$, with maximal RLU shown. Dashed lines indicate the average maximal RLU values of the WT. C-F, All experiments were performed in biological replicates (*n* = 3), and error bars represent standard deviations of the mean. Analysis of variance, followed by Bonferroni's multiple comparisons test, was used to compare log-transformed max. RLU values between mutant strains and the wild type (BW25113) (*P ≤ 0.05, ***P ≤ 0.001, ****P ≤ 0.0001).

affected the promotor activity of *gadBC* (Fig. 4D). Although the LysR-type regulator YdcI has been shown to affect pH stress regulation in *Salmonella enterica* serovar Typhimurium and *E. coli*, its precise role is still unclear (86–88). Based on the data presented here, we hypothesize that the decreased survival of the *ydcI* mutant under severe acid stress is due to decreased expression of the Gad system. The absence of YdeO resulted in an eightfold stimulation of the *adiA* promoter activity (Fig. 4E). Thus, YdeO not only activates the Gad system (89) but also appears to be a repressor for the Adi system. This implies that the Adi system is regulated not only by the XylS/AraC-type regulator AdiY but also by YdeO. Thus, YdeO is the first example of a transcriptional activator shown to be involved in the regulation of more than one AR system in *E. coli* and might play a role in the heterogeneous activation of the Adi and Gad systems within a population. Although we observed a slight decrease in *cadBA* promoter activity in the *ydcI* mutant, the decrease was not statistically significant. Therefore, none of the tested TFs affected the Cad system (Fig. 4F).

In conclusion, based on the differential expression data and lower survival of mutants during acid shock, we identified two novel TFs, namely, MhpR and IscR, which are crucial under severe acid stress (Fig. 4C), but are not associated with the Gad, Adi, and Cad systems (Fig. 4D through F). This implies that these regulators ensure the survival of *E. coli* in acidic habitats by inducing other defense mechanisms. Of particular interest is MhpR, which had the highest increase in RPFs of all TFs at pH 4.4 (Fig. 4B), and the corresponding mutant had the lowest survival at pH 3 (Fig. 4C). Further studies are needed to determine whether MhpR, which is a specific regulator of the *mhpABCDFE* operon-encoding enzymes for the degradation of phenylpropionate (90, 91), directly or indirectly contributes to acid resistance.

Differential expression of known and novel sORFs under mild and severe acid stress

In recent years, the annotation of many bacterial genomes has been extended by previously unknown small proteins (29), many of which are located in the membrane (92). This progress has been achieved primarily through the development of optimized detection strategies using adapted ribosome profiling and mass spectrometry protocols (28, 33, 93). Recently, additional sORFs were identified in *E. coli* using antibiotic-assisted Ribo-Seq, which captures initiating ribosomes at start codons (94, 95). Advanced detection strategies also revealed novel small proteins in other species, such as the archaeon *Haloferax volcanii*, the nitrogen-fixing plant symbiont *Sinorhizobium meliloti*, *Salmonella* Typhimurium, and *Staphylococcus aureus* (33–35, 96).

Among the previously known sORFs in *E. coli* K-12 and those discovered by Storz and colleagues (94), pH-dependent differential RPF levels were observed in our data sets for 12 and 29 small proteins at pH 5.8 and pH 4.4, respectively (Table S8). These findings validate the expression of these sORFs and highlight their physiological relevance in the acid stress response of *E. coli*. For example, induction of *mdtU*, an upstream ORF of *mdtJI*, was observed under mild acid stress (Fig. 5A) and corresponds to the observed upregulation of the multidrug/spermidine exporter MdtJI (Table 1). A previous study has shown that translation of MdtU is crucial for spermidine-mediated expression of the MdtJ subunit under spermidine supplementation at pH 9 (97). A similar mechanism could operate under acid stress conditions. The strongest induction of sORFs under severe acid stress was detected for *ydgU* (located in the same transcriptional unit as the acid shock protein-encoding gene *asr*) and *azuC* (Fig. 5B). AzuCR acts as a dual-function RNA and encodes a 28-amino acid protein, but it can also base pair as an sRNA (AzuR) with two target mRNAs, including *cadA* (98). AzuCR modulates carbon metabolism through interactions with the aerobic glycerol-3-phosphate dehydrogenase GlpD (98).

In addition to known sORFs, we aimed to uncover further hidden small proteins on the basis that our Ribo-Seq data were acquired under stress conditions to which *E. coli* is exposed in its natural habitat, the gastrointestinal tract. In particular, we searched for novel sORFs that remained undetected in previous Ribo-Seq approaches when *E. coli* was grown at a neutral pH.

Initial predictions for novel sORF candidates were acquired using the neural network-based prediction tool *DeepRibo* (99). All potential candidates were filtered based on coverage (rpkm >30 across all Ribo-Seq samples) and codon count [10–70 amino acids (aa)], with the exception of sORF15 (93 amino acids) (Table S7), which was manually discovered by inspecting the 3′ UTR of *gadW*. To further refine our search, we focused on sORF candidates that were significantly induced at either pH 5.8 or pH 4.4 (RPF $\log_2$ FC >2 and *P*-adjust <0.05) compared to pH 7.6. Predictions that overlapped with annotated genes on the same strand were excluded because Ribo-Seq signals were indistinguishable. This workflow yielded 152 candidates that were visually inspected using the web-based genome browser JBrowse2 (100). Candidates with continuous coverage across the predicted sORF, matching the ORF boundaries, and promising Shine-Dalgarno sequences were considered high-confidence candidates. In total, we identified 18 acid-induced sORF candidates (Table S7) that had not been previously detected. Of note, most of the
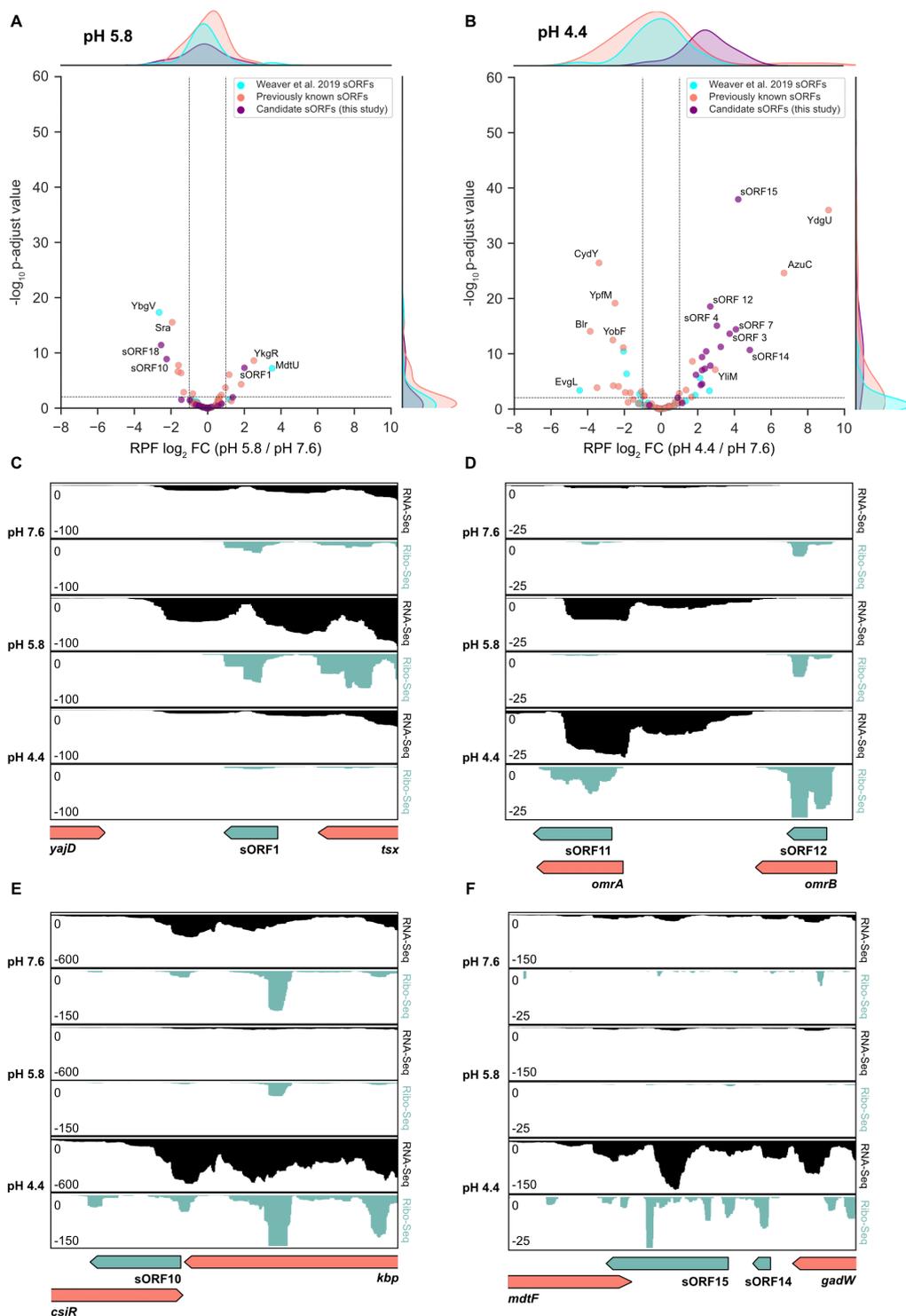
FIG 5 Differentially expressed sORF candidates upon exposure of *E. coli* to acid stress. Volcano plots illustrating differential RPF levels at (A) pH 5.8 vs 7.6 and (B) pH 4.4 vs 7.6 for sORF candidates identified in this study (Table S7), sORFs identified by Weaver *et al.* (94), and previously known sORFs. (C through F) JBrowse2 screenshots of read coverage from Ribo-Seq (green tracks) and RNA-Seq (black tracks) libraries at pH 7.6, pH 5.8, and pH 4.4. The schematic illustrations below indicate the genomic locations of enriched sORF candidates under acidic stress and adjacent annotated genes. Novel sORF candidates sORF1 (13 aa), sORF10 (38 aa), sORF11 (28 aa), sORF12 (11 aa), sORF14 (13 aa), and sORF15 (93 aa) are shown in green. aa, amino acids

candidates are encoded as part of operons or are located in the 3′ UTR of annotated genes. In addition, we detected one independent antisense sORF (sORF2 encoded antisense to *tesA*) and two upstream ORFs (leader peptides): sORF18, located upstream of the translation start site of the periplasmic chaperone encoding *osmY*, and sORF8, located close to the glucokinase-encoding gene *glk* (Table S7).

Of these 18 acid-induced candidate small proteins (Table S7), 17 had higher RPF counts at pH 4.4 than at pH 5.8. This suggests that the contribution of sORFs to acid defense in *E. coli* is more relevant under severe acid stress. Only sORF1 showed a higher expression level in cells exposed to mild acid stress (Fig. 5C). sORF1 is located in the 3′ UTR of *tsx*, which encodes a nucleoside-specific channel-forming protein. This finding is consistent with the observed increased requirement for nucleotides by *E. coli* at pH 5.8 (Fig. 3).

For the first time, we identified two sORF candidates located within genes encoding the redundant small regulatory RNAs OmrA and OmrB (Fig. 5D). *omrA* and *omrB* are highly identical at the 3′ and 5′ ends, differ mainly in their central parts, and regulate the expression of numerous outer membrane proteins (101). Our analysis suggests that both OmrA and OmrB act as dual-function RNAs under severe acid stress and encode small proteins: a 28-amino acid protein OmrA (sORF11) and an 11-amino acid protein OmrB (sORF12) (Fig. 5D). Due to the sequence variation in the central parts, the translation of OmrB ends at an earlier stop codon. Notably, both *omrA* and *omrB* displayed higher RPF levels at pH 4.4, whereas transcription of *omrA* but not *omrB* was induced at pH 4.4 (Fig. 5D). Thus, despite the high sequence similarity, *omrA* and *omrB* do not encode identical small proteins under severe acid stress and are differentially regulated at the transcriptional and translational levels. We also detected an acid-induced sORF candidate (sORF3) in *rybB* (Table S7), another sRNA involved in the regulation of outer membrane proteins (102). To our best knowledge, the presence of OmrA, OmrB, and RybB peptides has not yet been reported.

Three new candidate sORFs potentially involved in the regulation of AR systems were detected. sORF10 is located in the 3′ UTR of a potassium-binding protein encoded by *kbp* and encoded antisense to the transcriptional regulator CsiR (Fig. 5E). The latter might be involved in the regulation of the Adi system (15, 103). Given the significant upregulation of sORF10 at pH 4.4 and its complete complementarity to the 3′ end of the *csiR* mRNA, we hypothesize that sORF10 plays a role in fine-tuning the expression of the Adi system. Strikingly, we also discovered two high-confidence candidates for sORFs located in the relatively long 3′ UTR of GadW, one of the major transcriptional regulators of the Gad system (Fig. 5F). sORF14 and sORF15 exhibit constant coverage across the predicted ORF and contain Shine-Dalgarno sequences (Table S7). These results suggest that the complex Gad system may consist of even more components.

To gain further insight into the subcellular location and features of the newly identified sORF candidates, we used PSORTb (104) and DeepTMHMM (105) for transmembrane topology prediction. Notably, sORF15 is predicted to be located in the inner membrane and has two transmembrane helices, which were predicted with a probability of >90% (Fig. S9A). Additionally, the sORF15 protein structure prediction using Alpha-Fold2 (106) in Google Colab (ColabFold) (107) revealed a potential third helix toward the C-terminal end (Fig. S9B). Using blastp and tblastn (108), we found homologs of sORF15 with >80% identity in *Vibrio*, *Shigella*, *Klebsiella*, *Salmonella*, *Enterococcus*, and *Escherichia* (Fig. S9C) and identified homologs with at least 60% identity for approximately half of the other candidate sORFs (sORF2, 4, 5, 6, 7, 9, 10, 11, 16, and 18). These results strengthen confidence in the correct prediction of these sORFs. However, homologs in other species often only displayed partial matches and were almost exclusively annotated as "hypothetical proteins," as illustrated for sORF15 (Figure S9C). Moreover, we evaluated whether sORF15 is translated in the absence of the upstream gene *gadW*. A pBAD24-sORF15:3xFLAG plasmid, which harbors the native Shine-Dalgarno sequence of sORF15 (Table S11), and a FLAG-tagged version of sORF15 were constructed. sORF15

translation was successfully verified by Western blotting (Fig. S9D), which exemplifies that sORFs detected in this study yield detectable protein products.

In conclusion, we identified 18 high-confidence candidates for novel sORFs that are significantly induced upon exposure of *E. coli* to mild or severe acid stress.

Differentiation of the acid stress and general stress responses using autoencoder-based machine learning

In general, stress response mechanisms can be broadly classified into two categories: global stress responses and adaptations to specific types of stress. Global stress responses can be triggered by various stimuli and provide protection against multiple other unrelated stress factors (109). The global response often involves the activation of alternative sigma factors that affect hundreds of genes. In contrast, adaptations to specific types of stress are tailored to the specific stressor and involve a regulator that senses an environmental cue and modulates the expression of a set of genes, which counteract the stress (109, 110).

Given the large number of differentially regulated genes and pathways in response to acid stress (Fig. 2 and 3), we asked which of these adaptive mechanisms are acid-specific and which are also triggered by other stressors. In order to distinguish acid-specific and general stress responses, we used denoising autoencoders (DAEs), deep learning models designed for meaningful dimensionality reduction (111, 112). DAEs accomplish this by passing data through an encoder that compresses it into activations of a bottleneck layer (Fig. 6A1), with each node in the bottleneck layer interpretable as a coordinated expression program (113). For our analysis, we employed an ensemble of deep DAEs (see Materials and Methods) (113), trained on the *E. coli* K-12 PRECISE 2.0 compendium (114), augmented with additional stress conditions (115), as well as the acid stress conditions of the current study (Fig. 6A1). Using this method and data set, we have conducted a comparative analysis of the transcriptional response of *E. coli* to pH 4.4 and pH 5.8, contrasted against an extensive range of other stress conditions, including heat stress (116), ethanol stress (117), osmotic stress (118), oxidative stress (119, 120), low oxygen (LOX) (115), and exposure to sublethal concentrations of chloramphenicol (CAM) (115) and trimethoprim (TMP) (115).

To identify biological processes associated with a particular stress condition, we passed the associated RNA-seq data set into the encoder of each network and identified bottleneck nodes that were uniquely turned on by that data set (Fig. 6A2). We then manually turned on these nodes to generate gene sets that are associated with that condition, which can be further analyzed through GO term enrichment (Fig. 6A3 and 4). Using this procedure, we identified groups of nodes that uniquely turn on for acid stress conditions and turn off for all other stress conditions, as well as groups that are simultaneously on for both acid and one additional stress condition. We observed that there are many nodes that turn on simultaneously upon both acid and ethanol exposure (Fig. 6B). The overlap between acid stress and ethanol stress responses has been noted previously and can be explained by the fact that ethanol fluidizes the cytosolic membrane and increases the permeability for protons (121). Furthermore, there are indications of an overlap between acid and antibiotic stress (122, 123), reflected in the high number of acid + CAM activating nodes (Fig. 6B).

To pinpoint which cellular adaptations cause acid-specificity for the 48 and 91 specific bottleneck nodes at pH 5.8 and 4.4 (Fig. 6B), respectively, we conducted GSEA for biological processes on each of the gene sets associated with acid-specific node groups (see Materials and Methods). The GO terms that were significantly enriched in the highest number of both pH 5.8- and pH 4.4-specific upregulating node gene sets were "siderophore transmembrane transport," "response to cold," "bacterial-type flagellum assembly," and "chemotaxis" (Fig. 6C), reflecting our previous differential RNA-seq analysis (Fig. 3). The appearance of the GO term "response to cold" might be a result of the lack of cold stress in our compendium of stressors. Additionally, it should be noted that genes associated with this GO term include cold shock proteins, which may have
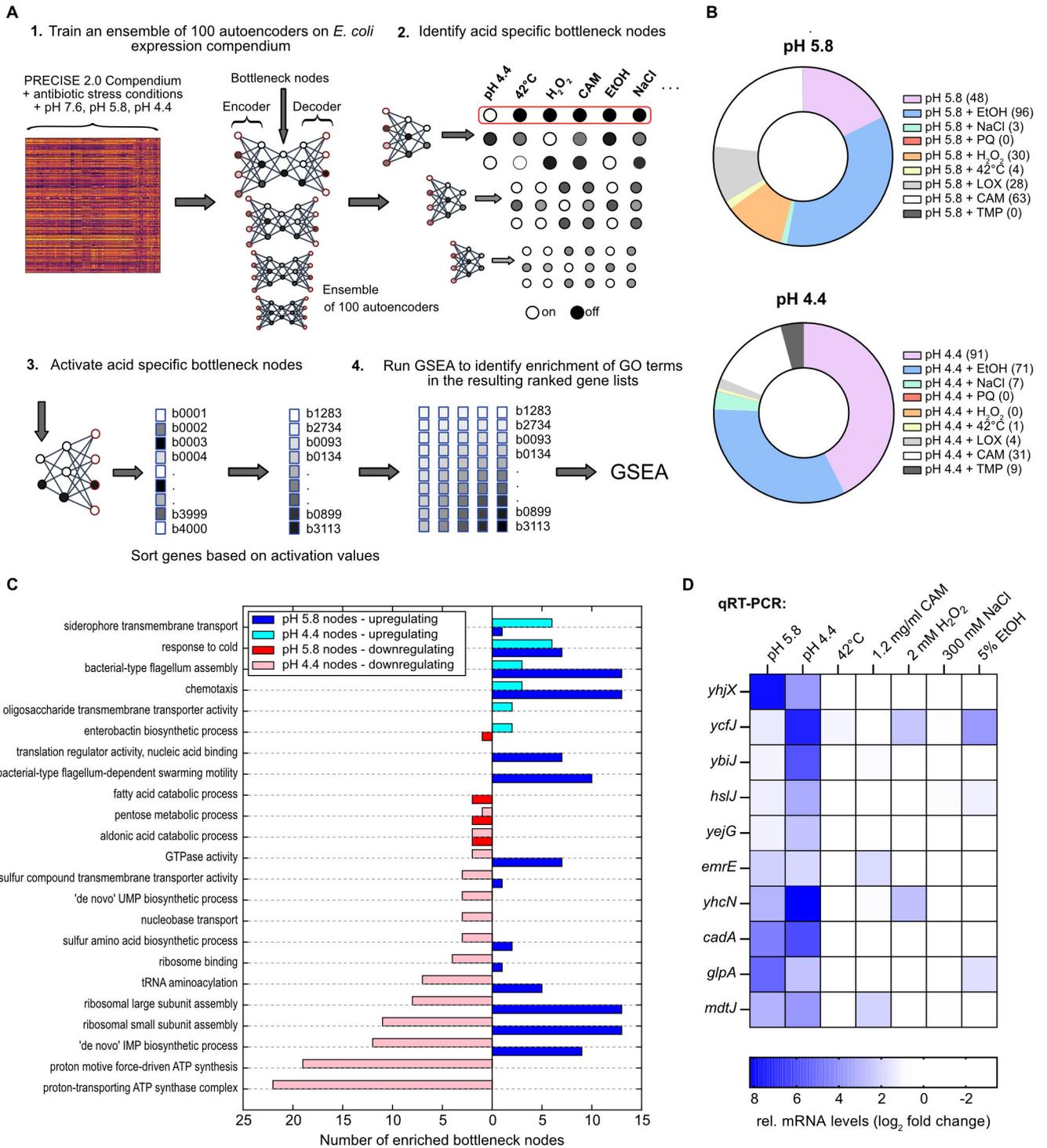
FIG 6 Differentiation between acid stress responses and general stress using autoencoders. (A) Schematic overview of the autoencoder ensemble training and subsequent bottleneck group identification pipeline. (B) Donut charts indicating the proportion of bottleneck nodes that turn on only under the specified conditions. Absolute numbers of specific bottleneck nodes are listed in brackets after each condition. (C) Significantly enriched GO terms associated with pH 4.4- and pH 5.8-specific nodes. Left-facing red bars indicate nodes that downregulate the corresponding GO term, while right-facing blue bars indicate upregulation. (D) Verification of acid stress-specific genes predicted by autoencoders. *E. coli* cells were either grown as indicated in Fig. 1A or exposed to heat (42°C), oxidative (H_2O_2), osmotic (NaCl), chloramphenicol (CAM), or ethanol (EtOH) stress. Total RNA was isolated, and relative mRNA levels were measured by RT-qPCR. Fold change values were determined relative to non-stress conditions and normalized using either *secA* or *recA* as reference genes. Standard deviations were calculated from three replicates ($n = 3$) and accounted for <10% of fold change values in all cases. PQ, paraquat; LOX, low oxygen; TMP, trimethoprim.

broader roles in the survival of stress conditions (124, 125), as well as several prophage genes and ribosome biogenesis factors. We found that mild acid stress turns on nodes, which correspond to gene sets associated with nucleotide and ribosome biosynthesis, including the GO terms "ribosome large subunit assembly," "ribosome small subunit assembly," and *"de novo* IMP biosynthetic process," while severe acid stress turns them off (Fig. 6C). These findings are consistent with our previous observations, namely, that *E. coli* induces nucleotide and ribosome biosynthesis to cope with mild acid stress but enters a metabolically inactive state under severe acid stress. The GO terms significantly affected in the highest number of acid-specific pH 4.4 downregulated bottleneck nodes were "proton motive force-driven ATP synthesis" and "proton-transporting ATP synthase complex" (Fig. 6C). These two GO terms exclusively involve genes encoding subunits of the F_OF_1 ATP synthase and can be considered paradigms for acid-specific adaptations since the F_OF_1 ATP synthase can also pump protons (126).

Considering that we detected a high number of genes induced by severe acid stress with unknown functions (Table 3) and lacking GO associations, we expanded our search for acid-specific adaptations from GO terms to single genes. In order to select acid-specific candidate genes, we investigated all genes associated with acid-specific bottleneck nodes and calculated the $\log_2$ FC between each acid stress and every other above-mentioned stress condition for these genes. Genes with the highest expression values under acidic conditions and $\log_2$ FCs of at least 0.5 for at least 95% of comparisons were then selected. This procedure yielded 10 candidate genes (Fig. 6D). To experimentally validate that these genes are indeed specifically upregulated under acid stress, we exposed *E. coli* to a variety of common stressors and performed qRT-PCR. *E. coli* cells were either grown under acid stress (Fig. 1A) or exposed to heat (42°C), oxidative (H_2O_2), osmotic (NaCl), antibiotic (chloramphenicol), or ethanol (EtOH) stress. For all investigated genes, the strongest upregulation was observed at either pH 4.4 or pH 5.8 relative to non-stress conditions (Fig. 6D), except for *ycfJ*, which was activated at pH 4.4 and under oxidative and ethanol stress. The remaining investigated genes were only upregulated under one other stress condition at most (Fig. 6D). Given that *emrE* and *mdtJ* encode multidrug exporters, the induction upon supplementation with sublethal concentrations of chloramphenicol is not surprising and further underscores the interplay between acid and antibiotic stress. The observed upregulation of *yhcN* under oxidative stress (Fig. 6D) was also reported previously (127). Nevertheless, we uncovered four *bona fide* examples of genes (*ybiJ*, *hslJ*, *yejG*, and *yhjX*) that displayed exclusive pH-dependent expression (Fig. 6D). Induction of *yhjX*, encoding a putative pyruvate transporter, might be related to the deamination of serine, which yields ammonia and pyruvate in uropathogenic *E. coli* (128). The precise molecular functions of YbiJ, YejG, and HslJ in the context of acid stress are currently unclear. These results highlight that our autoencoder pipeline is complementary to differential gene expression analysis, yielding biologically consistent results while also identifying expression patterns that uniquely discriminate acid stress from other stress responses.

Conclusions

Here, we present the first comprehensive study on the global transcriptome- and translatome-wide response of *E. coli* exposed to varying degrees of acid stress. Our investigation goes beyond previous research, which focused on comparing *E. coli* transcriptomes across different pH levels during growth (18, 21). Instead, we report on rapid changes occurring upon sudden pH shifts, which are relevant for bacteria such as *E. coli*, during passage of the gastrointestinal tract (129).

Using both Ribo- and RNA-Seq, we uncovered not only well-known acid defense mechanisms but also numerous previously undiscovered relevant genes and pathways to combat mild and severe acid stress (Fig. 7). The latter include siderophore production, glycerol-3-phosphate conversion, copper export, *de novo* nucleotide biosynthesis, and spermidine/multidrug export (Fig. 3 and 7). A striking number of membrane proteins and H$^+$-coupled transporters were found to be downregulated under both mild and
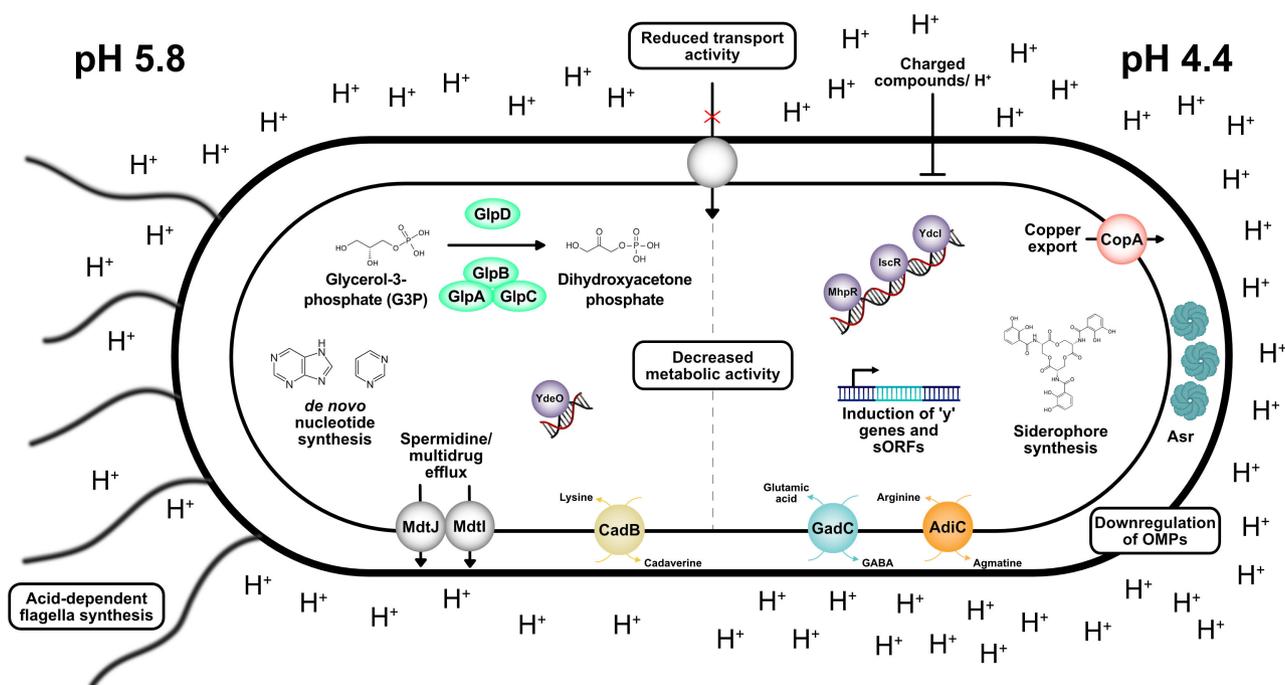
FIG 7 Overview of the fine-tuned response of *E. coli* to mild (pH 5.8) and severe (pH 4.4) acid stress. Acid stress counteracting mechanisms, including those revealed by Ribo-Seq and RNA-seq, are indicated.

severe acid stress (Fig. 7; Tables 2 and 4), underscoring the importance of the cytosolic membrane and its composition as a barrier for protons. Moreover, under severe stress, many outer membrane proteins were downregulated (Fig. 7; Table 4). Notably, a large proportion of genes with yet unknown functions were strongly induced, particularly under severe acid stress (Table 3). Our approach implies that exposing *E. coli* to culture conditions mimicking near-lethal habitats can offer valuable insights into the molecular functions of genes with low expression levels under standard growth conditions.

Our analysis revealed two new TFs, MhpR and IscR, involved in acid stress adaptation. Furthermore, we gained new insights into the role of the TFs YdeO and MarR. YdeO controls not only the transcription of genes in the Gad system but also *adiA* in the Adi system (Fig. 4), suggesting that YdeO connects the regulation of two AR systems in *E. coli*. The observed upregulation of MarR under acid stress, but the low contribution of this TF to acid resistance (Fig. 4B), may provide a link to antibiotic resistance and solvent stress tolerance in *E. coli* (130).

In addition to the pH-dependent differential expression levels of previously identified small proteins, such as YdgU, MdtU, and AzuC, we identified 18 high-confidence, not yet annotated, sORF candidates (Fig. 5). Of particular interest are sORF14 (13 amino acids) and sORF15 (93 amino acids), which are located in a transcriptional unit with *gadW* and *gadX*, suggesting their association with the Gad AR system and a potential involvement in glutamate transport and/or glutamate decarboxylation to gamma-aminobutyrate (GABA). Considering the predicted membrane location of sORF15 and its adjacent gene *mdtF*, an association with either the glutamate/GABA antiporter GadC and/or the multidrug efflux pump MdtF is conceivable.

The autoencoder-based comparison with other common stressors allowed us to distinguish acid stress-specific adaptations from general stress response programs (Fig. 6). Therefore, it was possible to differentiate between direct and indirect effects triggered by protonation and/or cellular damage. Considering the growing volume of next-generation sequence data, denoising autoencoders will be an increasingly important tool for interpreting future studies in the full context of accumulating RNA-seq data sets. Colonizing the intestinal tract is a complex process that includes not only rapid pH

changes but also alterations in oxygen and nutrient availability as well as competition with other bacteria. The ability of pathogenic *E. coli* strains to respond to such rapidly changing environments ensures their fitness advantage. We have shown here, for acid stress, the complexity of the regulatory network for ensuring survival and adaptation. The use of autoencoders, successfully tested here, could allow for the identification of physiological weak points associated with the survival of specific stresses. Targeting such weak points could lead to new classes of antibiotics or antivirulence treatments that take advantage of the unique expression patterns induced by natural stress conditions encountered in the host environment.

MATERIALS AND METHODS

Strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table S9. *E. coli* strains were cultivated in LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) and incubated aerobically in a rotary shaker at 37°C. When appropriate, media were supplemented with 15 µg/mL gentamycin, 100 µg/mL carbenicillin, or 50 µg/mL chloramphenicol.

For ribosome profiling and RNA-Seq experiments, the pH of the medium was adjusted at the indicated time points by the direct addition of 5 M HCl to the growing cultures (Fig. 1A).

For comparison with other stress conditions (heat stress, osmotic stress, oxidative stress, antibiotic stress, and ethanol stress), *E. coli* was initially grown in LB medium to $OD_{600} = 0.5$. Stress conditions were initiated either by moving flasks to a pre-heated 42°C incubator or by the addition of either H_2O_2 (2 mM), NaCl (300 mM), chloramphenicol [1.2 µg/mL (wt/vol)], or ethanol [5% (vol/vol)]. In all cases, samples were collected after 15 min of stress treatment.

Plasmid construction

Molecular methods were performed according to standard protocols or according to the manufacturer's instructions. Kits for the isolation of plasmids and the purification of PCR products were purchased from Süd-Laborbedarf. Enzymes and HiFi DNA Assembly Master Mix were purchased from New England Biolabs. To construct the reporter plasmid pBBR1-MCS5-P*gadBC:lux* (Table S11), 335 nt of the upstream region of *gadBC* were amplified by PCR using primers KSO-0131/KSO-0132 (Table S10) and MG1655 genomic DNA as a template. For the construction of the pBAD24-sORF15:3xFLAG plasmid (Table S11), the sORF15 coding region (excluding the stop codon) and an additional 15 nt upstream of the start codon (harboring the native Shine-Dalgarno sequence) were amplified using primers KSO-183/KSO-184 (Table S10) from MG1655 genomic DNA. After purification, promoter fragments were assembled into PCR-linearized pBBR1-MCS5 or pBAD24-3xFLAG vectors via Gibson assembly (131). The pCA24N-control plasmid was obtained by excising the *ydcI* insert from a pCA24N-*ydcI* vector using restriction enzymes XhoI and SalI (NEB) and subsequent ligation using T4 DNA Ligase (NEB). Correct insertions were verified by colony PCR and sequencing.

Sample collection for Ribo-Seq and RNA-Seq

Three sets of biological triplicates of MG1655 cells were inoculated to a starting OD_{600} of 0.05 from overnight cultures and grown to exponential phase ($OD_{600} = 0.5$) in 200 mL of unbuffered LB medium (pH 7.6). Two sets of cultures were adjusted to pH 5.8 by direct addition of 5 M HCl, while one set was further grown for 30 min at pH 7.6 (Fig. 1A). After 15 min, one set of biological triplicates was further adjusted from pH 5.8 to pH 4.4 by the addition of 5 M HCl, while the other cultures remained at pH 5.8 or pH 7.6, respectively (Fig. 1A). Cells were grown for another 15 min, samples were collected, and Ribo-Seq and RNA-Seq were performed as described below. pH values before and after pH shifts, as

well as final optical densities, were monitored throughout the experiment (TablesS1 and S2).

Ribosome profiling

Whole-culture flash freezing, cell lysis using a freezer mill, pelleting ribosomes over sucrose cushions, and ribosomal footprint isolation using a size selection gel were performed following the published protocol from Mohammad and Buskirk (40). MNase treatment, monosome recovery, RNA isolation, end-labelling by T4 polynucleotide kinase, and cDNA library construction were conducted by adapting the protocol from Latif and colleagues (39).

Briefly, 100 mL of liquid cultures and 10× lysis buffer [200 mM Tris pH 8, 1.5 M MgCl$_2$, 1 M NH$_4$Cl, 50 mM CaCl$_2$, 4% (vol/vol) Triton X-100, 1% (vol/vol) NP-40] were flash frozen in liquid nitrogen. For each sample, 90 g of frozen cells was mixed with 10 g of frozen 10× lysis buffer and lysed by cryogenic grinding in a SPEX SamplePrep 6875 Freezer/Mill (10 cycles, 10 Hz, 5 min precool, 1 min run, 1 min cool). The pulverized samples were thawed, and the lysate was pre-cleared by centrifugation (9,800 × g, 10 min, 4°C) in a Beckman Coulter Optima XE-90 Ultracentrifuge using a 50.2 Ti Rotor. The supernatant was used to pellet ribosomes over sucrose cushions by centrifugation in a Beckman Coulter Optima XE-90 Ultracentrifuge using a 70.1 Ti Rotor (330,000 × g, 1.5 h, 4°C). After resuspension of pellets in resuspension buffer (20 mM Tris pH 8, 15 mM MgCl$_2$, 100 mM NH$_4$Cl, 5 mM CaCl$_2$), nuclease digestion was performed using MNase (NEB) (2 h, 25°C). Monosomes were recovered using MicroSpin S-400 HR columns (GE Healthcare), and RNA was isolated using the miRNeasy Mini Kit (QIAGEN) in combination with the RNase-Free DNase Set (QIAGEN). The isolated RNA was loaded on a 15% TBE urea size selection gel, and after staining with SYBR Gold (Invitrogen), ribosomal footprints between 15 and 45 nt were excised from the gel. For elution of RNA, gel pieces were crushed by poking a hole with an 18 G needle in a 0.5-mL tube, placing it in a 1.5-mL tube, and subsequent centrifugation. RNA was recovered by precipitation after adding elution buffer (300 mM NaOAc pH 5.5, 1 mM EDTA pH 8) to the crushed gel, overnight incubation (4°C), and centrifugation in Corning-Costar Spin-X Centrifuge Tube Filters (20,000 × g, 3 min, room temperature). The isolation of RNA fragments corresponding to ribosomal footprints of 15–45 nt size was verified using the RNA 6000 Nano Kit (Agilent) and the Agilent 2100 Bioanalyzer. Then, 5′ phosphorylation of RNA fragments was achieved using T4 polynucleotide kinase (NEB) and ATP (NEB). After RNA recovery using an RNA MinElute Cleanup Kit (QIAGEN), footprint fragments were again evaluated using the Agilent 2100 Bioanalyzer as described above. cDNA libraries were constructed using the NEBNext Small RNA Library Prep Set for Illumina (NEB) with 14 PCR amplification cycles. cDNA library quality was assessed using a High Sensitivity DNA Kit (Agilent). cDNA libraries were purified using the QIAquick PCR Purification Kit (QIAGEN) and sequenced using a HiSeq 1500 machine (Illumina) in single-read mode with a 50-bp read length.

RNA-Seq analysis

After flash-freezing the cultures for Ribo-Seq, 6 mL of the remaining culture volume was mixed with 1.2 mL of Stop Mix solution [95% (vol/vol) ethanol, 5% (vol/vol) phenol] to terminate ongoing transcription and translation. Samples were frozen in liquid nitrogen and stored at −80°C until RNA isolation. Cells were pelleted (3,000 × g, 15 min, 4°C), and total RNA was isolated using the miRNeasy Mini Kit (QIAGEN) in combination with the RNase-Free DNase Set (QIAGEN). The integrity of RNA samples was evaluated using the RNA 6000 Nano Kit (Agilent) and the Agilent 2100 Bioanalyzer. RNA was quantified using the Qubit RNA HS Assay Kit (Invitrogen). Ribosomal RNA depletion was performed using the NEBNext rRNA Depletion Kit for bacteria (NEB), and directional cDNA libraries were prepared using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB). cDNA library quality was assessed using a High Sensitivity DNA Kit (Agilent). Finally,

cDNA libraries were sequenced using a NextSeq 1000 machine (Illumina) in single-read mode with a 60-bp read length.

Next-generation sequencing data analysis

E. coli Ribo-seq and RNA-seq raw sequencing libraries were processed and analyzed using the published HRIBO workflow (version 1.6.0) (37), which has previously been used for analysis of bacterial Ribo-seq data (35). HRIBO is a snakemake (132) workflow that downloads all required tools from Bioconda (133) and Singularity (134). All necessary processing steps are automatically determined by the workflow.

Adapter and quality trimming of the reads was performed using cutadapt (version 2.1) (135). The trimmed reads were then mapped against the *E. coli* K-12 MG1655 reference genome (ASM584v2) with segemehl (version 0.3.4) (136). Reads corresponding to ribosomal RNA (rRNA), transfer RNA (tRNA), and multi-mapping reads were removed with SAMtools (version 1.9) (137) using rRNA and tRNA annotations.

Quality control was performed by creating read count statistics for each processing step and RNA class with Subread featureCounts (1.6.3) (138). All processing steps were analyzed with FastQC (version 0.11.8) (139), and results were aggregated with MultiQC (version 1.7) (140). Additionally, a PCA was performed to determine whether the major source of variance in the data stems from the different experimental conditions. The PCA ensures the correctness of the downstream differential expression analysis. To generate the PCA plots, normalized read counts for all samples were generated using DESeq2 (141) normalization. To improve the clustering, a regularized log transform was applied to the normalized read counts. Standard deviations and variance were subsequently calculated using DESeq2, and the first three principle components were plotted using plotly (142).

Read coverage files were generated with HRIBO using different full read mapping approaches (global or centered) and single-nucleotide mapping strategies (5′ or 3′ end). Read coverage files were normalized using the counts per million (mil) normalization. For the mil normalization, read counts were normalized by the total number of mapped reads within the sample and scaled by a per-million factor.

Metagene analysis of ribosome density at start codons was performed as described previously (143). Here, annotated start codons of coding sequences are collected, and the density of the read coverage is determined for every position in a pre-determined window around the collected start codons.

A differential expression and translation analysis was performed using the tool deltaTE (43). The tool combines both Ribo-seq and RNA-seq data by calculating the TE of genes in order to capture changes in translational regulation when comparing different growth conditions.

Gene set enrichment analysis

GSEA was conducted using the tool *clusterProfiler* (52). To this end, the genome-wide annotation database for *E. coli* K-12 MG1655 (144) was used in combination with the results of the differential expression analysis. The analysis was focused on the biological process domain, omitting the cellular component and molecular function domains. Furthermore, the minimum gene set size was set to 5 and the maximum to 30. To avoid redundancy within the results, GO terms were filtered, and only terms at the bottom (lowest branch level) of the GO hierarchy were analyzed.

Detection and differential expression of novel acid-induced sORF candidates

sORF candidates were initially detected using the neural network *DeepRibo* (99). Summary statistics, including TE, rpkm, codon counts, and nucleotide and amino acid sequences, for annotated and potential novel sORFs were computed using HRIBO (version 1.6.0) (37). Moreover, GFF track files were created for detailed manual inspection using the web-based genome browser JBrowse2 (100).

DeepRibo was reported to produce high numbers of false positives (145). Therefore, the high number of initial predictions (>25,000) was reduced by introducing cut-off criteria based on sORF length and Ribo-Seq coverage. Predictions with average rpkm (reads per kilobase of transcript per million mapped reads) values <30 across all Ribo-Seq samples and outside of the codon count range of 10–70 amino acids were excluded from further analysis. To specifically detect sORFs, which are only detectable under acidic conditions and involved in the acid response of *E. coli*, the search was further restricted to predictions with deltaTE Ribo- and RNA $\log_2$ FC values of ≥2, in combination with *P*-adjust values of ≤0.05, at either pH 5.8 or pH 4.4, compared to pH 7.6. Additionally, *DeepRibo* predictions of overlapping annotated genes on the same strand were excluded. The remaining 152 candidates were manually inspected in JBrowse2, and novel sORFs were included in our final candidate list (Table S7) if the coverage was even over the predicted ORF, restricted to the ORF boundaries, and potential Shine-Dalgarno sequences were detectable. The remaining candidates were manually curated, and, in a few cases, alternative sORFs in the genomic vicinity of DeepRibo predictions were selected, which matched better to the Ribo-Seq coverage signal. Differential expression values, comparing pH 7.6, pH 5.8, and pH 4.4, for novel sORF candidates, previously known sORFs, and sORFs detected by Storz and colleagues (94), were calculated using *deltaTE* (43).

Homologs for novel sORF candidates were identified using blastp and tblastn (108) within the "BLAST at NCBI" function in the CLC Main Workbench v. 20.0.4 (QIAGEN). For tblastn, the following parameters were used: an *E*-value (expected value) ≤0.05, a seed length that initiates an alignment (word size) of 6, and the filter for low-complexity regions off. Potential cellular localization was evaluated with PSORTb v3.0.3 (https://www.psort.org/psortb/) (104), and transmembrane topology was assessed using DeepTMHMM v1.0.24 (105). The protein structure of sORF15 was predicted by running AlphaFold2 (106) via Google Colab (ColabFold v1.5.2) (107) with default settings.

RNA isolation and RT-qPCR analysis

RNA was isolated using the Quick-RNA Miniprep Kit (Zymo Research) or miRNeasy Mini Kit (QIAGEN) in combination with the RNase-Free DNase Set (QIAGEN) according to the manufacturer's instructions. Total RNA was DNase digested for 30 min at 37°C using 1 µL TURBO DNase (2 U/µL) (Invitrogen). A 500-ng aliquot of the isolated RNA was converted to cDNA with the iScript Advanced Kit (Bio-Rad) according to the manufacturer's instructions. Then, 1 µL of a 1:10 dilution in nuclease-free water of the cDNA samples was mixed with 5 µL of SsoAdvanced Univ SYBR Green Supermix (Bio-Rad) and 0.8 µL of 5 µM forward and reverse primers (Table S10). The total reaction volume was adjusted to 10 µL with nuclease-free water, dispensed in triplicates in a 96-well PCR plate (Bio-Rad), and subjected to qPCR in a Bio-Rad CFX real-time cycler. Evaluation of the obtained data were performed according to the ΔΔCt method (146), using *recA* or *secA* genes as internal references.

Acid shock assay

Acid resistance was determined based on previously described protocols (16, 147) with the following modifications: *E. coli* BW25113 cells were grown at 37°C in LB pH 7.6 to $OD_{600} = 0.5$, adjusted to $OD_{600} = 1$, and then shifted for 15 min each, first to LB pH 5.8 and then to LB pH 4.4. Then, cells were shifted to LB pH = 3 for 1 h at 37°C. As a control, cells were cultivated at pH 7.6 throughout the experiment. After 1 h at pH 7.6, or pH 3, samples were serially diluted in 1× phosphate-buffered saline (PBS) and plated on LB agar plates to count the number of colonies. Percent survival was calculated as the ratio of colony-forming units at pH 3 and pH 7.6. For complementation of mutants from the Keio collection (84), BW25113 strains were transformed with IPTG-inducible pCA24N plasmids from the ASKA collection (Table S11) (85). Acid shock survival rates were determined as described above, with the exception that media were supplemented with 50 µg/mL chloramphenicol and different concentrations of IPTG to mimic native

expression levels. pCA24N-control, *gadE*, and *gadW* vectors were induced with 100 µM IPTG, pCA24N-*rcsB*, and *iscR* with 10 µM IPTG, and pCA24N-*iscR*, *ydcI*, and *mhpR* with 1 µM IPTG.

Promoter activity assay

In vivo promoter activities of *gadBC*, *adiA*, and *cadBA* were determined using luminescence-based reporter plasmids harboring fusions of the respective promoter regions to the *luxCDABE* genes from *Photorhabdus luminescens*. BW25113 wild-type cells or corresponding mutants from the Keio collection (84) were transformed with plasmids pBBR1-P*gadBC:lux*, pBBR1-P*adiA:lux*, or pBBR1-P*cadBA:lux*. All strains were cultivated in LB medium supplemented with gentamycin overnight. The overnight cultures were inoculated to an OD_{600} of 0.05 in fresh LB medium (pH 7.6), aerobically cultivated until exponential phase ($OD_{600} = 0.5$), and shifted to LB pH 5.8. To assess the promoter activity of *adiA* and *gadBC*, the cultures were shifted again to LB pH 4.4 after 15 min of growth in LB pH 5.8. In the next step, the cells were transferred to a 96-well plate and aerobically cultivated at 37°C in LB medium at different pH values supplemented with gentamycin. Growth and bioluminescence were measured every 10 min in the microtiter plates using a CLARIOstar Plus plate reader (BMG Labtech). Data are reported as relative light units in counts per second of OD_{600}.

Autoencoder-based identification of acid-specific genes and biological processes

The denoising autoencoders in this study were implemented using the Python package Keras (148). Details of the various hyperparameter and network architecture choices were taken from reference (113). In brief, an ensemble of 100 DAEs with two hidden layers of 2,000 and 1,000 nodes between the input layer and the bottleneck was used. The bottleneck layer of each network consists of 50 nodes, as this was found to be within an optimal range for DAEs trained on the PRECISE 2.0 expression compendium (114) by Kion-Crosby and Barquist (113). All layers have sigmoid activation functions; the weights of each layer were randomly initialized based on the Glorot distribution, and the bias vectors were zeros. The weight matrices that make up the decoder of each network are tied together such that they consist of the transpose of the corresponding weight matrices of each encoder.

All networks were trained in each ensemble using the Adam optimization algorithm. Data corruption during training was employed to improve generalizability, such that 10% of the entries of each input data point were randomly set to zero during each training step. Additionally, early stopping was employed during training; the data were randomly portioned into an 80% training set and a 10% validation set, and training was stopped once the validation score began to worsen. A 10% test set was also portioned to determine the optimal training parameters. A local search over all training parameters, including the learning rate and batch size, was performed using the test score as a metric, and training was done with batch shuffling enabled.

For the training of the autoencoder ensemble, the PRECISE 2.0 compendium (114) was used, as well as six additional data points (115) representing *E. coli* K-12 MG1655 strains grown in M9 medium and treated with various antibiotics, in addition to the nine data points from the current study. All data were converted to log transcripts per million, and features were normalized after the train/validation/test split such that the expression of each gene was scaled between 0 and 1.

Following the procedure described by Kion-Crosby and Barquist (113), the encoder of each of the 100 trained networks was used to determine which nodes turn on for the specific stress conditions of interest (e.g., pH 4.4) and simultaneously turn off for alternative stress conditions (e.g., oxidative stress, heat stress, and ethanol). This was done by passing each data point into each encoder while observing the activations of nodes at the bottleneck layer. After identifying which nodes are specific to the stress condition of interest, each corresponding decoder was utilized to generate gene

expression predictions for all *E. coli* genes by activating each of these bottleneck nodes individually and propagating this signal through the decoder. After sorting all genes based on the average decoder output from all identified nodes, the top of this sorted vector was used to define a gene set associated with the condition of interest.

Additionally, GSEA was run on the outputs of each decoder for each node to determine which biological processes were associated with the condition of interest. Since these groups often consist of ~100 nodes and thousands of GO terms are evaluated, a conservative threshold for the adj. *P*-value of 0.0005 was set. The adj. *P*-value was found using the BH method, and each *P*-value was computed based on 1,000,000 permutations. Finally, for the selection of the high-confidence acid-specific gene candidates, first the log_2 FC between each acidic condition and every other stress condition was taken for all genes in the acid-specific gene sets. Genes corresponding to a log_2 FC of at least 0.5 for at least 95% of comparisons were then selected.

Propidium iodide viability staining

Cells were grown either at pH 7.6, 5.8, or 4.4 in the same manner as described for Ribo-Seq and RNA-Seq (Fig. 1A). As a positive control, cells were grown to an OD_{600} of 1 and subsequently heat-shocked for 5 min at 80°C. After cultivation, 1 mL of each culture was centrifuged (15,000 × *g*, room temperature) and washed with PBS. Propidium iodide (Invitrogen) was added with a final concentration of 2 µg/mL, followed by a 5-min incubation at room temperature in the dark to label dead cells. After another wash step using PBS, 2 µL of the culture was spotted on 1% (wt/vol) agarose pads, placed onto microscope slides, and covered with a coverslip. Microscopic images were taken using a Leica DMi8 inverted microscope equipped with a Leica DFC365 FX camera. An excitation wavelength of 546 nm and a 605-nm emission filter with a 75-nm bandwidth were used to detect fluorescence with an exposure of 500 ms, a gain of 5, and 100% intensity in the Leica LAS X 3.7.4 software.

To quantify the relative fluorescent intensities (RF) of single cells, phase contrast and fluorescent images were analyzed using the ImageJ (149) plugin for MicrobeJ (150). Default settings of MicrobeJ were used for cell segmentation (Fit shape, rod-shaped bacteria) apart from the following settings: area, 0.1-max $µm^2$; length, 1.2–5 µm; width, 0.1–1 µm; curvature, 0.–0.15; and angularity, 0.–0.25 for *E. coli* cells. In total, ≥1,000 cells were quantified per strain and condition, and the background of the agarose pad was subtracted from each cell per field of view. Cells with RF values ≥300 after subtraction of the background were considered dead.

Western blot analysis

To verify the translation of sORF15, *E. coli* MG1655 cells harboring a pBAD24-sORF15:3xFLAG plasmid were cultivated in LB medium. Once an OD_{600} of 0.5 was reached, expression of sORF15 was induced for 1 h by supplementation of a final concentration of 0.2% (vol/vol) L-arabinose. Next, culture aliquots normalized to an OD_{600} of 1 were collected by centrifugation (1 min, 13,000 × *g*, 4°C). Supernatants were removed, and pellets were resuspended in 100 µL of protein loading buffer [12% SDS (wt/vol), 30% glycerol (wt/vol), 0.05% Coomassie blue (wt/vol), 150 mM Tris-HCl pH 7]. Prior to loading, protein samples were denatured at 95°C for 5 min and then chilled on ice. Protein fractions were separated by Tricine-SDS-PAGE on a 16% gel containing 6 M urea (151). After separation, proteins were transferred onto a nitrocellulose membrane in a semidry blot chamber. The membrane was blocked for 1 h in 5% (wt/vol) skim milk in 1× TBS. After short washing with Tris-buffered saline with Tween (TBS-T), blots were hybridized with the primary antibody (α-FLAG, Invitrogen) for 1 h at room temperature. After three washing steps in TBS-T for 10 min each, membranes were incubated with an alkaline phosphatase-conjugated secondary antibody (α-rabbit, Rockland Immunochemicals) for 1 h at room temperature. After three washing steps conducted as described above, proteins were visualized using colorimetric detection of alkaline phosphatase activity with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride.

As a ladder, the PageRuler Prestained Protein Ladder (10–180 kDa, Thermo Fisher) was used.

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DATA AVAILABILITY

The raw sequencing data for both the Ribo-seq and RNA-seq were deposited at Gene Expression Omnibus (GEO) under the accession number GSE219022. A JBrowse2 (100) online genome browser instance containing annotation files and read coverage files of the processed data is available via the RIBOBASE (http://www.bioinf.uni-freiburg.de/ribo-base). Additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental material (mSystems01037-23-S0001.docx). Fig. S1 to S9; Tables S1, S2, and S6.
Supplemental resource table (mSystems01037-23-S0002.docx). Table of key resources.
Table S3 (mSystems01037-23-S0003.xlsx). Ribo-Seq and RNA-Seq exclusive genes.
Table S4 (mSystems01037-23-S0004.xlsx). Genes with differential translation efficiency under acid stress.
Table S5 (mSystems01037-23-S0005.xlsx). Complete HRIBO results.
Table S7 (mSystems01037-23-S0006.xlsx). Acid-induced novel candidate sORFs.
Table S8 (mSystems01037-23-S0007.xlsx). Differential expression of sORFs under acid stress.
Table S9 (mSystems01037-23-S0008.xlsx). *Escherichia coli* strains used in this study.
Table S10 (mSystems01037-23-S0009.xlsx). Oligonucleotides used in this study.
Table S11 (mSystems01037-23-S00010.xlsx). Plasmids used in this study.

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2.2 Motility-activating mutations upstream of *flhDC* reduce acid shock survival of *Escherichia coli*

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Motility-activating mutations upstream of *flhDC* reduce acid shock survival of *Escherichia coli*

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ABSTRACT Many neutralophilic bacterial species try to evade acid stress with an escape strategy, which is reflected in the increased expression of genes coding for flagellar components. Extremely acid-tolerant bacteria, such as *Escherichia coli*, survive the strong acid stress, e.g., in the stomach of vertebrates. Recently, we were able to show that the induction of motility genes in *E. coli* is strictly dependent on the degree of acid stress, i.e., they are induced under mild acid stress but not under severe acid stress. However, it was not known to what extent fine-tuned expression of motility genes is related to fitness and the ability to survive periods of acid shock. In this study, we demonstrate that the expression of FlhDC, the master regulator of flagellation, is inversely correlated with the acid shock survival of *E. coli*. We encountered this phenomenon when analyzing mutants from the Keio collection, in which the expression of *flhDC* was altered by an insertion sequence element. These results suggest a fitness trade-off between acid tolerance and motility.

IMPORTANCE *Escherichia coli* is extremely acid-resistant, which is crucial for survival in the gastrointestinal tract of vertebrates. Recently, we systematically studied the response of *E. coli* to mild and severe acidic conditions using Ribo-Seq and RNA-Seq. We found that motility genes are induced at pH 5.8 but not at pH 4.4, indicating stress-dependent synthesis of flagellar components. In this study, we demonstrate that motility-activating mutations upstream of *flhDC*, encoding the master regulator of flagella genes, reduce the ability of *E. coli* to survive periods of acid shock. Furthermore, we show an inverse correlation between motility and acid survival using a chromosomal isopropyl β-D-thio-galactopyranoside (IPTG)-inducible *flhDC* promoter and by sampling differentially motile subpopulations from swim agar plates. These results reveal a previously undiscovered trade-off between motility and acid tolerance and suggest a differentiation of *E. coli* into motile and acid-tolerant subpopulations, driven by the integration of insertion sequence elements.

KEYWORDS motility, flagella, acid resistance, acid shock survival, insertion sequences

B acteria colonize habitats such as the human stomach and acidic soils, which have a low pH (1, 2). In these environments, protective mechanisms against acidity are crucial to ensure the growth and/or survival of neutralophilic bacteria. Particularly, *Escherichia coli* exhibits substantial tolerance to acidity and has a corresponding infective dose, which is several orders of magnitude lower in comparison to other enteropathogens (3). Pathogenic *E. coli* strains, as well as non-pathogenic K-12 lab strains, survive at extremely low pH (<2.5) (4). This is crucial for pathogenic strains in order to endure the low pH of the stomach, which constitutes the major bactericidal barrier of the human gastrointestinal tract (5, 6). As a consequence, a multitude of defensive strategies have evolved in *E. coli* in order to adapt to such extreme conditions, including H+-consum-

ing acid resistance systems, acid shock proteins, restrained membrane permeability by altered fatty acid compositions, proton pumps, and many others (3, 7–9).

At moderate acid stress, many neutralophilic bacterial species use an escape strategy to avoid acidity, which is reflected by the increased expression of flagellar genes (10, 11). Accordingly, our RNA-Seq and Ribo-Seq data indicated that chemotaxis and motility genes were induced only under mildly acidic (pH 5.8) but not under severely acidic conditions (pH 4.4) (12). Most of the pH-affected motility genes belonged to the FlhDC regulon (13, 14). FlhD and FlhC form heterohexamers, and the complex acts as the master regulator of flagella synthesis and motility in *E. coli* (15–17). Genes regulated by FlhDC involve class II flagellar operons encoding basal body components, flagella export systems, the alternative sigma factor FliA, and non-flagellar operons (14, 18, 19). The *flhDC* operon is under the transcriptional control of a large number of transcription factors and small RNAs, which implement environmental cues such as osmolarity, synthesis of fimbriae, catabolite repression, and quorum sensing (20–23).

Notably, several motility-activating mutations in the promoter region of *flhDC* have been described. These include insertion sequence (IS) elements and point mutations, which disrupt the binding sites of transcriptional repressors like OmpR or LrhA (24–27). The presence of such mutations varies among various *E. coli* K-12 strains and affects motility rates due to consequently enhanced *flhDC* expression levels (24–27). Strikingly, motility-activating mutations in the *flhDC* regulatory region have also been detected in several single-gene knockout mutants of the Keio collection (28).

In our previous study, we noted significantly increased transcript and ribosome footprint levels for FliA and FlhDC at pH 5.8 (12). However, the highest increase among all annotated transcription factors in *E. coli* was found for the IclR-type regulator MhpR at pH 4.4 (12). Moreover, an *mhpR* mutant obtained from the Keio collection (29) showed the lowest survival rate among all tested knockout mutants of crucial acid resistance regulators under acid shock (12). MhpR regulates the expression of an operon involved in the degradation of cinnamic acid derivatives such as phenylpropionate (PP), 3-(2,3-dihy-droxyphenyl)propionate (DHPP), and 3-hydroxyphenylpropionate (3HPP) (30, 31). These aromatic compounds can bind individually or synergistically to MhpR and stimulate the interaction of MhpR with its operator sequence in the *mhpABCDFE* promoter region (32). Upon induction of the *mhpABCDFE* genes, 3-HPP and PP can be converted first to DHPP, which is ultimately degraded to intermediates of the citric acid cycle (30, 33). 3HPP and PP are commonly ingested through the uptake of plant material. For instance, several hydroxycinnamic acid derivatives are found to large extents in blueberries and huckleberries (34) and occur as secondary metabolites in other plants (35). However, the contribution of MhpR to acid resistance in *E. coli* is unclear.

In this study, we show that the reduced survival rate of an *mhpR* mutant from the Keio collection is caused by the integration of an IS5 element in the promoter region of *flhDC* and not by *mhpR* itself. RNA-Seq and soft agar swim assays confirmed that this mutant is hypermotile. The presence or absence of IS elements in *flhDC* promoter regions of different *E. coli* strains affected not only motility but also survival under acid shock. Using a strain with a chromosomal isopropyl β-D-thio-galactopyranoside (IPTG)-inducible *flhDC* promoter, we found an inverse correlation between motility and the ability to survive acid shock periods. These results indicate that induced expression of flagellar components, which is beneficial in mildly acidic environments, is detrimental for *E. coli* under severe acid stress and requires tight regulation of FlhDC target genes.

RESULTS

Examination of a putative role of MhpR under severe acid stress

We have previously detected enriched transcript and ribosome occupancy levels of *mhpR* in *E. coli* K-12 MG1655 at pH 4.4 (Fig. 1A) (12). To verify whether *mhpR* is upregulated under severe acid stress and to distinguish whether the regulation occurs at the transcriptional or post-transcriptional level, we examined *mhpR* mRNA levels and promoter activity under acid stress and non-stress conditions in *E. coli* MG1655. qRT-PCR
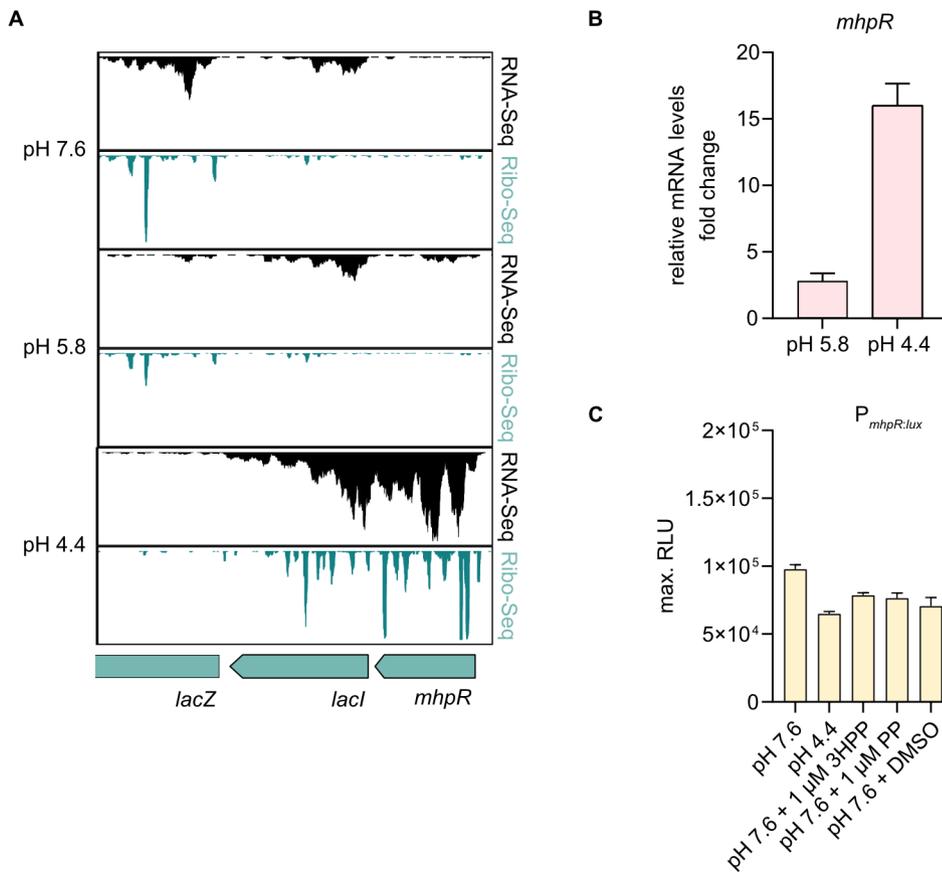
FIG 1 *mhpR* is post-transcriptionally upregulated under severe acid stress. (A) JBrowse2 (36) screenshots of read coverage from Ribo-Seq (green tracks) and RNA-Seq (black tracks) libraries at pH 7.6, 5.8, and 4.4. Schematic illustrations below indicate the genomic locations of *mhpR* and adjacent genes. Ribo- and RNA-Seq data were obtained from Schumacher et al. (12). (B) Verification of increased *mhpR* mRNA levels under acid stress using RT-qPCR. Cells were cultivated as described by Schumacher et al. (12). Fold change values were calculated relative to pH 7.6 and normalized using *secA* as a reference gene. Error bars indicate the standard deviation of three independent biological replicates (*n* = 3). (C) Luciferase-based promoter assay. *E. coli* MG1655 wild-type cells were transformed with the plasmid pBBR1-MCS5-P$_{mhpR:lux}$ and grown in LB medium (pH 7.6) until OD$_{600}$ = 0.5. The pH of the medium was then either adjusted stepwise to 5.8 and to pH 4.4, or 1 mM 3HPP, 1 mM PP, or dimethyl sulfoxide (DMSO) was added. Luminescence and growth were determined every 10 min in microtiter plates using a CLARIOstar plus plate reader (BMG Labtech). Data are reported as relative light units (RLUs) in counts per second per OD$_{600}$, with maximal RLU shown. All experiments were performed in biological replicates (*n* = 3), and error bars represent standard deviations of the mean.

analysis revealed that *mhpR* transcript levels increased only slightly at pH 5.8, but 15-fold at pH 4.4 compared to pH 7.6 (Fig. 1B).

Next, we constructed a luciferase-based promoter-activity reporter plasmid (pBBR1-MCS5-P$_{mhpR:lux}$). The *mhpR* promoter was constitutively active regardless of the extracellular pH and the presence of PP or 3HPP (Fig. 1C). This implies that the *mhpR* promoter is not affected by acidification, which is in line with previous findings (31). These results suggest that the upregulation of *mhpR* under severe acid stress is mediated by a post-transcriptional mechanism.

We then tested whether the described activation of a catabolic pathway for cinnamic acid derivatives (30–32) is related to MhpR under acid stress. However, this hypothesis could be neglected as we did not detect any activation of the *mhpABCDFE* promoter at pH 4.4 (Fig. S1). We also investigated whether cinnamic acid derivatives (3HPP, PP, and DHPP) are present in the LB medium and whether their abundance increases in a pH-dependent manner. For this purpose, we analyzed the LB medium and sterile-filtered
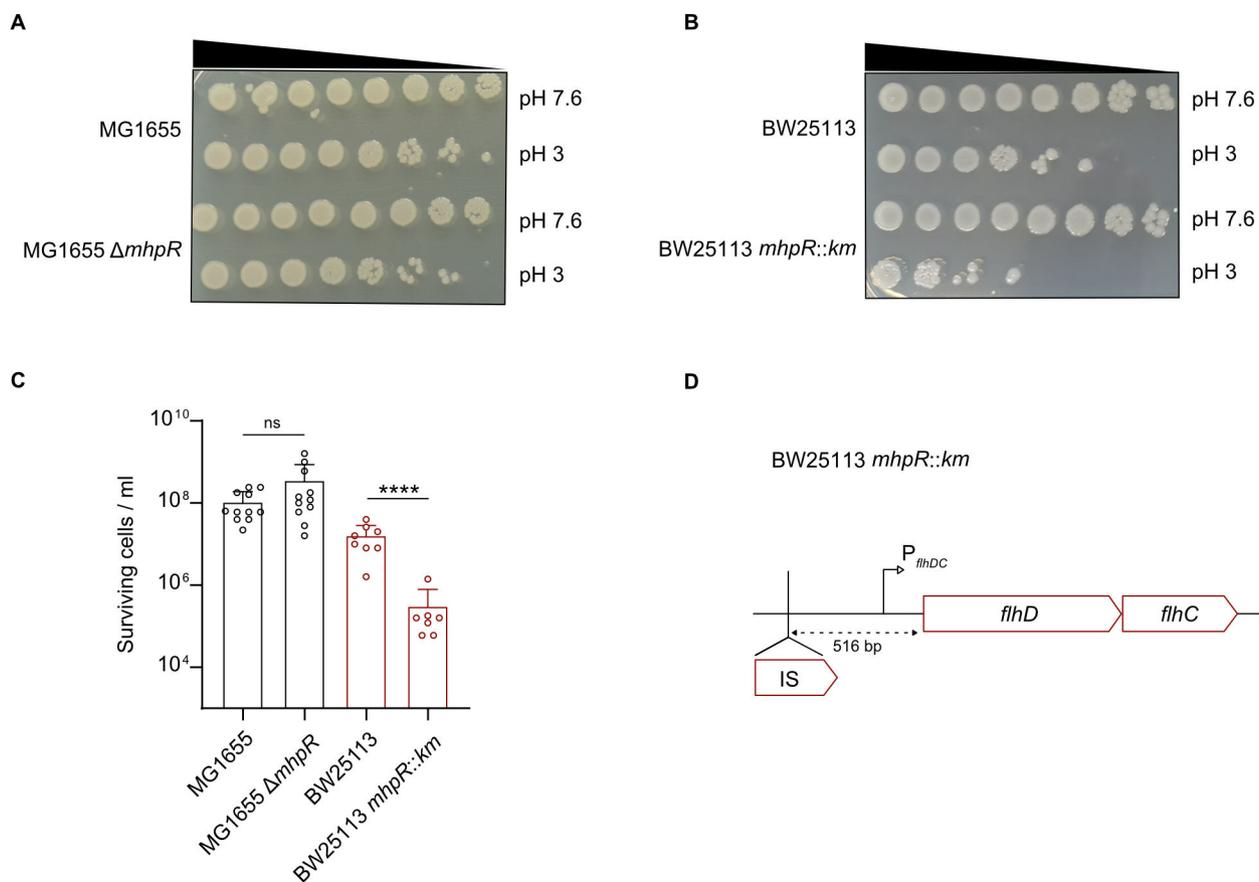
FIG 2 Survival under acid shock is exclusively affected in an *mhpR* mutant from the Keio collection. (A and B) Acid shock assays to evaluate the survival of *E. coli* MG1655 and MG1655 Δ*mhpR* (A) and BW25113 and BW25113 *mhpR::km* (B) at pH 3. Cells were grown in LB pH 7.6 to $OD_{600} = 0.5$ and cell numbers were adjusted to 10^9/mL. Cultures were split and then either grown at pH 7.6 or stepwise stressed (15 min pH 5.8 and 15 min pH 4.4) before being exposed to LB pH 3 for 1 h. Cultures were serially diluted by a factor of 10 in 1× PBS and plated on LB agar plates. Images were taken after overnight incubation. (C) Quantitative assessment of acid shock survival of *E. coli* MG1655 and MG1655 Δ*mhpR* as well as BW25113 and BW25113 *mhpR::km* strains. Cells were cultivated as described above, and the total number of colony-forming units at pH 3 was counted after overnight incubation. All experiments were performed in biological replicates ($n \geq 6$), and the error bars represent standard deviations of the mean. Significance was evaluated by performing a one-way ANOVA test followed by Bonferroni's multiple comparisons test to compare log-transformed numbers of surviving cells (ns, not significant and ****$P < 0.0001$). (D) Schematic representation of the genomic *flhDC* locus and the distance between the *flhD* start codon and the inserted IS5 element. The presence of the IS5 sequence was determined by colony PCR and sequencing.

supernatants of cultures grown at pH 7.6, 5.8, or 4.4 by LC-MS (see Materials and Methods for details). Neither PP, DHPP, nor 3HPP were detectable in any of the analyzed samples (data not shown). Thus, we conclude that both the enzymes responsible for 3HPP, PP degradation (MhpA-F) and the corresponding substrates for the pathway are not present in pH-neutral and acidified LB media. In summary, MhpR-induced catabolism of 3HPP and PP does not play a role under acid stress adaptation.

Phenotypic characterization of *E. coli* *mphR* mutants with different genetic backgrounds

Nevertheless, as previously shown (12) and reconfirmed here again, we observed a strong phenotype of *E. coli* BW25113 *mhpR::km*, a knockout mutant from the Keio collection in an acid shock experiment (Fig. 2B and C). Based on this result, which somewhat contradicted all the results obtained with *E. coli* MG1655 described above, we decided to construct an MG1655 Δ*mhpR* mutant. We performed the acid shock assay (survival of cells at pH 3 for 1 h) and did not detect any significant difference between the MG1655 wild type and the Δ*mhpR* mutant (Fig. 2A and C).

To determine why an acid shock phenotype associated with the knockout of *mhpR* is exclusively detectable in *E. coli* BW25113 but not in MG1655, we searched for strain-specific differences between the two strains. It is important to note that some BW25113 mutants from the Keio collection have IS elements or point mutations in the regulatory region upstream of *flhDC*, which cause upregulation of motility genes (28). Therefore, we sequenced the corresponding region of the BW25113 *mhpR::km* mutant and found an IS5 element in the *flhDC* promoter (Fig. S2). The IS5 element was integrated 516 bp upstream of the *flhDC* start codon (Fig. 2D).

Upregulation of motility and chemotaxis genes is not associated with MhpR

We then conducted an RNA-Seq experiment to elucidate whether differentially expressed genes (DEGs) are detectable in *mhpR* mutants or whether the IS5 insertion 516 bp upstream of the *flhDC* start codon (Fig. 2D) leads exclusively to the induction of chemotaxis and motility genes. To this end, we compared the transcriptomes of *E. coli* MG1655 Δ*mhpR* and BW25113 *mhpR::km* under severe acid stress conditions (pH 4.4) with the respective parental strains. We did not detect differentially expressed genes (fold change > 2, FDR-adjusted *P*-value < 0.01) between MG1655 and MG1655 Δ*mhpR,* with the exception of the deleted *mhpR* gene (Fig. 3A).

In contrast, 62 genes were differentially expressed in the *mhpR* mutant from the Keio collection (Fig. 3B). Almost all upregulated DEGs in BW25113 *mhpR::km* were related to motility and chemotaxis (Table 1) and are part of the FlhDC regulon (13, 14). This result indicates that the IS5 insertion upstream of *flhDC* leads to the induction of motility genes. The only DEGs in BW25113 *mhpR::km* that were not related to flagellar biosynthesis and chemotaxis were *mhpA* and *mhpB* (Table 1). However, it has already been described (37) that the *mhpC* gene in strain BW25113 contains an IS30 element, which leads to a strong expression of *mhpCDEF* and probably to the deregulation of the expression of *mhpAB* in the absence of *mhpR*.

To confirm the RNA-Seq results, we tested a representative selection of DEGs (Table 1) by RT-qPCR. As expected, genes encoding flagella components (*fliA*, *fliC*, *flgA*, *flhB*, and *motA*) and chemotaxis proteins (*cheA* and *tar*) showed enriched mRNA levels in BW25113 *mhpR::km* but not in MG1655 Δ*mhpR* (Fig. 3C). In parallel, we tested the motility of the two strains on soft agar [0.3% (wt/vol)] plates. In agreement with the RNA-Seq data (Fig. 3B), BW25113 *mhpR::km* showed significantly increased spreading (*P* < 0.001) compared to MG1655 Δ*mhpR* (Fig. 3D).

The presence or absence of an IS element upstream of *flhDC*, but not MhpR, determines survival under severe acid stress

To evaluate whether the presence of the motility-activating IS5 element is indeed the cause of reduced survival under severe acid stress, we removed the IS5 element from the *flhDC* promoter of BW25113 *mhpR::km*. We found that the strain obtained from the Keio collection (29) contained a mixture of cells with and without IS5 insertion in the *flhDC* regulatory region (Fig. S3). By re-streaking this strain several times on LB agar plates and screening individual colonies, we obtained a BW25113 *mhpR* mutant without IS5 insertion in the *flhDC* promoter (Fig. S3) and designated this strain as BW25113 *mhpR::km**. As expected, removal of the IS element from the *flhDC* regulatory region restored the motility of BW25113 back to wild-type levels (Fig. S4). Similar to MG1655 Δ*mhpR*, BW25113 *mhpR::km**, with the restored *flhDC* regulatory region, showed no differential acid shock survival compared to the wild type (Fig. 4A and B). Furthermore, we constructed another *mhpR* knockout in BW25113 via double homologous recombination, which also contains the native *flhDC* promoter locus (BW25113 Δ*mhpR*). This strain also showed no reduced survival in the acid shock assay (Fig. 4A and B). These findings indicate that survival under acid shock is not affected by MhpR but depends directly on the presence or absence of an IS element in the intergenic region between *flhDC* and *uspC*.
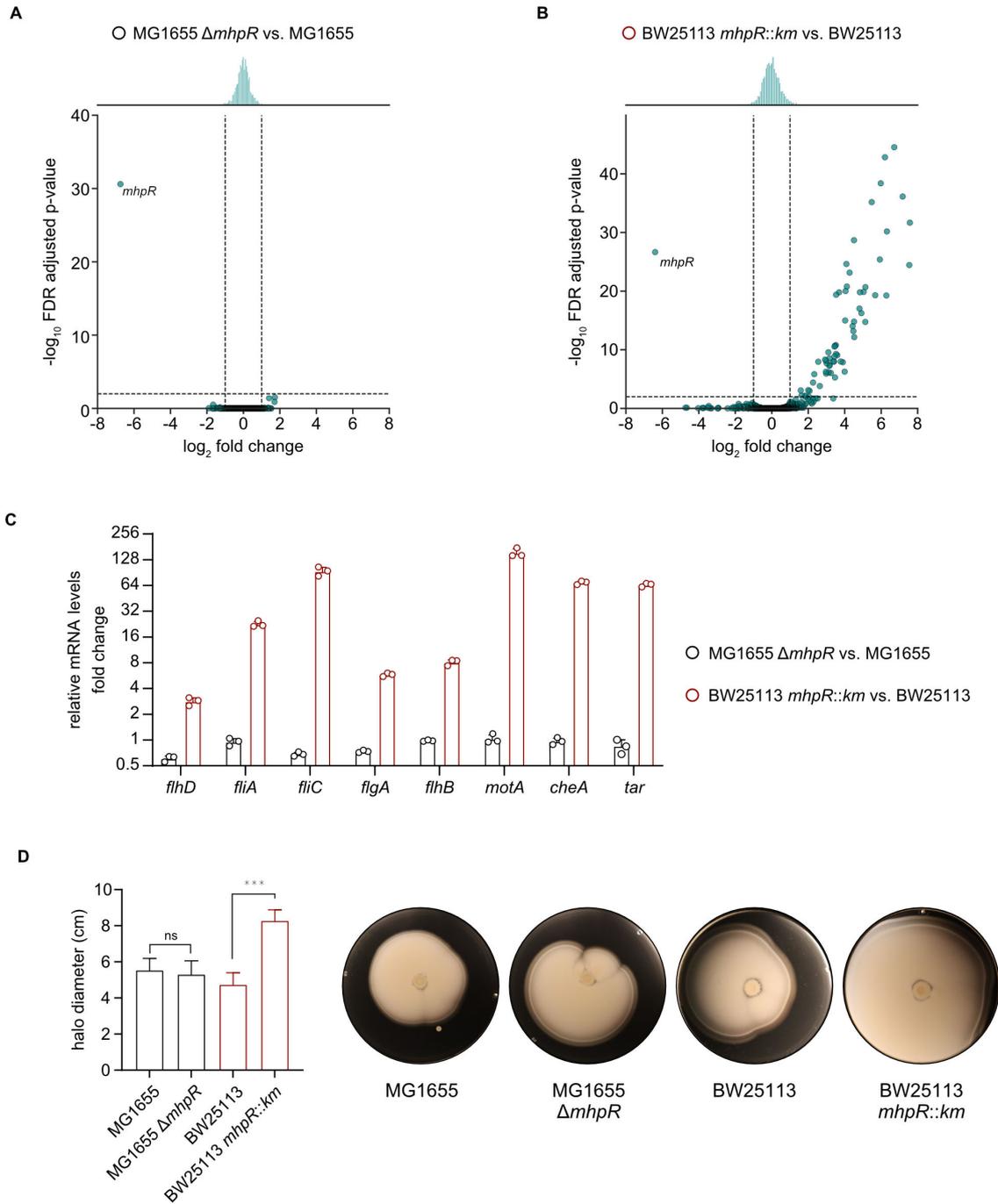
FIG 3 Motility and chemotaxis genes are upregulated in *E. coli* BW25113 *mhpR::km* but not in MG1655 Δ*mhpR*. (A and B) Volcano plots illustrating differential mRNA levels determined by RNA-Seq for MG1655 Δ*mhpR* compared to MG1655 (A) and BW25113 *mhpR::km* compared to BW25113 (B). Cells were grown to an OD_{600} of 0.5 in LB pH 7.6 before being shifted to LB pH 5.8 for 15 min and LB pH 4.4 for another 15 min. Dashed lines indicate log_2 fold change values of +1 or −1 and *P*-adjusted values of 0.01. (C) Verification of a selection of differentially expressed genes in BW25113 *mhpR::km* compared to BW25113 at pH 4.4 by RT-qPCR. Cells were cultivated as described above. Fold change values were calculated relative to MG1655 or BW25113 and normalized using *secA* as a reference gene. Error bars indicate the standard deviation of independent biological replicates ($n = 3$). (D) Soft agar swim assay to evaluate the strains described in panels A–C. Overnight cultures normalized to an OD_{600} of 1 were spotted on LB soft agar [0.3% (wt/vol)] plates and incubated for 16 h. Halo diameters were measured, and all experiments were performed in biological replicates ($n \geq 4$). Error bars represent standard deviations of the mean, and significance was evaluated by performing a one-way ANOVA test followed by Bonferroni's multiple comparisons test (ns, not significant and ***$P < 0.001$). Representative images are shown.

TABLE 1 Top 20 genes with increased mRNA levels in BW25113 *mhpR::km* compared to BW25113, sorted in descending order by RNA-Seq fold change values

Gene name	Fold change	*P*-adjust	Annotation
motA	190.27	2.12×10^{-32}	Motility protein A
tar	186.37	3.58×10^{-25}	Methyl-accepting chemotaxis protein
tap	144.98	7.40×10^{-37}	Methyl-accepting chemotaxis protein—dipeptide-sensing
motB	105.21	2.90×10^{-45}	Motility protein B
yhjH	79.58	6.55×10^{-31}	Cyclic di-GMP phosphodiesterase
fliC	77.91	5.53×10^{-20}	Flagellar filament structural protein
cheA	73.86	1.49×10^{-43}	Chemotaxis protein
mhpB	62.98	4.13×10^{-39}	3-Carboxyethylcatechol 2,3-dioxygenase
cheW	60.85	4.06×10^{-26}	Chemotaxis protein
cheB	51.00	5.08×10^{-20}	Protein-glutamate methylesterase/protein glutamine deamidase
mhpA	44.64	6.71×10^{-36}	3-(3-hydroxyphenyl)propanoate hydroxylase
flxA	35.15	2.11×10^{-21}	Qin prophage, PF14282 family protein
tsr	35.05	1.80×10^{-15}	Methyl-accepting chemotaxis protein—serine-sensing
fliS	32.82	1.38×10^{-20}	Flagellar biosynthesis protein
fliT	30.18	5.67×10^{-17}	Flagellar biosynthesis protein
cheY	28.51	1.58×10^{-20}	Chemotaxis protein
ycgR	28.03	9.35×10^{-18}	Flagellar brake protein
fliE	22.97	7.04×10^{-13}	Flagellar protein
yjcZ	22.81	1.59×10^{-15}	Regulator of diguanylate cyclase
fliD	22.77	2.06×10^{-29}	Flagellar filament capping protein

Considering that the removal of motility-activating insertions improved the survival of *E. coli* under severe acid stress, we wondered whether we could provoke the reverse phenomenon by introducing an IS element into a strain with a native *flhDC* promoter. Notably, variations in terms of the presence of IS elements upstream of *flhDC* have been observed in different *E. coli* K-12 MG1655 wild-type strains (25). As shown in Fig. S2, the MG1655 wild-type strain used in our laboratory does not harbor IS elements in the *flhDC* promoter and is correspondingly less motile (Fig. 3D). In contrast, another sequenced MG1655 wild-type version (CGSC 7740) contains an IS element in the *flhDC* promoter and is highly motile (25). We thus ordered this strain, detected the presence of the IS element (Fig. S5), and confirmed that motility is indeed significantly increased (Fig. S6). As expected, acid shock survival rates of MG1655 CGSC 7740 were significantly lower (Fig. 4C and D), suggesting that the IS insertion increased motility at the cost of acid tolerance.

Survival under severe acid stress and *flhDC* expression levels are inversely correlated

Due to the inherent capacity of IS elements for genomic transpositions, we cannot exclude the possibility of loss or gain of IS elements upstream of *flhDC* during our experimental procedures. To circumvent the issue of IS transposition and gain the ability to fine-tune *flhDC* expression levels over a wide range, we next used an MG1655 strain in which the regulatory *flhDC* region was chromosomally replaced by a synthetic IPTG-inducible P$_{tac}$ promoter (Fig. 5A) (38). This strain contains a *flu* deletion to minimize cell aggregation (38). Intriguingly, we observed an inverse correlation between the supplemented IPTG concentrations, i.e., FlhDC levels, and the number of cells that survived the acid shock (Fig. 5B). The addition of 10 µM IPTG corresponds to the native *flhDC* expression level (V. Sourjik, personal communication), and no decrease in survival after acid shock was observed under this condition (Fig. 5B and C). On the other hand, overexpression of *flhDC* as a result of the addition of 50 or 100 µM IPTG led to a
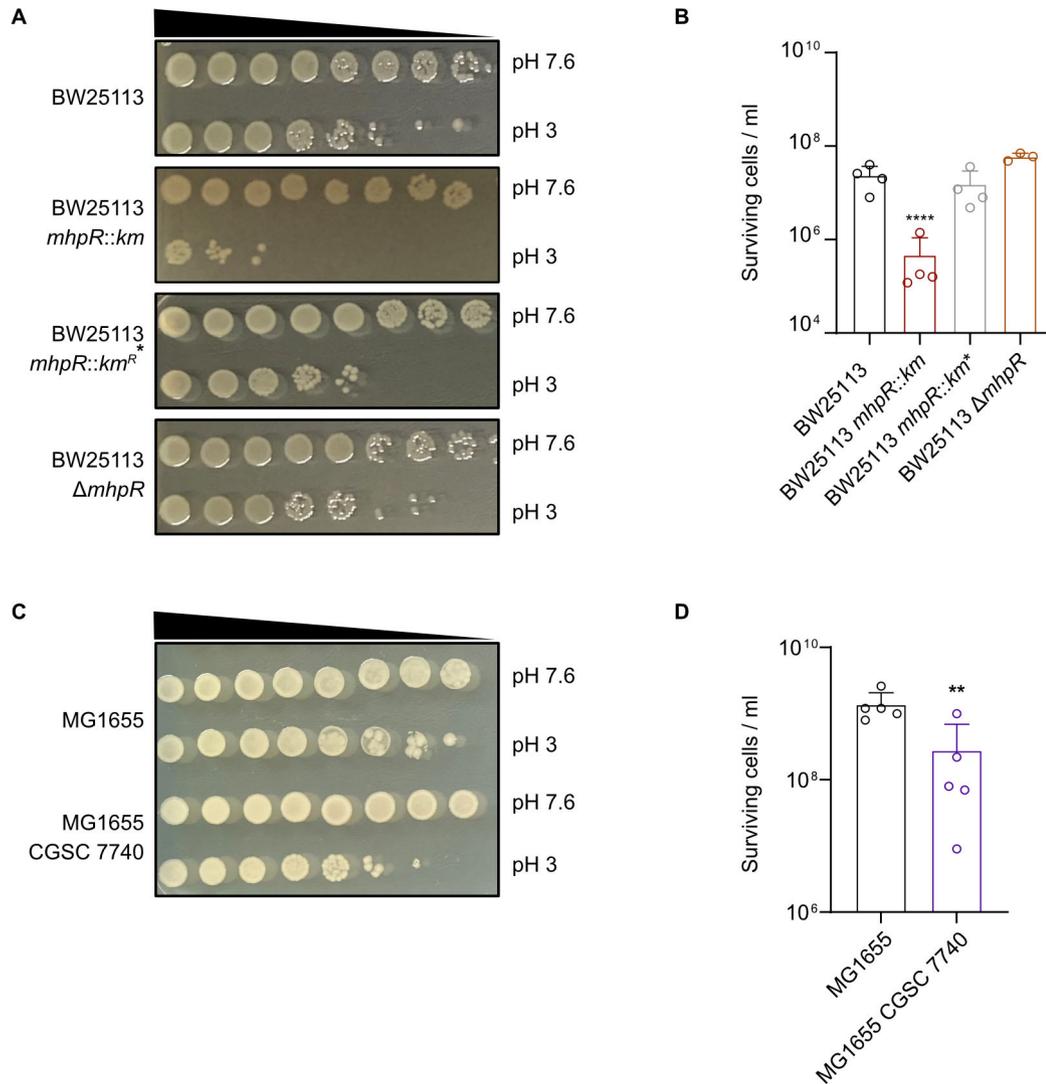
FIG 4 The presence or absence of insertion elements upstream of *flhDC* determines survival under severe acid stress. (A and C) Acid shock assays to evaluate the survival of *E. coli* BW25113, BW25113 *mhpR::km*, BW25113 *mhpR::km**, BW25113 Δ*mhpR*, MG1655, and MG1655 CGSC 7740 at pH 3. Cells were grown in LB pH 7.6 to OD_{600} = 0.5 and cell numbers were adjusted to 10^9/mL. Cultures were split and then either grown at pH 7.6 or stepwise stressed (15 min pH 5.8 and 15 min pH 4.4) before being exposed to LB pH 3 for 1 h. Cultures were serially diluted by a factor of 10 in 1× PBS and plated on LB agar plates. Images were taken after overnight incubation. (B and D) Quantitative assessment of acid shock survival of *E. coli* BW25113, BW25113 *mhpR::km*, BW25113 *mhpR::km**, BW25113 Δ*mhpR*, MG1655, and MG1655 CGSC 7740. Cells were cultivated as described in panel A, and the total number of colony-forming units at pH 3 was counted after overnight incubation. All experiments were performed in biological replicates (*n* ≥ 3), and error bars represent standard deviations of the mean. Significance was evaluated by performing a one-way ANOVA test followed by Bonferroni's multiple comparisons test (B) or an unpaired *t*-test (D) to compare log-transformed numbers of surviving cells (**$P < 0.01$ and ****$P < 0.0001$).

significant reduction in survival (Fig. 5C). Of note, neither the deletion of *flhC* nor the absence of an inducer for the P_{tac} promoter (0 µM IPTG) had any effect on acid tolerance compared to the Δ*flu* reference strain (Fig. 5B and C).

To further explore the inverse correlation between acid tolerance and the degree of motility/flagellation, we isolated subpopulations from soft-agar plates and subjected them to severe acid stress. As described previously (39), we sampled MG1655 wild type grown on soft agar [0.3% (wt/vol)] from three different halo positions with increasing distance to the center (1, center; 2, intermediate; and 3, edge) (Fig. 6A). After exposure to
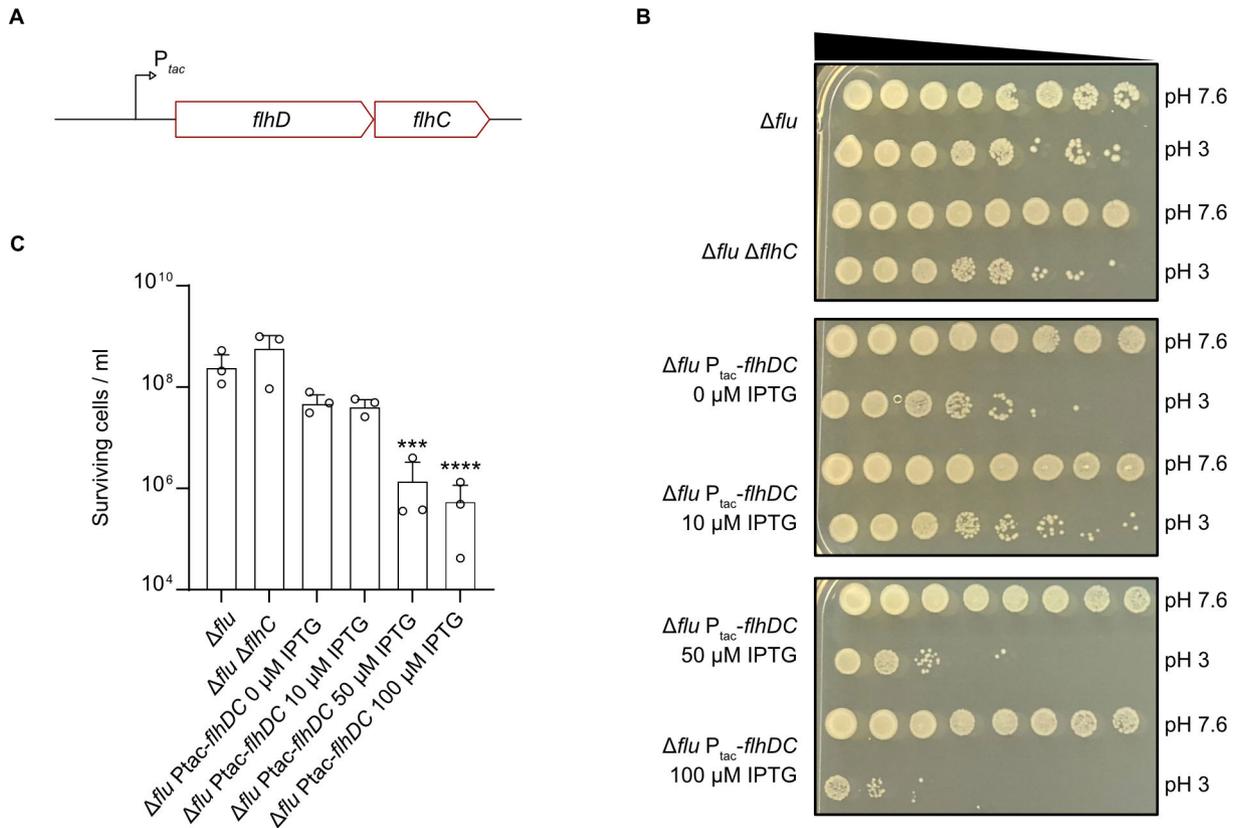
FIG 5 Survival under acid shock and the level of *flhDC* induction are inversely related. (A) Schematic representation of the *flhDC* locus with a replacement of the canonical *flhDC* promoter by an IPTG-inducible P$_{tac}$ promoter. (B) Acid shock assays to evaluate the survival of *E. coli* Δ*flu*, Δ*flu* Δ*flhC*, and Δ*flu* P$_{tac}$-*flhDC* strains supplemented with different concentrations of IPTG. Cells were grown in LB pH 7.6 containing the indicated IPTG concentrations to OD$_{600}$ = 0.5 and cell numbers were adjusted to 10^9/mL. Cultures were split and then either grown at pH 7.6 or stepwise stressed (15 min pH 5.8 and 15 min pH 4.4) before being exposed to LB pH 3 for 1 h. Cultures were serially diluted by a factor of 10 in 1× PBS and plated on LB agar plates. Images were taken after overnight incubation. (C) Quantitative assessment of acid shock survival of *E. coli* Δ*flu*, Δ*flu* Δ*flhC*, and Δ*flu* P$_{tac}$-*flhDC* strains supplemented with different concentrations of IPTG. Cells were cultivated as described in panel B, and the total number of colony-forming units at pH 3 was counted after overnight incubation. All experiments were performed in biological replicates (*n* = 3), and error bars represent standard deviations of the mean. Significance was evaluated by performing a one-way ANOVA test followed by Bonferroni's multiple comparisons test to compare log-transformed numbers of surviving cells (***P < 0.001 and ****P < 0.0001).

acid shock, the cells collected from the center showed very high acid survival rates (Fig. 6B), even exceeding the values usually observed for *E. coli* MG1655 in liquid culture. Strikingly, cells from the intermediate position and especially those from the edge of the halo were characterized by a significantly lower survival rate (Fig. 6C). It should be noted that the tested subpopulations are in different growth stages and that the extreme resistance of the subpopulation from the center overlaps with the adaptation to the stationary phase (40). In conclusion, (hyper)flagellation mediates a survival deficit in very low pH habitats.

DISCUSSION

The RNA-Seq and Ribo-Seq data from our previous study revealed that the MhpR synthesis levels increased by about one order of magnitude at pH 4.4 and that survival of an *mhpR* mutant from the Keio collection was impaired under severe acid stress (12). Here, we confirmed a higher transcript level of *mhpR* under severe acid stress but found no effects of the transcription factor itself, the regulated 3HPP/PP-dependent catabolic operon, or the cinnamic acid derivatives on acid tolerance.

Thus, despite the upregulation of MhpR at pH 4.4, MhpR does not confer a survival benefit for *E. coli* when challenged with acid at pH 3, at least not in our experimental setup. It is important to note that we could not detect MhpR-inducers such as 3HPP or PP
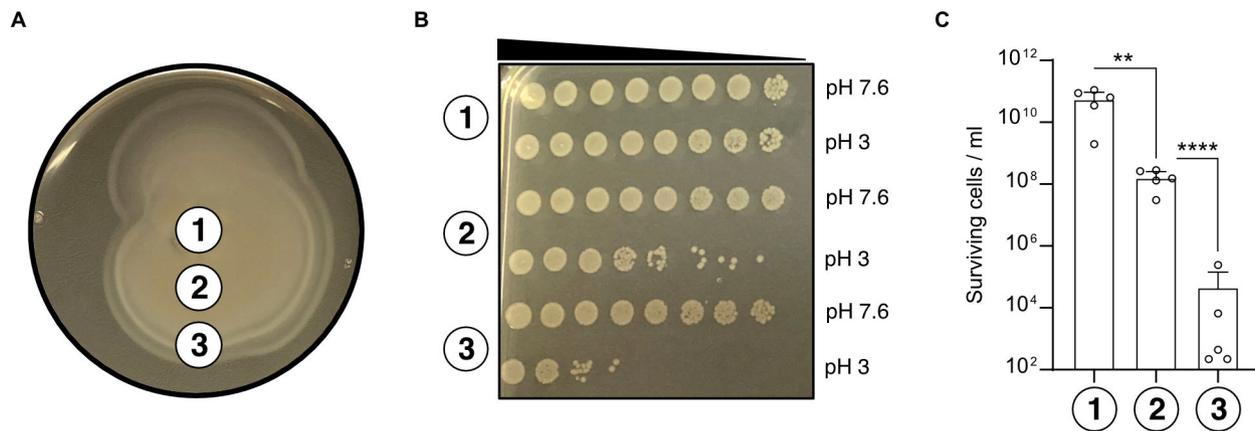
FIG 6 Subpopulations from swim agar plates have different abilities to survive acid shock. (A) Illustration of the MG1655 radial expansion on a soft agar [0.3% (wt/vol)] plate. Three microliters of exponentially grown MG1655 cultures were spotted on soft agar plates and incubated for 16 h. The labeled circles indicate the positions where the cells were collected (1, center; 2, intermediate; and 3, edge) and subsequently exposed to acid shock. (B) Acid shock survival assays of samples taken as described in panel A. Cells were punched out from soft agar plates using P1000 pipette tips and exposed to either LB pH 7.6 or 3 for 1 h. Cultures were serially diluted by a factor of 10 in 1× PBS and plated on LB agar plates. Images were taken after overnight incubation. (C) Quantitative assessment of acid shock survival of cells collected from sampling positions described in panel A. Cells were cultivated as in panels A and B, and the total number of colony-forming units at pH 3 was counted after overnight incubation. All experiments were performed in biological replicates ($n = 5$), and error bars represent standard deviations of the mean. Significance was evaluated by performing a one-way ANOVA test followed by Bonferroni's multiple comparisons test to compare log-transformed numbers of surviving cells (**$P < 0.01$ and ****$P < 0.0001$).

in pH-neutral or acidified LB media via LC-MS. Therefore, we cannot neglect that MhpR and the degradation of cinnamic acid derivatives are useful in nature. PP and 3HPP are present in the human gastrointestinal tract due to the metabolism of aromatic amino acids and plant-derived flavonoids (35), so *E. coli* is exposed to these molecules under natural conditions. Indeed, the expression of *mhp* catabolic genes in enterohemorrhagic *E. coli* (EHEC) increases during growth in the cecal contents of human gut microbiota-associated rats (41). Under these conditions, the cinnamic acid derivatives 3HPP and PP are utilized as carbon sources (30). *mhpR* expression was also significantly increased in EHEC upon infection of mice, and an *mhpR* mutant was outcompeted by a wild type during colonization of the mouse intestine (42). However, our RNA-Seq analysis of *E. coli* K12 provided no evidence that MhpR regulates a gene important for acid tolerance.

BW25113 mutants harboring both, an IS5 insertion in the *flhDC* promoter and an *mhpR* deletion, showed an acid shock phenotype, whereas the BW25113 Δ*mhpR* and *mhpR::km** strains with the native *flhDC* upstream region did not (Fig. 4). This indicated that the motility-inducing IS5 insertion is the cause of the observed phenotype in the BW25113 *mhpR::km* strain (Fig. 2). Similar IS insertions and point mutations were described in other mutants of the Keio collection. In fact, 49 of 71 tested strains had mutations in the upstream region of *flhDC,* and as a result, showed increased expression of genes involved in flagellar biosynthesis (28). It is known that the integration of IS elements in the *flhDC* promoter is favored under resting (nonshaking) conditions (28), and mutants from the Keio collection were constructed by culturing bacteria overnight without shaking (29). Moreover, it is hypothesized that IS insertion in this genomic locus is triggered by the cellular environment and depends on whether the encountered conditions permit motility (26).

Our results clearly indicate that IS5 integration upstream of *flhDC* reduces population survival under acid shock in a manner that is inversely correlated with *flhDC* expression levels (Fig. 5). Likewise, acid shock tolerance was dependent on the location of swimming cells on the soft-agar surface, and cells at the edge of the halo could barely survive at pH 3 (Fig. 6). As indicated by RNA-Seq and RT-qPCR, the reason for the reduced acid shock survival was increased flagellar and motility gene expression (Fig. 3). However, it remains to be clarified why increased flagellation and/or motility reduces survival under

severe acid stress. It appears plausible that hypermotile cells have increased energy demand resulting from flagellar synthesis, including the motor and filaments (43); the cost of synthesizing and operating the flagella accounts for up to 3.5% of the total cell energy (44–46). Therefore, cells with IS insertions upstream of *flhDC* are characterized by a reduced growth rate (26). Furthermore, a high number of rotating flagella affects not only the integrity of the membrane but also causes a high flux of protons back into the cytoplasm, which might be disadvantageous at low pH.

Under severe acid stress (pH 4.0–4.5), flagella are rapidly shed (47, 48), chemotaxis to attractants ceases, and the bacteria are no longer motile (49). On the other hand, several studies reported an increased expression of motility genes under mild acid stress (10–12). We propose that under mildly acidic conditions, *E. coli* uses an escape strategy to migrate to environments with optimal pH. Under severe acidic conditions, *E. coli* abandons the escape strategy, switches to an energy-saving mode, and also prevents the flux of protons into the cytoplasm. Considering that motility genes are still induced at pH 5.0 (10), we hypothesize that the inversion point at which motility stops to be beneficial is between pH 5.0 and 4.4.

Motility-activating mutations in the regulatory region of *flhDC* have already been shown to represent a trade-off between growth and biofilm formation (26). Strains containing IS5 upstream of *flhDC* produced more biofilms at the expense of overall growth (26). In this study, we present another trade-off associated with the presence of IS elements, namely the relationship between acid tolerance and the expression of motility genes. It is important to note that the transposition of IS elements can lead to phenotypic heterogeneity within bacterial populations. For example, the phenotypic variability of cells in a biofilm increased as a function of the frequency of IS5 insertions upstream of *flhDC* (26). Moreover, IS-mediated motility heterogeneity within a biofilm was beneficial for bacteria to increase biofilm mass (50). Also, in this study, we found a heterogeneous population of strain CGSC 7740 MG1655 with respect to cells carrying an IS element upstream of *flhDC* (Fig. S5). We have already shown the advantage of phenotypic heterogeneity under acid stress for the three major AR systems using a triple fluorescent reporter strain that enables bet-hedging and division of labor in *E. coli* (51). It is possible that the cytoplasmic membrane protein HdeD represents a link between the acid resistance Gad system and flagellar synthesis in *E. coli*. The expression of *hdeD* is controlled by GadE and HdeD represses the flagella biosynthesis via LrhA (52). Heterogeneous distribution of HdeD in the *E. coli* population (53) and heterogeneous expression of flagella in *Salmonella* are known (54, 55). Using single-cell RNA-Seq (M3-Seq), an acid-resistant subpopulation was found in stationary phase *E. coli* cells (56). According to the results of this study, the transposition of IS elements could be another important factor leading to heterogeneity under acid stress, and it is tempting to speculate that there might also be a motile subpopulation in stationary phase *E. coli* cells (56). This assumption is supported by the data collected here, which suggest an anti-correlation between motility and acid tolerance mediated by IS integrations upstream of *flhDC*. Of note, motility was also found to be correlated with oxygen availability (57). Given the different levels of acidity in the gastrointestinal tract, with a pH of <2 in the stomach (5, 58) and ~pH 6 in the duodenum (59, 60), it would certainly be advantageous for an *E. coli* population to diversify into a motile and an acid-tolerant subpopulation. Indeed, colonization of the mouse intestine was affected by the presence of IS upstream of *flhDC* in *E. coli* MG1655, indicating niches where motility is advantageous (61). In light of the presence of different intestinal niches where either acid tolerance or motility is beneficial, IS transposition upstream of *flhDC* could be crucial to ensure that *E. coli* is able to colonize both niches by dividing into a motile and an acid-tolerant subpopulation.

Taken together, this study demonstrates that the presence or absence of motility-activating mutations upstream of the master regulatory genes *flhDC* is important for *E. coli* to survive severe acid stress. The FlhDC levels are found to be anticorrelated with survival at pH 3, and motile subpopulations exhibit extremely low acid tolerance. These

findings highlight a fitness trade-off between acid tolerance and motility and suggest an IS-mediated differentiation of *E. coli* into motile and acid-tolerant subpopulations.

MATERIALS AND METHODS

Bacterial strains and growth conditions

E. coli MG1655 (62) and BW25113 strains (63) and plasmids used in this study are listed in Tables S2 and S3. Cells were grown in LB medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) and incubated aerobically in a rotary shaker at 37°C. When appropriate, media were supplemented with 15 µg/mL gentamicin or 50 µg/mL kanamycin. For RNA-Seq experiments, the pH of the medium was adjusted by the direct addition of 5 M HCl to growing cultures, as described in Schumacher et al. (12).

Plasmid construction

Molecular methods were performed according to standard protocols or according to the manufacturer's instructions. Kits for the isolation of plasmids and the purification of PCR products were purchased from Süd-Laborbedarf. Enzymes and HiFi DNA Assembly Master Mix were purchased from New England Biolabs. To construct the reporter plasmids pBBR1-MCS5-P$_{mhpR:lux}$ and P$_{mhpABCDFE:lux}$, 200 nt of the upstream regions of the respective genes were amplified by PCR using primers KSO-0169–KSO-0172 and MG1655 genomic DNA as a template. For the construction of the pNPTS-R6KT-Δ*mhpR* plasmid, 1,000 nt upstream and downstream of the *mhpR* coding region were amplified. After purification, fragments were assembled into PCR-linearized pBBR1-MCS5 or pNPTS-R6KT vectors via Gibson assembly (64). Correct insertions were verified by colony PCR and sequencing.

Construction of chromosomal *mhpR* deletions

Construction of the marker-less in-frame deletion strains of *mhpR* in *E. coli* MG1655 and BW25113 was achieved using the suicide plasmid pNPTS138-R6KT Δ*mhpR*. The plasmid pNPTS138-R6KT Δ*mhpR* was introduced into *E. coli* MG1655 and BW25113 by conjugative mating using *E. coli* ST18 (65) as a donor in LB medium containing 50 µg/mL 5-aminolevulinic acid (Ala). Single-crossover integration mutants were selected on LB plates containing kanamycin but lacking Ala. Single colonies were grown over a day without antibiotics and plated onto LB plates containing 10% (wt/vol) sucrose and lacking NaCl to select for plasmid excision. Kanamycin-sensitive colonies were investigated in terms of *mhpR* deletion by colony PCR using primers up- and downstream of the site of the insertion. Deletion of *mhpR* was verified by sequencing.

Acid shock assay

Acid shock assays were conducted as described (12). Briefly, cells were incubated at 37°C in LB medium (pH 7.6) until an OD$_{600}$ of 0.5 was reached. Upon adjustment to an OD$_{600}$ of 1, cells were either grown at pH 7.6 throughout the experiment or stepwise pH adjusted (15 min pH 5.8 and 15 min pH 4.4) before being shifted to pH 3 for 1 h. Next, samples were serially diluted by a factor of 10 in 1× PBS and plated on LB agar plates. Colony-forming units were counted the next day, and significance was evaluated by performing a one-way ANOVA test followed by Bonferroni's multiple comparisons test.

To evaluate acid shock survival of MG1655 Δ*flu* P$_{tac}$-*flhDC*, cells were grown as described above. All media were supplemented with either 0, 10, 50, or 100 µM IPTG, throughout the experiment.

Acid shock survival of MG1655 subpopulations obtained from different sampling areas of soft agar plates was evaluated using 3 µL of cells grown to OD$_{600}$ of 0.4. Cells were spotted on soft agar plates [0.3% (wt/vol)] and incubated for 16 h at 37°C as described by Bubendorfer and colleagues (39). Subsequently, cells from three different

sampling areas (1, center; 2, intermediate; and 3, edge) (Fig. 6A) were punched out using a P1000 pipette tip and immediately resuspended in either 1 mL LB pH 7.6 or pH 3. Serial dilution and data analysis were conducted as described above.

RNA-Seq analysis

Biological triplicates of *E. coli* MG1655 wild type or $\Delta mhpR$, as well as BW25113 wild-type or *mhpR::km* cells, were inoculated to a starting OD_{600} of 0.05 from overnight cultures and grown in 200 mL of unbuffered LB medium (pH 7.6) until an OD_{600} of 0.5 was reached. Cultures were shifted first for 15 min to LB pH 5.8 and subsequently for 15 min to LB pH 4.4. pH shifts were achieved by adding 5 M HCl directly to growing cultures. pH values before and after pH shifts, as well as final optical densities, were monitored (Table S1). Upon exposure to pH 4.4, 1.6 mL of stop mix [95% (vol/vol) ethanol and 5% (vol/vol) phenol] was added to 8 mL aliquots of the respective cultures to terminate ongoing transcription and translation. Samples were flash frozen in liquid nitrogen and stored at −80°C until RNA isolation. Cells were pelleted (3,000 × *g*, 15 min, 4°C), and total RNA was isolated using the miRNeasy Mini Kit (Qiagen) in combination with the RNase-Free DNase Set (Qiagen). RNA samples were evaluated in terms of integrity using an RNA 6000 Nano Kit (Agilent) and quantified using a Qubit RNA HS Assay Kit (Invitrogen). Ribosomal RNA depletion was performed using the NEBNext rRNA Depletion Kit for bacteria (NEB), and directional cDNA libraries were prepared using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB). cDNA library quality was evaluated using a High Sensitivity DNA Kit (Agilent). Finally, cDNA libraries were sequenced using a NextSeq 1000 machine (Illumina) in single-read mode with a 60 bp read length.

The demultiplexed read files in fastq format were imported into the CLC Genomics Workbench v20.0.4 (Qiagen) and trimmed for quality and adaptors. Reads were mapped to the *E. coli* MG1655 and BW25113 reference genomes (NCBI accession numbers: NC_000913.3 and CP009273.1) using the "RNA-Seq Analysis" tool with default parameters. Reads that mapped to annotated genes were normalized (reads per kilobase per million reads mapped rpkm) and transformed ($\log_2$). Low-expression transcripts were filtered out, and we focused our analysis on genes with rpkm values ≥ 5 in at least one replicate. Differential expression was evaluated using the "Empirical Analysis of DGE" tool. Genes with a fold change ≥ 2 and an FDR-adjusted *P*-value ≤ 0.01 were considered as differentially expressed. Volcano plots were created using the seaborn.jointplot function in Python 3.8.8. A comprehensive overview of all expression values is available in Tables S5 and S6.

RNA isolation and RT-qPCR analysis

RNA isolation and RT-qPCR analysis were conducted as described (12). In brief, RNA was isolated using the miRNeasy Mini Kit (Qiagen) in combination with the RNase-Free DNase Set (Qiagen) according to the manufacturer's instructions. A 500 ng aliquot of isolated RNA was converted to cDNA with the iScript Advanced Kit (Bio-Rad) according to the manufacturer's instructions. Next, 1 µL of a 1:10 dilution of the cDNA samples in nuclease-free water was mixed with 5 µL of SsoAdvanced Univ SYBR Green Supermix (Bio-Rad) and 0.8 µL of 5 µM forward and reverse primers (Table S4), and the total reaction volume was adjusted to 10 µL with nuclease-free water. The mixture was dispensed in triplicates in a 96-well PCR plate (Bio-Rad) and subjected to qPCR in a Bio-Rad CFX real-time cycler. Data were analyzed according to the $\Delta\Delta Ct$ method (66), using the *secA* gene as a reference.

Promoter activity assay

Promoter activities of *mhpR* and *mhpABCDFE* were determined using luminescence-based reporter plasmids harboring fusions of the respective promoter regions to the *luxCDABE* genes from *Photorhabdus luminescens* encoded on a pBBR-MCS5 vector. MG1655 cells were transformed with plasmids pBBR1-P*mhpR:lux* or pBBR1-P*mhpABCDFE:lux*.

All strains were cultivated in LB medium supplemented with gentamicin overnight, and day cultures were inoculated to an OD_{600} of 0.05 in fresh LB medium (pH 7.6) and aerobically cultivated until the exponential phase (OD_{600} = 0.5). Cultures were then either shifted to LB pH 5.8 for 15 min and LB pH 4.4 for 15 min or further cultivated at pH 7.6 and supplemented with 1 mM PP (Sigma-Aldrich), 1 mM 3HPP (Fisher Scientific), or DMSO. PP and 3HPP were solved in DMSO. In the next step, cells were transferred to a 96-well plate and cultivated at 37°C in the above-mentioned media supplemented with gentamicin. Growth and bioluminescence were measured every 10 min in microtiter plates using a CLARIOstar Plus plate reader (BMG Labtech). Data are reported as relative light units in counts per second of OD_{600}.

Swim agar assay

To determine colony expansion, overnight cultures grown in LB medium were diluted in fresh LB medium and normalized to an OD_{600} of 1 before being dropped in the center of an LB soft agar plate [0.3% (wt/vol)]. After incubation at 37°C for 16 h, the diameter of the halo was measured. Significance was evaluated by performing a one-way ANOVA test followed by Bonferroni's multiple comparisons test.

Analysis of cinnamic acid derivatives via LC-MS

For sample preparation, 100 µL culture media was diluted with 100 µL of acetonitrile. After vigorously shaking the samples for 1 min, the samples were centrifuged at 10,000 rpm for 10 min at 10°C. The supernatants were analyzed by means of LC-MS/MS with a 5500 QTrap (AB Sciex) coupled to an ExionLC AD UPLC (AB Sciex). For chromatographic separation, a Kinetex 1.7 µm C18 100 × 2.1 mm (Phenomenex) was used with 0.1% formic acid as solvent A and acetonitrile with 0.1% formic acid as solvent B. First, 5% B was held for 0.5 min and then a linear gradient was used from 5% B to 100% B in 5 min. Afterward, the column was flushed and equilibrated to starting conditions. The separation was performed using a 400 µL/min flow rate at 40°C column oven temperature. Ions were analyzed by MS in the negative ionization mode. The spray voltage was set to −4,500 V at a source temperature of 400°C using nitrogen as collision gas. The parameters for the collision-activated dissociation were medium, curtain gas: 35 psi, ion source gas 1: 55 psi, ion source gas 2: 65 psi, entrance potential: −10 V, and the dwell time: 100 ms. The MRM (multiple reaction monitoring) transition for each compound was optimized by direct infusion of the reference standards. The MRM settings were as follows: 3-(4-hydroxyphenyl)propionic acid: 162.931 ≥ 90.9 (quantifier), declustering potential (DP) −35 V, collision energy (CE) −36 V, cell exit potential (CXP) −1 V; 162.931 ≥ 64.8 (qualifier), DP −35 V, CE −44 V, and CXP −9 V; trans-3-hydroxycinnamic acid: 164.917 ≥ 120.8 (quantifier), DP −40 V, CE −16 V, CXP −19 V; 164.917 ≥ 80.0 (qualifier), DP −40 V, CE −40 V, CXP −3 V; and hydrocinnamic acid: 148.949 ≥ 149.0 (quantifier), DP −65 V, CE −10 V, CXP −7 V; 148.949 ≥ 105.0 (qualifier), DP −65 V, CE −14 V, and CXP −5 V. Analyst 1.7. was used to acquire the data, and Multiquant 3.0.3 was used to analyze the data (both AB Sciex).

D.B., K.G., and K.J. reviewed and edited the manuscript. K.J. acquired funding, provided resources, and supervised the study.

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DATA AVAILABILITY

The RNA-Seq raw data were deposited at Gene Expression Omnibus (GEO) under the accession number GSE260455. Additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental material (Spectrum00544-24-S0001.pdf). Fig. S1 to S6; Table S1 to S4.
Table S5 (Spectrum00544-24-S0002.xlsx). RNA-Seq results MG1655 dmhpR vs MG1655 - pH 4.4.
Table S6 (Spectrum00544-24-S0003.xlsx). RNA-Seq results BW25113 mhpR-km vs BW25113 - pH 4.4.

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2.3 Eukaryotic catecholamine hormones influence the chemotactic control of *Vibrio campbellii* by binding to the coupling protein CheW

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Eukaryotic catecholamine hormones influence the chemotactic control of *Vibrio campbellii* by binding to the coupling protein CheW

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In addition to their well-known role as stress-associated catecholamine hormones in animals and humans, epinephrine (EPI) and norepinephrine (NE) act as interkingdom signals between eukaryotic hosts and bacteria. However, the molecular basis of their effects on bacteria is not well understood. In initial phenotypic studies utilizing *Vibrio campbellii* as a model organism, we characterized the bipartite mode of action of catecholamines, which consists of promotion of growth under iron limitation and enhanced colony expansion on soft agar. In order to identify the molecular targets of the hormones, we designed and synthesized tailored probes for chemical proteomic studies. As the catechol group in EPI and NE acts as an iron chelator and is prone to form a reactive quinone moiety, we devised a photoprobe based on the adrenergic agonist phenylephrine (PE), which solely influenced colony expansion. Using this probe, we identified CheW, located at the core of the chemotaxis signaling network, as a major target. In vitro studies confirmed that EPI, NE, PE, and labetalol, a clinically applied antagonist, bind to purified CheW with affinity constants in the submicromolar range. In line with these findings, exposure of *V. campbellii* to these adrenergic agonists affects the chemotactic control of the bacterium. This study highlights an effect of eukaryotic signaling molecules on bacterial motility.

interkingdom signaling | bacterial chemotaxis | chemical proteomics | bacterial pathogenicity | epinephrine

Catecholamine hormones are widespread signaling molecules present in animals and humans, where they act as neurotransmitters and stress hormones. They include epinephrine (EPI), norepinephrine (NE), and dopamine, which all bear a characteristic catechol motif and a side-chain amine. It is well known that stress of the mammalian host increases its susceptibility to bacterial infections, and EPI and NE, for example, stimulate growth of the enterobacteria *Salmonella enterica* serovar Typhimurium, *Escherichia coli*, and *Vibrio cholerae* in serum-based media (1–4). In addition, EPI and NE affect biofilm formation, siderophore production, invasion of epithelial cells, and the expression of virulence factors (5–8). It is thus assumed that some bacteria use these hormones as cues to recognize the eukaryotic host environment and to occupy a niche in it (9).

In eukaryotes, catecholamines bind to G protein–coupled receptors. The observation that certain antagonists of the human receptors also antagonize adrenergic effects in bacteria corroborates the hypothesis that receptors with a similar specificity may have evolved in prokaryotes. Indeed, binding of NE and EPI to the two-component system histidine kinases QseC and QseE was reported in enterohemorrhagic *E. coli* O157:H7 (10, 11). However, not all adrenergic responses depend on QseC and/or QseE, as mutants lacking the corresponding homologous genes in *Salmonella* and *V. cholerae* were still

responsive to catecholamines (4, 7, 12). Thus, other, as yet unexplored bacterial pathways may contribute to the catecholamine signaling and virulence.

Furthermore, catecholamines act as chemical signals in bacterial chemotaxis, a process in which bacteria navigate along chemical gradients toward attractants and away from repellents (13–15). While the individual components in the chemotaxis signaling cascades vary across different species, the core of the signaling complex typically consists of a transmembrane chemoreceptor (methyl-accepting chemotaxis protein, MCP) and a histidine kinase CheA, the two of which are bridged by the coupling protein CheW (16). In response to chemical stimuli, this complex controls the autophosphorylation of CheA, which consequently transfers its phosphoryl group to the response regulator CheY. Phosphorylated CheY induces clockwise rotation of the flagellar motor and thereby increases the frequency of tumbling in *E. coli*. Notably, NE behaved as a weak attractant at low concentrations, and as a repellent at higher concentrations (1 mM) and this response appeared not to be mediated by specific binding to an MCP in *E. coli* (17).

Significance

Host-emitted stress hormones significantly influence the growth and behavior of various bacterial species; however, their cellular targets have so far remained elusive. Here, we used customized probes and quantitative proteomics to identify the target of epinephrine and the α-adrenoceptor agonist phenylephrine in live cells of the aquatic pathogen *Vibrio campbellii*. Consequently, we have discovered the coupling protein CheW, which is in the center of the chemotaxis signaling network, as a target of both molecules. We not only demonstrate direct ligand binding to CheW but also elucidate how this affects chemotactic control. These findings are pivotal for further research on hormone-specific effects on bacterial behavior.

Vibrio campbellii (previously *V. harveyi*) ATCC BAA-1116 (18) is an important model organism for quorum sensing (19–21). It is a marine, motile, bioluminescent γ-proteobacterium and an opportunistic pathogen for fish, shrimp, squid, and other marine invertebrates (22). The presence of NE and dopamine increased not only its growth in serum-supplemented medium but also siderophore production, swimming motility, and expression of genes involved in biofilm formation and virulence (5). Interestingly, antagonists of the mammalian α-adrenoreceptors such as labetalol (LAB), inhibited the effect of NE on motility, whereas β-adrenoreceptor antagonists such as propranolol (PRO) had no effect, suggesting that catecholamines act via a specific receptor in *V. campbellii* (5). Motility has long been recognized to be important for both commensals and pathogens to colonize their host. In particular, nonmotile mutants of different pathogenic *Vibrio* species showed reduced virulence (23). While the effect of catecholamines on growth most likely stems from the iron-sequestering properties of the catechol siderophore (24), diverging reports exist about the mechanism underlying the altered virulence (4, 5, 25).

In this study, we applied chemical proteomics to identify protein targets of catecholamine hormones in the model organism *V. campbellii*. First, we studied the effects of NE, EPI, and a set of chemically related structures as well as the adrenergic antagonists LAB and PRO on growth and motility. The α-adrenoreceptor agonist phenylephrine (PE) promoted colony expansion without facilitating iron uptake. Therefore, a corresponding chemical probe was used for subsequent photoaffinity labeling, which revealed the chemotaxis coupling protein CheW as a major target. Binding of catecholamines to purified CheW was confirmed by microscale thermophoresis (MST). The adrenergic agonists EPI, NE, and PE also influenced the chemotactic control of *V. campbellii*, suggesting a role of eukaryotic host signals on the physiology of bacteria.

Results

Effects of Catecholamines and Related Compounds on Colony Expansion of *V. campbellii* on Semisolid (Soft) Agar.
We first tested the effect of the hormones EPI and NE and structurally related compounds on a readily detectable phenotype of *V. campbellii*. As previously shown for NE (5), NE, but even more strongly EPI, stimulated colony expansion of *V. campbellii* on semisolid (soft) agar. This assay reports swimming motility but also growth and chemotaxis. PE, a synthetic agonist of human α-adrenoreceptors, elicited a similar increase of colony expansion as the natural hormones, whereas its para-substituted analog, octopamine (OA), a neurotransmitter of invertebrates, had no significant effect (Fig. 1 *A* and *B*). In addition, we included the adrenergic antagonists LAB (mammalian α- and β-adrenoreceptor specificity) and PRO (β-adrenoreceptor specificity). LAB has been reported to antagonize adrenergic effects in *V. campbellii*, while PRO showed no effect (5). Consistent with this literature report, the stimulation of colony expansion by EPI, NE, and PE was blocked by LAB but not by PRO (Fig. 1*C*). These results indicate the presence of a specific adrenergic sensor that, analogously to the mammalian receptor, can be activated by NE, EPI, and PE and blocked by LAB.

Design and Synthesis of Tailored Probes for Chemical Proteomic Studies.
To decipher the cellular targets of catecholamine hormones, we designed and synthesized chemical probes containing an alkyne handle suitable for target protein enrichment via affinity-based protein profiling (26–28). As we could not exclude that the probe scaffold binds reversibly, we appended an alkyne-containing minimalist photocrosslinker to the EPI-based probe EPI-P1 via alkylation of NE at the amine (Fig. 1*D*; *SI Appendix*, Fig. S1A) (29). Here, the synthesis was challenged

by the instability of catecholamines. At neutral to basic pH, the catechol group is easily oxidized to an ortho-quinone, which can be attacked by the amine (30). This required purification under acidic conditions, resulting in a residual amount of acetic acid in the product needed for stability. Thus, in a second generation of probes, we used direct acylation of catecholamines, which yielded more stable products. Moreover, as we observed an inherent photoreactivity of the adrenergic compounds, we omitted the diazirine and appended an alkyne handle to the terminal amine of NE, norphenylephrine, and OA by standard amide coupling, yielding probes EPI-P2, PE-P, and OA-P, respectively (Fig. 1*D*; *SI Appendix*, Fig. S1B). Next, the derivatives were tested in soft agar motility assays in which enhanced colony expansion was confirmed for probes EPI-P1 (6 to 60 μM), EPI-P2 (10 to 25 μM), and PE-P (10 to 50 μM), suggesting that these probes retain suitable biological activity to unravel their cellular targets (Fig. 1*D*; *SI Appendix*, Fig. S2). For EPI-P2 and PE-P, a drop in activity was observed at concentrations above 25 and 50 μM, respectively (*SI Appendix*, Fig. S2 *B* and *C*). In contrast to its parent compound OA, OA-P proved slightly active (25 to 100 μM), although still to a lesser extent than the other probes (*SI Appendix*, Fig. S2D).

Catechol-bearing Probes Enhance Growth Under Iron-limited Conditions.
In the body fluids of eukaryotic hosts, bacterial growth is limited by extremely low levels of available iron, a phenomenon known as nutritional immunity (31). Iron limitation is achieved by high-affinity iron-binding proteins such as transferrin and lactoferrin. Nevertheless, bacteria are able to acquire iron via iron-binding molecules, so-called siderophores, either synthesized by the bacteria or scavenged from the environment. Among the most common classes of siderophores are catecholates, which include the bacterial enterobactin, but also catecholamine hormones (24, 32–34). We, therefore, tested the siderophore effects of our probes and their parent compounds on the growth of *V. campbellii*. These assays were performed in mineral salt medium supplemented with apo-transferrin, a setup typically used to mimic the iron-limited conditions encountered by the bacteria in eukaryotic hosts (35). As expected, catechol-containing compounds EPI-P1, EPI-P2, and EPI enhanced growth, while all other compounds, including PE-P, did not have an effect. The determined doubling times are summarized in *SI Appendix* Table S1. A comparable growth stimulation was not observed when the bacteria were grown in the absence of apo-transferrin, indicating that the growth-stimulating effect of catecholamines is due to their ability to sequester iron (Fig. 1*E*). The catechol-bearing compounds also stimulated growth of *V. campbellii* in LB35 medium containing 30% (vol/vol) adult calf serum (*SI Appendix*, Fig. S3).

Photolabeling Reveals the Chemotaxis Protein CheW as a Potential Adrenergic Target.
Prior to mass spectrometry (MS)-based proteome profiling, we investigated general protein labeling of intact *V. campbellii* cells with EPI-P1, EPI-P2, PE-P, and OA-P, respectively, via gel-based fluorescence analysis of the proteomes. Live cells were incubated with 50 μM of probes and irradiated with ultraviolet (UV) light to enable covalent bond formation of the probe to the target proteins. Following cell lysis, the proteome was separated into a phosphate-buffered saline (PBS)-soluble fraction, containing primarily cytosolic proteins, and a PBS-insoluble fraction, containing cytosolic and membrane proteins, and both fractions were subjected to copper-catalyzed azide–alkyne cycloaddition (CuAAC) to append a fluorescent tag to the labeled proteins (36, 37). Labeled proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by in-gel fluorescence scanning. Interestingly, labeling was observed in both fractions, indicating cell permeability of the
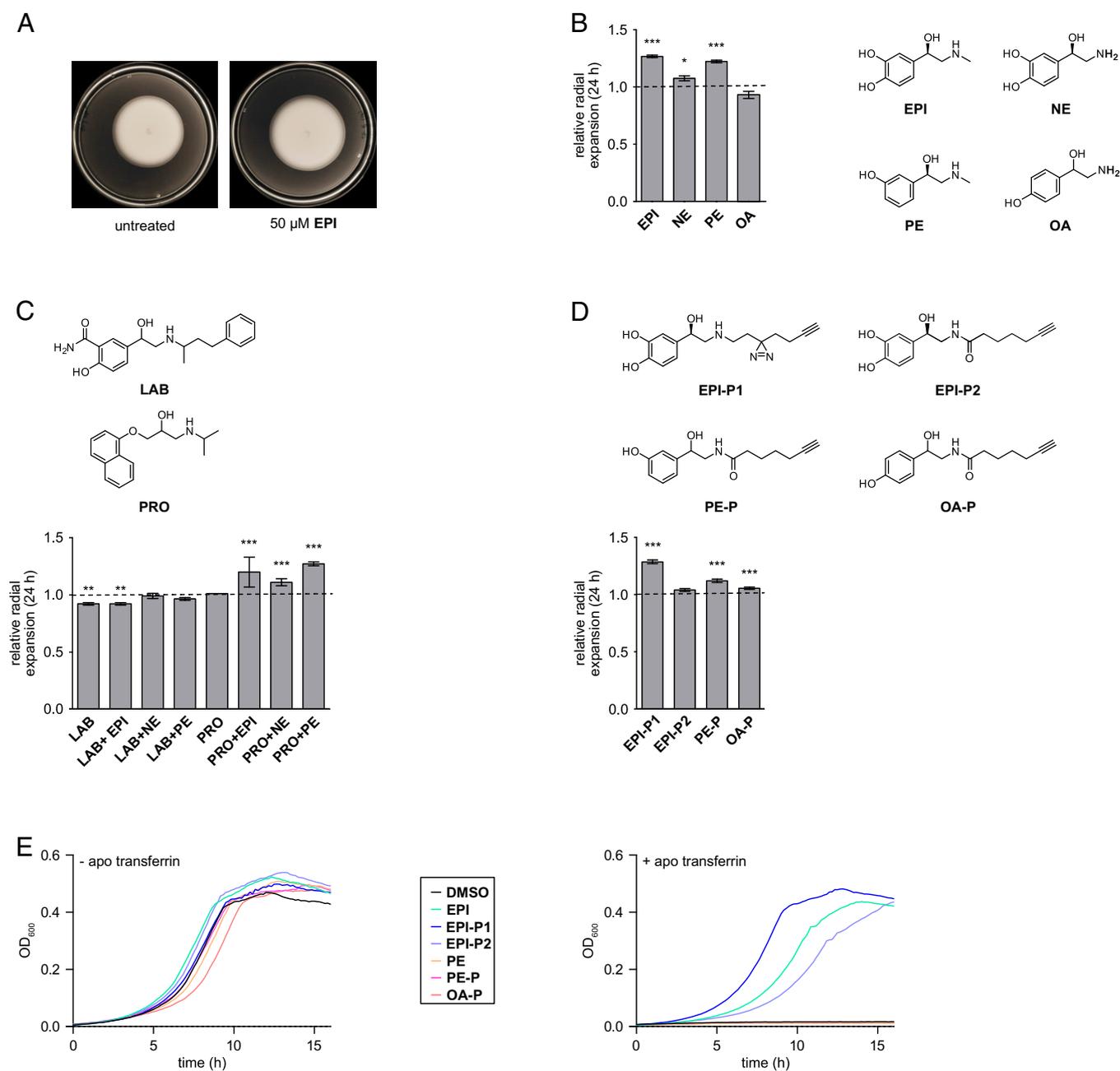
Fig. 1. Biological activity of catecholamines and related compounds on soft agar colony expansion and growth under iron limitation. (*A*) Example soft agar plates of *V. campbellii* treated with 50 μM EPI compared to an untreated control. (*B*) Structure and activity in soft agar expansion assay of parent compounds EPI, NE, PE, and OA. (*C*) Structure and activity in soft agar expansion assay of adrenergic antagonists LAB and PRO added alone or in combination with EPI, NE, or PE. (*D*) Structure and activity in soft agar expansion assay of chemical probes EPI-P1, EPI-P2, PE-P, and OA-P. All compounds were added at 50 μM except EPI-P1 at 60 μM. Radial expansions were normalized to an untreated control. Error bars represent SD, $n = 6$ independent experiments. Significance was determined performing a one-way ANOVA with Tukey's post hoc test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (*E*) Bacterial growth in KE medium (*Left* plot) or in KE medium supplemented with 100 μg/mL human apo-transferrin to generate iron limitation (*Right* plot) in a 96-well microtiter plate with continuous shaking at 30 °C. Compounds were added at 50 μM. Data show the mean of triplicates; SDs between replicates account for less than 10% in all growth experiments. For doubling times, see *SI Appendix*, Table S1.

probes (*SI Appendix*, Fig. S4). Fluorescent SDS-PAGE analysis of EPI-P1– and EPI-P2–treated cells showed labeling both in the presence and absence of UV, albeit to a lesser extent in the latter case. The general reactivity of EPI-P1 and EPI-P2 toward proteins is likely attributed to the catechol moiety, which is known to form an electrophilic ortho-quinone upon oxidation even in the absence of UV light (30). Consistent with its lack of a catechol moiety, PE-P strongly labeled proteins solely upon UV irradiation, suggesting photoreactivity of the molecule.

Almost no protein reactivity was observed for OA-P, which is in line with its weak bioactivity, and it was therefore excluded from further analysis. We selected EPI-P1 and PE-P for further studies, as both exhibited a distinct labeling profile and activity in the motility assays. With two adrenergic probes at hand, we studied their cellular target proteins via quantitative liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis (Fig. 2*A*). Treatment of live *V. campbellii* with EPI-P1 or PE-P was followed by UV irradiation, cell lysis,
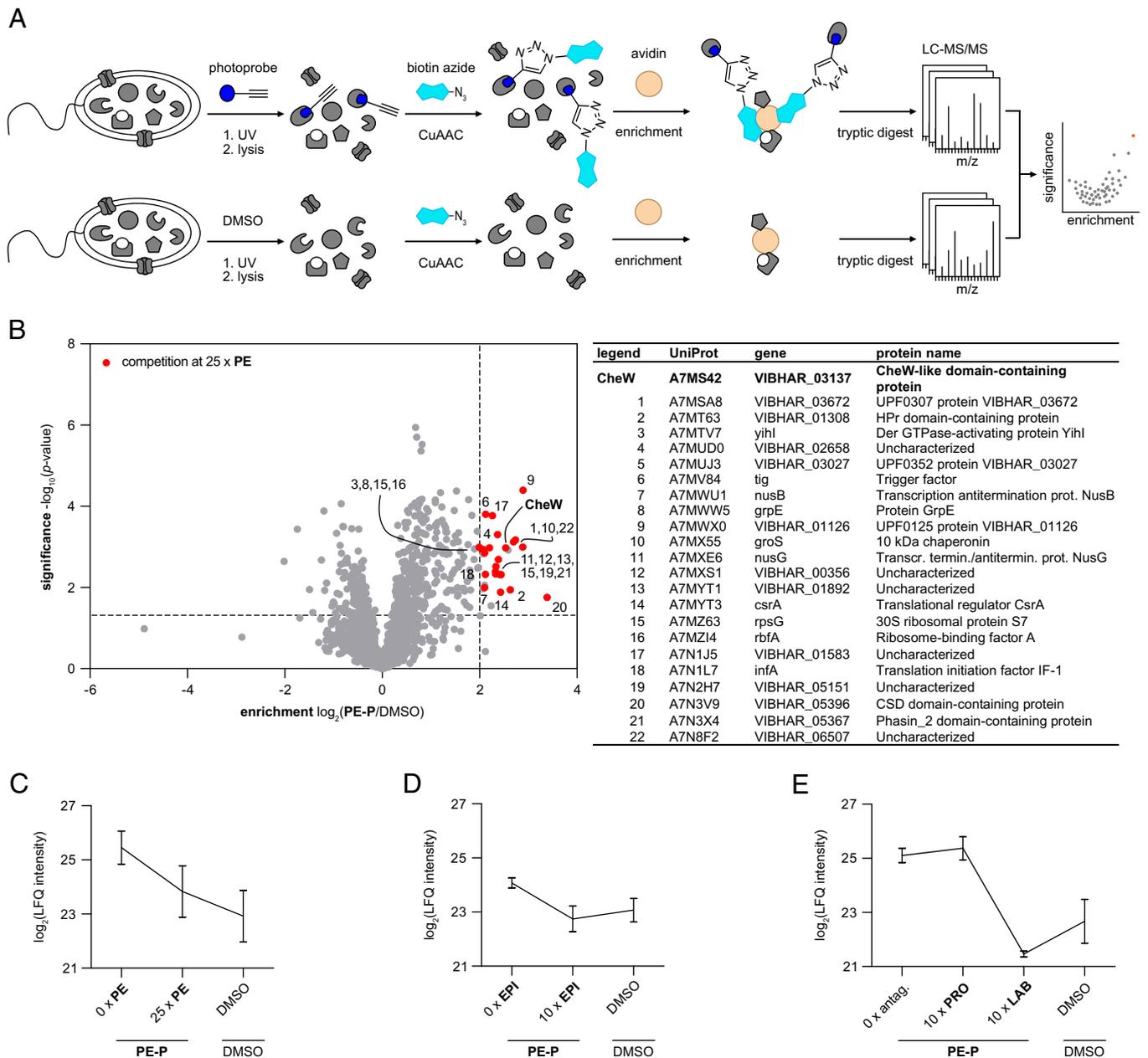
Fig. 2. CheW is identified as a potential adrenergic target by photoaffinity labeling with PE-P. (*A*) Chemical proteomics workflow applied for target identification. Live bacteria were treated with 10 μM PE-P or DMSO as control or PE-P plus increasing concentrations of competitors (PE, EPI, LAB, PRO), irradiated, lysed, separated into soluble (PBS) and insoluble proteins, ligated by CuAAC to biotin azide, enriched on avidin beads, and digested, and peptides were analyzed by LC-MS/MS. (*B*) Volcano plot showing proteins enriched in samples treated with 10 μM PE-P over DMSO controls. Proteins outcompeted by a 25-fold excess of PE are highlighted in red; the experiment was done in five biological replicates. MS data were analyzed by MaxLFQ (38) and filtered for proteins identified in four replicates, and missing values were imputed. Samples were compared using a two-sample permutation-corrected *t* test. (*C*) Profile plot of CheW LFQ intensities from competition with a 25-fold excess of PE. Error bars denote SD, *n* = 5 biological replicates. (*D*) Profile plot of CheW LFQ intensities from competition with a 10-fold excess of EPI, *n* = 4 biological replicates. (*E*) Profile plot from competition with a 10-fold excess of PRO or LAB, *n* = 4 biological replicates. All data shown here are from the insoluble fraction. See *SI Appendix*, Figs. S6–S8 for more detailed competition data and soluble fractions.

CuAAC to biotin azide, enrichment on avidin beads, and tryptic digest, resulting in peptides, which were measured by LC-MS/MS with label-free quantification (LFQ) (38). All proteins that were significantly [$-\log_{10}(P$ value$) \geq 1.3$] enriched by at least twofold in the probe-treated samples compared to the dimethyl sulfoxide (DMSO) controls are visualized in the upper right quadrant of the corresponding volcano plot. EPI-P1 enriched, among others, an outer membrane receptor protein for ferrienterochelin and colicins (A7MZS4) and an iron-

hydroxamate ABC transporter substrate binding protein (A7MSY4), indicating that this probe indeed functions as a xenosiderophore (*SI Appendix*, Fig. S5). In fact, CheW was also enriched 3.9-fold in this dataset, although it was not among the most prominent hits. We hypothesized that the abundance of iron-uptake proteins interfered with the identification of further adrenergic targets. We therefore switched to labeling studies with PE-P, devoid of the catechol moiety (Fig. 2*B*). Here, proteins associated with iron uptake were no longer

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significantly enriched. Importantly, the chemotaxis coupling protein CheW turned out to be one of the most prominent hits. Based on its role in chemotaxis, CheW is an intriguing candidate for catecholamine binding, and it was thus further investigated for displacement of probe binding in the presence of excess concentrations of the unmodified catecholamines. Cells were preincubated with different concentrations (100 to 250 μM) of PE, EPI, LAB, or PRO before adding PE-P (10 μM). EPI, PE, and LAB reduced enrichment of CheW by probe PE-P, indicating competitive binding and corroborating CheW as a specific target of these compounds (Fig. 2 *C–E*; *SI Appendix*, Figs. S6–S8). PRO, however, did not compete for binding, consistent with its lack of antagonism in the motility assays (Fig. 2E; *SI Appendix*, Fig. S8C). We identified additional proteins for which, as with CheW, the binding of PE-P was outcompeted by EPI, PE, and LAB, but not by PRO (*SI Appendix*, Table S2). In the soluble fraction, we found three uncharacterized proteins (A7MXS1, A7N2H7, A7N8F2). In the insoluble fraction, we found the Der GTPase-activating protein YihI (A7MTV7), the 30S ribosomal protein S7 (A7MZ63), the translation initiation factor IF-1 (A7N1L7), and four other uncharacterized proteins (A7MYT1, A7MXS1, A7N8F2, A7MUD0). Notably, of all differentially outcompeted proteins, only CheW was also significantly enriched by EPI-P1.

Insights Into the Mechanism of PE-P Binding. To elucidate the UV-dependent binding mode of PE-P, we performed labeling experiments in the presence of radical scavengers. UV-dependent labeling by PE-P could be fully quenched by the addition of thiourea and tiron, suggesting a light-induced fragmentation of the molecule to form reactive radical intermediates (*SI Appendix*, Fig. S9). To further assess the nature of radical binding to proteins, we applied a mass-spectrometry method, which unravels the modified residues within proteins as well as the type of modification. This technology is based on isotopically labeled desthiobiotin azide (isoDTB) tags, which are clicked to probe-labeled proteins after lysis (39). Proteins are subsequently digested, followed by peptide enrichment on avidin beads and detection of modified peptides via LC-MS/MS analysis (Fig. 3A) (40). In an unbiased analysis (41), we detected modified peptides with the added mass of the adduct with PE-P plus a light or heavy isoDTB tag, respectively (Fig. 3B). Interestingly, we observed a high selectivity for tyrosine, which constituted 90% of all detected modified residues, corroborating a radical mechanism of binding (Fig. 3 *C* and *D*). Furthermore, MS-based site identification revealed two tyrosine residues, Y_{44} and Y_{112}, within CheW, which were modified by the probe (Fig. 3E; *SI Appendix* Fig. S10 *A* and *B*). As there is no crystal structure of *V. campbellii* CheW available, we used AlphaFold for prediction (42). We found that the two tyrosine residues frame the conserved arginine at position 64, which is assumed to be necessary for modulating CheA activity (*SI Appendix*, Fig. S10C) (43).

Validation of Catecholamine Binding to CheW and Analysis of the CheW Interaction Network. To validate catecholamine binding to CheW, we measured the affinity of the ligands for CheW using MST (44). For this purpose, CheW was recombinantly expressed in *E. coli*, purified, and fluorescently labeled. Temperature-induced changes in fluorescence (temperature-related intensity changes and/or temperature dependent movements) were determined as a function of ligand concentration in glass capillaries. The parent compounds EPI, NE, and PE, the adrenergic antagonist LAB, and OA as negative control were tested as putative ligands. PRO could not be tested because of interference from its intrinsic fluorescence. Interestingly, EPI, NE, PE, and LAB caused concentration-dependent effects on the fluorescently labeled CheW with dissociation

constants K_d ranging from 300 to 740 nM (Fig. 4A), indicating strong affinity binding. These observations are consistent with the results from the colony expansion and competitive labeling experiments (Figs. 1 *B* and *C* and 2 *C–E*). In line with its lack of activity in the motility assays (Fig. 1B), OA showed no binding affinity for CheW.

Next, protein interaction partners of CheW were studied by coimmunoprecipitation (Co-IP) in cells treated first with 100 μM EPI or DMSO as control and then with disuccinimidyl sulfoxide to stabilize interaction partners by covalent cross-links (45). Numerous proteins annotated to be involved in chemotaxis (Gene Ontology [GO] or Kyoto Encyclopedia of Genes and Genomes [KEGG] database) were enriched compared to an isotype control, including CheA, CheZ, MCPs, and a CheW-like domain-containing protein, confirming the validity of the antibody and the methodology (*SI Appendix*, Fig. S11 *A* and *B*). The presence of EPI, however, did not result in any obvious catecholamine-dependent changes of interaction partners (*SI Appendix*, Fig. S11C). These results suggest that under the conditions tested, binding of catecholamines to CheW does not alter the associated protein networks. This is in line with the observation that transmembrane chemoreceptor arrays remain intact upon activation (46).

Adrenergic Compounds Affect the Chemotactic Control of *V. campbellii*. Colony expansion in soft agar is a complex phenotype that is driven by a combination of motility behavior, chemosensing, chemoattractant consumption, and growth (47). The increased colony expansion on soft agar plates with EPI and PE (Fig. 1B) could, in principle, result from growth benefits (*SI Appendix*, Fig. S3), chemoattraction, or changes in individual motility behavior such as the turning frequency. To gain deeper insights into the effect of EPI and PE on motility and chemotaxis, we first used 3D tracking to examine the motility of untreated *V. campbellii* cells of the midexponential growth phase. Similar to *Vibrio alginolyticus* (48), the cells exhibited a run-reverse-flick pattern at typical swimming speeds of 54 ± 2 μm/s and a steady-state turning frequency of 0.52 ± 0.03 s^{-1} (Fig. 4B). We did not detect any significant effects of EPI on the average speed or steady-state turning frequency (*SI Appendix*, Fig. S12). Since EPI and PE bind to CheW, we next examined the motility of a ΔcheW mutant. The ΔcheW mutant had a similar average swimming speed as the wild-type (48 ± 4 μm/s) but proved to be a smooth swimmer with a very low turning frequency (Fig. 4C). This mutant was unable to spread on soft agar (*SI Appendix*, Fig. S13), a phenomenon previously observed in other *Vibrio* species (49). Next, we used a multiscale 3D chemotaxis assay (50) that combines high-throughput 3D bacterial tracking with a microfluidically created linear chemical gradient. A 100 μM/mm EPI gradient elicited a weak chemoattractant response (positive chemotactic drift). Serine (50 μM/mm) and glucose (1 mM/mm), respectively, were recognized as stronger chemoattractants, and the presence of EPI or PE had no effect on the chemotactic drift (Fig. 4D).

Finally, we used a capillary chemotaxis assay originally developed for *E. coli* (51). Briefly, in this assay, a glass capillary filled with an attractant is inserted into a cell suspension, and the number of cells in the capillary is counted after 60 min of incubation (Fig. 4E). Using this assay, we detected a chemotactic response of *V. campbellii* to chitin, glucose, serine, and also EPI (*SI Appendix*, Fig. S14). Although this assay is not as sensitive as the 3D microfluidic chemotaxis assay (thus requiring higher concentrations of the chemoattractant), we found a significant reduction in cell numbers in the glucose-filled capillary in the presence of EPI and PE, whereas NE, LAB, PRO, and OA did not show an effect (Fig. 4E). Furthermore, the effect of EPI was suppressed in the presence of LAB. These results are consistent with the specific effect of EPI,

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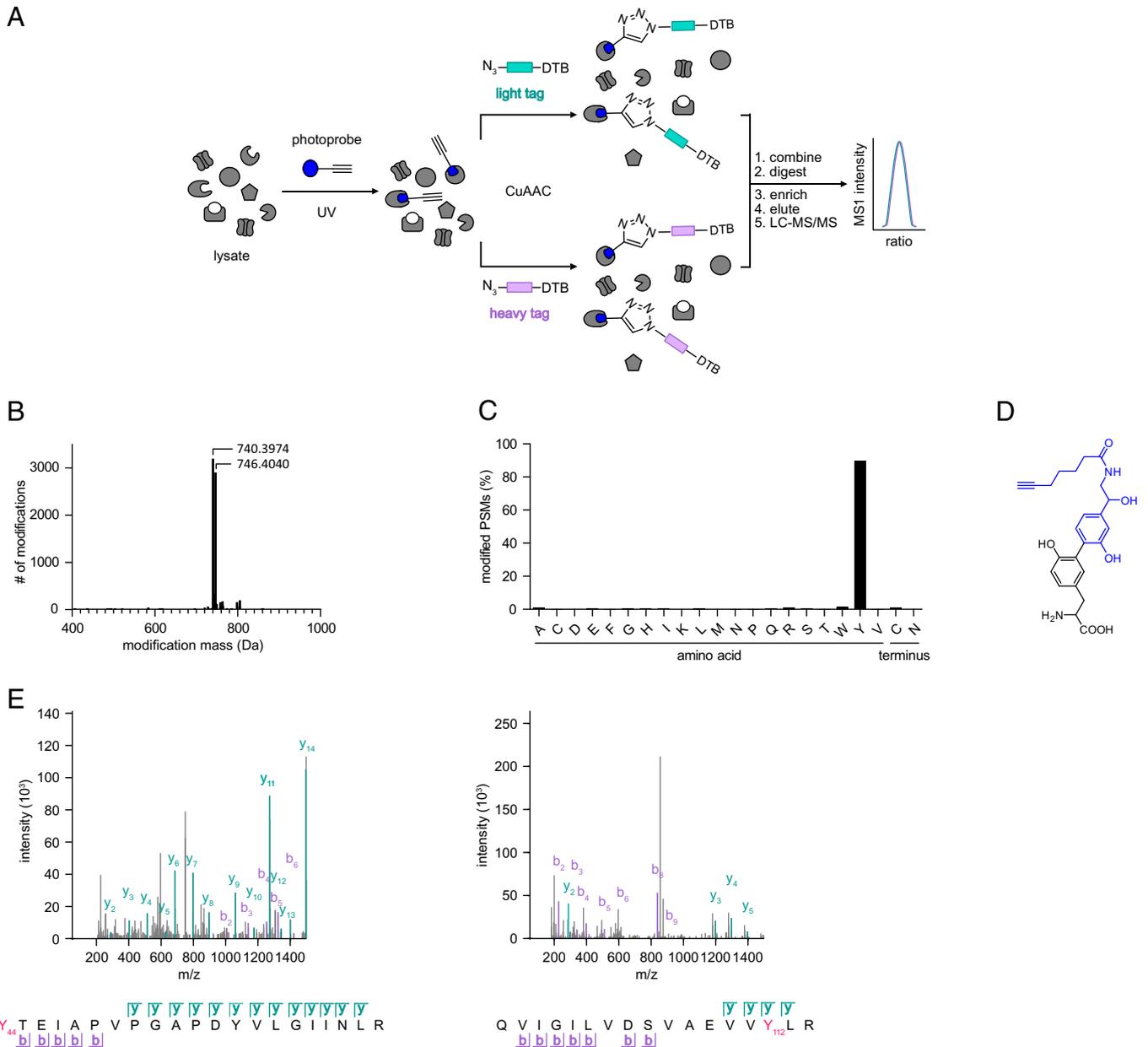
Fig. 3. Analysis of the modifications introduced by PE-P proteome-wide. (*A*) Workflow applied to study binding mode and sites of PE-P using isoDTB tags (39, 41). *V. campbellii* lysate was treated with 10 μM PE-P (blue circle), irradiated, split, and subjected to CuAAC with either light- (turquoise rectangle) or heavy-labeled (purple rectangle) isoDTB azide. Differentially labeled lysates were combined in a ratio of 1:1, and proteins were precipitated and digested. Modified peptides were enriched on avidin, eluted, and analyzed by LC-MS/MS. Peptides detected with a ratio of close to 1:1 heavy/light tag were considered true hits. (*B*) Unbiased, proteome-wide analysis of the masses of modification introduced by PE-P and the light or heavy isoDTB tag, respectively. (*C*) Analysis of the amino acid selectivity of the detected modification with PE-P. PSM, peptide spectrum matches. (*D*) Potential structure of the modified tyrosine regioisomer corresponding to the observed modification mass. (*E*) MS2 spectra of CheW peptides modified by PE-P. Identified b- and y-ions are labeled with purple and turquoise, respectively. The PE-P binding sites and their position in the sequence are indicated in red. All data are based on technical duplicates.

PE, and EPI + LAB on colony expansion (Fig. 1 *B* and *C*) and suggest that binding of EPI or PE to CheW affects the swimming behavior of *V. campbellii* in chemical gradients over long distances.

Discussion

In this study, we report the direct binding of the eukaryotic stress hormone EPI to CheW, the coupling protein between MCPs and CheA, located in the core of the chemotaxis signaling network in *V. campbellii*. It is known that mammalian host

stress associated with the release of catecholamine hormones not only increases susceptibility to bacterial infection but that EPI and NE also stimulate the growth and motility of enterobacteria such as *Salmonella* Typhimurium, *E. coli*, and *V. cholerae* (1–4). We used an untargeted chemical proteomics approach to identify the cellular targets of EPI. We chose PE, which is a structural homolog of EPI, for probe design as it still promotes colony expansion but cannot bind iron. In these studies, the chemotaxis coupling protein CheW proved to be one of the most prominent hits. This finding was confirmed by competitive labeling in the presence of EPI, PE, and LAB, the

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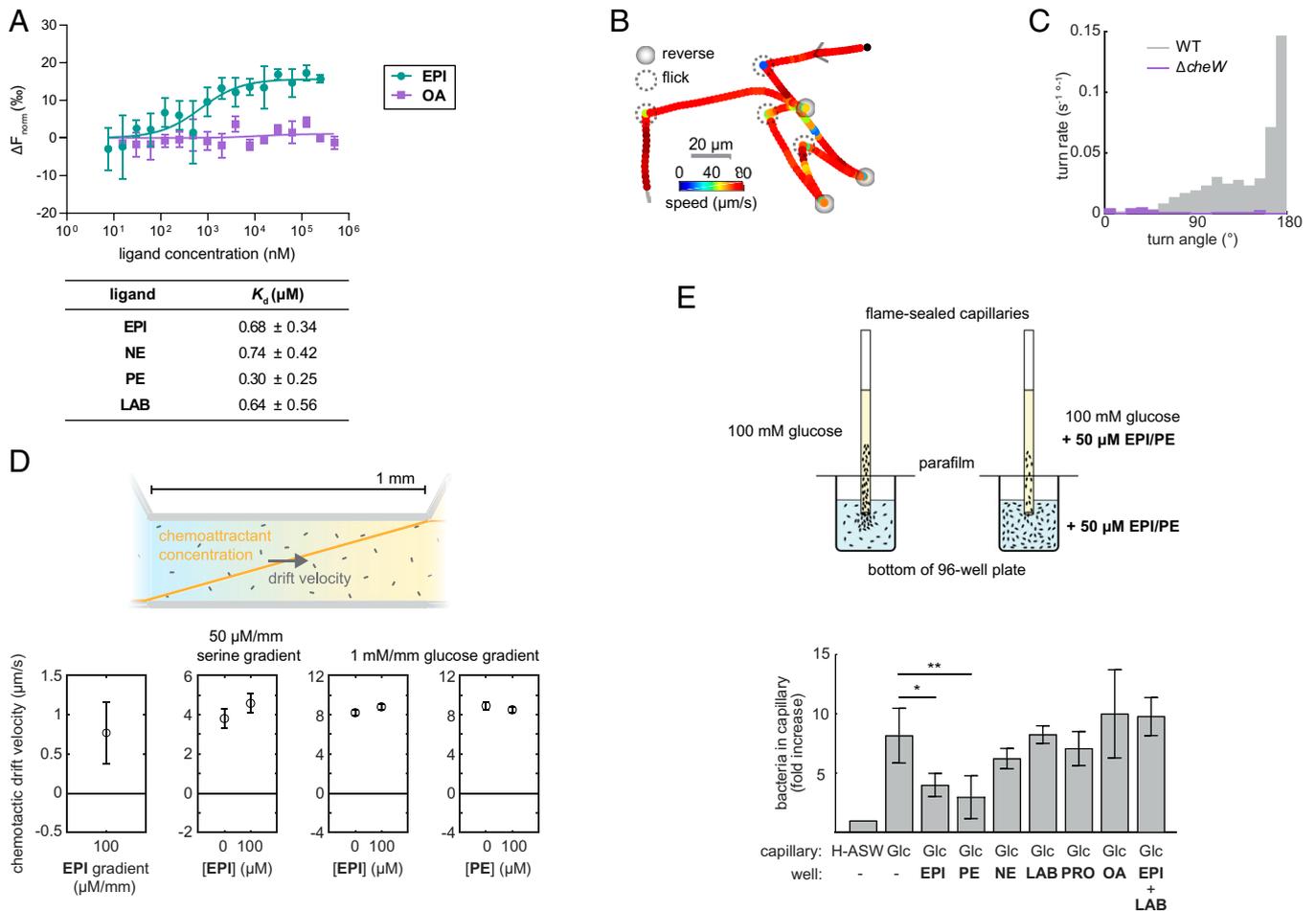
Fig. 4. Binding of adrenergic compounds to CheW in vitro and their effect on motility and chemotaxis of *V. campbellii* in vivo. (*A*) Binding of adrenergic compounds to purified CheW was determined using MST. Increasing concentrations of the test compounds (c = 7.63 nM to 500 μM) were titrated to a constant concentration of fluorescently labeled CheW (c = 50 nM), and temperature-induced changes in fluorescence were monitored in glass capillaries using the Monolith NT.115 (NanoTemper Technologies). K_d values were determined using the Thermophoresis + T-Jump signal for data analysis (NT Analysis software version 1.5.41, NanoTemper Technologies, *n* = 3 independent measurements, error bars represent SD). (*B*) Example 3D trajectory of *V. campbellii* showing run-reverse-flick motility with turns alternating between reversals and flicks. (*C*) Rate of turn events as a function of turn angle for wild-type (WT) and the Δ*cheW* mutant, based on 1,447 turn events detected in 1,295 s of trajectory time for WT and 29 events in 3,250 s of trajectory time for Δ*cheW*. (*D*) The 3D chemotaxis assay was performed by 3D tracking of cells in linear chemical gradients established in a 1-mm–long channel between two reservoirs of different chemical but matched bacterial concentration (concept is schematically shown in the *Upper* part). The drift velocity was determined as the average speed of motile cells along the gradient direction. Error bars represent SEM. The drift measured in a 100 μM/mm EPI gradient (left panel) is statistically significant at a *P* value of 0.029 in a one-tailed Student's *t* test against the null hypothesis of no drift. Differences in the drift to other compounds in the presence and absence of EPI or PE (other panels) are not significant. (*E*) In the capillary chemotaxis assay, bacteria swim from a reservoir in a microtiter plate into a glass capillary filled with an attractant (100 mM glucose) (concept is schematically shown in the *Upper* part). After 60 min, the number of bacteria in the capillary is determined by plating the cells on agar plates. The concentration of EPI and structurally related compounds was constant (50 μM) in the capillary and reservoir. Values were normalized to their respective control (H-ASW). Error bars represent SD, *n* = 4 biological replicates. Statistical significance was determined using an unpaired two-tailed *t* test (**P* < 0.05, ***P* < 0.01).

latter being a clinically applied antagonist of α-adrenergic receptors in eukaryotes. We also demonstrated direct binding of EPI, NE, PE, and LAB to purified CheW. The binding of EPI to CheW is an unexpected discovery but nevertheless consolidates previous reports seeking to find a specific chemoreceptor for catecholamines. For example, a *V. cholerae* mutant lacking the *qseC*-like gene still responded to catecholamines (4). Based on studies on the influence of catecholamines on chemotaxis, Sourjik and coworkers proposed that in *E. coli*, these hormones are sensed by a mechanism other than specific binding to an MCP (17).

The Δ*cheW* mutant of *V. campbellii* is motile but has an extremely low turning frequency, and it is nonchemotactic (Fig. 4C; *SI Appendix*, Fig. S14C). This finding is consistent with results for many other bacterial species that lack *cheW*

(summarized in Alexander et al.; ref. 43). Our 3D tracking assays revealed that EPI does not affect the turning rate or swimming speed of wild-type *V. campbellii* but acts as a weak attractant. These results indicate that binding of EPI to CheW does not phenocopy the behavior of the Δ*cheW* mutant (Fig. 4). However, in the capillary assay, which depends on the motility and chemotactic behavior of a population over large distances that span a wide range of gradient conditions, the presence of EPI and PE led to a significant decrease in the accumulation of cells in the glucose-filled capillary. While the 3D tracking experiments in linear gradients did not identify an effect of EPI or PE on chemotaxis to other compounds under the conditions tested, the very steep concentration gradients likely present in the immediate vicinity of the capillary are not accessible in the linear-gradient device. The effect of

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EPI might thus be restricted to steep gradients or other specific conditions that are not recapitulated in the linear-gradient assay. Another open question is whether the interaction between EPI and CheW is causal either for the chemoattraction to EPI or for the reduced chemoattractive effect of glucose in the capillary assay. The latter effect, however, is specific for EPI and PE because the presence of NE, LAB, PRO, and OA did not affect the movement of the cells along the glucose gradient. Interestingly, LAB, which binds to CheW, prevents the effect of EPI and PE both in the capillary-based chemotaxis and in the soft agar assays. The lack of activity of LAB itself is reminiscent of the behavior of classical pharmacological antagonists, which typically block agonist activity by binding to their target proteins but without exerting any effects themselves. Similar to its weak effect on colony expansion, NE was barely active in the capillary-based chemotaxis assay. As determined by MST, NE had a lower affinity for CheW compared to all tested compounds, which could explain these observations. Overall, our results suggest that binding of EPI and PE to CheW influences the chemotactic control of the bacterium.

Both chemotaxis and net motility are known to affect the infectivity of *V. cholerae* (52). The results described here could potentially provide an explanation for the hyperinfective transient phenotype of stool-derived *V. cholerae* (53–55). Although the stool-derived *V. cholerae* are highly motile, many chemotaxis genes, including *cheW* and *cheR*, are repressed compared to in vitro grown cells, resulting in a smooth-swimming state. Our finding that EPI targets CheW suggests a previously unknown mechanism for such host-triggered phenotypes. This work reveals a previously unknown role of CheW as adrenergic receptor that, given its conserved position in the core of chemotaxis complexes, could have broad implications for many other bacterial species.

Materials and Methods

Chemical Synthesis. For synthetic procedures and compound characterization data, see *SI Appendix*, Supplementary Methods and Figs. S1, S15–S19.

General Information. Detailed information on compounds, bacterial strains, culture conditions, preparation of overnight cultures, and media composition are provided in the *SI Appendix*, Supplementary Methods and Tables S3–S6.

Growth Assays. A *V. campbellii* overnight culture was diluted 1:100 into Kim-Epstein (KE) medium (10 mL) (56) or lysogeny broth containing 3.5% (wt/vol) NaCl (LB35) medium (60 mL) and grown until early exponential growth phase (optical density at 600 nm [OD_{600}] 0.5, KE medium) or early stationary phase (OD_{600} 5.0, LB35). The KE medium was modified as follows: The pH was adjusted to 7.6 with the corresponding phosphate buffer, the salt concentration was increased to 2% (wt/vol) NaCl, and $FeSO_4$ was omitted. Compounds were added from DMSO stocks to 50 μM into a clear flat-bottom 96-well plate. Next, 200 μL bacterial culture previously diluted to OD_{600} 0.01 in KE medium + 20 mM $NaHCO_3$ or in KE medium + 20 mM $NaHCO_3$ supplemented with 100 μg/mL human apo-transferrin (Sigma-Aldrich) or to OD_{600} 0.005 in LB35 or in LB35 supplemented with 30% (vol/vol) of adult bovine serum (Sigma-Aldrich) was added. The plates were incubated in an Infinite M200 Pro plate reader (Tecan) at 30 °C with continuous shaking (KE medium) or with 20 s shaking every 5 min (LB35 medium). The optical density at 600 nm was measured every 10 to 30 min. Blank values (only medium) were subtracted from data values, and data were plotted using GraphPad Prism.

Soft Agar Colony Expansion Assay. Soft agar colony expansion assays were performed as described previously on LB35 plates containing 0.3% (wt/vol) agar (5). Catecholamines and antagonists were dissolved in water and added as supplements directly to the autoclaved medium before pouring plates. As control, the appropriate volume of water was added to the plates. *V. campbellii* overnight culture was diluted in fresh LB35 (OD_{600} 1), and 5 μL was dropped in the center of the plate with six independent replicates for each condition. After an incubation of 24 h at 30 °C, the expansion of the colony (diameter) was measured. Radial expansions were normalized to an untreated control, and significance was determined performing a one-way ANOVA with Tukey's post hoc test.

Preparative Photolabeling with PE-P. Overnight cultures of *V. campbellii* were diluted 1:100 into 60 mL fresh medium and grown until early stationary phase (30 °C, 200 rpm, 7 h, OD_{600} ~5.0 to 5.2). Bacteria were harvested by centrifugation (6,000 × *g*, 10 min, 4 °C), washed with PBS (10 mL), and adjusted to OD_{600} 4.0 in 10 mL PBS. Competitors PE, EPI, LAB, PRO, or DMSO were added from 1,000-fold concentrated DMSO stocks to the final concentrations as indicated, and the suspensions were incubated 15 min, 30 °C, 200 rpm in 50-mL falcons with the lids fixed loosely. Next, DMSO or the photoprobe PE-P was added from a 1,000-fold concentrated stock (10 mM) to a final concentration of 10 μM and incubated 1 h, 30 °C, 200 rpm. Samples were transferred to 10-cm dishes and irradiated for 10 min with UV light (UV low-pressure mercury-vapor fluorescent lamp, Philips TL-D 18W BLB, 360 nm maximum) on a cooling pack. Labeled bacteria were centrifuged (6,000 × *g*, 10 min, 4 °C), and the pellet was washed twice with cold PBS (1 mL). Pellets were flash frozen and stored at −80 °C. Pellets were resuspended in 1 mL PBS + ethylenediaminetetraacetic acid-free protease inhibitor (Roche) and sonicated 2 × 15 s, 60% intensity, on ice. Following centrifugation (16,060 × *g*, 30 min, 4 °C), the supernatant was removed ("soluble"), and the pellet was resuspended in 1% (wt/vol) SDS/PBS with sonication for 2 × 15 s, 40% intensity. Cell debris was pelleted (16,060 × *g*, 10 min, room temperature [RT]), and the supernatant was transferred into a new tube ("insoluble"). For analytical scale photolabeling and preparative photolabeling with EPI-P1, see *SI Appendix*.

CuAAC, Preparative Scale Photolabeling. Protein concentration was determined using the Roti-Quant kit (Carl Roth) and adjusted to ~1 μg/μL in 500 μL. SDS was added to 0.8% (wt/vol) in the "soluble" samples. Click reagents were added to the lysate from a premix to the following concentrations: 100 μM rhodamine-biotin-azide tag (10 mM stock in DMSO) (57), 1 mM $CuSO_4$ (50 mM stock in water), 1 mM Tris(2-carboxyethyl)phosphine hydrochloride (52 mM stock in water), and 100 μM Tris((1-benzyl-4-triazolyl)methyl)amine [1.667 mM stock in 20% (vol/vol) DMSO/*t*-BuOH] and incubated 1 h, 25 °C, 400 rpm. Proteins were precipitated in 2 mL acetone at −20 °C overnight, pelleted (20,450 × *g*, 15 min, 4 °C), and washed twice with 1 mL ice-cold methanol with sonication (1 × 10 s, 10% intensity). Pellets were air-dried, and proteins were resolubilized in 1 mM dithiothreitol (DTT), 0.2% (wt/vol) SDS/PBS with sonication (1 × 10 s, 10% intensity) and transferred to LoBind microcentrifuge tubes.

General MS Sample Preparation. Protein LoBind microcentrifuge tubes and MS-grade reagents were used throughout MS sample preparation.

Enrichment, Alkylation, and Digest for Photoaffinity Labeling Experiments. Per sample, 50 μL avidin slurry (Sigma) was dispensed into a microcentrifuge tube and washed 3× with 0.2% (wt/vol) SDS/PBS (3 min, 400 × *g*). Protein samples were centrifuged (21,000 × *g*, 10 min, RT) to remove particulates, then added to the beads and incubated at RT under constant rotation for 1 to 2 h. Beads were pelleted, the supernatant was discarded, and beads were washed with 0.5 to 1 mL of the following solutions: 2 × 1% (wt/vol) SDS/PBS, then 3 × 4 M urea/PBS, and 3 × 50 mM triethylammonium bicarbonate buffer (TEAB). The beads were resuspended in 100 μL 50 mM TEAB and reduced with 10 mM DTT (from 250 mM stock in water) at 55 °C for 30 min with shaking. Next, beads were washed with 0.5 mL TEAB and resuspended in 100 μL TEAB, and thiols were alkylated with 20 mM iodoacetamide (from 500 mM stock in TEAB) at 25 °C from 30 min with shaking. Beads were washed twice with 100 μL TEAB and resuspended in 100 μL TEAB, and 1 μg trypsin was added (from 0.5 μg/μL in 50 mM acetic acid, Promega). Proteins were digested at 37 °C for 14 h under vigorous shaking. The digest was quenched with formic acid [1% (vol/vol) final concentration (pH 2 to 3)], beads were washed twice with 100 μL 0.1% (vol/vol) formic acid, and the washes were combined with the supernatant.

Desalting on Stage Tip for Photoaffinity Labeling Experiments. Stage tips consisted of three layers of C-18 material (Empore C18 disk-C18, 47 mm, Agilent Technologies) plunged into p200 tips and were inserted into holes in the lids of microcentrifuge tubes. The following solutions were added and the stage tips centrifuged (≤1 to 2 min, 500 × *g*) after every addition: Stage tips were washed with 1 × 80 μL methanol and then equilibrated with 1 × 80 μL 80% (vol/vol) acetonitrile, 0.5% (vol/vol) formic acid and with 2 × 100 μL 0.5% (vol/vol) formic acid. Next, peptides were loaded and desalted with 1 × 150 μL 0.1% (vol/vol) formic acid. Stage tips were transferred to fresh LoBind microcentrifuge tubes, and the peptides were eluted with 100 μL 80% (vol/vol) acetonitrile, 0.5% (vol/vol) formic acid. Solvents were removed in a speed vac, and dry peptides were stored at −80 °C until analysis.

Chemoproteomic Experiments with isoDTB Tags. A pellet of *V. campbellii* grown to stationary phase (OD_{600} ~5.0, ~24 mL) was washed 3× with PBS and stored at −80 °C before lysis in 1.5 mL PBS with sonication (5 × 15 s, 60%

intensity, on ice), and insoluble proteins were removed by centrifugation (16,060 × g, 30 min, 4 °C). Protein concentration was adjusted to 1 mg/mL, and 2 mL lysate was labeled with 10 μM PE-P (2 μL of a 10 mM stock in DMSO) at 30 °C for 1 h, 200 rpm. Following 10 min UV irradiation (UV low-pressure mercury-vapor fluorescent lamp, Philips TL-D 18W BLB, 360 nm maximum) in a 6-well plate (Thermo Fisher Scientific), the lysate was split into 2 × 800 μL and adjusted to 1% (wt/vol) SDS [from a 10% (wt/vol) stock in PBS] before adding the Click reagents as for the photoaffinity labeling experiments, except using either heavy- or light-labeled isoDTB azide (100 μM final from a 5 mM DMSO stock, isoDTB azide synthesized as reported previously; ref. 39). After the Click reaction, heavy- and light-labeled lysates (800 μL each) were combined in 8 mL cold acetone and precipitated overnight at −20 °C. Precipitated proteins were centrifuged (10,178 × g, 10 min, 4 °C), the supernatant decanted, and the pellet resuspended in 1 mL cold methanol with sonication (10% intensity) and pelleted again (13,000 × g, 10 min, 4 °C). The methanol wash was repeated, and protein pellets were air-dried and resuspended in 300 μL 8 M urea in 0.1 M TEAB with sonication (10% intensity). Samples were centrifuged (16,249 × g, 3 min) and reduced with 10 mM DTT (15 μL of 201 mM stock in water) for 45 min at 37 °C, 850 rpm. Next, thiols were alkylated with 20 mM iodoacetamide (15 μl from a 400 mM stock in water) for 30 min at 25 °C, 850 rpm (protected from light), and the remaining iodoacetamide was quenched with 10 mM DTT for 30 min at 25 °C, 850 rpm. Then, 900 μ 0.1 M TEAB was added (to achieve pH ~8 and 2 M urea), and proteins were digested with 20 μg trypsin (40 μL from 0.5 μg/μL in 50 mM acetic acid, Promega) overnight at 37 °C with intense shaking. Per sample, 2 × 25 μL avidin slurry (Sigma-Aldrich) in Protein LoBind tubes was washed with 3 × 1 mL 1% (vol/vol) Nonidet P-40 in PBS with centrifugation (400 × g, 2 min). The tryptic digest was split into two portions, added to 600 μL 0.2% (vol/vol) Nonidet P-40 and then to the avidin beads, and incubated for 2.5 h with constant rotation. Beads were then centrifuged (1,000 × g, 2 min), the supernatant was discarded, and the beads were resuspended in 600 μL 0.1% (vol/vol) Nonidet P-40 and transferred to a centrifuge column (Thermo Fisher Scientific) recombining the two portions of one sample. Beads were washed with 2 × 600 μL 0.1% (vol/vol) Nonidet P-40, then with 3 × 600 μL PBS and with 3 × 600 μL water; after every washing step, the solutions were removed by suction. The columns were transferred into LoBind tubes, and peptides were eluted with 400 μL (in three batches) 50% (vol/vol) acetonitrile, 0.1% (vol/vol) formic acid in water with centrifugation (5,000 × g, 3 min). Solvents were removed in a speed vac, and dry peptides were stored at −80 °C until analysis.

Co-IP. For details on Co-IP experimental procedures and antibody generation, see *SI Appendix*, Supplementary Methods.

Peptide Reconstitution (All Proteomics Experiments). Dry peptides were reconstituted in 30 μL 1% (vol/vol) formic acid with vortexing and in a sonication bath (10 min) and filtered through centrifugal filters (0.22 μm, Durapore, polyvinylidene fluoride, Merck KGaA) pre-equilibrated with 300 μL 1% (vol/vol) formic acid (16,249 × g, 2 min, RT). For details on MS instrument settings and MS data analysis, see *SI Appendix*.

Plasmid Construction and Protein Purification. For a detailed description of the construction of the *V. campbellii* ΔcheW knockout strain, the plasmid coding for N-terminally His6-tagged CheW, and CheW purification, see *SI Appendix*, Supplementary Methods and Tables S6 and S7.

Microscale Thermophoresis. PD-10 desalting columns packed with Sephadex G-25 resin (GE Healthcare) were used to exchange 6His-CheW protein buffer to MST buffer [PBS with 0.05% (vol/vol) Tween 20]. Purified CheW was labeled using the RED-Tris-NTA Labeling kit (NanoTemper Technologies) according to the manufacturer's instructions. Ligands were dissolved in MST buffer and serially diluted. For thermophoresis, a constant concentration of fluorescently labeled 6His-CheW (100 nM) was mixed with increasing ligand concentrations, resulting in a final concentration of 50 nM labeled 6His-CheW and final ligand concentrations in a range of 7.63 nM to 500 μM. After 10 min incubation at RT, followed by centrifugation (10,000 × g, 10 min) to remove aggregates, the solution was soaked into Monolith NT.115 Series Standard Treated Capillaries. MST measurements were carried out using a Monolith NT.115 instrument (NanoTemper Technologies) with 60% light-emitting diode/excitation power and medium MST power (40%). Three independent measurements were analyzed (NT Analysis software version 1.5.41, NanoTemper Technologies) using the signal from Thermophoresis + T-Jump.

3D Motility Assay. First, 20 μL bacterial culture was added to 1 mL Tris/Mg/NaCl buffer (TMN) (50 mM Tris/HCl, pH 7.4, 5 mM glucose, 100 mM NaCl, 5 mM MgCl2) containing EPI at the specified concentration, mixed gently, and

left on the bench for 30 min. The solutions were then flowed into sample chambers, consisting of three layers of parafilm as spacers between a microscopy slide and a #1 coverslip that had been heated and pressed to seal. After filling, the ends of the filled chamber were sealed with molten valap (a mixture of vaseline, lanolin, and paraffin) and immediately brought to the microscope for recording, all within 60 min of dilution from the day culture. EPI was diluted into TMN from a 50 mM stock solution in DMSO stored at −20 °C within 3 h before the experiment.

3D Chemotaxis Assays. Cells were prepared as for 3D motility assays. For glucose chemotaxis experiments, glucose was omitted in the motility medium TMN. Then, 3D chemotaxis experiments were performed using a high-throughput chemotaxis assay (50) using a commercially available microfluidic device (Ibidi) consisting of two 65-μL reservoirs connected by a 1-mm–long channel with a height of 70 μm and a width of 1 mm. *V. campbellii* cultures were diluted into chemotaxis buffer without (creating solution A) or with the putative chemoattractant (creating solution B) to a target OD600 of 0.008 for EPI gradients or OD600 0.005 for serine and glucose gradients. Chemotaxis buffer consisted of TMN, with an added background of EPI or PE for some experiments as specified, and without glucose for glucose gradients. Chemoattractants included EPI, L-serine, and D-glucose at the specified concentrations. First, the entire microfluidic device was overfilled with solution A. Then, the content of one reservoir was exchanged by solution B. A linear chemical gradient was established in the narrow channel between reservoirs within ~30 min and stable for several hours. About 40 to 60 min after closing the device, 3D bacterial trajectories were acquired in the middle of this gradient. For experiments with EPI gradients, EPI was prepared as a 40 mM stock in TMN within 3 h before the experiment. For serine chemotaxis experiments, EPI was prepared as 20 mM stock in TMN within 3 h before the experiments. For glucose chemotaxis experiments, EPI and PE were prepared as 60 mM stock in water within 3 h before the experiment. For data acquisition and analysis of 3D trajectories, see *SI Appendix*.

Chemotaxis Capillary Assay. The capillary assay was performed following a published protocol (51) adapted for *Vibrio* species (58, 59). Briefly, *V. campbellii* overnight cultures were diluted into LB35 medium (1:100) and grown to OD600 0.5. The cells were gently washed three times (10 min, 2,000 × g) and resuspended in Hepes-buffered artificial seawater [H-ASW: 100 mM MgSO4, 20 mM CaCl2, 20 mM KCl, 400 mM NaCl, and 50 mM Hepes (pH 7.5)] (60). The OD600 was adjusted to 0.1, and 200 μL culture was transferred into a 96-well plate. The plate was covered with three layers of parafilm, and the open end of a flame-sealed 1-μL capillary (64 mm, Drummond Scientific) was inserted into the bacterial suspension. The capillaries were filled with either H-ASW alone or with attractants dissolved in H-ASW. Solutions containing attractants and cell suspensions were supplemented with catecholamines and antagonists as indicated. The hormones were either dissolved in H-ASW or diluted from a 100-fold concentrated stock solution in 0.1 M HCl prepared immediately before the experiment (EPI and NE) and added directly after the wash steps. After 60 min incubation at room temperature, the contents of the capillaries were expelled and plated in appropriate dilutions on LB agar plates containing carbenicillin. The plates were incubated at 30 °C overnight, and colony-forming units were enumerated. Each experiment was conducted at least three times with four technical replicates per condition. Statistical significance was determined using an unpaired two-tailed t test (*P < 0.05, **P < 0.01).

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2.4 Division of labor and collective functionality in *Escherichia coli* under acid stress

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Division of labor and collective functionality in *Escherichia coli* under acid stress

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The acid stress response is an important factor influencing the transmission of intestinal microbes such as the enterobacterium *Escherichia coli*. *E. coli* activates three inducible acid resistance systems - the glutamate decarboxylase, arginine decarboxylase, and lysine decarboxylase systems to counteract acid stress. Each system relies on the activity of a proton-consuming reaction catalyzed by a specific amino acid decarboxylase and a corresponding antiporter. Activation of these three systems is tightly regulated by a sophisticated interplay of membrane-integrated and soluble regulators. Using a fluorescent triple reporter strain, we quantitatively illuminated the cellular individuality during activation of each of the three acid resistance (AR) systems under consecutively increasing acid stress. Our studies highlight the advantages of *E. coli* in possessing three AR systems that enable division of labor in the population, which ensures survival over a wide range of low pH values.

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The acquisition of acid resistance (AR) is an important property of *Escherichia coli* and many other neutralophilic bacteria that enables survival in acidic environments, such as the human gastrointestinal tract or acidic soils[1,2]. Although the cytoplasmic membrane is impermeable for protons, some protons enter the cytoplasm through protein channels, transient water chains, or damaged membranes[3,4]. Bacteria are generally able to maintain a fairly constant internal pH when grown in a wide range of media at different external pH values[5,6]. Despite this, pH homeostasis varies among individual bacterial cells as reported amongst others for *E. coli* and *Bacillus subtilis*[7]. Interestingly, persister cells of *E. coli* display a lower intracellular pH allowing survival after antibiotic treatment[8]. Nevertheless, after exposure of *E. coli* to an external pH of 5.8, the pH of its cytoplasm drops transiently; however, the pH rapidly returns to a neutral level due to the intrinsic buffering capacity of the cytoplasm or alterations in the flux of ions[9]. A lower external pH such as 4.4 causing a lower intracellular pH of around 6.0 has adverse effects on all macromolecules of a cell, which might result in lowered enzyme activity, acid-induced protein unfolding, membrane damage, and DNA damage.

Besides its passive mechanisms, *E. coli* possesses several inducible AR systems to counteract acidic environments[1,3]. The major systems are the glutamate decarboxylase (Gad) system, arginine decarboxylase (Adi) system, and lysine decarboxylase (Cad) system (AR2, AR3, and AR4, respectively)[10–12]. AR1 does not require an amino acid and is regulated by the alternative σ-factor (RpoS) and the cAMP receptor protein[10,13]. The ornithine decarboxylase system (AR5) plays only a minor role in *E. coli* MG1655, yet has a more important role in avian pathogenic *E. coli*[14]. The core components of each of the three major AR systems are a proton-consuming amino acid decarboxylase and a cognate antiporter to excrete the decarboxylated (more alkaline) compounds in exchange with the corresponding extracellular amino acid. In this way, both the intracellular and extracellular pH values can be increased[3,11,15,16]. Each system is active at different external pH values and growth phases, and induction is regulated by a sophisticated interplay of membrane-integrated and soluble regulators.

Activation of the Gad System occurs during the transition of an *E. coli* culture to stationary phase and during exponential growth in acidified media. Furthermore, this system is essential for cell survival at an extremely low pH of 2.5[10,17–19]. The Gad system employs GadA and GadB, two pyridoxalphosphate-dependent decarboxylases that catalyze the proton-consuming decarboxylation of L-glutamate to generate γ-aminobutyrate (GABA), and GadC, the cognate antiporter that performs the import of L-glutamate and the export of GABA[17,20]. It is important to note that the activity of GadC is pH-dependent, and the antiporter preferentially exchanges protonated glutamate (Glu^0) in exchange for protonated GABA ($GABA^+$) at an external pH of 3.0 and lower[21–23]. The induction of the *gad* genes is rather complex. The main transcriptional regulator is GadE, whose expression is regulated by the transcription factors EvgA, GadW, GadX, and YdeO as well as σ-factor RpoS. RpoS is activated and stabilized in response to different conditions, including the stationary phase, various stress treatments, and for example by the signal transduction cascade proceeding from PhoQ/PhoP to RssB and IraM[24,25]. Furthermore, the EvgS/EvgA histidine kinase/response regulator system is a primary detector of mild acidic environments (pH 5) and additionally regulates the activation of the Gad system through a cascade of EvgA-YdeO-GadE regulators[26,27] (Fig. 1). In addition, RcsB is a critical partner of GadE and the binding of both regulators as a heterodimer to the GAD box activates *gadA* transcription[28–30].

The Adi system is responsible for the conversion of arginine to agmatine under the consumption of one proton (Fig. 1). The main components are the transcriptional activator AdiY, a member of the AraC-family; the arginine decarboxylase AdiA; and the arginine/agmatine antiporter AdiC. The genes encoding the Adi system have an unusual genomic arrangement in *E. coli*, as the gene of *adiY* is located downstream of *adiA* and upstream of *adiC*. The Adi system is maximally induced under the conditions of acidic pH (pH 4.4), anaerobiosis, and a rich medium[31]. The activity of AdiC is regulated in response to acidic pH and remains fully active at a pH of ≤6.0[32]. The expression of *adiY* seems to be indirectly influenced by the transcriptional repressor CsiR, as its overexpression results in the repression of *adiY* and *adiA*[16]. Under anaerobic conditions, the *adiY* mRNA can be base-paired by the small RNA SgrS resulting in post-transcriptional downregulation[33].

The Cad system, which is activated when *E. coli* is exposed to pH 5.8 in the presence of lysine, uses external lysine, which is converted to cadaverine by the lysine decarboxylase CadA under the consumption of one proton. The lysine/cadaverine antiporter CadB imports lysine and excretes the more alkaline cadaverine, thereby elevating the external pH[34]. Expression of the *cadBA* operon is activated by the membrane-integrated one-component regulator CadC, which is a representative of the ToxR-family[35] (Fig. 1). The pH-sensory function and the feedback inhibition by cadaverine could be assigned to distinct amino acids within the periplasmic sensory domain of CadC[36,37]. The availability of external lysine is transduced to CadC via the co-sensor and inhibitor LysP, which is a lysine-specific transporter[38,39]. Recently, we demonstrated that not only the copy number of CadC affects the dual-stress response[40], but also the noise of the target protein abundance. Using fluorophore-tagged CadB, a heterogeneous output in the single cells of *E. coli* occurred under mild acidic stress. Moreover, an increase in the CadC copy number was correlated with decreased heterogeneous behavior[41].

The ON/OFF behavior of the *E. coli* population in activating the Cad system prompted us to ask whether the "Cad-OFF" cells activate the Gad and/or Adi systems. Here, we used a fluorescent triple reporter to study the induction of the three inducible AR systems, Gad, Adi, and Cad, in individual *E. coli* cells under consecutively increasing acid stress. We herein report extensive heterogeneity and division of labor in the acid stress response of individual *E. coli* cells. For the first time, we present a model that explains the cellular individuality that occurs during activation of each of the three AR systems in the context of ensuring AR for the whole population over a wide range of acidic pH values.

Results

Heterogenous activation of the three inducible AR systems by acid stress in *E. coli*. In our previous studies, we demonstrated that heterogenous activation of the Cad system results in ~70% ON and ~30% OFF cells[41]. Here, we constructed a three-color reporter strain *E. coli* (*gadC:eGFP-adiC:mCerulean-cadB:mCherry*) to study whether the "Cad-OFF" cells activate the Gad and/or the Adi systems and whether these two systems are activated heterogeneously. In this strain, each of the antiporter genes (*gadC*, *adiC*, and *cadB*) is fused with a different fluorophore gene coding for eGFP, mCerulean, or mCherry, respectively (Fig. 1). These fusions were chromosomally integrated to avoid a copy number effect, and the functionality of the hybrid proteins was confirmed (Supplementary Fig. 1).

First, the three-color reporter strain was exposed to consecutively increasing acidic conditions (pH 7.6, pH 5.8, and pH 4.4) in a well-mixed environment (Fig. 2a). Then, at the indicated time
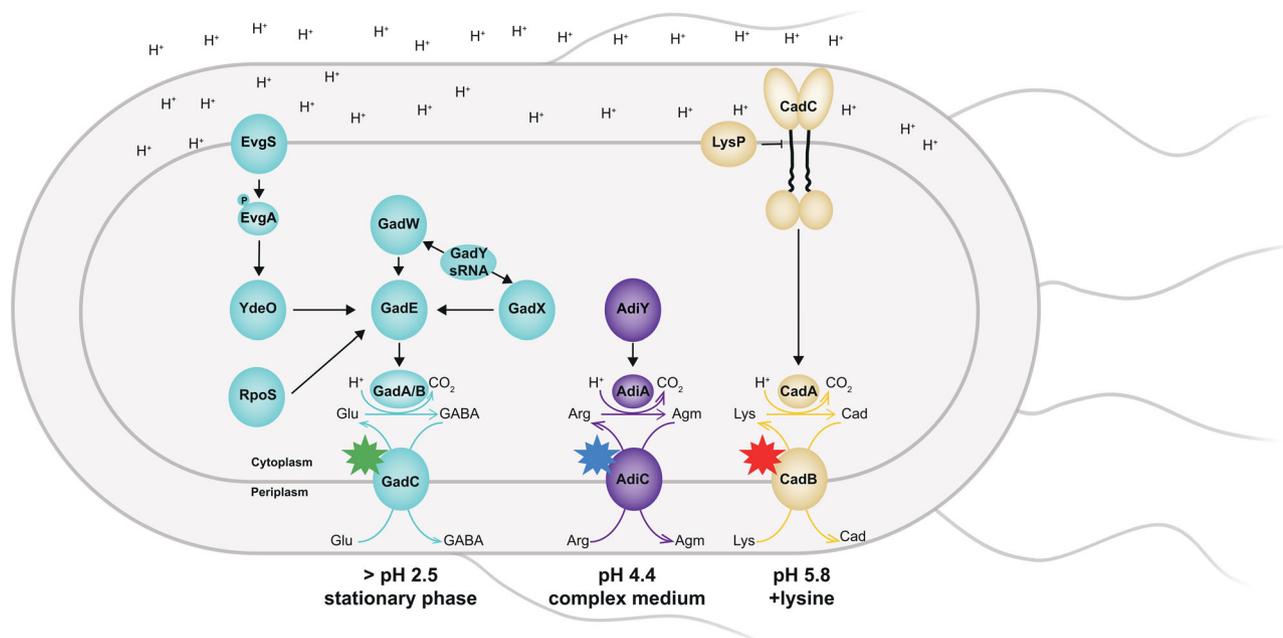
Fig. 1 The regulatory network of inducible amino acid decarboxylase-antiporter systems in _E. coli_ three-color strain. Regulated induction of the Gad (blue symbols), Adi (purple symbols), and Cad (yellow symbols) systems requires a network of membrane-integrated pH-sensors, namely EvgS and CadC; interconnected transcription factors; and the sRNA GadY. The glutamate decarboxylases GadA and GadB convert glutamate (Glu) into γ-aminobutyrate (GABA), which is excreted by GadC. The arginine decarboxylase AdiA converts arginine (Arg) into agmatine (Agm), which is excreted by AdiC. The lysine decarboxylase CadA converts lysine (Lys) into cadaverine (Cad), which is excreted by CadB. Adapted from[3, 15, 24, 25, 47, 74]. The stimuli leading to an induction of each of the three AR systems is indicated below each system. Each fluorescent hybrid of the three antiporter is indicated with a star symbol: GadC:eGFP (green star), AdiC:mCerulean (blue star), and CadB:mCherry (red star) according to the hybrid with the fluorophore eGFP, mCerulean, and mCherry, respectively.

points, cells were imaged, and the fluorescence intensities were quantified (Fig. 2b).

GadC:eGFP showed the highest production during the stationary phase and under the acidic conditions of pH 5.8 and pH 4.4 (Fig. 2b–d, t_{300}). In the stationary phase at physiological pH, GadC:eGFP was produced two times less than under acidic conditions; however, its heterogenous distribution increased, as indicated by noise values of 0.11 (defined as the standard deviation divided by the mean of log-transformed intensity values[42]) (t_{300}) (Fig. 2c; left panel). When cells were exposed to mild acid stress (pH 5.8), GadC:eGFP was homogenously distributed among individual cells in the _E. coli_ population and exhibited low noise values of 0.04–0.08 (Fig. 2b, c; left panel).

The Cad system is activated by mild acidic stress in the presence of lysine; therefore, CadB:mCherry became visible after 2.5 h of growth in a minimal medium supplemented with lysine at pH 5.8. At this time point, CadB:mCherry was heterogeneously distributed, as reflected by a high noise value of 0.37 (Fig. 2b–d), which is in perfect agreement with previous experiments of CadB tagged with eGFP[41]. The distribution of CadB:mCherry remained heterogeneous at pH 4.4 in complex medium, but the mean value increased 1.5-fold (Fig. 2c; right panel, t_{300}).

The Adi system is only activated under stronger acidic conditions, such as pH 4.4 in a complex medium with a noise value of 0.12 (Fig. 2b, c; middle panel).

Overall, the distribution of CadB:mCherry among single _E. coli_ cells under acid stress was not a symmetric Gaussian-like distribution but rather an asymmetric right-skewed distribution (Fig. 2d), as up to 83% of the population produced CadB:m-Cherry at varying high levels, and the remaining cells were in the OFF state, which was in agreement with previous findings using CadB:eGFP[41]. The distribution of GadC:eGFP and AdiC:mCerulean follows a symmetric Gaussian-like distribution (Fig. 2d),

which indicated that individual cells produce different amounts of GadC:eGFP and AdiC:mCerulean. However, almost all cells produced varying amounts of GadC:eGFP (99%), whereas only half of the population (47.7%) produced AdiC:mCerulean at pH 4.4 (Fig. 2d; right panel). As a control, non-tagged _E. coli_ MG1655 cells were examined under the same conditions; these cells exhibited low background fluorescence and extremely low noise values (Supplementary Table 1).

To exclude pH-effects on the fluorophores, each of the three fluorophores (eGFP, mCherry, and mCerulean) was fused to the antiporter gene _gadC_, because GadC is produced under all tested external pH values. Neither the different fluorophores nor the external pH affected the output or noise of the fluorescent hybrids (Supplementary Fig. 2; Supplementary Table 1).

Overall, the three inducible AR systems were heterogeneously activated with a different degree of noise. The Cad system showed the highest heterogeneous distribution (pH 5.8 t_{150}) of all three systems, whereas the Gad and Adi systems showed comparable noise values (0.11 and 0.12, respectively), but under different conditions. The Gad system was the most heterogenous during the stationary phase, whereas the Adi system was activated heterogeneously by stronger acid stress (pH 4.4).

Simultaneous activation of the AR systems in single _E. coli_ cells. Since the three AR systems showed a heterogenous output, although each had a different strength of heterogeneity, we examined their simultaneous activation within individual _E. coli_ cells under exposure to consecutively increasing acid stress (Fig. 2a).

The correlation between GadC:eGFP and CadB:mCherry was analyzed at the time point of the highest degree of heterogeneity of the Cad system (pH 5.8 t_{150}; Fig. 3a); however, no correlation occurred, thus indicating that there was a lack of dependency
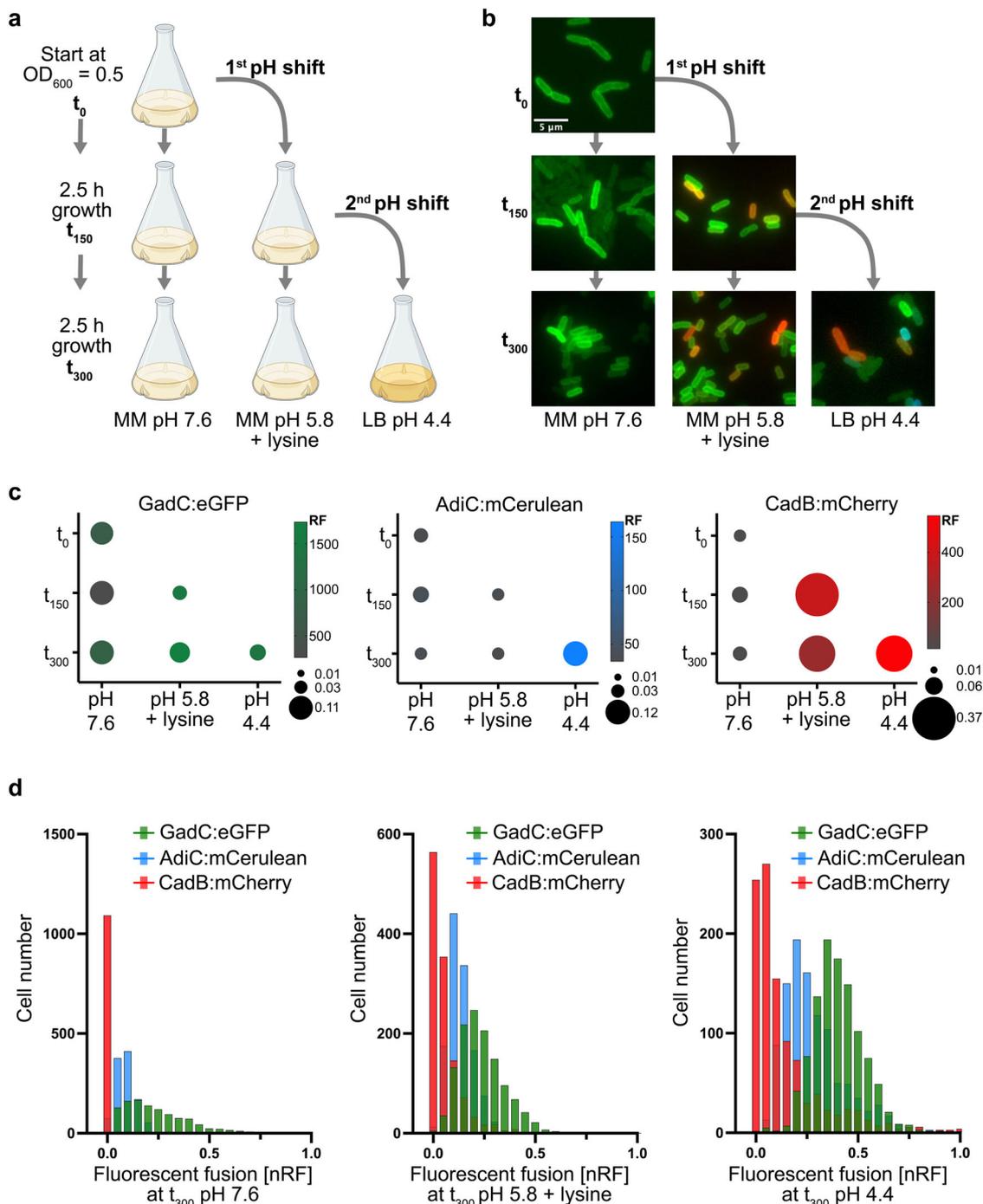
Fig. 2 Heterogenous activation of the three inducible AR systems in *E. coli* in response to acid stress. a Schematic representation of the experimental setup: at the exponential phase (t_0), the three-color reporter strain *E. coli gadC:eGFP-adiC:mCerulean-cadB:mCherry* was shifted from the MM at pH 7.6 to MM at pH 5.8 supplemented with 10 mM lysine, followed by a second shift to the LB medium at pH 4.4. In parallel, half of the culture was incubated under the original pH condition. **b** Fluorescent microscopic images of the three-color reporter strain *E. coli gadC:eGFP-adiC:mCerulean-cadB:mCherry*, cultivated as presented in (**a**), were taken at the indicated time points. Representative fluorescence overlay images are shown. Scale bar, 5 µm. **c** Quantified noise and mean RF were calculated for 1000 cells per condition and time point of the cultivated three-color reporter strain *E. coli gadC:eGFP-adiC:mCerulean-cadB:mCherry*, as presented in (**a**). Noise (standard deviation/mean of log-transformed values) is presented by the size of the dots (the higher the noise, the larger the size of the dot), and the average of the RF is presented with a color code for each fluorescent hybrid, GadC:eGFP (left panel), AdiC:mCerulean (middle panel), and CadB:mCherry (right panel). RF was quantified by using the MicrobeJ plugin of the ImageJ software of the fluorescent microscopic images. **d** Histogram presentation of the nRF quantified for 1000 cells per fluorescent hybrid, GadC:eGFP, AdiC:mCerulean, and CadB:mCherry, grown at t_{300} in MM pH 7.6 (left panel), MM at pH 5.8 supplemented with 10 mM lysine (middle panel), and LB medium at pH 4.4 (right panel), respectively. A comparison of the frequencies of CadB:mCherry with AdiC:mCerulean and with GadC:eGFP using the Chi-square test showed a *p*-value < 0.0001 for each time point. LB lysogeny broth, MM minimal medium, nRF normalized relative fluorescence intensity, RF relative fluorescence intensity.
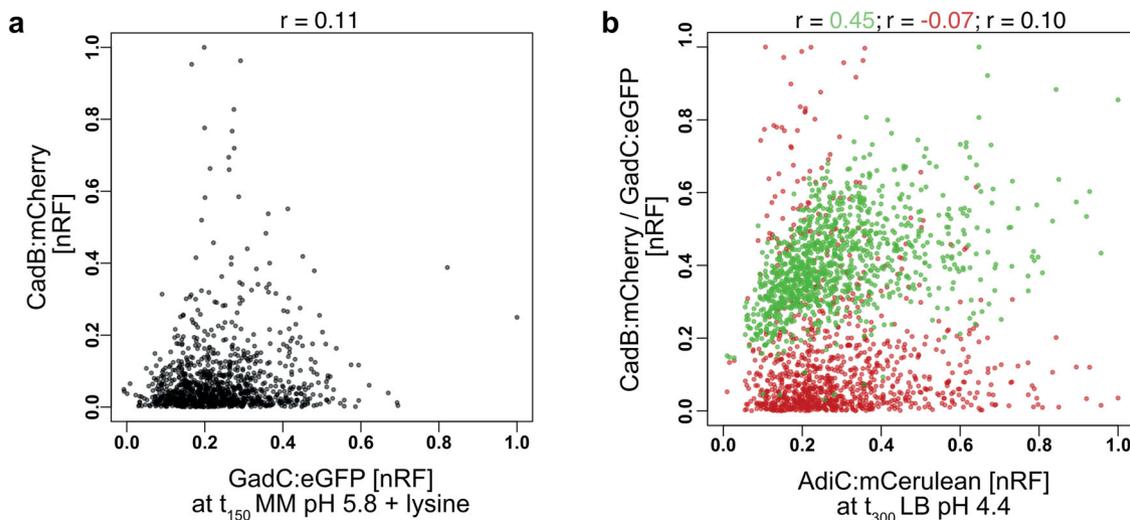
Fig. 3 Simultaneous activation of the AR systems in single *E. coli*. a Normalized fluorescence intensity (nRF) presented in a correlation plot of CadC:eGFP versus CadB:mCherry of the strain *E. coli gadC:eGFP-adiC.mCerulean-cadB:mCherry* in MM at pH 5.8 supplemented with lysine at t_{150}. Pearson's correlation coefficient (r) is shown on top of the graph: $r = 0.11$ for GadC:eGFP and CadB:mCherry with a *p*-value of $3.28e^{-4}$. **b** nRF presented in a correlation plot of AdiC:mCerulean versus GadC:eGFP (green dots) and versus CadB:mCherry (red dots) of *E. coli gadC:eGFP-adiC.mCerulean-cadB:mCherry* in LB medium at pH 4.4 at t_{300}. Pearson's correlation coefficient is shown on top of the graph: $r = 0.45$ for GadC:eGFP and AdiC:mCerulean (green) with a p-value of 4.4^{-56}; $r = -0.07$ for AdiC:mCerulean and CadB:mCherry (red) with a *p*-value of <0.05; $r = 0.10$ for GadC:eGFP and CadB:mCherry (black) with a *p*-value of <0.05. Pearson's correlation coefficient was calculated using GraphPad Prism 9.1.0. Correlation plots were created using R 4.0.3. LB lysogeny broth, MM minimal medium, RF relative fluorescence intensity, nRF normalized RF values, noise standard deviation/mean of log-transformed values.

between these two systems (Pearson's correlation coefficient, $r = 0.11$). During the stationary phase under strong acid stress (pH 4.4 t_{300}), when all three systems were activated, GadC:eGFP and AdiC:mCerulean showed a positive correlation ($r = 0.45$; green dots); however, no correlation occurred between CadB:mCherry and GadC:eGFP or AdiC:mCerulean ($r = 0.10$ and $r = -0.07$, respectively) (Fig. 3b). In summary, in stationary phase under strong acid stress, almost all cells activated the Gad system; however, simultaneous activation of the Cad and Adi systems did not occur.

Phylogenetic distribution of the components of the three AR systems within the bacterial kingdom. To identify connecting regulators between the three AR systems and to understand the segregation of the Adi and the Cad systems in *E. coli*, we used a bioinformatic approach to investigate the presence and distribution of regulatory components of the three AR systems within the bacterial kingdom. We used the antiporters GadC, AdiC, and CadB as the basis for the construction of phylogenetic trees and focused on discovering a potential co-occurrence of their specific regulators (Fig. 1; Supplementary Table 2).

According to our study, there are 1112 homologs of *E. coli* GadC that mainly occur in the phylum Proteobacteria (42.4%), such as *Enterobacteriaceae* (31.1%, with 27.8% belonging to *Escherichia*); *Morganellaceae* (4.5%); and *Yersiniaceae* (3.0%). Moreover, 51.3% of the GadC homologs belong to the bacteria of Firmicutes (56.0%), such as *Listeriaceae* (14.8%), *Enterococcaceae* (14.1%), *Clostridaceae* (9.8%), *Lactobacillaceae* (7.3%) and *Streptococcaceae* (5.2%) (Fig. 4a). Although GadC and GadB homologs are widely occurring, the regulators responsible for the sophisticated regulation of the Gad system of *E. coli* are mainly present in *Enterobacteriaceae* (Fig. 4a). GadW, GadX, GadY, GadE, and YdeO are predominantly conserved in *Escherichia* (89.3%) and, to some extent, in *Shigella* (0.04%) species. GadE is found in *Escherichia* and *Shigella* but also in other *Enterobacteriaceae* species, such as *Citrobacter freundii* and *Kluyvera ascorbate*. (Fig. 4a; Supplementary Table 2). The carbon induced

starvation transcriptional regulator CsiR, a member of the GntR-family, mainly occurs within γ-proteobacteria in 30.3% of species containing a GadC homolog, especially in *Enterobacteriaceae* (94.9%), such as *E. coli* (Fig. 4a). In *E. coli*, CsiR presumably affects all three AR systems: overexpression of CsiR stimulates binding to promoters of the Gad system, *gadX* and *gadW*; of the Cad system, *cadC*; and leads to lower expression of the *adiY* and *adiA* genes[16]. The sensor kinase EvgS is mainly found in *Enterobacteriaceae* (85.6%) but also in members of *Hafniaceae*, like *Hafnia alvei*, and *Yersiniaceae*, such as *Yersinia fredericksenii* and *Serratia fonticola*, all of which belong to γ-proteobacteria (Fig. 4a).

The components of the Adi and Cad systems are mainly conserved within γ-proteobacteria. In total, 756 AdiC homologs were identified within *Enterobacteriaceae* (70.8%), *Lysobacteraceae* (11.6%), *Yersiniaceae* (7.5%), and *Morganellaceae* (4.4%) (Fig. 4b). Of species containing an AdiC homolog, the Adi system-specific transcriptional activator AdiY is present in 69.0%, all of which, like *Escherichia* (68.9%), *Salmonella* (20.1%), and *Shigella* (4.9%), belong to the *Enterobacteriaceae* family. CsiR is distributed in a similar manner and is present in 70.4% of species containing an AdiC homolog. The 533 CsiR homologs that were identified mainly belonged to *Enterobacteriaceae* and *Hafniaceae* species, such as *Escherichia* (67.7%), *Salmonella* (19.7%), *Shigella* (4.9%), and *Hafnia* (2.8%) species. Moreover, CsiR homologs co-occur to a high degree with AdiY homologs, as 96.4% of species containing AdiY also have a CsiR homolog, except for *Hafina* species, which lack AdiY homologs (Fig. 4b; Supplementary Table 2).

The antiporter CadB of the Cad system was found mainly in γ-proteobacteria. 857 homologs were identified belonging to *Enterobacteriaceae* (55.8%), *Vibrionaceae* (20.0%), *Aeromonadaceae* (7.0%), *Hafniaceae* (5.6%), and *Yersiniaceae* (4.7%). CadB mainly co-occurs with CadA and CadC homologs. Of the species that have a CadB homolog, 94.3% also possess a CadA homolog of the cognate lysine decarboxylase of the Cad system, and 95.5% also have a homolog of the cognate pH-sensor CadC (Fig. 4c). In total, 573 homologs of the lysine-permease LysP were identified,
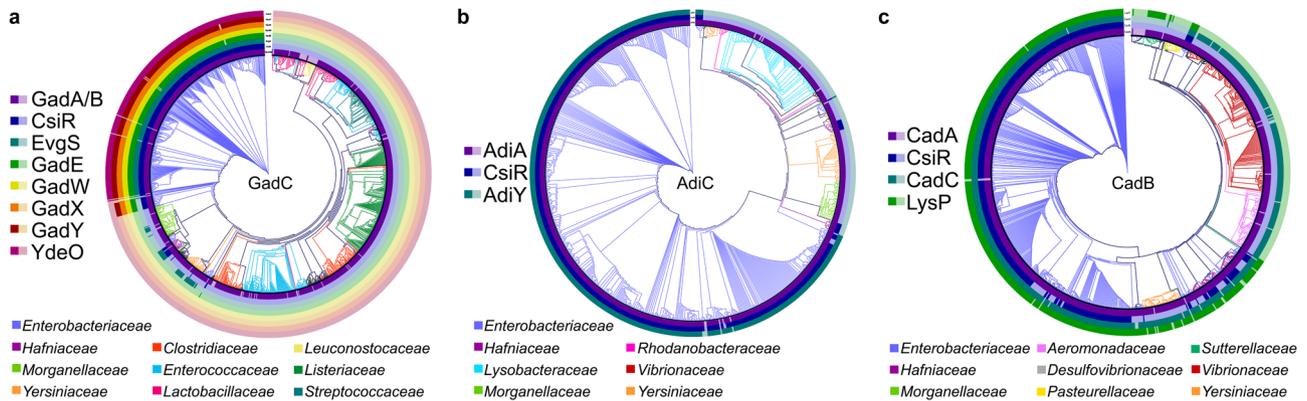
Fig. 4 Phylogenetic trees of the *E. coli* antiporters GadC, AdiC, and CadB and co-occurring regulators of the three AR systems within the bacterial kingdom. a The protein sequences of 1112 of *E. coli* GadC homologs were aligned, and a phylogenetic tree was generated, which is shown as a circular cladogram. The branches of the tree were colored according to the family of the organisms containing a GadC homolog. The presence of different regulatory components CsiR, EvgS, GadE, GadW, GadX, GadY, and YdeO and the decarboxylases GadA and GadB (GadA/B) is indicated by solid colors in the respective ring layer. Translucent colors represent components that were not detected. **b** The protein sequences of 756 *E. coli* AdiC homologs were aligned, and a phylogenetic tree was generated, which is shown as a circular cladogram. The branches of the tree are colored according to the family of organisms containing an AdiC homolog. The presence of the different regulatory components CsiR and AdiY as well as the decarboxylase AdiA is indicated by solid colors in the respective ring layer. If these components were not found, the colors are translucent. **c** The protein sequences of 857 *E. coli* CadB homologs were aligned, and a phylogenetic tree was generated, which is shown as a circular cladogram. The branches of the tree are colored according to the family of organisms containing a CadB homolog. The presence of the different regulatory components CsiR, CadC, and LysP as well as the decarboxylase CadA are indicated by solid colors in the respective ring layer. Translucent colors represent components that were not detected. Identifiers of all GadC, AdiC, and CadB homologs with an indication of the presence of their main regulators are summarized in Supplementary Data 1.

66.9% of which were found in species containing a CadB homolog. As previously reported, LysP predominantly co-occurs with the Cad system within the *Enterobacteriaceae* family (82.3%) and is mostly absent in *Vibrionaceae* (Fig. 4c; Supplementary Table 2)[41]. CsiR homologs are less coupled with CadB compared to other components of the Cad system. However, similar to LysP, CsiR is mainly found in *Enterobacteriaceae* and *Hafniaceae*, such as *Escherichia* (49.1%), *Klebsiella* (23.4%), *Salmonella* (14.6%), and *Hafnia* (4.1%) species. In summary, the regulatory components specific to *E. coli* of the three AR systems that were studied were predominantly conserved in *Enterobacteriaceae*. The Gad system was the most widespread AR system, whereas the Adi and Cad systems were restricted to γ-proteobacteria. Interestingly, the decarboxylases, together with their cognate antiporters, were more widely distributed than other regulatory components. The central regulators of the Cad system and the Adi system were more specific to the *Enterobacteriaceae* family.

Mechanisms of heterogenous activation of the three AR systems. To gain insight into the molecular mechanisms of heterogeneous activation of the AR systems in *E. coli*, we analyzed the importance of different transcriptional regulators. We focused mainly on the Adi and Cad systems, as these systems exhibited a higher degree of heterogeneity. First, we assessed the role of the regulator CsiR, which co-occurs with the components of the Gad, Adi, and Cad systems, mainly in *Enterobacteriaceae* (Fig. 4), and is supposed to modulate AR in *E. coli*[16]. However, artificially increased CsiR levels affected only the Adi system and not the Gad or Cad systems (Supplementary Table 1). Higher intracellular CsiR levels caused a 2.4-fold decrease in the amount of AdiC:mCerulean but did not affect the heterogenous distribution. The measured noise value was 0.10 under these conditions and is consistent with the wild type (Supplementary Table 1). These results are in good agreement with the suggested repressing impact of CsiR on the Adi system according to ChiP-Seq analysis[16].

Second, we already know that the native low CadC copy number generates heterogenous activation of the Cad system, as

an increase in the CadC copy number correlates with a decrease in heterogeneous distribution of the Cad system[41]. In addition, CadC itself is distributed heterogeneously[43]. Because of the mutually exclusive activation of the Cad and Adi systems (Fig. 3b), we tested whether CadC also has an influence on the Adi system. When we artificially elevated the copy number of CadC, we found four-times lower mean fluorescence of AdiC:mCherry (Fig. 5a). Moreover, the heterogenous distribution of AdiC:mCherry was much lower under this condition (Fig. 5a). It should be noted that the higher level of CadC decreased the amount of GadC:mCerulean by only two-fold in cells exposed to pH 5.8, and the already less heterogenous distribution of the Gad system was not affected (noise value of 0.05, which is comparable to that of the wild type) (Supplementary Table 1). In agreement with previous observations, the higher level of CadC increased the production of CadB:eGFP while reducing its heterogeneous distribution (Supplementary Table 1).

Third, AdiY also belongs to low copy number regulators with approximately 11–37 copies per cell[44]. Based on our previous experience with CadC[41], we analyzed the influence of the copy number of AdiY on heterogenous activation of the Adi system in pH 4.4-stressed cells. We increased the copy number by placing *adiY* under the control of the P_{BAD} promoter on a plasmid. The artificially increased copy number of AdiY resulted in a two-fold increase in the mean fluorescence and thus a higher amount of AdiC:mCerulean, which is not surprising, since AdiY is the main transcriptional activator of *adiC*. Furthermore, the heterogeneous distribution of AdiC:mCerulean was strongly reduced (Fig. 5b). However, *E. coli* cells producing a higher copy number of AdiY had a growth disadvantage under acid stress compared with wild-type cells (Fig. 5c). A similar effect on growth was previously demonstrated for *E. coli* cells producing a higher CadC copy number[41] (Supplementary Fig. 3).

The effect of CadC on the Adi system could be direct or indirect. A direct effect, i.e., that CadC binds upstream of *adiC* as a transcriptional regulator, can be excluded, as we did not find a second binding site for CadC in *E. coli*[43,45,46]. Therefore, we hypothesized that an indirect effect was caused by a CadC-
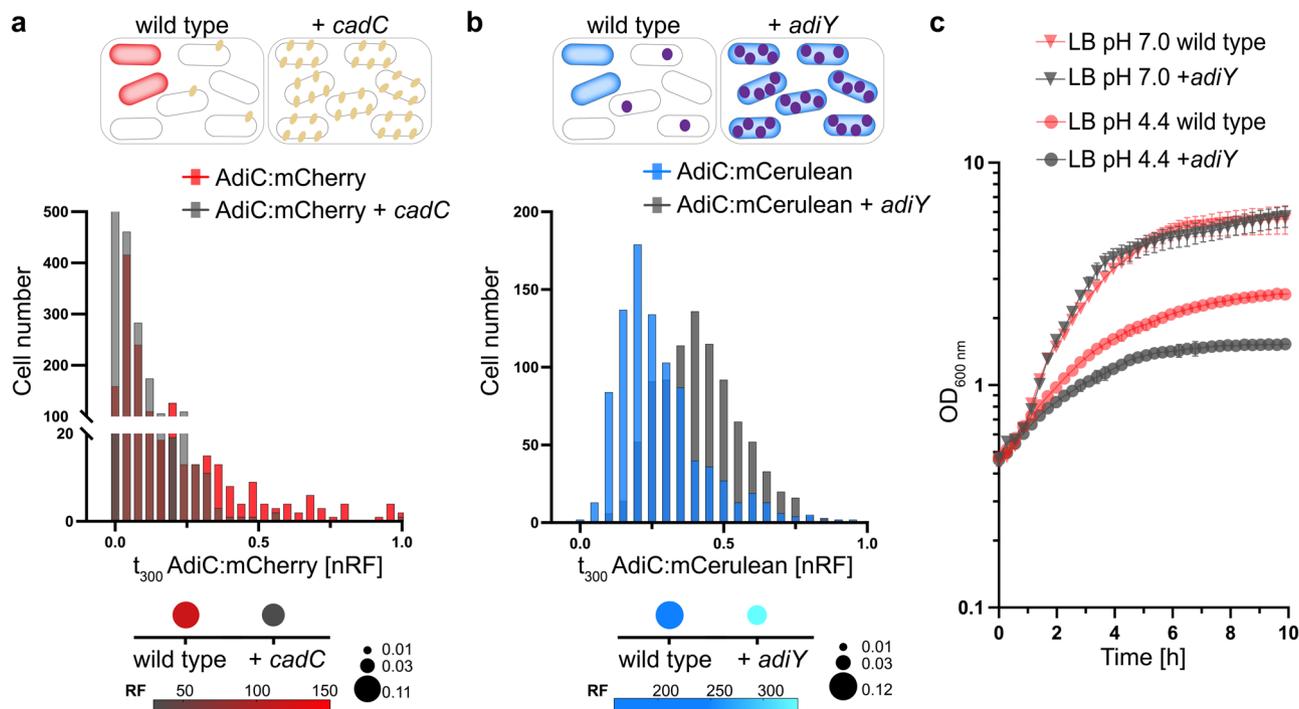
Fig. 5 An increase of the copy number of AdiY or CadC affects the degree of heterogeneity of the Adi system. a nRF values of cells expressing AdiC:mCherry in the wild type background (red) or with an elevated CadC copy number (gray) at pH 4.4 in LB medium at t_{300}. +$cadC$, the expression of $cadC$ under the control of the arabinose (0.1%)-inducible promoter in plasmid pBAD24. The comparison of frequencies of AdiC:mCherry in wild type or +$cadC$ cells using the Chi-square test showed a p-value < 0.001. On top of the histogram: red cells represent the AdiC:mCherry ON state; +$cadC$ is represented by yellow dots. Below the histogram: noise is presented by the size of the dots; average RF is represented by color intensity (Supplementary Table 1). RF was quantified using the MicrobeJ plugin of the ImageJ software of the fluorescent microscopic images for 1000 cells per condition. nRF normalized RF values. **b** nRF values of cells expressing AdiC:mCerulean in the wild type background (blue) or with an elevated AdiY copy number (gray) and cultivated as in (a). +$adiY$, expression of $adiY$ under the control of the arabinose (0.1%)-inducible promoter in plasmid pBAD24. Comparison of the frequencies of AdiC:mCerulean in wild type or +$adiY$ cells using the Chi-square test showed a p-value < 0.0001. On top of the histogram: blue cells represent the AdiC:mCerulean ON state; +$adiY$ is represented by purple dots. Below the histogram: noise level is represented by the size of the dots; average RF is represented by color intensity (Supplementary Table 1). RF was quantified using the MicrobeJ plugin of the ImageJ software of the fluorescent microscopic images for 1000 cells per condition. nRF normalized RF values. **c** E. coli MG1655 wild type transformed with the plasmid pBAD24-$adiY$ or with empty pBAD24 were grown in minimal medium at pH 5.8 supplemented with lysine and then shifted to LB medium of the indicated pH values. Growth (OD_{600}) was determined every 10 min in microtiter plates with continuous shaking. All experiments were performed three times ($n = 3$), and error bars represent standard deviation of the means. LB lysogeny broth.

induced increase in H^+-consuming lysine decarboxylase CadA, thus conferring moderate AR to all cells (homogeneous response; Fig. 6a). To test this hypothesis, we deleted $cadA$ from our reporter strain ($\Delta cadA$ $gadC:eGFP$-$adiC:mCerulean$-$cadB:m$-$Cherry$). Deletion of $cadA$ reduced the heterogenous distribution of AdiC:mCerulean and strongly increased the mean fluorescence of AdiC:mCerulean (by 11-fold), thus suggesting that many more cells produced AdiC:mCerulean compared with the wild type (Fig. 6a). Deletion of $cadA$ did not affect the heterogenous distribution of either GadC:eGFP or CadB:mCherry in pH-4.4-stressed cells. Only the overall mean level of CadB:mCherry was increased under this condition, which was due to the lack of negative feedback inhibition of cadaverine on CadC (Supplementary Table 1)[34,36].

To further support these results, we analyzed AdiY-driven pH-dependent activation of the $adiA$ promoter at the population level. In wild-type cells, $adiA$ expression began when cells were exposed to an acidic environment of pH < 4.8 (Fig. 6b). The increased number of CadC molecules did not change the pH-dependent induction profile but caused downregulation of the $adiA$ promoter activity. In contrast, in the $cadA$ mutant, the promoter activity of $adiA$ not only increased, but the onset of induction shifted to a higher external pH such as 5.8 (Fig. 6b). These data suggest that activation of the Adi system is influenced

by the intracellular pH, as a manipulation of the cytosolic proton concentration by either deletion of $cadA$ or overproduction of $cadA$ (triggered by increased CadC copy numbers) leads to increased activation or downregulation, respectively, of the Adi system in E. coli.

Discussion

To counteract acidic environments, many bacteria possess inducible AR systems that rely on H^+-consuming amino acid decarboxylases and their corresponding antiporters. The latter function as importers for corresponding amino acids and exporters for decarboxylated products. The number and complexity of the three inducible Gad, Adi, and Cad AR systems vary among bacteria and reflect an adaptation to the needs of their individual natural habitat[47]. Our phylogenetic analysis of the distribution of antiporters GadC, AdiC, and CadB revealed that the Gad system is the most widely distributed AR system, whereas the Adi system and Cad system are restricted to γ-proteobacteria (Fig. 4). Consistent with previous reports, Salmonella has Adi and Cad systems, whereas Shigella has the Gad system[47]. In Shigella, we also identified the Adi system, and in some species, the Cad system, i.e., in S. boydii and S. flexneri (Supplementary Table 2, Supplementary Data 1). However, not only does the number of
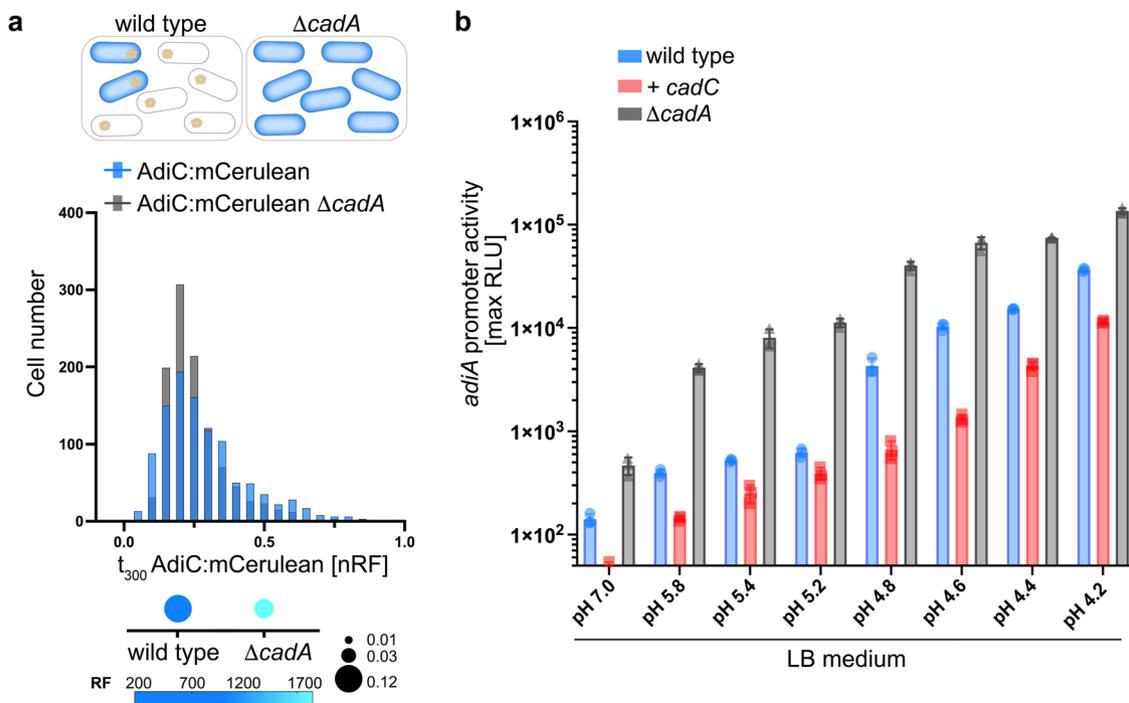
Fig. 6 Manipulation of intracellular stress resistance by either eliminating ($\Delta cadA$) or increasing ($+cadC$) the activity of the Cad system affects the degree of heterogeneity and pH-dependent induction of the Adi system. a Normalized RF values of cells expressing AdiC:mCerulean in the *E. coli* wild type background (blue) or the *cadA* mutant (gray). Cells were monitored after growth at pH 4.4 in LB medium at t_{300}. On top of the histogram, the cells are shown in a schematic overview, with blue cells representing the AdiC:mCerulean ON state and yellow dots representing CadA. Below the histogram, the noise (standard deviation/mean of log-transformed values) is presented by the size of the dots (the higher the noise, the larger the size of the dot), and the average RF is represented by a blue color (the more intense the blue, the higher the average relative fluorescence intensity). RF was quantified using the MicrobeJ plugin of the ImageJ software of the fluorescent microscopic images for 1000 cells per condition. The calculated mean RF and noise values are summarized in Supplementary Table 1. nRF normalized RF values. **b** *E. coli* MG1655 wild type and the *cadA* mutant (each transformed with the plasmid pBBR1-MCS5-P$_{adiA}$-lux) were grown in MM at pH 5.8 supplemented with 10 mM lysine and then shifted to LB medium of the indicated pH values. Luminescence and growth were determined every 10 min in microtiter plates with a Tecan Infinite F500 system (Tecan, Crailsheim, Germany). Data are reported as relative light units (RLUs) in counts per second per milliliter per OD$_{600}$, and maximal RLU at 1.9 h is shown. All experiments were performed three times ($n = 3$), and error bars represent standard deviation of the means. Growth over 10 h is presented in Supplementary Fig. 3 at selected pH values. LB lysogeny broth, MM minimal medium, RF relative fluorescence intensity.

AR systems possessed by bacteria differ, but the complexity of their regulation varies (Fig. 4). The most sophisticated regulatory network of AR systems is found in *E. coli*. Its close relatives *E. albertii* have almost all components as well but lack the EvgSA two-component system and YdeO.

For the first time, we investigated the activation of the three major AR systems in *E. coli* simultaneously at the single-cell level under consecutive increasing acid stress. By using the three-color reporter strain *E. coli gadC:eGFP-adiC:mCerulean-cadB:mCherry*, we confirmed that each of the three AR systems is specialized for a certain strength of acid stress under certain environmental conditions. The Gad system was activated under mild acid strength and during the stationary phase. The Cad system was induced at pH 5.8 and required the presence of lysine. The Adi system was induced at pH 4.4 and required the lysogeny broth medium, which contains tryptone and yeast extract. However, activation of the three systems differs greatly from cell to cell (Figs. 2 and 7). The Cad system showed the highest heterogeneous distribution of all three systems, when activated at pH 5.8 in the presence of lysine. Under almost all conditions, all cells of the population activate the Gad system but to varying degrees. The Adi system is activated heterogeneously under strong acid stress (pH 4.4) (Fig. 2).

Phenotypic variations can be beneficial for the *E. coli* population under acid stress. Our results provide a model of how the three inducible AR systems overlap and generate a division of labor and functional cooperation in the population (Fig. 7). All cells of the *E. coli* population individually adapt to mild acid stress by activating the Gad system to varying degrees, and the glutamate decarboxylases GadA and GadB might initially utilize intracellularly available glutamate under the consumption of protons. As described below, the antiporter GadC becomes more important under extreme acid stress. It should also be noted here that the decarboxylase GadB also undergoes a pH-dependent conformational change and exhibits an activity optimum at low pH[48]. Furthermore, glutamate is the most abundant intracellular metabolite, with a concentration of 100 mM, and its concentration is much higher compared to that of lysine (0.41 mM) and arginine (0.57 mM)[49]. Under stronger acid stress (pH 5.8 to pH 4.4), some cells in the population activate the Cad system, whereas others activate the Adi system. These two subpopulations each contribute to acid stress relief by secreting the more alkaline cadaverine and agmatine, respectively (Fig. 7). These two polyamines are considered common goods and contribute to an increase of the extracellular pH, which benefits the whole population. This behavior can be considered as an example of true division of labor in bacteria, as the metabolic burden of the individual cells to produce both the Adi and Cad systems is prevented, but nevertheless, the whole population benefits from the elevation of external pH by secretion of cadaverine and agmatine (Fig. 7). The fact that homogenous production of the components of the Adi system in all cells would be a burden for
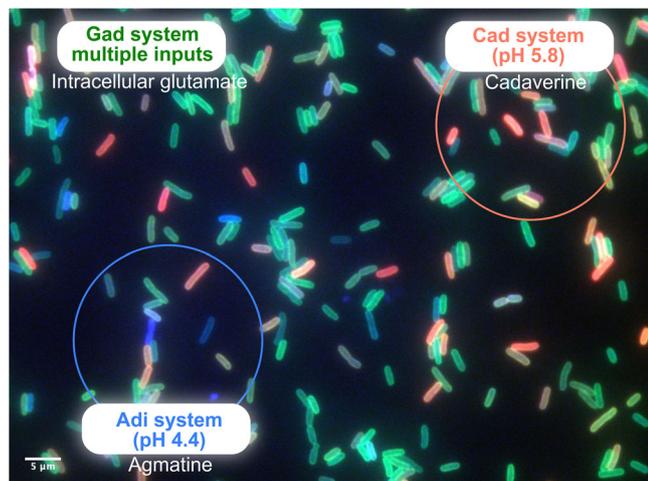
Fig. 7 Model of the heterogenous activation of three inducible acid resistance systems and division of labor in the *E. coli* population.
Fluorescent microscopic images of the three-color reporter strain *E. coli gadC:eGFP-adiC:mCerulean-cadB:mCherry* at pH 4.4 in a complex medium at t$_{300}$. All cells of the *E. coli* population individually adapt to acid stress by activating the Gad system to varying degrees (green fluorescent cells) due to multiple extracellular and intracellular inputs. These cells utilize primarily intracellularly available glutamate under consumption of protons to increase their internal pH. Under stronger acid stress (pH 5.8 to pH 4.4), some cells in the population activate the Cad system (red fluorescent cells), while others activate the Adi system (blue fluorescent cells). These cells secrete (as indicated by the rings) the more alkaline products cadaverine and agmatine, respectively, thereby contributing to acid-stress relief with an increase of the extracellular pH, which benefits the whole population. In addition, their internal pH is elevated by consuming protons due to the conversion of lysine to cadaverine and arginine to agmatine, respectively.

the population is clearly shown by the reduced growth rate under this condition (Fig. 6c), which was similar for a homogenous production of the Cad system[41] (Supplementary Fig. 3).

We were then interested in the molecular mechanisms that not only lead to this phenotypic heterogeneity but that are also heritable. CadC and AdiY are low copy number proteins; therefore, they are stochastically distributed among the cells of the population[44]. Here and in our previous studies[41], we showed that an increase of their copy number was sufficient to shift the population into homogeneous behavior. Cells that activated the Cad system are protected against acid stress due to the H$^+$-consuming activity of the CadA decarboxylase. It should be noted that CadA is produced on average with 60,000 copies per cell, which accounts for at least 2% of all cytoplasmic proteins in CadA-producing cells[50]. In cells lacking the Cad system, the intracellular pH decreases under strong external acid stress, such as pH 4.4. Our results have shown that any manipulation of the activity of the Cad system, such as its elimination (Δ*cadA*) or increase (+*cadC*), affected the degree of heterogeneity and pH-dependent induction of the Adi system, which suggests that the regulator of the Adi system is an intracellular pH sensor that senses a decrease of cytoplasmic pH.

Induction of the Gad system of *E. coli* is controlled by an unusually high number of transcription factors, the stationary sigma factor, and a small RNA GadY (Figs. 1 and 4a). Moreover, its activation is strongly influenced by the medium composition and growth phase[10,17]. The transcription factors GadX and GadW were already associated with higher noise levels of their target genes of unstressed cells[42] and potentially contribute to the heterogenous distribution of GadC:eGFP (Fig. 2b). In addition, the stationary sigma factor RpoS is known generate extensive

transcriptional heterogeneity[51–53]. Thus, the Gad system integrates multiple extracellular and intracellular inputs, which results in quantitative differences and continuous variation of phenotypic traits in the individual cells of the population. The noisy activation of the *gadBC* promoter enables prediction of the survival of single cells[54]. Usually, high levels of noise are found for genes that are involved in either the energy metabolism of carbon sources or adaptation to stress, like osmotic pressure, temperature extremes, starvation response, pH response, and mechanical, nutritional, or oxidative stress. Indeed, the Gad system is a major system under extreme acid stress (pH $\leq$ 2.5)[10]. The antiporter GadC preferentially transports protonated glutamate (Glu$^+$) in exchange for protonated GABA (GABA$^+$) at an external pH of 3.0 and lower[21–23]. This transport activity is crucial for protecting cells against incoming protons. It should be noted that at extremely low pH values, such as pH 2.5, only <0.01% of a non-preadapted population survives[27], and therefore, for maintenance of the population, it is extremely important that all *E. coli* cells produce the Gad system, albeit with different levels.

We can only speculate to what extent this model might hold up in a natural epitope of *E. coli*, such as digestive system of humans or animals. Before *E. coli* arrives in the intestine, it is exposed to an extreme acid stress with HCl in the stomach that this bacterium can only survive with the help of the Gad system. The mean pH in the proximal small intestine is 6.6 and increases in the terminal ileum to 7.5. Then, there is a sharp fall in pH to 6.4 in the caecum, and an increase in colon with a final value of 7.0[55]. Depending on the availability of the amino acids lysine and arginine, the Cad and Adi systems might be switched on under these conditions. The latter system is increasingly activated due to the anaerobic conditions[31]. Both systems will still be heterogeneously distributed due to the low copy number of the main regulators CadC and AdiY. Fermentation products, such as acetate, also activate the Gad system[56]. The extent to which microcolonies of *E. coli* exist in the colon, in which common goods such as cadaverine and agmatine can change the micromilieu, is also unclear, as is the extent to which the host plays a role in acid stress. In fact, the levels of putrescine, a conversion product of agmatine, and cadaverine were found to be 15- and 3-fold, increased, respectively, in the inflamed mouse intestine mono-colonized with *E. coli* compared to an equally colonized healthy mouse cohort[57].

Considered together, our results provide a model for the advantage of *E. coli* to have three AR systems that allow for division of labor in the population and ensure its survival over a wide range of low pH values, thus making this bacterium highly acid-stress resistant.

Methods

Bacteria and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were cultivated in LB medium (10 g/liter NaCl, 10 g/l tryptone, 5 g/l yeast extract) or in Kim-Epstein (KE) minimal medium[58] adjusted to pH 5.8 (MM pH 5.8) or pH 7.6 (MM pH 7.6), using the corresponding phosphate buffer. *E. coli* strains were incubated aerobically in a rotary shaker at 37 °C. KE medium was always supplemented with 0.2% (w/v) glucose. When indicated lysine was added to a final concentration of 10 mM.

If necessary, media were supplemented with 100 µg/ml ampicillin, or 50 µg/ml kanamycin sulfate. To allow the growth of the conjugation strain *E. coli* WM3064, *meso*-diamino-pimelic acid (DAP) was added to a final concentration of 300 µM.

Construction of plasmids. Molecular methods were carried out according to standard protocols or according to the manufacturer's instructions. Kits for the isolation of plasmids and the purification of PCR products were purchased from Süd-Laborbedarf (SLG; Gauting, Germany). Enzymes and HiFi DNA Assembly Master Mix were purchased from New England BioLabs (Frankfurt, Germany).

To construct different fluorescent fusions with *gadC, adiC* and *cadB*, to be inserted in-frame chromosomally, the corresponding flanking regions (600 bp upstream and downstream) of *gadC, adiC* or *cadB* were amplified by PCR using MG1655 genomic DNA as template. Plasmid pET-*mCherry-cadC*[43] was used as

Table 1 Strains and plasmids used in this study.

Strains	Relevant genotype or description	Reference
E. coli MG1655	K-12 F⁻ λ⁻ ilvG⁻ rfb-50 rph-1	[68]
E. coli DH5αλpir	endA1 hsdR17 glnV44 (≡ supE44) thi-1 recA1 gyrA96 relA1 φ80'lacΔ(lacZ)M15 Δ(lacZYA-argF)U169 zdg−232::Tn10 uidA::pir +	[69]
E. coli WM3064	thrB1004 pro thi rpsL hsdS lacZΔM15 RP4-1360 Δ(araBAD)567 ΔdapA1341::[erm pir]	W. Metcalf, Univ. of Illinois, Urbana
E. coli MG1655 cadB:egfp	Chromosomally integrated C-terminal cadB:egfp fusion in E. coli MG1655	[41]
E. coli MG1655 adiC:mCherry-cadB:egfp	Chromosomally integrated C-terminal adiC:mCherry and cadB:egfp fusion in E. coli MG1655	This work
E. coli MG1655 gadC:mCerulean-adiC:mCherry-cadB:egfp	Chromosomally integrated C-terminal gadC:mCeruleanᵃ, adiC:mCherry and cadB:egfp fusion in E. coli MG1655	This work
E. coli MG1655 adiC:mCerulean-cadB:egfp	Chromosomally integrated C-terminal adiC:mCerulean and cadB:egfp fusion in E. coli MG1655	This work
E. coli MG1655 gadC:mCherry-adiC:mCerulean-cadB:egfp	Chromosomally integrated C-terminal gadC:mCherryᵃ, adiC:mCerulean and cadB:egfp fusion in E. coli MG1655	This work
E. coli MG1655 cadB:mCherry	Chromosomally integrated C-terminal cadB:mCherry fusion in E. coli MG1655	This work
E. coli MG1655 adiC:mCerulean-cadB:mCherry	Chromosomally integrated C-terminal adiC:mCerulean and cadB:mCherry fusion in E. coli MG1655	This work
E. coli MG1655 gadC:egfp-adiC:mCerulean-cadB:mCherry	Chromosomally integrated C-terminal gadC:egfpᵃ, adiC:mCerulean and cadB:mCherry fusion in E. coli MG1655	This work
E. coli MG1655 ΔcadA gadC:eGFP-adiC:mCerulean-cadB:mCherry	In-frame deletion of cadA in E. coli MG1655 gadC:egfpᵃ-adiC:mCerulean-cadB:mCherry	This work
E. coli ΔgadC	Deletion of gadC (JW1487), gadC::Km	[70]
E. coli ΔadiC	Deletion of adiC (JW4076), adiC::Km	[70]
E. coli ΔcadA	Deletion of cadA (JW4092), cadA::Km	[70]
Plasmids		
pBAD24	Arabinose-inducible P_BAD promoter, pBR322 ori, Ampᴿ	[71]
pBAD-cadC	cadC under control of arabinose inducible promoter in pBAD24, Ampᴿ	[40]
pNTPS138-R6KT	mobRP4 + ori-R6K sacB; suicide plasmid for in-frame deletions, Kmᴿ	[72]
pNPTS138-R6KT-cadB:egfp-EC	pNPTS-138-R6KT-derived suicide plasmid for in-frame insertion of cadB:egfp in E. coli MG1655, Kmᴿ	[41]
pNPTS138-R6KT-gadC:egfp	pNPTS-138-R6KT-derived suicide plasmid for in-frame insertion of gadC:egfpᵃ in E. coli MG1655, Kmᴿ	This work
pNPTS138-R6KT-cadB:mCherry	pNPTS-138-R6KT-derived suicide plasmid for in-frame insertion of cadB:mCherry in E. coli MG1655, Kmᴿ	This work
pNPTS138-R6KT-adiC:mCerulean	pNPTS-138-R6KT-derived suicide plasmid for in-frame insertion of adiC:mCerulean in E. coli MG1655 strains, Kmᴿ	This work
pNPTS138-R6KT-gadC:mCherry	pNPTS-138-R6KT-derived suicide plasmid for in-frame insertion of gadC:mCherryᵃ in E. coli MG1655, Kmᴿ	This work
pNPTS138-R6KT-gadC:mCerulean	pNPTS-138-R6KT-derived suicide plasmid for in-frame insertion of gadC:mCeruleanᵃ in E. coli MG1655, Kmᴿ	This work
pNPTS138-R6KT-adiC:mCherry	pNPTS-138-R6KT-derived suicide plasmid for in-frame insertion of adiC:mCherry in E. coli MG1655, Kmᴿ	This work
pBAD-His₆-csiR	N-terminal His₆-tagged csiR in pBAD24, Ampᴿ	This work
pBAD-His₆-adiY	N-terminal His₆-tagged adiY in pBAD24, Ampᴿ	This work
pBBR1-MCS5-TT-RBS-lux	luxCDABE and terminators lambda T0 rrnB1 T1 cloned into pBBR1-MCS5 for plasmid-based transcriptional fusions, Gmᴿ	[73]
pBBR1-MCS5-P_adiA-lux	adiA promoter controlling expression of luxCDABE, in pBBR1-MCS5-TT-RBS-lux, Gmᴿ	This work
pNPTS138-R6KT-ΔcadA	pNPTS-138-R6KT-derived suicide plasmid for in-frame deletion of cadA in MG1655 gadC:eGFPᵃ-adiC:mCerulean-cadB:mCherry, Kmᴿ	This work
pET-mCherry-cadC	N-terminal fusion of CadC with mCherry in pET16b, Ampᴿ	[43]
pK18mob2-TriFluoR	pK18mob2 Km carrying triple reporter construct with MCS I-cerulean, PT5-mCherry, MCS II-mVenus	[59]

EC E. coli MG1655.
ᵃAll gadC genes encode a truncated protein (amino acids 1–470) that lacks the C-plug[21].

template (720 bp) to amplify mCherry. Plasmid pK18mob2-TriFluoR[59] was used as template (720 bp) to amplify mCerulean. Plasmid pNPTS138-R6KT-cadB:egfp-EC was used as template (720 bp) to amplify egfp. After purification of the different fragments, the respective combinations were assembled via Gibson assembly[60] into EcoRV-digested pNPTS138-R6KT plasmid, resulting in the plasmids pNPTS138-R6KT-gadC:egfp, pNPTS138-R6KT-gadC:mCherry, pNPTS138-R6KT-gadC:mCerulean, pNPTS138-R6KT-cadB:mCherry, pNPTS138-R6KT-adiC:mCerulean and pNPTS138-R6KT-adiC:mCherry. Each plasmid was verified by colony PCR and sequencing.

Functional hybrid proteins of GadC with each of the three different fluorophores was only achieved by using a shorter version of GadC (amino acids 1–470), in which the C-terminal C-plug is removed. Truncation of this C-plug does not affect the transport activity, but shifts the pH-optimum to a higher pH[21].

To construct a marker-less in-frame deletion of cadA in E. coli MG1655 gadC:eGFP-adiC:mCerulean-cadB:mCherry, the suicide plasmid pNPTS138-R6KT-ΔcadA was generated. Briefly, flanking regions (1000 bp upstream and downstream) of cadA were amplified by PCR using MG1655 gadC:eGFP-adiC:mCerulean-cadB:mCherry genomic DNA as template. After purification of the different fragments, the DNA fragments were assembled via Gibson assembly[60] into by PCR

linearized pNPTS138-R6KT plasmid, resulting in the plasmid pNPTS138-R6KT-ΔcadA. Correct insertion was verified by sequence analysis using primer pNTPS_Seq_fwd.

For construction of the reporter plasmid pBBR1-MCS5-P$_{adiY}$-lux, 200 bp of the region upstream of adiA was amplified by PCR using primers (PadiA_XbaI_fwd and PadiA_XmaI_rev) and MG1655 genomic DNA as template, and cloned into plasmid pBBR1-MCS5-TT-RBS-lux using restriction sites XbaI and XmaI. Correct insertion was verified by colony PCR and sequencing.

For construction of N-terminal His$_6$-tagged AdiY and His$_6$-tagged CsiR, adiY and csiR were amplified by PCR using MG1655 genomic DNA as template. The codons for the His$_6$-tag were introduced by using a respective forward primer. After purification of the different fragments, the DNA fragments were assembled via Gibson assembly[60] into SmaI-digested pBAD24 plasmid, resulting in the plasmids pBAD24-His$_6$-adiY and pBAD24-His$_6$-csiR. Correct insertion was verified by colony PCR and sequencing.

Construction of chromosomally integrated fluorescent fusions and deletion strains. The genes encoding the three different fluorophores, egfp, mCherry and mCerulean, are separately C-terminally fused to the genes gadC, adiC and cadB encoding the antiporter of the three AR systems. Expression of each fusion is under the control of the respective native promoter.

To generate the three-color E. coli MG1655 gadC:mCerulean-adiC:mCherry-cadB:egfp strain, at first the suicide plasmid pNPTS138-R6KT-adiC:mCherry was introduced into E. coli MG1655 cadB:egfp by conjugative mating using E. coli WM3064 as a donor in LB medium containing DAP. Single-crossover integration mutants were selected on LB plates containing kanamycin but lacking DAP. Single colonies were grown over a day without antibiotics and plated on LB plates containing 10% (w/v) sucrose but lacking NaCl to select for plasmid excision. Kanamycin-sensitive colonies were checked for targeted deletion by colony PCR using primers bracketing the site of the insertion. Insertion of mCherry was verified by colony PCR and sequencing resulting in the strain E. coli MG1655 adiC:mCherry-cadB:egfp. In the next step tagging of gadC with mCerulean was achieved using the suicide plasmid pNPTS138-R6KT-gadC:mCerulean. The plasmid pNPTS138-R6KT-gadC:mCerulean was introduced into E. coli MG1655 adiC:mCherry-cadB:egfp by conjugative mating using E. coli WM3064 as a donor in LB medium containing DAP as described above. Insertion of mCerulean was verified by colony PCR and sequencing, resulting in the strain E. coli MG1655 gadC:mCerulean-adiC:mCherry-cadB:egfp.

To generate the three-color E. coli MG1655 gadC:mCherry-adiC:mCerulean-cadB:egfp strain, at first the suicide plasmid pNPTS138-R6KT-adiC:mCerulean was introduced into E. coli MG1655 cadB:egfp by conjugative mating using E. coli WM3064 as a donor in LB medium containing DAP as described above. Insertion of mCerulean was verified by colony PCR and sequencing, resulting in the strain E. coli MG1655 adiC:mCerulean-cadB:egfp. In the next step tagging of gadC with mCherry was achieved using the suicide plasmid pNPTS138-R6KT-gadC:mCherry. The plasmid pNPTS138-R6KT-gadC:mCherry was introduced into E. coli MG1655 adiC:mCerulean-cadB:egfp by conjugative mating using E. coli WM3064 as a donor in LB medium containing DAP as described above. Insertion of mCherry was verified by colony PCR and sequencing resulting in the strain E. coli MG1655 gadC:mCherry-adiC:mCerulean-cadB:egfp.

To generate the two-color E. coli MG1655 gadC:mCerulean-cadB:mCherry strain, at first the suicide plasmid pNPTS138-R6KT-cadB:mCherry was introduced into E. coli MG1655 by conjugative mating using E. coli WM3064 as a donor in LB medium containing DAP as described above. Insertion of mCherry was verified by colony PCR and sequencing, resulting in the strain E. coli MG1655 cadB:mCherry. In the next step tagging of gadC with mCerulean in this strain was achieved using the suicide plasmid pNPTS138-R6KT-gadC:mCerulean. The plasmid pNPTS138-R6KT-gadC:mCerulean was introduced into E. coli MG1655 cadB:mCherry by conjugative mating using E. coli WM3064 as a donor in LB medium containing DAP as described above. Insertion of mCerulean was verified by colony PCR and sequencing resulting in the strain E. coli MG1655 gadC:mCerulean-cadB:mCherry.

To generate the three-color E. coli MG1655 gadC:egfp-adiC:mCerulean-cadB:mCherry strain, at first the suicide plasmid pNPTS138-R6KT-adiC:mCerulean was introduced into E. coli MG1655 cadB:mCherry by conjugative mating using E. coli WM3064 as a donor in LB medium containing DAP as described above. Insertion of mCerulean was verified by colony PCR and sequencing, resulting in the strain E. coli MG1655 adiC:mCerulean-cadB:mCherry. In the next step tagging of gadC with egfp was achieved using the suicide plasmid pNPTS138-R6KT-gadC:egfp. The plasmid pNPTS138-R6KT-gadC:egfp was introduced into E. coli MG1655 adiC:mCerulean-cadB:mCherry by conjugative mating using E. coli WM3064 as a donor in LB medium containing DAP as described above. Insertion of egfp was verified by colony PCR and sequencing, resulting in the strain E. coli MG1655 gadC:egfp-adiC:mCerulean-cadB:mCherry.

Construction of the marker-less in-frame deletion strain of cadA in E. coli MG1655 gadC:eGFP-adiC:mCerulean-cadB:mCherry was achieved using the suicide plasmid pNPTS138-R6KT-ΔcadA. The plasmid pNPTS138-R6KT-ΔcadA was introduced into E. coli MG1655 gadC:eGFP-adiC:mCerulean-cadB:mCherry by conjugative mating using E. coli WM3064 as a donor in LB medium containing DAP as described above. Deletion of cadA was verified by colony PCR and sequencing, resulting in the strain E. coli MG1655 ΔcadA gadC:eGFP-adiC:mCerulean-cadB:mCherry.

In vivo fluorescence microscopy and data analysis. To analyze the spatial temporal localization of the different fluorescent hybrids in E. coli, overnight cultures were prepared in KE medium pH 7.6 and aerobically cultivated at 37 °C. The overnight cultures were used to inoculate day cultures (OD$_{600}$ of 0.1) in fresh medium at pH 7.6. At an OD$_{600}$ of 0.5 (t$_0$), half of the culture was gently centrifuged and resuspended in KE medium pH 5.8 + lysine as the first pH shift. The rest of the culture continued growing in KE medium pH 7.6 at 37 °C. Then the cultures were cultivated aerobically at 37 °C for 2.5 h (t$_{150}$), and half of the culture was gently centrifuged and resuspended in LB medium pH 4.4 as the second pH shift. The rest of the culture continued growing in KE medium pH 5.8 + lysine at 37 °C (t$_{300}$). At the beginning of the experiment (t$_0$) and at every pH shift (t$_{150}$ and t$_{300}$), 2 µl of the culture was spotted on 1% (w/v) agarose pads (prepared with the respective media), placed onto microscope slides and covered with a coverslip. Subsequently, images were taken on a Leica DMi8 inverted microscope equipped with a Leica DFC365 FX camera (Wetzlar, Germany). An excitation wavelength of 546 nm and a 605-nm emission filter with a 75-nm bandwidth was used for mCherry fluorescence with an exposure of 500 ms, gain 5, and 100% intensity for mCherry-tagged strains. An excitation wavelength of 485 nm and a 510-nm emission filter with a 75-nm bandwidth was used for eGFP fluorescence with an exposure of 500 ms, gain 5, and 100% intensity for eGFP-tagged strains. An excitation wavelength of 436 nm and 480-nm emission filter with a 40-nm bandwidth was used for mCerulean fluorescence with an exposure of 500 ms, gain 5, and 100% intensity for mCerulean-tagged strains.

To analyze the influence of an increased copy number of CsiR, the E. coli strains gadC:mCerulean-cadB:mCherry and adiC:mCerulean-cadB:mCherry were transformed with plasmid pBAD-His$_6$-csiR by electroporation. 0.1% (w/v) L-arabinose was added throughout the experiment and cells were cultivated as described above.

To analyze the influence of an increased copy number of AdiY, the E. coli strain adiC:mCerulean-cadB:mCherry was transformed with plasmid pBAD-His$_6$-adiY by electroporation. 0.1% (w/v) L-arabinose was added throughout the experiment and cells were cultivated as described above.

To analyze the influence of an increased copy number of CadC, the E. coli strains gadC:mCerulean-cadB:mCherry and adiC:mCherry-cadB:eGFP were transformed with plasmid pBAD-His$_6$-adiY by electroporation. 0.1% (w/v) L-arabinose was added throughout the experiment and cells were cultivated as described above.

To quantify relative fluorescent intensities (RF) representing fluorescent hybrids of single cells, phase contrast and fluorescent images were analyzed using the ImageJ[61] plugin MicrobeJ[62]. Default settings of MicrobeJ was used for cell segmentation (Fit shape, rod-shaped bacteria) apart from the following settings: area: 0.1-max µm^2; length: 1.2–5 µm; width: 0.1–1 µm; curvature 0.−0.15 and angularity 0.−0.25 for E. coli cells. In total >1000 cells were quantified per strain and condition and time point. The background of the agarose pad was subtracted from each cell per field of view.

Mean and standard deviation (std) of the relative fluorescence (RF) were quantified using MicrobeJ and background was subtracted. Moreover, the RF of the GFP channel was corrected for the overlapping signal of the CFP channel via linear regression with the experimentally determined proportionality factor of 0.456. The noise was defined as std/mean of log-transformed values per sample. Log-transformed values were used because the coefficient of variation, as a measure of noise, is based on the assumption that the underlying data are normally distributed. However, not all data were normally distributed under all conditions, therefore the log-transformed values were used (log(x + 1)) as previously published for CadB:eGFP[41]. In addition, this helped to overcome the problem that the noise becomes higher with lower gene expression, which is often observed[42].

In order to compare fluorescence intensity values of the different fluorophores, the RF values were normalized (nRF) according to the highest values, as each of the three fluorophores has a different brightness (eGFP: 33; mCerulean: 16; mCherry: 15)[63].

Statistical analysis and presentation were performed using GraphPad Prism 9.1.0 and R 4.0.3[64].

Localization of fluorescently tagged GadC, AdiC, and CadB hybrids. To verify the localization of fluorescently tagged GadC, AdiC and CadB in the membrane, the three-color E. coli strains gadC:egfp-adiC:mCerulean-cadB:mCherry, gadC:mCherry-adiC:mCerulean-cadB:eGFP and gadC:mCerulean-adiC:mCherry-cadB:eGFP were cultivated as described above for fluorescence microscopy. After the second pH shift to pH 4.4 (t$_{300}$) cells were harvested and then adjusted to OD$_{600}$ = 30. Cells were disrupted by passage through a high-pressure cell disrupter (Constant Systems, Northants, United Kingdom) in ice-cold disruption buffer (50 mM Tris-HCl pH 7.5, 10% (v/v) glycerol, 10 mM MgCl$_2$, 100 mM NaCl, 1 mM dithiothreitol, 0.5 mM PMSF and 0.03 mg ml^{-1} DNase). After removal of intact cells and cell debris via centrifugation (5000 × g, 30 min, 4 °C), membrane vesicles were collected by ultracentrifugation (45,000 × g, 60 min, 4 °C), whereas the pellet contained the membrane fraction and the supernatant the cytoplasm. The membrane fractions were separated by SDS-PAGE[65] on 12.5% acrylamide gels and transferred to a nitrocellulose membrane. Fluorophore-tagged proteins were labeled either with the primary polyclonal α-mCherry antibody (Invitrogen), α-GFP antibody (Roche) or the α-mCerulean antibody PABG1 (Chromotek). The α-

rabbit or α-mouse alkaline phosphatase-conjugated antibody (Rockland Immunochemicals) was used as secondary antibody according to the manufacturer's recommendations. Localization of the secondary antibody was visualized using colorimetric detection of alkaline phosphatase activity with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride. As ladder the PageRuler Prestained Protein Ladder (10–180 kDa, Thermo Fisher) was used.

Functionality of fluorescent antiporter hybrids. The functionality of the Cad-B:eGFP hybrid was previously confirmed using a liquid-based colorimetric assay with a pH indicator[41]. To test the functionality of the fluorescent hybrid proteins GadC:eGFP and AdiC:mCerulean, an acid survival assay was performed as described previously[10,41] with the following modifications. E. coli strains were cultivated in LB medium (pH 7.6) to an OD_{600} of 0.6–0.8, then the cultures were adjusted to an OD_{600} of 0.5 and resuspended in LB medium with a pH of 3.0 or 4.4. The low pH challenge was conducted at 37 °C and samples were collected immediately after resuspension ($t = 0$) and then hourly for 3 h. Samples were serially diluted and plated onto LB agar plates to assess the number of colonies surviving the acid challenge. As controls, the parental E. coli MG1655 and the gadC and adiC deletion mutants were used.

Measurement of adiA promoter activity in vivo. In vivo promoter activity of adiA was probed with a luminescence-based reporter (P_{adiA}-luxCDABE). The influence of the Cad system and the AdiY copy number was tested in cells exposed to different external pH values in LB medium using luminescence as readout. The strains E. coli MG1655 carrying pBBR1-MCS5-P_{adiA}-lux, E. coli MG1655 ΔcadA carrying pBBR1-MCS5-P_{adiA}-lux, E. coli MG1655 carrying pBBR1-MCS5-P_{adiA}-lux co-transformed with pBAD-cadC were incubated in KE medium pH 7.6 supplemented with the respective antibiotics overnight. The overnight cultures were inoculated to an OD_{600} of 0.1 in fresh KE medium pH 7.6, aerobically cultivated until exponential phase and then shifted to KE medium pH 5.8 + 10 mM lysine and cultivated for another 2.5 h at 37 °C (comparable to the experimental setup described in Fig. 2a). In the next step, the cultures were shifted into a 96-well plate and aerobically cultivated at 37 °C in LB medium at different pH values supplemented with the respective antibiotics. Bioluminescence and growth were determined every 10 min in the microtiter plates with a Tecan Infinite F500 system (Tecan, Crailsheim, Germany). Data are reported as relative light units (RLU) in counts per second per milliliter per OD_{600}.

To analyze the effect of an increased copy number of CadC or AdiY, 0.1% (w/v) L-arabinose was added during the growth at pH 5.8 + 10 mM lysine and at pH 4.4. Thereby the CadC copy number is elevated to about 100 CadC molecules per cell, whereas the E. coli MG1655 wild type strain contains only ≤4 CadC molecules per cell[40].

Alignment and construction of phylogenetic trees. To identify non-redundant orthologues of the components of the three AR systems in relation to each antiporter GadC, AdiC and CadB, a Protein BLAST search of the NCBI RefSeq protein database[66] using the full-length sequence of either GadC (XasA), AdiC and CadB from E. coli MG1655 as the query sequence was carried out. Different expect thresholds were used for the Protein BLAST search (e < 10^{-100} for GadC, e < 10^{-120} for AdiC and CadB), in order to include all homologous yet functional similar proteins (June 2020). A tolerance of 10% of the amino acid length was set as default parameters.

Three alignments and phylogenetic trees were constructed, each based on the antiporter GadC, AdiC or CadB with the different components, and visualized as metadata. Therefore, a pairwise alignment of 1112 sequences for GadC, of 755 sequences for AdiC and of 857 sequences for CadB was done with a progressive algorithm from the software CLC Main Workbench 20.0.3 (CLC Bio Qiagen, Hilden, Germany) using the following parameters: gap open cost 10, gap extension cost 1, high accuracy[67]. The results served as the basis for the construction of three phylogenetic trees by the software's high-accuracy, distance-based neighbor-joining algorithm (100 bootstrap replicates and the Jukes-Cantor distance correction as default parameters). The branch lengths, therefore, represent the degree of evolutionary divergence between any two nodes in the tree.

We screened the organisms containing a GadC orthologue for the presence of the GadB, GadE, GadX, GadW, GadY, CsiR, EvgS and YdeO by searching for orthologues of E. coli MG1655 with NCBI Protein BLAST (e < 10^{-20} for GadE, e < 10^{-50} for CsiR, e < 10^{-60} for YdeO, e < 10^{-80} for GadB, e < 10^{-100} for GadW and GadX, e < 10^{-170} for EvgS). Homologs of the sRNA GadY were determined by a NCBI Nucleotide BLAST[66] search using gadY from E. coli MG1655 as the query sequence with an expect threshold of e < 10^{-20}.

We screened the organisms containing a AdiC orthologue for the presence of the AdiA, AdiY and CsiR by searching for orthologues of E. coli MG1655 with NCBI Protein BLAST (e < 10^{-170} for AdiA, e < 10^{-100} for AdiY, e < 10^{-50} for CsiR).

We screened the organisms containing an CadB orthologue for the presence of the CadA, CadC, LysP and CsiR by searching for orthologues of E. coli MG1655 with NCBI Protein BLAST (e < 10^{-170} for CadA, e < 10^{-120} for LysP, e < 10^{-50} for CsiR). For the phylogenetic distribution of CadC, the data from our previous publication[41] were used.

All data for the construction of these phylogenetic trees are available in Supplementary Data 1.

Data availability

Supplementary Data 1 contains a list of all GadC, AdiC, and CadB homologs identified by a local alignment search based on the full-length sequence of E. coli GadC, AdiC, and CadB underlying Fig. 4. All source data underlying the graphs and charts presented in Figs. 2, 3, 5 and 6 are presented in Supplementary Data 2. Plasmids and primer sequences are available on request.

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2.5 Bacterial battle against acidity

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Bacterial battle against acidity

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Abstract

The Earth is home to environments characterized by low pH, including the gastrointestinal tract of vertebrates and large areas of acidic soil. Most bacteria are neutralophiles, but can survive fluctuations in pH. Herein, we review how *Escherichia, Salmonella, Helicobacter, Brucella*, and other acid-resistant Gram-negative bacteria adapt to acidic environments. We discuss the constitutive and inducible defense mechanisms that promote survival, including proton-consuming or ammonia-producing processes, cellular remodeling affecting membranes and chaperones, and chemotaxis. We provide insights into how Gram-negative bacteria sense environmental acidity using membrane-integrated and cytosolic pH sensors. Finally, we address in more detail the powerful proton-consuming decarboxylase systems by examining the phylogeny of their regulatory components and their collective functionality in a population.

Keywords: Gram-negative bacteria, protons, glutamate acid-dependent acid resistance, lysine-dependent acid resistance, pH-sensor

Introduction

Compared to acidophilic organisms, which grow optimally at a pH near 3.0, and alkaliphiles, which prefer a pH between 8 and 10.5, most bacteria are neutralophilic and grow best at pH values between 7.0 and 7.8 (Slonczewski et al. 2009). However, neutralophilic bacteria are able to adapt and grow in environments with pH values ranging from 5.5 to 9.0, and thereby occupy a wide range of environmental niches including acidic environments such as human and animal digestive systems, acidic soils, and fermented foods (Fig. 1), which has important effects on the environment and public health. The evolution of molecular systems conferring acid tolerance and/or acid resistance (AR) is an important survival strategy for these bacteria, including the pathogenic ones (Castanié-Cornet et al. 1999).

The pH of the human stomach ranges between 1 and 3 under fasting conditions and increases during digestion. The stomach, thus is a major bactericidal barrier against enterobacteria (Martinsen et al. 2005, Kalantzi et al. 2006). Its low pH causes growth arrest and even death of bacteria (e.g. only 10% of the highly resistant *E. coli* population survives in the stomach). Under this condition, an acidification of the bacterial cytoplasm occurs as protons (H^{+}) use water bridges or weak organic acids to traverse the cytoplasmic membranes (Brahmachari et al. 2019). This acidification has two important effects: slowing biological activity of macromolecules and decreasing or abolishing the pH difference at the cytoplasmic membrane. The latter is an important component of the proton motive force and crucial for bioenergetics (Foster 2004, Lund et al. 2014).

Neutralophilic enterobacteria that survive the stomach also confront mild acid stress (pH 6.4 or lower) in the colon due to the presence of short-chain fatty acids (Hirshfield et al. 2003), such as butyric, propionic, lactic, acetic, and formic acids, which are produced by anaerobic fermenting bacteria (Evans et al. 1988, Boets

et al. 2015). Intracellular pathogens, such as *Salmonella*, also encounter low-pH (pH 4–5) environments in the macrophage phagosome (Rathman et al. 1996, Steinberg et al. 2007) (Fig. 1).

On Earth there are large areas of soil with an acidic pH-value (pH 6.5–3.5; Wieder et al. 2014; Fig. 1). Many processes contribute to soil acidification. For example, carbon dioxide (CO_2) emissions combine with water in the atmosphere to form carbonic acid (H_2CO_3), and the burning of fossil fuels releases oxides of sulfur and nitrogen into the atmosphere that react with water to form sulfuric and nitric acids. This acidic rain then accumulates in the soil. Root respiration and decomposition of organic matter by microorganisms also release CO_2, which increases the level of H_2CO_3 in the soil. Simultaneously, cations are exchanged resulting in leaching of base cations, such as Ca^{2+}, Mg^{2+}, from the soil and an increase of the percentage of Al^{3+} and H^{+} (vanLoon 1984). Severely acidic conditions also can form in soils located near acid mine drainage sites (Johnson and Hallberg 2005).

In food industry, acidity plays an important role for bacteria (Fig. 1). Homofermentative and heterofermentative lactic acid bacteria and propionic bacteria, which are predominant in dairy products, trigger the production of lactic acid, propionic acid, ethanol, and CO_2, all of which enhance flavor, digestibility, nutritional properties, and preservation (Fröhlich-Wyder et al. 2017, Siedler et al. 2019). As a consequence, these bacteria are exposed to acidity of their own fermentation activities. On the other hand, weak acid preservatives such as potassium sorbate, calcium propionate, and sodium benzoate are often added to foods to inhibit the growth of bacteria and to extend the shelf life of products (Guynot et al. 2005).

In this review, we focus on the manifold adaptive responses of neutralophilic Gram-negative proteobacteria and the molecular mechanisms of sensing acid stress. For detailed information on
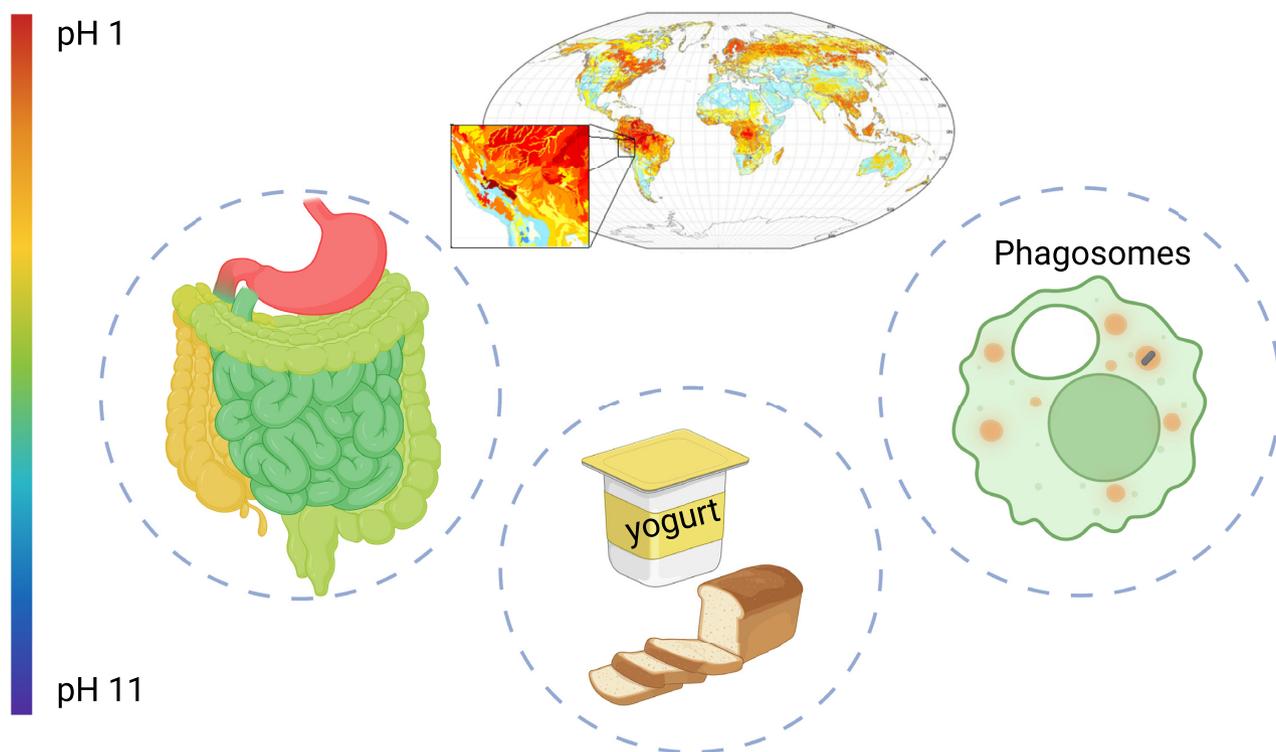
Figure 1. Schematic overview of acidic environments encountered by bacteria.

the acid-stress response of neutralophilic Gram-positive bacteria, see reviews by Guan and Li (2020) and Arcadi et al. (2020).

Acid stress adaptation of Gram-negative γ-proteobacteria

pH homeostasis and ion transport

In response to rapid drops in external pH, prokaryotic cells can retain a fairly constant internal pH from 7.5 to 8.0 for neutralophiles, 6.5 to 7.0 for acidophiles, and 8.4 to 9.0 for alkaliphiles (Booth 1985). In contrast to the periplasm, the cytoplasm transiently acidifies and restores neutral pH within short time intervals due to numerous cellular acid stress adaptions (Wilks and Slonczewski 2007, Krulwich et al. 2011). An important factor for preserving a constant internal pH is the intrinsic buffering capacity of the cytoplasm (Fig. 2). Excess intracellular protons can be sequestered by pH-titratable cellular components, such as amino acid sidechains, polyamines, polyphosphates, or inorganic phosphates, as summarized in Slonczewski et al. (2009).

The cytoplasmic membrane constitutes a major barrier to protons due to the intrinsic impermeability of the lipid bilayer and specifically controlled ion influx (Booth 1985). However, protons can pass this natural barrier using transient water chains, channels, or damaged membranes (Deamer 1987, Lund et al. 2014). Different types of acid stress counteracting membrane-integrated transporters exist, such as monovalent cation-proton antiporters, or proton pumps associated to the respiratory chain (Fig. 2).

The active transport of H^+ is a constitutive mechanism that maintains a constant internal pH, but the activity of H^+ pumps is constrained by the generation of the proton motive force (Mitchell 1973). In low-pH conditions, *E. coli* downregulates synthesis of the F_oF_1-ATP synthase to limit proton re-entry (Maurer et al. 2005, Sun et al. 2012). In various other organisms, the F_oF_1-ATP synthase re-

lieves acid stress via ATP-dependent extrusion of protons from the cytoplasm (Kobayashi et al. 1986, Foster and Hall 1991, Cotter et al. 2000, Sun et al. 2012). Net potassium uptake is the dominant means of maintaining ion homeostasis (Booth 1985).

Acidophilic bacteria also can relieve acid stress by reversing the usually negative transmembrane potential ($\Delta\psi$) Matin 1999). An inside-positive membrane potential helps prevent further proton uptake due to charge repulsion (Matin et al. 1982). A similar mechanism has been suggested for neutralophilic bacteria (Foster 2004, Richard and Foster 2004). In addition, in *E. coli*, two genes (*eriC* and *mriT*) coding for two chloride channels are induced at extremely low pH levels. Export of Cl^- prevents H^+ uptake and restores an inside-negative $\Delta\psi$ once the external pH is recovered (Iyer et al. 2002, Accardi and Miller 2004).

Enzyme-based H^+-consuming acid stress resistance systems

A total of four different H^+-consuming AR systems have been identified in *E. coli* (Figs. 2 and 3). These are the glutamate–dependent AR2 (Gad) system (also known as GDAR), the arginine-dependent AR3 (Adi) system (also known as ADAR), the lysine-dependent AR4 (Cad) system (also known as LDAR) and the ornithine-dependent AR5 (Orn) system (also known as ODAR). Each of these four systems relies on a cytoplasmic amino acid decarboxylase and a corresponding antiporter. In an H^+-consuming reaction, the pyridoxal-5'-phosphate (PLP)-dependent decarboxylase converts a specific amino acid (e.g. glutamate, arginine, lysine, and ornithine) into a more alkaline product, which is then exported by a specific antiporter in exchange for the amino acid. Through these biochemical reactions, the internal and external pH levels rise. The AR2–AR5 systems are activated at different pH ranges (< 6) Fig. 3) with varying strengths in individual cells, and each decarboxylase has its own optimum pH (Gale 1940, 1946, Lin
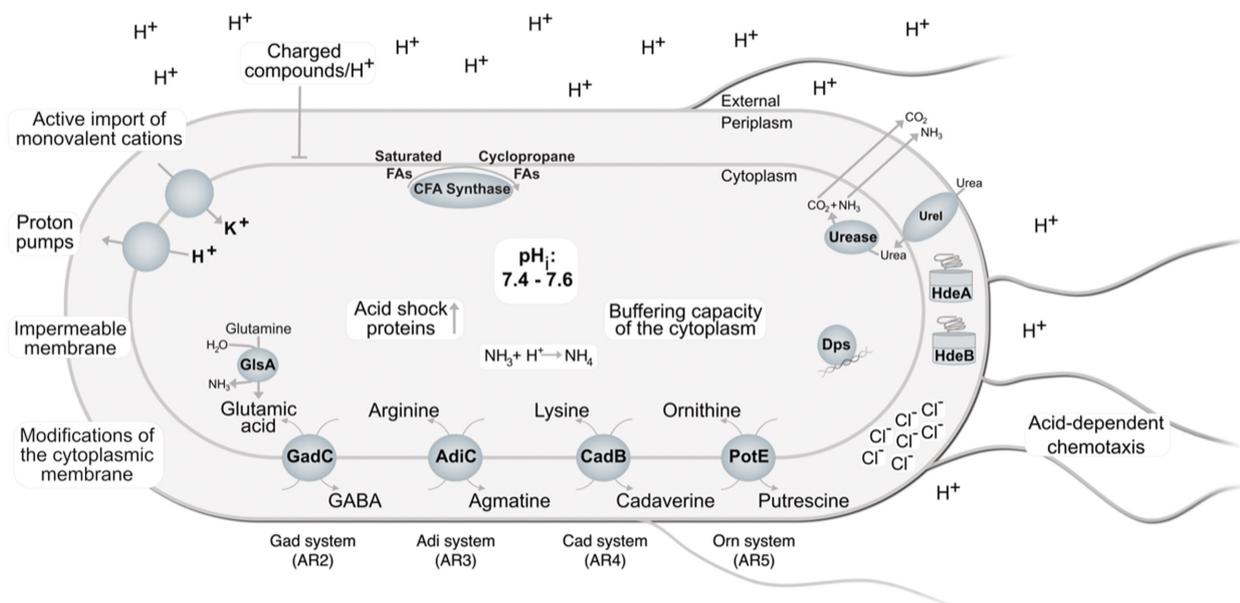
Figure 2. Overview of mechanisms in Gram-negative proteobacteria to cope with acidic environments. The various mechanisms to counteract acidic stress are discussed in detail in the text.
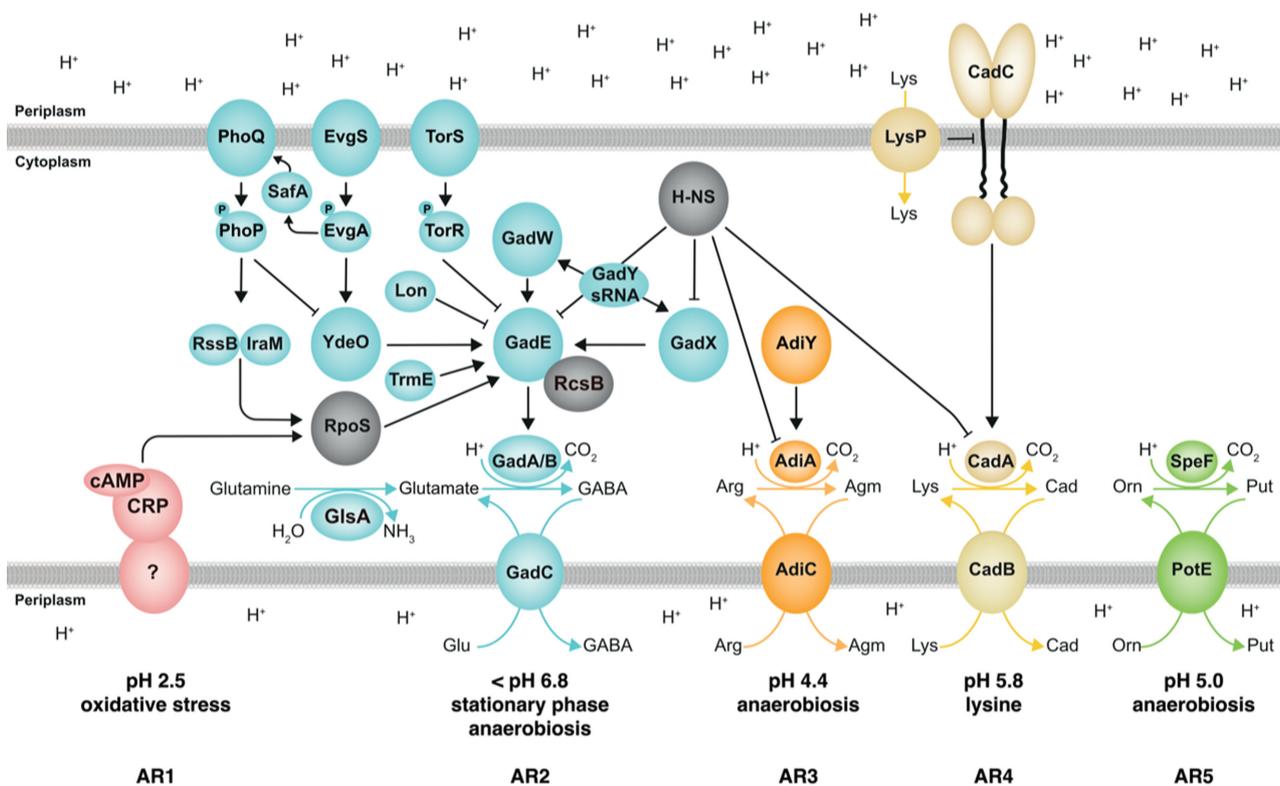
Figure 3. The regulatory network of the enzyme-based acid stress resistance systems in *E. coli*. Regulated induction of AR1 (in red), the Gad (AR2; in light blue), Adi (AR3; in orange), Cad (AR4; in pale yellow), and Orn (AR5; in green) systems requires a network of membrane-integrated pH-sensors, namely EvgS, PhoQ, and CadC; interconnected transcription factors; and the sRNA GadY. The glutamate decarboxylases GadA and GadB convert glutamate (Glu) into γ-aminobutyrate (GABA), which is excreted by GadC. The glutaminase GlsA (formerly YbaS) converts glutamine in glutamate and NH_3. The arginine decarboxylase AdiA converts arginine (Arg) into agmatine (Agm), which is excreted by AdiC. The lysine decarboxylase CadA converts lysine (Lys) into cadaverine (Cad), which is excreted by CadB. The ornithine decarboxylase SpeF converts ornithine (Orn) into putrescine (Put), which is excreted by PotE. RpoS, RcsB, and H-NS (gray symbols) are involved in the regulation of several enzyme-based acid stress resistance systems. Adapted from Lin et al. (1996), Castanié-Cornet et al. (1999), Foster (2004), Richard and Foster (2004), Bougdour et al. (2008), Zhao and Houry (2010), Eguchi et al. (2011), and Lund et al. (2014). The stimuli leading to an induction of each of the five AR systems are indicated below each system.

et al. 1995, Bearson et al. 1997, Castanié-Cornet et al. 1999, Foster 2004, Kanjee et al. 2011b, Kanjee and Houry 2013, Aquino et al. 2017). Little is known about AR1, an oxidative or glucose repressed system, which is independent of an external amino acid.

Acid-resistance system 1 (AR1)

The AR1 system is induced in stationary phase cells in complex media, or in minimal medium under extremely acidic stress conditions (pH 2.5) (Lin et al. 1996, Castanié-Cornet et al. 1999, Foster 2004). Its expression is regulated by alternative sigma factor σ^S (RpoS) and the cyclic adenosine monophosphate (cAMP) receptor protein (CRP). Part of the protective function of the AR1 system is the F_oF_1-ATP-synthase, which hydrolyzes ATP to pump protons out of the cell and lower internal pH (Richard and Foster 2004). Aquino et al. (2017) predicted a model according to which the AR1 system uses the enzymes of the Gad system, which are described in the next chapter, to decarboxylate intracellular glutamate.

Glutamate-dependent AR (Gad) system (AR2)

The core components of the Gad system are the decarboxylase isoenzymes GadA and GadB and the glutamate/γ-amino butyrate antiporter GadC in *E. coli*. Both GadA and GadB convert L-glutamate into γ-amino butyrate (GABA), consuming a proton in this process. The *gadBC* genes form one operon, while *gadA* is located inside the acid fitness island in *E. coli* (Biase and Pennacchietti 2012). The Gad system is induced during transition to the stationary phase and during exponential growth in acidified media (Biase et al. 1999, Castanié-Cornet et al. 1999, Castanié-Cornet and Foster 2001, Weber et al. 2005). In addition, the Gad system is essential for survival at pH levels below 3, but is also induced during anaerobiosis and salt stress (Biase et al. 1999, Hayes et al. 2006).

GadA and GadB form hexamers, have maximal activities at pH 3.7–3.8 (Shukuya and Schwert 1960, Lawson and Quinn 1967) and are 98% identical at the nucleotide level (Smith et al. 1992). More functional and structural details of GadB are summarized in excellent reviews of Biase and Pennachietti (2012) and Zhao and Houry (2010).

The high-resolution X-ray crystal structure of GadC, determined by Ma et al. (2012), reveals detailed insights into substrate binding and pH-dependent transport activity. GadC antiport activity has been observed at pH ≤ 6 *in vitro*, and *in vivo* measurements of GABA export suggest maximum activity at pH ≤ 3 (Richard and Foster 2004, Ma et al. 2012). Notably, GadC recognizes substrates by their net electric charge and exclusively transports $Glu^{0/-1}$ or Gln^0 in exchange for $GABA^{+1}$ (Ma et al. 2013, Tsai et al. 2013). Under nonactivating conditions, a C-terminal plug in GadC prevents substrate-binding. Correspondingly, mutants lacking the C-plug display a shift in pH-dependent transport activity towards higher pH-units (Ma et al. 2012).

In addition to glutamate, GadC also transports the amino acid glutamine (Ma et al. 2012, 2013), which is subsequently converted to glutamate by the glutaminase GlsA (formerly YbaS; Figs. 2 and 3). During this reaction, NH_3 is released, which itself sequesters protons while the produced glutamate serves as further substrate for GadA and GadB (Lu et al. 2013). Correspondingly, *glsA* is upregulated under the same conditions as *gadBC* (Biase and Pennacchietti 2012). In contrast to substrates and reaction products from other AR systems, glutamate and GABA both contribute to cytoplasmatic buffering due to carboxyl groups with suitable pK$_a$ values (Richard and Foster 2004, Lund et al. 2014).

The transcriptional regulation of *gadA*, *gadB*, and *gadC* represents one of the most complex examples thereof within bacteria. More than 20 regulatory elements with partially interconnected circuits and several small noncoding RNAs are involved in controlling their expression (Foster 2004, Zhao and Houry 2010).

The central activator of the Gad system is the transcriptional regulator GadE, which belongs to the LuxR-family (Ma et al. 2003; Fig. 3). GadE binds to the so-called *gad* box, a 20-nt region 63 bp upstream of the *gadA* and *gadBC* transcriptional start site (Castanié-Cornet and Foster 2001, Ma et al. 2002). In stationary phase *E. coli* cells, the 3´end of the GadE mRNA is processed and a 91-nt small regulatory RNA, GadF, is generated, which is involved in regulation of acid resistant genes (Melamed et al. 2016). To initiate expression of *gadA*, GadE forms a heterodimer with RcsB (Castanié-Cornet et al. 2007, 2010). RcsB is the response regulator (RR) of the Rcs phosphorelay system, which is responsible for adapting to cell envelope stress in *Enterobacteriaceae* (Meng et al. 2021). Beyond RcsB, the Rcs system consists of the transmembrane hybrid histidine kinase (HK) RcsC, another transmembrane protein RcsD and the outer membrane protein RcsF. RcsC and RcsD dephosphorylate RcsB in the absence of envelope stress. RcsB is not only able to form heterodimers with GadE, but also with RcsA, MatA, DctR, BglJ, and presumably EvgA, to control numerous target genes (Castanié-Cornet et al. 2010, Venkatesh et al. 2010, Johnson et al. 2011, Pannen et al. 2015).

gadE transcription is regulated by the AraC/XylS-like regulator YdeO of the EvgS/EvgA-PhoQ/PhoP-YdeO circuit (Fig. 3). YdeO and EvgA have additive effects on *gadE* transcription (Ma et al. 2004). EvgA also contributes indirectly to *gadE* transcription, on the one hand through activation of YdeO by phosphorylation and on the other hand by induction of the PhoQ/PhoP HK/RR system via the small connector protein SafA (Ma et al. 2004, Eguchi et al. 2012, Ishii et al. 2013). The PhoQ/PhoP system in turn activates the anti-adaptor protein IraM, which inhibits activity of the protease adaptor RssB. RssB is responsible for degradation of RpoS by ClpXP, which implies that an active PhoP leads to increased RpoS levels (Eguchi et al. 2011).

The GadX-GadY-GadW regulatory circuit is required for induction of the Gad system in stationary phase cells, independently of the culture conditions (Ma et al. 2002), as shown in Fig. 3. GadW and GadX both belong to the AraC/XylS family and activate *gadA* and *gadBC* directly and indirectly through transcriptional activation of *gadE* (Ma et al. 2002, Tramonti et al. 2006, Sayed et al. 2007). Both regulators exert a dual role and can also repress the above-mentioned genes when bound to the *gad* box (Tramonti et al. 2002). Another element adding even more complexity to this circuit is the small RNA GadY. GadY directly base pairs and thereby stabilizes the 3´ untranslated region of the *gadX* mRNA (Opdyke et al. 2004). Another factor linked to AR via GadW is the LuxR-like protein SdiA, which presumably regulates *gadW* and *gadY* at the transcriptional level (Ma et al. 2020). This effect is further influenced by N-acyl homoserine lactones, which stabilize the SdiA protein and increase its binding affinity to DNA (Nguyen et al. 2015, Ma et al. 2020).

Other elements, including CRP and several sRNAs, influence induction of the Gad system through RpoS. Increased amounts of RpoS promotes *gadE*, *gadX*, and *gadY* expression (Castanié-Cornet and Foster 2001, Ma et al. 2002). For example, cAMP-CRP negatively controls *rpoS* transcription during exponential growth (Lange and Hengge-Aronis 1994). The DsrA small noncoding RNA stabilizes the *rpoS* mRNA and simultaneously inhibits *hns* mRNA translation via RNA–RNA interactions, which is expected to reduce gene silencing caused by the histone-like nucleoid-structuring protein

(H-NS) (Lease et al. 2004). This contributes to AR as H-NS represses *gadA*, *gadE*, and *gadX* (Giangrossi et al. 2005). The expression of *rpoS* is also positively regulated by the GcvB, RprA, and ArcZ small RNAs (Jin et al. 2009, Bak et al. 2014).

The expression of *gadE* is also induced during anaerobic growth of *E. coli* via the MnmE (TrmE) circuit. The Era-like GTPase MnmE can indirectly activate *gadE* in stationary-phase cells in rich medium supplemented with glucose (Gong et al. 2004). This activation also appears to be associated with anaerobic conditions and the ArcA/ArcB HK/RR system (Deng et al. 2013).

A combined ChIP-Seq RNA-Seq approach by Aquino et al. (2017) reports binding of the carbon starvation–induced regulator CsiR to *gadX*, *ydeO*, and *gadW* promoter regions, as well as effects of CsiR on gene expression of multiple *gad* genes. In addition to vast amounts of direct and indirect activators, examples of negative regulation of the Gad system also have been reported. In addition to H-NS, the RR TorR also negatively regulates *gad* genes, and the Lon protease is involved in GadE proteolysis (Bordi et al. 2003, Heuveling et al. 2008).

In summary, the Gad system is the most important AR system under extremely acidic conditions. In *E. coli* and other bacteria (see below), this system includes several decarboxylase isoenzymes. Up to now, no differences have been demonstrated in the induction of *gadA* and *gadB* and protein function.

It would be interesting to find out why two nearly identical decarboxylase genes have evolved or to find a condition in which only one of the two enzymes is produced. It also remains puzzling why the network of transcriptional regulators and small RNAs controlling the Gad system reaches such enormous complexity in *E. coli*. In contrast, the molecular design of the Cad, Adi, and Orn systems is simpler.

Arginine-dependent AR (Adi) system (AR3)

The arginine-dependent AR (Adi) system, shown in Fig. 3, consists of the arginine decarboxylase AdiA, the arginine/agmatine transporter AdiC, and the AraC/XylS-like regulator AdiY. The *adiA*, *adiY*, and *adiC* genes are clustered but not arranged in an operon, as *adiY* separates *adiA* and *adiC* on the chromosome. This system is maximally activated in *E. coli* grown in rich medium with an external pH of 4.4 under anaerobic conditions (Melnykovych and Snell 1958, Blethen et al. 1968, Kanjee and Houry 2013). AdiA decarboxylates arginine to produce agmatine, which is then secreted by AdiC via an exchange with extracellular arginine.

AdiA activity is pH-dependent and regulated via changes in oligomerization. At pH 5.2, AdiA mainly forms decamers. Oligomers dissociate into inactive dimers at higher pH values, and this transition is enhanced through electrostatic repulsion because AdiA has many negatively charged surface residues (Nowak and Boeker 1981).

Like AdiA, the activity of AdiC is pH-dependent, increasing at extremely acidic pH levels (< ~4) compared to physiological pH (Fang et al. 2007). The outward- and inside-facing domains of the antiporter also are sensitive to pH. AdiC functions as a homodimer, but each monomer can exchange one arginine to one agmatine (Fang et al. 2007, Gao et al. 2009, 2010, Kowalczyk et al. 2011, Krammer et al. 2016, 2018, Ilgü et al. 2018). The maximum uptake for arginine from the periplasm is reached at pH < 4, and the optimal pH for the cytoplasm is 5.5, showing that AdiC is a pH sensor of the periplasm and the cytoplasm (Gao et al. 2009, Zomot and Bahar 2011, Tsai and Miller 2013, Wang et al. 2014, Krammer et al. 2018).

Transcription of the *adi* genes is influenced by the growth phase, the switch to anaerobic conditions and several transcriptional regulators. Nevertheless, the main transcriptional regulator of the Adi system is AdiY, a member of the AraC/XylS-family, which activates the transcription of its target genes *adiA* and *adiC* (Stim-Herndon et al. 1996). *adiY* transcription itself is downregulated upon entry into the stationary phase of *E. coli* (Smith et al. 2018) and *adiY* mRNA can be base-paired by the small RNA SgrS, resulting in post-transcriptional downregulation under anaerobic conditions (Bobrovskyy and Vanderpool 2015). In addition to the main activator AdiY, CysB, CsiR, and H-NS have modulating effects on the expression of the Adi system (Shi and Bennett 1994, Aquino et al. 2017).

Recently, it was shown that pH-dependent activation of the *adiA* promoter can be manipulated by changes in intracellular pH, as activation of the Adi system can be influenced by manipulation of the cytosolic proton concentration either by deletion of *cadA* or by overproduction of *cadA* (triggered by increased CadC copy numbers) (Brameyer et al. 2022). These data suggest that AdiY acts as an intracellular pH sensor that detects a decrease in cytoplasmic pH (Brameyer et al. 2022). The molecular mechanism of how AdiY responds to low pH is still unclear. But it is an interesting hypothesis that low cytosolic pH, which normally has deleterious effects, is sensed by one sensor to activate another AR system.

Lysine-dependent AR (Cad) system (AR4)

The Cad system consists of the lysine decarboxylase CadA, the lysine/cadaverine antiporter CadB and the regulatory protein CadC, a member of the ToxR-family (Fig. 3). This system only moderately protects the cells from extreme acid shock, but it is important for growth in mildly acidic environments (Watson et al. 1992, Dell et al. 1994). Together with the Gad system, the Cad system contributes to cell survival in anaerobic environments with fermentation acids and phosphate starvation, such as the small intestine (Moreau 2007).

CadC functions as a periplasmic pH sensor and regulates the expression of *cadA* and *cadB*. Like all members of the ToxR-family, CadC is a membrane–integrated sensor with DNA-binding capacity. The *cadA* and *cadB* genes are organized in one operon, which is activated by CadC in *E. coli* cells exposed in mildly acidic conditions (pH 5.8) and in the presence of lysine (> 1 mM) (Watson et al. 1992, Neely et al. 1994, Neely and Olson 1996, Soksawat-maekhin et al. 2004, Tetsch et al. 2008, Fritz et al. 2009). Detection of low pH leads to dimerization of the periplasmic sensory domain of CadC. A disordered CadC linker on the cytoplasmic side is required to transduce the pH-dependent response into a structural rearrangement that facilitates dimerization of the cytoplasmic DNA-binding domain (Haneburger et al. 2011, Buchner et al. 2015, Schlundt et al. 2017). By recruiting the RNA polymerase, CadC binds to the *cadBA* promoter at two specific binding sites, with each binding site occupied by a CadC homodimer (Neely et al. 1994, Kuper and Jung 2005, Eichinger et al. 2011, Haneburger et al. 2011, Lindner and White 2014, Schlundt et al. 2017).

The availability of external lysine is transduced to CadC by the cosensor and inhibitor LysP, a lysine-specific permease in *E. coli*. CadC is inactivated by an interaction with LysP in low-lysine conditions. This interaction is released when lysine is abundant (Tetsch et al. 2008, Rauschmeier et al. 2014). Additionally, CadC activity is negatively feedback-inhibited by cadaverine (Fritz et al. 2009, Haneburger et al. 2011, 2012). LysP and H-NS act as negative regulators of the Cad system in *E. coli*. H-NS binds under noninducing conditions to four potential binding sites within the *cadBA*

promoter region and forms a repressor complex. This complex dissolves when CadC binds to the Cad1 site. Binding of CadC to Cad2 initiates transcription of *cadBA* (Shi et al. 1993, Neely et al. 1994, Kuper and Jung 2005, Buchner et al. 2015, Schlundt et al. 2017).

In *E. coli*, *cadC* transcription is constitutive (Watson et al. 1992, Dell et al. 1994), but *cadC* translation depends on the elongation factor P (EF-P) due to the presence of a polyproline motif. EF-P is an elongation factor that enhances translation of polyproline-containing proteins through alleviating of the ribosome stalling. This translational regulation keeps the copy number of CadC low at only 1–3 copies per cell, on average (Ude et al. 2013). Brameyer et al. (2019) showed that a fluorophore-tagged CadC appears uniformly in the membrane of cells exposed to physiological pH. Under CadC-activating conditions, a distinct cluster appears at the lateral cellular position, which immediately disappears under nonstress conditions. The results suggest that binding of CadC to DNA determines its localization in the cell. This finding is confirmed using various CadC variants that mimic the active state of CadC, independent of environmental stimuli, or in mutants in which the DNA-binding site is relocated (Brameyer et al. 2019, Martini et al. 2021). Due to the low copy number and consequent stochastic distribution of CadC in single *E. coli* cells, the Cad system is heterogeneously activated, resulting in about two thirds of cells in an ON state and the rest in an OFF state (Brameyer et al. 2020). For more details on the molecular mechanism of pH-sensing by CadC, see below.

CadA catalyzes the decarboxylation of L-lysine into the more alkaline polyamine cadaverine and into CO_2 by consuming a proton. The enzymatic activity is regulated by oligomerization and the alarmone guanosine $3',5'$-bis(diphosphate) (ppGpp). Active CadA comprises five dimers that form a decamer in a double-ringed structure with a distinct 5-fold symmetry homologous to AdiA (Snider et al. 2006). A total of 10 molecules of the stringent response molecule ppGpp can bind to a CadA decamer to inhibit activity (Kanjee et al. 2011a), enabling cells to react quickly to changing environmental conditions. The maximum activity for CadA occurs at pH 5.7 (Gale and Epps 1944, Sabo et al. 1974, Kanjee and Houry 2013).

The structure of the lysine/cadaverine antiporter CadB is presently unknown. CadB performs an electrogenic exchange of lysine with cadaverine. At physiological pH, CadB mediates uptake of cadaverine, i.e. driven by proton-motive force (Soksawat-maekhin et al. 2004, 2006).

The Cad system is also important for survival and growth of other pathogenic bacteria in acidic conditions, such as *S. Typhimurium* (Park et al. 1996) and *V. cholerae* (Merrell and Camilli 1999). However, regulation of the Cad system varies among species. For example, in *Vibrio campbellii*, the Cad system is the major pH stress response system, but its Cad module is missing the cosensor LysP. In *Vibrio* spp., *cadC* expression is activated by the LysR-type transcriptional activator AphB (Rhee et al. 2006, Kovacikova et al. 2010). Another difference compared to *E. coli* is that the translation of CadC does not depend on EF-P. Consequently, CadC has higher copy numbers of about 41–69 copies per cell under activating conditions. The Cad system, thus is homogeneously activated in all cells of the *V. campbellii* population (Brameyer et al. 2020).

Ornithine-dependent AR (Orn) system (AR5)

The Orn system is an exceptional AR system because it converts a nonproteinogenic amino acid. The system consists

of the inducible ornithine decarboxylase SpeF and the ornithine/putrescine antiporter PotE (Fig. 3). The main activity of this system occurs during anaerobic growth of *E. coli* at low pH and high ornithine levels (Gale 1946, Applebaum et al. 1977, Kashiwagi et al. 1991, 1992). The genes *speF* and *potE* are arranged in one operon and induced at pH ~5.0. Dimerization and GTP regulate the decarboxylase activity, and the alarmone ppGpp binds weakly to SpeF (Applebaum et al. 1977, Kanjee et al. 2011b). The optimal enzymatic activity for SpeF occurs at pH 7.0. This relatively high optimal pH, in comparison to other amino acid decarboxylases, highlights the efficiency of these four systems to deal with extremely acidic conditions (Gad > Adi > Cad >> Orn) (Foster 2004, Kanjee and Houry 2013). Under mildly acidic conditions, PotE exchanges ornithine or lysine with putrescine. At neutral pH, PotE catalyzes the proton motive force–dependent uptake of putrescine (Kashiwagi et al. 1992, 1997). Regulation of the *speF-potB* operon is unusual and involves ribosome stalling and antitermination (Ben-Zvi et al. 2019, Herrero Del Valle et al. 2020). Upstream of *speF* is a short open reading frame (ORF) that encodes the 34-amino acid peptide SpeFL (also known as *orf34*). This ORF is conserved in many γ-proteobacteria. In the absence of ornithine, translation of *speFL* causes ribosome stalling and transcription of *speF* pauses. In the presence of ornithine, translation of SpeFL activates *speF* expression by preventing Rho-dependent transcription termination (Herrero Del Valle et al. 2020).

Enzyme-based ammonia-producing AR systems

Deiminase and deaminase systems

Different bacterial species, such as *Helicobacter pylori, Pseudomonas aeruginosa*, and many Gram-positive bacteria like *Streptococcus mutans*, use deiminase and deaminase systems to withstand acidic environments. These enzymes produce ammonia (NH_3) to neutralize internal protons, which are used to create ammonium ions (NH_4^+) and in turn increase the internal pH. Different deiminase and deaminase systems are present in different groups of bacteria in varying combinations.

The agmatine deiminase (AgD) belongs to the family of guanidino-group modifying enzymes (GMEs) (Linsky and Fast 2010). It catalyzes the conversion of agmatine to N-carbamoyl putrescine and NH_3. AgD mediates acid resistance in *Streptococcus mutans*, *P. aeruginosa* and *H. pylori* (Jones et al. 2010).

Another GME is the L-arginine deiminase, it belongs to the arginine deiminase system (Linsky and Fast 2010), which protects cells against acid damage (Cunin et al. 1986, Marquis et al. 1987, Liu et al. 1995, D'Hooghe et al. 1997, Angelis et al. 2002). This system is present in a variety of bacteria, including *Mycoplasma, Pseudomonas, Bacillus, Rhizobium*, and lactic acid bacteria. A total of three enzymes are responsible for its acid tolerance: (i) arginine deiminase, which degrades arginine into citrulline and NH_3, (ii) ornithine carbamoyl transferase (OTC), which cleaves citrulline into carbamoyl phosphate and ornithine, and (iii) carbamate kinase, which produces ATP, NH_3, and CO_2 through dephosphorylation of carbamoyl phosphate. These enzymes show maximal activity at a pH of 3.1 or lower (Cunin et al. 1986, Marquis et al. 1987, Casiano-Colón and Marquis 1988). The generation of NH_3 and of one ATP per arginine contributes to the internal pH homoeostasis through the F_oF_1-ATPase, which extrudes cytoplasmic protons.

In *E. coli*, the glutaminase GlsA (formerly YbaS) is part of the Gad system, i.e. activated under acidic conditions (Figs 2 and 3). L-glutamine is imported by GadC and then converted by GlsA to

L-glutamate and NH$_3$ (Ma et al. 2012, 2013, Sun et al. 2012, Lu et al. 2013). Another deaminase system in *E. coli* is based on adenosine. Internal pH can increase when adenosine is converted into inosine and NH$_3$, a reaction catalyzed by the adenosine deaminase Add (Maurer et al. 2005, Sun et al. 2012).

Urease

Another enzyme frequently used to combat acidification is urease, which is present in a wide variety of bacterial genera (e.g. *Helicobacter*, *Brucella*, *Klebsiella*, *Pseudomonas*, *Yersinia*, *Proteus*, *Staphylococcus*, and *Streptococcus*) (Lund et al. 2014), as well as members of the human gut microbiota (Mora and Arioli 2014). Urease catalyzes the hydrolysis of its substrate urea to NH$_3$ and carbamate, which subsequently decomposes into another molecule of NH$_3$ and CO$_2$ (Mobley et al. 1995). NH$_3$ contributes to cytoplasmic and periplasmic buffering by neutralizing protons and yielding NH$_4^+$ (Scott et al. 2010), and CO$_2$ is converted to bicarbonate by alpha-carbonic anhydrase (Marcus et al. 2005). The *ureAB* genes encode the urease subunits, whereas the protein products of *ureDEFG* are involved in urea metabolism, urease biogenesis and incorporation of nickel (Lund et al. 2014). Nickel ions are essential cofactors of urease activity and are inserted into the active site of the enzyme. Additionally, the NikR transcription factor of *H. pylori* regulates nickel uptake and induces expression from the *ureA* promoter in low-pH conditions (van Vliet et al. 2002, Li and Zamble 2009).

The importance of urease activity is best studied in *H. pylori*, in which the proton-gated urea channel UreI mediates urea uptake into the cytoplasm. Urea is present in 1–3 mM concentrations in gastric juice. UreI activity is strictly pH dependent, which prevents urea import and alkalization of the cytoplasm during physiological pH conditions (Weeks et al. 2000). Correspondingly, the previously mentioned genes encoding urease subunits are regulated in a pH-responsive manner, mediated by the ArsS/ArsR HK/RR system (see below) and the cytoplasmic HK FlgS (Wen et al. 2009). *Helicobacter pylori* produces urease up to 10% of its total protein content (Sachs et al. 2005). In animal models, urease-negative mutants are unable to colonize, further confirming the essential role of urease in gastric habitation (Eaton et al. 1991, Skouloubris et al. 1998, Mollenhauer-Rektorschek et al. 2002).

In several *Brucella* spp., urease activity is crucial to increase infectivity (Bandara et al. 2007, Sangari et al. 2007). Urea is also present at 3–10 mM concentrations in saliva and constitutes a nitrogen source for oral plaque microbiota (Mora and Arioli 2014). *Streptococcus salivarius* and *Actinomyces naeslundii* have been demonstrated to be urease positive (Morou-Bermudez and Burne 1999, Chen and Burne 2003), and viability of *S. salivarius* at extremely low pH increases in a urea concentration dependent manner (Chen et al. 2000).

Cellular remodeling to withstand acid stress
Synthesis of chaperones

Acidification leads to protein unfolding, denaturation, or damage as more amino acid residues become fully protonated (Goto et al. 1990). *Escherichia coli* has efficient chaperone-based mechanisms to protect proteins from acid-dependent denaturation and damage, especially in the periplasm (Fig. 2). HdeA and HdeB are two structurally related periplasmic chaperones that function in an ATP-independent manner and minimize protein aggregation due to high proton and chloride concentrations in the periplasm during acid stress (Fig. 2). They also assist in refolding the substrate after acid stress relief in *E. coli*, *Shigella flexneri*, *Brucella abortus*,

and other species (Waterman and Small 1996, Gajiwala and Burley 2000, Valderas et al. 2005, Kern et al. 2007, Malki et al. 2008, Stull et al. 2018). The corresponding genes are usually part of the acid fitness island, where genes are induced in cells exposed to acid stress (Tucker et al. 2002).

In vitro, both chaperones are inactive dimers at neutral pH. At pH 2, the HdeA dimer rapidly converts into partially disordered, chaperone-active monomers. In its monomeric form, HdeA binds to proteins that were unfolded at low pH, effectively preventing them from aggregating (Tapley et al. 2009b). Upon return to neutral pH, HdeA slowly releases the bound proteins, allowing them to refold while minimizing the concentration of aggregation-prone intermediates (Tapley et al. 2009a). HdeA then converts back to its chaperone-inactive dimer.

In vivo, HdeA functions optimally in cells exposed to extreme acid stress of pH 2–3, and HdeB functions optimally at a moderately acidic pH of 4 (Dahl et al. 2015). The cytoplasmic chaperone Hsp31 (*hchA*) also contributes to acid stress resistance of stationary-phase *E. coli* cells (Mujacic and Baneyx 2006). Its transcription is controlled by sigma factor σ^S, but its exact function remains unclear. Some evidence indicates that cytoplasmic chaperones such as GroE, DnaK, and IbpB are upregulated in some Gram-negative bacteria under acid stress (Lund et al. 2014). Further experimental evidence is needed to determine the extent to which strong acidification of the cytoplasm leads to significant protein unfolding in the cytoplasm of these organisms.

Modifications of the cytoplasmic membrane

The cytoplasmic membrane is a major barrier to proton influx in acid exposed cells. To protect this membrane against acid damage, bacteria modulate its integrity, fluidity, and lipid composition. Under acid stress, the ratio of saturated to unsaturated fatty acids in the membrane increases (Yang et al. 2014). Furthermore, cyclopropane fatty acids (CFAs) are synthesized and incorporated into the membrane (Brown et al. 1997) (Fig. 2). In this process, unsaturated fatty acids are converted to CFAs by the postsynthetic addition of a methyl group from S-adenosyl-methionine to a double bond (Chang and Cronan 1999). An *E. coli* mutant lacking *cfa*, the gene encoding CFA synthase, is highly sensitive to acid stress, but this sensitivity can be partially overcome by an exogenous supply of CFAs (Chang and Cronan 1999). In addition, *E. coli* membranes lacking CFAs are more permeable to protons, suggesting that the presence of CFAs is important to maintain membrane integrity under acid stress (Shabala and Ross 2008). The transcription of the *cfa* gene is upregulated at low external pH, indicating that alteration of the cytoplasmic membrane fatty acid composition is an adaptive response mechanism (Chang and Cronan 1999). Changes in the cytoplasmic membrane composition in response to acid stress have also been observed in *Salmonella* and *Helicobacter* (Haque et al. 1996, Kim et al. 2005, Alvarez-Ordóñez et al. 2009).

In addition to modifications of fatty acids, the modification of lipid types has been described in acid-stressed bacteria. Common responses involve hopanoid formation and the introduction of hydroxyl groups into existing membrane lipids such as ornithine lipids, lipid A, or sphingolipids (Sohlenkamp 2019).

Synthesis of acid-shock proteins

Of the previously mentioned amino-acid decarboxylase systems, the Gad system contributes most to the survival of an *E. coli* population in extremely acidic conditions at or below a pH of 2.5 (Biase et al. 1999, Biase and Pennacchietti 2012). Such conditions however, as they are encountered in the human gastrointestinal tract,

generally require adaptions on a global scale beyond amino-acid dependent AR systems (Kalantzi et al. 2006). Proteomic and microarray analyses have identified several candidates as acid shock proteins in cells exposed to acidified media (Blankenhorn et al. 1999, Audia et al. 2001, Stancik et al. 2002, Maurer et al. 2005) (Fig. 2). One prominent example is the periplasmatic Asr protein, which is crucial for survival in extreme acidity (Šeputienė et al. 2003). Expression of the *asr* gene can be induced by at least three transcriptional regulators, RstA, RcdA, and PhoB (Sužiedėlienė et al. 1999, Ogasawara et al. 2007, Shimada et al. 2012). Upon expression, Asr is post-translationally cleaved into an 8- and a 5-kDa protein, however, the biological significance of this processing is still unclear. Maximum induction occurs at pH 4.0–4.5, and *asr* null mutants display an acid-sensitive phenotype (Šeputienė et al. 2003). Other strongly induced potential acid shock proteins in *E. coli* have been identified in moderate acidity of pH 4.3–5.0 (Foster and Hall 1991), including several poorly characterized proteins and known heat shock proteins (Heyde 1990, Blankenhorn et al. 1999, Stancik et al. 2002, Maurer et al. 2005). Several acid shock proteins have also been identified in *S.* Typhimurium. For most acid shock proteins, the exact mechanisms of protection during acid shock remain elusive and require further characterization.

Another important cellular component that protects DNA from damage is the Dps protein. In *dps* mutants, acid-induced DNA damage is significantly increased (Choi et al. 2000, Jeong et al. 2008). In stationary-phase cells, Dps becomes the most abundant nucleoid component (180 000 molecules per cell) and causes compaction of DNA characteristics of this growth phase (Ali Azam et al. 1999). Dps also protects DNA from oxidative cleavage (Martinez and Kolter 1997). Both oxidative and acid stress are associated with the production of hydroxyl radicals (OH$^{\bullet}$) via Fenton-type reactions, which mainly lead to DNA single and double strand breaks by oxidizing the sugar and base moieties. Dps proteins inhibit formation of these toxic hydroxyl radicals via a multistep process of removing Fe(II) (Ceci et al. 2003, 2004). In conclusion, further proteome and transcriptome wide studies and an in-depth characterization of the most promising candidate proteins at various pH levels are needed to unravel the full spectrum of acid shock proteins in bacteria.

Acid-dependent chemotaxis

Beyond the above-mentioned AR mechanisms, chemotactic movements have been described in several bacterial species as a direct adaptation to physically avoid low pH environments (Fig. 2) (Kihara and Macnab 1981, Repaske and Adler 1981, Tohidifar et al. 2020). When bacteria sense acidic pH or weak organic acids such as acetate, they migrate to more desirable environments (Tsang et al. 1973, Tso and Adler 1974). For instance, weak acids induce prolonged tumbling in *E. coli* and *Salmonella* cells at pH 5.5 (Kihara and Macnab 1981). Moreover, a microarray study conducted at various pH levels revealed that flagellar and chemotaxis genes in *E. coli* are regulated in response to the pH (Maurer et al. 2005). Correspondingly, genes encoding the flagellar motility apparatus are upregulated in *H. pylori* at low pH (Merrell et al. 2003, Wen et al. 2003). The swimming behavior of bacterial cells is controlled by the direction of the flagellar motor, which alternates between clockwise and counterclockwise rotations (Berg 1974). If repellents such as acids are perceived, tumbling is enhanced by prolonged clockwise flagella rotation (Berg and Brown 1972, Silverman and Simon 1974).

In *E. coli*, alkaline and acidic environments are sensed by two methyl-accepting chemotaxis proteins (MCPs), the aspartate binding receptor, Tar, and the serine binding receptor, Tsr (Repaske and Adler 1981, Krikos et al. 1985, Khan et al. 1995). Using a fluorescence resonance energy transfer-based reporter, Yang and Sourjik (2012) reported a "push–pull" mode of action in which Tar initiates an attractant response to low pH and a repellent response to high pH, whereas Tsr exerts the opposing mode of action. This bidirectional response helps *E. coli* avoid extremely low or high pH by transitioning from acid-seeking to base-seeking and *vice versa* at specific pH inversion points. Another example for an MCP involved in pH taxis has been found in *H. pylori* (Croxen et al. 2006, Sweeney et al. 2012). Besides perception of the quorum-sensing molecule AI-2 (Rader et al. 2011), the chemotaxis receptor TlpB is indispensable for pH taxis and colonization of the gastric mucosa (Croxen et al. 2006). See below for further discussion of Tar, Tsr, and TlpB. To date, the strategy of avoiding acidic environments by chemotaxis has only been observed in *E. coli* and *H. pylori*. It remains unclear whether this strategy is specific to these species or whether it represents a universal stress response within bacteria. Further research is needed to clarify whether the number of flagella per cell is increased under low pH conditions, as upregulation of genes encoding the flagellar motility apparatus was observed.

Molecular mechanisms of pH-sensing

In the next chapters, we will discuss the molecular processes of pH-responsive receptors that are able to sense changes in pH outside and inside bacteria. In most cases, stimulus perception is coupled to transmembrane signaling.

Hybrid HK EvgS

The hybrid HK EvgS senses low external pH and transmits this signal to its cognate RR EvgA in *E. coli* (Utsumi et al. 1994, Eguchi and Utsumi 2014) (Figs. 3 and 4). The multidomain cytoplasmic part of EvgS comprises three signal transduction modules, including a transmitter domain (composed of an H-box containing dimerization and histidine phosphotransfer domain and a catalytic domain), a receiver domain, and a histidine-containing phosphotransfer (HPr) domain. The periplasmic part is connected via a cytoplasmatic linker and includes two Venus flytrap (VFT) domains (Perraud et al . 1998, Sen et al. 2017). Upon signal perception, the phosphoryl group at the first histidine residue (His721) in EvgS is transferred to an aspartate residue (Asp1009) in the receiver domain and then transmitted to another histidine residue (His1137) in the HPr domain. To complete the four-step His-Asp-His-Asp phosphorelay, the phosphoryl group is transferred to the aspartate residue (Asp52) in EvgA (Utsumi et al. 1996, Perraud et al. 1998, Tanabe et al. 1998, Kinoshita-Kikuta et al. 2015).

EvgS displays maximum activity at pH 5.0–5.5, and a leucine residue at position 152 in the first VFT domain is crucial for activation (Fig. 4) (Eguchi and Utsumi 2014). Sen et al. (2017) also elucidated the structure of the periplasmic domain of EvgS. Additionally, the residues His226 and Pro522 are presumably involved in sensing acidity and signal transduction (Fig. 4).

In a proposed model, the stimulus is transduced from the VFT domains to the cytoplasmatic Per-Arnt-Sim (PAS) domain via structural rearrangements mediated by the region comprising Leu152, His226, and Pro522. In combination with further stimuli sensed in the cytoplasm, the structural change putatively triggers a weakening of subunit interactions in an inactive tight EvgS dimer. Subsequently, the loose dimer, which constitutes the active EvgS conformation, induces autokinase activity and initiates the described phosphorelay (Johnson et al. 2014, Sen et al. 2017). This model is further supported by the fact that several amino
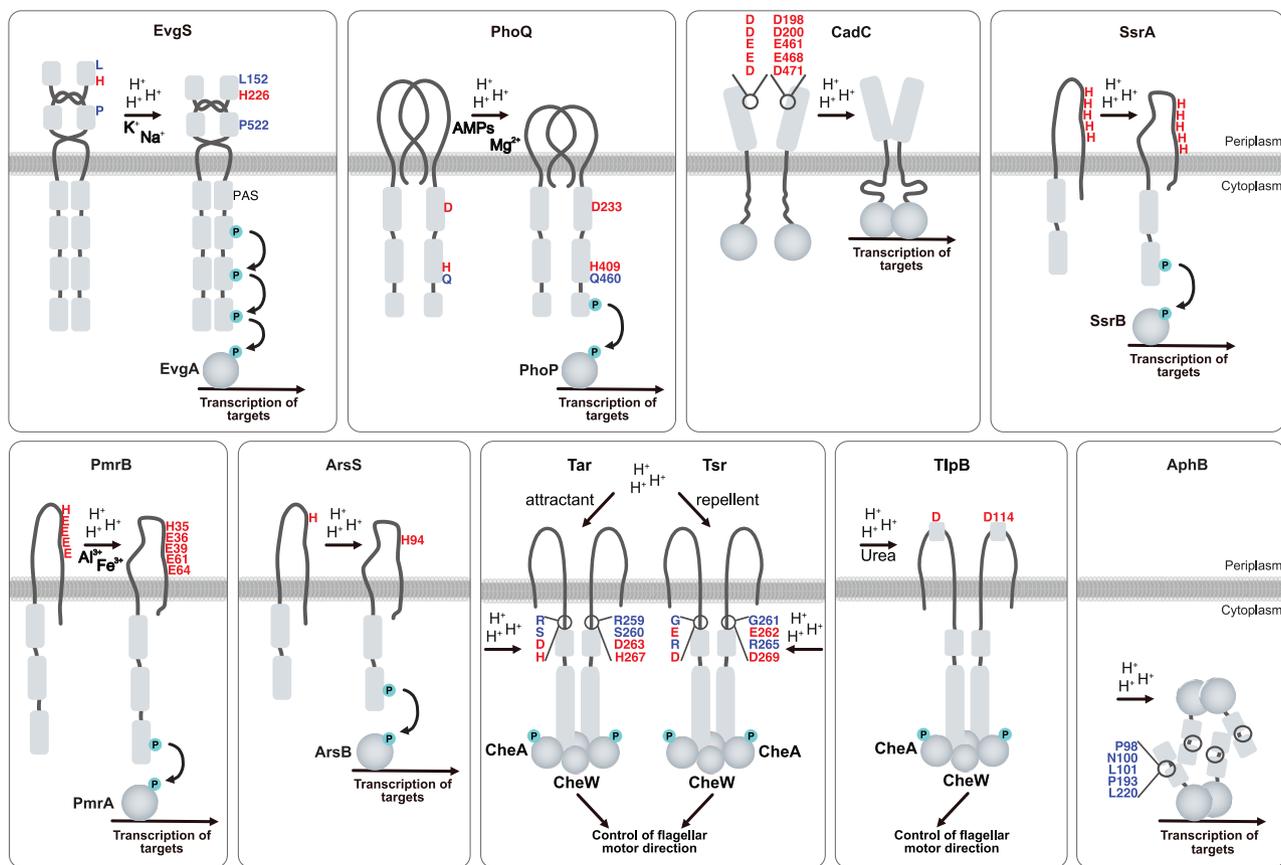
Figure 4. Schematic overview of the mechanisms of acid sensing of membrane-integrated and soluble pH sensors. The overall structure and mode of activation by low pH and various cations are indicated for the membrane-integrated sensors EvgS (Eguchi and Utsumi 2014, Hicks et al. 2015, Choi and Groisman 2016, Sen et al. 2017), PhoQ (Choi and Groisman 2016), CadC (Haneburger et al. 2011), SsrA (Mulder et al. 2015), PmrB (Perez and Groisman 2007), ArsS (Müller et al. 2009), Tar and Tsr (Umemura et al. 2002), as well as TlpB (Sweeney et al. 2012) and for the soluble regulator AphB (Taylor et al. 2012). Amino acid residues of the regulators are marked in red when described to be important for the recognition of low pH. For SsrA, only five histidine residues in the C-terminal end are described to be important for the recognition of low pH. Amino acid residues marked in blue are connected with the adaptation to low pH. Low pH is indicated via "H$^+$." AMPs, antimicrobial peptides. PAS, Per-Arnt-Sim domain.

acid replacements, which constitutively activate EvgS, have been identified in the PAS domain. These amino acid replacements might mimic the rearrangement from an inactive tight dimer to a more dissociated conformation, which constitutes the active state (Johnson et al. 2014).

In addition to the required mild acidity, alkali metals with a concentration of at least 150 mM are necessary to activate the EvgS/EvgA system (Eguchi and Utsumi 2014). To sense acidic pH, both the periplasmatic region and the PAS domain are required, while alkali metals (e.g. K$^+$) can be sensed by either domain individually (Eguchi and Utsumi 2014, Johnson et al. 2014). Beyond these findings, EvgS is active in mildly acidic environments under aerobic but not in anaerobic growth conditions. The EvgS PAS domain is hypothesized to act as an intermediate redox switch that transmits information from the periplasm to the cytoplasmic catalytic core, depending on the oxidation state of ubiquinone in the electron transport chain (Inada et al. 2021).

Due to substantial amino acid polymorphisms across different *E. coli* isolates, EvgS displays large variability in its pH-sensing capacity, including pH nonresponsive variants (Roggiani et al. 2017). The kinase forms clusters in *E. coli* (Sommer et al. 2013). Once EvgA is phosphorylated, it activates expression of two genes coding for regulators of the Gad system, *ydeO* and *gadE* (Ma et al. 2004, Itou et al. 2009). EvgA acts as a homodimer; however, it may also form heterodimers with RcsB (Johnson et al. 2011, Pannen et al.

2015). Moreover, the EvgS/EvgA system regulates the expression of multiple multidrug efflux pumps, including EmrYK and MdtEF (formerly YhiUV) (Kato et al. 2000, Nishino and Yamaguchi 2001, Eguchi et al. 2003).

HK PhoQ

Together with EvgS, PhoQ is responsible to transmit the activating signal of the Gad system in *E. coli*. In addition, the kinase has also been extensively studied in *S. Typhimurium*. The amino acid sequences of PhoQ in both bacterial species are 86% identical (Kasahara et al. 1992). As shown in Fig. 4, the bifunctional PhoQ is membrane-integrated and comprises a periplasmic sensor domain, a transmembrane TM 4-helix bundle, cytoplasmic HAMP domain, DHp domain, and a kinase domain (Goldberg et al. 2010). Under physiological conditions, PhoQ acts as a homodimer (Goldberg et al. 2008). Activation by various stimuli leads to autophosphorylation of PhoQ at a conserved histidine residue and subsequent phosphotransfer to the RR PhoP (Groisman et al. 1989, Miller et al. 1989). Under noninducing conditions, PhoQ displays phosphatase activity and dephosphorylates PhoP (Gunn and Miller 1996, Shin and Groisman 2005).

In *E. coli*, the phosphorelay between PhoQ and PhoP is fine-tuned by the small membrane protein SafA, which accelerates PhoQ autophosphorylation and accumulation of phosphorylated PhoP by direct interaction between its C-terminal domain and

the periplasmic sensor domain of PhoQ (Ishii et al. 2013). Transcription of *safA* itself is under the control of EvgA and thus connects the PhoQ/PhoP to the EvgS/EvgA system (Masuda and Church 2003, Eguchi et al. 2007, Itou et al. 2009). This connection to the EvgS acid-sensor is crucial, as direct pH-sensing has not been demonstrated for PhoQ in *E. coli*. Similar to *E. coli*, an additional horizontally acquired gene (*ugtL*) is needed to enhance autophosphorylation of PhoQ in S. Typhimurium (Choi and Groisman 2017). Both UgtL and SafA are required for the response of the PhoQ/PhoP system in mildly acidic conditions but not for sensing low Mg^{2+} levels and also do not affect phosphotransfer from PhoQ to PhoP (Groisman et al. 2021). SsrB (see below) is another factor involved in S. Typhimurium's response to acid stress mediated by PhoQ/PhoP, as it binds to the *ugtL* promoter region and counteracts the repressing effects of H-NS (Choi and Groisman 2020).

In *E. coli*, the PhoQ/PhoP system is regulated at the post-transcriptional level by two Hfq-dependent small RNAs, MicA and GcvB. Both independently base-pair with the translation initiation region of the *phoPQ* mRNA, which impairs ribosomal binding and promotes transcript degradation (Coornaert et al. 2010, 2013). In addition, the PhoQ/PhoP system also involves a negative feedback inhibition loop via the small-membrane protein MgrB (formerly YobG). PhoP initiates expression of *mgrB*, and MgrB in turn inhibits the kinase activity of PhoQ (Lippa and Goulian 2009, Salazar et al. 2016). Once the PhoP RR is phosphorylated, it transcriptionally controls hundreds of target genes (5% of all genes) in S. Typhimurium (Belden and Miller 1994, Soncini et al. 1996, Zwir et al. 2005, Harari et al. 2010, Zwir et al. 2012). The PhoQ/PhoP system reaches maximum activity around pH 5.0, which corresponds to the pH of the macrophage phagosomes (Alpuche Aranda et al. 1992, Rathman et al. 1996, Bearson et al. 1998). PhoP-dependent gene regulation itself depends on phosphorylated PhoP amounts, which are affected by numerous factors including PhoP acetylation, feedback loops, proteolysis by ClpSAP, the Mg^{2+} transporter MgtA, EIIA^{Ntr}, and others, as reviewed in Groisman et al. (2021). In *E. coli,* the PhoP regulon includes several AR genes, including *hdeD, hdeAB*, and the central Gad system regulators *gadW* and *gadE* (Belden and Miller 1994, Zwir et al. 2005, Eguchi et al. 2011).

Only in S. Typhimurium, protons have been shown to be directly sensed by PhoQ (Prost et al. 2007, Hicks et al. 2015, Choi and Groisman 2016). Nuclear magnetic resonance studies show that the PhoQ periplasmic sensor domain undergoes a conformational change at mildly acidic pH levels and involves a network of residues surrounding His157 (Prost et al. 2007). Moreover, α-helices 4 and 5 in this domain may act as potential pH-responsive structural elements altering the dynamic behavior of PhoQ in an acid-dependent manner. Introducing a nonphysiological disulfide bond between the substitutions Trp104Cys and Ala128Cys restrains flexibility in α-helices 4 and 5 of the periplasmic domain and subsequently impairs PhoP-mediated gene expression during acid stress (Hicks et al. 2015). However, those results have been challenged by the findings of Choi and Groisman (2016), who discovered three residues in the cytoplasmic PhoQ domain (Asp233, His409, and Gln460) that are indispensable for pH sensing (Fig. 4). Their results also indicate that PhoQ senses acidic pH exclusively in the cytoplasm, as *in vivo* experiments demonstrate that mutants lacking the PhoQ transmembrane and periplasmatic regions, as well as chimeras with the EnvZ periplasmic domain, still respond to acidity. Nevertheless, it remains elusive whether protons itself are sensed by the critical amino acids in the cytoplasm or rather a different stimulus which is triggered by intracellular acidification (Choi and Groisman 2016, Groisman et al. 2021).

In addition to sensing low pH, PhoQ in S. Typhimurium and *E. coli* can sense a wide variety of other signals, including divalent cations (Mg^{2+}, Mn^{2+}, and Ca^{2+}), hyperosmotic stress, the periplasmatic redox state, and acetate (Groisman et al. 2021). Antimicrobial peptides (AMPs) and long-chain unsaturated fatty acids can be bound by the periplasmic PhoQ domain in *Salmonella* (Bader et al. 2003, Shprung et al. 2012, Viarengo et al. 2013, Carabajal et al. 2020). Protons and AMPs are presumably sensed via distinct signaling mechanism as they have additive effects on PhoQ activation, whereas divalent cations and AMPs compete for binding in the periplasmic domain (Bader et al. 2005, Prost et al. 2007, Hicks et al. 2015). Remarkably, in the nonpathogenic strain *Salmonella bongori,* Mg^{2+} and AMPs can be sensed, but mildly acidic pH levels do not trigger PhoP-dependent gene expression, presumably because the crucial residues involved in cytoplasmic pH sensing (Fig. 4) are not conserved in S. *bongori* (Choi and Groisman 2016). In *E. coli*, only His409 but not Asp233 and Glu460 are conserved, which supports the idea that protons are not directly, but rather indirectly sensed via EvgS and SafA. A further critical residue for signaling in *E. coli* PhoQ is Asn202 in the second transmembrane helix. A substitution with other hydrophobic residues after exposure to Mg^{2+} results in disrupted signal propagation and failed expression of PhoP-activated genes (Goldberg et al. 2010).

ToxR-like receptor CadC

Unlike EvgS and PhoQ, CadC belongs to the one-component signal transduction systems (ToxR-like family) of transcriptional regulators. These low-copy membrane-integrated receptors combine sensory and transcriptional regulatory functions in one polypeptide (Miller et al. 1987). ToxR controls virulence in *V. cholerae*. Nevertheless, other members of this family contribute to virulence, acidic stress response and biofilm and type VI pilus formation (DiRita and Mekalanos 1991, Merrell and Camilli 2000, Stelzer et al. 2006). These receptors have a conserved modular composition: a C-terminal periplasmic sensor domain that senses external stimuli and a single transmembrane helix to transduce the signal to the N-terminal cytoplasmic winged helix-turn-helix DNA-binding domain, which then regulates gene expression. This signal transduction is independent from chemical modifications (Miller et al. 1987).

In 2011, the crystal structure of the periplasmatic domain of *E. coli* CadC was solved (Eichinger et al. 2011). This sensory domain consists of two subdomains: the N-terminal subdomain comprises a mixture of parallel and antiparallel β-sheets and a three-helix-bundle, and the C-terminal subdomain is a pure bundle of 11 α-helices. Distinct amino acids within the periplasmic sensory domain of CadC are responsible for dimerization, pH sensory function, and feedback inhibition by cadaverine. To detect alterations in external pH, a cluster of negatively charged amino acids, namely Asp198, Asp200, Glu461, Glu468, and Asp471, are essential (Fig. 4). This negatively charged patch extends across the N- and the C-terminal subdomains. It is proposed that protonation of this cluster at the dimer interface within the periplasmic domain of CadC enables conformational changes resulting in dimerization and signal transduction (Eichinger et al. 2011, Haneburger et al. 2011, 2012, Buchner et al. 2015, Schlundt et al. 2017).

HK SsrA

The HK/RR system SsrA/SsrB of S. Typhimurium controls, amongst others, the virulence genes encoded in the *Salmonella pathogenicity* island 2 (SPI-2), which are required for intracellular survival in host cells. SsrA signaling requires acidification of

the cytoplasm of *S.* Typhimurium due to the acidic environment of the macrophage vacuole of the host in which the bacteria reside. Response to external acidification is integrated through SsrA, whereas acidification of the cytoplasm affects the RR SsrB. As shown in Fig. 4, five histidine residues in the C-terminal end of the periplasmic sensor domain are important for the full acid-responsiveness of SsrA (Mulder et al. 2015). Yet, an SsrA variant lacking the important histidine residues of the periplasmic domain shows basal activity at neutral pH, similar to the activity of wild type. As no single histidine is responsible for the full acid activation of SsrA, Mulder et al. (2015) concluded that the concerted action of multiple histidine residues is needed, as with the diphtheria toxin T domain (Perier et al. 2007).

Acidification of the cytosol induces conformational changes in SsrB, increasing its affinity to bind to DNA (Liew et al. 2019). This pH-dependent DNA-binding of SsrB has been shown by atomic force microscopy and single-particle tracking assays. Binding of PhoP or OmpR is not pH-sensitive; thus, acid-dependent DNA binding of SsrB is not an intrinsic property of transcriptional regulators (Liew et al. 2019). Nevertheless, *S.* Typhimurium acidifies its cytoplasm in an OmpR-dependent manner by repressing the CadC-regulated *cadBA* operon (Chakraborty et al. 2015). Moreover, the HK/RR system EnvZ/OmpR upregulates the SsrA/SsrB system upon stimulation by acid stress by directly activating the *ssrAB* promoter (Lee et al. 2000, Garmendia et al. 2003, Fass and Groisman 2009, Chakraborty et al. 2015, Liew et al. 2019). The EnvZ/OmpR system integrates acidic pH, absence of Ca^{2+} and low osmolarity (Garmendia et al. 2003) and is required for the replication and survival (Lee et al. 2000) of *Salmonellae* in macrophages. It also is necessary for full virulence in mice (Dorman et al. 1989, Chatfield et al. 1991). Overall, instead of recovering from acid stress, *S.* Typhimurium use the acidification of its cytoplasm as a signal to activate on the one hand PhoQ (Choi and Groisman 2016) and on the other hand its pathogenesis via SsrB that upregulates the expression and secretion of SPI-2 effector proteins (Kenney 2019).

HK PmrB

In addition to the SsrA/SsrB and PhoQ/PhoP systems, *S.* Typhimurium also possess the PmrB/PmrA HK/RR system, which is involved in perceiving extracellular acidic conditions. The HK PmrB is activated in mildly acidic conditions (pH 5.8), and the RR PmrA promotes transcription of several genes (Perez and Groisman 2007). PmrB and PmrA are required for various pathways, such as virulence in mice (Gunn et al. 2000), infection of chicken macrophages (Zhao et al. 2002), growth in soil (Chamnongpol et al. 2002), resistance to the cationic peptide antibiotic polymyxin B (Roland et al. 1993), and resistance to Fe^{3+} (Wösten et al. 2000) and Al^{3+} (Nishino et al. 2006). For full acid-responsiveness of PmrB, a histidine and four glutamic acid residues in the periplasmic domain of PmrB are required (Fig. 4) (Perez and Groisman 2007). Submillimolar levels of extracellular Fe^{3+} or Al^{3+} are perceived directly by PmrB (Wösten et al. 2000), and low concentrations of extracellular Mg^{2+} (Soncini and Groisman 1996) trigger transcription of PmrA-activated genes. The activation of PmrA by low extracellular Mg^{2+} levels occurs via activated PhoQ, which directly senses the extracellular Mg^{2+}, followed by phosphorylation of its cognate RR PhoP, which then activates transcription of *pmrD*. PmrD then promotes phosphorylation of PmrA and inhibits PmrB-promoted dephosphorylation of phosphorylated PmrA (Kox et al. 2000, Kato and Groisman 2004). Multiple signals are integrated into two-

component systems similar to the signal transduction cascades in eukaryotes.

HK ArsS

The human gastric pathogen *H. pylori* is extremely well-adapted to the fluctuating low-pH conditions in the human stomach. This adaptation is mainly mediated via the NH_3-producing enzyme urease, which is essential for maintaining neutral pH in both the cytoplasm and periplasm. Urease-independent mechanisms are likely to contribute to acid adaptation. In response to low pH, hundreds of genes are regulated, including the urease gene cluster, as identified by four independent studies performing genome-wide transcriptional profiling (Merrell et al. 2003, Wen et al. 2003, Bury-Moné et al. 2004, Pflock et al. 2006).

The ArsS/ArsR HK/RR system is the main regulator for the acid response. An acidic pH of 5.0 triggers autophosphorylation of ArsS, followed by the subsequent phosphorylation of its cognate RR, ArsR. Phosphorylated ArsR then acts both as an activator and repressor of pH-responsive genes (Pflock et al. 2006). To perceive low pH, the histidine residue at position 94 of ArsS is crucial (Fig. 4). Moreover, depending on the degree of protonation of several acidic amino acids in the periplasmic domain, ArsS can adopt different conformations resulting in different activation states to allow a gradual transcriptional response to moderate low pH conditions (pH 5.0). Müller et al. (2009) concluded that pH sensing by ArsS does not rely exclusively on protonation of histidine residues but rather on protonatable amino acids other than histidine that contribute substantially to acidity. The acid-induced upregulation of the urease gene cluster requires the sensor kinase ArsS but not phosphorylation of the RR ArsR. Although ArsR phosphorylation increases expression of the urease gene cluster, it is not required for acid survival.

Chemoreceptors Tar and Tsr

Tar and Tsr are required to navigate *E. coli* to a desired intermediate pH level and to physically avoid habitats with extreme pH levels. Both receptors form clusters that associate with the adaptor protein CheW and the HK CheA, resulting in initiation of a signal transduction cascade and ultimately in regulation of the flagellar motor to navigate the cell to desired environments (Maddock and Shapiro 1993, Kentner et al. 2006, Hazelbauer et al. 2008). Both MCPs can sense internal as well as external pH. Ambient pH is sensed by the periplasmatic domains, and residues for sensing the internal pH have been identified in cytoplasmic regions (Krikos et al. 1985). By characterizing chimeras, residues in Tsr (Gly261, Glu262, Arg265, and Asp269) and Tar (Arg259, Ser261, Asp263, and His267) have been identified as crucial in the chemotactic response to alterations of the internal pH (Fig. 4). Interchanging these residues located in the linker region, which connects the second transmembrane helix to the first methylation helix, affects the pH response of Tar and Tsr. While a replacement of Arg259 by the corresponding residue of Tsr reversed the swimming behavior, a substitution of Asp263 or His267 only altered the time course of pH response (Umemura et al. 2002).

Especially for Tsr but also for Tar, adaptive receptor methylation was indicated to fine-tune the strength of the pH response due to a weakened response upon methylation of either receptor. At low pH levels, Tsr is the dominant receptor as it has fewer methylated glutamyl residues and guides the cells toward higher pH values, whereas at alkaline pH levels, Tar is less methylated and dominates the pH response. Moreover, the pH inversion point is affected by culture density-dependent alterations in Tar and Tsr

expression levels (Yang and Sourjik 2012). At high cell densities, the preferential pH point is shifted toward lower pH units as the ratio of Tar/Tsr increases, presumably to avoid nutrient-depleted regions of high cell numbers, which were demonstrated to display more alkaline pH values (Salman and Libchaber 2007, Kalinin et al. 2010, Yang and Sourjik 2012).

Chemoreceptor TlpB

Similar to Tar and Tsr in *E. coli*, TlpB is the crucial receptor for pH taxis in *H. pylori*. In a mouse infection model, *tlpB* mutants were shown to be pH nonresponsive and defective for colonization (Croxen et al. 2006). An atomic resolution structure of the periplasmic domain has been solved by Sweeney et al. (2012), showing that TlpB forms dimers via extracellular urea-binding PAS-domains and contains two transmembrane regions per subunit and a cytoplasmic HAMP domain.

Asp114 in the extracellular PAS-domain of the TlpB dimer is proposed to be the key titratable residue for signaling, while urea acts as a cofactor for acid sensing. In a proposed model, urea binding is weakened via protonation of Asp114 at low pH levels and a structural rearrangement within the PAS-domain is induced. Moreover, urea protects the TlpB periplasmic domain against thermal denaturation in a pH-dependent manner. Thereby, Asp114 accepts two hydrogen bonds from the amide nitrogen atoms of urea at neutral pH and stabilizes the PAS domain. This interaction, however, is disrupted or weakened in acidic environments due to protonation of Asp114. This in turn may cause a loosening of urea binding and partial unfolding of the PAS-domain. Subsequent movement of TM2 relative to TM1 could affect the phosphorylation state of CheA (Sweeney et al. 2012). Little is known about the transcriptional regulation of *tlpB*. The only thus far identified protein to bind the promoter region of *tlpB* is the orphan RR HP1043 (Delany et al. 2002).

Transcriptional regulator AphB

In addition to membrane integrated proteins, also cytosolic regulators like AphB have been described to harbor pH-sensing residues. AphB is an important regulator in several *Vibrio* spp. for adapting to environmental conditions and pathogenicity. AphB belongs to the family of LysR-type transcriptional regulators (LTTR), one of the largest families of regulators in prokaryotes (Maddocks and Oyston 2008). Together with the quorum sensing-regulated activator AphA, AphB$_{Vc}$ activates the virulence cascade via *tcpPH* expression of the human pathogen *V. cholerae* (Kovacikova and Skorupski 1999). AphB$_{Vc}$ also regulates several genes involved in pH regulation like CadC (Kovacikova et al. 2010). In *V. cholerae*, low pH and anaerobiosis are the two environmental signals leading to activation of AphB (Kovacikova et al. 2010).

Taylor et al. (2012) solved the crystal structure of full-length AphB$_{Vc}$, showing that AphB forms a tetramer by dimerization of dimers like other members of the LTTR family. AphB consists of an N-terminal winged helix DNA-binding domain and a C-terminal regulatory domain. The DNA-binding domain is formed by three α-helices (α1–α3) followed by two β-strands that form the wing. The C-terminal regulator domain has two subdomains, RD-I and RD-II, connected by two extended antiparallel β-strands. Their interface harbors a putative ligand-binding pocket formed by hydrophobic and polar amino acids. A total of five residues in the ligand-binding pocket of AphB can render the activity of the protein to nearly constitutive activity, with respect to pH and oxygen, when they are replaced by negatively charged amino acids. These residues are Pro98, Asn100, and Leu101, which are located in the

loop between β3 and α5; residue Pro193, located in the loop between β7 and β8, and residue Leu220 (Fig. 4).

AphB$_{Vc}$ was crystallized alone (wild type) and bound to the DNA (Asn100Glu variant). A comparison of these two structures shows that AphB$_{Vc}$ undergoes a major conformational change to alter the position of the DNA-binding domain (Taylor et al. 2012). These findings also have been observed for AphB in *Vibrio vulnificus*, where AphB$_{Vv}$ affects the expression of more than 10% of the genome, including *cadC* (Jeong and Choi 2008). The crystal structure of the regulatory domain of AphB$_{Vv}$ has been solved and shows high similarity to AphB$_{Vc}$. The putative ligand binding pocket consists of 12 amino acids (Pro98, Asp100, Leu101, Ser128, Val144, Asp162, His192, Pro193, Leu220, Pro237, Met240, and Arg262). An additional small cavity on the backside of the regulatory domain also has been identified (Lys103, Arg104, Asn221, Asn224, Met240, and Tyr244). Further study is needed to determine if it also contributes to ligand binding (Park et al. 2017).

Phylogenetic occurrence of the Gad, Adi, Cad, and Orn systems in the bacterial kingdom

Enteropathogenic bacteria (e.g. *Escherichia*, *Salmonella*, and *Yersinia*) generally possess several enzyme-based AR systems to survive in highly acidic environments, such as the stomach, and mild acidic ones, such as the caecum and ascending colon, however, this is not the usual case. Overall, the number, architecture and the complexity of regulation of the four main systems, Gad, Adi, Cad, and Orn, vary amongst bacterial species and often reflect an adaptation to their (acidic) habitat (Zhao and Houry 2010). The most widespread systems are the Gad and Orn systems, which co-occur in many Proteobacteria and Firmicutes. Bacterial species such as *Escherichia*, *Shigella*, *Citrobacter*, *Hafnia*, and *Serratia* have all four enzyme-based AR systems; *Salmonellae* have the Adi, Cad, and Orn systems; whereas the Gram-positive *Lactococcus* (Amachi et al. 1998, Sanders et al. 1998, O'Sullivan and Condon 1999) and *Listeria* (Davis et al. 1996, Cotter et al. 2001a, Arcari et al. 2020) possess the Gad system only (Zhao and Houry 2010). Species, like *Vibrio* spp. that generally are more sensitive to acid (Brenneman et al. 2014, Brameyer et al. 2020), contain only the Cad and Orn systems.

The different number of enzyme-based AR systems in the various bacteria is also reflected by the number of bacteria required to cause infection in humans. For example, *Shigella* spp. have a uniquely low infective dose of only 10–100 *Shigella* cells, whereas *Vibrio cholerae* infection requires about 10^8 organisms and *Salmonella* infection requires 10^5 to 10^7 (Table 1). *Escherichia* and *Shigella* spp. are the most acid-resistant neutralophilic bacteria. The *E. coli* K12 lab strains DH5α and MC4100 are as acid resistant as naturally occurring *E. coli* isolates (Gorden and Small 1993).

Pseudomonas spp. contain only the Orn system (Brameyer et al. 2022), which generally plays a minor role in AR, but are additionally equipped with deiminase and urease systems to withstand acidic environments.

The Gad system plays an important role for commensal and pathogenic bacteria, such as *E. coli* and *S. flexneri* (Waterman and Small 2003, Bhagwat and Bhagwat 2004), and it was recently identified in *Yersinia* (Foster 2004, Biase and Pennacchietti 2012, Pennacchietti et al. 2018, Brameyer et al. 2022), *Clostridium* (Villarreal et al. 2000, Tennant et al. 2007, Biase and Pennacchietti 2012), *Listeria monocytogenes* (Cotter et al. 2001a, 2001b), *Lactobacillus brevis* (Gong et al. 2019) and *Lactococcus lactis* (Sanders et al. 1998, O'Sullivan and Condon 1999). See Table 2 and Fig. 5 for details

Table 1. Infective doses of food-borne pathogens.

Pathogen	Infective dose	Reference
Enterohemorrhagic *E. coli* (EHEC)	< 50	Tilden et al. (1996)
Brucella spp.	< 10	Román et al. (2013)
Shigella spp.	10–100	Sansonetti (2001)
Salmonella spp.	10^5–10^7	D'Aoust et al. (2001)
Vibrio cholerae	10^4–10^8	Cash et al. (1974)
Yersinia enterocolitica	10^4–10^9	Bottone (1997)
Campylobacter jejuni	500–800	Robinson (1981)
Clostridium perfringens	10^7	Brynestad and Granum (2002)
Listeria monocytogenes	> 100 bacteria/g*	Swaminathan (2001)

*Number in contaminated food responsible for foodborne human cases.
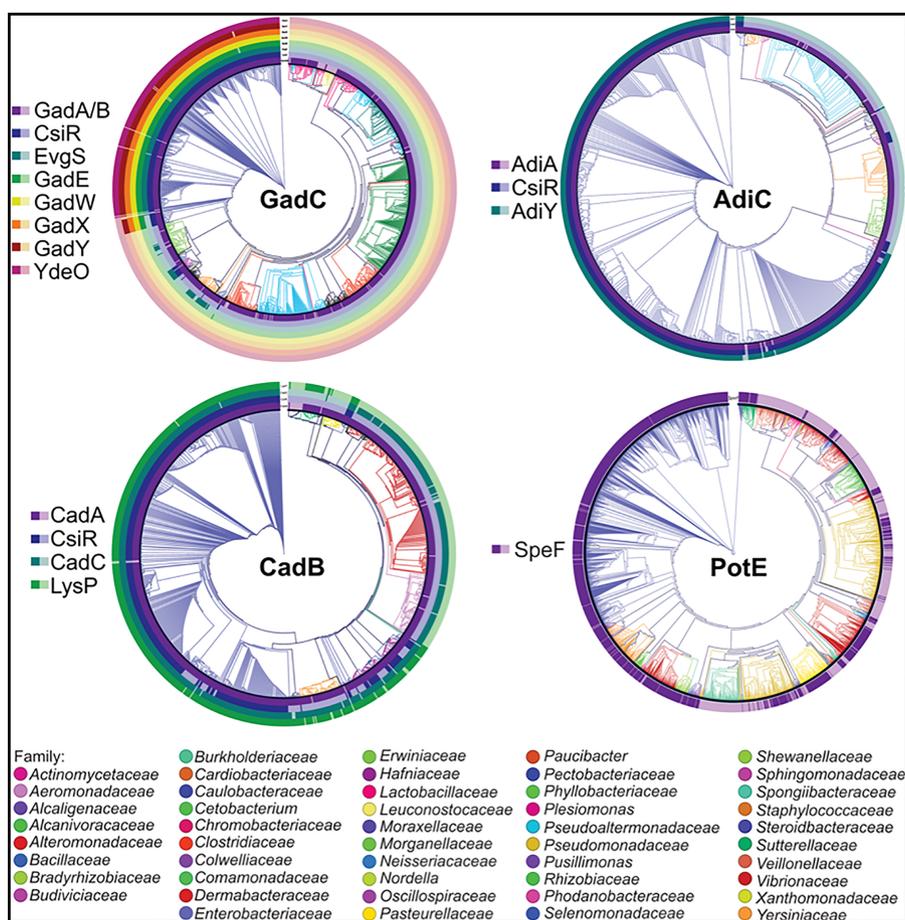
Figure 5. Phylogenetic trees of the *E. coli* antiporters GadC, AdiC, CadB, and PotE and co-occurring regulators within the bacterial kingdom. The protein sequences of 1112 of *E. coli* GadC homologs (upper left) were aligned, and a phylogenetic tree was generated. The presence of different regulatory components CsiR, EvgS, GadE, GadW, GadX, GadY, and YdeO and the decarboxylases GadA and GadB (GadA/B) is indicated by solid colors in the respective ring layer. Translucent colors represent components that were not detected. The protein sequences of 756 *E. coli* AdiC homologs (upper right) were aligned, and a phylogenetic tree was generated. The presence of the different regulatory components CsiR and AdiY as well as the decarboxylase AdiA is indicated by solid colors in the respective ring layer. If these components were not found, the colors are translucent. The protein sequences of 857 *E. coli* CadB homologs (lower left) were aligned, and a phylogenetic tree was generated. The presence of the different regulatory components CsiR, CadC, and LysP as well as the decarboxylase CadA are indicated by solid colors in the respective ring layer. Translucent colors represent components that were not detected. The protein sequences of 2577 *E. coli* PotE homologs (lower right) were aligned, and a phylogenetic tree was generated. The presence of the decarboxylase SpeF is indicated by solid colors in the respective ring layer. See legend of Table 2 for more details. Each phylogenetic tree is shown as a circular cladogram. For each phylogenetic tree the branches of the tree were colored according to the family of the organisms containing a GadC, AdiC, CadB, or PotE homolog, respectively.

on these and other bacteria discussed in this section.

Regulation of the Gad system has been studied in great detail in *E. coli* (see above). The regulators responsible for the sophisticated regulation of the Gad, Adi, and Cad systems of *E. coli* (Fig. 3) are present mainly in the *Enterobacteriaceae* family. The central regulator of the Gad system, GadE, is found in *Escherichia* and *Shigella*, as well as other *Enterobacteriaceae* species, such as *Citrobacter freundii* and *Kluyvera ascorbate* (Fig. 5) (Brameyer et al. 2022). The carbon-

Table 2. Occurrence of the main components of the Gad, Adi, Cad, and Orm system within selected species of the proteobacteria and firmicutes. Homologs of the components of the Gad, Adi, Cad, and Orm system in the selected species were identified based on the proteins from *E. coli* MG1655. Phylogenetic distribution of the Gad, Adi, Cad, and Orm system is presented in Fig. 5. "x" indicates presence of a homolog of the proteins GadA/B, GadC, CsiR, EvgS, GadE, GadW, GadY, YdeO, AdiA, AdiC, AdiY, CadA, CadB, CadC, LysP, SpeF, and PotE. This table is modified and extended from Brameyer et al. (2022) for the occurrence of the Orm system. Briefly, homologs of the Orm system were identified as described previously (Brameyer et al. 2022) with the modified expect thresholds for the Protein BLAST search (e < 10^{-40} for PotE and e < 10^{-170} for SpeF) resulting in the identification of 2577 PotE homologs. These are present with Proteobacteria, such as Enterobacteriaceae (36.4%, with 20.0% belonging to *Escherichia*); *Pseudomonaceae* (17.6%), *Vibrionaceae* (15.0%), and *Burkholderiaceae* (4.0%); and within Firmicutes, such as *Streptococcaceae* (5.2%), *Yersiniaceae* (4.3%), *Lactobacillaceae* (1.2%), and *Clostridaceae* (0.5%).

	GadA/B	GadC	CsiR	EvgS	GadE	GadW	GadX	GadY	YdeO	AdiA	AdiC	AdiY	CadA	CadB	CadC	LysP	SpeF	PotE
Proteobacteria																		
Escherichia coli	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Escherichia albertii	X	X	X		X	X	X	X		X	X	X	X	X	X	X	X	X
Shigella boydii	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X
Shigella flexneri	X	X	X	X	X	X	X	X	X	X	X	X					X	X
Salmonella enterica		X	X							X	X	X	X	X	X	X	X	X
Citrobacter freundii	X	X	X		X					X	X	X	X	X		X	X	X
Hafnia alvei	X	X	X	X						X	X		X	X	X	X	X	X
Serratia fonticola	X	X		X						X	X		X	X	X	X	X	X
Yersinia spp.	X	X		X						X	X		X	X	X	X	X	X
Stenotrophomonas maltophilia										X	X							
Edwardsiella tarda													X	X	X	X	X	X
Pseudomonas fluorescens																	X	X
Morganella morganii		X											X	X		X	X	X
Brucella spp.	X	X																
Vibrio parahaemolyticus													X	X	X		X	X
Vibrio cholerae													X	X	X		X	X
Vibrio campbellii													X	X	X		X	X
Firmicutes																		
Clostridium perfringens	X	X														X		
Lactococcus lactis	X	X														X		
Listeria monocytogenes	X	X														X		
Enterococcus faecium	X	X																
	Gad system									Adi system			Cad system				Orm system	

induced starvation transcriptional regulator CsiR is a member of the GntR-family and mainly occurs in γ-proteobacteria. A total of 30.3% of species containing a GadC homolog possess an CsiR homolog (Fig. 5). Comparison of GadB homologs from Gram-positive and Gram-negative enteric bacteria shows that 84 residues (18% of the *E. coli* GadB sequence) are strictly conserved, including residues at critical positions in or near the active site of *E. coli* GadB or at sites of conformational change. Moreover, eight residues that are shared with group II decarboxylases may serve as signatures for bacterial glutamate-dependent AR (Biase and Pennacchietti 2012).

Besides using ureases to encounter acidic threats, *Brucella microti* also uses the Gad system to increase AR and oral infection in mice (Occhialini et al. 2012). An intact *gadBC* operon helps *B. microti* to survive in acidic soils and supports intracellular survival, such as within host macrophages (Audic et al. 2009). In 2015, Damiano et al. (2015) investigated the functionality of the Gad system among a collection of strains belonging to the *Brucella* genus and showed that not all species could export GABA and survive at extremely low pH levels; the authors thus concluded that the Gad system may reflect the adaptive evolution of *Brucella* spp.

In addition to the variations in bacterial AR systems, the architecture of individual AR systems also can vary. For example, the number of glutamate decarboxylases (up to three) and number of antiporters (up to two) varies in some bacteria, such as *Edwardsiella tarda*, *L. brevis*, and *L. monocytogenes* (Cotter et al. 2001a, 2001b, Cotter et al. 2005, Karatzas et al. 2010, Feehily and Karatzas 2013). Moreover, *E. coli* (Smith et al. 1992) and *S. flexneri* (Waterman and Small 2003) each contain two glutamate decarboxylases, GadA and GadB (Feehily and Karatzas 2013).

The Adi and Cad systems are not as widely distributed as the Gad system and occur mainly within γ-proteobacteria in the *Enterobacteriaceae*, *Lysobacteraceae*, *Yersiniaceae*, and *Hafniaceae* families (Brameyer et al. 2022) (see Table 2 and Fig. 5 for details).

However, *S.* Typhimurium is one well-studied example that harbors the Adi (Kieboom and Abee 2006), Cad (Lee et al. 2007), and Orn systems (Viala et al. 2011) (Table 2 and Fig. 5), and its virulent strains are more acid tolerant than its nonvirulent strains (Wilmes-Riesenberg et al. 1996, 1997, Berk et al. 2005). These three systems confer AR in different ways in this serovar. Specifically, the Adi system is effective at extremely acidic pH levels but ineffective during growth at moderately acidic pH levels. The Orn system improves growth at moderately acidic pH levels in the absence of oxygen, but it plays a minor role in survival. The Cad system has a broad range of actions and confers both to significant survival at pH 2.3 and to growth improvement at pH 4.5 in an oxygen-independent manner (Viala et al. 2011). Unlike *E. coli*, in which transcription of *cadC* is constitutive, in *S.* Typhimurium, *cadC* expression is induced by mildly acidic pH levels and by lysine (Lee et al. 2007). Instead of recovering from acid stress, *S.* Typhimurium uses acidity as a signal to control pathogenesis via the action of OmpR, which represses the *cadC/cadBA* module, thereby preventing neutralization of the bacterial cytoplasm (Chakraborty et al. 2015, Kenney 2019). Both the Cad and Orn systems enhance *S.* Typhimurium's acid tolerance. The *spe* genes (*speB, speC, speE,* and *speF*) have been shown to contribute to intracellular survival and bacterial replication for 18 h in human epithelial cells and for 21 h in macrophages (Espinel et al. 2016).

Functionality and pH-dependent regulation of the Orn system are less studied, however, the presence of a system does not necessarily indicate its contribution to AR in these species. For example, putrescine is linked to the formation of biofilm (Patel et al. 2006), expression of virulence genes in *S. flexneri* (Durand and

Björk 2003) and the counteraction of toxic effects caused by reactive oxygen species in *E. coli* (Jung and Kim 2003). Nevertheless, the cognate ornithine decarboxylase SpeF of the Orn system has been identified in about two-thirds (63.6%) of species harboring the ornithine/putrescine antiporter PotE, such as *E. coli*, *Shigella boydii*, *S. enterica*, *C. freundii*, and *Serratia fonticola*. Homologs of the ornithine/putrescine antiporter PotE, which belongs to the Orn system, are widely distributed and occur in species belonging to 48 different families of Proteobacteria (e.g. *Enterobacteriaceae*, *Pseudomonaceae*, *Vibrionaceae*, and *Burkholderiaceae*) and several from the phylum Firmicutes (e.g. *Streptococcaceae*, *Yersiniaceae*, *Lactobacillaceae*, and *Clostridaceae*) (see Table 2 and Fig. 5 for details). The Orn system often occurs in conjunction with the other three inducible AR systems, however, it seems to play a minor role in acid adaptation. For example, it plays only a minor role in *E. coli* MG1655 but has a more important function in avian pathogenic *E. coli*, in which growth attenuation occurs after the addition of membrane stress by sodium dodecyl sulfate. This finding suggests that PotE is more involved in membrane stress tolerance than acidic tolerance (Guerra et al. 2018). As previously mentioned, the Orn systems plays only a minor role compared to the Cad system during acid adaptation of *V. cholerae* (Merrell and Camilli 1999) (Table 2 and Fig. 5). Mutation analyses have revealed a significant role of the lysine decarboxylase *cadA*, but not the ornithine decarboxylase *speF* in both inorganic and organic acid adaptation of *V. cholerae* (Merrell and Camilli 1999). In summary, the *E. coli*-specific regulation of the Gad, Adi, Cad, and Orn systems are predominantly conserved in the *Enterobacteriaceae* family. The Gad and Orn systems occur in many bacteria, whereas the Adi and Cad systems are restricted to γ-proteobacteria. Interestingly, the decarboxylases together with their respective cognate antiporters in each system are more widely distributed than known regulatory components. The central regulators of the Cad and Adi systems identified in *E. coli* are more specific to the *Enterobacteriaceae* family.

Functional diversification of bacterial populations under acid stress

Escherichia coli possesses all four enzyme-based H$^+$-consuming AR systems (Gad, Adi, Cad, and Orn) and is one of the best studied organisms with respect to acid tolerance (Table 1). Individual cells do not always respond uniformly to different external pH values. The responses can be highly variable depending on the AR system, and each system is activated at different external pH values and growth phases (Fig. 3). This phenotypic heterogeneity can be beneficial for populations under acid stress. Between individual *E. coli* cells pH homeostasis varies (Martinez et al. 2012). Furthermore, persister cells of *E. coli* have a lower intracellular pH that allows survival after antibiotic treatment (Goode et al. 2021). Moreover, the induction of enzyme-based AR systems is regulated by a sophisticated interplay of membrane-integrated and soluble regulators. For example, the noisy activation of the *gadBC* promoter is highly variable under acid stress and supports the survival of single *E. coli* cells by maintaining a sufficient high intracellular pH (Mitosch et al. 2017).

Activation of the Gad, Adi, and Cad systems has been quantitatively analyzed using a fluorescent triple reporter strain under consecutively increasing acid stress conditions in *E. coli* (Brameyer et al. 2022). As shown in Fig. 6, the Cad system shows the highest heterogeneous distribution of the three systems when activated at pH 5.8 in the presence of lysine. The Adi system is activated heterogeneously under moderate acid stress (pH 4.4), though simultaneous activation of the Cad and Adi systems does not occur.
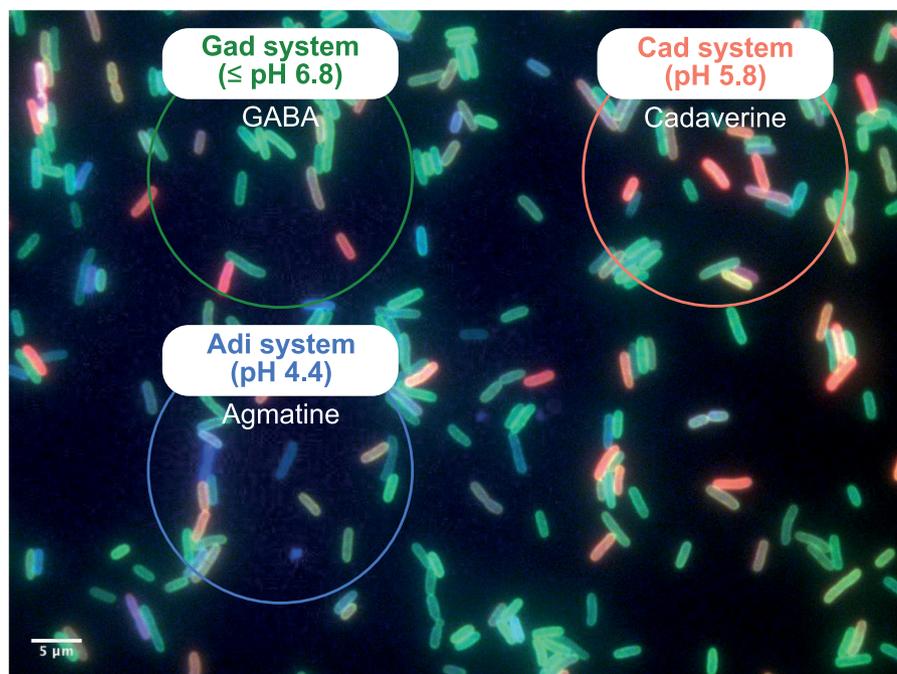
Figure 6. Model of the heterogenous activation of enzyme-based AR systems and division of labor in the *E. coli* population. Fluorescent microscopic image of the three-color reporter strain *E. coli gadC:eGFP-adiC:mCerulean-cadB:mCherry* at pH 4.4 in a complex medium. All cells of the *E. coli* population individually adapt to acid stress by activating the Gad system to varying degrees (green fluorescent cells), due to multiple extracellular and intracellular inputs, and secrete GABA. Under mild to moderate acid stress (pH 5.8–4.4), some cells in the population activate the Cad system (red fluorescent cells), while others activate the Adi system (blue fluorescent cells). These cells secrete (as indicated by the rings) the more alkaline products cadaverine and agmatine, respectively, thereby contributing to acid-stress relief with an increase of the extracellular pH, which benefits the whole population (Brameyer et al. 2022).

Under almost all conditions, bacterial cells activate the Gad system to varying degrees, primarily using intracellularly available glutamate by consuming protons to raise their internal pH. In response to high acid stress, glutamic acid is imported and GABA excreted. Heterogenous activation of the Cad and Adi systems is driven by the low copy numbers of their main regulators CadC and AdiY (Brameyer et al. 2020, 2022). Furthermore, CadC itself is distributed heterogeneously (Brameyer et al. 2019). An artificial increase in the copy number of these regulators leads to reduced heterogeneity and a growth disadvantage under acid stress, representing a metabolic burden for individual cells (Brameyer et al. 2020, 2022).

Although Adi and Cad system activation occurs only in a subpopulation of cells, the whole population benefits from the elevated external pH via secretion of the polyamine compounds cadaverine and agmatine, which are considered common goods (Fig. 6). The extent to which microcolonies of *E. coli* exist in the colon, where cadaverine and agmatine can change the micromilieu, remains unclear. Additionally, the internal pH of these cells is elevated by consuming protons during the conversion of lysine to cadaverine and arginine to agmatine, respectively (Brameyer et al. 2022). Overall, the heterogeneous activation of these three enzyme-based AR systems enables division of labor in the bacterial population, which ensures its survival over a wide range of low pH values (Fig. 6).

In contrast to *E. coli*, *V. campbellii* is more acid sensitive and uses mainly the Cad system to counteract acidic conditions. Additionally, its CadC signaling system is simpler, as CadC lacks the regulatory inputs by LysP and the polyproline motif known from *E. coli* CadC (see above). Without this tight regulation, all the cells of the *V. campbellii* population activate the Cad system, which is important, as the Cad system is the main AR system in this bacterium (Brameyer et al. 2020). Although the presence and function of the variety of AR systems are well-described in several bacterial species, their behavior at single cell level and specific niches is highly unexplored yet.

Concluding remarks

Bacteria have sophisticated and varying mechanisms for sensing and adapting to acidity. Depending on the bacterial genus, the number and complexity of AR mechanisms vary greatly, which is best reflected in the number of bacteria that survive challenging environments, such as the human stomach, and thus contribute to bacterial pathogenesis (Table 1). For example, *Escherichia* and *Shigella* spp. have the most complex regulatory network and are extremely acid-resistant, followed by *Salmonella* and *Brucella* spp., whereas *Yersinia* and *Vibrio* spp. are less acid-resistant.

At the population level, there is strong evidence for the heterogenous behavior of individual cells in response to acid stress (Mitosch et al. 2017, Smith et al. 2018, Brameyer et al. 2020, 2022). In *E. coli*, functional diversification of several AR mechanisms in single cells allows division of labor and energy conservation to ensure that at least a small percentage of the population survives even the most acidic conditions (e.g. pH 1–3). The cells' regulatory networks also affect their behavior. For example, implementing additional components or reducing the network to basic components allows heterogeneous activation of the Cad system in *Escherichia* spp., whereas it is homogeneous in *Vibrio* spp. (Brameyer et al. 2020). Further studies are required to analyze the single-cell acid stress response in more species, preferably in mixed bacterial communities and in natural environments. This analysis also could identify differences in responses to inorganic and organic acids (Wilks and Slonczewski 2007).

pH is one of the most important parameters for bacterial interactions (Ratzke and Gore 2018). The human and animal caecum and colon are characterized by dense communities of numerous obligate anaerobic fermenting bacteria that produce a variety of short-chain fatty acids resulting in a low pH environment. Depending on the acidity and availability of amino acids (e.g. glutamic acid, lysine, arginine, and ornithine), decarboxylase-dependent systems are activated in Proteobacteria phylum members. These systems produce GABA (an important neurotransmitter); cadaverine, agmatine, and putrescine (polyamines used by prokaryotes and eukaryotes) and histamine (an allergen). However, it is unclear how these molecules change the microenvironment and to what extent they affect the host. In fact, the levels of putrescine and cadaverine are found to increase 15-fold and 3-fold, respectively, in inflamed mouse intestine monocolonized with *E. coli*, compared to an equally colonized healthy mouse cohort (Kitamoto et al. 2020). Further studies are needed to better understand the adaptation of complex communities to partially self-caused acid stress and the effects of the involved metabolites on the host.

Neutralophilic bacteria can maintain a constant intracellular pH under most conditions. However, under strong acid stress (pH < 4.4) or amplification of stress by weak organic acids, such as acetate, the cytoplasmic pH decreases (e.g. by about one pH unit in *E. coli*) (Wilks and Slonczewski 2007). A lower intracellular pH affects all macromolecules in cells and can reduce enzyme activity, acid-induced protein unfolding, membrane damage, and DNA damage. Some bacteria use low intracellular pH as a stimulus to induce other mechanisms. For example, in the acidic environment of the macrophage vacuole, *S.* Typhimurium induces virulence genes encoded in its pathogenicity island SPI-2. This induction depends on the SsrA/SsrB system, which requires acidification of the cytoplasm for activation (Liew et al. 2019). Once in the vacuole, *S.* Typhimurium acidifies its cytoplasm in an OmpR-dependent manner by repressing the CadC-regulated *cadBA* operon (Chakraborty et al. 2015). Thus, intracellular acidification of *Salmonella* spp. provides an important signal for expression of the SPI-2 type III secretion system. Recent evidence also shows that acidification of the cytoplasm in *E. coli* is linked to antibiotic resistance (Reyes-Fernández and Schuldiner 2020). The authors analyzed two mutants, derived by laboratory evolution, that are resistant to norfloxacin and chloramphenicol. They found that intracellular pH was significantly lower in the mutants than of the parental strain. The mediators of this relationship between cytoplasmic acidification and antibiotic resistance are not yet known. Interestingly, persister cells of *E. coli* display a lower intracellular pH, allowing survival after antibiotic treatment (Goode et al. 2021). Further work is needed to elucidate the mechanism by which acidification is related to virulence and antibiotic resistance.

Finally, we would like to highlight an important applied aspect for future research on acid stress resistance in bacteria. Probiotic bacteria are becoming increasingly important for their pharmaceutical value in maintaining and improving gut microbiota. So-called superfoods are marketed as products that provide nutritional value and health benefits. However, the incorporation of probiotics into food remains a challenge as the number of live bacteria declines during manufacturing and storage and especially during gastrointestinal transit (Palanivelu et al. 2022).

In summary, a better understanding of the mechanisms of AR of bacteria that have evolved during evolution has great potential for infection biology, microbiome research, and ecology.

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2.6 Bacterial acid stress response: from cellular changes to antibiotic tolerance and phenotypic heterogeneity

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Bacterial acid stress response: from cellular changes to antibiotic tolerance and phenotypic heterogeneity

Kilian Schumacher, Sophie Brameyer and Kirsten Jung

Most bacteria are neutralophiles but can survive fluctuations in pH in their environment. Herein, we provide an overview of the adaptation of several human, soil, and food bacteria to acid stress, mainly based on next-generation sequencing studies, highlighting common and specific strategies. We also discuss the interplay between acid stress response and antibiotic tolerance, as well as the response of individual cells.

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Introduction

Bacteria have evolved sophisticated acid resistance systems to survive and even multiply in acidic environments (Figure 1a). Acid stress sensing and adaptation enable neutralophilic bacteria to maintain constant intracellular pH under moderate acid stress [1]. As acidification of the cytoplasm has severe effects on all macromolecules of a cell that might lead to protein unfolding, decreased enzyme activity, membrane damage, and DNA damage [2–4], bacteria have evolved a variety of acid stress resistance systems to counteract and survive in mild and highly acidic environments [5]. Detailed information on the molecular mechanisms of acid resistance in bacteria can be found in reviews by Cotter & Hill 2003, Foster 2004, Kanjee & Houry 2013, Lund et al. 2014, Guan & Li 2020, Arcari et al. 2020, Schwarz et al. 2022, and Rai et al. 2022 [5–12].

Here, we focus on systemic studies of the acid response of various bacteria and describe common and species-specific acid resistance mechanisms. We will also discuss how acid stress resistance facilitates development of antibiotic resistance [13]. Although most studies of stress response dynamics have focused on the population level and thus on the average response of millions of cells, recent work has also examined the acid stress response of individual cells, revealing phenotypic heterogeneity, which is also discussed.

Acid stress-dependent alterations of the transcriptome and proteome of various bacteria

Significant advances have been made in understanding how bacteria interact with their environment, particularly after the development of powerful next-generation sequencing techniques such as bacterial single-cell RNA-Seq, dual RNA-Seq, and triple RNA-Seq [14–17]. This trend of rapid large-scale data collection has now also impacted the field of acid stress research, as more and more next-generation sequencing techniques have become routine workflows in many laboratories. Since 2020, over a dozen studies, which are reviewed here, have been published with the aim of revealing genome-wide responses to acidity in a broad range of bacterial species (Figure 1), using a variety of techniques. These included mainly bulk RNA-Seq to analyze bacterial transcriptomes, with fewer studies using mass spectrometry to examine the translational level [18,19] (Table 1). Additionally, ribosome profiling (Ribo-Seq) has been employed to evaluate global protein synthesis rates under mild and severe acid stress in *E. coli* K-12 MG1655 (designated as *E. coli*) [20]. Ribo-Seq is a powerful tool that provides a global overview of protein synthesis rates and enables the detection of unidentified small open-reading frames [21,22]. Further insights into the microbial defense against low pH were provided by transposon-insertion-directed sequencing (TraDis), also called Tn-Seq. In the respective studies, transposon libraries were used for deep sequencing to provide information on the contribution of single genes to fitness in acidic habitats in uropathogenic *E. coli* (UPEC) and *Salmonella derby* [23,24].

Recent multi-omics studies focused on bacterial species from a broad range of phylogenetic classes, including *Acidithiobacillia*, *Actinomycetes*, *Alphaproteobacteria*, *Bacilli*, *Deltaproteobacteria*, *Epsilonproteobacteria*, and *Gammaproteobacteria* (Table 1). These organisms colonize a variety of environmental niches, including the

Figure 1
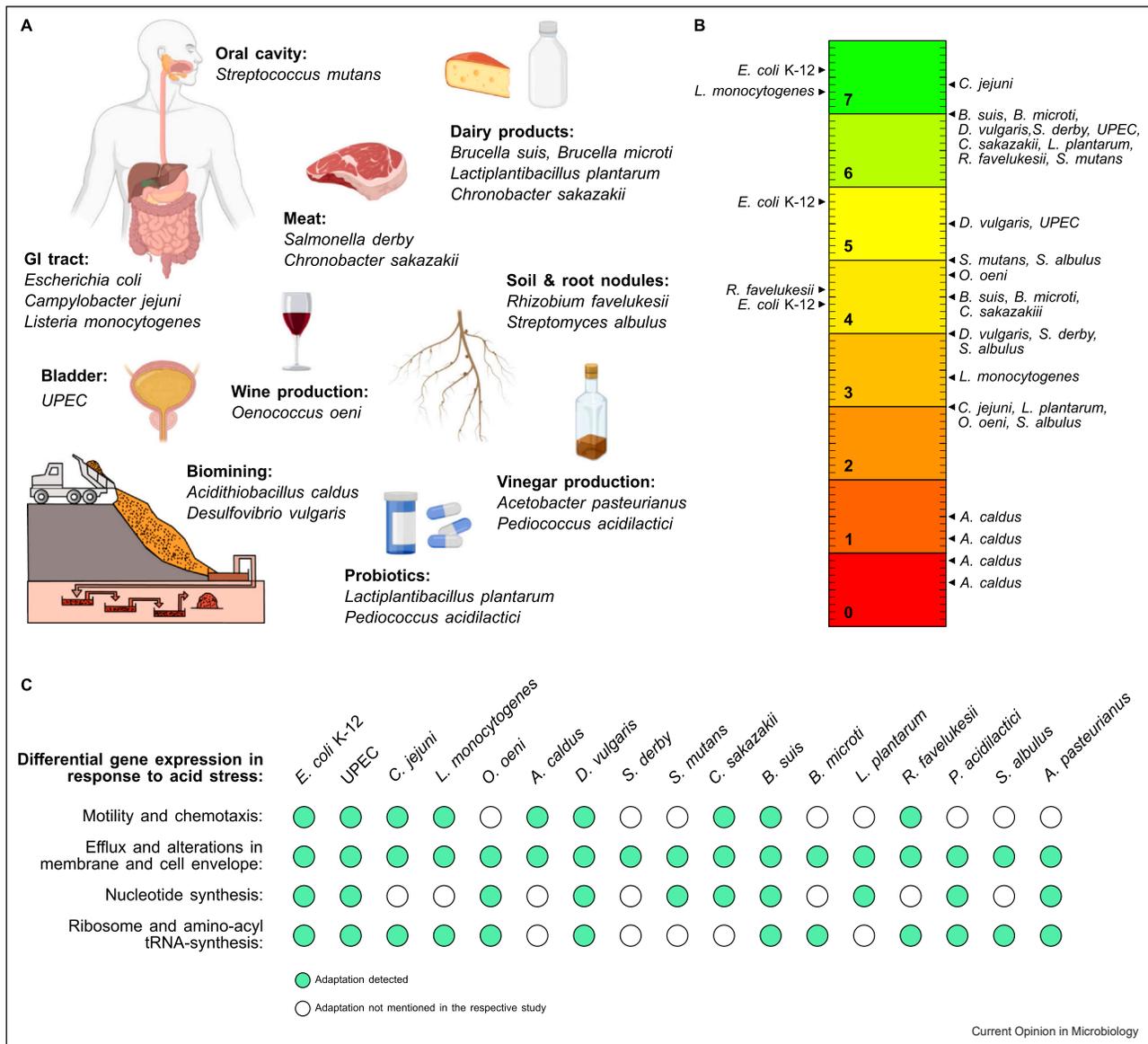
Overview of *omics* studies from 2020 to 2023, investigating global acid stress responses in various bacteria. **(a)** Habitats and industrial applications of bacterial species being studied using next-generation sequencing. **(b)** Visual representation of pH values selected as acid stress and controls for experiments listed in Table 1. **(c)** Schematic overview of acid stress responses. Common adaptions detected in several bacterial species are highlighted in green, adaptations that were not mentioned in the respective studies are marked in white. Figure **(a)** is created using BioRender.com and Affinity Designer 1.10.4.

gastrointestinal tract (GI tract), the human oral cavity and bladder, processed and unprocessed foods, acidic soils and root nodules, as well as biomining sites (Figure 1a). Among the strains listed in Table 1, *Escherichia coli*, *Campylobacter jejuni*, and *Listeria monocytogenes* colonize the human GI tract and can induce diseases. Further investigated bacteria include *Streptococcus mutans*, which is associated with dental caries and causes dysbiosis in the oral cavity [19], and UPEC, which is the primary cause of urinary tract infections. In

addition, genome-wide acid responses of other food-borne pathogens, including *Salmonella derby*, *Chronobacter sakazakii*, *Brucella suis*, and *Brucella microti* have been characterized [24–26]. Other bacteria that have been the subject of recent multi-omics studies were *Rhizobium favelukesii* [27], an alfalfa-nodulating rhizobia frequently found in low-pH soils, and *Streptomyces albulus*, which is used for the industrial production of ε-poly-L-lysine [28]. Additionally, *Oenococcus oeni*, *Pediococcus acidilactici*, and *Acetobacter pasteurianus* are

Table 1

Bacterial species, methods, and growth media used to study genome-wide responses to acid stress.

Strain	Family	Class	Methods	Growth medium	Reference
Acetobacter pasteurianus Ab3	Acetobacteraceae	Alphaproteobacteria	RNA-Seq	Yeast extract–peptone–dextrose (YPD) medium + 20 g/l ethanol	[45]
Acidithiobacillus caldus CCTCC M 2018054	Acidithiobacillaceae	Acidithiobacillia	RNA-Seq	Modified Starkey medium	[37]
Brucella suis ATCC 23444, Brucella microti CCM4915	Brucellaceae	Alphaproteobacteria	RNA-Seq	Tryptic Soy broth (TSB) and Gerhardt's minimal medium (GMM)	[25]
Campylobacter jejuni NCTC11168	Campylobacteraceae	Epsilonproteobacteria	Microarray	Mueller Hinton (MH)	[38]
Cronobacter sakazakii CICC 21544	Enterobacteriaceae	Gammaproteobacteria	RNA-Seq	TSB	[26]
Desulfovibrio vulgaris ATCC 7757	Desulfovibrionaceae	Deltaproteobacteria	RNA-Seq	Self-created medium containing yeast extract	[36]
Escherichia coli K-12 MG1655	Enterobacteriaceae	Gammaproteobacteria	RNA-Seq, Ribo-Seq	Luria–Bertani (LB)	[20]
Escherichia coli serotype ST131 strain EO499 (UPEC)	Enterobacteriaceae	Gammaproteobacteria	RNA-Seq, TraDIS	M9 medium supplemented with glucose and cas-amino acids + 100 mM 3-(N-morpholino)propanesulfonic acid (MOPS) /100 mM 2-(N-morpholino)ethanesulfonic acid (MES)	[23]
Lactiplantibacillus plantarum ZDY2013	Lactobacillaceae	Bacilli	RNA-Seq	De Man, Rogosa, and Sharpe (MRS) broth	[39]
Listeria monocytogenes 6179 and R479a	Listeriaceae	Bacilli	RNA-Seq	TSB	[40]
Oenococcus oeni SD-2a	Leuconostocaceae	Bacilli	RNA-Seq, metabolomics	Fructose–malate–acid–tomato juice broth medium	[48]
Pediococcus acidilactici CGMCC 17856	Lactobacillaceae	Bacilli	Proteomics	MRS	[18]
Rhizobium favelukesii LPU83	Rhizobiaceae	Alphaproteobacteria	RNA-Seq	Sucrose–glutamate minimal medium + 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) /MES	[27]
Salmonella derby 14T	Enterobacteriaceae	Gammaproteobacteria	Tn-Seq	LB	[24]
Streptococcus mutans UA159	Streptococcaceae	Bacilli	Proteomics	Tryptone yeast medium + 1% (w/v) glucose	[19]
Streptomyces albulus M-Z18	Streptomycetaceae	Actinomycetes	RNA-Seq	Agar slant medium	[28]

See text for details.

exposed to acidity during industrial applications (Figure 1a). *Oenococcus oeni* is commonly used in wine production for malolactic fermentation, during which L-malate is converted to L-lactate and carbon dioxide under low-pH conditions [29]. *Pediococcus acidilactici* is utilized in the production of vinegar and as a probiotic food supplement [18,30]. Acid tolerance is crucial for these applications because acetic and lactic acids accumulate during vinegar production [31], and probiotic supplements are supposed to exhibit robustness toward acidity encountered in the stomach [32]. While most transcriptome and proteome analyses were conducted with neutralophilic bacteria, *Acidithiobacillus caldus* is acidophilic and commonly used for bioleaching to recover valuable metals from reduced sulfides [33]. During this process, metal ions are oxidized, and sulfuric acid is produced, resulting in an ambient pH of < 1.5 [34]. Despite being exposed to extreme acid stress, acidophilic organisms are capable of maintaining an internal pH of approximately 6.0 [35]. In this regard, the transcriptome of *A. caldus* was studied within the pH range of 0.6–1.5, whereas other bacteria were typically subjected to experiments performed within the range of pH 3.0–4.5, or 5.0–5.8 (Figure 1b). In addition to *A. caldus*, *Desulfovibrio vulgaris*, a sulfate-reducing bacterium, can be applied during biomining to remediate acid mine wastewater [36].

A comparison of these global systemic studies (Table 1) reveals both common changes in the expression of specific genes and species-specific adaptation strategies. Remarkably, despite substantial differences in sampling time points, stress treatment, stress duration, growth medium, pH (Figure 1b), oxygen availability, strain physiology, and habitats (Figure 1a), a number of common acid stress adaptions are detectable, even among distantly related bacteria. These adaptations include differential expression of genes associated with motility and chemotaxis, efflux and alterations in membrane and cell envelope, nucleotide synthesis, and ribosome and amino-acyl tRNA synthesis (Figure 1c). For example, differential expression of motility and chemotaxis-associated genes is found in *E. coli*, UPEC, *A. caldus*, *B. suis*, *C. jejuni*, *D. vulgaris*, *C. sakazakii*, *L. monocytogenes*, and *R. favelukesii* [20,23,25–27,36–40]. pH-dependent expression of genes involved in flagellar assembly and locomotion, as well as pH taxis, has previously been observed in both *E. coli* and *B. subtilis* and represents a strategy to migrate to territories with desired proton concentrations [41–43]. In response to low pH, modifications in inner and outer membrane composition are noted, which include differential synthesis of transporters, efflux pumps, and fatty acids. These adaptations restrict proton uptake and accelerate proton extrusion, and are detectable in all studied species (Figure 1c). A common adaptation of this type is the upregulation of genes encoding the subunits of F_oF_1-

adenosine triphosphate (ATP) synthase, which exports protons in an ATP-dependent manner [44], in *E. coli*, *A. caldus*, *A. pasteurianus*, *B. microti*, and *S. mutans* [19,25,37,45]. Bacterial nucleotide metabolism is also undergoing significant changes (Figure 1c). Particularly in *B. suis*, *E. coli*, and *S. mutans*, genes involved in purine biosynthesis are upregulated under acid stress [19,20,25]. It is worth noting that DNA damage is accelerated by acid stress, and DNA depurination increases proportionally with lower pH [46]. Depurination refers to the loss of purine caused by hydrolysis of *N*-glycosyl bonds, which occurs more rapidly compared with depyrimidination [47]. Thus, the observed increase in purine biosynthesis in acid-stressed bacteria might provide additional purine nucleotides for DNA repair. This hypothesis is supported by metabolomics data from *O. oeni*, which suggest a decrease in the abundance of nucleotides upon acid treatment [48]. In the same study, increased expression of genes related to translation and post-translational modifications is suggested. Indeed, genes encoding ribosomal subunits are differentially expressed upon acid stress, for example, in *E. coli*, *C. jejuni*, *D. vulgaris*, *P. acidilactici*, *O. oeni*, *R. favelukesii*, and *S. albulus* (Figure 1c) [18,20,27,28,36,38,48]. Accordingly, amino-acyl tRNA synthesis in *E. coli*, UPEC, and *L. monocytogenes* is affected by low pH [20,23,40].

In addition to common adaptation strategies, there are also different species-specific mechanisms. The plasmid-encoded putative metal-ion sensing riboswitch is specific for the acid response of *L. monocytogenes* [40]. Acid stress in *B. microti* induces production of the cold-shock protein CspA. A deletion of the corresponding gene decreases the survival of these bacteria in acidified media [25]. Intriguingly, *cspA* is a pseudogene in *B. suis*, a feature that could explain the observed difference in acid tolerance between *B. suis* and *B. microti* [25]. Among other transcriptional changes in the acidophilic *A. caldus*, a decrease in external pH resulted in downregulation of several transcriptional regulators of the LysR family, which have been demonstrated to be essential for maintaining cytoplasmic pH [37]. In *E. coli* K-12, about 2% of all ribosome profiling reads at pH 4.4 mapped to the *asr* region, which encodes an acid-shock protein [20]. This highlights the crucial role of Asr, a periplasmic chaperone that contributes to membrane integrity by preventing aggregation of proteins with positive charges [49]. Moreover, altered pyruvate metabolism was observed in *P. acidilactici* and *O. oeni* [18,48]. UPEC in acidic habitats, is protected by serine deamination resulting in increased levels of pyruvate and ammonia. Presumably, the produced pyruvate is taken up by the putative pyruvate transporter YhjX [50].

In conclusion, while many mechanisms of acid resistance are conserved in a variety of bacterial genera, there are also species- and strain-specific adaptations, which is

ultimately reflected in the different degrees of acid tolerance among bacteria [5].

Acid stress and antibiotic tolerance

The activation of stress response programs often protects bacteria from subsequent higher levels of the same stress, or other environmental stresses. Acid-induced changes in the size of outer membrane porins, and membrane fluidity and lipid composition, as a mechanism against proton penetration, contribute to a cross-protection against antibiotics of bacterial cells [9,13] (Figure 2). Moreover, the proton-pumping ATPase (H^+-ATPase) is necessary for pumping protons out of bacterial cells and even H^+-ATPase inhibitors are known to enhance antibiotic activity against several Gram-negative bacteria [51–53] (Figure 2). Additionally, multidrug efflux pumps, which contribute to antibiotic tolerance, have been associated with acid stress response systems and are discussed for their role in pH homeostasis [54]. Under acidic conditions, the expression of numerous efflux-encoding and efflux-associated genes is altered. For example, in *E. coli*, the expression of the multidrug locus *mdtEF* is upregulated by pH-induced transcription factors such as GadX–GadW [55] and EvgA–YdeO [56,57] (Figure 2). During the exposure to extreme acidic conditions (pH 2.0), the efflux pump MdtEF–TolC confers a fitness advantage for *E. coli* cells [58]. The expression of *emrKY*, coding for a drug efflux pump, in *Shigella flexneri* is enhanced under acidic pH and high K^+ concentrations to mediate survival during macrophage infection [59]. The expression of *norB*, coding for a drug efflux pump of *Staphylococcus aureus*, is derepressed under moderate acidic conditions via the transcriptional regulator MgrA, leading to a decrease in bacterial killing by the antibiotic moxifloxacin [60].

In recent years, more attention has been paid to the link between intracellular pH and its variability and the bacterial susceptibility to antibiotics. Adaptation to acidic conditions leads to a shift in pH homeostasis toward intracellular acidification that prevents antibiotic killing of bacteria, such as *S. aureus*, *E. coli*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis*, as well as *M. smegmatis* [61]. For example, when *E. coli*

Figure 2
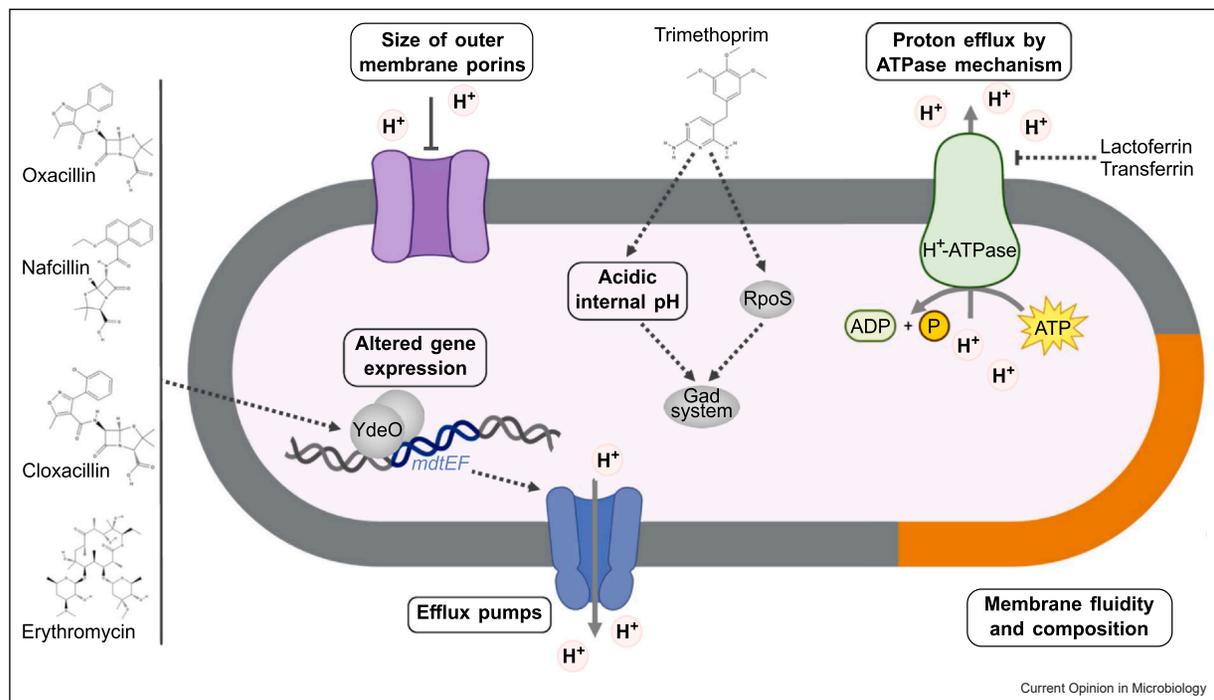
The interplay between acid stress response and antibiotic tolerance. Examples of the interconnectivity between acid stress and antibiotic tolerance include changes in cell membrane fluidity and composition, altered gene expression, acidification of cytoplasmic pH, size of outer membrane porins, regulation of efflux pumps spanning the inner and outer membranes, and proton efflux by H^+-ATPase (all circled in black boxes). Notably, the pH-responsive transcriptional regulator YdeO is also affected by several antibiotics such as oxacillin, nafcillin, cloxacillin, and erythromycin, leading to upregulation of the *mdtEF* operon (blue), which encodes an efflux pump [56]. TMP influences the intracellular pH and copy number of RpoS, leading to heterogeneous activation of the Gad system [65]. Lactoferrin and transferrin inhibit the H^+-ATPase activity and impair proton efflux [53]. The cell envelope is shown as a gray, partially orange ring. Solid gray arrows indicate transport, dashed arrows indicate influences of compounds and their consequences. The figure is created with BioRender.com and Affinity Designer 1.10.4, and MolView v2.4 was used to draw the chemical structures.

is cultivated under conditions that cause intracellular acidification, its susceptibility to antibiotics, including kanamycin, norfloxacin, and carbenicillin, has been reduced [61]. Furthermore, a reduction in transcripts, all regulated by RpoS and involved in multiple stress response cascades, including acid resistance, multidrug resistance, and osmotic resistance, led to an increase in persistence of *E. coli* [62]. Persister cells survive the antibiotic challenge and allow regrowth after antibiotic removal [63]. *E. coli* cells that become persisters have a more acidic intracellular pH than cells that are either susceptible or viable but nonculturable. The key to this differential pH regulation in persister cells seems to be the enzyme tryptophanase, encoded by *tnaA* [63]. In *Salmonella*, vacuolar acidification and nutritional deprivation induced persistence immediately after uptake by macrophages of the host [64]. Furthermore, acidification of the intracellular pH of *E. coli* cells contributes to the full implementation of resistance to antibiotics, such as mediated via the *marRAB* operon [2].

Mitosch and colleagues [65] analyzed quantitatively at the single-cell level, using fluorescence microscopy, the survival of *E. coli* cells under extreme acid stress (pH 3.0) when the cells were prestressed with antibiotics. Of the tested antibiotics, the folate biosynthesis inhibitor trimethoprim (TMP) activated an acid stress response and thus cross-protection of bacteria from subsequent extreme acid stress (Figure 2). This response to acidity is mediated via the Gad system by the expression of *gadBC*, which in turn is highly variable under acid stress. The highly variable *gadBC* expression correlated with single-cell survival by maintaining higher intracellular pH (see below). It has been hypothesized that the acid stress response to TMP is activated as a downstream effect of the depletion of purine bases [65]. In a subsequent study [66], they showed that the acid stress response caused by TMP in single cells does not have a strict temporal order with the DNA damage repair response, known as SOS response.

In conclusion, the correlation between the degree of intracellular acidification of individual cells and a resulting antibiotic tolerance/resistance of subpopulations needs to be further investigated.

The heterogeneous acid stress response
Under acid stress, bacteria often show heterogeneous behavior to increase the fitness of a population by enabling division of labor. Phenotypic heterogeneity arises from internal factors such as individual physiological state and stochastic gene expression and is strongly influenced by external factors such as physical and chemical stresses, availability of nutrients, and cell density [67,68].

E. coli, for example, possesses the four enzyme-based H^+-consuming acid-resistant systems Gad, Adi, Cad, and Orn. Recently, activation of the Gad, Adi, and Cad systems was quantitatively analyzed using a fluorescent triple *E. coli* reporter strain under consecutively increasing acid stress conditions [69]. The Cad system (activated at pH 5.8 in the presence of lysine) and the Adi system (activated at pH 4.4) were found to be mutually exclusively activated, with a high level of ON–OFF cells. These phenotypic variations are not only due to differences in the pH homeostasis of individual *E. coli* cells [70], but also to the low copy number of the transcriptional regulators CadC and AdiY [69,71,72]. It should be noted that activation of the Cad or Adi system leads to an increase in internal pH based on the consumption of protons in the decarboxylation of lysine to cadaverine or arginine to agmatine, respectively, in individual cells, but the entire population also benefits due to the secretion of the more alkaline cadaverine and agmatine, which raise the external pH [69].

The Gad system (activated at moderate acid stress and essential under extreme acid stress) was activated in all *E. coli* cells, albeit to varying degrees [69]. Activation of two promoters of the Gad system, namely *gadBC* and *gadX*, is highly dynamic and variable, leading to cell-to-cell heterogeneity [65,73] that ensures the survival of at least a subpopulation of *E. coli* under extreme acid stress. A recent study revealed that *gadE*, encoding the master regulator of the Gad system, is heterogeneously expressed even in the absence of acid stress, thereby preemptively generating acid-resistant subpopulations within a clonal *E. coli* population [74]. The heterogeneous activation of these three enzyme-based acid resistance systems enables division of labor in the bacterial population and survival over a wide range of low-pH values.

Vibrio species have only the Cad and Orn systems to counteract acidic conditions. In contrast to *E. coli*, the Cad system was shown to be activated in all cells in *V. campbellii* [72]. Apparently, all cells in the *Vibrio* population need the Cad system to increase their intracellular pH by inducing the proton-consuming lysine decarboxylase.

In *Salmonella*, which resides in acidic vacuoles of macrophages, transcriptional heterogeneity can have functional consequences for host–pathogen interactions [75,76]. This phenotype involves regulation by the transcription factors SsrB and PhoP [77], but further studies are required to understand the exact mechanism.

Phenotypic heterogeneity is certainly inherent in many other neutralophilic bacteria that survive in acidic environments, but has been less documented. In addition, the underlying molecular mechanisms that give rise to it and maintain it evolutionarily need to be further investigated.

Conclusions and perspectives

In the past decades, great progress has been made in understanding the different molecular mechanisms of acid stress response in model bacteria, such as *E. coli*, *Salmonella*, and *Listeria*, which have diverse acid resistance systems. New opportunities are now emerging using next-generation sequencing methods that allow the systemic analysis of acid stress response of various bacteria originating from humans, soil, and food. As described above, many mechanisms of acid stress response, such as differential expression of genes involved in transport, H^+-efflux, and membrane permeability, as well as motility and chemotaxis, but also nucleotide synthesis, and translation, are conserved in phylogenetically distantly related bacteria. Nevertheless, bacterial species differ in terms of additional components and degree of acid tolerance, which in turn reflect optimal adaptation to pH fluctuations in the habitats they colonize.

An exciting new aspect is that the acid stress response, in particular the reduction in membrane fluidity, the change in membrane channel size, proton flux through H^+-ATPases, the activation of other proton pumps, and the differential gene induction due to decreased intracellular pH are associated with antibiotic tolerance/resistance (Figure 2). In the future, proteins involved in acid stress adaptation could be explored as targets to sensitize pathogenic bacteria to antibiotics.

pH plays a crucial role in bacterial interactions [78]. It is important to note that low pH in nature is often caused by weak organic acids such as acetic acid, lactic acid, and propionic acid, also known as short-chain fatty acids. These acids originate from fermentation processes under anaerobic growth conditions and accumulate in high concentrations, for example, in the human large intestine (100 mM) [79] or rumen. Even mild acid stress (pH 6–7) strongly enhances the uptake of acetic acid (pKa value = 4.75), since this acid is membrane-permeable in the protonated form. In the cytoplasm, acetic acid dissociates, leading to anion accumulation but also to dissipation of the electrochemical gradient. In the future, it will be important to focus on the stress response to organic acids in prominent intestinal bacteria belonging, for example, to the phyla Actinobacteria, Firmicutes, Bacteriodetes, and Verrucomicrobiota. These studies will help to better understand the complexity of the human gut microbiota.

Finally, there are already examples of transient acidification of an environment to coordinate effective symbiosis between bacteria and eukaryotes [80], a still underestimated phenomenon with major implications for host–bacterial interactions that needs to be further explored.

In conclusion, acidification and adaptation to acid stress are exciting areas of research with far-reaching implications for human health, food industry, and agriculture.

CRediT authorship contribution statement

Kilian Schumacher: Conceptualization, Writing – original draft, preparation of figures. **Sophie Brameyer**: Conceptualization, Writing – original draft, preparation of figures. **Kirsten Jung:** Conceptualization, Writing – original draft and editing.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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- •• of outstanding interest

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3 Concluding discussion

Parts of this chapter were adapted from:

Schumacher, K., Gelhausen, R., Kion-Crosby, W., Barquist, L., Backofen, R., and Jung, K. (2023). Ribosome profiling reveals the fine-tuned response of *Escherichia coli* to mild and severe acid stress. mSystems.10.1128/msystems.01037-23.

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3.1 Novel adaptations of *Escherichia coli* to acid stress uncovered by Ribo-Seq

Acidic environments are frequently occurring on earth and involve acidic soils, macrophage phagosomes, and gastrointestinal tracts (Figure 1). In recent decades, a magnitude of studies both on the systematic and molecular levels have been conducted to understand which defense mechanisms against acidity evolved in bacteria. Thereby several strategies for restricting proton uptake, accelerating extrusion, and limiting damage to cellular components have been detected. Nevertheless, most studies were limited to microarrays, proteomics, or only mildly acidic conditions. Using a combined ribosome profiling and RNA-Seq approach, entirely new adaptations to mildly and severely acidic conditions were uncovered (Chapter 2.1). These previously overlooked adaptations were revealed by leveraging the advantages of the Ribo-Seq technique which include the distinction between coding and non-coding RNAs, the detection of novel sORFs, and its independence of protein biochemistry (139, 144, 146, 157). Moreover, Ribo-Seq is less sensitive to acid treatment compared to proteomics (133). Conclusively, Ribo-Seq allowed monitoring of protein synthesis rates also at pH 4.4 and revealed the induction of a large spectrum of so far uncharacterized genes (Chapter 2.1). Moreover, the differential synthesis of membrane proteins, restricted cellular metabolism, induction of siderophore and iron uptake, and the differential expression of sORFs were highlighted (Figure 7). These novel acid stress adaptations are further discussed below.

3.1.1. Differential synthesis of membrane proteins and putatively altered membrane fluidity

Ribo-Seq revealed restricted synthesis of a high proportion of membrane proteins under acidic conditions. In particular, 18 of the 20 genes with the most reduced ribosome footprint levels at pH 5.8 encoded inner- or outer membrane proteins. Correspondingly, 75% of the Top 20 genes with reduced RPFs under severe acid stress encoded non-cytosolic proteins (Chapter 2.1). It appears plausible, that synthesis of these membrane components is restricted to limit proton intake. Examples of integral membrane components with reduced RPF levels involved, amongst others, the DppABCDF dipeptide transport system, the MalEFGK maltose uptake system, the melibiose/H^+ symporter MelB, and the 4-aminobutanote/H^+ symporter GabP (pH 5.8). Along this line, examples of transporters that were synthesized in lower amounts at pH 4.4 included the tryptophan/H^+ symporter TnaB, as well as several OMPs such as OmpW, OmpF, NmpC, and LamB (Chapter 2.1).

As indicated by the restricted synthesis of H^+ symporters such as GabP and MelB, proton influx is restricted under acidity. GabP and MelB are driven by the membrane potential and transport protons as co-substrates (169, 170). While decreased production of H^+ co-transporting symporters was detected regardless of the stress intensity, diminished synthesis of OMPs was exclusively noted at pH 4.4 (Chapter 2.1). OMPs consist of antiparallel beta-strands that form

a barrel-like pore in the outer membrane and facilitate the transport of nutrients, ions, and other molecules (171). The exclusive alteration of OMP synthesis at pH 4.4 (Figure 7) demonstrates that *E. coli* rather modifies the inner membrane under mild acid stress whereas both the inner- and outer membrane composition are adjusted under severe acid stress.

It is well known that *E. coli* incorporates CFAs into the membrane under acid stress (32, 33). Furthermore, the polar phospholipid content can be modified and derivates of phosphatidylglycerol, hopanoids, and sphingolipids are formed in these conditions (35). Together with the downregulation of OMPs and other transporters (Figure 7), such adaptations likely also affect the fluidity of the membrane. Of note, Ribo-Seq also revealed a significant increase of ClsA, ClsB, and ClsC synthesis at pH 4.4 (Figure 7), with the most drastic induction being noted for ClsB (12-fold). These proteins are responsible for synthesizing cardiolipin, which is formed by the condensation of two phosphatidylglycerol molecules (172–174). Cardiolipin is a unique phospholipid with a dimeric structure containing four acyl chains and its presence in the membrane alters the packing arrangement of phospholipids due to its conical shape and increased molecular size compared to other phospholipids (175, 176). Elevated cardiolipin levels contributed to membrane stabilization under osmotic conditions in *E. coli*, indicating a reduction of membrane fluidity and permeability (177). For future studies, it will be important to directly measure and confirm changes in membrane fluidity under acid stress, for instance via excimer fluorescence of pyrene lipid probes (178).

The hypothesis of alterations in phospholipid content in *E. coli* under acid stress was further underlined as a significant induction of a catabolic pathway for glycerol and glycerol 3-phosphate (G3P), encoded by the *glp* regulon, was detected (Figure 7). Glycerol and G3P can be released during polar phospholipid breakdown, especially during turnover of phosphatidylglycerol (179), and act as inducers for the *glp* regulon (180, 181). Thus, it is tempting to speculate that in addition to elevated cardiolipin synthesis, *E. coli* can gain energy under acid stress via the catabolic breakdown of glycerol and G3P, which are putatively released from phospholipid turnover.

Collectively, these results highlight the superiority of Ribo-Seq over LC-MS in terms of membrane protein analysis. The underlying reason for this is that membrane proteins have a tendency to aggregate and precipitate in solution and are oftentimes lacking target residues (lysine and arginine) which are essential for tryptic cleavage, an essential step within bottom-up proteomic approaches (133). This difference between the methods mentioned above is an important consideration point for future studies since ~ 20–30% of genes in an organism encode integral membrane components (182).
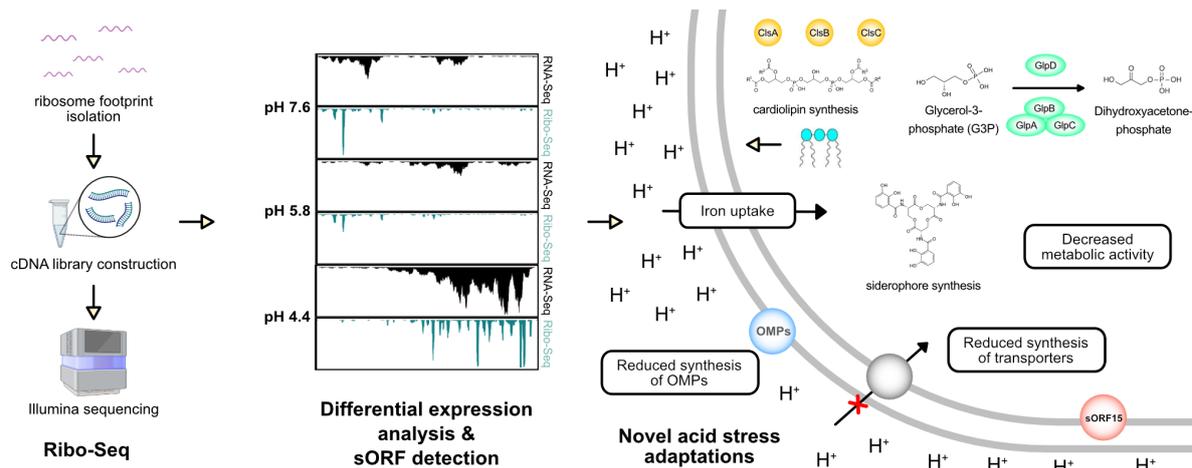
Figure 7: Overview of the final steps of the Ribo-Seq workflow which yielded the discovery of novel adaptations of *E. coli* to acid stress. These included the reduced synthesis of transport proteins, catabolism of glycerol-3-phosphate, decreased metabolic activities, iron uptake, siderophore synthesis, and the discovery of novel sORFs. Parts of this figure were created using Biorender.com.

3.1.2 Restriction of cellular metabolism

In addition to restricted membrane protein synthesis, reduced global metabolism was detected at both pH 5.8 and pH 4.4 (Chapter 2.1). This finding was mainly revealed by gene set enrichment analysis (GSEA). GSEA is a bioinformatic approach determining whether predefined gene sets show statistical differences in high-throughput sequencing data (183). Using the tool *clusterProfiler* (184) and the RNA-Seq and Ribo-Seq data as input, differences in gene ontology (GO) terms for biological processes were evaluated. Thereby a large spectrum of metabolic and catabolic processes was detected to be restricted under low pH (Figure 7). This included for instance putrescine catabolism, threonine catabolism, xylose metabolism, and glucan catabolism (Chapter 2.1).

Particularly interesting was the downregulation of arginine catabolism. This GO term is mainly associated with a pathway involving the protein products of the *astEBDAC* operon, responsible for the L-arginine degradation II (AST) pathway. The AST pathway is the main arginine-degrading pathway in *E. coli* and yields two molecules of glutamate and ammonia (185). Importantly, reduced catabolism of arginine was detected at pH 5.8, but not at pH 4.4. Thus, *E. coli* may preserve the intracellular arginine pool under mild acid stress to ensure enough substrate for the Adi system is available during severe acid stress (see Chapter 2.5). This is important as the conversion of arginine to agmatine via the Adi system is a crucial acid stress mechanism, which is maximally induced at pH 4.4 (186).

The strongest downregulation among all biosynthetic operons at pH 4.4 was observed for *tnaABC*. *tnaA* encodes a tryptophanase, which cleaves L-tryptophan into indole, pyruvate, and NH4$^+$, *tnaB* encodes a tryptophan/H$^+$ symporter, and TnaC functions as a leader peptide (187,

188). Importantly, *E. coli* persister cells were previously indicated to display a lower intracellular pH. This effect was linked to the activity of TnaA, as the pH difference was abolished in a Δ*tnaA* mutant (189). Considering the detected pH-dependent regulation of *tnaABC* (Chapter 2.1), the link between pH and bacterial persistence is further emphasized.

3.1.3 Induction of siderophore and iron uptake

GSEA also identified GO terms related to iron- and siderophore uptake as statistically enriched at pH 4.4 (Figure 7). These included 'siderophore-dependent iron import into cell', 'siderophore transmembrane transport', 'enterobactin biosynthetic process', and 'ferric-enterobactin import' (Chapter 2.1). Specifically, the complete enterobactin biosynthesis pathway, comprising the *entCEBAH* operon, *entF*, *entH*, and *ybdZ*, was induced at pH 4.4. Enterobactin is a key component for *E. coli* to scavenge iron from the environment, mainly by binding Fe(III) ions and enhancing solubility (190).

Furthermore, all subunits of the Ton complex were enriched at pH 5.8 and pH 4.4. The Ton complex functions as a proton motive force-dependent molecular motor that facilitates the import of iron-bound siderophores (191, 192). Along this line, several other iron uptake systems were also induced under acidic conditions. These included a ferric dicitrate ABC transport system (*fecABCDE*), an iron (III) hydroxamate ABC transport system (*fhuACDB*), and a ferric enterobactin ABC transport system (*fepA*, *fepB*, *fepCGD*). Moreover, upregulation of genes of the *isc* and *suf* operons was detected, encoding components of the complex machinery responsible for iron-sulfur cluster assembly in *E. coli* (193).

These results are somewhat contradictory considering the increased solubility of iron under low pH which is expected to increase bioavailability (194). The above described induction of iron and siderophore uptake systems might also be caused by restricted heme transport under acidity. Indeed, GSEA revealed significant downregulation of the GOterm 'heme transport' (Chapter 2.1). Heme is a molecule consisting of an iron atom contained in the center of a large organic ring and can serve as an iron source (195). Specifically, synthesis of the DppABCDF dipeptide transporter was detected. This protein complex is responsible for transporting heme from the periplasm into the cytoplasm in *E. coli* (196).

Considering the enhanced solubility of iron under low pH (194), it is worth discussing whether *E. coli* synthesizes siderophores to respond to iron limitation or rather protects itself against putative iron excess. The latter was demonstrated for *Pseudomonas aeruginosa*, where siderophores protected cells from the harmful effects of ROS. In this case, the cells no longer secreted siderophores but instead stored them intracellularly (197). Overall, the questions raised above are of high importance for future work since iron acquisition is a crucial factor for pathogenesis due to limited availability in host environments (198).

3.1.4 Discovery of sORFs that are exclusively detectable under acid stress conditions

A unique advantage of Ribo-Seq is the possibility to detect sORFs which were previously overlooked due to their small size (155–157). Upon conducting Ribo-Seq at pH 7.6, pH 5.8, and pH 4.4, 18 novel candidate sORFs were identified in *E. coli* (Chapter 2.1). Moreover, it was demonstrated that several previously known sORFs such as YdgU, AzuC, and MdtU, are differentially expressed under acidic conditions. These results indicate that small proteins play a significant role in stress adaptation. Considering that the majority of sORFs are located in the membrane (161), small proteins may affect the membrane permeability for protons.

Ribo-Seq yielded novel sORFs in the UTRs of annotated genes, antisense sORFs, and dual-function sRNAs (Chapter 2.1). For instance, the sRNAs OmrA and OmrB, which were previously thought to be non-coding, encoded small peptides under severe acid stress (Chapter 2.1). This finding may be of high relevance considering that the OmrA and OmrB sRNAs regulate the expression of numerous OMPs (199). Particularly striking however was the discovery of two novel candidate sORFs in the 3'UTR of the major acid resistance regulator GadW (sORF14 and sORF15). While the expression of sORF14 was not confirmed, sORF15 protein production could be successfully detected via western blotting (Chapter 2.1). This suggests that the complex Gad system (see Chapter 2.5) consists of even more components than thus far assumed. sORF15 is predicted to be membrane-bound (Figure 7), and considering that most sORFs interact with other cellular components rather than exerting *bona fide* enzymatic activities (155), sORF15 may associate with other crucial membrane proteins for acid resistance. Since sORF15 is located in a transcriptional unit with *gadW* and *gadX*, it could bind to GadC, the transporter of the Gad system. Alternatively, sORF15 may associate with MdtF, which is adjacently encoded and partially overlapping the *sORF15* gene. MdtF is the inner membrane subunit of the tripartite MdtEF-TolC efflux pump responsible for the extrusion of bile salts, dyes, and antibiotics such as novobiocin, ciprofloxacin, and erythromycin (200, 201). Notably, an overlap between antibiotic resistance mediated by efflux pumps and acid resistance in bacteria was previously demonstrated (see Chapter 2.6). Thus, it will be of great relevance for future investigations to determine binding partners of sORF15, for instance via Co-IP (202).

Examples of successful functional descriptions of sORFs have been provided in recent years. For instance, the VdcP small protein of *Vibrio cholerae* binds to the GltA citrate synthase of the citric acid cycle and thereby reroutes carbon metabolism (203). In *Methanosarcina mazei*, the small protein sP36 regulates nitrogen metabolism as it inhibits the activity of the $AmtB_1$ ammonium transporter (204) and in *Haloferax volcanii*, HVO_2753 mediates biofilm formation and swarming behaviors (205).

Nevertheless, the lack of conserved domains due to restricted ORF lengths complicates the search for evolutionary relationships of small proteins. Theories for sORF evolution involve the degeneration of larger sORFs, partial ORF duplications, the evolution of a promoter upstream of a non-coding gene, and the evolution of a protease cleavage site towards the 5'- or 3' end of a canonical longer ORF (155). Moreover, a proto-gene model involving the *de novo* birth of sORFs from random non-coding genomic DNA sequences via pervasive transcription and subsequent ribosome association is discussed (206–208).

In sum, the discovery of sORF15 and other small proteins demonstrates that alterations in genomic coverage as a consequence of stress adaptations can guide the discovery of new small proteins, even in well-studied model organisms such as *Escherichia coli*. This exemplifies that certain sORFs previously not only remained hidden due to their small size but also because they are exclusively expressed under stress conditions.

3.2 Stress-dependent adaptations of chemotaxis and motility

Chemotaxis and the ability to regulate flagella biosynthesis are essential for *E. coli* to react to external stimuli and environmental alterations (209). Such adaptations have already been described under acidity but were expanded within this thesis. The following chapters will discuss modifications of the signal transduction cascade responsible for propagating the signals received via MCPs to the flagellar motor. In particular, the effects of insertion sequence (IS) element integration upstream of *flhDC*, encoding the master regulators for motility and chemotaxis (Chapter 2.2), as well as binding of eukaryotic catecholamine hormones to the chemotaxis coupling protein CheW (Chapter 2.3), will be discussed.

3.2.1 pH-dependent differential expression of motility genes

The Ribo- and RNA-Seq data gathered at varying pH levels revealed strong induction of motility genes under mild acidity (pH 5.8) but not under severe acidity (pH 4.4) (Chapter 2.1). Conclusively, the induction of the motility machinery in *E. coli* appears to be strictly dependent on the degree of acid stress. It is tempting to speculate that bacteria use an escape strategy to migrate to habitats with optimal pH under mild acid stress but abandon this strategy under severely acidic conditions and switch to an energy-saving mode (Figure 8). These assumptions are supported by independent findings suggesting that flagella are rapidly shed at pH 4.0 – 4.5 (77, 78) and reports noting diminished chemotaxis and overall motility in severely acidic conditions (210). The switch to an energy-saving mode at pH 4.4 is in alignment with the overall reduction of metabolic activity (see Chapter 3.1.3). Importantly, a microarray study conducted in *E. coli* reported that motility was still induced at pH 5.0 (37). Thus, the inversion point at which motility stops to be beneficial is likely situated between pH 5.0 and pH 4.4 (Figure 8), at least in *E. coli*.

Mild acid stress

induction of motility gene expression
escape strategy
migration to environments with desired pH levels

Severe acid stress

restriction of flagella gene expression
shedding of flagella
energy savor mode

pH   5.8   4.4

degree of acid stress
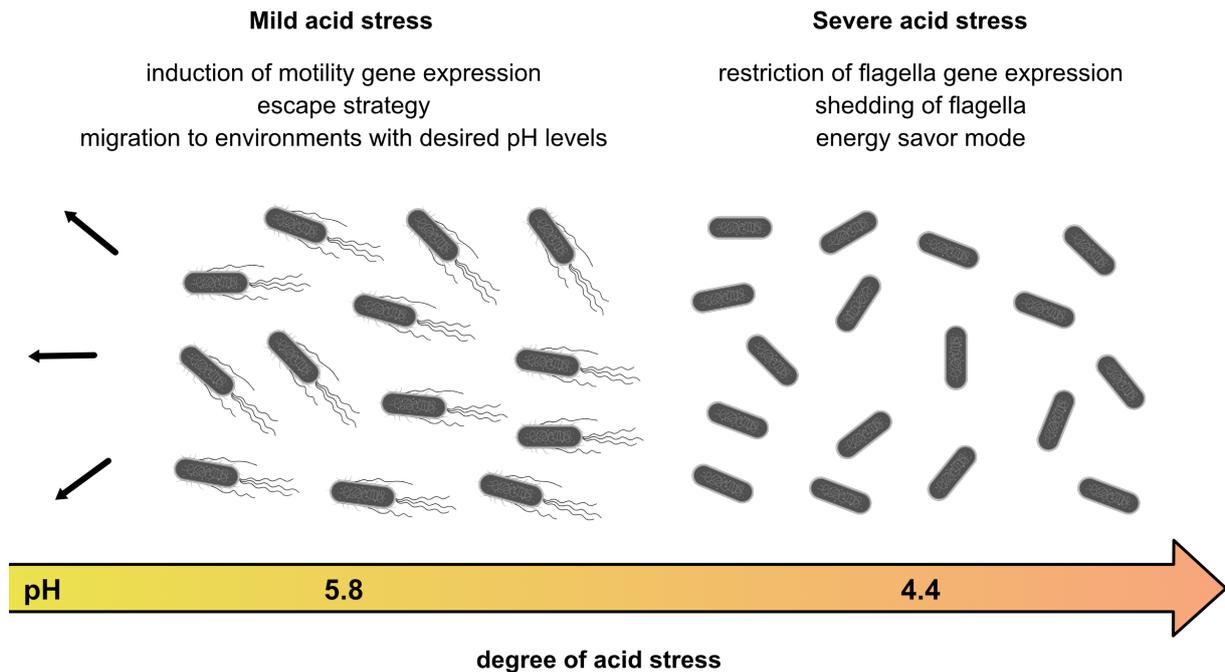
Figure 8: Adaptation of motility in *E. coli* depending on the degree of acid stress. Responses to mild and severe acid stress, as well as migration behaviors are indicated. Parts of this figure were created using Biorender.com.

Along this note, several NGS studies were recently performed in a broad spectrum of bacteria from distinct habitats (see Chapter 2.6). In nearly all investigated species, differential motility and chemotaxis gene expression were reported in response to acidity. Thus, the effect appears to be a universal phenomenon in motile bacteria. However, the respective RNA-Seq and proteomic studies detected in some cases upregulation and in others downregulation of motility genes (Chapter 2.6). For instance, motility was induced at pH 3 in *Campylobacter jejuni* (211) but restricted in *Listeria monocytogenes* at pH 3.4 (212). This suggests, that the inversion point at which motility stops to be beneficial is correlated to the overall acid tolerance of a species.

3.2.2 Insertion sequence element-controlled regulation of motility

Another link between motility and acid stress was discovered upon analysis of an *mhpR* mutant from the Keio collection (213). The acid shock phenotype observed in this strain was surprisingly not mediated by the MhpR knockout itself, but by the integration of an IS element in the promoter region of *flhDC* (Chapter 2.2). IS integration and point mutations in the upstream region of *flhDC* have previously been described to activate motility due to disruption of transcriptional repressor binding sites (214–217). Notably, motility-activating mutations in the *flhDC* regulatory region have been detected in the majority of strains from the Keio collection (218). IS elements are mobile genetic elements and consist of two inverted repeat sequences flanking a central region containing genes responsible for transposition (219, 220). Moreover, they are usually less than 2.5 kb in length, exclusively encode genes involved in translocation, and are major drivers for genomic plasticity in prokaryotes (221).

Escherichia coli:

Figure 9: Effects on motility mediated by mild acid stress and IS elements integrated in the *flhDC* regulatory region in *E. coli*. Schematic illustrations of induced class 2 and class 3 flagellar genes by FlhDC and FliA are included. Parts of this figure were created using BioRender.com.

Chapter 2.2 demonstrated that motility and the ability to survive acid shock periods are inversely correlated in *E. coli*. In particular, the presence of IS elements in the upstream region of *flhDC* increased motility at the expense of acid tolerance (Figure 9). Further, FlhDC levels were shown to be increased by mild acid stress (Chapter 2.1). Elevated FlhDC levels in turn lead to the activation of class II flagellar operons encoding basal body components, flagella export systems, and the alternative sigma factor FliA (222, 223). FliA is responsible for controlling class III genes which are essential for late flagellar assembly (Figure 9) (224, 225).

The above mentioned findings were underlined by using a strain with a chromosomal isopropyl β-D-thio-galactopyranoside (IPTG)-inducible *flhDC* promoter (226), which revealed that increased FlhDC levels reduce acid shock survival in a dose-dependent manner. Furthermore, differentially motile subpopulations were isolated from swim agar plates and subjected to acid stress. Intriguingly, cells collected from the center had very high survival rates whereas motile cells isolated from halo edges were characterized by poor survival (Chapter 2.2). These results revealed a fitness trade-off between motility and acid tolerance. Nevertheless, it remains to be clarified why increased flagellation and/or motility reduced survival under severe acid stress. One possible explanation is that hypermotile cells have an increased energy demand due to flagellar component synthesis such as the motor, or filaments. Specifically, the cost of synthesizing and operating the flagella apparatus was measured to account for up to 3.5 % of the total cell energy (227–229). Correspondingly, cells with IS insertions in the promoter of *flhDC* exhibited a reduced growth rate (216). Further, a high number of rotating flagella affects the integrity of the membrane and causes a high flux of protons back into the cytoplasm, which is disadvantageous at low pH.

Motility-activating mutations in the regulatory region of *flhDC* have also been shown to introduce a trade-off between growth and biofilm formation (216). This indicates, that the consequences of IS integration upstream of *flhDC* are not restricted to acid survival but entail further consequences. This is in agreement with findings of FlhDC regulating also non-motility genes involved in processes including carbon source metabolism, anaerobic respiration, and cell division (230, 231). Moreover, transposition frequencies were previously indicated to be affected by different stressors such as UV light, oxidative stress, and DNA damage (232–234). Thus, it will be of interest for future studies to investigate whether the likelihood of IS integration also increases relative to the degree of acid stress.

It is worth noting that IS transposition can cause phenotypic heterogeneity within bacterial populations. For instance, phenotypic variability in a biofilm increased relative to the frequency of IS5 insertion upstream of *flhDC* (216) and IS-related heterogeneity was beneficial for bacteria as it increased biofilm mass (235). Importantly, a heterogeneous population of MG1655 CGSC 7740 carrying an IS element upstream of *flhDC* was detected (Chapter 2.2), suggesting the existence of subpopulations that are either acid-tolerant or motile (Chapter 3.3).

3.2.3 Binding of stress hormones to the motility machinery

In addition to alterations in gene expression levels, chemotactic behaviors can be affected by modifications of the signal transduction cascade connecting MCPs to the flagellar motor. The latter was demonstrated for eukaryotic catecholamine hormones which bound to the chemotaxis coupling protein CheW in *Vibrio campbellii* (Chapter 2.3). CheW is located at the core of the chemotaxis signaling network and was demonstrated to be targeted by stress-related catecholamine hormones of animals and humans, such as EPI and NE (Figure 10). This interaction, with affinity constants in the submicromolar range, was confirmed by an untargeted chemical proteomics approach and MST (Chapter 2.3).

The binding of the mammalian compounds also affected the chemotactic control of *V. campbellii* (Figure 10). In particular, supplementation of 50 µM EPI or PE significantly reduced the chemotactic response to glucose in a capillary chemotaxis assay (Chapter 2.3). This assay measures the number of cells migrating into attractant-containing glass capillaries after fixed time intervals (210). The results demonstrated, that the capacity to sense attractants is disturbed when eukaryotic catechol hormones are bound to CheW in *V. campbellii* (Figure 10).

CheW is a crucial determinant of the chemotaxis signaling network and connects MCPs to the HK CheA (Figure 9) (236). However, the exact mechanism of catecholamine binding to CheW regarding signal transduction interference remained unclear. One possible explanation is that the binding of stress hormones interferes with the autophosphorylation of CheA, which transfers its phosphoryl group to the RR CheY (89). Thus, the interaction with catecholamine

hormones may mimic a Δ*cheW* phenotype, i.e. smooth swimming and non-chemotactic (237). This would explain why taxis towards glucose was abolished in the presence of eukaryotic catecholamine hormones. Nevertheless, 3D tracking experiments revealed that the turning rate and swimming speed of *V. campbellii* are unaffected by EPI (Chapter 2.3), contradicting the statement above and the capillary assay data. This discrepancy may be explained by the fact that 3D tracking experiments rely on linear gradients while the capillary assay operates with steep gradients in the capillary vicinity. Thus, the effects of catecholamines on chemotactic behaviors could be restricted to steep gradients or other conditions specifically encountered in the capillary assay. Another noteworthy difference between the capillary assay and the 3D chemotaxis assay conducted in microfluidic devices (Chapter 2.3) is the measured swimming distance covered by the bacteria, which is significantly higher in the capillary assay.
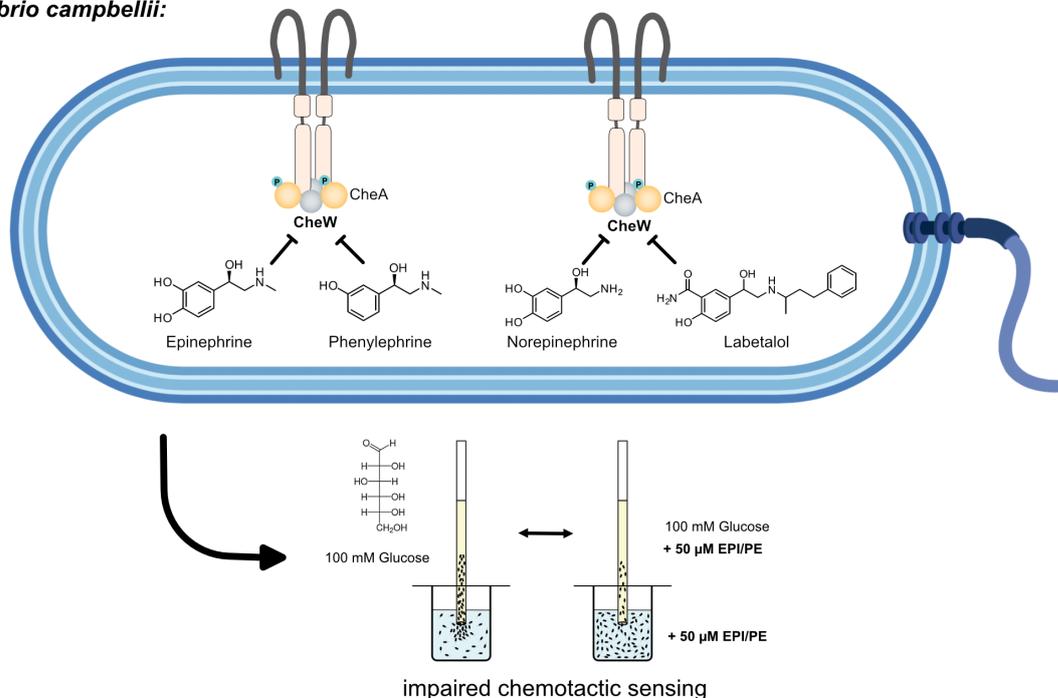
Figure 10: Impaired chemotactic sensing in *Vibrio campbellii* as a consequence of the binding of eukaryotic catecholamine hormones to the chemotaxis coupling protein CheW. Significantly lower numbers of bacteria were detected in glass capillaries filled with 100 mM glucose upon supplementation with 50 µM EPI or PE (Chapter 2.3). Parts of this figure were created using Biorender.com.

Even though the impact on chemotactic signaling should be elaborated, the results further emphasize that stress hormones such as EPI and NE act as interkingdom signals between eukaryotic hosts and bacteria. In eukaryotes, catecholamines bind to G protein-coupled receptors, and certain antagonists of human receptors can also antagonize adrenergic effects in bacteria (88). This suggests that similar receptors may have also evolved in prokaryotes, which indeed has been demonstrated in EHEC where EPI and NE bound to the two-component system HKs QseC and QseE (95, 96). Thus, binding of catecholamines in *V. campbellii* to the

coupling protein CheW, and not to a receptor, was a somewhat surprising finding. However, *Salmonella* and *V. cholerae* mutants lacking QseC and/or QseE still responded to catecholamines (87, 97, 98). It will be important for follow-up studies to pinpoint the exact residues in CheW where catecholamine hormones bind to elucidate the regulatory mechanism exerted on chemotactic control. Importantly, the findings (Chapter 2.3) also have notable physiological implications considering that chemotaxis and net motility affected the infectivity of *V. cholerae* (238). Stool-derived *V. cholerae* cells commonly display a smooth-swimming state and exhibit repression of chemotaxis genes such as *cheR* and *cheW* (239–241). Thus, chemotactic control mediated by binding to CheW may not only be important for the perception of interkingdom signals, but also affect the overall infectivity of bacterial cells.

3.3 Phenotypic heterogeneity under consecutively increasing acid stress

An example of heterogeneity under acid stress was presented using a chromosomal fluorescent triple reporter strain in Chapter 2.4. The antiporters of the three major ARs in *E. coli* (GadC, AdiC, CadB) were fused to fluorophores (eGFP, mCherry, mCerulean) and exposed to consecutively increasing acidity. This approach revealed a heterogeneous activation of the three systems with a different degree of noise and suggested a division of labor among the *E. coli* population (Chapter 2.4). The Adi system (induced at pH 4.4) and the Cad system (induced at pH 5.8 in the presence of lysine) were activated in a mutually exclusive manner, with a large proportion of ON-OFF cells (Figure 11). On the other hand, the Gad system was heterogeneously activated in exponential phase, but nearly all cells activated the system in stationary phase. The low copy numbers of the transcriptional activators CadC and AdiY (122, 242) and their stochastic distribution were suggested as the underlying cause for heterogeneous activation among single cells. Correspondingly, an artificial increase in CadC and AdiY copy numbers shifted the populations to homogeneity (Chapter 2.4).

It is also worth noting that the number of ARs in *E. coli* is higher than in other γ-proteobacteria, reflected in significantly increased acid tolerance (Chapter 2.5). The benefit of possessing several ARs but activating them heterogeneously among the population can be explained, for instance, by the efficient differential consumption of amino acids. Glutamate is the most abundant amino acid in the cell (100 mM), while arginine (0.57 mM) and lysine (0.41 mM) are less abundant (243). Since the Gad system is homogeneously distributed under mild acid stress (Chapter 2.4), all cells of the population can initially use the available glutamate in the cytoplasm. When the stress increases, only a limited percentage of cells activate either the Cad or Adi system (Figure 11). These resulting subpopulations however contribute to combat acidity by releasing the more alkaline cadaverine and agmatine, which serve as common goods for the entire population. This strategy can be considered as 'bet-hedging', as producing both the Cad and Adi system would represent a major metabolic burden for an individual cell.

This assumption is underlined by findings demonstrating reduced growth rates upon artificial overexpression of the Adi or Cad system respectively (Chapter 2.4). Moreover, preemptive expression of AR genes under mild acid stress in a limited cell percentage may also be important to prepare the population against future strong acid stresses. The latter was indicated for *gadA* and *gadB*, which are essential for acid shock survival but confer a growth deficit upon overexpression (244). Nevertheless, limited expression in a subpopulation will not affect the overall growth rate of the population drastically, but ensure the survival of a limited number of cells in case of sudden unexpected strong stress, such as encountered in the human stomach.

Importantly, the Ribo- and RNA-Seq approach (Chapter 2.1) revealed further crucial regulators for the adaptation of *E. coli* to acidity such as YdcI, which affected the transcription of the Gad and Cad systems. It will be of interest for future studies to investigate whether copy number manipulations of YdcI may also affect the heterogeneity of AR systems in a manner as described for AdiY and CadC (Chapter 2.4).

Phenotypic heterogeneitiy of the three major ARs in *E. coli*

Gad system (≤ pH 6.8)
GABA

Cad system (pH 5.8)
Cadaverine

Adi system (pH 4.4)
Agmatine
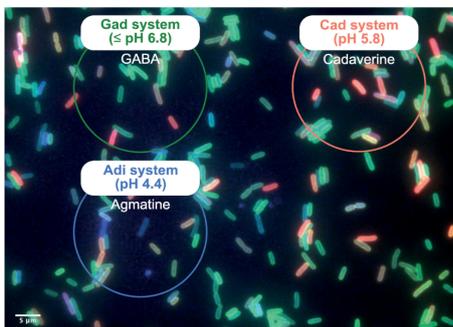
Gad system:
homogeneously activated in stationary phase
heterogeneously activated in acidified media in exponential phase
complex transcriptional regulation

Adi system:
max. induction at pH 4.4
activated by AdiY
heterogeneously distributed

Cad system:
max. induction at pH 5.8 in the presence of lysine
activated by CadC
heterogeneously distributed

mutually exclusive

Differential integration of IS elements in the *flhDC* regulatory region

P_{flhDC}

flhD   flhC

IS elements

+ IS   - IS

Motile and acid susceptible subpopulation

Poorly motile and acid tolerant subpopulation
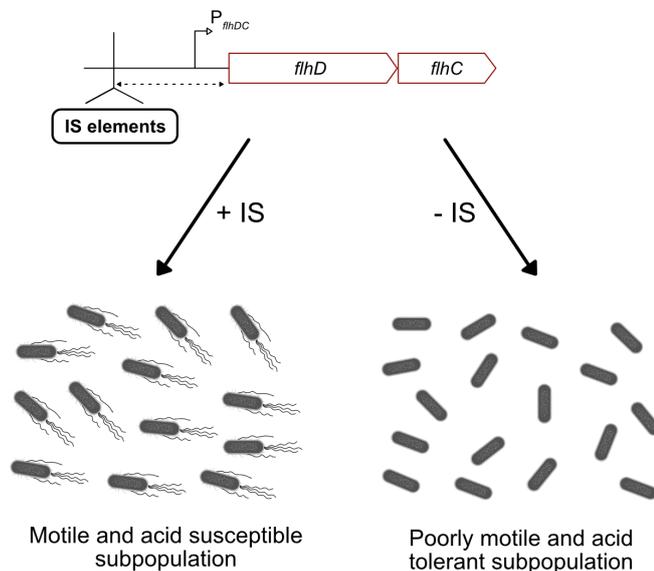
Figure 11: Examples of phenotypic heterogeneity detected in *E. coli* under acid stress. Division of labor among an *E. coli* population in terms of heterogeneous AR activation was determined by fluorescence microscopy using a triple-fluorescent reporter strain (Chapter 2.4). Differential integration of IS elements into the regulatory region of *flhDC* is responsible for the differentiation into subpopulations that are either motile and acid-susceptible, or poorly motile and acid-tolerant (Chapter 2.2).

As mentioned in Chapter 3.2.2, heterogeneity was also detected in terms of MG1655 cells harboring a motility-activating IS element upstream of *flhDC*. Considering that this insertion induces motility while reducing the capacity to survive acid shock (Chapter 2.2), it is tempting to speculate that *E. coli* diversifies into a motile and an acid tolerant subpopulation under acid stress in a manner dependent on IS elements (Figure 11). This hypothesis is supported as bacterial heterogeneity in flagella expression was previously demonstrated (245, 246). Furthermore, colonization efficiencies of mouse intestines were heavily affected by differential IS element integration upstream of *flhDC* in *E. coli*, indicating existing niches in the gut where motility is beneficial (247). Given distinct intestinal niches where either acid tolerance or motility is advantageous, IS transposition may be crucial to ensure that *E. coli* can colonize both niches by diversifying into motile and acid-tolerant subpopulations (Figure 11).

Importantly, the study of collective behaviors in *E. coli* was so far mainly restricted to AR components (121, 122). Thus, it will be crucial for future investigations to study the potential heterogeneity of other cellular defense proteins such as chaperones, enzymes responsible for membrane modifications, or efflux pumps. Moreover, the potential heterogeneity of the Orn system should be addressed. For instance, the triple-fluorescent reporter described in Chapter 2.4 could be utilized and PotE, the transporter of the Orn system, labeled with a fourth fluorophore. A potential approach to address all above raised suggestions on a systematic level is scRNA-Seq, which is further discussed in Chapter 3.4.

3.4 Conclusions and outlook

Reacting to pH fluctuations is essential for the survival of prokaryotes in various habitats on earth (Figure 1). Within this thesis, the spectrum of acid defense mechanisms in *E. coli* was extended by leveraging the advantages of the Ribo-Seq technique. This approach revealed novel adaptation strategies, detected previously overlooked sORFs, and provided a genome-wide overview of mRNA and RPF levels under mild and severe acid stress (Chapter 2.1). Furthermore, a novel fitness trade-off between motility and acid survival was demonstrated (Chapter 2.2). An alteration in chemotactic signaling was noted as mammalian stress hormones bound to the chemotaxis coupling protein CheW in *V. campbellii* (Chapter 2.3). Finally, division of labor and phenotypic heterogeneity were detected in *E. coli* upon conducting fluorescence microscopy with a triple-fluorescent reporter strain (Chapter 2.4).

Fluorescence microscopy is a valuable approach for gathering spatial information and assessing the distribution of a small number of proteins in single cells. Nevertheless, the method is limited in terms of information beyond the labeled proteins, introduces biases due to delayed fluorophore folding, and is restricted in terms of throughput due to limitations of cell numbers per experiment (114, 248). To overcome these limitations, scRNA-Seq could be conducted under consecutively increasing acid stress. Unlike bulk RNA-Seq, which provides

an average expression profile of cells in a sample, scRNA-Seq allows monitoring global gene expression patterns at the single-cell level (114, 249). The immense potential of scRNA-Seq was demonstrated in numerous recent studies (249–252), and a recent scRNA-Seq approach (M3-Seq) revealed an acid resistant subpopulation in stationary phase *E. coli* cells which was characterized by very high expression levels of *gadA* and *gadB* (244). Moreover, scRNA-Seq and/or Ribo-Seq could be applied upon bacterial exposure to mammalian stress hormones.

The continuously growing amount of sequencing data is an important consideration for future NGS studies. For instance, the storage capacity for human genomic data alone is estimated to account for up to 40 exabytes by 2025 (253). Thus, it is essential to implement workflows for automated prioritization of important findings from large-scale genomic datasets. The latter was successfully demonstrated within this thesis through machine learning via denoising autoencoders (Chapter 2.1). This self-supervised method can generate new hypotheses from expression data and identify genes uniquely expressed in a certain condition (254).

The significance of phenotypic heterogeneity in isogenic bacterial populations is another key finding of this work (Chapters 2.2 and 2.4) and underlines that addressing bacterial heterogeneity is crucial for the upcoming antibiotic crisis. Links and overlaps exist between the acid and antibiotic stress responses (63, 64), and severe near-lethal acid stress caused a drastic reduction in bacterial metabolism (Chapter 2.1). Thus, acid shock periods may prepare *E. coli* for a persistent state which in turn confers antibiotic resistance. Along this line, highly resistant strains towards chloramphenicol were recently characterized by a significantly decreased internal pH (65). The above-described overlap between acidity and antibiotic stress could be leveraged for drug design with the ultimate aim of modifying the cytoplasmic pH of bacteria. Such drugs would automatically also affect the antibiotic tolerance of pathogens and could be prescribed together with conventional antibiotics.

While the reduction of acid tolerance is desirable for pathogens, limited acid susceptibility is important for certain bacteria with industrial relevance. For example, probiotic bacteria have significant pharmaceutical value while their incorporation into food remains challenging (255). Conferring acid resistance to probiotics would enhance live cell numbers upon gastrointestinal transition and improve the value and shelf-life of these products.

It is worth noting, that the Ribo-Seq study (Chapter 2.1) was conducted by the addition of HCl to growing cultures. However, environments such as the human gastrointestinal tract usually comprise a mixture of different acids. For instance, bile acids are present in the small intestine (256), and SCFAs are produced by gut bacteria in the large intestine upon fermentation of dietary fibers (257, 258). Thus, it will be of interest to study the response to a cocktail of acids including organic acids, which rather resembles the naturally encountered conditions of bacteria when traversing the gastrointestinal tract.

In summary, pH is a crucial determinant for bacterial survival on earth and shapes the composition of environments such as gastrointestinal tracts, soils, or phagosomes (see Chapter 1). Thereby, the degree of acid tolerance dictates whether a strain can occupy a certain niche or not. The results obtained in this thesis suggest that bacterial responses to acidity are more intricate than thus far assumed and showcase the tremendous complexity of bacterial integration of information from distinct environments. Thinking one step ahead, this complexity will be even more pronounced when studying bacteria in mixed biofilms, or communities. Such experiments are needed to understand the effect of acidity on a community level encountered, for example, in the human microbiome, which is ultimately linked to the overall health of a person. Correspondingly, studying stress hormones within the scope of mixed bacterial populations will provide further insights into the interplay of eukaryotes and prokaryotes.

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Supplemental information – Chapter 2.1

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Supplementary information

Ribosome profiling reveals the fine-tuned response of *Escherichia coli* to mild and severe acid stress

Kilian Schumacher, Rick Gelhausen, Willow Kion-Crosby, Lars Barquist, Rolf Backofen and Kirsten Jung
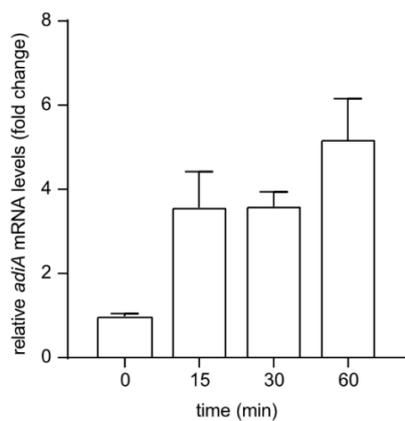
Figure S1: Temporal dynamics of *adiA* transcription under acid stress (pH 4.4). Cells were cultivated as described in Figure 1. After the shift to pH 4.4, cells were collected after 0, 15, 30 and 60 min and total RNA was prepared. Relative levels of *adiA* mRNA were quantified by RT-qPCR. Fold-change values were determined relative to the 0-min time point and normalized using *recA* as a reference gene. Error bars indicate the standard deviation of three independent biological replicates (n=3).
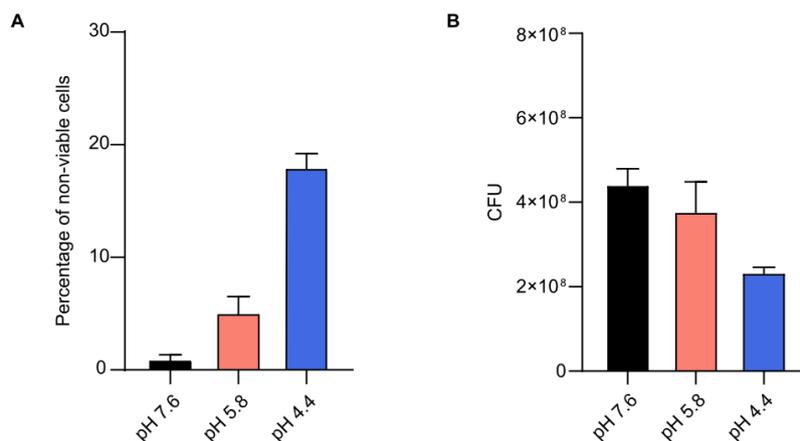
Figure S2: Quantification of viable and non-viable cells at varying pH conditions. (A) *E. coli* MG1655 cells were cultured as outlined in Figure 1A. Following sample collection, dead cells were distinguished by propidium iodide staining. Microscopy was performed using a Leica DMi8 inverted microscope equipped with a Leica DFC365 FX camera. A minimum of 1,000 cells were evaluated per condition, and relative fluorescence was measured using the MicrobeJ plugin for the ImageJ software. Cells exhibiting relative fluorescence values ≥300 after subtraction of the background were considered non-viable. **(B)** Quantification of colony forming units (CFU) at varying pH conditions. *E. coli* MG1655 cells were cultured as outlined in Figure 1A. Upon sample collection, samples were serially diluted in 1x PBS and plated on LB agar plates. Following overnight incubation, CFUs were enumerated. CFUs, colony forming units.
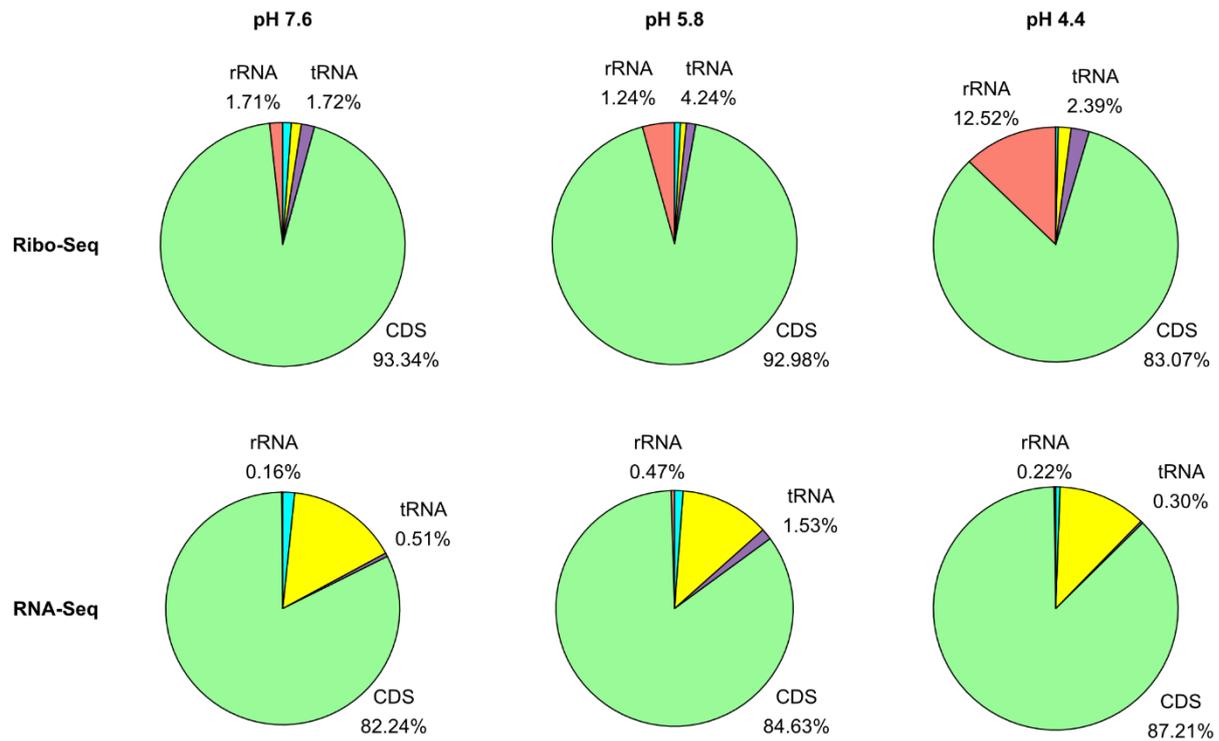
Figure S3: Read mapping statistics for Ribo-Seq and RNA-Seq data. Pie charts illustrate the percentage of uniquely mapped reads either to CDS (green), rRNA (salmon), tRNA (purple), ncRNA (yellow), or pseudogenes (lightblue). The percentages provided indicate the average ratios relative to the total number of uniquely mapped reads per condition, which were calculated from biological triplicates. CDS, coding sequence; rRNA, ribosomal RNA; tRNA, transfer RNA; nc RNA, non-coding RNA.
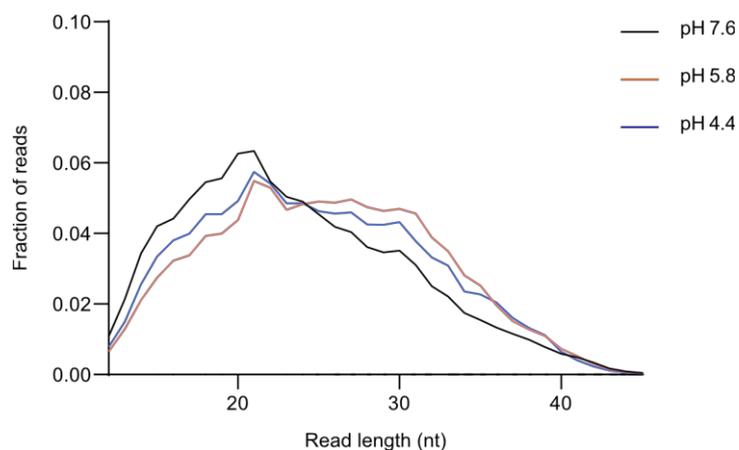
Figure S4: Read length distribution of ribosome-protected mRNA fragments (RPF) at different degrees of acidity.
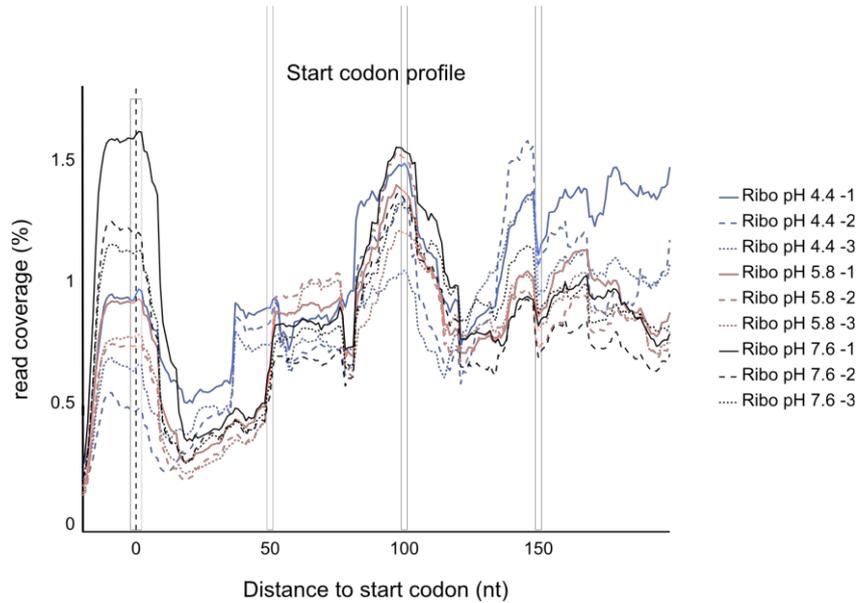
Figure S5: Reduction of ribosome occupancy in translation initiation regions at pH 5.8 and pH 4.4. Alignments of reads from ribosome-protected fragments in the 5'UTR and the first 200 nucleotides of the coding sequence from all *E. coli* genes. The percentage of read coverage is shown relative to the total number of mapped reads per condition for each specific nucleotide position. The position of the first nucleotide of the start codon is indicated by a vertical dashed line.
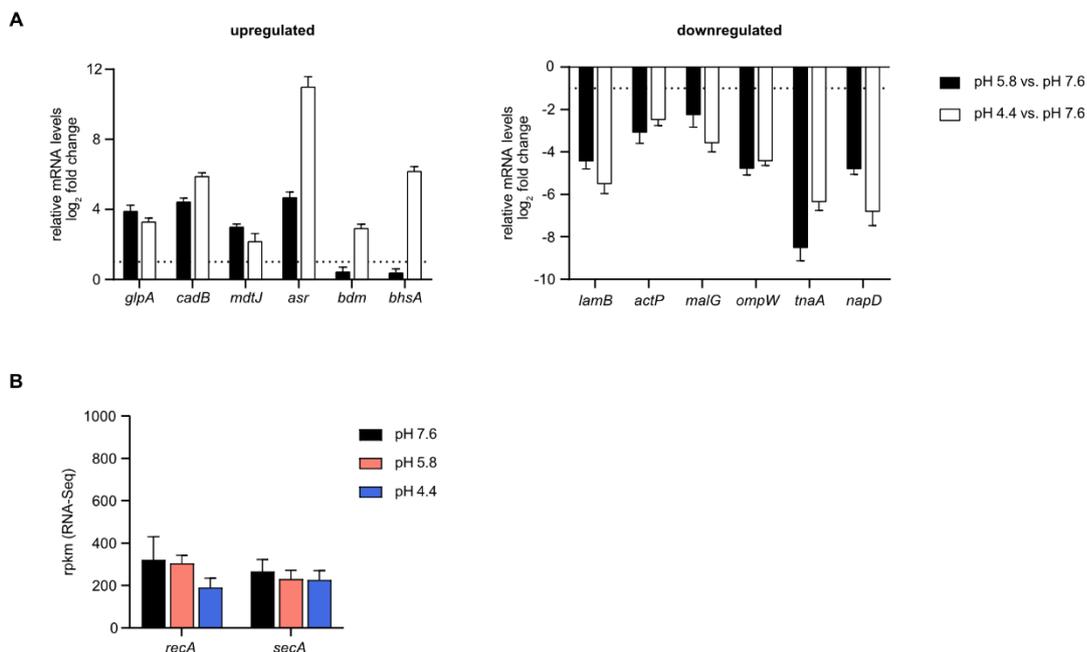
Figure S6: Verification of differentially expressed genes under acid stress using RT-qPCR. (A) Cells were cultivated as described in Figure 1. Relative mRNA levels were measured by RT-qPCR and fold change values were calculated relative to pH 7.6 and normalized using *recA*, or *secA* as reference genes. Error bars indicate the standard deviation of three independent biological replicates (n=3). **(B)** Eligibility of *recA* and *secA* as reference genes for RT-qPCR data under acid stress conditions. rpkm values determined by RNA-Seq are shown. Error bars indicate the standard deviation of three independent biological replicates (n=3). rpkm = reads per kilobase of transcript per million mapped reads.
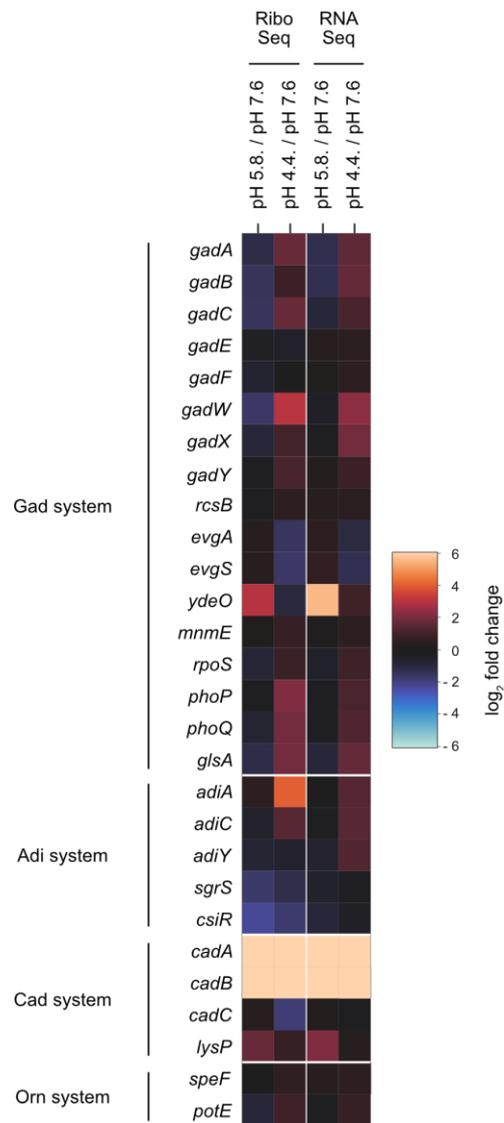
Figure S7: Transcriptional and translational expression profiles of genes associated with enzyme-based H⁺-consuming acid resistance (AR) systems in *E. coli*. Heatmap displaying RNA-Seq and Ribo-Seq log₂ fold change values of genes encoding amino acid decarboxylases, antiporters, or regulatory elements associated with either the Gad, Adi, Cad, or Orn system. Log₂ fold changes of normalized expression values at pH 4.4 and 5.8 were calculated relative to the normalized expression values at pH 7.6.
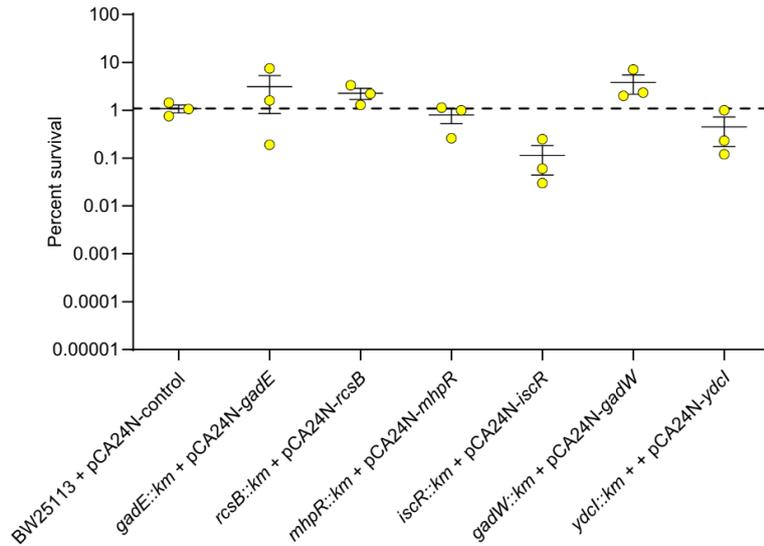
Figure S8: Survival of complemented mutants under acid shock. BW25113 wildtype, or indicated mutant strains from the Keio collection were complemented with pCA24N plasmids from the ASKA collection, or a pCA24N control vector. Cells were grown in LB pH 7.6 to OD_{600} = 0.5. The cultures were split and then either grown at pH 7.6, or stepwise stressed (15 min pH 5.8, 15 min pH 4.4) before being exposed to LB pH 3 for 1 h. Media were supplemented with 50 µg/ml (w/v) chloramphenicol and IPTG. Upon overnight incubation, colony forming units were counted and the ratio of surviving cells was calculated. The dashed line indicates the average percentage of surviving WT cells.

Species	Min E-value	Max %identity	Annotation
Vibrio parahaemolyticus	4.48E-39	100	hypothetical protein
Shigella flexneri	2.46E-27	100	hypothetical protein
Klebsiella pneumoniae	3.42E-44	98.67	hypothetical protein
Salmonella enterica	2.14E-41	98.61	hypothetical protein
Shigella boydii	7.4E-30	98.21	hypothetical protein
Shigella sonnei	1.98E-56	97.85	hypothetical protein
Enterococcus faecium	1.06E-37	97.01	hypothetical protein
Escherichia albertii	2.57E-43	88.1	hypothetical protein
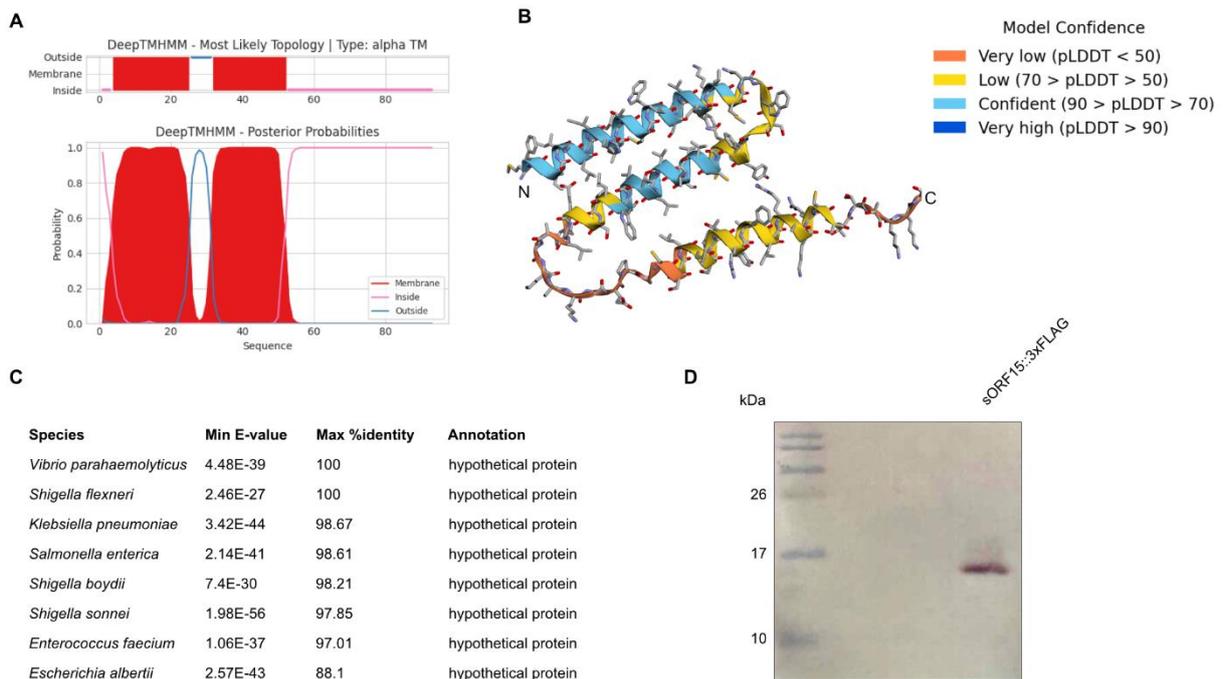
Figure S9: Subcellular location, homology, and verification of sORF15. (A) Transmembrane topology of sORF15 predicted by DeepTMHMM. **(B)** Predicted protein structure of sORF15 using ColabFold. N- and C-termini are indicated. **(C)** Homologs of sORF15 identified using blastp. The sORF15 amino acid sequence was used as the query sequence and homologs with an E-value < 0.05 and Max % identity of > 80% are listed. **(D)** Validation of sORF15 via Western Blot using a pBAD24-sORF15::3xFLAG construct. *E. coli* MG1655 cells harboring a pBAD24-sORF15:3xFLAG plasmid were cultivated to an OD_{600} of 0.5, before expression of sORF15 was induced for 1 h by addition of 0.2% (v/v) L-Arabinose. Protein levels of sORF15:3xFLAG were monitored via Tricine-SDS-PAGE followed by detection using primary α-FLAG and secondary alkaline phosphatase conjugated α-rabbit antibodies.

Table S1: OD$_{600}$ values determined at t$_{30}$ (Fig. 1A) prior to sample collection for Ribo-Seq and RNA-Seq experiments.

	Replicate I			Replicate II			Replicate III		
	pH 7.6	pH 5.8	pH 4.4	pH 7.6	pH 5.8	pH 4.4	pH 7.6	pH 5.8	pH 4.4
OD$_{600}$ (t$_{30}$)	1.05	0.93	0.69	1.18	1.08	0.66	1.13	1.04	0.72

Table S2: pH values monitored during cultivation of *E. coli* for Ribo-Seq and RNA-Seq experiments (Fig. 1A). pH-shifts were initiated by direct addition of 5 M HCl to the cultures and are indicated by (*).

	Replicate I			Replicate II			Replicate III		
	pH 7.6	pH 5.8	pH 4.4	pH 7.6	pH 5.8	pH 4.4	pH 7.6	pH 5.8	pH 4.4
pH 0 min (t$_0$)	7.24	5.90*	5.84*	7.29	5.84*	5.82*	7.28	5.81*	5.87*
pH 15 min (t$_{15}$)	7.17	5.95	4.41*	7.21	5.87	4.37*	7.24	5.83	4.33*
pH 30 min (t$_{30}$)	7.15	6.01	4.43	7.17	5.93	4.39	7.16	5.88	4.39

Table S6: Candidate transcriptional regulators evaluated in Figure 4. Transcription factors were chosen based on Ribo-Seq log$_2$ fold change values at either pH 4.4 or pH 5.8 compared to pH 7.6.

Regulator	log$_2$ fold change
YdeO	2.96 (pH 5.8)
MhpR	4.72 (pH 4.4)
IscR	4.52 (pH 4.4)
MarR	3.10 (pH 4.4)
GadW	3.03 (pH 4.4)
YdcI	2.92 (pH 4.4)

Supplemental information – Chapter 2.2

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Supplementary information

Motility-activating mutations upstream of *flhDC* reduce acid shock survival of *Escherichia coli*

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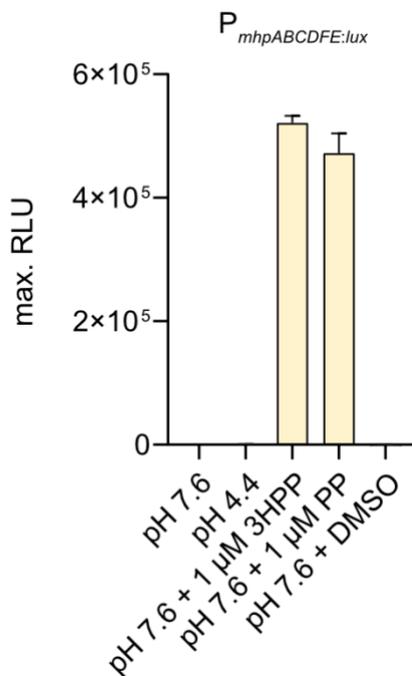
Figure S1: Promoter activity of the *mhpABCDFE* operon. *E. coli* MG1655 wild type cells were transformed with plasmid pBBR1-MCS5-P$_{mhpABCDFE:lux}$ and grown in LB medium (pH 7.6) until OD$_{600}$ = 0.5. The medium pH was then either stepwise adjusted to 5.8 and pH 4.4, or 1 mM 3HPP, 1 mM PP, or DMSO were added. Luminescence and growth were determined every 10 min in microtiter plates using a CLARIOstar plus plate reader (BMG Labtech). Data are reported as relative light units (RLUs) in counts per second per OD$_{600}$, with maximal RLU shown. All experiments were performed in biological replicates (n = 3), and error bars represent standard deviations of the mean.
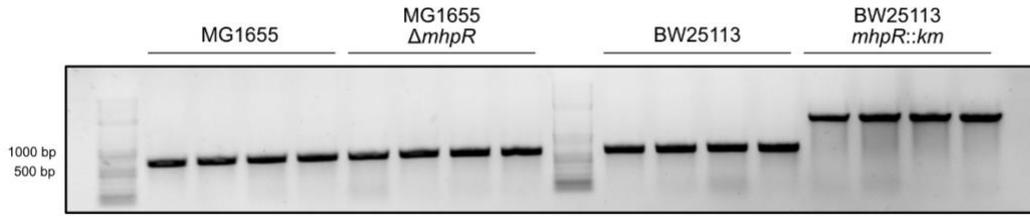
Figure S2: Transposon insertions in the promoter region of *flhDC*. The presence of IS elements in the intergenic region between *flhD* and *uspC* was verified by colony PCR using primers KSO-244 and KSO-245. The expected PCR product sizes were 767 bp in the absence of an IS element and ~2000 bp in the presence.
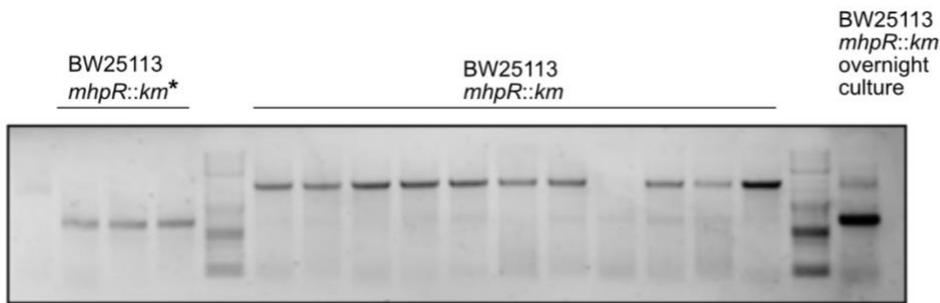
Figure S3: Verification of a BW25113 *mhpR*::*km strain without IS insertion in the promoter region of *flhDC*.** BW25113 *mhpR*::*km* was streaked on LB agar plates, and single colonies were screened by colony PCR using primers KSO-244 and KSO-245 for an intact *flhDC* promoter. The expected PCR product sizes were 767 bp in the absence of an IS element and ~2000 bp in the presence.
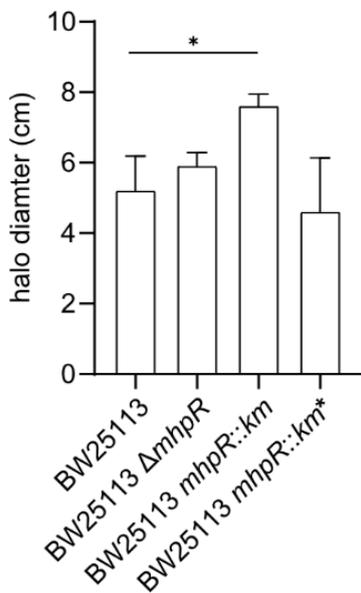
Figure S4: IS element dependent motility of BW25113. Soft agar assay evaluating the motility of BW25113 wild type, Δ*mhpR*, *mhpR*::*km*, and, *mhpR*::*km** strains. Overnight cultures normalized to an OD_{600} of 1 were spotted on LB soft agar [0.3 % (w/v)] and incubated for 16 h. Halo diameters were measured and all experiments were performed in biological replicates (n ≥ 4). Error bars represent standard deviations of the mean and significance was evaluated by performing a one-way ANOVA test followed by Bonferroni's multiple comparisons test (* $p < 0.05$).
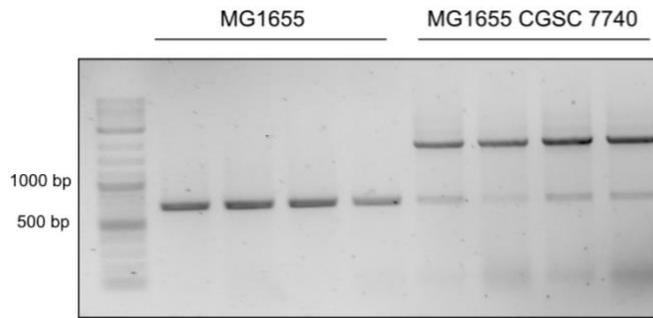
Figure S5: Alterations in the *flhDC* promoter region of two different MG1655 strains. The presence of IS elements in the intergenic region between *flhD* and *uspC* was verified by colony PCR using primers KSO-244 and KSO-245. The expected PCR product sizes were 767 bp in the absence of an IS element and ~2000 bp in the presence.
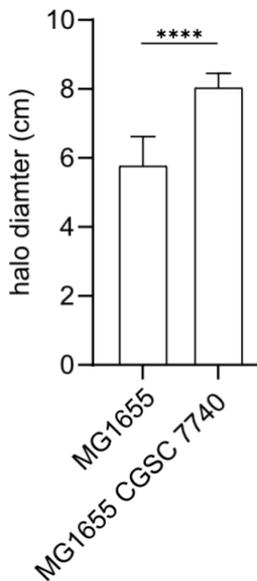
Figure S6: IS element dependent motility of MG1655. Soft agar assay evaluating the motility of MG1655 and MG1655 CGSC7740. Overnight cultures normalized to an OD_{600} of 1 were spotted on LB soft agar [0.3% (w/v)] and incubated for 16 h. Halo diameters were measured and all experiments were performed in biological replicates (n ≥ 4). Error bars represent standard deviations of the mean and significance was evaluated by performing an unpaired t-test (**** $p < 0.0001$).

Table S1: OD_{600} and pH values determined throughout the experiment conducted for RNA-Seq; pH-shifts were initiated by direct addition of 5 M HCl to growing cultures.

Strain	Replicate I				Replicate II				Replicate III			
	MG1655	MG1655 ΔmhpR	BW25113	BW25113 mhpR::km	MG1655	MG1655 ΔmhpR	BW25113	BW25113 mhpR::km	MG1655	MG1655 ΔmhpR	BW25113	BW25113 mhpR::km
OD600 (0.5)	0.50	0.46	0.47	0.48	0.50	0.50	0.42	0.44	0.45	0.41	0.46	0.49
OD600 (end)	0.88	0.74	0.74	0.91	0.76	0.64	0.62	0.61	0.65	0.78	0.71	0.87
pH (0 min)	7.24	7.24	7.23	7.21	7.26	7.27	7.27	7.27	7.28	7.25	7.23	7.20
pH (15 min)	5.72	5.75	5.71	5.65	5.78	5.78	5.81	5.75	5.83	5.82	5.77	5.79
pH (30 min)	4.38	4.33	4.33	4.27	4.34	4.34	4.32	4.32	4.32	4.33	4.47	4.46

Table S2: *Escherichia coli* strains used in this study.

Strain	Relevant genotype or description	Reference
MG1655	K-12, F-, λ- *ilvG-*, *rfb*-50, *rph*-1	(62)
BW25113	F-, Δ(*araD-araB*)567, Δ*lacZ*4787(::*rrnB*-3), λ-, *rph*-1, Δ(*rhaD-rhaB*)568, *hsdR*514	(63)
ST18	S17 λ*pir*Δ*hemA*	(65)
MG1655 Δ*mhpR*	K-12, F-, λ- *ilvG-*, *rfb*-50, *rph*-1, Δ*mhpR*	This study
BW25113 *mhpR*::*km*	F-, Δ(*araD-araB*)567, Δ*lacZ*4787(::*rrnB*-3), λ-, *rph*-1 , Δ*mhpR*786::*kan*, Δ(*rhaD-rhaB*)568, *hsdR*514, IS5 insertion 516 bp upstream of *flhDC*	(29)
BW25113 Δ*mhpR*	F-, Δ(*araD-araB*)567, Δ*lacZ*4787(::*rrnB*-3), λ-, *rph*-1, Δ(*rhaD-rhaB*)568, *hsdR*514 Δ*mhpR*	This study
BW25113 *mhpR*::*km**	F-, Δ(*araD-araB*)567, Δ*lacZ*4787(::*rrnB*-3), λ-, *rph*-1, Δ*mhpR*786::*kan*, Δ(*rhaD-rhaB*)568, *hsdR*514	This study
MG1655 CGSC 7740	K-12, F-, λ- *ilvG-*, *rfb*-50, *rph*-1, IS insertion upstream of *flhDC*	(62)
MG1655 Δ*flu*	K-12, F-, λ- *ilvG-*, *rfb*-50, *rph*-1, Δ*flu*::.FLP	(37)
MG1655 Δ*flu* Δ*flhC*	K-12, F-, λ- *ilvG-*, *rfb*-50, *rph*-1, Δ*flu*::.FLP, Δ*flhC*::.FLP	(37)
MG1655 Δ*flu* P$_{tac}$-*flhDC*	K-12, F-, λ- *ilvG-*, *rfb*-50, *rph*-1, Δ*flu*::.FLP-*lacI*-P$_{tac}$-*flhDC*	(37)

Table S3: Plasmids used in this study.

Name	Reference
pBBR1-MCS5-P*mhpR:lux*	This study
pBBR1-MCS5-P*mhpABCDFE:lux*	This study
pNPTS-R6KT-Δ*mhpR*	This study

Table S4: Oligonucleotides used in this study.

Name	Sequence
KSO-0025	CCCGGGCTGCAGGAATTC
KSO-0026	TCTAGAGAATAGGAACTTCGGAAT
KSO-0033	ATCTGGATCCACGAATTCGC
KSO-0034	ATCCTGCAGAGAAGCTTGG
KSO-0041	CAGGAAACAGCTATGACC
KSO-0042	TGTAAAACGACGGCCAGT
KSO-0149	AGCCGCTTATCCTTTCACC
KSO-0150	TTCCTTGCGGTCTTGTTCC
KSO-0151	CAGATATTGTGCTCGGTGGTAG
KSO-0152	GACAGGTAGAAACGGGAAGAAC
KSO-0169	CGAAGTTCCTATTCTCTAGATTCAGTACCTCACGACTCGG
KSO-0170	TCGAATTCCTGCAGCCCGGGATTAATTGACATTTCTATAGTTAAAACAAC
KSO-0171	CGAAGTTCCTATTCTCTAGAATTAATTGACATTTCTATAGTTAAAACAAC
KSO-0172	TCGAATTCCTGCAGCCCGGGTTCAGTACCTCACGACTCGG
KSO-0190	GCCAAGCTTCTCTGCAGGATCCAGCAGGCGAAAATCCTG
KSO-0191	CACCATCGAATGGCGCAAAAC
KSO-0192	TTTTGCGCCATTCGATGGTGATTAATTGACATTTCTATAGTTAAAACAAC
KSO-0193	GCGAATTCGTGGATCCAGATGGCAGCACTTTGCTTAACAG
KSO-0194	CCCGCGTTTTCGCAGAAAC
KSO-0195	TTCAGTACCTCACGACTCGG
KSO-0214	GCCAAGCTTCTCTGCAGGATGAGTTGCAGCAAGCGGTC
KSO-0224	AGAGATTGAGACGCACGAAAG
KSO-0225	CCATTACAGCCGCAACAATAC
KSO-0226	TATTTGCACCTGGCTTCTCC
KSO-0227	CCGCCCATCTTCTGGATATTAC
KSO-0228	TCGCCGTATGTTGCTTACTC
KSO-0229	CCGTCAATGGTCAGGGTATTC
KSO-0230	TGAAGTGGCACAGGCAATAG
KSO-0231	TCGGCAATATCGATCCCTAAAC
KSO-0232	GGAAGCTGGCAATGTCAAAC
KSO-0233	ATAGATCGCGCAGGCTAATG
KSO-0234	GTCATTCCAATGGTGGGATTTG
KSO-0235	GGTCACCTTCGCTTTGTTTG
KSO-0236	ACTGGTTTATGACCTGGGAAC
KSO-0237	CATCGACGCCATTACACAAAC
KSO-0238	GTTAAGCTGGCAGAAACCAATC
KSO-0239	ATCGTCAACGCGGGAATC
KSO-0240	ACCTGAACAACACCACTACC
KSO-0241	GATCTGCGCTTTCGACATATTG
KSO-0242	CGGTACGTCGCTTTGAATTTATG
KSO-0243	GATTAGGCAGCACTTTGCTTAAC
KSO-0244	GTTTCACCGCACCCCGTG
KSO-0245	GGAGAAACGACGCAATCCCAAC

Supplemental information – Chapter 2.3

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Supplementary Information for

Eukaryotic catecholamine hormones influence the chemotactic control of *Vibrio campbellii* by binding to the coupling protein CheW

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Other supplementary materials for this manuscript include the following:

Datasets S1 to S6

Supplementary methods

Biological methods

Bacterial strains and cultivation

Bacteria were stored as 50% (v/v) glycerol stocks at -80 °C. Overnight cultures for growth assays, photolabeling, and Co-IP experiments were grown from 50% (v/v) glycerol stocks inoculated 1:1000 into 20 mL medium for 15 h at the indicated temperatures and 200 rpm. Overnight cultures were then diluted 1:100 into fresh medium (60 mL) and grown to early stationary phase. For 3D motility and chemotaxis assays, overnight cultures were inoculated from individual *V. campbellii* colonies, grown on 2% (w/v) agar MB plates streaked from a glycerol stock, and grown to saturation in 2 mL MB at 30°C, 200 rpm. Day cultures were inoculated with the overnight cultures at 1:200 dilution in 10 mL MB and grown at 30°C, 250 rpm, until they reached OD_{600} 0.3.

Strain construction

Construction of the Δ*cheW* marker-less in-frame deletion in *V. campbellii* ATCC-BAA 1116 was achieved using the suicide plasmid pNPTS138-R6KT-*cheW* as described previously (1). Briefly, 600 bp upstream and downstream of *cheW* were amplified by PCR using *V. campbellii* ATCC-BAA 1116 genomic DNA as template. After PCR product purification, the fragments were fused by overlap PCR. The overlap PCR fragment was cloned into plasmid pNPTS138-R6KT using BamHI and EcoRI as restriction sites. The resulting plasmid pNPTS138-R6KT-Δ*cheW* was introduced into *V. campbellii* ATCC-BAA 1116 by conjugative mating using *E. coli* WM3064 as a donor in LB medium containing 2,6-diaminopimelic acid (DAP). Single-crossover integration mutants were selected on LB plates containing kanamycin but lacking DAP. Single colonies were grown over a day without antibiotics and plated onto LB plates containing 10% (w/v) sucrose to select for plasmid excision. Kanamycin-sensitive colonies were checked for targeted deletion by colony PCR using primers bracketing the site of the deletion.

Construction of a plasmid coding for N-terminally His6-tagged CheW

The *cheW* gene of *V. campbellii* (VIBHAR_RS14640; old locus tag VIBHAR_03137) was cloned into vector pET28a using BamHI and XhoI as restriction sites, resulting in an extension of the sequence by codons for a N-terminal His_6 tag.

Purification of 6His-CheW

To purify CheW of *V. campbellii*, *E. coli* BL21(DE3) carrying the plasmid pET28a-*cheW* was cultivated in LB supplemented with kanamycin (50 mg/mL) at 37 °C. At OD_{600} 0.6, 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture to induce *cheW* expression at 30 °C for 4 h. Cells were harvested (20 min, 5,000 x *g*, 4 °C), resuspended, and disrupted by high-

pressure cell disrupter (*Constant Systems Limited*) in ice-cold disruption buffer [20 mM Tris/HCl pH 7.5, 500 mM NaCl, 10 mM imidazole, 3 mg DNase, and 0.5 mM phenazine methosulfate (PMSF) in double-distilled water (ddH$_2$O)]. After removal of intact cells and cells debris via centrifugation (5,000 x *g*, 30 min, 4 °C), membrane vesicles were removed by ultracentrifugation (45,000 x *g*, 60 min, 4°C), and the cell lysate was loaded onto a Ni-nitrilotriacetic acid (NTA) column (*Qiagen*). After a washing step (20 mM Tris/HCl pH 7.5, 500 mM NaCl, 50 mM imidazole), the recombinant protein was eluted with elution buffer (20 mM Tris/HCl pH 7.5, 500 mM NaCl, 250 mM imidazole).

Generation of polyclonal rabbit antibody against 6His-CheW

Customized polyclonal rabbit antibody against 6His-CheW was obtained from *Kaneka Eurogentec*. For this purpose, heterologously produced and purified 6His-CheW was provided as antigen in a Speedy 28-day immunization programme with two rabbits as hosts. 5 mL of rabbit serum was purified after the immunization by affinity purification to separate IgG antibodies from crude serum. Specificity of the polyclonal antibody against CheW was verified by Western blot analyses.

Data acquisition and analysis of 3D trajectories

Phase contrast microscopy recordings were obtained at room temperature (~21 °C) on a Nikon Ti-E inverted microscope using an sCMOS camera (PCO Edge 4.2) and a 40x objective lens (Nikon CFI SPlan Fluor ELWD 40x ADM Ph2, correction collar set to 1.2 mm to induce spherical aberrations) (2). For motility experiments, it was focused 135 µm above the bottom surface of the sample chamber. One to four 1 or 1.5-min long recordings were obtained at 30 fps per condition in motility experiments, alternating between conditions. A typical 1.5-min motility recording yields 1,500 - 2,000 bacterial trajectories. For chemotaxis experiments, it was focused at the center of the 70 µm-tall channel in all three dimensions. Two to three 2 - 2.5-min long recordings were obtained at 30 fps per condition in chemotaxis experiments. Three biological replicates were performed for chemotaxis in a 100 µM/mm **EPI** gradient, one otherwise. Biological replicates used cultures grown from different colonies.

3D trajectories were extracted from phase contrast recordings using a high-throughput 3D tracking method based on image similarity between bacteria and a reference library (2). Trajectories shorter than 5 frames were discarded. Positions were smoothed using 2nd order ADMM-based trend-filtering with regularization parameter λ = 0.3, and speeds computed as forward differences in positions divided by the time interval between frames. All trajectories with an average speed below a 20 µm/s threshold were considered non-motile and discarded. The range of 3D bacterial trajectories was ~350 µm x 300 µm laterally (*x*, *y*) and 200 µm (*z*) in motility chambers, or the entire 70 µm height (*z*) of the channel in the chemotaxis device.

For motility experiments, trajectories with a minimum duration of 1 s were analyzed for turn events. The turn event detection was based on the local rate of angular change of direction, computed from

the dot product between the sums of the two consecutive velocity vectors preceding and subsequent to a time point. The threshold for a turn to begin was an α-fold rate relative to the median rate of angular change rate of the run segments, as determined in three iterations of the procedure. We determined by visual inspection of trajectories that a factor α = 10 gave satisfactory results. A new run begins with at least two time points (at least 0.066 s) below this threshold.

For chemotaxis experiments, the *z* position of the top and bottom of the chemotaxis chambers were identified by visual inspection of trajectory data, and all trajectory segments within 10 µm of the top or bottom of the central channel were removed to avoid surface interaction effects. The drift velocity is the average of the *x* component of all instantaneous 3D speed vectors from all bacteria, *x* being the gradient direction. We estimate the noise on the drift measurement by a jackknife resampling procedure consisting of dividing the data into subsets of 150 trajectories and computing the standard error of the mean drift obtained for different subsets.

Proteomics methods

General

For proteomics experiments, synthesized probes were stored as DMSO stocks at -20 °C; unmodified parent compounds were dissolved in DMSO on the day of the experiment.

Preparative photolabeling with **EPI-P1**

Preparative scale photolabeling experiments with **EPI-P1** were performed as with **PE-P** with the following exceptions: Labeling was performed in 5 mL culture and **EPI-P1** was added to 7.5 µM (25 µL from a 1.5 mM DMSO stock), the same volume of 5.25 mM acetic acid in DMSO was added as control. Samples were incubated for 30 min at 30 °C, 200 rpm and cultures were irradiated for 5 min in 6 cm dishes. Bacteria were lysed in 450 µL PBS + protease inhibitor with sonication (2 x 15 s, 60% intensity), then TX100 was added to 1% (v/v) and sonicated (1 x 10 s, 10% intensity) and samples were incubated for 30 min on ice. Insoluble debris was removed by centrifugation (16,060 x *g*, 20 min, 4 °C).

In situ analytical scale photolabeling

Analytical scale photolabeling experiments were performed as preparative scale experiments with the following exceptions: Probe concentration was 50 µM (0.5% (v/v) DMSO). Irradiation of 1 mL labeled bacteria was performed in a 12-well dish. Lysis and fractionation into soluble and insoluble proteins was performed as for preparative experiments with **PE-P** (in 200 µL).

Analytical photolabeling in lysate in presence of radical scavengers

Lysate (in PBS) was adjusted to a protein concentration of 1 mg/mL and DMSO or **PE-P** was added to 50 µM (0.1% (v/v) DMSO) in 60-100 µL lysate in a 96-well plate. Samples were incubated for 1 h at 30 °C, 200 rpm and radical scavengers were added to 1 mM or 10 mM as indicated (11 µL from a freshly made 10 mM or 100 mM stock in water), mixed thoroughly, and irradiated for 10 min. Proteins were precipitated in acetone and washed with methanol to remove the radical scavengers (as described above), resuspended in 1% (w/v) SDS/PBS (with vortexing and sonication) to the same protein concentration, and subjected to CuAAC with TAMRA-azide.

CuAAC, analytical scale photolabeling

CuAAC for analytical labeling experiments was performed with rhodamine-azide (tetramethylrhodamine 5-carboxamido-(6-azidohexanyl), 5-isomer, *Base Click*). Click reaction was quenched by addition of the same volume of 2 x sample loading buffer (63 mM Tris/HCl, 10% (v/v) glycerol, 2% (w/v) SDS, 0.0025% (w/v) bromophenol blue, 5% (v/v) 2-mercaptoethanol).

SDS-PAGE

Stacking gels consisted of 4% (w/v) acrylamide (in 50 mM Tris, pH 6.8) and resolving gels of 12.5% (w/v) acrylamide (in 300 mM Tris, pH 8.8) and were run in a Tris-glycine buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3). Typically, 30 µL sample (~15 µg protein), 8 µL fluorescent marker (BenchMark™ Fluorescent Protein Standard, *Thermo Fisher*), and 12 µL protein marker (Roti®-Mark Standard, *Carl Roth*) were loaded and gels were run at 150-300 V (depending on gel size) on a EV265 Consort power supply (*Hoefer*). Fluorescence was scanned in a LAS-4000 imaging system equipped with a Fujinon VRF43LMD3 lens and a 575DF20 filter (*Fujifilm*). Gels were stained in Coomassie staining solution (0.25% (w/v) Coomassie Brilliant Blue R-250, 9.2% (v/v) concentrated acetic acid, 45.4% (v/v) ethanol) overnight and destained in 10% (v/v) acetic acid, 40% (v/v) ethanol).

Co-IP

Bacteria from an overnight culture were diluted in LB35 medium and grown until early stationary phase, pelleted, washed with PBS, and resuspended in PBS to OD_{600} 4.0 as for preparative photolabeling experiments with **PE-P**. Four replicates were used starting from independent overnight cultures. Next, 10 mL of the suspension in a 50 mL falcon was treated with 100 µM **EPI** (from 100 mM stock in DMSO) or DMSO and incubated 30 min, 30 °C, 200 rpm. Bacteria were harvested (6,000 x *g*, 5 min, RT) and resuspended in 2 mL PBS containing 100 µM **EPI** or the equivalent volume of DMSO. The DSSO crosslinker was added to 2 mM (from a 100 mM stock in DMSO, DSSO synthesized as described previously (3)), and samples were incubated at 30 °C, 200 rpm for 30 min. Bacteria were pelleted (6,000 x *g*, 10 min, 4 °C), washed with 2 x 1 mL cold 50

mM Tris/HCl, pH 8.0 to quench the DSSO, and the pellet was flash-frozen and stored at -80 °C. The pellet was resuspended in 1 mL lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5% (v/v) glycerol, 0.1% (v/v) NP-40) and sonicated 3 x 15 s, 60% intensity, on ice. The lysate was cleared by centrifugation (21,000 x g, 30 min, 4 °C) and the supernatant was sterile filtered (0.2 µm). Protein amount was adjusted to 1 mg (1 µg/µL). Per sample, 30 µL protein A/G bead slurry (*Pierce Biotechnology, Thermo Fisher Scientific*) in LoBind microcentrifuge tubes was equilibrated with 1 x 1 mL cold wash buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5% (v/v) glycerol, 0.05% (v/v) NP-40) and centrifuged (1,000 x g, 1 min, 4 °C). The supernatant was discarded and the lysate was added to the beads. Next, 2.5 µg antibody (167 µL from a 0.015 µg/µL stock in 50% (v/v) glycerol) or a rabbit mAb IgG XP® isotype control (1 µL, 2.5 µg/µL, *Cell Signaling Technology*) was added and incubated overnight at 4 °C under constant rotation. Samples were centrifuged (30 s, 500 x g, 4 °C), the supernatant discarded, and the beads washed with 2 x 1 mL cold wash buffer and 3 x 1 mL cold basic buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5% (v/v) glycerol). Proteins were reduced and digested by the addition of 25 µL IP elution buffer I (50 mM Tris/HCl, pH 8.0, 5 ng/µL trypsin, 2 M urea, 1 mM DTT) at 25 °C, 1,000 rpm for 30 min. To alkylate cysteines, 100 µL IP elution buffer II (50 mM Tris/HCl, pH 8.0, 2 M urea, 5 mM iodoacetamide) was added, and the samples were incubated overnight (~16 h) at 37 °C, 1,000 rpm. Formic acid was added to 1% (v/v) (pH 2-3) to quench the digestion and samples were desalted on stage tips. Desalting was performed as for photolabeling experiments with slight modifications: two layers of C-18 material was used, and stage tips were equilibrated with 1 x 80 µL methanol, then 1 x 80 µL 80% (v/v) acetonitrile, 0.5% (v/v) formic acid, and with 3 x 70 µL 0.5% (v/v) formic acid. Samples were centrifuged (16,249 x g, 2 min) and loaded, the beads were washed with 2 x 150 µL 0.5% (v/v) formic acid and washes were loaded too. Peptides were desalted with 3 x 70 µL 0.5% (v/v) formic acid and eluted with 2 x 30 µL 80% (v/v) acetonitrile, 0.5% (v/v) formic acid. Solvents were removed in a speed-vac and dry samples were stored at -80 °C.

<u>LC-MS/MS measurements</u>

Co-IP peptide samples were analyzed on an UltiMate 3000 nano HPLC system (*Dionex*) equipped with an Acclaim C18 PepMap100 (75 µm ID × 2 cm) trap column and an Aurora Series Emitter Column with Gen2 nanoZero fitting (75 µm ID × 25 cm, 1.6 µm FSC C18) separation column (column oven heated to 40 °C) coupled to an Orbitrap Fusion (*Thermo Fisher*) in EASY-spray setting. Peptides were loaded on the trap column and washed with 0.1% (v/v) TFA before being transferred to the analytical column and separated using a 152 min gradient (buffer A: 0.1% (v/v) formic acid in water, buffer B: 0.1% (v/v) formic acid in acetonitrile, gradient: 5-22% (v/v) buffer B in 112 min, then to 32% (v/v) buffer B in 10 min, then to 90% (v/v) buffer B in 10 min and hold 90% (v/v) buffer B for 10 min, then to 5% (v/v) buffer B in 0.1 min and hold 5% (v/v) buffer B for 9.9 min) with a flow rate of 400 nL/min. The Orbitrap Fusion was operated in a TOP10 data dependent mode

and full scan acquisition in the orbitrap was performed with a resolution of 120,000 and an AGC target of 2e5 (maximum injection time of 50 ms) in a scan range of 300−1,500 m/z. Monoisotopic precursor selection as well as dynamic exclusion (exclusion duration: 60 s) was enabled. Most intense precursors with charge states of 2-7 and intensities greater than 5e3 were selected for fragmentation. Isolation was performed in the quadrupole using a window of 1.6 m/z. Precursor ions were collected to an AGC target of 1e4 (maximum injection time of 100 ms). Fragments were generated using higher-energy collisional dissociation (HCD, normalized collision energy: 30%) and detected in the ion trap operating at a rapid scan rate.

Photoaffinity labeling peptide samples were analyzed on an UltiMate 3000 nano HPLC system (*Dionex*) equipped with an Acclaim C18 PepMap100 (75 µm ID × 2 cm) trap column and a 25 cm Aurora Series emitter column (25 cm × 75 µm ID, 1.6 µm FSC C18) (Ionoptics) separation column (column oven heated to 40 °C) coupled to a Q Exactive Plus (*Thermo Fisher*) in EASY-spray setting. Peptides were loaded on the trap column and washed with 0.1% (v/v) TFA before being transferred to the analytical column and separated using a 152 min gradient (buffer A: 0.1% (v/v) formic acid in water, buffer B: 0.1% (v/v) formic acid in acetonitrile. Gradient of buffer B: 5% (v/v) for 7 min, increase to 22% (v/v) in 105 min, then to 32% (v/v) in 10 min, then to 90% (v/v) in 10 min, hold at 90% (v/v) for 10 min, decrease to 5% (v/v) in 0.1 min and hold at 5% (v/v) for 9.9 min) with a flow rate of 400 nL/min. The Q Exactive Plus was operated in a TOP10 data dependent mode full scan acquisition in the orbitrap was performed with a resolution of 140 000 and an AGC target of 3e6 (maximum injection time of 80 ms) in a scan range of 300−1,500 m/z. Most intense precursors with charge states of > 1, a minimum AGC target of 1e3, and intensities greater than 1e4 were selected for fragmentation. Peptide fragments were generated using higher-energy collisional dissociation (HCD, normalized collision energy: 27%) and detected in the orbitrap with a resolution of 17 500 m/z. The AGC target was set to 1e5 (maximum injection time 100 ms) and the dynamic exclusion duration to 60 s. Isolation in the quadrupole was performed with a window of 1.6 m/z.

Differential isotopic labeling samples were measured on a Q Exactive Plus spectrometer with a different gradient (buffer B: 5% (v/v) for 7 min, increase to 40% (v/v) in 105 min, then to 60% (v/v) B in 10 min, and to 90% (v/v) B in 10 min, hold at 90% (v/v) for 10 min, then decrease to 5% (v/v) in 0.1 min and hold at 5% (v/v) for another 9.9 min) at the same flow rate. All parameters were the same as for photoaffinity labeling experiments except full MS scans were collected at a resolution of 70 000.

<u>Analysis of MS data from photoaffinity labeling and Co-IP experiments</u>

MS data were analyzed using MaxQuant (4, 5) version 1.6.5.0 and peptides were searched against the UniProt database for *Vibrio campbellii* ATCC BAA-1116 / BB120 (taxon identifier 338187,

downloaded on 17.02.2020). Cysteine carbamidomethylation was set as fixed modification and methionine oxidation and N-terminal acetylation as variable modifications. Trypsin (without N-terminal cleavage to proline) was set as proteolytic enzyme with a maximum of two allowed missed cleavages. Label-free quantification (LFQ) mode (6) was performed with a minimum ratio count of 2. The "match between runs" (0.7 min match and 20 min alignment time window) and second peptide identification options were activated. All other parameters were used as pre-set in the software. LFQ intensities were further processed with Perseus (7) version 1.6.1.1. Peptides of the categories "only identified by site", "reverse", or "potential contaminant" were removed and LFQ intensities were $\log_2$-transformed. Data were filtered to retain only protein groups identified in at least 3/4 valid values (experiments: competitive labeling **PE-P** vs. **EPI**; competitive labeling **PE-P** vs. **PRO**/**LAB**; Co-IP), 4/5 valid values (competitive labeling **PE-P** vs. **PE**), or 3/3 valid values (labeling with **EPI-P1**) in at least one group and missing values were imputed (width 0.3, downshift 1.8, total matrix). A two-sample Student´s t-test with permutation-based FDR (FDR 0.05) was performed and the significance cut-off was set at p-value = 0.05 (-log10(p-value) = 1.3) and an enrichment factor of 2 ($\log_2(x)$ = 1) or 4 ($\log_2(x)$ = 2) as indicated in the plots. Protein IDs were matched to annotations downloaded from annotations.perseus-framework.org on 18.02.2020. Proteins with the annotation "chemotaxis" in the GOBP or KEGG database were highlighted in the Co-IP plots.

Analysis of isoDTB data

Analysis software was set up as previously described (8) using the MSconvert tool (version: 3.0.19172-57d620127) of the ProteoWizard software (version: 3.0.19172 64bit) (9), the FragPipe interface (version: 14.0) (10, 11), MSFragger (version: 3.1.1) (10, 11), Philosopher (version: 3.3.10) (12), IonQuant (version 1.4.6) (13), and Python (version: 3.7.3). The FASTA file (*Vibrio campbellii* ATCC BAA-1116/BB120; taxon identifier 338187, downloaded on 17.02.2020) was modified by adding the reverse sequences manually. Modifications were analyzed as previously described (8). Amino acid selectivity was analyzed and data were evaluated and filtered as previously published (8) performing an Offset Search in MSFragger (10, 11) with mass offsets set as 740.3974 or 746.4040. Run MS1 quant was enabled with Labeling based quant with masses set as 740.3974 or 746.4040. Specific amino acids were quantified and data were evaluated and filtered as previously published (8) performing a Closed Search in MSFragger with variable modifications set to 740.3974 or 746.4040 on Tyr. Run MS1 quant was enabled with Labeling based quant with masses set as 740.3974 or 746.4040.

AlphaFold structure prediction

The sequence of CheW was retrieved from UniProt (Uniprot Code: A7MS42) and used as basis for the Alphafold prediction on a local installation of the Alphafold algorithm as released by Jumper *et*

al. (14). Visualization and alignment with the CheW structure from *E. coli* (PDB:2HO9, solution NMR) was done using the open source version of PyMOL 2.4 (15).

Chemical synthesis

<u>General</u>

Chemicals with reagent or higher grade as well as dry solvents were purchased from *Sigma Aldrich*, *Acros Organics*, or *Alfa Aesar*. 2-(3-But-3-ynyl-3*H*-diazirin-3-yl)-ethanol was purchased from *Ark Pharm Inc*.

Analytical thin layer chromatography was performed on aluminum-coated TLC silica gel plates (silica gel 60, F254, *Merck KGaA*) with visualization by UV light (λ = 254 nm) or $KMnO_4$-stain (3.0 g $KMnO_4$, 20.0 g K_2CO_3 and 5 mL 5% (w/v) NaOH in 300 mL ddH_2O). Column chromatography was carried out using silica gel (40-63 µm (Si 60), *Merck KGaA*). High-resolution mass spectra (HRMS) were measured on a LTQ-FT Ultra (*Thermo Fisher*) equipped with an ESI ion source.

NMR spectra were measured at room temperature on Avance-III HD NMR systems with 300, 400, or 500 MHz (*Bruker Co.*). Chemical shifts are reported in parts per million (ppm) and residual proton signals of deuterated solvents were used as internal reference (^{1}H NMR: $CDCl_3$ δ = 7.26 ppm, DMSO-*d*6 δ = 2.50 ppm, 0.04% (v/v) DCl in D_2O referenced to D_2O δ = 4.79 ppm. ^{13}C-NMR: $CDCl_3$ δ = 77.16 ppm, DMSO-*d*6 δ = 39.52 ppm, 50% (v/v) AcOD in D_2O referenced to AcOD δ = 178.990 ppm). Coupling constants (*J*) are reported in Hertz (Hz). Signal multiplicities are denoted with the following abbreviations: s – singlet, d – doublet, dd – doublet of doublets, ddd – doublet of doublet of doublets, t – triplet, td – triplet of doublets, p – pentet, and m – multiplet. NMR data were analyzed using MestReNova (*Mestrelab Research*).

<u>3-(But-3-yn-1-yl)-3-(2-iodoethyl)-3*H*-diazirine (**PCL-I**)</u>
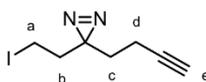
PCL-I
$C_7H_9IN_2$
248.07 g/mol

The reaction was performed following a published protocol (16). A solution of imidazole (74 mg, 1.08 mmol, 3.0 eq.) and triphenylphosphine (104 mg, 0.398 mmol, 1.1 eq.) in anhydrous DCM (2 mL) was cooled to 0 °C. Pestled iodine (110 mg, 0.434 mmol, 1.2 eq.) was added and the solution was stirred at 0 °C for 5 min. 2-(3-(But-3-ynyl)-3*H*-diazirin-3-yl)ethanol (50 mg, 0.361 mmol, 1.0 eq.) was added in DCM (~1 mL) and the reaction mixture was stirred 7 h under exclusion of light. The reaction was quenched with saturated aqueous $Na_2S_2O_3$ (2 mL) and the aqueous layer was extracted with EtOAc (2 x 5 mL). Combined organic layers were washed with brine (1 x 5 mL) and dried over Na_2SO_4. Solvents were removed under reduced pressure ($\geq$ 50 mbar) and the residue

was purified by column chromatography (EtOAc/n-hexane 1:20). Solvents were removed under reduced pressure (≥ 50 mbar) and the product was obtained as a colorless liquid (55 mg, 0.222 mmol, 62%).

TLC: R_f = 0.53 (EtOAc/n-hexane 1:20) [UV/KMnO$_4$]. **^{1}H-NMR** (500 MHz, CDCl$_3$) δ [ppm]: 2.89 (t, J = 7.6 Hz, 2H, H-a), 2.12 (t, J = 7.6 Hz, 2H, H-d), 2.06-2.00 (m, 3H, H-b, H-e), 1.69 (t, J = 7.2 Hz, 2H, H-c). **^{13}C-NMR** (75 MHz, CDCl$_3$) δ [ppm]: 82.56, 69.57, 37.66, 31.96, 28.73, 13.39, -3.88. Analytical data are in accordance with literature reports (16).

(*R*)-4-(2-((2-(3-(But-3-yn-1-yl)-3*H*-diazirin-3-yl)ethyl)amino)-1-hydroxyethyl)-benzene-1,2-diol (**EPI-P1**)

EPI-P1
C$_{15}$H$_{19}$N$_3$O$_3$
289.34
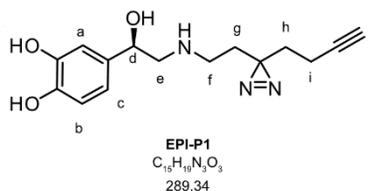
NE (105 mg, 0.621 mmol, 3.5 eq.) was added to a solution of **PCL-I** (44 mg, 0.177 mmol, 1.0 eq.) in anhydrous DMF (6 mL) and it was stirred at 70 °C (with reflux) under argon for 17 h. Solvents were removed under reduced pressure and the residue was purified by column chromatography (MeOH/AcOH/DCM 1:3:10) and solvents were removed under reduced pressure with toluene co-evaporation. The product **EPI-P1** was obtained as a mixture with acetic acid (**EPI-P1**:AcOH 1:3.5) as a brown solid (53 mg, 0.106 mmol, 62%).

TLC: R_f = 0.43 (MeOH/AcOH/DCM 1:3:7) [UV/KMnO$_4$]. **^{1}H-NMR** (500 MHz, DCl/D$_2$O 38:962) δ [ppm]: 6.66 – 6.63 (m, 2H, H-a and H-b), 6.57 (dd, J = 8.2, 2.1 Hz, 1H, H-c), 4.63 (dd, J = 7.9, 5.2 Hz, 1H, H-d), 3.01 – 2.95 (m, 2H, H-e), 2.78 (td, J = 8.7, 2.1 Hz, 2H, H-f), 2.10 (t, J = 2.7 Hz, 1H, H-j), 1.80 – 1.74 (m, 13H, AcOH + H-i), 1.59 (ddd, J = 10.6, 6.4, 2.7 Hz, 2H, H-g), 1.42 (t, J = 7.1 Hz, 2H, H-h). **^{13}C-NMR** (101 MHz, AcOD/D$_2$O 1:1) δ [ppm]: 147.11, 147.04, 134.59, 120.99, 118.78, 116.23, 86.08, 72.85, 71.27, 56.17, 45.43, 33.56, 32.06, 29.07, 15.19. **HRMS:** (ESI) C$_{15}$H$_{20}$N$_3$O$_3^+$ [M+H]$^+$ calculated: 290.1499; found: 290.1499.

Amide coupling general protocol

To a solution of 6-heptynoic acid (127 µL, 0.950 mmol, 1.0 eq.) in DMF (10 mL), EDC•HCl (182 mg, 0.95 mmol, 1.0 eq.) and HOBt (128 mg, 0.95 mmol, 1.0 eq.) was added. The clear solution was stirred at 0 °C for 30 min and then at room temperature for 4 h. Anhydrous TEA (395 µL, 2.85 mmol, 3.0 eq.; or 4 eq. if amine was HCl salt) was added followed by the amine (0.95 mmol, 1.0 eq.). The mixture was stirred overnight. Water (100 mL) was added, extracted with 3 x EtOAc (30 mL), and combined organic phases were washed with brine (30 mL) and dried over Na$_2$SO$_4$. Solvents were

removed under reduced pressure and the crude mixture was purified by SiO$_2$ chromatography (MeOH/DCM 8:92).

<u>(R)-N-(2-(3,4-Dihydroxyphenyl)-2-hydroxyethyl)hept-6-ynamide (**EPI-P2**)</u>

EPI-P2
C$_{15}$H$_{19}$NO$_4$
277.32
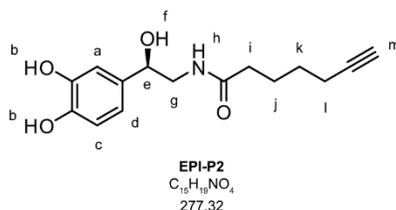
The reaction was performed with L-norepinephrine (161 mg, 0.950 mmol, 1.0 eq.) and the product was obtained as a pale orange amorphous solid (86 mg, 0.310 mmol, 16%).

TLC: R_f = 0.37 (MeOH/DCM 8:92) [UV/KMnO$_4$]. **^{1}H-NMR:** (500 MHz, DMSO-d$_6$) δ [ppm]: 8.78 (s, 1H, H-b), 8.68 (s, 1H, H-b), 7.77 (t, J = 5.6 Hz, 1H, H-h), 6.71 (d, J = 1.7 Hz, 1H, H-a), 6.65 (d, J = 8.0 Hz, 1H, H-c), 6.54 (dd, J = 8.0, 1.7 Hz, 1H, H-d), 5.18 (d, J = 4.1 Hz, 1H, H-f), 4.39 (dt, J = 8.3, 4.4 Hz, 1H, H-e), 3.23 – 3.17 (m, 1H, H-g), 3.00 (ddd, J = 13.1, 7.9, 5.1 Hz, 1H, H-g), 2.74 (t, J = 2.6 Hz, 1H, H-m), 2.13 (td, J = 7.0, 2.6 Hz, 2H, H-l), 2.08 – 2.04 (m, 2H, H-i), 1.54 (p, J = 7.4 Hz, 2H, H-j), 1.39 (p, J = 7.1 Hz, 2H, H-k). **^{13}C-NMR:** (101 MHz, DMSO-d$_6$) δ [ppm]: 172.10, 144.83, 144.19, 134.87, 116.88, 115.05, 113.46, 84.43, 71.29, 71.20, 46.96, 34.69, 27.52, 24.42, 17.48. **HRMS:** (ESI) C$_{15}$H$_{20}$NO$_4^+$ [M+H]$^+$ calculated: 278.1387, found: 278.1388.

<u>N-(2-Hydroxy-2-(3-hydroxyphenyl)ethyl)hept-6-ynamide (**PE-P**)</u>

PE-P
C$_{15}$H$_{19}$NO$_3$
261.32
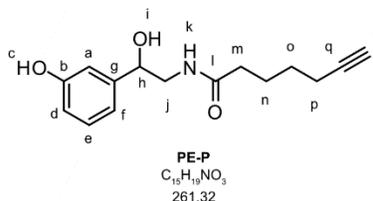
The reaction was performed with DL-norphenylephrine•HCl (180 mg, 1.00 mmol, 1.0 eq.) and the product was obtained as a white powder (169 mg, 0.648 mmol, 65%).

TLC: R_f = 0.63 (MeOH/DCM 8:92) [UV/KMnO$_4$]. **^{1}H-NMR:** (500 MHz, DMSO-d$_6$) δ [ppm]: 9.27 (s, 1H, H-c), 7.83 (t, J = 5.8 Hz, 1H, H-k), 7.09 (t, J = 7.8 Hz, 1H, H-e), 6.74 (s, 1H, H-a), 6.71 (d, J = 7.6 Hz, 1H, H-f), 6.61 (dd, J = 8.0, 2.5 Hz, 1H, H-d), 5.35 (d, J = 4.3 Hz, 1H, H-i), 4.48 (dt, J = 8.5, 4.5 Hz, 1H, H-h), 3.28 – 3.22 (m, 1H, H-j), 3.06 – 3.00 (m, 1H, H-j), 2.75 (t, J = 2.6 Hz, 1H, H-r), 2.13 (td, J = 7.0, 2.6 Hz, 2H, H-p), 2.07 (t, J = 7.4 Hz, 2H, H-m), 1.55 (p, J = 7.4 Hz, 2H, H-n), 1.38 (p, J = 7.1 Hz, 2H, H-o). **^{13}C-NMR:** (101 MHz, DMSO-d$_6$) δ [ppm]: 172.56, 157.62, 145.83, 129.35, 117.08, 114.35, 113.29, 84.86, 71.90, 71.68, 47.31, 35.12, 27.97, 24.85, 17.93. **HRMS:** (ESI) C$_{15}$H$_{12}$NO$_3^+$ [M+H]$^+$ calculated: 262.1438, found: 262.1438.

<u>*N*-(2-Hydroxy-2-(4-hydroxyphenyl)ethyl)hept-6-ynamide (**OA-P**)</u>

OA-P
$C_{15}H_{19}NO_3$
261.32
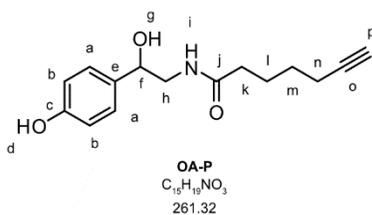
The reaction was performed with DL-octopamine•HCl (241 mg, 1.27 mmol, 1.0 eq.) and the product was obtained as a white powder (189 mg, 0.723 mmol, 54%).

TLC: R_f = 0.47 (MeOH/DCM 9:91) [UV/KMnO$_4$]. **^{1}H-NMR:** (300 MHz, DMSO-d$_6$) *δ* [ppm]: 9.22 (s, 1H, H-d), 7.76 (t, *J* = 5.7 Hz, 1H, H-i), 7.15 – 7.04 (m, 2H, H-a), 6.75 – 6.64 (m, 2H, H-b), 5.22 (d, *J* = 4.2 Hz, 1H, H-g), 4.47 (dt, *J* = 8.6, 4.6 Hz, 1H, H-f), 3.26 – 3.01 (m, 2H, H-h), 2.74 (t, *J* = 2.7 Hz, 1H, H-p), 2.13 (td, *J* = 7.0, 2.7 Hz, 2H, H-n), 2.06 (t, *J* = 7.3 Hz, 2H, H-k), 1.60 – 1.48 (m, 2H, H-l), 1.44 – 1.32 (m, 2H, H-m). **^{13}C-NMR:** (75 MHz, DMSO-d$_6$) *δ* [ppm]: 172.01, 156.34, 134.06, 127.10, 114.68, 84.37, 71.18, 71.14, 46.81, 34.63, 27.48, 24.38, 17.43. **HRMS:** (ESI) $C_{15}H_{18}NO_3^-$ [M-H]$^-$ calculated: 260. 1292; found: 260.1292.

Supplementary figures
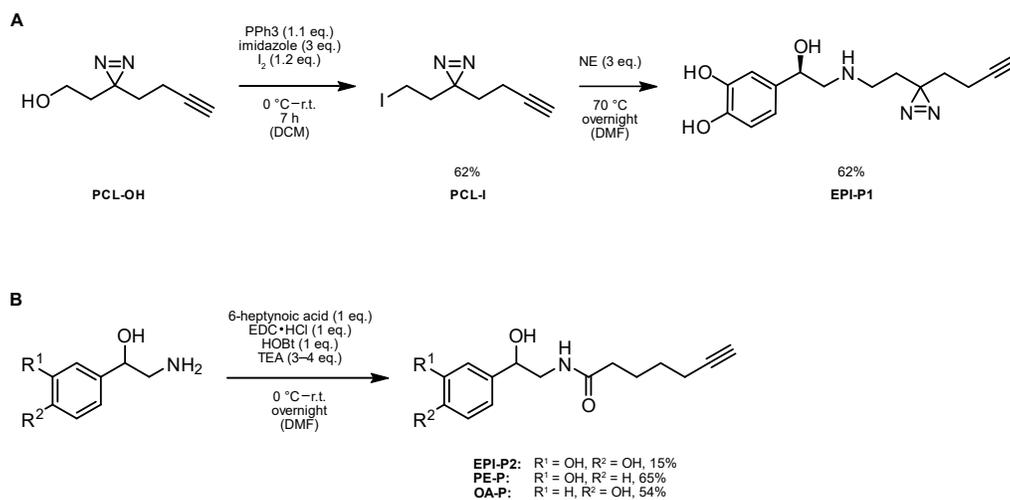
Fig. S1. Synthesis of photoprobes. (*A*) Probe **EPI-P1** was synthesized *via* alkylation of **NE**. (*B*) Probes **EPI-P2**, **PE-P**, and **OA-P** were obtained by acylation of **NE**, **PE**, and **OA**, respectively using amide coupling.

A

relative radial expansion (24 h)

EPI-P1 (μM): 0 6 15 30 60

B

relative radial expansion (24 h)

EPI-P2 (μM): 0 10 25 50 100

C

relative radial expansion (24 h)

PE-P (μM): 0 10 25 50 100

D

relative radial expansion (24 h)

OA-P (μM): 0 10 25 50 100
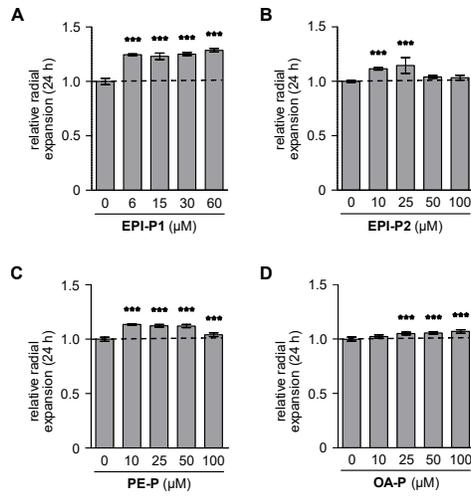
Fig. S2. Soft agar colony expansion assays with (*A*) **EPI-P1** (diameter of control: 5.68 cm), (B) **EPI-P2** (diameter of control: 4.28 cm), (*C*) **PE-P** (diameter of control: 6.15 cm), and (*D*) **OA-P** (diameter of control: 6.15 cm) at different concentrations as indicated. Radial expansions were normalized to an untreated control. Error bars represent the standard deviation from six independent experiments. Significance was determined performing a one-way ANOVA with Tukey´s post hoc test (*** = p < 0.001).
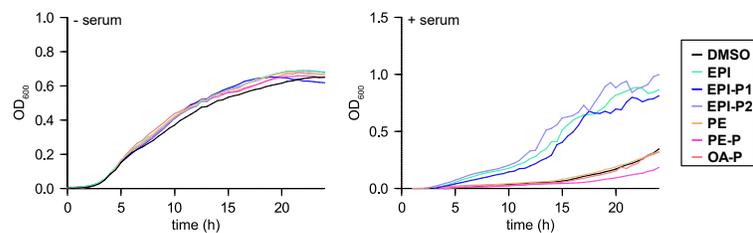
Fig. S3. Bacterial growth in LB35 (left plot) or in LB35 supplemented with 30% (v/v) adult bovine serum to generate iron limitation (right plot) in a 96-well microtiter plate with shaking for 20 s every 5 min at 30 °C. Compounds were added at 50 µM. Data show the mean of triplicates; standard deviations between replicates account for not more than 15% in all growth experiments.
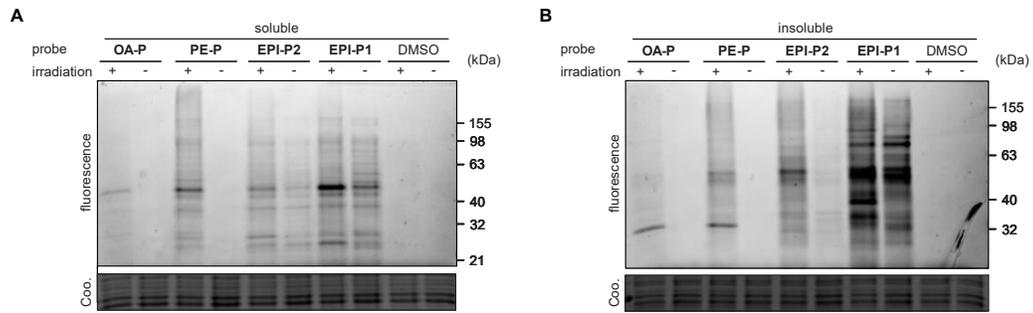
Fig. S4. Comparison of soluble and insoluble fractions from *in situ* photoaffinity labeling. Treatment of live bacteria with the probes (50 µM) or DMSO was followed by UV-irradiation, lysis, and CuAAC to rhodamine azide. Proteins were separated by SDS-PAGE and scanned for fluorescence. Total protein loading (15 µg per lane) was visualized by Coomassie staining (Coo.) (*A*) Soluble (PBS) fraction and (*B*) insoluble fraction.

legend	UniProt	gene	protein name
CheW	**A7MS42**	**VIBHAR_03137**	**CheW-like domain-containing protein**
1	A7MSY4	VIBHAR_00007	OMP_b-brl domain-containing protein
2	A7MT36	VIBHAR_01351	SDH cytochrome b556 subunit
3	A7MUH3	VIBHAR_03044	Protease 4
4	A7MV17	VIBHAR_01530	Uncharacterized protein
5	A7MWN4	VIBHAR_00869	Uncharacterized protein
6	A7MWW5	grpE	Protein GrpE
7	A7MX99	VIBHAR_00062	Peptidyl-prolyl cis-trans isomerase
8	A7MXZ7	frr	Ribosome-recycling factor
9	A7MY24	upp	Uracil phosphoribosyltransferase
10	A7MY64	VIBHAR_01273	OmpA-like domain-containing protein
11	A7MY66	VIBHAR_01269	Porin_4 domain-containing protein
12	A7MYY0	VIBHAR_03442	PNPLA domain-containing protein
13	A7MZS4	VIBHAR_01404	Uncharacterized protein
14	A7N0S3	VIBHAR_02285	Putrescine-binding periplasmic protein
15	A7N146	VIBHAR_03705	Single-stranded DNA-binding protein
16	A7N1M5	VIBHAR_01558	Porin_4 domain-containing protein
17	A7N1N3	VIBHAR_01548	Uncharacterized protein
18	A7N1T8	VIBHAR_03287	Uncharacterized protein
19	A7N283	VIBHAR_06741	Porin_4 domain-containing protein
20	A7N3X4	VIBHAR_05367	Phasin_2 domain-containing protein
21	A7N5U4	VIBHAR_04785	Uncharacterized protein
22	A7N6I6	VIBHAR_06262	OmpA-like domain-containing protein
23	A7N7I3	VIBHAR_05401	FtsX domain-containing protein
24	A7N7I4	VIBHAR_05402	Uncharacterized protein
25	A7N7K9	VIBHAR_07111	Uncharacterized protein
26	A7N8H3	VIBHAR_05966	HTH tetR-type domain-containing protein

Fig. S5. Volcano plot of photolabeling with **EPI-P1**. Bacteria were labeled with 7.5 µM **EPI-P1**, irradiated, and lysed in 1% (v/v) TX100 (without fractionation). The plot shows proteins enriched by **EPI-P1** compared to the solvent-treated control. The experiment was performed in three independent replicates. Proteins enriched above the cut-off (-$\log_{10}$(p-value) > 1.3, $\log_2$(enrichment) > 2) are highlighted in red and listed in the table.
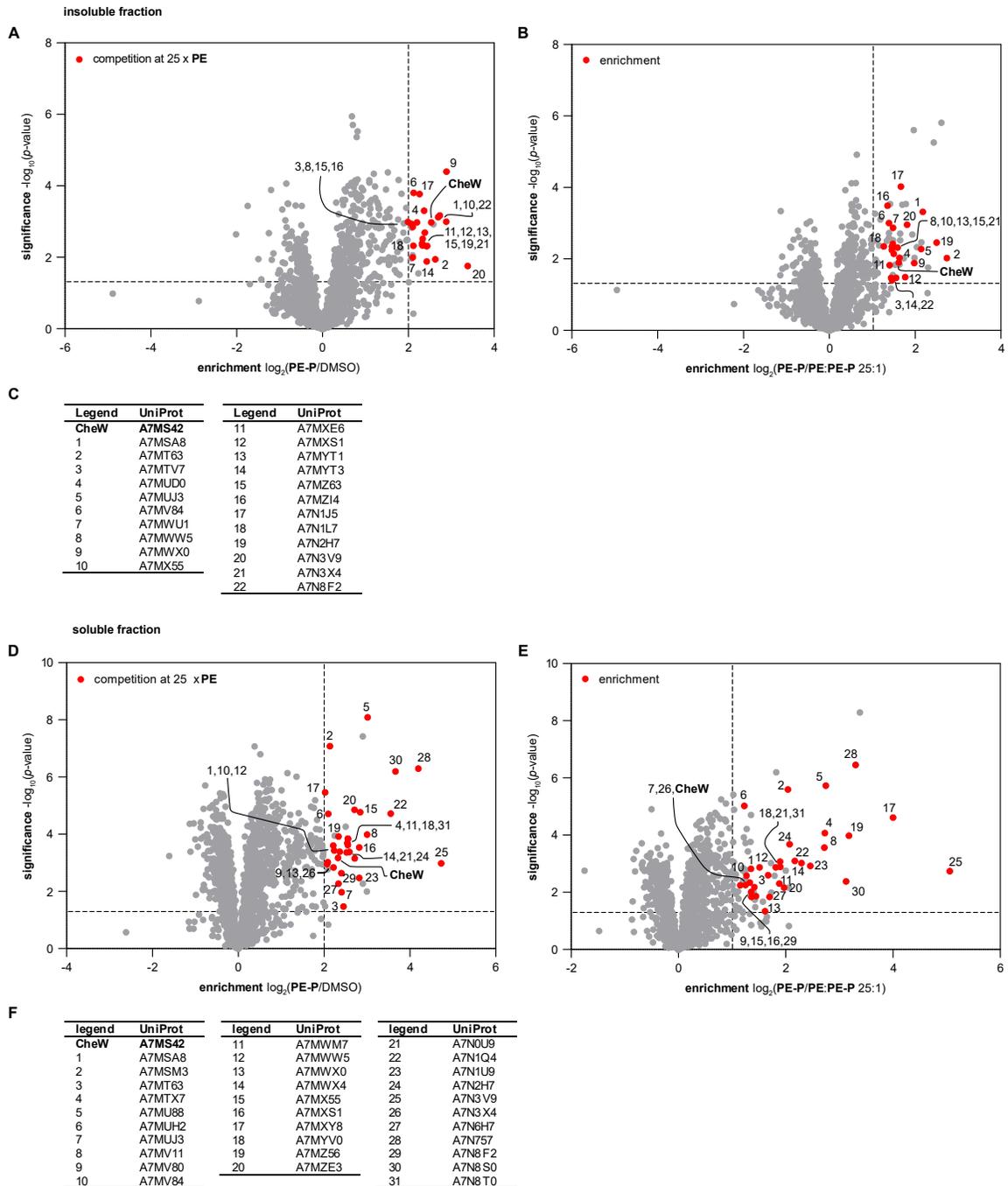
Fig. S6. Volcano plots of competitive labeling experiments with **PE-P** and **PE**. (*A*) Proteins enriched by **PE-P** compared to the DMSO-treated control. Proteins also outcompeted by a 25-fold excess of **PE** are highlighted in red. Insoluble fraction. (*B*) Proteins enriched by **PE-P** compared to samples treated with **PE-P** plus a 25-fold excess of **PE**. Proteins also found in the enrichment over DMSO are highlighted in red. Insoluble fraction. (*C*) UniProt identifiers of proteins enriched by **PE-P** over DMSO and outcompeted by a 25-fold excess of **PE**. Insoluble fraction. (*D*) Proteins enriched by **PE-P**, soluble fraction (*E*) Proteins enriched by **PE-P** and outcompeted by **PE**, soluble fraction. (*F*) UniProt identifiers of proteins enriched by **PE-P** and outcompeted by **PE**, soluble fraction. All experiments were performed with 10 µM **PE-P** in five independent replicates.

C

Legend	UniProt	Legend	UniProt
CheW	**A7MS42**	11	A7MXE6
1	A7MSA8	12	A7MXS1
2	A7MT63	13	A7MYT1
3	A7MTV7	14	A7MYT3
4	A7MUD0	15	A7MZ63
5	A7MUJ3	16	A7MZI4
6	A7MV84	17	A7N1J5
7	A7MWU1	18	A7N1L7
8	A7MWW5	19	A7N2H7
9	A7MWX0	20	A7N3V9
10	A7MX55	21	A7N3X4
		22	A7N8F2

F

legend	UniProt	legend	UniProt	legend	UniProt
CheW	**A7MS42**	11	A7MWM7	21	A7N0U9
1	A7MSA8	12	A7MWW5	22	A7N1Q4
2	A7MSM3	13	A7MWX0	23	A7N1U9
3	A7MT63	14	A7MWX4	24	A7N2H7
4	A7MTX7	15	A7MX55	25	A7N3V9
5	A7MU88	16	A7MXS1	26	A7N3X4
6	A7MUH2	17	A7MXY8	27	A7N6H7
7	A7MUJ3	18	A7MYV0	28	A7N757
8	A7MV11	19	A7MZ56	29	A7N8F2
9	A7MV80	20	A7MZE3	30	A7N8S0
10	A7MV84			31	A7N8T0

Fig. S7. Volcano plots of competitive labeling experiments with **PE-P** and **EPI**. (*A*) Proteins enriched by **PE-P** compared to the DMSO-treated control. Proteins also outcompeted by a 10-fold excess of **EPI** are highlighted in red. Insoluble fraction. (*B*) Proteins enriched by **PE-P** compared to samples treated with **PE-P** plus a 10-fold excess of **EPI**. Proteins also found in the enrichment over DMSO are highlighted in red. Insoluble fraction. (*C*) UniProt identifiers of proteins enriched by **PE-P** over DMSO and outcompeted by **EPI**. Insoluble fraction. (*D*) Proteins enriched by **PE-P**, soluble fraction (*E*) UniProt identifiers of proteins enriched by **PE-P** and outcompeted by **EPI**, soluble fraction. All experiments were performed with 10 µM **PE-P** in four independent replicates.

C

| Legend	UniProt ID	Legend	UniProt ID
CheW	A7MS42	17	A7N0E8
1	A7MRS9	18	A7N0U9
2	A7MSM3	19	A7N1L7
3	A7MSV2	20	A7N1N3
4	A7MTV7	21	A7N1Q4
5	A7MU88	22	A7N1T8
6	A7MUD0	23	A7N4I9
7	A7MWY7	24	A7N4M9
8	A7MX37	25	A7N503
9	A7MX55	26	A7N5C3
10	A7MXS1	27	A7N5L8
11	A7MXY8	28	A7N5R2
12	A7MYT1	29	A7N6B5
13	A7MZ56	30	A7N757
14	A7MZ63	31	A7N8F2
15	A7MZR7	32	A7N8H3
16	A7N0D8		

E

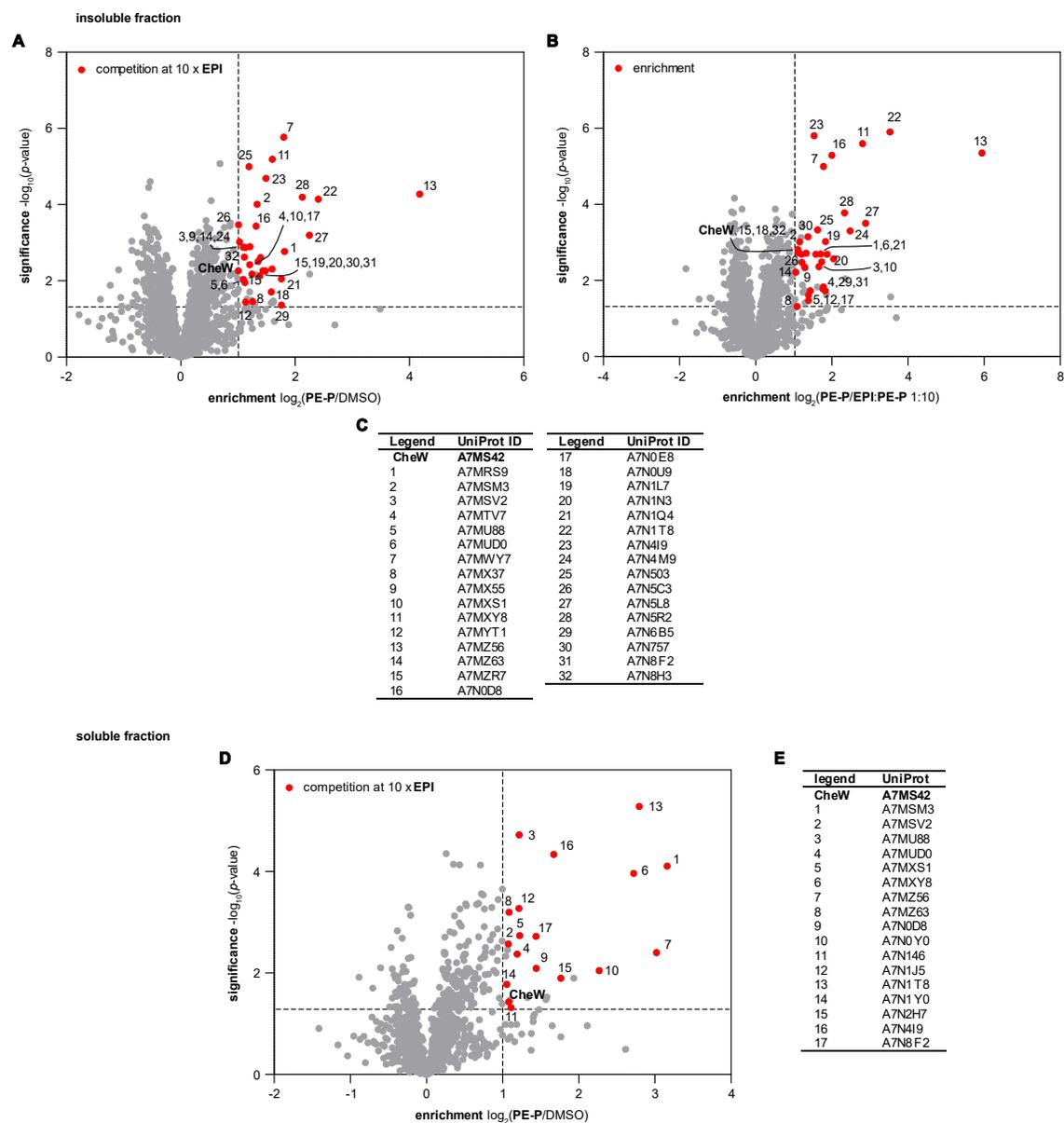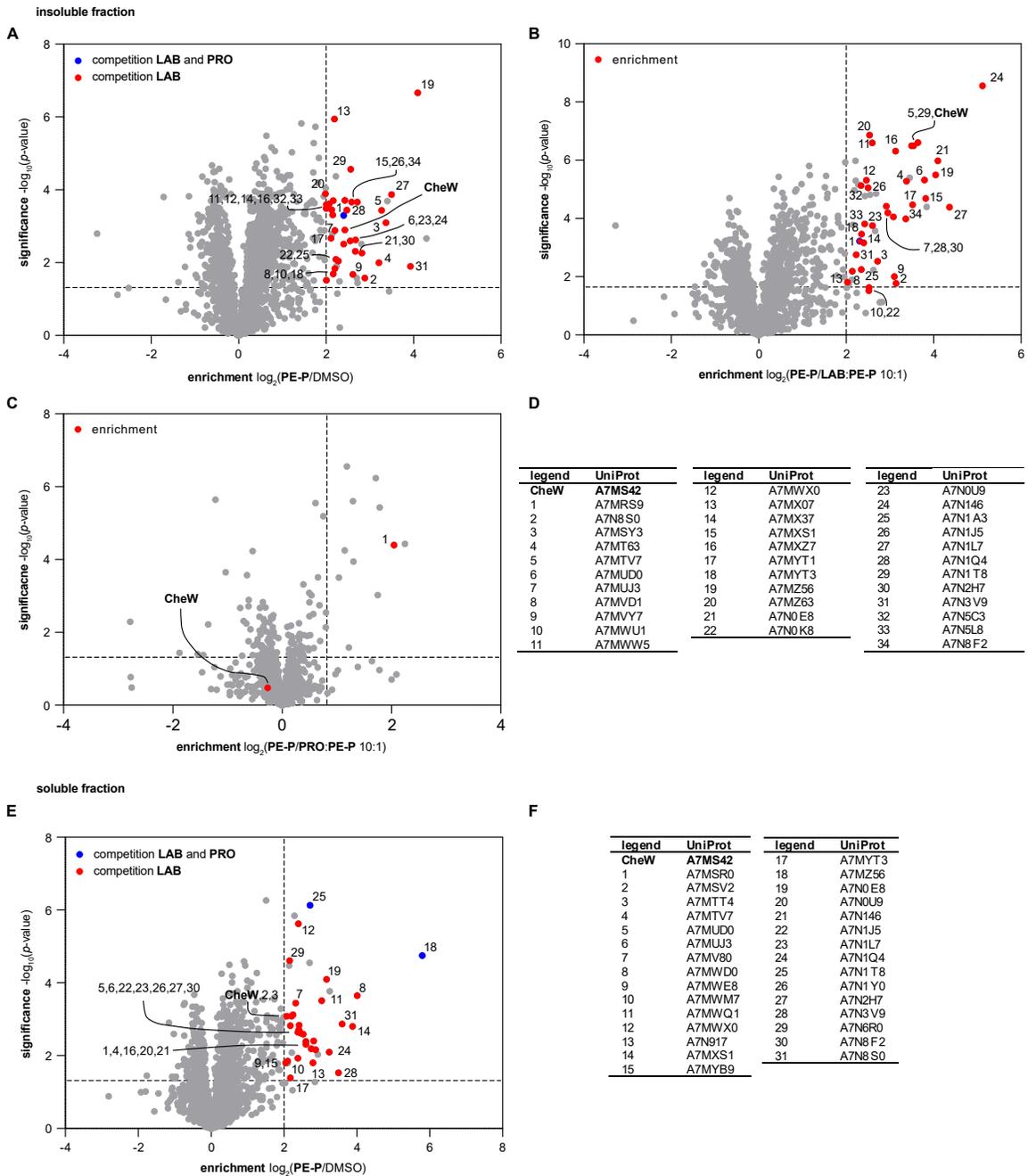| legend	UniProt
CheW	A7MS42
1	A7MSM3
2	A7MSV2
3	A7MU88
4	A7MUD0
5	A7MXS1
6	A7MXY8
7	A7MZ56
8	A7MZ63
9	A7N0D8
10	A7N0Y0
11	A7N146
12	A7N1J5
13	A7N1T8
14	A7N1Y0
15	A7N2H7
16	A7N4I9
17	A7N8F2

Fig. S8. Volcano plots of competitive labeling experiments with **PE-P** and adrenergic antagonists. (*A*) Proteins enriched by **PE-P** compared to the DMSO-treated control. Proteins also outcompeted by a 10-fold excess of **LAB** are highlighted in red, proteins outcompeted by both 10-fold **LAB** and **PRO** are highlighted in blue. Insoluble fraction. (*B*) Proteins enriched by **PE-P** compared to samples treated with **PE-P** plus a 10-fold excess of **LAB**. Proteins also found in the enrichment over DMSO are highlighted in red. Insoluble fraction. (*C*) Proteins enriched by **PE-P** compared to samples treated with **PE-P** plus a 10-fold excess of **PRO**. Proteins also found in the enrichment over DMSO and CheW are highlighted in red. Insoluble fraction. (*D*) UniProt identifiers of proteins outcompeted by **LAB** or **PRO**. Insoluble fraction. (*E*) Proteins enriched by **PE-P** compared to the DMSO-treated control. Proteins also outcompeted by a 10-fold excess of **LAB** are highlighted in red, proteins outcompeted by both 10-fold **LAB** and **PRO** are highlighted in blue. Soluble fraction. (*F*) UniProt

Panel D

legend	UniProt	legend	UniProt	legend	UniProt
CheW	A7MS42	12	A7MWX0	23	A7N0U9
1	A7MRS9	13	A7MX07	24	A7N146
2	A7N8S0	14	A7MX37	25	A7N1A3
3	A7MSY3	15	A7MXS1	26	A7N1J5
4	A7MT63	16	A7MXZ7	27	A7N1L7
5	A7MTV7	17	A7MYT1	28	A7N1Q4
6	A7MUD0	18	A7MYT3	29	A7N1T8
7	A7MUJ3	19	A7MZ56	30	A7N2H7
8	A7MVD1	20	A7MZ63	31	A7N3V9
9	A7MVY7	21	A7N0E8	32	A7N5C3
10	A7MWU1	22	A7N0K8	33	A7N5L8
11	A7MWW5			34	A7N8F2

Panel F

legend	UniProt	legend	UniProt
CheW	A7MS42	17	A7MYT3
1	A7MSR0	18	A7MZ56
2	A7MSV2	19	A7N0E8
3	A7MTT4	20	A7N0U9
4	A7MTV7	21	A7N146
5	A7MUD0	22	A7N1J5
6	A7MUJ3	23	A7N1L7
7	A7MV80	24	A7N1Q4
8	A7MWD0	25	A7N1T8
9	A7MWE8	26	A7N1Y0
10	A7MWM7	27	A7N2H7
11	A7MWQ1	28	A7N3V9
12	A7MWX0	29	A7N6R0
13	A7N917	30	A7N8F2
14	A7MXS1	31	A7N8S0
15	A7MYB9		

identifiers of proteins outcompeted by **LAB** or **PRO**. Soluble fraction. All experiments were performed with 10 µM **PE-P** in four independent replicates.
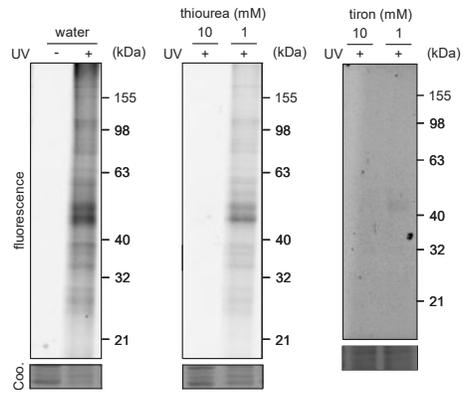
Fig. S9. Photoaffinity labeling in *V. campbellii* lysate with 50 µM **PE-P** in the presence of radical scavengers. (*A*) Labeling in the presence of different concentrations of thiourea. (*B*) Labeling in the presence of tiron at different concentrations. In all experiments, labeled proteins were clicked to rhodamine azide, separated by SDS-PAGE, and visualized for fluorescence. Total protein loading (15 µg protein per lane) was visualized by Coomassie staining (Coo.).

A

replicate 1

ID
78.8

78.8
+3.8 ppm

78.9
+0.6 ppm

replicate 2

ID
78.9

78.9
+1.5 ppm

78.9
+2.9 ppm

Y$_{44}$TEIAPVPGAPDYVLGIINLR

— 1006.5524+++ (L)
— 1004.5500+++ (H)

B

replicate 1

ID
85.1

85.0
+0.6 ppm

85.0
+3.4 ppm

replicate 2

85.1
+0.7 ppm

85.1
+4.7 ppm

QVIGILVDSVAEVVY$_{112}$LR

— 873.8328+++ (L)
— 871.8303+++ (H)
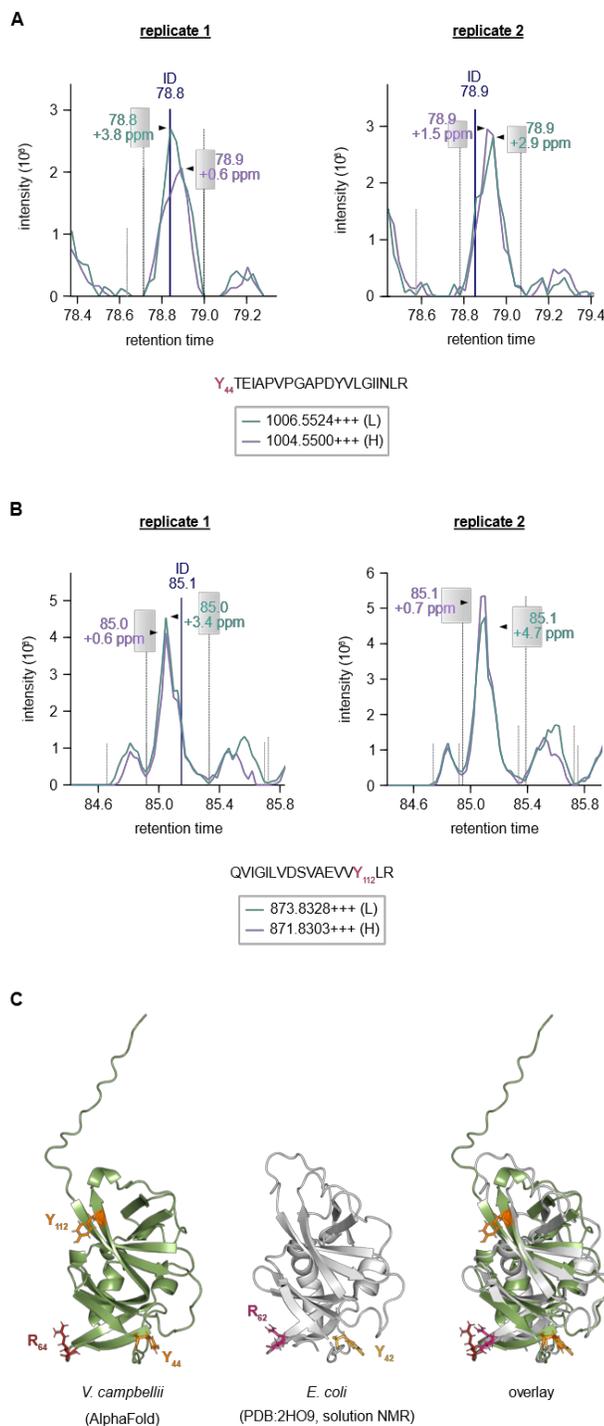
C

V. campbellii
(AlphaFold)

E. coli
(PDB:2HO9, solution NMR)

overlay

Fig. S10 Binding sites of **PE-P** in CheW. (*A*) Extracted MS1 ion chromatograms of **PE-P**-labeled peptides. The heavy- and light-labeled peptide containing the Y$_{44}$ binding site are shown in purple and in cyan, respectively; charge state = 3. (*B*) Chromatograms of the heavy- (purple) and light-labeled (cyan) peptide containing the Y$_{112}$ binding site; charge state = 3. Both replicates are shown. (*C*) Structure prediction of CheW from *V. campbellii* (green) generated with AlphaFold (14) and visualized with PyMOL (15). Binding sites Y$_{44}$ and Y$_{112}$ are highlighted in orange, the conserved arginine R$_{64}$ is shown in red. The structure of CheW from *E. coli* (gray) was retrieved from PDB (PDB:2HO9, solution NMR) and the alignment was performed in PyMOL; RMSD =

2.217 (1448 to 1448 atoms of 1771 total). In the *E. coli* structure, Y_{42} (orange) and R_{62} (red) are conserved.

A

B

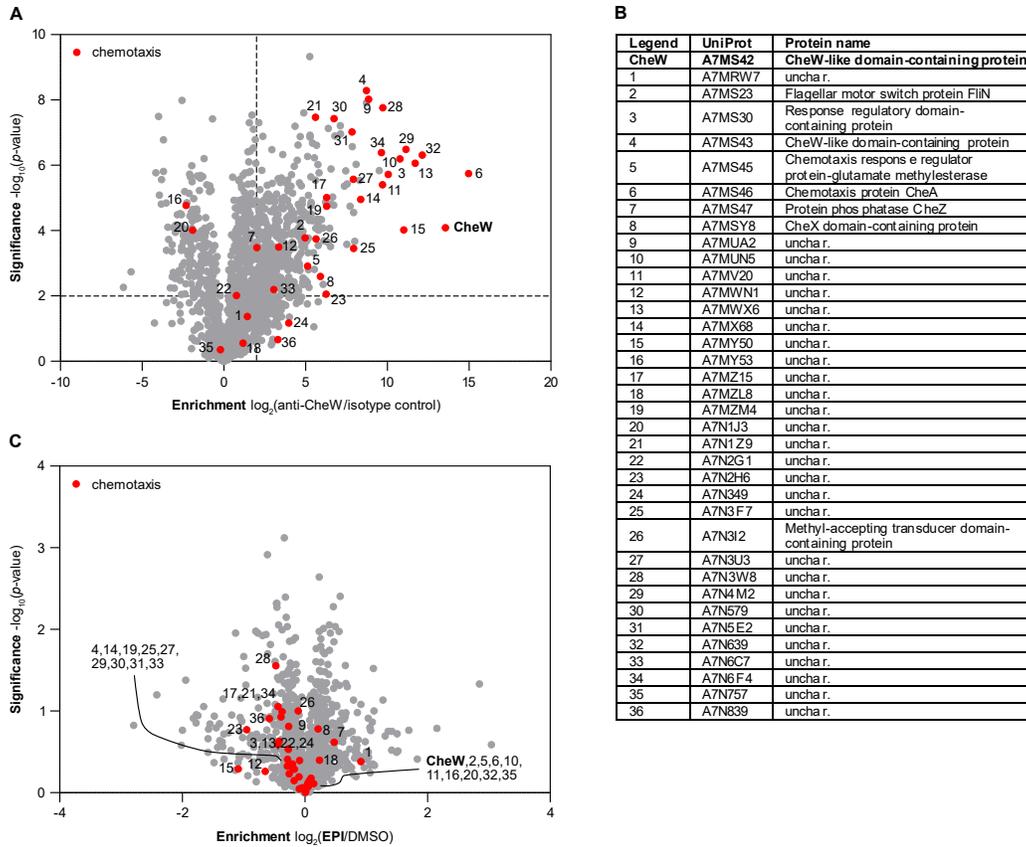| Legend	UniProt	Protein name
CheW	A7MS42	CheW-like domain-containing protein
1	A7MRW7	unchar.
2	A7MS23	Flagellar motor switch protein FliN
3	A7MS30	Response regulatory domain-containing protein
4	A7MS43	CheW-like domain-containing protein
5	A7MS45	Chemotaxis response regulator protein-glutamate methylesterase
6	A7MS46	Chemotaxis protein CheA
7	A7MS47	Protein phosphatase CheZ
8	A7MSY8	CheX domain-containing protein
9	A7MUA2	unchar.
10	A7MUN5	unchar.
11	A7MV20	unchar.
12	A7MWN1	unchar.
13	A7MWX6	unchar.
14	A7MX68	unchar.
15	A7MY50	unchar.
16	A7MY53	unchar.
17	A7MZ15	unchar.
18	A7MZL8	unchar.
19	A7MZM4	unchar.
20	A7N1J3	unchar.
21	A7N1Z9	unchar.
22	A7N2G1	unchar.
23	A7N2H6	unchar.
24	A7N349	unchar.
25	A7N3F7	unchar.
26	A7N3I2	Methyl-accepting transducer domain-containing protein
27	A7N3U3	unchar.
28	A7N3W8	unchar.
29	A7N4M2	unchar.
30	A7N579	unchar.
31	A7N5E2	unchar.
32	A7N639	unchar.
33	A7N6C7	unchar.
34	A7N6F4	unchar.
35	A7N757	unchar.
36	A7N839	unchar.

C

Fig. S11. Volcano plots of Co-IP of CheW binding partners in live *V. campbellii*. Bacteria were treated with 100 µM **EPI** or DMSO for 30 min and proteins were chemically crosslinked with 2 mM DSSO. Bacteria were lysed and proteins were pulled down using an anti-CheW antibody or an isotype control. (*A*) Proteins enriched by the anti-CheW antibody compared to the isotype control in the DMSO-treated samples. (*B*) UniProt identifiers of proteins involved in chemotaxis. Listed are proteins with the annotation "chemotaxis" in the GOBP or KEGG database. (*C*) Proteins enriched by the anti-CheW antibody in samples treated with 100 µM **EPI** compared to DMSO-treated controls. The abundance of chemotaxis proteins did not change significantly (cut-off criteria: $0.5 \le$ $\log_2$ enrichment ≥ 2; $-\log_{10}(p\text{-value}) \ge 1.3$). Volcano plot represents t-test results of four independent replicates. Chemotaxis proteins are highlighted in red.
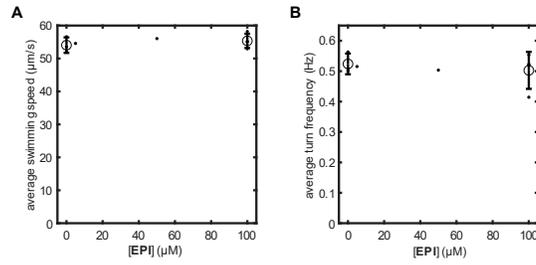
Fig. S12. Motility of *V. campbellii* after 30 min in the absence and presence of **EPI** assessed by 3D tracking. *(A)* Average motile swimming speed as a function of **EPI** concentration. Motile bacteria were defined as those with an average swimming speed of at least 20 µm/s. *(B)* Average turning frequency as a function of **EPI** concentration. Technical replicates are shown as points. Open circles with error bars denote average and standard deviation across technical replicates, when at least three replicates are available. The analysis was performed for motile bacteria with a minimum trajectory duration of 1s.
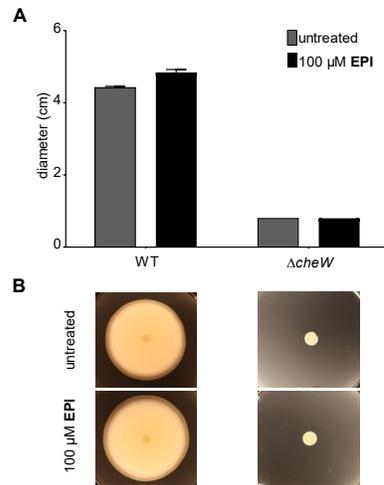
Fig. S13. Soft agar colony expansion assay with the *V. campbellii* Δ*cheW* mutant. (*A*) Plot shows means of expansion of the wild-type (WT) and the Δ*cheW* mutant on soft agar in the absence and presence of 100 µM **EPI** after 24 h. Error bars represent the standard deviation (wild-type n = 7 and mutant n = 6 independent replicates, *** = p < 0.001). (*B*) Appearance of *V. campbellii* WT and Δ*cheW* mutant on soft agar plates with and without **EPI** after 24 h incubation.
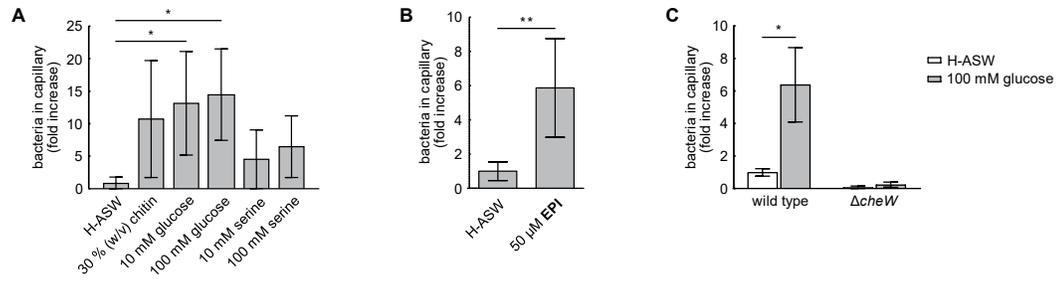
Fig. S14. Capillary assay to determine chemotactic movement of *V. campbellii*. (*A*) Relative increase of bacterial cell numbers in the capillary containing chitin, glucose, or serine, after 60 min. (*B*) Relative increase of bacterial cell numbers in the capillary containing EPI after 60 min. Values were normalized to their respective control (HEPES-buffered artificial sea water, H-ASW). (*C*) Relative increase of *V. campbellii* wild type and Δ*cheW* cell numbers in the capillaries filled with H-ASW or 100 mM glucose. Error bars indicate standard deviations; n = 4 replicates. Statistical significance was determined using an unpaired two-tailed *t*-test (* = $p < 0.05$, ** = $p < 0.01$).
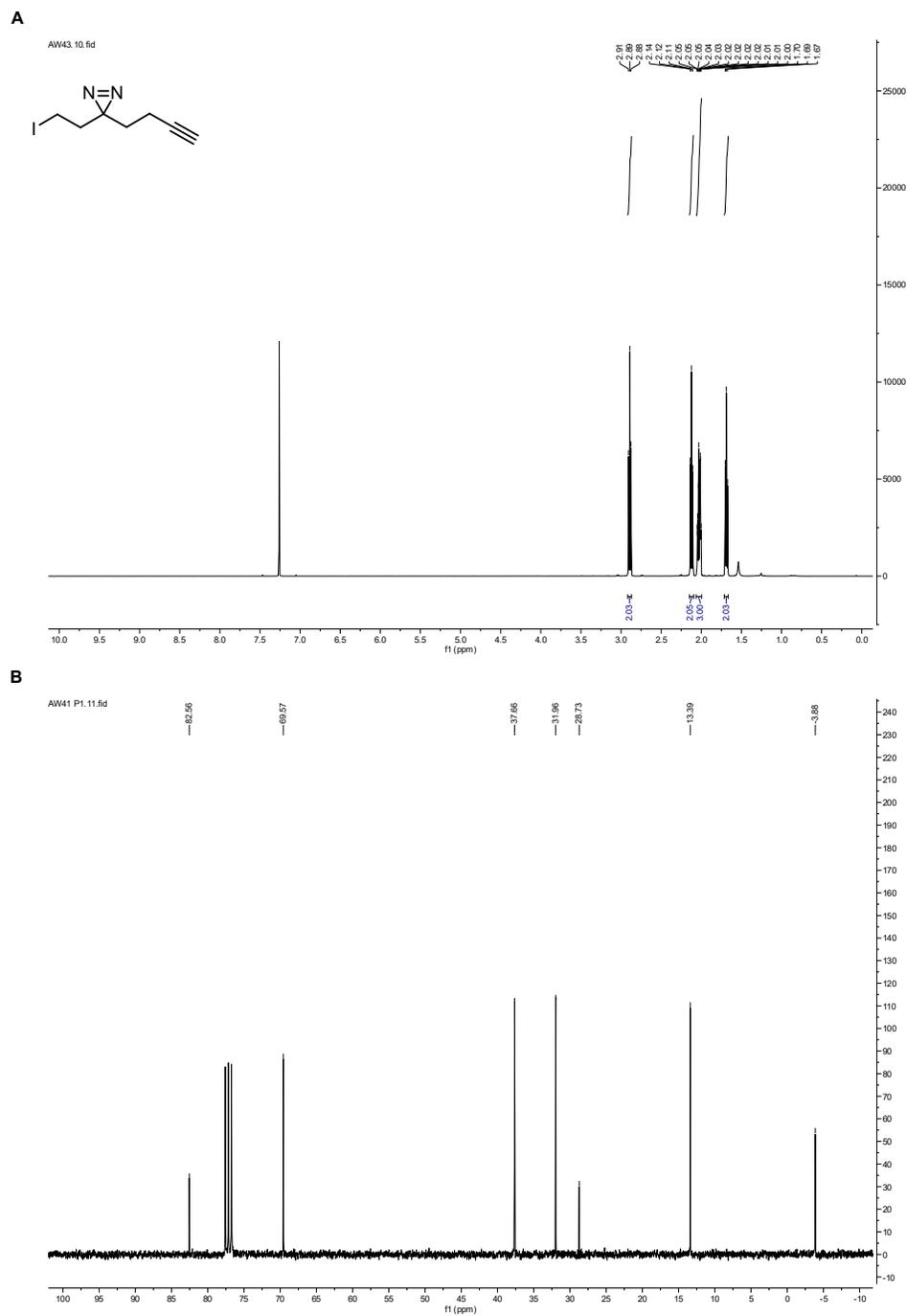
A

B

Fig. S15. NMR spectra of 3-(but-3-yn-1-yl)-3-(2-iodoethyl)-3*H*-diazirine (**PCL-I**) in CDCl₃. (*A*) ¹H-NMR (500 MHz). (*B*) ¹³C-NMR (75 MHz).
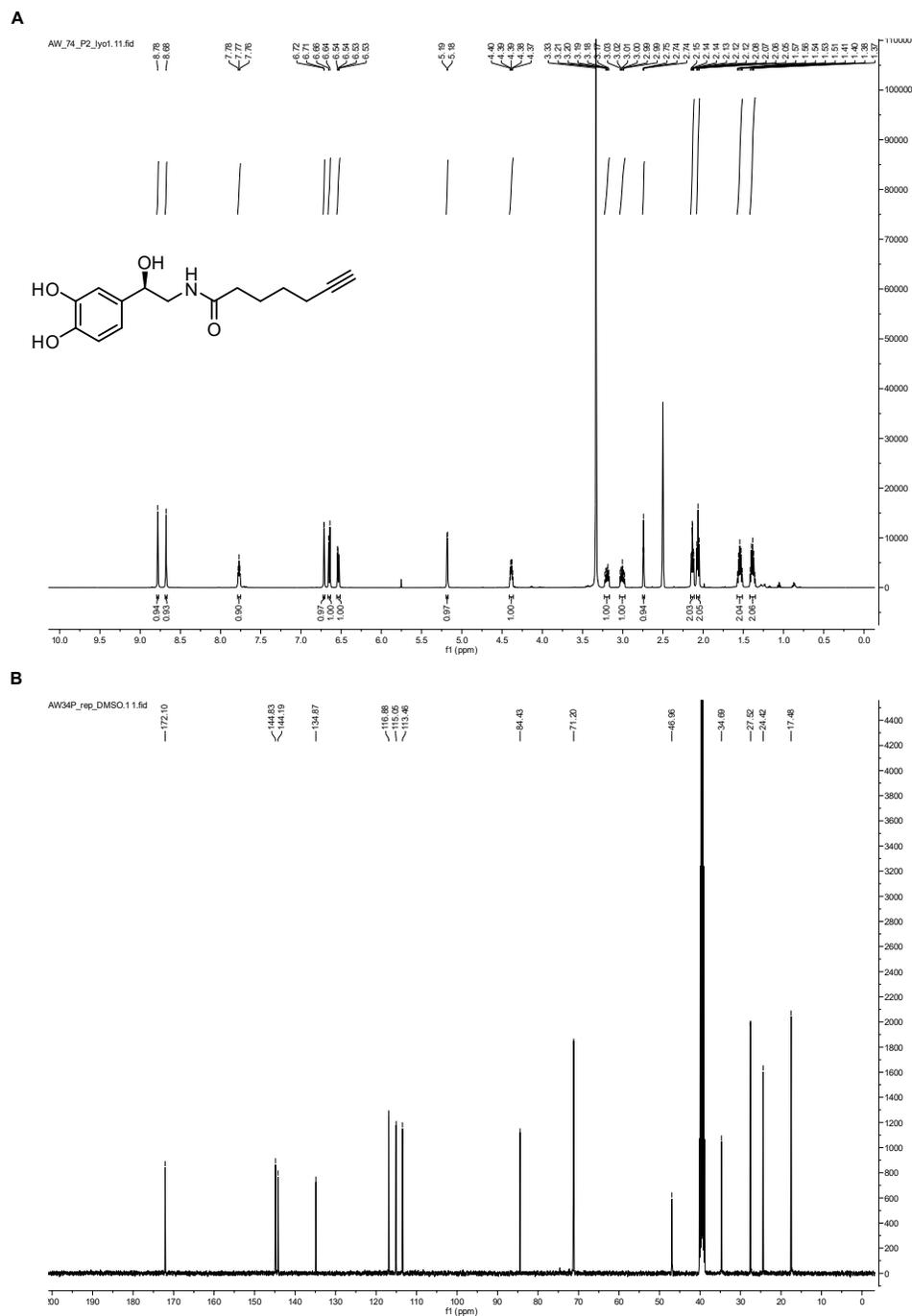
A

B

Fig. S16. NMR spectra of (*R*)-4-(2-((2-(3-(but-3-yn-1-yl)-3*H*-diazirin-3-yl)ethyl)amino)-1-hydroxyethyl)-benzene-1,2-diol (**EPI-P1**) as a 1:3.5 mixture with acetic acid. (*A*) ¹H-NMR (500 MHz, DCl:D₂O 38:962). (*B*) ¹³C-NMR (101 MHz, AcOD/D₂O 1:1).

Fig. S17. NMR spectra of (*R*)-*N*-(2-(3,4-Dihydroxyphenyl)-2-hydroxyethyl)hept-6-ynamide (**EPI-P2**) in DMSO-d$_6$. (*A*) ^{1}H-NMR (500 MHz). (*B*) ^{13}C-NMR (101 MHz).
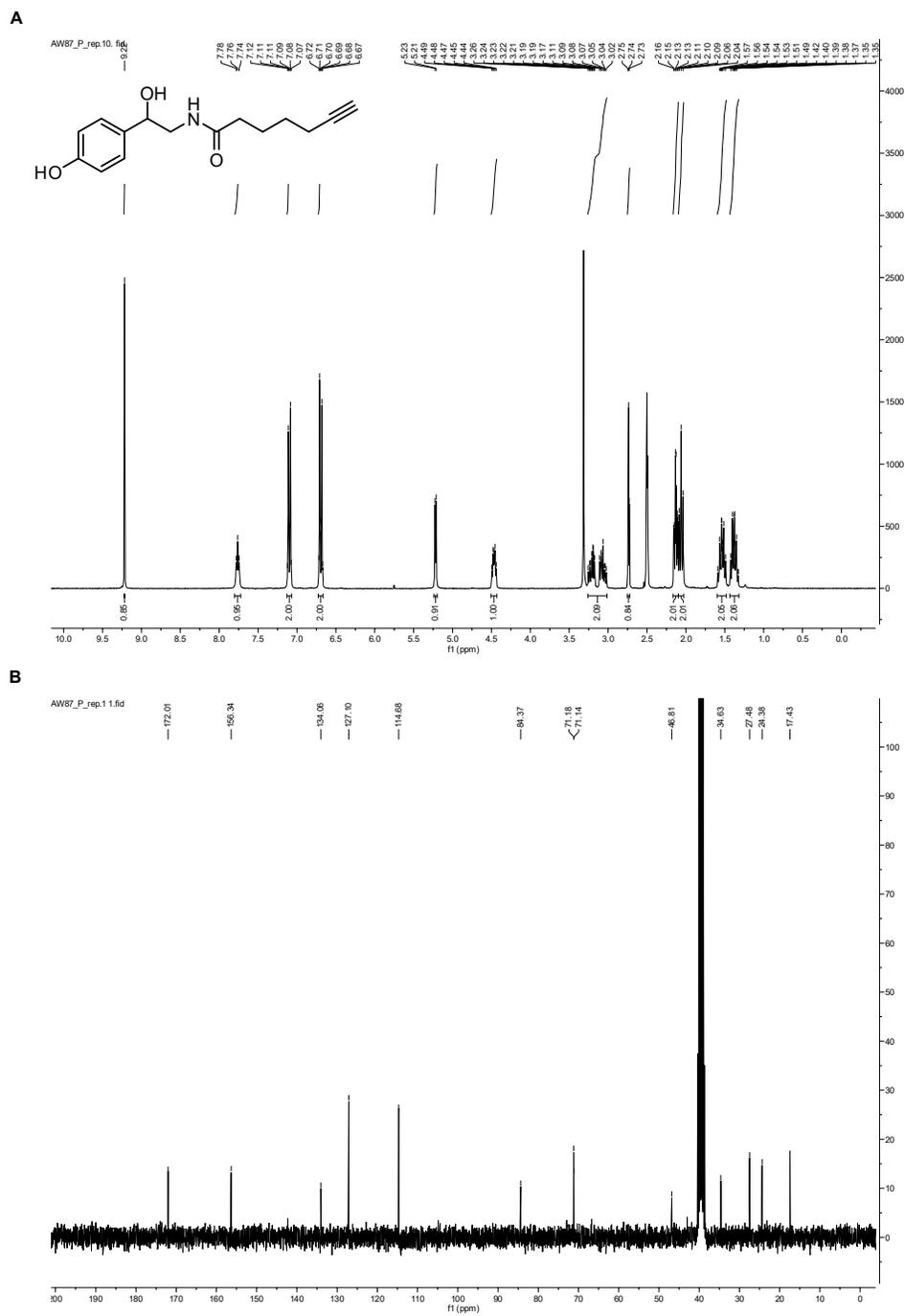
A

B

Fig. S18. NMR spectra of *N*-(2-hydroxy-2-(4-hydroxyphenyl)ethyl)hept-6-ynamide (**OA-P**) in DMSO-d$_6$. (*A*) ^{1}H-NMR (300 MHz). (*B*) ^{13}C-NMR (75 MHz).
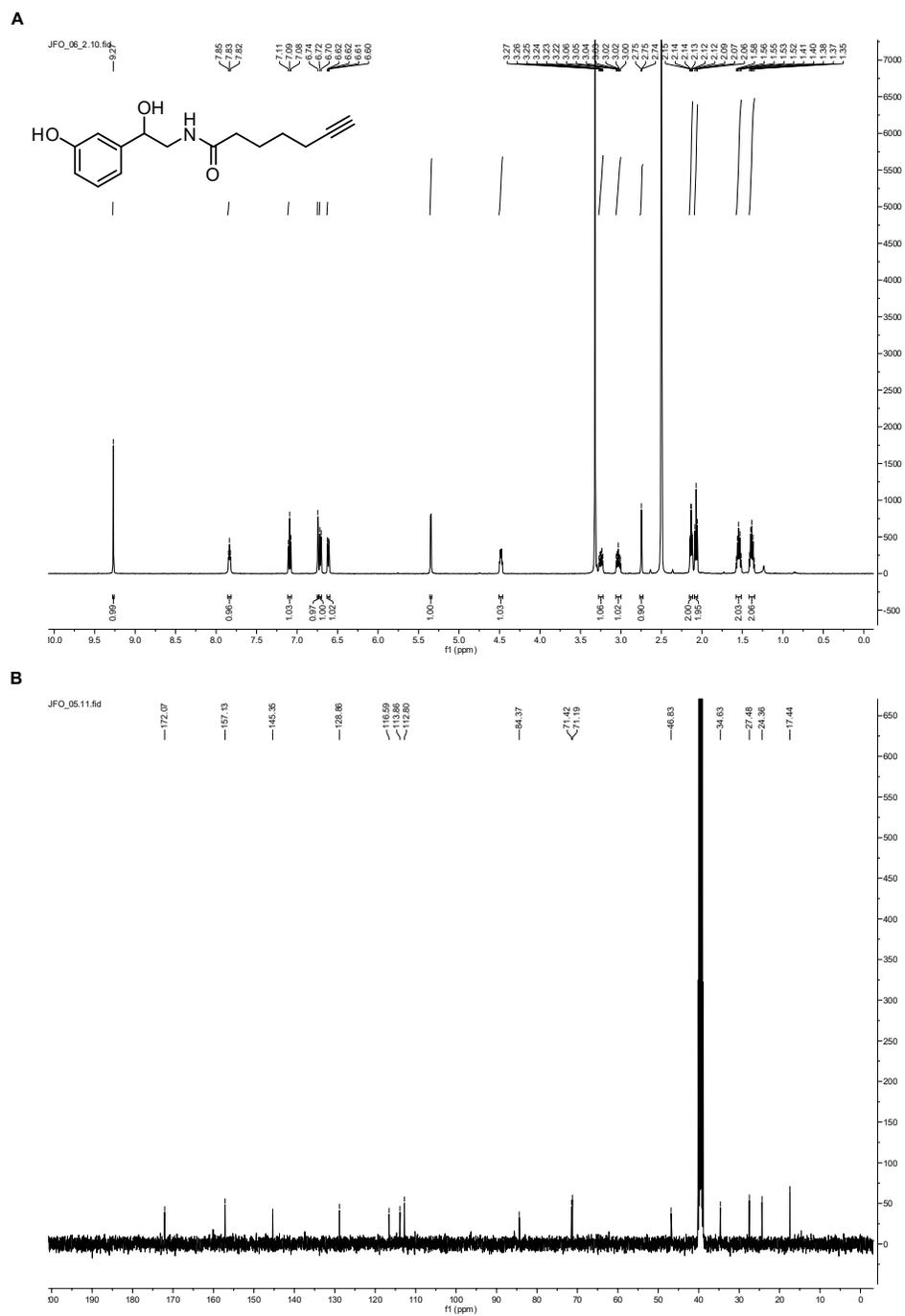
A

B

Fig. S19. NMR spectra of *N*-(2-hydroxy-2-(3-hydroxyphenyl)ethyl)hept-6-ynamide (**PE-P**) in DMSO-d$_6$. (*A*) ^{1}H-NMR (500 MHz). (*B*) ^{13}C-NMR (101 MHz).

Supplementary tables

Table S1: Doubling times (t_d) of *V. campbellii* cultured in KE minimal medium under iron-limited and non-limited conditions in the presence of the indicated compounds (50 µM).

compound	t_d (min)	
	iron limitation (+ human apo-transferrin)	**no iron limitation**
EPI	150	95
EPI-P1	102	100
EPI-P2	184	98
PE	No growth	93
PE-P	No growth	97
OA-P	No growth	96
DMSO	No growth	93

Table S2: Proteins at which **PE-P** was outcompeted by **EPI**, **PE**, and **LAB** but not by **PRO**.

UniProt ID	gene name	protein name
soluble fraction		
A7MXS1	VIBHAR_00356	Uncharacterized protein
A7N2H7	VIBHAR_05151	Uncharacterized protein
A7N8F2	VIBHAR_06507	Uncharacterized protein
A7MS42	VIBHAR_03137	CheW-like domain-containing protein
insoluble fraction		
A7MYT1	VIBHAR_01892	Uncharacterized protein
A7MXS1	VIBHAR_00356	Uncharacterized protein
A7MTV7	yihI	Der GTPase-activating protein YihI
A7MZ63	rpsG	30S ribosomal protein S7
A7N8F2	VIBHAR_06507	Uncharacterized protein
A7MUD0	VIBHAR_02658	Uncharacterized protein
A7N1L7	infA	Translation initiation factor IF-1
A7MS42	VIBHAR_03137	CheW-like domain-containing protein

Table S3. Compounds used in this study.

abbreviation	substance	supplier
EPI	DL-Epinephrine hydrochloride	*TCI Chemicals*
NE	L-Norepinephrine	*Alfa Aesar*
PE	(*R*)-(-)-Phenylephrine hydrochloride	*Sigma*
LAB	Labetalol hydrochloride	*Sigma*
PRO	DL-Propranolol hydrochloride	*Sigma*

Table S4. Bacterial culture conditions.

strain	medium	temperature
Vibrio campbellii (all strains)	LB35, MB, KE	30 °C
Escherichia coli (all strains)	LB	37 °C

Table S5. Media used in this study.

medium	ingredients
KE	100 mM K_2HPO_4/KH_2PO_4, 0.058% (w/v) trisodium citrate dihydrate, 0.106% (w/v) $(NH_4)_2SO_4$, 400 µM $MgSO_4$, 0.2% (w/v) glucose, 2% (w/v) NaCl, pH 7.6
LB	1% (w/v) peptone, 0.5% (w/v) NaCl, 0.5% (w/v) yeast extract, pH 7.5
LB35	LB with 3.5% (w/v) NaCl
MB	Difco Marine Broth 2216
TMN	50 mM Tris-HCl, 300 mM NaCl, 5 mM $MgCl_2$, 5 mM glucose, pH 7.5

Table S6. Strains and plasmids used in this study.

strain or plasmid	relevant genotype or description	reference
	E. coli	
DH5__αλpir	*endA1 hsdR17 glnV44 (= supE44) thi-1 recA1 gyrA96 relA1* φ80'*lac*Δ*(lacZ)*M15 Δ*(lacZYA-argF)*U169 *zdg*-232::Tn10 *uidA::pir+*	(17)
WM3064	*thrB*1004 *pro thi rpsL hsdS lacZ*ΔM15 RP4-1360 Δ(*araBAD*)567 Δ*dapA*1341::[*erm pir*]	W. Metcalf, Univ. of Illinois, Urbana
BL21(DE3)	F⁻ *ompT gal dcm lon hsdSB*(rB⁻ mB⁻) λ(DE3)	(18)
	V. campbellii	
V. campbellii ATCC BAA-1116	wild type	(19)
V. campbellii Δ*cheW*	clean deletion of *cheW*	This work
	plasmids	
pET28a	Vector for expression of N-terminally 6xHis-tagged proteins with a thrombin site, Km^R	*Novagen*
pET28a-*cheW*	*cheW* cloned in the BamHI and XhoI sites of pET28a*,* Km^R	This work
pNTPS138-R6KT	*mobRP4*⁺ *ori*-R6K *sacB*; suicide plasmid for in-frame deletions, Km^R	(20)
pNPTS138-R6KT-*cheW*	pNPTS-138-R6KT-derived suicide plasmid for clean deletion of *cheW* in *V. campbellii,* Km^R	This work

Table S7. Oligonucleotides used in this study.

name	sequence (restriction enzyme cutting site in blue)	description
fwd_BamHI_up_cheW	TAGCCGGATCCTTGAACAGCACACTGAGACAGC	Generation of the **pNPTS138-R6KT** *cheW* plasmid for clean deletion of *cheW* in *V. campbellii*
rev_EcoRI_down_cheW	GCGTAGAATTCGCGAGAGGAATATGTCGGGTC	
rev_start cheW _up	TGAGCCATAAAAGCTTGAGACATAGTTAATCCTCGTTAATG	
fwd_start cheW _down	AAGCTTTTATGGCTCACCTGTAATTGGCTGATGAATCATGG	
fwd_BamHI_cheW	TAGCCGGATCCATGTCTCAAGCTTTTGAAG	Generation of the overexpression plasmid **pET28a-*cheW*** coding for N-terminal His$_6$-tagged *cheW*
rev_cheW_Stop_XhoI	TAGCCCTCGAGTTACAGGTGAGCCATCTCATC	

Legends for supplementary datasets (separate files)

Dataset S1. Proteomics data of **EPI-P1** photolabeling containing detailed protein annotation, MS data, statistics, and LFQ intensities.

Dataset S2. Proteomics data of **PE-P** photolabeling in competition with **PE** containing detailed protein annotation, MS data, statistics, and LFQ intensities

Dataset S3. Proteomics data of **PE-P** photolabeling in competition with **EPI** containing detailed protein annotation, MS data, statistics, and LFQ intensities

Dataset S4. Proteomics data of **PE-P** photolabeling in competition with **LAB** or **PRO** containing detailed protein annotation, MS data, statistics, and LFQ intensities

Dataset S5. Proteomics data of isoDTB labeling experiment containing amino acid modification, selectivity, and quantification results.

Dataset S6. Proteomics data of Co-IP containing detailed protein annotation, MS data, statistics, and LFQ intensities

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Supplemental information – Chapter 2.4

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Supplementary information

Division of labor and collective functionality in *Escherichia coli* under acid stress

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Supplementary Table 1: Quantification of fluorescent microscopic images of different *E. coli* strains during acidic conditions. Quantified noise and mean of relative fluorescence intensity (RF) were calculated for 1,000 cells per condition and time point. Cells were cultivated as described in Fig. 2a. Single-cell fluorescence intensity was acquired by microscopy and the use of the MicrobeJ plugin of the ImageJ software. Relative fluorescence intensity (RF) is the first value. Noise, standard deviation/mean of log-transformed values in the second value. Cells cultivated in KE medium pH 5.8 are always supplemented with 10mM lysine. Copy numbers of AdiY (+ *adiY*), CadC (+ *cadC*) or CsiR (+ *csiR*) were elevated by placing the corresponding genes under control of the arabinose (0.1%)-inducible promoter in plasmid pBAD24. All source data is summarized in Supplementary data 2.

gadC:eGFP-adiC:mCerulean-cadB:mCherry			
	pH 7.6	pH 5.8 + lysine	pH 4.4
t_0	GadC:eGFP 735 RF - 0.10 AdiC:mCerulean 35 RF - 0.04 CadB:mCherry 14 RF – 0.03		
t_{150}	GadC:eGFP 265 RF - 0.11 AdiC:mCerulean 42 RF - 0.05 CadB:mCherry 17 RF – 0.05	GadC:eGFP 1512RF - 0.04 AdiC:mCerulean 39 RF - 0.03 CadB:mCherry 386 RF – 0.37	
t_{300}	GadC:eGFP 836 RF - 0.11 AdiC:mCerulean 40 RF - 0.03 CadB:mCherry 15 RF – 0.04	GadC:eGFP 1734 RF - 0.08 AdiC:mCerulean 34 RF - 0.03 CadB:mCherry 246 RF – 0.26	GadC:eGFP 1401 RF - 0.05 AdiC:mCerulean 164 RF - 0.12 CadB:mCherry 547RF – 0.26
E. coli **MG1655 wildtype**			
LB pH 4.4 t_{300}			
eGFP channel		mCerulean channel	mCherry channel
25 RF - 0.07		37 RF - 0.05	12 RF - 0.09
Δ*cadA* *gadC:eGFP-adiC:mCerulean-cadB:mCherry* LB pH 4.4 t_{300}			
GadC:eGFP 1848 RU - 0.06		AdiC:mCerulean 306 RU - 0.07	CadB:mCherry 1979 RU - 0.23
+ *csiR* *gadC:mCerulean-cadB:mCherry* LB pH 4.4 t_{300}			
GadC:mCerulean 1636 RF - 0.12		CadB:mCherry 495 RF - 0.29	
+ *csiR* *adiC:mCerulean-cadB:mCherry* LB pH 4.4 t_{300}			

AdiC:mCerulean 92 RF - 0.10	CadB:mCherry 509 RF - 0.31
***+ cadC** gadC:mCerulean-cadB:mCherry* **KE pH 5.8 t₃₀₀**	
GadC:mCerulean 856 RF - 0.05	CadB:mCherry 1164 RF - 0.08
***+ cadC** adiC:mCherry-cadB:eGFP* **LB pH 4.4 t₃₀₀**	
AdiC:mCherry 30 RF - 0.08	CadB:eGFP 1026 RF - 0.07
***+ adiY** adiC:mCerulean-cadB:mCherry* **LB pH 4.4 t₃₀₀**	
AdiC:mCerulean 333 RF - 0.06	CadB: mCherry 396 RF - 0.29

gadC:mCherry-adiC:mCerulean-cadB:eGFP		
KE pH 7.6 t₃₀₀	KE pH 5.8 t₃₀₀	LB pH 4.4 t₃₀₀
GadC:mCherry 767 RF - 0.12	GadC:mCherry 1332 RF - 0.08	GadC:mCherry 1466 RF - 0.06

gadC:mCerulean-adiC:mCherry-cadB:eGFP		
KE pH 7.6 t₃₀₀	KE pH 5.8 t₃₀₀	LB pH 4.4 t₃₀₀
GadC:mCerulean 680RF - 0.11	GadC:mCerulean 1997 RF - 0.07	GadC:mCerulean 1532 RF - 0.09

Supplementary Table 2: Occurrence of the main components of the Gad, Adi and Cad system within selected species of the proteobacteria and firmicutes. Phylogenetic distribution is presented in Fig. 4 and sequences are summarized in Supplementary data 1. x, indicates present of a homolog of the protein GadA/B, GadC, CsiR, EvgS, GadE, GadW, GadY, YdeO, AdiA, AdiC, AdiY, CadA, CadB, CadC and LysP.

|		Gad system									Adi system			Cad system			
		GadA/B	GadC	CsiR	EvgS	GadE	GadW	GadX	GadY	YdeO	AdiA	AdiC	AdiY	CadA	CadB	CadC	LysP
Proteobacteria	*Escherichia coli*	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
	Escherichia albertii	x	x	x		x	x	x	x		x	x	x	x	x	x	x
	Shigella boydii	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x
	Shigella flexneri	x	x	x	x	x	x	x	x	x	x	x	x				
	Salmonella enterica			x							x	x	x	x	x	x	x
	Citrobacter freundii	x	x	x		x					x	x	x	x	x		x
	Hafnia alvei	x	x	x	x						x	x		x	x	x	x
	Serratia fonticola	x	x		x						x	x		x	x	x	x
	Xenorhabdus bovienii										x	x					x
	Stenotrophomonas maltophilia										x	x					
	Vibrio parahaemolyticus													x	x	x	
	Vibrio campbellii													x	x	x	
Firmicutes	*Clostridium perfringens*	x	x														x
	Lactococcus lactis	x	x														x
	Lactobacillus reuteri	x	x														x
	Listeria monocytogenes	x	x														x
	Enterococcus faecium	x	x														
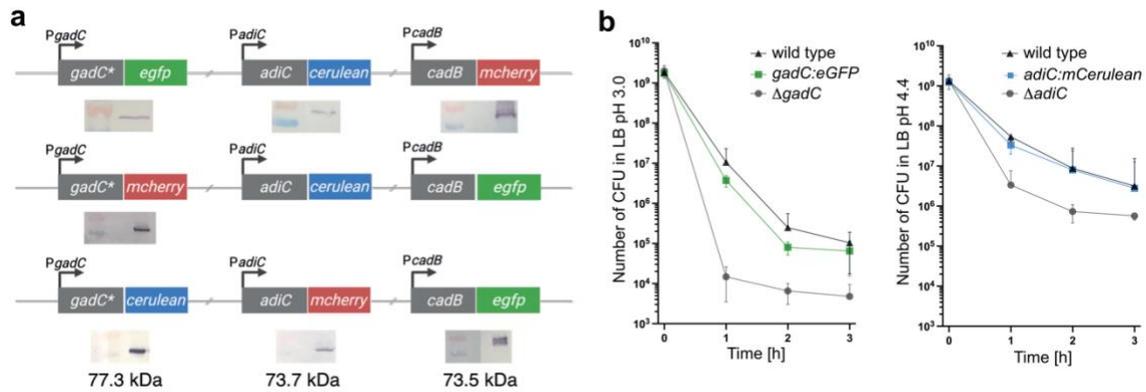
Supplementary Figure 1: Fluorescently-tagged antiporter hybrid proteins are functional in *E. coli*. (**a**) Schematic overview of the genomic organization of the different combinations of the three-color reporter strains: *gadC:eGFP-adiC:mCerulean-cadB:mCherry*, *gadC:mCherry-adiC:eGFP-cadB:mCerulean* and *gadC:mCerulean-adiC:mCherry-cadB:eGFP*. To verify the location of the different fluorescent hybrid proteins, the different three-color *E. coli* strains were pelleted at an OD_{600} = 1 at t_{300} in LB pH 4.4, fractionated and separated in a 12.5 % SDS-PAGE that was then transferred to nitrocellulose membrane. The different fluorescent hybrid proteins were labeled with either the primary polyclonal α-GFP antibody, α-mCerulean antibody or α-mCherry antibody and the α-rabbit alkaline phosphatase-conjugated antibody was used as secondary antibody. As ladder the PageRuler Prestained Protein Ladder (10 to 180 kDa) was used and only the membrane fraction is shown. The red band of the PageRuler Prestained Protein Ladder corresponds to 70 kDa. The molecular weight of each fluorescent hybrid protein is indicated below the Western blot. (**b**) Acid survival assay to test functionality of GadC:eGFP in LB medium pH 3.0 (left panel) and AdiC:mCerulean in LB medium pH 4.4 (right panel) via colony forming units during incubation for 3 h. The three-color strain *gadC:eGFP-adiC:mCerulean-cadB:mCherry* was cultivated and as control *E. coli* MG1655 cells and a deletion of either *gadC* or *adiC* were used. Functionality of the CadB:eGFP fusion was already assed via an liquid-based colorimetric assay using a pH indicator [1].
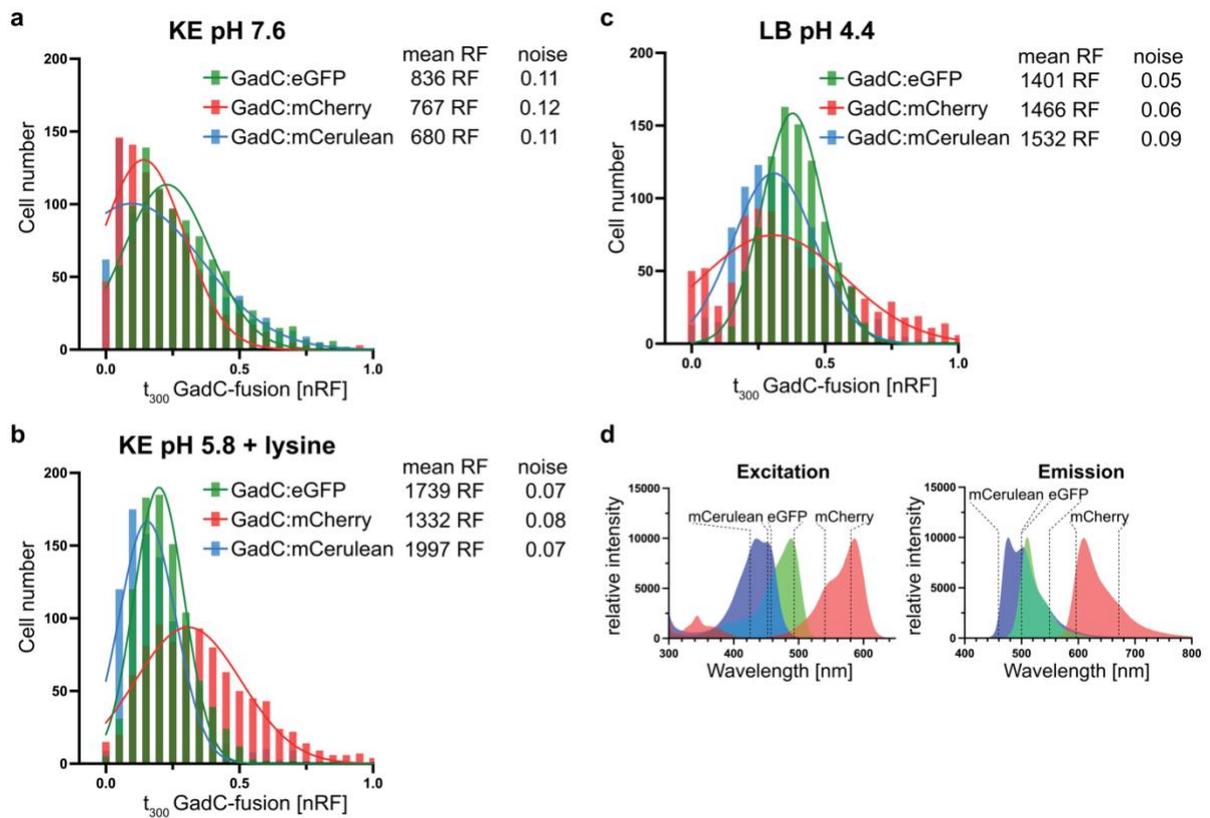
Supplementary Figure 2: Comparison of the production and distribution of the different fluorescent fusions with GadC at different acidic conditions. Histogram presentation of the normalized relative fluorescence intensity (nRF) quantified for 1,000 cells per fluorescent fusion, GadC:eGFP, GadC:mCherry or GadC:mCerulean, at t_{300} in (**a**) KE pH 7.6, (**b**) KE pH 5.8 supplemented with lysine and (**c**) LB pH 4.4. Calculated mean RF and noise values are summarized in Table S1. nRF, normalized RF values. (**d**) Comparison of the excitation (left panel) and emission (right panel) wavelength of the fluorophores mCerulean, eGFP and mCherry. Dashed lines indicate the ranges of the fluorescence filter cubes for the three fluorophores of the used Leica DMi8 inverted microscope. Spectra were obtained from the FPbase [2] and visualized using GraphPad Prism 9.1.0.
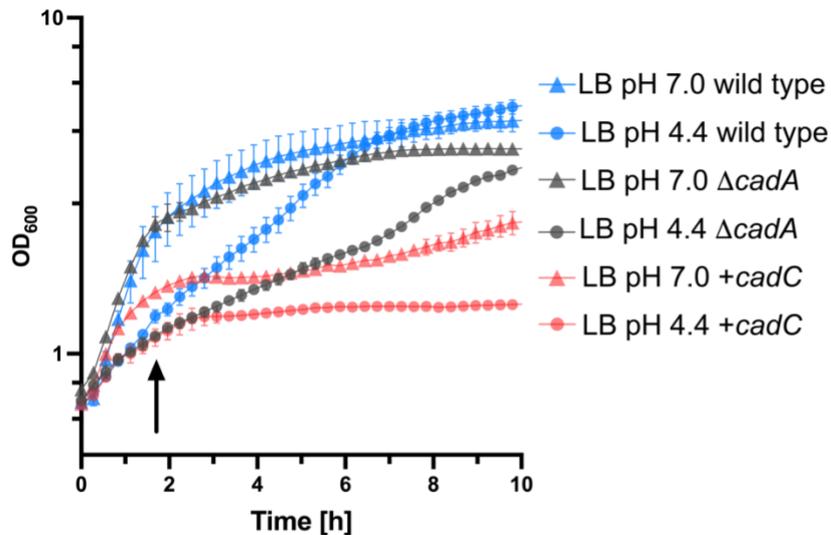
Supplementary Figure 3: High CadC copy number and *cadA* deletion slows down growth of *E. coli* at low pH. The *E. coli* strains, MG1655Δ*cadC* containing plasmid pBAD-*cadC,* and *E. coli* MG1655 wild type containing the empty plasmid pBAD24, were cultivated in KE medium pH 7.6 until exponential phase, then shifted to KE medium pH 5.8 + 10 mM lysine and finally shifted to LB medium at pH 7.0 or pH 4.4 (each supplemented with 0.1% (w/v) arabinose). Cultures were aerobically cultivated in 96-well plates at 37 °C in a Tecan Infinite F500 system (Tecan, Crailsheim, Germany) and growth (OD_{600}) was measured every 10 min at 37 °C. In strain MG1655Δ*cadC* + pBAD-*cadC*, the copy number of CadC is elevated to about 100 CadC molecules per cell, whereas the *E. coli* MG1655 pBAD24 strain produces only ≤4 CadC molecules per cell as reported previously [3]. The black arrow indicates the time point of the values displayed as a bar graph in Fig. 6b.

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