Systematic analysis of the glutamate-dependent acid resistance system in *Escherichia coli*

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Lingyun Guo

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München, den 18.11.2022

Lingyun Guo

Table of Contents

Abbreviations

ADAR	Arginine-dependent acid resistance
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
cDNA	Complementary DNA
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
Ethylenediaminetetraacetic acid	EDTA
FAD	Flavin adenine dinucleotide
GABA	γ-aminobutyric acid
GDAR	Glutamate-dependent acid resistance
gDNA	Genomic DNA
GTP	Guanosine triphosphate
HPLC	High-performance liquid chromatography
IPTG	isopropyl β-D-1-thiogalactopyranoside
Kan^R	Kanamycin resistance
LDAR	Lysine-dependent acid resistance
mRNA	Messenger RNA
m^6A	N6-Methyladenosine
NADH	Nicotinamide adenine dinucleotide (Reduced form)
OD_{600}	Optical density measured at a wavelength of 600 nm
ODAR	Ornithine-dependent acid resistance
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PCI	phenol-chloroform-isoamyl alcohol
PLP	pyridoxal-5'-phosphate
qRT-PCR	Quantitative reverse transcription polymerase chain reaction

RFU	Relative fluorescence unit
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SCFAs	Short-chain fatty acids
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCE	2,2,2-Trichloroethanol
TLC	Thin-layer chromatography
tRNA	Transfer RNA
TCS	Two-component signal transduction system
v/v	Volume per volume
WT	Wild-type strain
w/v	Weight per volume

Nomenclature

Gene deletions are marked by “Δ”.

Gene insertion mutant are marked by “::”.

Promoter of a gene: “$P_{gene\ name}$”.

Promoter fusion with fluorescence tags: “$P_{gene\ name}$:*gene name of fluorescence*”.

Protein fusion with fluorescence tags: “Protein name: Protein name of fluorescence”.

Contribution

Figure 3.3F was contributed by the collaborators: Dr. Anna-Lena Heins and Manh Dat Hoang from Technische Universität München.

1. Introduction

1.1 The acid stress in the human digestive tract

Acid stress, defined as the combined biological effect of low pH and organic acids present in the environment, can affect the charge, structure, and function of biological molecules in bacteria (Arcari, Feger, Guerreiro, Wu, & O'Byrne, 2020; Bearson, Bearson, & Foster, 1997). Consequently, acid stress can lead to acid-induced protein unfolding, decrease of enzymatic activity, and cause membrane damage and DNA damage (Lund, Tramonti, & De Biase, 2014).

To colonize the human digestive tract, the orally acquired enteric bacteria, including the food-borne probiotics, have to cope with extreme acid stress (typical pH range 1.5 – 3.5, usually considered pH < 2.5) caused by the inorganic acid (hydrochloric acid) in the stomach (Figure 1.1), which is the major bactericidal barrier of the gastrointestinal tract. In addition, the enteric bacteria have to cope with mild acid stress (pH 5.0 – 7.0) in the colon (Figure 1.1) caused by short-chain fatty acids (SCFAs). SCFAs primarily include formate, acetate, propionate, and butyrate, which are the main fermentation products of dietary carbohydrates, specifically resistant starches and dietary fiber, by gut bacteria, mostly obligate anaerobes belonging to the Bacteroidetes and Firmicutes phyla (Lund et al., 2014; Macfarlane & Gibson, 1997; Morrison & Preston, 2016; Tobe, Nakanishi, & Sugimoto, 2011).

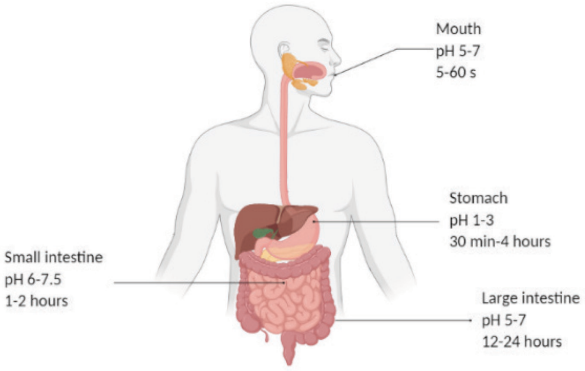


Figure 1.1. Schematic diagram of the acidic conditions in different regions of the human digestive tract that orally acquired bacteria have to face. Firstly, the orally acquired bacteria need to cope with the extremely acidic pH of 2.5 in the stomach for 30 mins – 4 hours. After passing the stomach, bacteria arrive and stay for 1 – 2 hours in the neutral small intestine (pH 6.0 – 7.5), to finally arrive to the colon, where they have to face a mildly organic acid stress (pH 5.0 – 7.0) for 12 – 24 hours (Lund et al., 2014; McClements & Li, 2010).

SCFAs in their uncharged, protonated forms diffuse more freely across the inner membrane of bacteria and disassociate in the cytoplasm, lowering the intracellular pH. Therefore, the SCFAs are more harmful at the same pH value than strong acids, which are in the dissociated form (Bearson et al., 1997; Lund et al., 2014).

1.2 The acid resistance systems in *Escherichia coli*

Escherichia coli belongs to the class Gammaproteobacteria, order Enterobacterales, family Enterobacteriaceae, is a well-studied Gram-stain-negative, rod-shaped, facultatively anaerobic, orally acquired enteric bacteria species, comprising numerous commensal and pathogenic strains, which are closely related to human health. To counteract different acid stresses in the human digestive tract, *E. coli* develops different strategies, including passive and active acid resistance systems. The passive systems rely on the buffering capacity of amino acids, proteins, polyphosphate, and inorganic phosphate in the cytoplasm. In contrast, the active systems rely on physiological, metabolic and proton-consuming protection strategies (Kanjee & Houry, 2013).

The physiological protections mainly depend on the unsaturated lipids and cyclopropane fatty acids present in the inner membrane, which can change the membrane fluidity and permeability to protons; the outer membrane porins which can reduce the proton influx by binding polyphosphate or cadaverine, and chaperone proteins in the periplasm, such as HdeA and HdeB, which can bind to acid-denatured proteins at low pH (Gajiwala & Burley, 2000; Kanjee & Houry, 2013; Kern, Malki, Abdallah, Tagourti, & Richarme, 2007). The metabolic protection against the mild acid stress is accomplished by the upregulation of genes involved in the electron transport chain, generating the proton motive force with the export of protons from the cytoplasm, and the upregulation genes involved in metabolism of secondary carbon sources, which produce fewer acids compared to glucose (Hayes et al., 2006; Kanjee & Houry, 2013; Maurer, Yohannes, Bondurant, Radmacher, & Slonczewski, 2005).

Another way of bacteria developed to cope with low pH is to consume protons via enzymatic reactions. The best examples of proton-consuming systems are amino acid decarboxylase acid stress resistant systems, containing two major components: pyridoxal-5'-phosphate (PLP)-dependent amino acid decarboxylases, which catalyze the conversion of one amino acid to a new product and one carbon dioxide (CO_2) consuming one proton, and one inner membrane substrate/product antiporter, which carries out the exchange of cytoplasmic products and the periplasmic substrates. Three major amino acid dependent acid stress resistant systems have been characterized in *E. coli*: the glutamate-dependent acid resistance (GDAR) system, consisting of two homologous inducible glutamate decarboxylases GadA and GadB and glutamate/γ-aminobutyric acid (GABA) antiporter GadC (Figure 1.2); the arginine-dependent acid resistance (ADAR) system, consisting of the arginine decarboxylase (AdiA) and the arginine/agmatine antiporter (AdiC) (Figure 1.2); and the lysine-dependent acid resistance (LDAR) system, consisting of the inducible lysine decarboxylase CadA and the lysine/cadaverine antiporter CadB (Figure 1.2) (Arcari et al., 2020; De Biase & Pennacchietti, 2012; Foster, 2004; Kanjee & Houry, 2013; Lund

et al., 2014). Activation conditions of the three systems are pH and growth phases dependent. The GDAR system is activated in *E. coli* mainly during the stationary phase regardless of pH, or exponential growth phase at acidic pH of 5.5 (De Biase, Tramonti, Bossa, & Visca, 1999; Gong, Ma, & Foster, 2004); the ADAR system is maximally induced in *E. coli* cultivated under anaerobiosis in the rich medium at pH 4.4 (Stim-Herndon, Flores, & Bennett, 1996); the LDAR system is activated in *E. coli* grown under acidic pH of 5.8 in the presence of external lysine (Fritz et al., 2009).

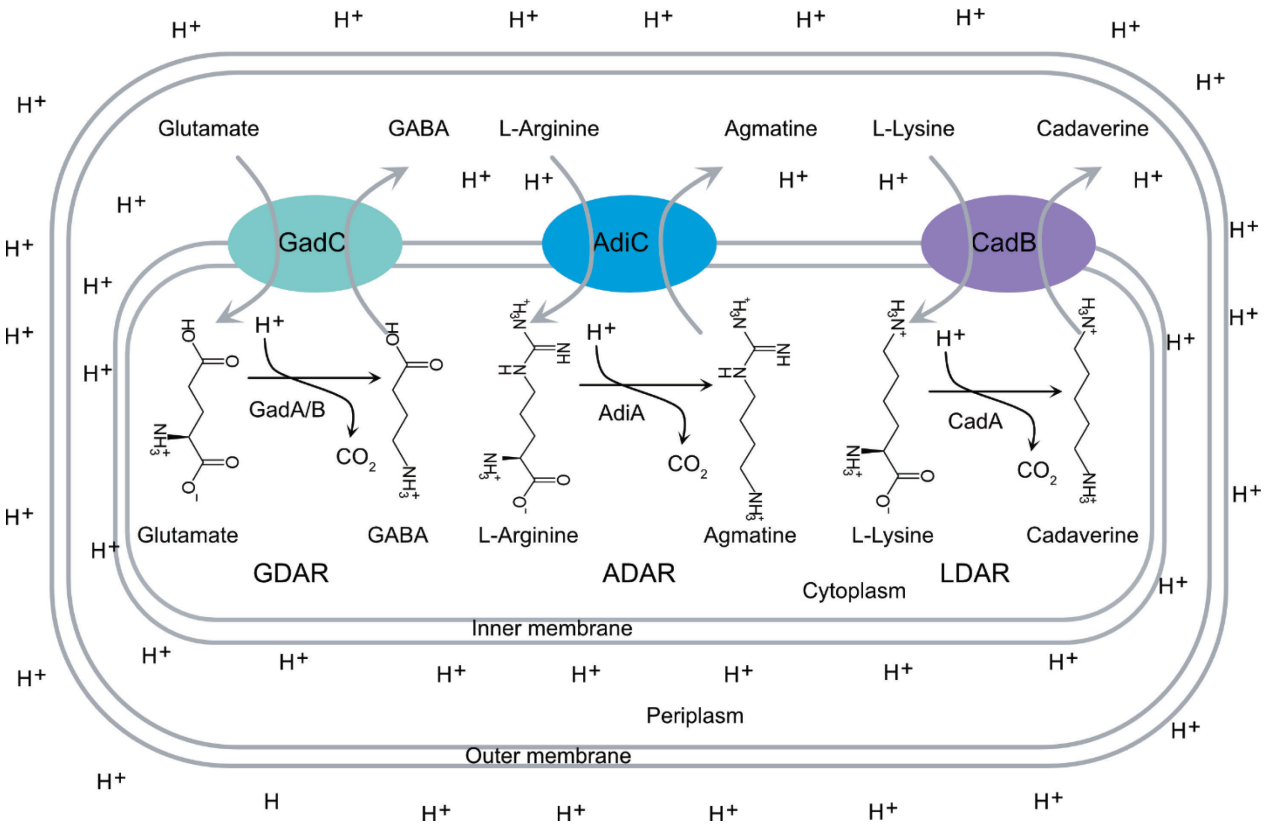


Figure 1.2. Schematic diagram of the three major amino acid dependent acid resistance systems in *E. coli*. The amino acid and decarboxylation products are shown in chemical notation and the proteins responsible for the reactions are shown under the reaction arrows. All the decarboxylation reactions consume a proton and release carbon dioxide. The ornithine-dependent acid resistance (ODAR) system is not shown due to lack of information. Adapted from Zhao and Houry (2010) (B. Zhao & Houry, 2010).

In addition to the three amino acid dependent acid resistance systems described above, the ornithine-dependent acid resistance (ODAR) system, consisting of the ornithine decarboxylase SpeF and the ornithine/putrescine antiporter PotE, might also play a role in *E. coli* under mild acid stress, but the contribution of the ODAR system to acid resistance in *E. coli* has not been proven (Arcari et al., 2020; Kanjee, Gutsche, Ramachandran, & Houry, 2011; Kashiwagi, Miyamoto, Suzuki, Kobayashi, & Igarashi, 1992; Kashiwagi et al., 1991). There is also one amino acid independent, glucose-repressed, RpoS-dependent, oxidative acid resistance (AR1) system in *E. coli*. However, the mechanism of the AR1 system is poorly understood (Castanie-Cornet, Penfound, Smith, Elliott, & Foster, 1999).

1.3 The mechanism of the glutamate-dependent acid resistance system

This study focuses on the GDAR system because it provides the most potent protection against extreme acidic stress in commensal and pathogenic *E. coli*, *Shigella flexneri*, *Listeria monocytogenes* and *Lactococcus lactis* (De Biase & Pennacchietti, 2012). The primary sequences of the homologous hexamer decarboxylases GadA and GadB only differ in 5 amino acids, and the comparison of the X-ray crystal structures demonstrated that they are structurally similar (Capitani et al., 2003; Dutyshev et al., 2005). GadA and GadB have optimal enzymatic activities at pH 3.7 – 3.8 and their activities decrease sharply as pH increases, indicating that they will be fully active under extreme acid stress (Arcari et al., 2020). The inner membrane antiporter GadC encounters glutamate in three states (Glu^-, Glu^0, Glu^+) and GABA in two oxidation states ($GABA^0$, $GABA^+$) under extremely acidic stomach conditions, which indicates that GadC has to cope with six possible combinations of the substrates. Notably, two research groups revealed that GadC selectively transports the substrates via a charge-based mechanism, which selectively imports the glutamate with no net charge (Glu^0) into the cytoplasm and exports GABA with one positive charge ($GABA^+$) to the periplasm, resulting in effective proton extrusion (Ma, Lu, & Shi, 2013; Tsai, McCarthy, & Miller,

2013). The activity of GadC is pH-dependent, which is inactive at neutral pH and active at acidic pH (extracellular pH is around 2 – 3, the cytoplasmic pH drops to 3.5 – 5.0), supporting the charge-based mechanism (Ma et al., 2013). Glutamine is another substrate that can be recognized and imported into the cytoplasm by the antiporter GadC, after which it will be deamidated to glutamate by the acid activated amidohydrolase YbaS, with the release of gaseous ammonia, which can neutralize proton and increase the intracellular pH. The YbaS/GadC system also confers the acid resistance in *E. coli* in the presence of the extracellular glutamine (Ma et al., 2013). In addition, the newly produced glutamate can also increase the intracellular pH via the GDAR system (Ma et al., 2013).

1.4 The regulation of the glutamate-dependent acid resistance system

The *gadB* and *gadC* genes are co-transcribed, whereas the *gadA* gene, which is 2.1 Mb away from *gadBC*, is transcribed either alone or co-transcribed with the downstream regulator *gadX* (De Biase et al., 1999; Tramonti, Visca, De Canio, Falconi, & De Biase, 2002). The induction of *gadA* and *gadBC* depends on the growth phase and media environments. *gadA* and *gadBC* are primarily transcribed during the stationary phase, and are significantly induced by acid and salt stress (De Biase et al., 1999). Induction of *gadA* and *gadBC* expression at the stationary phase occurs in both complex and minimal media, while it is more condition-dependent at the exponential growth phase. It has been proven that the protein production of GadA/GadB was induced in cells of the exponential growth phase only in a minimal medium, however, these cells are still sensitive to the extreme acid stress (Castanie-Cornet et al., 1999). The expression of *gadA* is predominately induced by acid stress, while expression of *gadB* is primarily stationary phase dependent (Castanie-Cornet et al., 1999). In addition, *gadA* and *gadBC* expression can be induced by sodium acetate at neutral pH (Arnold, McElhanon, Lee, Leonhart, & Siegele, 2001).

The regulation of the GDAR system is extremely intricate, mainly containing the alternative sigma factor δ^S (encoded by *rpoS*) -dependent and -independent pathways. The δ^S-dependent pathway is associated with *gadA* and *gadBC* expression at the stationary phase, whereas the δ^S-independent pathway is related to *gadA* and *gadBC* expression at the exponential growth phase in an acidic minimal medium (Castanie-Cornet et al., 1999; Waterman & Small, 2003). The regulatory network of the GDAR system consists of regulatory proteins, two-component signal transduction systems (TCSs) and small RNAs (Figure 1.3). The LuxR-family member GadE (formerly YhiE) is the central regulatory protein of the GDAR system, which can directly bind to the 20 bp GAD box, located in the promoter regions of both *gadA* and *gadBC* (Z. Ma et al., 2003). RcsB, the response regulator of the Rcs (Regulator of Capsule Synthesis) TCS, is essential for the expression of *gadA* and *gadB*, forming functional heterodimers with GadE (Castanie-Cornet et al., 2010; Castanié-Cornet, Treffandier, Francez-Charlot, Gutierrez, & Cam, 2007; Johnson, Burton, Gutiérrez, Painter, & Lund, 2011). In addition, a *gadE* mutant also showed defective expression of the AR1 and ADAR system (Ma et al., 2003). Three major regulatory circuits EvgS/EvgA, GadX/GadW and MnmE regulate *gadA* and *gadBC* expression via the central regulatory protein GadE (Foster, 2004). The regulatory network was further extended with newer research, and the extended regulatory network is shown in Figure 1.3.

The clearest regulatory circuit is the EvgS/EvgA circuit, which contains the histidine kinase EvgS and the response regulator EvgA of the EvgS/EvgA TCS, and an AraC-like regulator YdeO (Ma, Masuda, & Foster, 2004; Masuda & Church, 2002, 2003). EvgS is activated via responding to mildly acidic pH in the presence of alkali metals (Na^+ or K^+) during the exponential growth phase, then the signal cascade will go through either EvgS→EvgA→YdeO→GadE or directly from EvgA to GadE without YdeO (Eguchi & Utsumi, 2014; Itou, Eguchi, & Utsumi, 2009). New studies have recently extended the EvgS/EvgA circuit with the PhoQ/PhoP TCS, which responses to external Mg^{2+}. The activated EvgS/EvgA system activates the PhoQ/PhoP TCS via

a connector protein SafA (Sensor-associating-factor A). Once active, the PhoQ/PhoP TCS induces another connector IraM, which directly binds to the regulator RssB and stabilizes σ-factor RpoS, lastly, RpoS activates the *gadE* expression (Figure 1.3) (Eguchi, Ishii, Hata, & Utsumi, 2011; Eguchi et al., 2007; Eguchi et al., 2004; Zwir et al., 2005).

The most complex is the GadX/GadW regulatory circuit, including RpoS (Castanie-Cornet et al., 1999), two AraC-like regulators GadX and GadW, (Castanie-Cornet et al., 1999; Z. Ma, Richard, Tucker, Conway, & Foster, 2002; Shin et al., 2001), and one small RNA GadY (Opdyke, Kang, & Storz, 2004). The *gadX* gene is either co-transcribed with the upstream *gadA* gene, or independently transcribed, or transcribed with the downstream *gadW* gene, which is also independently transcribed from its indigenous promoter (Tramonti, De Canio, & De Biase, 2008; Tramonti, De Canio, Delany, Scarlato, & De Biase, 2006). The GadX/GadW circuit displays a condition-dependent dual role, which activates *gadA* and *gadBC* expression indirectly via the activation of *gadE* (Sayed, Odom, & Foster, 2007), or represses the *gadA* and *gadBC* expression by directly binding to the *gad* box of the promoter regions of *gadA* and *gadBC* (Figure 1.3) (Ma et al., 2002; Tramonti et al., 2002). GadX was reported to be a positive regulator on the expression of *gad* genes in acidic pH conditions, while acting as a negative regulator in LB at pH 8.0 (Shin et al., 2001). GadW negatively regulates *gadA* and *gadBC* expression by repressing *gadX*, and positively activates *gadA* and *gadBC* in the absence of GadX (Figure 1.3) (Z. Ma et al., 2003). The small RNA GadY was proven to be involved in the GadX/GadW circuit by stabilizing the *gadX* mRNA and affecting the processing of *gadXW* transcript (Opdyke, Fozo, Hemm, & Storz, 2011; Opdyke et al., 2004; Tramonti et al., 2008). *gadY* gene sequence overlaps the 3' end of the *gadX* mRNA encoded on the opposite strand, and *gadY* transcription is negatively affected by *gadW* transcript (Figure 1.3) (Opdyke et al., 2004; Tramonti et al., 2008). In addition, the cyclic AMP receptor protein CRP was reported to negatively regulate the *gadA* and *gadB* expression via repressing the *gadX* expression but not

gadW, through its control over RpoS, in exponential growth phase cells cultivated in LB medium (Ma et al., 2002). The nucleoid-associated protein H-NS is reported to repress the *gadX* expression by directly binding to the specific sites in the promoter regions of *gadA* and *gadX* (Figure 1.3) (Giangrossi, Zattoni, Tramonti, De Biase, & Falconi, 2005). It was proposed that GadX, GadW and CRP sense different signals and the ratio of these signals alters the balance in this regulatory circuit (Foster, 2004).

The least studied regulatory circuit is the MnmE circuit, which has the Era-like GTPase MnmE (also known as TrmE) as the only defined component. This circuit was first reported as one MnmE-dependent, pH-independent and glucose induced pathway, which regulates the *gadA* and *gadBC* expression via the *gadE* activation, and the translational control of *gadA* (Gong et al., 2004). This research group also proved that the GTPase activity is essential for the *gadA* and *gadBC* expression at both transcriptional and translational levels (Gong et al., 2004). A second research group revealed that MnmE is critical for the expression of the *gadE-mdtEF* multidrug efflux operon in stationary phase *E. coli* cells cultivated in glucose minimal medium under anaerobic conditions, while the EvgA, YdeO, RpoS, and GadX were not required under this condition (Deng, Shan, Pan, Gao, & Yan, 2013). In addition, they also found that MnmE activates the *gadE-mdtEF* promoter in non-glucose media (LB and glycerol medium supplement with nitrate), which is inconsistent with the previous aerobic conclusion that MnmE affects the *gadE* expression only in the presence of glucose (Z. Deng et al., 2013; Gong et al., 2004). Because of the GTPase MnmE lacks the DNA binding property, the two research groups both hypothesized that MnmE activates *gadE* transcription indirectly by influencing the synthesis or activity of an unknown regulator that binds the *gadE* control region and regulates its transcription (Deng et al., 2013; Gong et al., 2004). A third research showed that the *mnmE* mutant only showed a slight growth defect in acidic LB (4.4), but it was slowly inactivated in apple juice (pH 4.6) and tomato juice (pH 4.8) (Vivijs, Aertsen, & Michiels, 2016). Even with these three studies, the MnmE regulatory circuit is still poorly understood.

MnmE is a guanine nucleotide binding protein (G protein). This type of protein acts as a molecular switch by cycling the GDP-bound and GTP-bound states, and plays essential roles in many cellular processes, such as protein synthesis and signal transduction (Meyer, Wittinghofer, & Versées, 2009). MnmE and its partner protein MnmG (also called GidA) always form a functional heterotrameric $\alpha_2\beta_2$ complex MnmE/MnmG, which catalyzes the formation of a carboxymethylaminomethyl (cmnm) group at the 5' position of the wobble uridine base U_{34} of the first anticodon position of particular tRNAs (Böhme et al., 2010; Fislage et al., 2014; Shi et al., 2009; Yim, Moukadiri, Björk, & Armengod, 2006). This post-transcriptional modification of the U_{34} enables codon recognition at the ribosomal peptidyl (P) site, expends the ability of a tRNA to read more degenerate codons, and increases the accuracy of the translation (Agris, Vendeix, & Graham, 2007; Yarian et al., 2002). The MnmE is a GTP- and tetrahydrofolate binding protein, while MnmG is a FAD- and NADH-binding protein. It has been proven that the GTP hydrolysis and the MnmE/MnmG interaction are required for the tRNA modification (Meyer et al., 2009). As mentioned above, the MnmE is involved in the regulatory network of the GDAR system. However, studies about the role of MnmG are lacking. We proposed a hypothesis that the MnmG and MnmE might also be both involved in the regulatory network of the GDAR system because of the functional MnmE/MnmG complex, and confirmed this hypothesis.

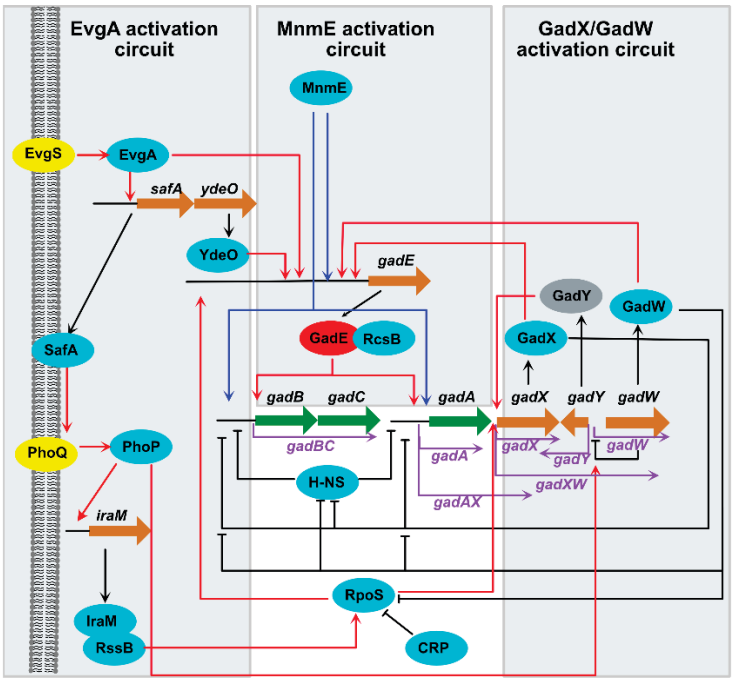


Figure 1.3. Three major activation circuits of the GDAR system in *E. coli*. Three major activation circuits via the central regulator GadE are presented here. Thick arrows represent genes, *gad* genes are colored in green, while the regulatory genes are colored in brown. Ellipses colored in blue represent regulatory proteins, ellipses colored in yellow represent sensor histidine kinases, ellipse in red represents the central regulator, ellipse in grey represents small RNA. Thin bent arrows in purple represent transcription units, thin bent arrows in blue indicate the positive regulation which might be indirect, small bent arrows in red indicate positive regulation, black bars indicate negative regulation. This figure is adapted from (Foster, 2004) and (Tramonti et al., 2008).

With the increasing number of studies, the regulatory network of the GDAR system is extending, however, the primary stimuli leading to activation of the system are relatively poorly understood. There is still a long way to go to clarify the regulatory network completely.

1.5 Scope of the thesis

The GDAR system is the most potent acid resistance system in *E. coli* under extremely acidic conditions. The regulatory network of the GDAR system is complex, and the external stimuli of the GDAR system are poorly understood. The main aims of this thesis are to systematically analyze the expression profile of the GDAR system and unravel the external stimuli and related regulatory network.

In this study, the time-dependent expression profile of the GDAR system is analyzed in *E. coli* cultivated in a bioreactor under different aeration conditions. Effects of oxygen-limitation, acid stress and stationary phase on the expression of the GDAR system are analyzed. The SCFAs are studied as external stimuli of the GDAR system, and the related regulatory circuit is investigated. Additionally, the regulatory network of the GDAR system in *E. coli* in response to SCFAs is analyzed. Finally, the role of mRNA m^6A modification on the expression of the GDAR system is also analyzed, as the m^6A modification has been reported in *gad* mRNAs.

2. Materials and Methods

2.1 Materials and chemicals

The materials and chemicals used in this study are listed in Table 1. All materials not listed were purchased from Sigma-Aldrich, Carl Roth, Serva, New England Biolabs, Bio-Rad or Invitrogen.

Table 1: Materials and chemicals used in this study

Material	Manufacturer
Acetic acid	Carl Roth, Germany
Agarose	Serva, Germany
Ammonium Persulfate (APS)	Roth, Germany
Aqua-Phenol/Chloroform/Isoamylalcohol (25/24/1)	Carl Roth, Germany
Bovine serum Albumin (BSA)	Sigma-Aldrich, Germany
Butyric acid	Sigma-Aldrich, Germany
Carbenicillin (disodium salt)	Carl Roth, Germany
Color Prestained Protein Standard (10 –250 kDa)	New England Biolabs, Germany
Diethylpyrocarbonate (DEPC)	Roth, Germany
DNA oligonucleotides	Sigma-Aldrich, Germany
DNA standard (2-Log DNA-Ladder)	New England Biolabs, Germany
dNTPs (deoxynucleotide triphosphates)	Invitrogen, Germany
Donkey Anti-Rabbit IgG H&L	Abcam, UK
Formic acid	Carl Roth, Germany
Glucose	Carl Roth, Germany
Glutamic acid monosodium salt monohydrate	Sigma-Aldrich, Germany
Glycerol	Carl Roth, Germany
Hi Yield gDNA Mini Kit	Süd-Laborbedarf GmbH, Germany
Hi Yield PCR Clean-Up & Gel-Extraction Kit	Süd-Laborbedarf GmbH, Germany

Hi Yield Plasmid Mini Kit	Süd-Laborbedarf GmbH, Germany
Hydrochloric acid (32%)	Carl Roth, Germany
iQ SYBR Green Supermix	Bio-Rad, Germany
iScript Select cDNA Synthesis Kit	Bio-Rad, Germany
Kanamycin (sulfate)	Carl Roth, Germany
N,N,N′,N′-Tetramethylethylenediamine (TEMED)	Carl Roth, Germany
Nitrocellulose membrane	GE Healthcare, Germany
PCRBIO VeriFi™ Polymerase	PCR Biosystems, UK
Propionic acid	Roth, Germany
Q5 High-Fidelity DNA Polymerase	New England Biolabs, Germany
Restriction Nuclease	New England Biolabs, Germany
ROTIPHORESE®Gel 30 (37.5:1)	Carl Roth, Germany
Sodium dodecyl sulphate (SDS)	Carl Roth, Germany
T4-DNA ligase	New England Biolabs, Germany
TLC Silica gel F254 plate	Merck, Germany
2,2,2-Trichloroethanol (TCE)	Carl Roth, Germany
TURBO DNA-free™ DNase Treatment and Removal Reagents	Invitrogen, Germany
Tween 20	Fisher Bioreagents, Germany

2.2 Nucleotides, plasmids and strains construction

Strains, plasmids and primers used in this study are listed in the Tables 2 – 4, respectively. All the enzymes and biology kits were used by following the standard protocols from the manufactures. Genomic DNA of *E. coli* MG1655 for the was extracted by using the Hi Yield gDNA Mini Kit. Plasmid extraction was performed by using the Hi Yield Plasmid Mini Kit. DNA fragments of our target genes (*gadA*, *gadB*, *gadE*, *rpoS*) were amplified by PCR with the specific primers (table 3) and the template genomic DNA of *E. coli* MG1655, and purified from agarose gels using the Hi Yield

PCR Cleanup and Gel Extraction Kit. The DNA fragment of *mCherry* was amplified from a triple reporter cassette (Schlüter et al., 2015). All fragments of gene fusion were generated by using overlap PCR. Then they were digested with restriction enzymes and ligated with T4 DNA ligase into the pBBR1-MCS4 plasmid (Kovach et al., 1995).

Table 2: Strains used in this study

Strain	Features	Source
E. coli DH5α	*fhuA2 lacΔU169 phoA glnV44 Φ80' lacZΔM15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Promega
E. coli MG1655	*E. coli* K.12 reference strain, F^-, λ^-, *rph*-1.	(Blattner et al., 1997)
E. coli MG1655 Δ*gadA*	*E. coli* MG1655 with in-frame deletion of *gadA*	This study
E. coli MG1655 Δ*gadC*	*E. coli* MG1655 with in-frame deletion of *gadC*	This study
E. coli MG1655 Δ*gadBC*	*E. coli* MG1655 with in-frame deletion of *gadBC*	This study
E. coli MG1655 Δ*gadE*	*E. coli* MG1655 with in-frame deletion of *gadE*	This study
E. coli MG1655 P(CCA)121P(CCT)	The amino acid code P121 (CCA) of *gadC* was replaced with P(CCT)	This study
E. coli MG1655 P(CCA)325P(CCT)	The amino acid code P325 (CCA) of *gadC* was replaced with P(CCT)	This study
E. coli MG1655 Δ*rlmF* Δ*rlmJ*	*E. coli* MG1655 with in-frame deletion of *rlmF* and *rlmJ*	(Petrov *et al*., unpublished)
E. coli MG1655 *ypdB* D53E	MG1655 *rpsL150 ypdB*-D53E; Kan^s Str^r	(Fried *et al*., 2013)
E. coli MG1655 *ypdB* D53N	MG1655 *rpsL150 ypdB*-D53N; Kan^s Str^r	(Fried *et al*., 2013)
E. coli MG1655 *rcsB* D56E	MG1655 *rcsB*-D56E; $KanA^s$	(Szczesny, *et al*., 2018)
E. coli MG1655 *rcsB* D56S	MG1655 *rcsB*-D56S; $KanA^s$	(Szczesny, *et al*., 2018)
E. coli K-12 BW25113	F^- Δ(*araD-araB*)567, ΔlacZ4787::*rrnB*-3, λ^-, *rph*-1, Δ(*rhaD-rhaB*)568, *hsdR*514	(Baba *et al.,* 2006)
JW3480	BW25113 *gadE767::kan*	(Baba *et al.,* 2006)
JW3484	BW25113 *gadX771::kan*	(Baba *et al.,* 2006)

JW3483	BW25113 *gadW770::kan*	(Baba *et al.,* 2006)
JW2366	BW25113 *evgA778::kan*	(Baba *et al.,* 2006)
JW2367	BW25113 *evgS779::kan*	(Baba *et al.,* 2006)
JW3684	BW25113 mnmE*E737::kan*	(Baba *et al.,* 2006)
JW3719	BW25113 *mnmG770::kan*	(Baba *et al.,* 2006)
JW1494	BW25113 *ydeO721::kan*	(Baba *et al.,* 2006)
JW1899	BW25113 *uvrY760::kan*	(Baba *et al.,* 2006)
JW2757	BW25113 *barA784::kan*	(Baba *et al.,* 2006)
JW1116	BW25113 *phoP790::kan*	(Baba *et al.,* 2006)
JW1115	BW25113 *phoQ789::kan*	(Baba *et al.,* 2006)
JW1487	BW25113 *gadC785::kan*	(Baba *et al.,* 2006)
JW2378	BW25113 *ypdB790::kan*	(Baba *et al.,* 2006)
JW5388	BW25113 *ypdA789::kan*	(Baba *et al.,* 2006)
JW2379	BW25113 *ypdC720::kan*	(Baba *et al.,* 2006)
JW4364	BW25113 *arcA726::kan*	(Baba *et al.,* 2006)
JW5536	BW25113 *arcB738::kan*	(Baba *et al.,* 2006)
JW2205	BW25113 *rcsB770::kan*	(Baba *et al.,* 2006)
JW5551	BW25113 *sthA750::kan*	(Baba *et al.,* 2006)
JW0075	BW25113 *leuO783::kan*	(Baba *et al.,* 2006)
JW0872	BW25113 *lrp-787::kan*	(Baba *et al.,* 2006)
JW5437	BW25113*rpoS746::kan*	(Baba *et al.,* 2006)

Table 3: Plasmids used in this study

Plasmid	Feature and construction comments	Source
pBBR1-MCS4	Amp^r-cassette, pBBR broad host range origin of replication, mob region for conjugative transfer, low copy number	(Kovach et al., 1995)
pBBR1-MCS4-P_{gadA}:*mcherry*	Fusion of *gadA* native promoter (-180bp – -1bp) to sequence encoding *mcherry* in pBBR1-MCS4 (*BamHI* + *SacI*)	This study
pBBR1-MCS4-GadA-mCherry	Translational GadA-mCherry fusion. Sequence of *gadA* (-180bp to +1398bp) + sequence encoding *mcherry* in pBBR1-MCS4 (*BamHI* + *SacI*)	This study
pBBR1-MCS4-P_{gadA}-*egfp*-P_{gadB}-*mcherry*	Fusion of *gadA* gene with promoter region (-288bp – -1bp) in pBBR1-MCS4 (*BamHI* + *EcoRI*), P_{gadA}-*egfp* and P_{gadB}-*mcherry* are in different direction	This study
pBBR1-MCS4-P_{gadE}-*mcherry*	Fusion of *gadE* native promoter (-798bp – -1bp) to sequence encoding *mcherry* in pBBR1-MCS4 (*BamHI* + *SacI*)	This study
pBBR1-MCS4-RpoS-mCherry	Translational RpoS-mCherry fusion. Sequence of *rpoS* (-801bp to +741bp) + sequence encoding *mcherry* in pBBR1-MCS4 (*BamHI* + *SacI*)	This study
pCA24N	Cm^r, $lacI^q$, pMB1 replication origin, high copy number	
pCA24N-*mnmE*	pCA24N $p_{T5\text{-}lac}$:*mnmE*	(Kitagawa *et al.,* 2005)
pCA24N-*mnmG*	pCA24N $p_{T5\text{-}lac}$:*mnmG*	(Kitagawa *et al.,* 2005)
pCA24N-*gadE*	pCA24N $p_{T5\text{-}lac}$:*gadE*	(Kitagawa *et al.,* 2005)
pCA24N-*rcsB*	pCA24N $p_{T5\text{-}lac}$:*rcsB*	(Kitagawa *et al.,* 2005)
pCA24N-*ypdB*	pCA24N $p_{T5\text{-}lac}$:*ypdB*	(Kitagawa *et al.,* 2005)

Table 4: Primers used in this study

Name	Sequence	Restriction site
Transcriptional and translational fusions		
gadA for BamHI	TAGCCGGATCCGGCGATTTTTATTACGAT AA	BamHI
gadA-pro-*egfp*-OL-Fwd	ATAAATTTAAGGAGTTCGAAATGCGTAAAGGA GAAGAACT	-
gadA-pro-*egfp*-OL-Rev	AGTTCTTCTCCTTTACGCATTTCGAACTCCTTA AATTTAT	-
egfp-Rev-EcoRI	TAGCCGAATTCTTATTTGTATAGTTCATCCA	EcoRI
gadA-pro-*mcherry*-OL-Fwd	ATAAATTTAAGGAGTTCGAAATGGTGAGCAAG GGCGAGGA	-
gadA-pro-*mcherry*-OL-Rev	TCCTCGCCCTTGCTCACCATTTCGAACTCCTT AAATTTAT	-
GadA-466aa-OL-Fwd	AGAACAGCTTTAAACACACCATGGTGAGCAAG GGCGAGGA	-
GadA-466aa-OL-Rev	TCCTCGCCCTTGCTCACCATGGTGTGTTTAAA GCTGTTCT	-
mcherry- SacI-Rev	TAGCCGAGCTCAGCTTTTACTTGTACAGCTCG TCC	SacI
gadB-for-BamHI-Fwd	TAGCCGGATCCTGCGTTCAAAATAATAATCA	BamHI
gadB-pro- *mcherry* -OL-Fwd	ATCATTTTAAGGAGTTTAAAATGGTGAGCAAG GGCGAGGA	-
gadB-pro- *mcherry* -OL-Rev	TCCTCGCCCTTGCTCACCATTTTAAACTCCTTA AAATGAT	-
gadE-pro-BamHI-Fwd	TAGCCGGATCC TTACCCCGGTTGTCACCCGG	BamHI
gadE-pro-*mcherry*-OL-Fwd	TAACGGCTAAGGAGCAAGTTATGGTGAGCAA GGGCGAGGA	-
gadE-pro-*mcherry*-OL-Rev	TCCTCGCCCTTGCTCACCATAACTTGCTCCTT AGCCGTTA	-
RpoS-801-BamHI Fwd	TAGCCGGATCC CGCCTGGATTACTGGCAACG	BamHI
RpoS-801-*mcherry*-OL-Fwd	AAAAAGAGAACGGTCCGGAAATGGTGAGCAA GGGCGAGGA	-
rpoS-801-mcherry-OL-Rev	TCCTCGCCCTTGCTCACCATTTCCGGACCGTT CTCTTTTT	-
Generation of mutants		
DEL_*gadA*_f1_for	GGCGCCAAGCTTCTCTGCAGGATAATTACGG CGGCGGATATTG	-

DEL_*gadA*_f1_rev	GTTCTGCTGGGCAATACCCTGCAGCTTCTGGTCCATTTCGAACTC	-
DEL_*gadA*_f2_for	CAGGGTATTGCCCAGCAGAAC	-
DEL_*gadA*_f2_rev	GCTAGCGAATTCGTGGATCCAGATCTCGAATTTGGCTTGCATCC	-
DEL_*gadB*_f1_for	GGCGCCAAGCTTCTCTGCAGGATATCCTGCAGCATGGACTGAG	-
DEL_*gadB*_f2_for	CAGGGTATTGCCCAACAGAAC	-
DEL_*gadC*_f1_for	GGCGCCAAGCTTCTCTGCAGGATGTGAAGATCCGGGATACACC	-
DEL_*gadC*_f1_rev	CATCACAATATAGTGTGGTGAACGACCTGTCTGTACTGATGTAG	-
DEL_*gadC*_f2_for	CGTTCACCACACTATATTGTGATG	-
DEL_*gadC*_f2_rev	GCTAGCGAATTCGTGGATCCAGATCATCGCCTGTTGTTGTACAC	-
DEL_*gadE*_f1_for	GGCGCCAAGCTTCTCTGCAGGATCCGTAAGCGTTGATGCTA	-
DEL_*gadE*_f1_rev	GGGTGACGATACTTGCTCCTTAGCCGTTATCG	-
DEL_*gadE*_f2_for	AGGAGCAAGTATCGTCACCCTGGGTATCAC	-
DEL_*gadE*_f2_rev	GCTAGCGAATTCGTGGATCCAGATAGCGAATCGCCCTGGTTCAC	-
DEL_*gadE*_chk_for	CTCTCCGCTACGCAGTGTTG	-
DEL_*gadE*_chk_rev	GAGCCTTTGGCGGAGTTTAG	-
qRT-PCR		
GadA-qPCR-Fwd	GGCGCAAAGGCCATTTCTACTATCG	-
GadA-qPCR-Rev	CGAGCGTTGCCATCAAGATATAATTC	-
GadE-qPCR-Fwd	GCAGTTGAAAGATAATCACGAAATG	-
GadE-qPCR-Rev	AATCGCTTCTTCATCAAGGATATG	-
GadA-Qpcr-Fwd-end	TTACCAGGTTGCCGCTTATC	-
gadA-qPCR-Rev-end	CCCGGATCTTCACCATCTTTC	-
gadA-qpcr-fwd-m^{6}A	TTACCAGGTTGCCGCTTATC	-
gadA-qpcr-rev- m^{6}A	CCGGATCTTCACCATCTTTC	-
recA-qPCR-Fwd	CGGTTCGCTTTCACTGGATATCG	-
recA-qPCR-Rev	CCTGCAGCGTCAGCGTGGT	-
16S--qPCR-Fwd	CGAACGGTAACAGGAAGAAG	-

16S--qPCR-Rev	GCACATCCGATGGCAAGAGG	-
Checking		
M13-Fwd	TGTAAAACGACGGCCAGT	-
M13-Rev	CAGGAAACAGCTATGAC	-
gadX-Fwd	TAA ATC CAG TCA TCC TGC CCG	-
gadX-Rev	CTTACTGAGAGCACAAAGTTTCC	-
gadW-Fwd	TCTGGCAGTTTTTGCGCTAA	-
gadW-Rev	TGCCGAGTCTTTTCCTCCTG	-
ydeO-Fwd	CAG AAA TGG GTC GCA TTG CA	-
ydeO-Rev	GCAACAGGTTATGCAAGTGC	-
evgA-Fwd	TGTCGAATTATCTTAAAGGAAGCTCA	-
evgA -Rev	ATTCCTTGTTGCTCAGACGT	-
evgS-Fwd	CATCAGCAACAAAACTGTCAGC	-
evgS -Rev	AGCATGGGGAACAAATTCGC	-
mnmE-Fwd	TGCATAGCCGCGAGAAGAAA	-
mnmE-Rev	TCGTTCGGGGAGCAATATTAA	-
uvrY -Fwd	ACTGTGAAACGATCCGGTAAG	-
uvrY-Rev	GCTTCGGTTTCTGTGTGAGTA	-
barA-Fwd	TCGAGGTCGTTGACTGAAAC	-
barA -Rev	CCGGTTTGAGACTGATGCTAA	-
mnmG-Fwd	CGACGCCGGTATGTTTCTAATA	-
mnmG -Rev	TACCGGCAGAGAGTGAGTAA	-
ypdB-Fwd	CCATCATCGCGTGAAGTTATTG	-
ypdB-Rev	CCCAGTAGTTGCGGGTTAAG	-
lrp-Fwd	ATGTCGATAGCTGCCACAAG	-
lrp -Rev	TTTCCTGCCCGATGCTAAA	-
leuO-Fwd	TTCCACGGCAATGGATTCT	-
leuO -Rev	AATAGAGAGCCCACACATTCAG	-

2.3 Medium and cultivation condition

Lysogeny broth (LB) and LB agar were used for strain maintenance and cloning procedure. The Kim-Epstein (KE) medium (100 mM phosphate buffer, 2 mM $Na_3C_6H_5O_7$, 8 mM $(NH_4)_2SO_4$, 6 µM $FeSO_4$, 0.4 mM $MgSO_4$ at pH 7.6 or pH 5.8 was used as the standard minimal medium (Epstein & Kim, 1971). 0.4% glucose or 30 mM glycerol were added to the KE medium as the carbon source. 0.04% glucose was used as carbon source for carbon-limitation experiment. In addition, 1.5 mM L-glutamic acid monosodium salt monohydrate was added into the KE minimal medium for all experiments. Antibiotics were added when necessary with the following concentrations: kanamycin (50 µg/mL), carbenicillin (100 µg/mL), chloramphenicol (34 µg/mL). The isopropyl β-D-1-thiogalactopyranoside (IPTG) was used for the induction of genes of the ASKA plasmids (Kitagawa et al., 2005).

For the systematic analysis of the activation profile of the GDAR system, the bioreactor (Infors HT minifors) was used to maintain and monitor the growth condition of *E. coli* MG1655 cells during the cultivation. For the aeration, the bioreactor was connected to atmospheric air (air pressure 1.0 bar, rotator 1.5 Nl/min). For the oxygen-excess, the bioreactor was connected to extra oxygen to maintain high oxygen level. The growth conditions for all the experiments were 37 °C and 200 rpm. For the oxygen-limitation, there was no external gas connected. *E. coli* cells were cultivated in 500 mL baffled flasks filled with 200 mL glucose KE medium with a starting OD_{600} of 0.2. After 2-hour incubation, cells were harvested via centrifugation (5,000 rpm, 10 min) at 37°C. Then cells were inoculated into bioreactor filled with 2 L KE minimal medium with a starting OD_{600} 0.05, which was considered as time 0. Samples were collected by centrifugation. The pellets were used for *gadA/gadB* mRNA and GadA/GadB protein analysis, while the supernatants were used for external metabolites analysis.

For the normal aerobic condition, the *E. coli* cells were cultivated in baffled flasks with aeration 200 rpm on a rotatory shaker. For the normal oxygen-limitation, *E. coli* cells were cultivated in the closed falcon tube fully filled with medium.

2.4 qRT-PCR

RNA was purified by using the phenol-chloroform-isoamyl alcohol (PCI) protocol (Ares, 2012) with modifications. The pellets of the bacteria were washed in 1 mL of ice-cold AE buffer (20 mM sodium acetate buffer, pH 5.2, 1 mM EDTA) and resuspended in 500 µL of the same buffer. Then 500 µL pre-warmed (60°C) PCI (Roth, X985) and 25 µL 10% (w/v) SDS in RNase free water were added. Then the mixture was incubated at 60°C for 5 minutes in Thermomixer shaking at 1000 rpm. After that, the samples were put on ice for 2 hours and centrifuged for 1 hour at 16,000g, 4°C. Then supernatant was transferred into phase-lock tubes (Quanta). 1.0x volume of PCI and 0.1x volume of 3 M sodium acetate (pH 5.2) were added. The mixture was mixed gently and centrifuged for 15 minutes at 16,000g, 4 °C. The supernatant was collected and mixed with cold ethanol (final concentration of 75%) and stored at -80°C overnight. The samples were centrifuged for 1 hour at 16,000g, 4°C, and the supernatant was discarded. Then the pellets were washed twice with 75% (v/v) ethanol, dried and resuspended in 100 µL of RNase-free water. RNA concentration and purity were checked using a Nanodrop spectrophotometer (ND-1000, PeqLab, Germany). Then the RNA samples were treated with the TURBO DNA-free Kit (Invitrogen, Germany) to remove the contained genomic DNA. The quality and concentration of the cleaned RNA samples were measured again by the Nanodrop spectrophotometer. Complementary DNA (cDNA) was synthesized from 2 µg of total RNA by using the iScript Advanced Script (Bio-Rad) according to the manufacturer's protocol. Then 3 µL of the 1:10 diluted cDNA mixed with 0.75 µL forward primer, 0.75 µL reverse primer, 7.5 µL of the 2x-SsoAdvanced Univ SYBR Green Supermix (Bio-Rad) and 3 µL of the RNase free water were dispensed into a 96-well PCR plate (Bio-Rad). The qPCR was

performed in a Bio-Rad CFX real-time cycler. The ribosome gene of 16S rRNA or *recA* was used as the internal reference to analyze the qRT-PCR data according to the ΔΔCt method (Schmittgen & Livak, 2008). To keep the Ct value in the same range, the template cDNA used to measure the 16S rRNA gene was 10,000 times diluted compared to the sample used to measure *gadA/gadB* mRNA.

2.5 Western blot

The collected cells were adjusted to OD_{600} of 10, then 16 µL of cells mixed with 4 µL loading buffer were boiled for 5 min at 100 °C. Then proteins were fractionated by 12.5% (w/v) sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). Then the proteins from the SDS-polyacrylamide gels were transferred to the nitrocellulose membranes (Amersham, GE Healthcare) using wet blotting. The specific anti-GadA/GadB antibody (Eurogentec) was used to detect GadA/GadB with 1: 5,000 dilution in 3% (w/v) bovine serum albumin solution in 1x TBS buffer [10 mM Tris/HCl pH 7.5, 500 mM NaCl, 0.05% (v/v) Tween 20, 0.2% (v/v) Triton X100]. The 1x TBS buffer and 1x TBST buffer [1x TBS buffer, 0.05% (v/v) Tween 20] was used for the washing steps. The Donkey Anti-rabbit IgG H&L (IRDye® 680RD) preadsorbed (Abcam, ab216779) was used as the secondary antibody with 1:20000 dilution. The Odyssey CLx imaging system (LI-COR Biosciences) was used for images and data analysis. The relative GadA/GadB amount was normalized by the reference protein of purified His-tagged GadA.

2.6 High performance liquid chromatography

Quantification of the organic compounds, such as acetate, citrate, formate, lactate, malate and succinate were performed by high performance liquid chromatography (HPLC, Prominence-i LC-2030C, Shimadzu, Kyoto, Japan) equipped with an ion exchange column (Aminex HPX-87H 300 mm x 7.8 mm, Bio-Rad, Hercules, CA, USA) and a refractive index detector (RID-20A, Shimadzu, Kyoto, Japan). For the analysis,

an isocratic flow rate of 0.6 mL min^{-1} with 5 mM H_2SO_4 as mobile phase and a temperature of 60 °C was applied. 10 µL of sample was injected. External standards were used for determining the organic compounds concentration. The HPLC measurement was done in collaboration with Dr. Anna-Lena Heins and Manh Dat Hoang from Technische Universität München.

2.7 Thin-layer chromatography

Quantification of the external glutamate was performed by thin-layer chromatography (TLC) (Krishna et al., 2010). *E. coli* MG1655 cells were cultivated in KE glucose glutamate minimal medium in a bioreactor under air-aeration at pH 7.6. The supernatant samples were collected after centrifugation every hour. The KE glucose glutamate medium was used as the standard. All the supernatant samples were filtered by 0.2 µL filter before performing the TLC measurement. 10 µL of the filtered supernatant samples were loaded per lane on the TLC Silica gel F_{254} plate (Merck, Germany) as 10 mM bands with 8 mM space. The TLC plate was pre-treated with methanol before loading samples. TLC was employed for detecting glutamate using a mobile phase methanol-chloroform-formic acid (5:5:1, v/v) in a TLC twin trough chamber under dark condition. After development, the plate was derivatized by 1% ninhydrin solution in acetone at 60 °C for 5 minutes.

2.8 Glutamate-dependent acid stress resistance assays

The assay was performed by the method of Masuda and Church with modifications (Masuda & Church, 2002). *E. coli* were inoculated into the KE glucose glutamate minimal medium at pH 2.5 with a start OD_{600} of 0.05. The survival was measured by plating serial diluted cultures onto LB agar before and after acid stress at pH 2.5 for 2 hours. The sample before acid stress was considered as time 0. The survival percentage was calculated by using the number of viable cells after acid stress for 2

hours divided by the number of viable cells of time 0. For different purpose, the preculture was prepared in different ways, which are described as follows.

To test the effects of the deletion of different *gad* genes on *E. coli* MG1655 survival of at pH 2.5, overnight cultures grown in 100 mL-baffled flasks in KE glucose glutamate minimal medium (pH 5.8) were inoculated to OD_{600} of 0.05 into fresh KE glucose glutamate minimal medium (pH 2.5).

To test the effects of the oxygen-limitation on the survival of *E. coli* MG1655 at pH 2.5, cells were cultivated under oxygen-limitation and aerobiosis. The oxygen-limitation was achieved by cultivating cells in a Falcon tube fully filled with medium, while the aerobiosis condition was achieved by cultivating cells in100 mL-baffled flasks filled with 25 mL medium. Overnight cultures were grown in KE glucose glutamate minimal medium (pH 5.8), then they were inoculated to OD_{600} of 0.05 into fresh KE glucose glutamate minimal medium (pH 2.5).

To test the effects of different acids on the survival of *E. coli* MG1655 at pH 2.5, overnight cultures were grown in KE glycerol glutamate minimal medium (pH 7.6), then they were diluted to OD_{600} of 0.05 into the fresh medium. After 5 hours, during the exponential growth phase 50 mM acid was added, 2 hours later *E. coli* cells incubated with different acids treatment were transferred into the fresh KE glucose glutamate minimal medium (pH 2.5).

To test the effect of different acids on the survival of *E. coli* under extreme acid stress, the glucose fermentation products and acid adaptation should be avoided. For this purpose, the glycerol minimal medium (pH 7.6) was chosen. Overnight cultures were diluted to OD_{600} of 0.05 into fresh medium. After 5-hour incubation, the cells were in the exponential growth phase. Then 50 mM acids were added into the cultures for 2-hour incubation.

2.9 Reporter fusion assay

E. coli cells transformed with reporter fusion were cultivated for 5 hours in KE glycerol glutamate minimal medium with start OD_{600} of 0.05 at pH 7.6 in baffled flasks with aeration 200 rpm on a rotatory shaker. Then 50 mM of different acids was added into the exponentially growing cultures. Then the expression of reporter fusions in *E. coli* cells was measured by using the a TECAN Spark 20 M plate reader or an infinite 200Pro plate reader measuring the optical density OD_{600} and fluorescence intensity in a 96 well plate at 37 °C with orbital shaking for 3 hours. The data were expressed as relative fluorescence unit (RFU) referring to Fluorescence Unit divided by optical density OD_{600}. The expression level of the reporter fusion was determined by the RFU.

The eGFP fluorescence intensity was measured with 485 nm excitation wavelength and 535 nm emission wavelengths. The mCherry fluorescence intensity was measured with 560 nm excitation wavelength and 612 nm emission wavelengths. The settings of the two machines are different. When using the TECAN Spark 20 M plate reader, the eGFP fluorescence intensity was measured with manual gain 45%. The mCherry fluorescence intensity was measured with manual gain 80%. When using the infinite 200Pro plate reader, the eGFP fluorescence intensity was measured with manual gain 50%. The mCherry fluorescence intensity was measured with manual gain 100%. The data can only be compared when they were measured in one machine, but not be compared between the two machines.

For the different purpose, the *E. coli* strains, reporter fusions, cultivation conditions and pretreatments of the cells were different. Notably, here is one case we used the glucose replace the glycerol as carbon source. To confirm the effect of the MnmE, MnmG, RcsB, YpdB on the expression of the GDAR system in *E. coli* in response to SCFAs, the mutants as well as the parental strain *E. coli* BW25113 harboring the reporter fusion were complemented with the pCA24N plasmid expressing the related gene from the ASKA collection (Kitagawa et al., 2005) were cultivated in the KE glucose glutamate

medium for 3 hours. *E. coli* strains transformed with two plasmids displayed the strong growth defect in glycerol medium, so, we used glucose instead of glycerol, and we shorted the cultivation time from 5 hours to 3 hours to keep the *E. coli* cells still in the exponential phase and lower level of excreted acetate. The IPTG was added after 2 hours of incubation to induce the expression of the target genes in the pCA24N plasmid.

2.10 Single-cell fluorescence microscopy and data analysis

The cells were taken after 3 hours of incubation with acetic acid, propionic acid or butyric acid in the TECAN reader. 4 µL of the culture was spotted on an agarose pad [1% agarose (wt/vol) in PBS] and covered with a coverslip. Images were taken on a Leica DMi8 inverted microscope with a DFC365 Fx camera (Leica). The eGFP fluorescence intensity was measured with 485 nm excitation and 510 nm emission wavelength, and the mCherry fluorescence was measured with excitation wavelength of 546 nm and a 605 nm emission. The eGPF and mCherry fluorescence intensity were both measured with exposure of 150 ms and gain of 2. The images were analyzed with the ImageJ (Schneider, Rasband, & Eliceiri, 2012) using the plug-in MicrobeJ (Ducret, Quardokus, & Brun, 2016). The settings of MicrobeJ followed the previous description from our group: the default settings for cell segmentation (fit shape, rod-shaped bacteria) with the exceptions of 0.1 to max m^2 area, 1.2 – 5 µm length, 0.1 – 1 µm width, 0 – 0.15curvature, and 0 – 0.25 angularity (Brameyer et al., 2020). The correlation between the expression of *gadA* and *gadB* was analyzed by computing Pearson correlation coefficient (r). The correlation plots were created by the Correlation matrix analyses using Graphpad Prism 8.4.3.

3. Results

3.1 Systematic analysis of the expression profile of the GDAR system in *E. coli*

3.1.1 The effects of different *gad* genes on the GDAR system

The GDAR system, consisting of the two homologous glutamate decarboxylases GadA and GadB and the glutamate/GABA antiporter GadC, is regulated by the central regulator GadE. To investigate the effect of different *gad* genes on the expression of the GDAR system, GadA/GadB protein production and the growth were measured in *E. coli* MG1655 wild type strain, as well as different mutants. The deletion of *gad* genes did not affect the growth of *E. coli* MG1655 at physiological pH of 7.6 in a glucose minimal medium (Figure 3.1A). The GadA/GadB was not detectable in the Δ*gadE* mutant (Figure 3.1B), which is consistent with the previous conclusion that *gadE* is required for the *gadA/gadB* expression (Z. Ma et al., 2003). The Δ*gadA* and Δ*gadBC* mutants both showed around 50% GadA/GadB protein production compared to the WT strain (Figure 3.1B), indicating that the *gadA* and *gadB* express equally during the stationary phase at physiological pH. In addition, the Δ*gadC* mutant did not show a significant effect on GadA/GadB production (Figure 3.1B), indicating the GadA/GadB expression is independent of GadC.

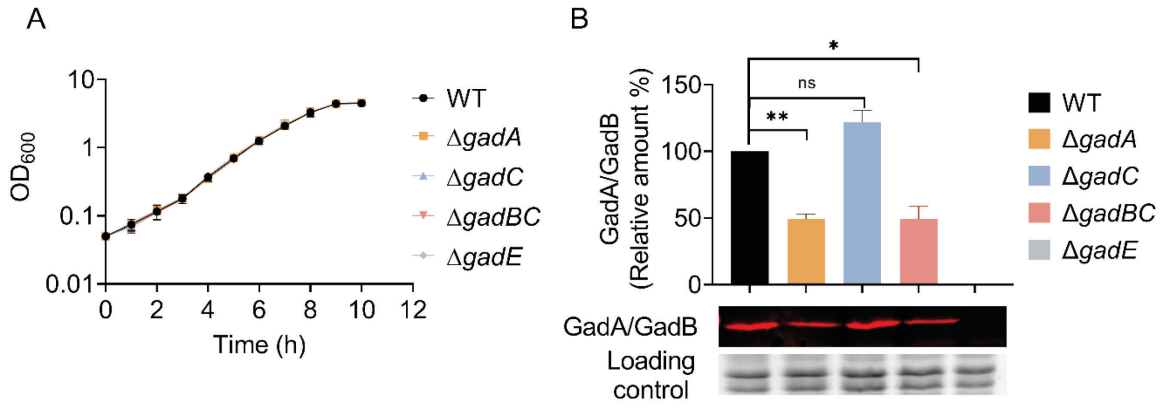


Figure 3.1. The effects of different *gad* genes on the GadA/GadB protein production in *E. coli* MG1655. (**A**) The growth curve of *E. coli* MG1655 and different mutants. Cells were cultivated in a 250 mL baffled flask filled with 50 mL KE glucose minimal medium supplemented with 1.5 mM glutamate at pH 7.6. All cultures were grown in baffled flasks with 200 rpm aeration on a rotatory shaker. (**B**) The GadA/GadB expression of *E. coli* MG1655 and different mutants was measured by Western Blot. Cells were collected for the Western Blot analysis after 10-hour incubation. 16 µL of cells adjusted to OD_{600} of 10 was loaded into each lane. The SDS PAGE was stained by TCE. The Western Blots were performed using the specific antibody against GadA/GadB. The relative amount of GadA/GadB was normalized to wild type strain (mutant / wild type strain). The experiments were done in triplicate. Statistical analysis was done by using a Two-tailed paired t-test. ****p<0.0001. Mean ± SD is shown. SD, standard deviation.

To confirm the biological function of the GDAR system on *E. coli* MG1655 under extreme acid stress (pH 2.5) in the presence of glutamate, we analyzed the survival of stationary phase cells of *E. coli* MG1655 wild type strain and different mutants, which were cultivated in KE glucose glutamate minimal medium (pH 5.8). Δ*gadE,* Δ*gadC* and Δ*gadBC* mutants were not able to survive at pH 2.5, and the Δ*gadA* mutant significantly lowed the survival level than the wild type strain (Figure 3.2). The survival data combined with the protein production results (Fig. 1B) indicate that the GDAR system is required for *E. coli* MG1655 survival under extreme acid stress, and the antiporter GadC is essential for the function of the GDAR system. The deletion of *gadA* only caused lower level survival, but did not abolish the survival, which indicates that both GadA and GadB play roles in the GDAR system and their function are complemented in some degrees. Our data is consistent with the previous study that the *gadC* mutant blocked the GDAR system (M. P. Castanie-Cornet et al., 1999). The lower survival level of *gadA* mutant is consistent with the previous study (Jung & Kim, 2003).

Taken together, our data indicated that the GDAR system plays a major role in *E. coli* MG1655 under extreme acid stress (pH 2.5). The central regulator GadE is essential

for the expression of *gad* genes, and the antiporter GadC is required for the biological function of the GDAR system. In addition, our data proved that homologous glutamate decarboxylases GadA and GadB express equally and independently of GadC in *E. coli* MG1655 when cultivated in glucose glutamate minimal medium at physiological pH (pH 7.6).

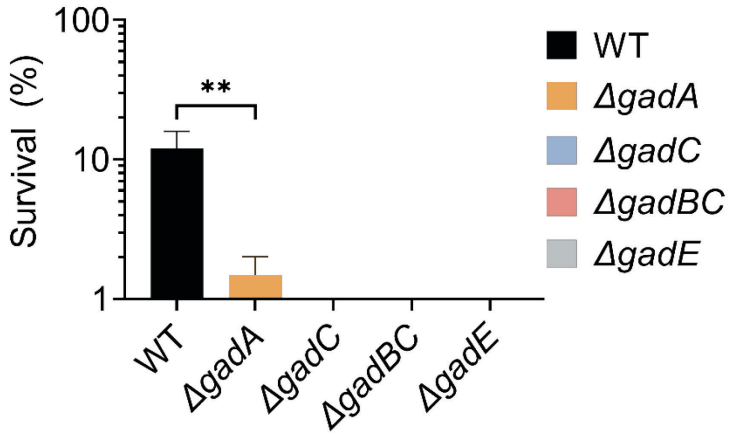


Figure 3.2. The effects of the deletion of different *gad* genes on the survival of *E. coli* MG1655 at pH 2.5. Overnight cultures were grown in 100 mL-baffled flasks in KE glucose glutamate minimal medium (pH 5.8). They were diluted into fresh KE glucose glutamate minimal medium (pH 2.5) with the starting OD_{600} of 0.05. Viable cells were measured by plating serial diluted cultures onto LB agar after 0 and 2 hours of pH 2.5 acid stress. The percentage survival was calculated using the reference of the viable cells of time 0. This experiment was done in triplicate. Statistical analysis was done by using a two-tailed unpaired t-test. ****p<0.0001. Mean ± SD is shown. SD, standard deviation.

3.1.2 The activation profile of the GDAR system in *E. coli* MG1655 under the aerobic condition at physiological pH

The GDAR system is the most powerful acid resistance system in *E. coli* under extreme acid stress. So, it is essential to reveal its activation profile, which can help researchers to reveal the primary stimuli, understand the activation mechanism and use the GDAR

system to deal with the disease caused by *E. coli* pathogens. Therefore, we studied the kinetic induction of the GDAR system during the growth of *E. coli* MG1655 in a bioreactor. *E. coli* cells were cultivated in KE glucose glutamate minimal medium (pH 7.6) in a bioreactor with 200 rpm air-aeration at 37°C. Every 60 min, samples were collected to quantify the levels of *gadA/gadB* mRNA and GadA/GadB proteins in relation to the optical density (OD_{600}), pH, dissolved oxygen, external glutamate and six major external metabolites. During the transition to the stationary phase, the oxygen was depleted from the medium and the pH dropped shortly after that to pH 6.8 (Figure 3.3A). As the growth rate slowed down, the oxygen consumption of the culture was lower, and due to the constant entrance of oxygen by stirring, the oxygen level increased. Similar pattern of oxygen and pH was proven during the cultivation of *E. coli* cells in glucose minimal medium in a 48-well plate with shaking (Heux, Philippe, & Portais, 2011). In addition, it has also been shown the oxygen drops during the exponential growth phase of *E. coli* cells cultivated in a well-controlled bioreactor (Paczia et al., 2012). Expression of *gadA/gadB* mRNA was transient at the transition to the stationary phase, which started after 5 hours incubation, and the *gadA/gadB* mRNA level was keeping high from time point 7 h to 8 h (Figure 3.3B). GadA/GadB protein expressed in cells also during the transition to the stationary phase, but one hour later than the mRNA expression (Figure 3.3C & D). Based on the data of the growth condition and the dynamics of the *gadA*/*gadB* mRNA and the GadA/GadB protein, we had the hypothesis that the stationary phase, oxygen limitation and the low pH are external stimuli of the GDAR system. Notably, external glutamate was depleted during stationary phase (Figure 3.3E), which is the same phase of the *gadA/gadB* expression. It seems like that the glutamate was processed to GABA by the GDAR system. The major external metabolites of *E. coli* under this condition were acetate, formate and a small amount of succinate, while lactate, citrate and malate were not detectable (Figure 3.3F). The acetate secretion occurred during the exponential growth phase of *E. coli*, and the extracellular acetate concentration reached to a maximum

value of 20 mM at the stationary phase (time point 7 h), then *E. coli* started to take up acetate until it was depleted (Figure 3.3F). The dynamics of acetate can be explained by the acetate switch (Wolfe, 2005). *E. coli* cells consume glucose and dissimilate acetate during the exponential growth phase, then the acetate switch occurs to assimilate both acetate and the remaining glucose before the glucose runs out. Furthermore, the pH increased to pH 7.2 at the stationary phase (Figure 3.3A), which is related to the dynamics of acetate. *E. coli* also secreted formate during the growth, and the concentration reached the maximum value of 17mM at the stationary phase (time point 7h) and was kept in a high level (Figure 3.3F). The dynamics of external acetate and formate match the *gadA/gadB* expression profile, indicating that acetate and formate might be external stimuli of the GDAR system.

In this part, we systematically analyzed the *gadA/gadB* expression at both transcriptional and translational levels in relation to the optical density, pH, dissolved oxygen, external glutamate, and six major external metabolites. This synchrony between the changes in the external conditions and the levels of *gadA/gadB* expression suggests that the stationary phase, low pH, and oxygen limitation may affect the activation of the GDAR system. These findings are supported by previous reports from other labs (Castanie-Cornet et al., 1999; De Biase et al., 1999; Hayes et al., 2006).

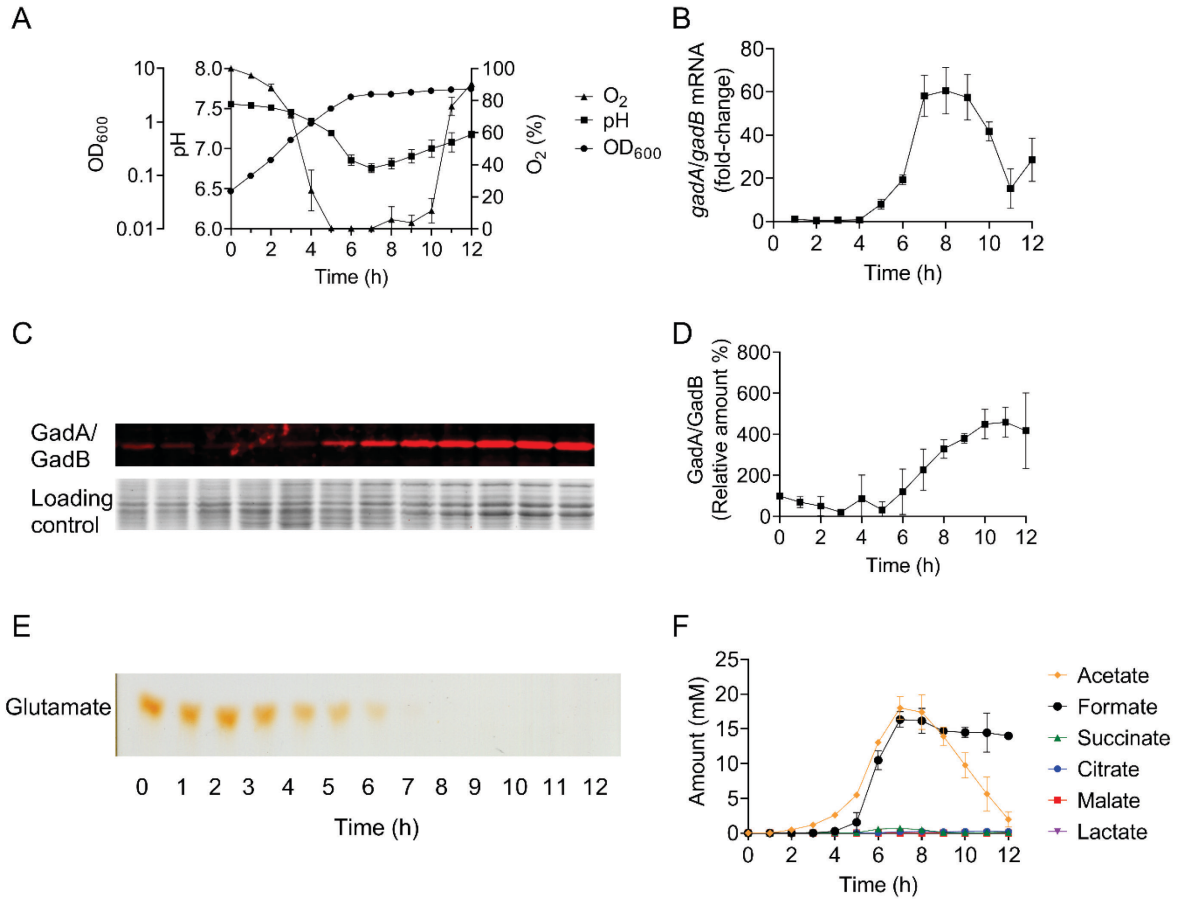


Figure 3.3 Expression profile of the GDAR system in *E. coli* MG1655 under air-aeration. (A) The dynamics of OD_{600}, external pH and dissolved oxygen. Firstly, the overnight culture was inoculated into the baffled flasks filled with KE glucose glutamate minimal medium at pH 7.6 starting at OD_{600} of 0.1 for 2 hours. Then the cells were collected by centrifugation and inoculated into the bioreactor under the air-aeration at 37 °C. The dissolved oxygen and pH were calibrated before inoculation and measured by the bioreactor automatically, while the optical density was measured by spectrophotometer. **(B)** The dynamics of the *gadA/gadB* mRNA. The *gadA/gadB* mRNA was analyzed by qRT-PCR, the 16S rRNA gene was used as the internal reference, fold-change was normalized to time 1. To keep the Ct value in the same range, the template cDNA used to measure the 16S rRNA gene was 10,000 times diluted compared to the sample used to measure *gadA/gadB* mRNA. **(C) & (D)**The dynamics of GadA/GadB measured by Western Blot. The SDS gel was stained by TCE. 16 μL of cells adjusted to OD_{600} of 10 was loaded into each lane. The GadA/GadB amount was analyzed by Western Blot with a specific anti-GadA/GadB antibody, the relative amount was normalized to time 0. **(E)** The dynamics of the external

glutamate. Quantification of the external glutamate was performed by thin-layer chromatography (TLC). The supernatant was collected after centrifugation and filtered by 0.2 µL filter. 10 µL of the filtered supernatant was loaded per lane on the TLC plate. TLC was employed for detecting glutamate using a mobile phase methanol-chloroform-formic acid (5:5:1, v/v). The plate was derivatized by 1% ninhydrin solution in acetone at 60 °C for 5 minutes. **(F)** The dynamics of the external metabolites. Quantification of the external metabolites was performed by high performance liquid chromatography (HPLC). An isocratic flow rate of 0.6 mL min^{-1} with 5 mM H_2SO_4 as mobile phase and a temperature of 60 °C was applied for the analysis. 10 µL of the supernatant sample was injected. External standards were used to determine the concentration of the organic compounds. These experiments were done in triplicate (except figure 3.3E). Mean ± SD is shown. SD, standard deviation.

3.1.3 The oxygen limitation, acid stress and stationary phase induce the expression of the GDAR system in *E. coli*

A systematic analysis is still lacking despite numerous studies about the GDAR system under different conditions. So, we analyzed the effect of oxygen limitation and low pH on the activation of the GDAR system in more detail. Firstly, *E. coli* MG1655 cells were cultivated in the bioreactor as described above, but either under oxygen-excess or oxygen-limitation at physiological pH of 7.6 or at an acidic pH of 5.8. The oxygen-excess was achieved by providing additional oxygen, which kept high oxygen level in the medium. The oxygen-limitation was achieved by cultivating *E. coli* in a degassed medium in the bioreactor with stirring. The last traces of oxygen were consumed by cells within the first hour of growth (Figure 3.4A). The oxygen-limitation has negative effects on the growth at both pH 7.6 and pH 5.8, and the impact of the oxygen-limitation is stronger than acid stress (Figure 3.4B). The medium pH dropped to 6.9 (time point 7h), then increased to pH 7.2 at the stationary phase under oxygen-excess. However, under oxygen-limitation and oxygen-excess at acidic pH of 5.8, the external pH only decreased during growth, but did not increase during the stationary phase (Figure 3.4C). GadA/GadB synthesis was strongly induced by oxygen-limitation at both

physiological pH of 7.6 and acidic pH of 5.8, however, the GadA/GadB expression level was relatively low under oxygen-excess even during the stationary phase and under acidic pH (Figure 3.4D & E), indicating the oxygen-limitation is required for the expression of the GDAR system. The GadA/GadB expression level was higher at pH 5.8 than pH 7.6 during the exponential growth phase under oxygen-limitation, which indicates that the acid stress is an external stimulus of the GDAR system. The expression level of GadA/GadB (at neutral pH) in stationary phase is higher than that in exponential growth phase (Figures 3.4D & E). This result also confirmed that the stationary induces the expression of the GDAR system at neutral pHs.

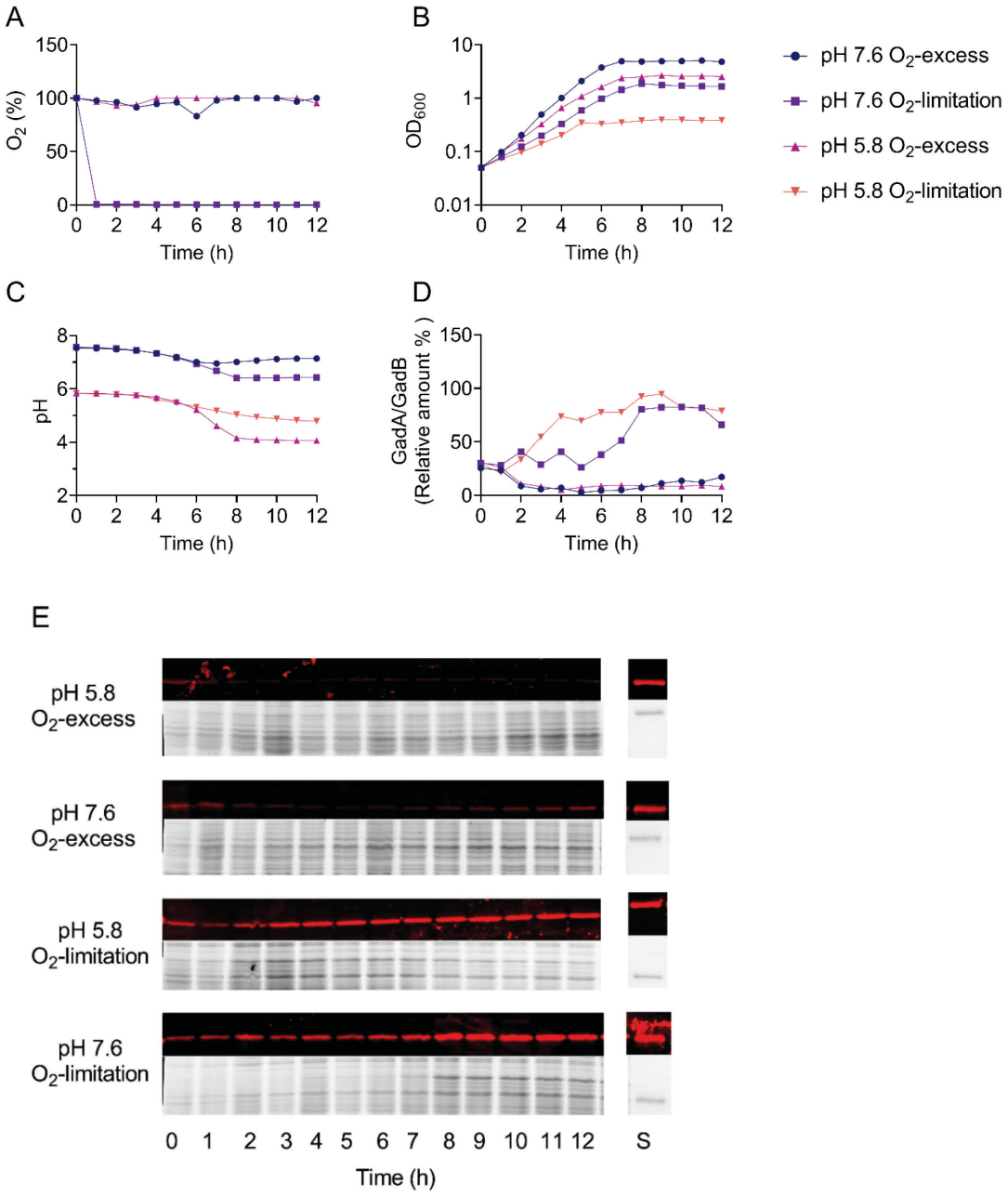


Figure 3.4 The influence of oxygen and acid stress on the GadA/GadB expression in *E. coli* MG1655 cultivated in a bioreactor. *E. coli* MG1655 cells were cultivated in KE glucose glutamate minimal medium at physiological pH of 7.6 or acidic pH of 5.8 in a bioreactor under oxygen-excess or oxygen-limitation. For the oxygen-excess, the bioreactor was connected to extra oxygen to maintain high oxygen level. For the oxygen-limitation, there was no external gas connected. **(A)** The dynamics of dissolved oxygen. **(B)** The growth curve of *E. coli* MG1655. **(C)** The dynamics of external pH. **(D)&(E)** The dynamics of the GadA/GadB protein production. S, standard. The dynamics of GadA/GadB were

measured by Western Blot. The SDS gel was stained by TCE. 16 µL of cells adjusted to OD_{600} of 10 was loaded into each lane. The GadA/GadB amount was analyzed by Western Blot with a specific anti-GadA/GadB antibody, the relative amount was normalized to the same amount of the standard purified His-GadA. The experiment was done in duplicate, the average values were presented.

In addition, the effect of the stationary phase was confirmed by the activation profile of the GDAR system in *E. coli* cultivated in the carbon-limitation medium in the bioreactor under the air-aeration at pH 7.6. The carbon-limitation induced an earlier onset of the stationary phase compared to cultivation in carbon-rich media (Figure 3.5A & Figure 3.3A). The GadA/GadB expression occurred at the stationary phase (time point 5 h) (Figure 3.5B), which is earlier than that in carbon-rich media (time point 6 h) (Figure 3.3). These results are consistent with the previous conclusion that the stationary phase induced *gadA/gadB* expression (De Biase et al., 1999).

In summary, here we proved that the oxygen-limitation, acidic pH and stationary phase all induce the expression of the GDAR system. Among these three conditions, the oxygen-limitation displayed the most potent induction ability, followed by the stationary phase, and the acidic pH.

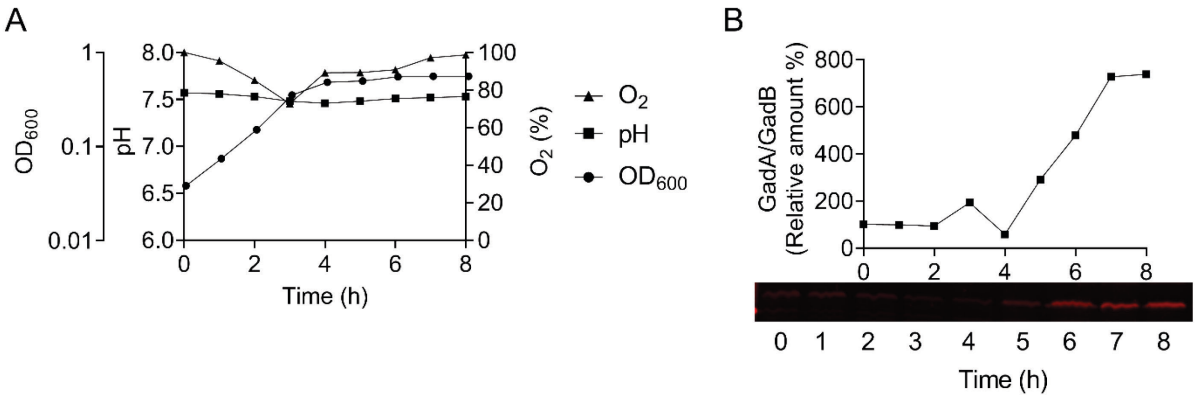


Figure 3.5 The activation profile of the GadA/GadB in *E. coli* MG1655 grown under carbon-limitation at pH 7.6 in a bioreactor. *E. coli* MG1655 cells were cultivated in KE glucose (0.04%) glutamate minimal medium at physiological pH of 7.6 under air-aeration conditions. **(A)** The dynamics of OD_{600}, external pH and dissolved oxygen. **(B)** The dynamics of GadA/GadB were measured by

Western Blot. 16 µL of cells adjusted to OD_{600} of 10 was loaded into each lane. The GadA/GadB amount was analyzed by Western Blot with a specific anti-GadA/GadB antibody, the relative amount was normalized to time 0. This was one single experiment.

To confirm the effect of oxygen-limitation on the expression of the GDAR system, we also measured the GadA/GadB expression in *E. coli* MG1655 cultivated in standard baffled flasks and Falcon tubes fully filled with a medium at both physiological pHs of pH 7.6 and acidic pH of 5.8, which mimicked the aerobiosis and oxygen-limitation condition, respectively. GadA/GadB expression was significantly increased under oxygen-limitation than aerobiosis at both physiological pH of 7.6 and acidic pH of 5.8 (Figure 3.6), which is consistence to the results of *E. coli* MG1655 cultivated in a bioreactor (Figure 3.4). In addition, the acid stress also induced the expression of GadA/GadB under the oxygen-limitation, confirming that the low pH is one external stimulus.

To test the correlation of the GadA/GadB expression and its physiological function as a resistance mechanism for extreme acid stress, we also analyzed the effect of oxygen-limitation on survival of *E. coli* MG1655 under extreme acidic stress at pH 2.5 in the presence of glutamate. The survival of *E. coli* MG1655 pre-cultivated under oxygen-limitation was 8, 35 and 130 times higher than it pre-cultivated under aerobiosis after the extreme acid stress of 2, 4 and 6 hours, respectively (Figure 3.7A). The survival of Δ*gadA* mutant also significantly increased after the pre-cultivation under oxygen-limitation compared to the aerobiosis (Figure 3.7B). In addition, the *gadA* deletion showed less effect on the survival after pre-cultivated under oxygen-limitation than aerobiosis (Figure 3.7B). So, the survival assay supports the conclusion that the oxygen-limitation induces the expression of the GDAR system.

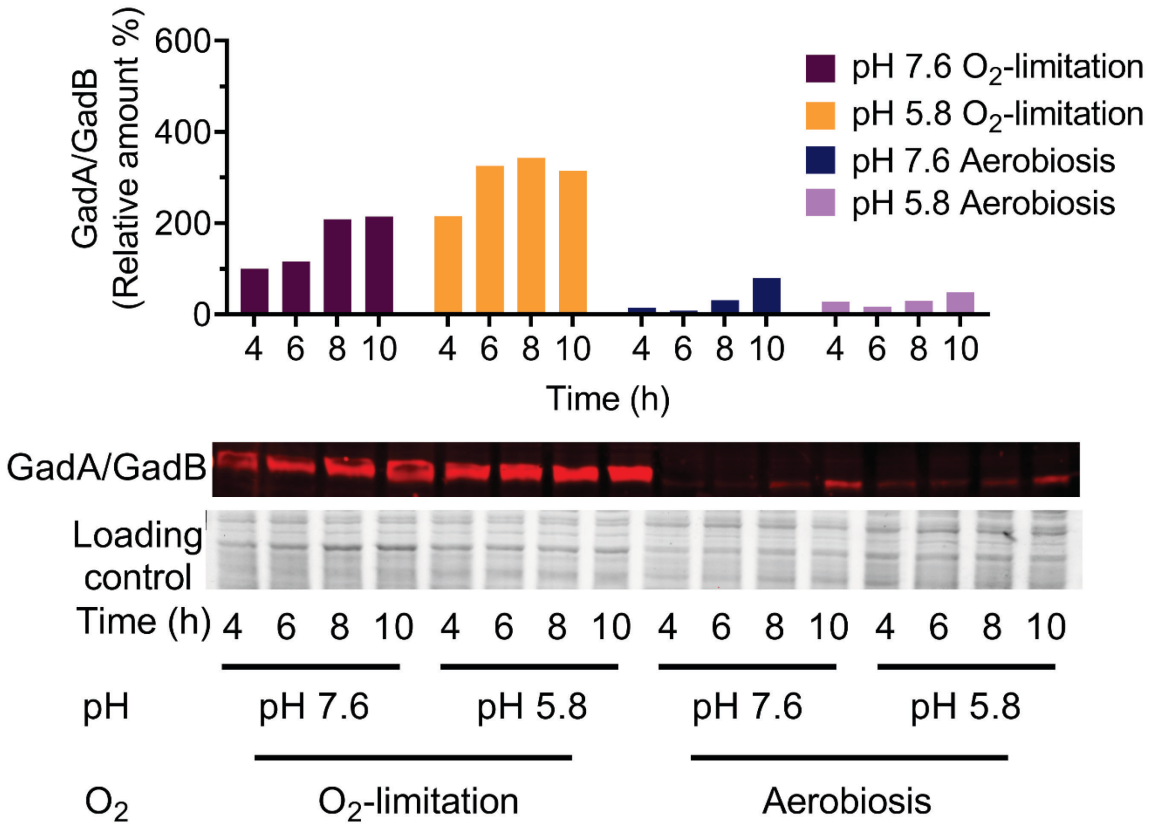


Figure 3.6. The influence of oxygen-limitation and acid stress on the expression of GadA/GadB in *E. coli* MG1655 cultivated in baffled flasks and Falcon tube. *E. coli* MG1655 cells were cultivated in KE glucose glutamate minimal medium at pH 7.6 or 5.8. The aerobiosis condition was achieved by growing *E. coli* MG1655 cells in a 250 mL baffled flask filled with a 50 mL medium with aeration (200 rpm). The oxygen-limitation was achieved by cultivating *E. coli* MG1655 in a Falcon tube fully filled with medium. Overnight culture from pH 7.6 was inoculated into the fresh medium (pH 7.6 or 5.8) with a start OD_{600} of 0.05. The samples were collected after 4, 6, 8, 10 hours of incubation. The Western Blot analysis was performed as described in Figure 3.1. The relative amount was normalized to pH 7.6 oxygen-limitation 4 h. The experiments were done in duplicate. The average value of the duplicate is shown.

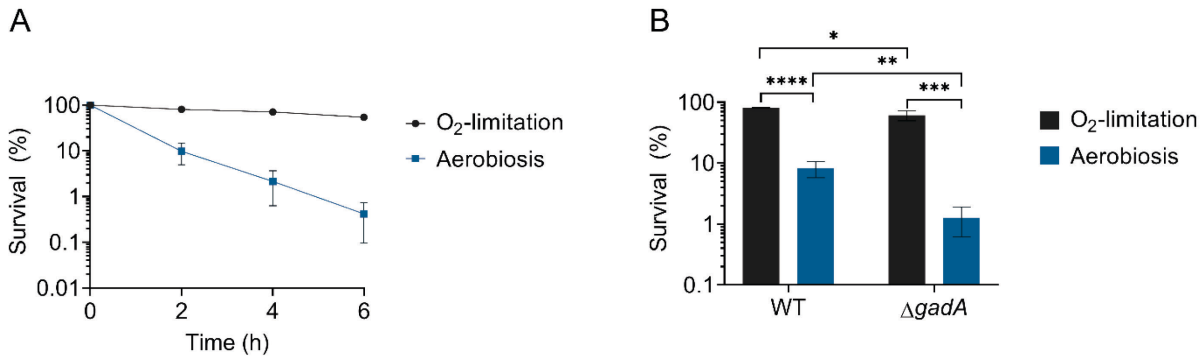


Figure 3.7. O_2-limitation increases the survival of *E. coli* MG1655 under extreme acid stress of pH 2.5. (A) The effect of the oxygen-limitation on the survival of *E. coli* MG1655 at pH 2.5. **(B)** The effect of oxygen-limitation on the survival of wild type strain and Δ*gadA* mutant of *E. coli* MG1655 at pH 2.5. The oxygen-limitation was achieved by cultivating cells in a Falcon tube fully filled with medium, while the aerobiosis was achieved by cultivating cells in100 mL-baffled flasks filled with 25mL medium. Overnight cultures were grown in KE glucose glutamate minimal medium (pH 5.8), then they were diluted into fresh KE glucose glutamate minimal medium (pH 2.5) with the starting OD_{600} of 0.05. Viable cells were measured by plating serial diluted cultures onto LB agar before and after the acid stress of pH 2.5. The percentage survival calculated by using time 0 as the reference. This experiment was done in triplicate. Statistical analysis was done by using two-tailed unpaired t test. ****p<0.0001. Mean ± SD is shown. SD, standard deviation.

Until now, we compared the GadA/GadB expression in *E. coli* MG1655 cultivated under oxygen-limitation and oxygen-excess in bioreactors, as well as the oxygen-limitation in closed Falcon tube filled with medium and normal aerobiosis condition in a baffled flask, and the effect of oxygen-limitation on the survival of *E. coli* MG1655 wild type strain and Δ*gadA* mutant at pH 2.5. All the data support that the oxygen-limitation strongly induced the expression of the GDAR system in *E. coli* MG1655.

3.2 The external stimuli of the GDAR system

3.2.1 The short chain fatty acids are external stimuli of the GDAR system

Under oxygen-limitation, *E. coli* converts glucose to mixed fermentation products, mainly including acetate, formate, ethanol, lactate, and smaller amounts of succinate (Clark, 1989). Our data have proved that the *E. coli* MG1655 cells met the oxygen-limitation during the cultivation in a bioreactor under air-aeration, and the major external metabolites are acetate and formate as well as small amounts of succinate (Figure 3.3F). Furthermore, we have proved that the GDAR system can be strongly induced under the oxygen-limitation, mainly because of the mixed fermentation. In addition to the organic acid produced by mixed fermentation of themselves, *E. coli* cells have to face the stress of propionic acid and butyric acid which are produced by the fermentation of other obligate bacteria in the human colon (Macfarlane & Gibson, 1997; Tobe et al., 2011). Based on the previous studies that acetate induced the *gad* genes expression (Arnold et al., 2001), and our activation profile data about the *gad* genes, we proposed the hypothesis that the fermentation products of the mixed acid fermentation of *E. coli* such as acetic acid and formic acid, might also be external stimuli of the GDAR system.

The transcript levels of *gadB* and *gadA* are almost indistinguishable (sequence identify of about 97.8%). *gadB*, located at 33 min (1.57 Mb), is a product of gene duplication, and *gadA* is located at 78 min (3.67 Mb) on the chromosome as part of an acid fitness island. Their upstream regulatory sequences are similar but not identical in structure (Bergholz, Tarr, Christensen, Betting, & Whittam, 2007). To distinguish the activation of the two genes, we constructed promoter fusions with two different fluorophores (P_{gadB}:*mcherry* and P_{gadA}:*egfp*). The two fusions were generated to one plasmid, named pBBR1MCS4- P_{gadB}:*mcherry*- P_{gadA}:*egfp*.

To test our hypothesis and find external stimuli of the GDAR system, we systematically analyzed the activation of *gadB* and *gadA* promoters in exponentially growing *E. coli* MG1655 cells after exposure to the short-chain fatty acids (SCFAs) acetic, propionic, and butyric acids, and as well as organic acids, namely succinic, lactic, formic, and citric acids. To avoid the effect of mixed fermentation product of glucose, we switched the carbon source from glucose to glycerol. To prevent the effect of stationary phase and oxygen-limitation, we chose the exponential growth phase cells instead of the stationary phase cells. *gadB* and *gadA* promoter activities were increased after all the tested acid treatments, confirming that the GDAR system responds to acid stress. *gadB* promoter activity was significantly increased in *E. coli* cells after adding SCFAs, namely acetic, propionic and butyric acid, compared to the control of adding the same amount of hydrochloric acid (Figure 3.8A), which indicated that the SCFAs in particular are external stimuli of the GDAR system. *gadA* promoter activity was significantly induced by acetic acid and propionic acid, but not by butyric acid (Figure 3.8B). The succinic acid and the formic acid caused the increase of the *gadB* or *gadA* promoter activities in the same range of the control hydrochloric acid, while the lactic acid and citric acid caused the increase of the *gadB* or *gadA* promoter activities under the range of the control hydrochloric acid. The pH values of the media remained constant between 5.9 and 6.8 (in more detail 6.64, 6.61, 6.71, 5.97, 6.61, 6.55, or 6.62, respectively) after adding 50 mM acetic, propionic, butyric, succinic, lactic, formic, or hydrochloric acid, respectively. Although the pH value after the addition of citric acid was even lower, pH 4.57, the P_{gadB}:*mcherry* and P_{gadA}:*egfp* activities were lower than the control hydrochloric acid. This result indicated that the decrease of the external pH by the SCFAs acetic acid, propionic acid, and butyric acid was not the main reason for activating the expression of the GDAR system. The SCFAs activate the GDAR system might work because of the property of the weak acid which causes the decrease of the intracellular pH or the molecular structure of the SCFAs.

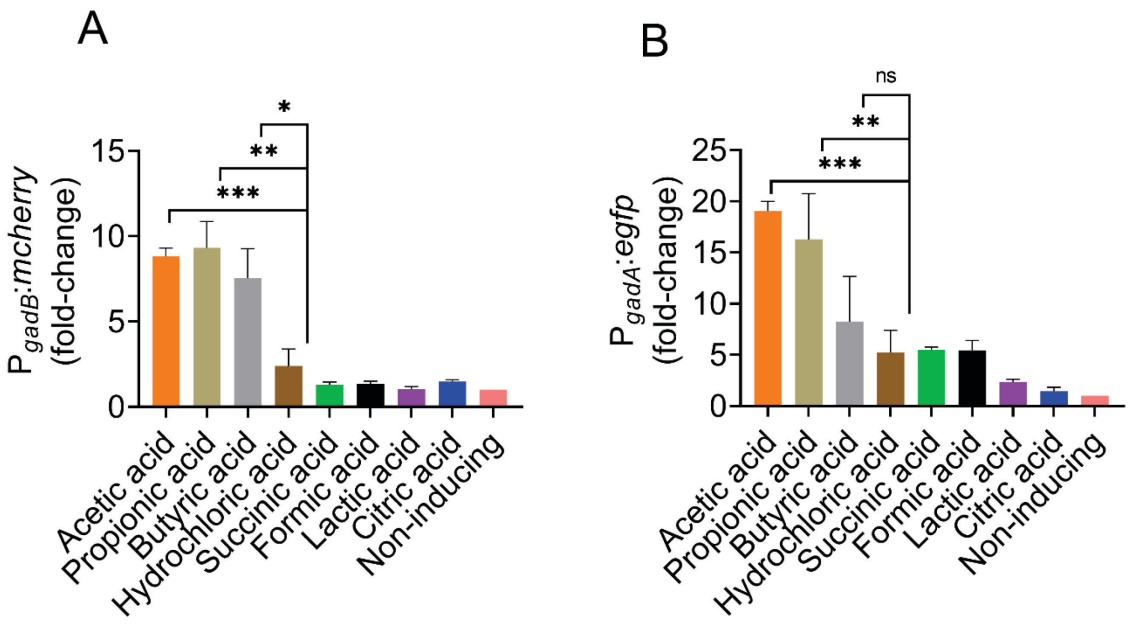


Figure 3.8. The effect of different acids on *gadB* and *gadA* promoter activity in *E. coli* MG1655. **(A)** The effect of different acids on *gadB* promoter activity. **(B)** The effect of different acids on *gadA* promoter activity. *E. coli* MG1655 cells were transformed with plasmid-based fluorescent, promoter fusion of P_{gadB}:*mcherry*-P_{gadA}:*egfp*. 50mM acid was added into the cultures after 5-hour incubation (exponential growth phase) with the start OD_{600} of 0.05 in glycerol glutamate minimal medium (pH 7.6). The *gadB* and *gadA* promoter activities were tested by measuring the fluorescence and OD_{600} using a TECAN Spark 20 M plate reader after 3-hours incubation. Relative fluorescence unit (RFU) refers to the Relative Fluorescence units divided by OD_{600}. The promoter activity was determined as RFU. The fold change values presented were normalized to non-inducing condition. The experiments were done in triplicate. Statistical analysis was done by using two-tailed unpaired t test. ****p<0.0001. Mean ± SD is shown. SD, standard deviation.

Then we tested the effect of different concentrations of the three SCFAs acetic acid propionic acid and butyric acid on the expression of *gadB* and *gadA* in *E. coli* MG1655. The *gadB* promoter activity increased with the increasing concentration of the SCFAs (Figure 3.9A). The *gadA* promoter activity also increased with the increasing concentration of acetic acid and propionic acid until 40 – 60 mM (Figure 3.9B). However,

butyric acid did not show strong induction on the P_{gadA}*:egfp* (Figure 3.9B). Combined the data of *gadB* and *gadA* promoter activities, we still sticked to the concentration of 50mM in the further analysis.

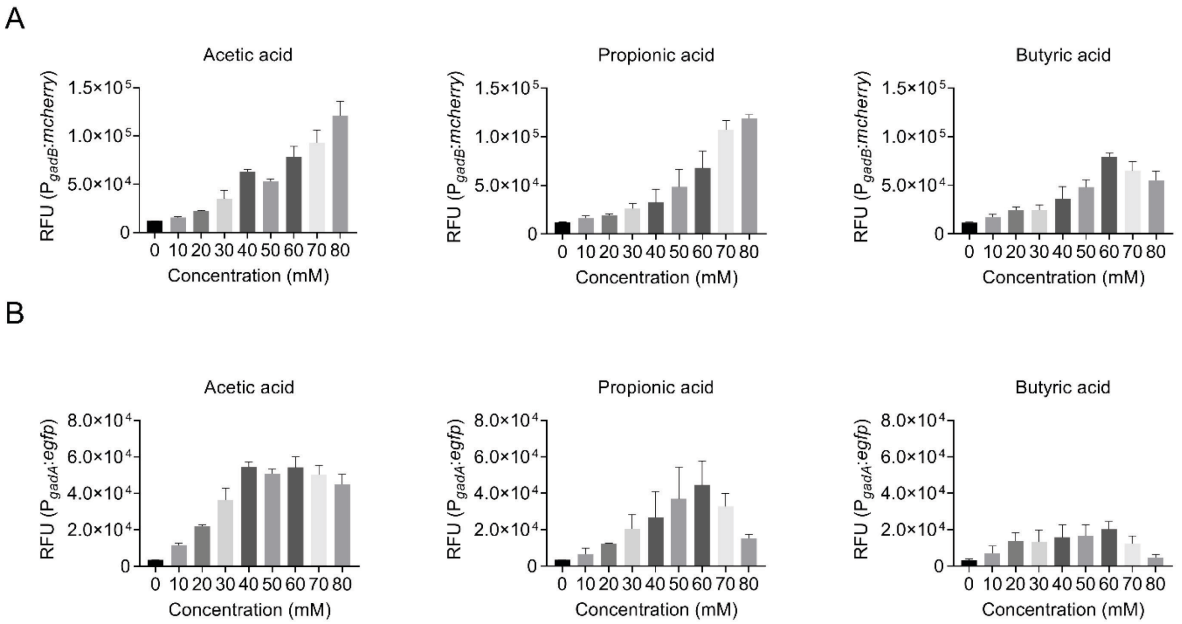


Figure 3.9. The effect of different concentration of the SCFAs on *gadB* and *gadA* promoter activities in *E. coli* MG1655. (A) The effect of different concentration of the SCFAs on *gadB* promoter activity. **(B)** The effect of different concentration of the SCFAs on *gadA* promoter activity. Different acid concentrations were added into the exponential growth phase of the *E. coli* cultures in glycerol glutamate minimal medium (pH 7.6). The *gadB* and *gadA* promoter activities were tested by measuring the fluorescence and OD_{600} using a TECAN Spark 20 M plate reader after 3-hours incubation. RFU, the Relative Fluorescence units divided by OD_{600}. The experiments were performed in triplicate. Mean ± SD is shown. SD, standard deviation.

Gene expression is not only controlled at the transcriptional level, but also controlled at the translational level and proprotein stability (Lange & Hengge-Aronis, 1994). To investigate whether there is post-transcriptional regulation on the *gad* genes, we compared the effect of SCFAs on the expression of *gadA* at both transcriptional and translational levels by using the transcriptional reporter plasmid pPBBR1MCS4-

P$_{gadA}$:*mcherry*, containing the promoter region between -180 bp and -1 bp, and the translational reporter pPBBR1MCS4-GadA:mCherry, including the promoter region and the whole *gadA* gene between -180 bp and +1398 bp. The expression of P$_{gadA}$:*mcherry* and GadA:mCherry in *E. coli* MG1655 was induced by acetic acid and propionic acid, and there was no difference between the transcriptional and translational levels (Figure 3.10), indicating that acetic acid and propionic acid are external stimuli of the GDAR system. The butyric acid significantly induced the expression of P$_{gadA}$:*mcherry* and GadA:mCherry compared to hydrochloric acid, which is different from the result measured by the plasmid pBBR1MCS4-P$_{gadB}$:*mcherry*-P$_{gadA}$:*egfp* (Figures 3.8 – 3.10). The intrinsic green autofluorescence of *E. coli* might affect the quantification of the fluorescence unit (Mihalcescu, Van-Melle Gateau, Chelli, Pinel, & Ravanat, 2015), which might be the reason of the effect of the butyric acid on *gadA* promoter activity was different measured based on the fluorescence of mCherry and eGFP. According to the above data, we proposed that the SCFAs acetic acid, propionic acid and butyric acid all induce the expression of the GDAR system at both transcriptional and translational level.

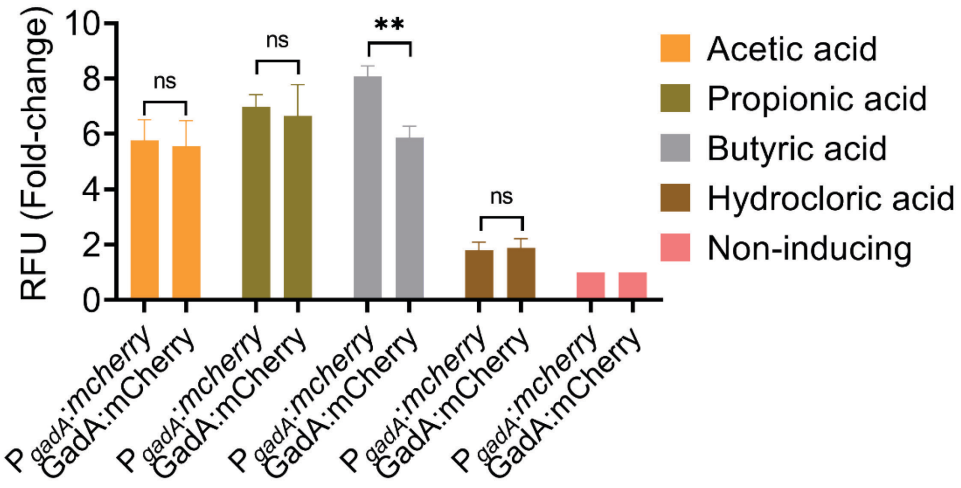


Figure 3.10. The expression of *gadA* at transcriptional and translational levels in *E. coli* MG1655 in response to the SCFAs. *E. coli* MG1655 cells were transformed with the fluorescent reporter plasmid

pPBBR1MCS4-P_{gadA}:*mcherry* or pPBBR1MCS4-GadA:mCherry. 50mM acid was added into the cultures after 5-hour incubation (exponential growth phase) with the start OD_{600} of 0.05 in glycerol glutamate minimal medium (pH 7.6). The fluorescence and OD_{600} were measured by using a TECAN Spark 20 M plate reader after 3-hours incubation. RFU, the Relative Fluorescence unit divided by OD_{600}. The fold change values presented were normalized to non-inducing condition. pPBBR1MCS4-P_{gadA}:*mcherry*, transcriptional fusion. pPBBR1MCS4-GadA:mCherry, translational fusion. The experiments were done in triplicate. Statistical analysis was done by using two-tailed unpaired t test. ****p<0.0001. Mean ± SD is shown. SD, standard deviation.

To test the correlation between the induction of *gadB* and *gadA* in *E. coli* MG1655 in response to different acids and the physiological function as resistance mechanism for extreme acid stress, we also measured the survival rate of *E. coli* MG1655 at pH 2.5 after being exposed to different acids for 2 hours. The pre-incubation of cells with SCFAs acetic acid and propionic acid, significantly increased the survival of *E. coli* MG1655 compared the control hydrochloric acid (Figure 3.11), which correlates with the promoter activities of *gadB* and *gadA* (Figure 3.8). However, the butyric acid showed the similar effect on the survival of *E. coli* MG1655 as well as the control hydrochloric acid, which might because of the growth defect of the *E. coli* cells after the butyric acid treatment compared to the acetic acid and propionic acid (data not shown). In addition, the pretreatment with succinic acid also significantly increased the survival of *E. coli* MG2655 (Figure 3.11), which is not consistent with that the succinic acid did not increase the *gadA* or *gadB* promoter activity (Figure 3.8). The succinic acid might play other roles on the acid stress resistance in *E. coli*. The survival of *E. coli* MG1655 was in the same range or lower after the pretreatment with citric, formic or lactic acids than the control hydrochloric acid. All of the different tested acids increased survival of *E. coli* MG1655 at pH 2.5 compared to the non-inducing condition (Figure 3.11), which is consistent the conclusion that the GDAR system in *E. coli* responds to low pH (M. P. Castanie-Cornet et al., 1999).

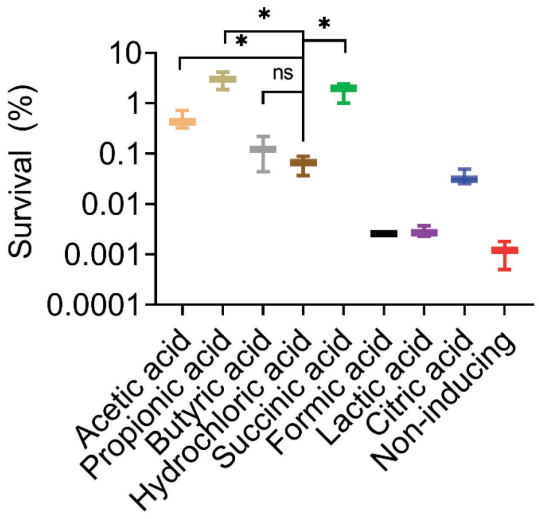


Figure 3.11. The effect of different acids on the survival of *E. coli* MG1655 at pH 2.5. Overnight cultures were grown in KE glycerol glutamate minimal medium (pH 7.6), then they were diluted into the fresh medium with the starting OD_{600} of 0.05. 50mM acid was added into the exponential growth phase culture after 5-hour incubation. After another 2-hour incubation with different acids treatment, *E. coli* cells were transferred into the fresh KE glucose glutamate minimal medium (pH 2.5) with the starting OD_{600} of 0.05. Viable cells were measured by plating serial diluted cultures onto LB agar before and after the acid stress of pH 2.5. The percentage survival calculated using time 0 as the reference. This experiment was done in triplicate. Statistical analysis was done by using two-tailed unpaired t test. ****p<0.0001. Mean ± SD is shown. SD, standard deviation.

Thus far, we have analyzed the effect of different acids on *gadB* and *gadA* promoter activities and the survival at pH 2.5 of *E. coli* in the population level. Because of the difficulty to distinguish the transcripts of *gadB* and *gadA*, there are few researches about the expression of *gadB* and *gadA* in individual cells. The two fusions of P_{gadB}:*mcherry* and P_{gadA}:*egfp* were generated into one plasmid, which gave us the chance to analyze the correlation of the expression of *gadB* and *gadA* at the single cell level. Compared to acetic acid, the butyric acid showed the lower induction on the P_{gadA}:*egfp* expression as the RFU measured by the Tecan in the population level, but

butyric acid showed higher induction on the P_{gadB}:*mcherry* (Figure 3.12A). The P_{gadB}:*mcherry* and P_{gadA}:*egfp* activities showed a highly positive correlation (r > 0.7) under butyric acid, propionic acid and acetic acid stress, indicating that both *gadA* and *gadB* expressed in *E. coli* in response to the SCFAs (Figure 3.12B). This data is also consistent with the result that the *gadA* and *gadB* expressed equally at the protein level (Figure 3.1A).

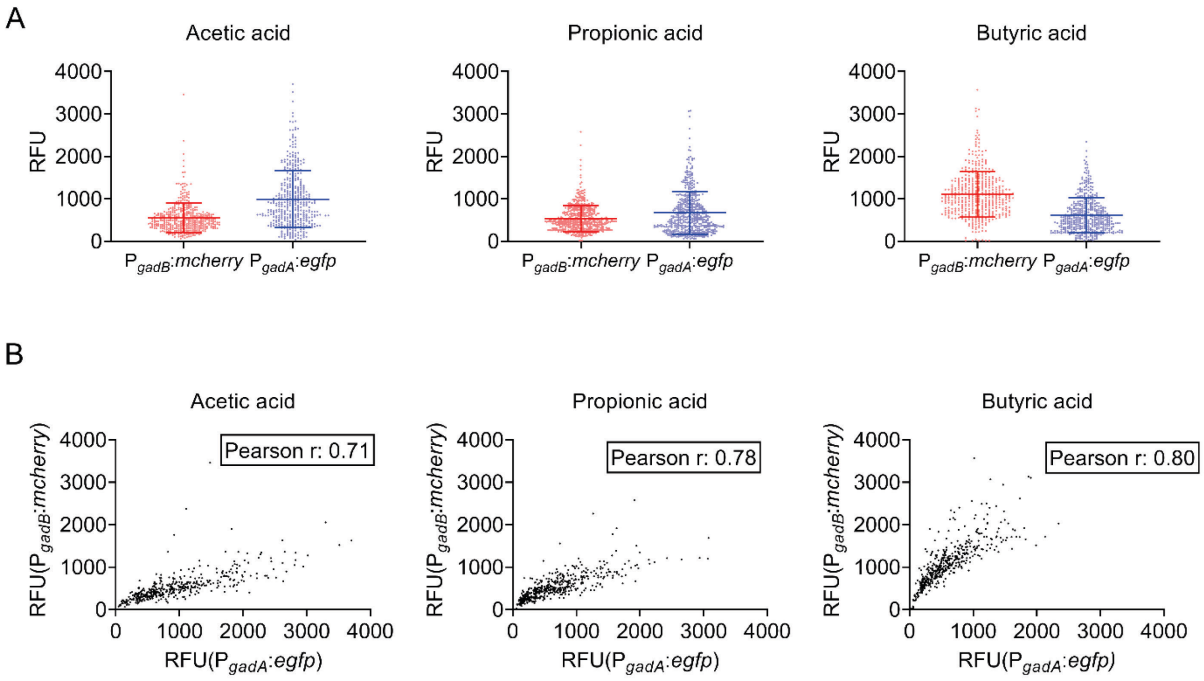


Figure 3.12. The correlation of *gadB* and *gadA* promoter activation under SCFAs stress. (A) Distribution of mCherry and eGFP fluorescence levels detected in at least 400 cells of *E. coli* MG1655 transformed with the plasmid-based fusions P_{gadB}:*mcherry* and P_{gadA}:*egfp*. **(B)** The correlation plots of mCherry and eGFP fluorescence levels. Pearson r, Pearson's correlation coefficient (r). The correlation plots were created by the Correlation matrix analyses using Graphpad Prism 8.4.3.

In this part, our data indicated that the SCFAs acetic acid, propionic acid and butyric acid induce the *gadB* and *gadA* expression in *E. coli* at both transcriptional and translational levels. The *gadB* and *gadA* promoter activities are positively correlated at

the single cell level in *E. coli* under SCFAs stress. Taken together, the SCFAs acetic acid, propionic acid and butyric acid are specific stimuli of the GDAR system.

3.2.2 SCFAs activate the GDAR system via the MnmG/MnmE-GadE circuit

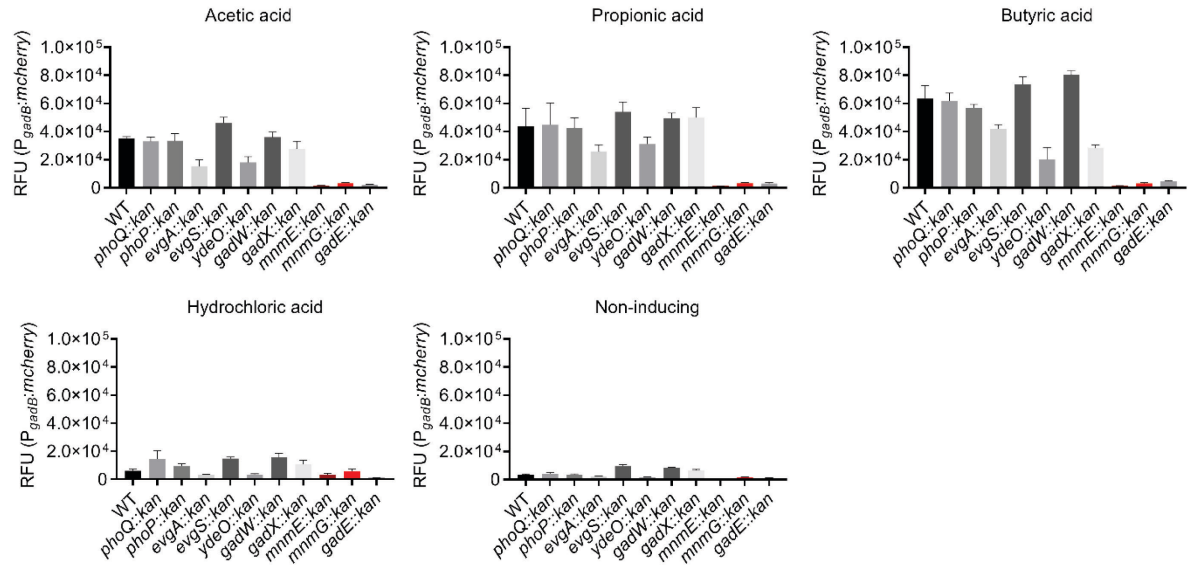
The regulation of the GDAR system is complex and its activation is mainly regulated by three circuits, namely the EvgS/EvgA, MnmE, GadX/GadW circuits (Foster, 2004). With more research on the GDAR system, the regulatory network got extended. The extended regulatory circuits are shown in Figure 1.3. As we have proved that the SCFAs are external stimuli of the GDAR system, our next aim was to identify the responsible regulatory circuit for the activation of the GDAR system by SCFAs in *E. coli*.

Activation of the P_{gadB}:*mcherry* and P_{gadA}:*egfp* reporters were analyzed in *phoQ*, *phoP*, *ydeO*, *evgA*, *evgS*, *gadW*, *gadX*, *mnmE*, *mnmG* mutants that were exposed to butyric acid, propionic acid, acetic acid during the exponential growth phase. The hydrochloric acid and non-inducing condition were used as the control conditions. The *gadE* mutant and the parental strain *E. coli* BW25113 were used as the negative and positive control strains, respectively. Among all the mutants tested, *mnmG::kan* and *mnmE::kan* showed the lowest *gadB* and *gadA* promoter activities in response to all butyric acid, propionic acid and acetic acid, which were in the range of the negative control *gadE* (Figures 3.13A & 3.14A). The fold-change values of the *gadB* and *gadA* promoter activities in all tested strains after different acids stress were normalized to the non-inducing (Figures 3.13B & 3.14B). *mnmE* and *mnmG* mutants even showed higher fold-change values of *gadB* promoter activity and a similar range of *gadA* promoter activity after the hydrochloric acid stress than wild type strain (Figures 3.13B & 3.14B), indicating that the *mnmE* and *mnmG* are mainly involved in the activation of *gadB* and *gadA* promoter in *E. coli* in response to SCFAs.

The *phoQ*, *phoP*, *evgS* and *gadW* mutants did not decrease *gadB* or *gadA* promoter activity in response to SCFAs (Figures 3.13A & B), indicating these regulators are not involved in sensing of SCFAs. The absence of *gadX* caused a decrease of *gadB* and *gadA* promoter activities in response to butyric acid, and a reduction of *gadA* promoter activity in response to acetic acid. Still, the decrease level was lower than *mnmE* and *mnmG* (Figures 3.13A & 3.14A), indicating that *gadX* is partially involved in the activation of the GDAR system in response to butyric acid. The absence of the *evgA* caused a decrease in *gadB* and *gadA* promoter activity in response to acetic acid and hydrochloric acid, but not the fold-change values (Figures 3.13 & 3.14), indicating that *evgA* is not involved in the activation of the GDAR system in *E. coli* in response to SCFAs. The absence of the *ydeO* caused a decrease in *gadB* promoter activity in response to butyric acid, acetic acid and hydrochloric acid, but not propionic acid (Figure 3.13 A).

The *ydeO::kan* did not affect the *gadA* promoter activity in response to all SCFAs. Still, it caused an increase in the *gadA* promoter activity in response to hydrochloric acid and non-inducing conditions (Figure 3.14). Potentially, the *ydeO::kan* induced *gadA* promoter activity independently of SCFAs. The absence of *gadX*, *evgA* or *ydeO* caused a partial decrease in *gadB* or *gadA* promoter activities in response to SCFAs, but not as strong as the absence of *mnmE* or *mnmG*, which were in the range of the negative control *gadE* mutant. Overall, these results indicated that the MnmE/MnmG activation circuit is a central regulatory circuit in *E. coli* in response to SCFAs leading to an activation of the GDAR system.

A



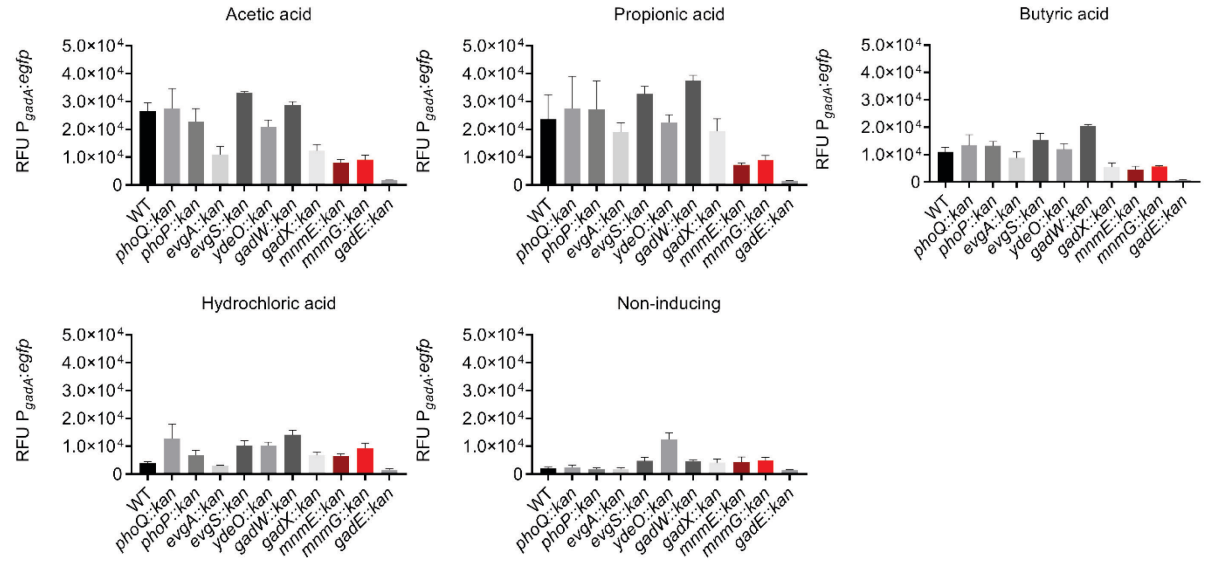
B

The fold change of the *gadB* promoter activity after acid stress compared to Non-inducing condition

P_{gadB}:*mcherry* (fold-change)	WT	*phoQ::kan*	*phoP::kan*	*evgA::kan*	*evgS::kan*	*ydeO::kan*	*gadW::kan*	*gadX::kan*	*mnmE::kan*	*mnmG::kan*	*gadE::kan*
Acetic acid	10.69	8.28	9.46	6.72	4.88	10.96	4.22	4.16	1.85	1.88	2.11
Propionic acid	13.07	10.59	11.78	11.15	5.81	19.19	5.81	7.43	1.78	1.87	2.95
Butyric acid	19.09	15.22	15.99	18.07	7.90	11.95	9.40	4.26	1.93	1.84	4.47
Hydrochoric acid	1.85	3.77	2.67	1.47	1.57	1.87	1.83	1.63	4.27	3.52	1.14
Non-inducing	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Figure 3.13. *gadB* promoter activity in various mutants involved in the GDAR system regulatory network of *E. coli* BW25113 exposed to SCFAs. (A) The *gadB* promoter activity in different mutants and the wild type strain of *E. coli* BW25113 after different acid stress. **(B)** The fold change of *gadB* promoter activity in different mutants and the wild type strain of *E. coli* BW25113 after different acid stress compared to the non-inducing condition. The wild-type strain and different mutants of *E. coli* BW25113 cells were transformed with the reporter plasmid pPBBR1MCS4-P_{gadB}:*mcherry*-P_{gadA}:*egfp*. 50 mM acid was added to the cultures after 5-hour incubation (exponential growth phase) in a minimal glycerol medium (pH 7.6). The *gadB* promoter activity was tested by measuring the fluorescence and OD_{600} using a TECAN Spark 20 M plate reader after 3-hours of incubation. RFU refers to the Relative Fluorescence Unit / OD_{600}. The experiments were performed in triplicate. Mean ± SD is shown. SD, standard deviation.

A



B

The fold change of the *gadA* promoter activity after acid stress compared to Non-inducing condition

P_{gadA}:*egfp* (fold-change)	WT	*phoQ::kan*	*phoP::kan*	*evgA::kan*	*evgS::kan*	*ydeO::kan*	*gadW::kan*	*gadX::kan*	*mnmE::kan*	*mnmG::kan*	*gadE::kan*
Acetic acid	12.82	11.37	13.55	7.01	7.22	1.75	6.33	3.31	2.02	1.87	1.19
Propionic acid	11.04	11.04	16.09	11.97	7.10	1.89	8.26	4.94	1.83	1.84	1.09
Butyric acid	5.31	5.70	7.98	5.20	3.31	0.97	4.49	1.49	1.09	1.18	0.54
Hydrochoric acid	1.89	6.09	4.02	1.82	2.23	0.85	3.11	1.76	1.66	1.97	1.08
Non-inducing	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Figure 3.14. *gadA* promoter activity in various mutants involved in the GDAR system regulatory network of *E. coli* BW25113 exposed to SCFAs. (A) The *gadA* promoter activity in different mutants and the wild type strain of *E. coli* BW25113 after different acid stress. **(B)** The fold-change of *gadA* promoter activity in different mutants and the wild type strain of *E. coli* BW25113 after different acid stress compared to the non-inducing condition. The wild-type strain and different mutants of *E. coli* BW25113 cells were transformed with the reporter plasmid pPBBR1MCS4-P_{gadB}:*mcherry*-P_{gadA}:*egfp*. 50 mM acid was added to the cultures after 5-hour incubation (exponential growth phase) in a minimal glycerol medium (pH 7.6). The *gadA* promoter activity was tested by measuring the fluorescence and OD_{600} using a TECAN Spark 20 M plate reader after 3-hours of incubation. RFU refers to the Relative Fluorescence Unit / OD_{600}. The experiments were done in triplicate. Mean ± SD is shown. SD, standard deviation.

Next, the *mnmE* and *mnmG* mutants were complemented with plasmid pCA24N-*mnmE* or pCA24N-*mnmG* under the control of the $P_{T5\text{-}lac}$ promoter, respectively. The

wild-type strain, *mnmE* and *mnmG* transformed with pCA24N empty plasmid were used as the positive and negative control, respectively. All the strains transformed with additional reporter plasmid pPBBR1MCS4-P_{gadB}:*mcherry*-P_{gadA}:*egfp* were used to analyze *gadB* and *gadA* promoter activation in response to different acids. The *E. coli* BW25113 cells with pPBBR and pCA24N two plasmids displayed a growth defect in the glycerol glutamate minimal medium. For this reason, we had to switch the carbon source from glycerol to glucose. To minimalize the effect of the mixed fermentation product of glucose, we started the acid stress after 3-hour incubation, where the external acetate concentration was still low (Figure 3.1C). Unfortunately, we could not complement the deletion of *mnmE* with the pCA24N-*mnmE* plasmid successfully (data not shown). The reason might be the copy number and the induction of the expression level of the *mnmE* from the plasmid. So, here we only show the data about the complementary experiment of the *mnmG.*

The activation of *gadB* promoter in response to the SCFAs was impaired in the negative control *mnmG* mutant transformed with the empty plasmid pCA24N and fully restored in the strain complemented with the pCA24N-*mnmG* plasmid (Figure 3.15A), confirming that the MnmG plays a significant role in the expression of the *gadB* in response to the SCFAs. The hydrochloric acid showed a stronger inducing ability on *gadB* promoter activity in the negative control strain *mnmG::kan*-pCA24N than SCFAs, confirming that the MnmG circuit mainly responds to the SCFAs.

However, GFP fluorescence was not detectable in *E. coli* strains transformed with pBBR1MCS4 and pCA24N (data not shown), which might occur because of the intrinsic green autofluorescence of *E. coli* (Mihalcescu et al., 2015). To solve this problem, the single reporter plasmid pPBBR1MCS4-P_{gadA}:*mcherry* was used to analyze the *gadA* promoter activity under the same conditions. Similar to the *gadB* promoter, the activation of the *gadA* promoter activity in response to the SCFAs was impaired in the *mnmG::kan*, and restored in the strain complemented with the pCA24N-

mnmG plasmid (Figure 3.16), confirming the role of MnmG in the activation of *gadA* in *E. coli* in response to the SCFAs.

The basal level expression of *mnmG* is enough to restore the *gadB* and *gadA* expression. However, the overexpression of the *mnmG* caused the defect of the *gadB* and *gadA* expression (data not shown). Overall, the MnmG is required to activate *gadB* and *gadA* in *E. coli* in response to SCFAs.

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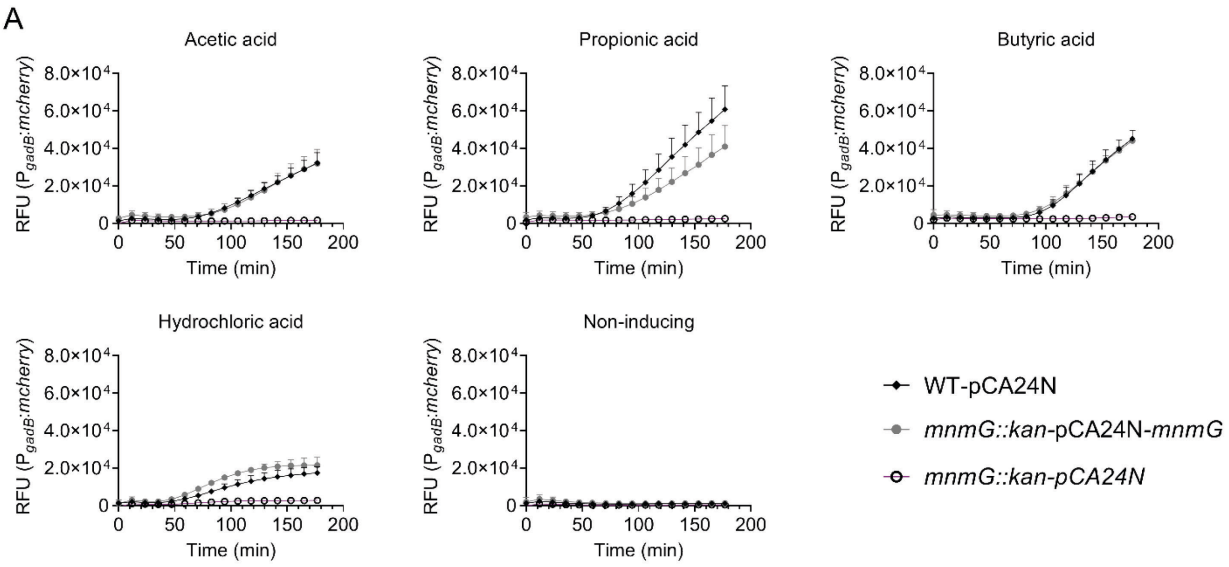


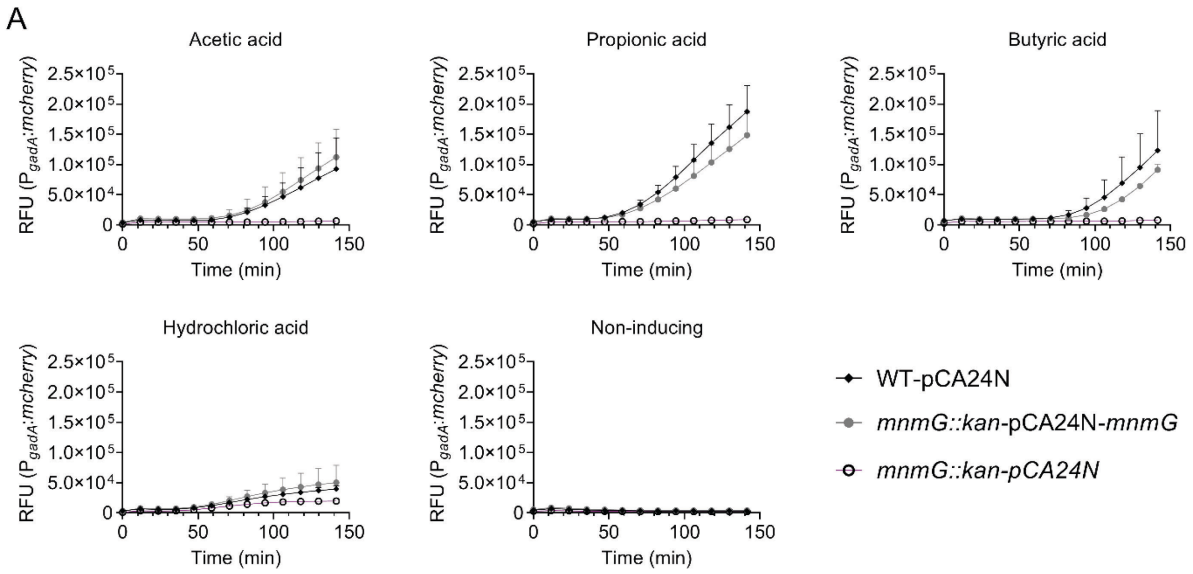
B

The fold change of the *gadB* promoter activity after acid stress compared to Non-inducing condition			
P_{gadB}:*mcherry* (fold-change)	WT-pCA24N	*mnmG::kan*-pCA24N-*mnmG*	*mnmG::kan*-pCA24N
Acetic acid	19.32	19.37	4.96
propionic acid	39.80	23.32	6.76
Butyric acid	26.87	24.71	10.17
Hydrocloric acid	14.63	18.67	9.46
Non-inducing	1.00	1.00	1.00

Figure 3.15. MnmG is required for the expression of *gadB* in *E. coli* in response to the SCFAs. (A) The dynamics of *gadB* promoter activity. **(B)** The fold-change of *gadB* promoter activity in *E. coli* strains after 3-hour treatment with different acids treatment compared to the non-inducing condition. The *mnmG* mutant was complemented with plasmid pCA24N-*mnmG* under the control of the $P_{T5\text{-}lac}$ promoter. The wild-type strain and *mnmG* transformed with pCA24N empty plasmid were used as the positive and negative control, respectively. All of the strains transformed with additional reporter plasmid pPBBR1MCS4-P_{gadB}:*mcherry*-P_{gadA}:*egfp* were used to analyze *gadB* promoter activity. Acids were

added to the exponential growth phase cells, cultivated in glucose glutamate minimal medium with a start OD_{600} 0.05 for 3 hours. The fluorescence and OD_{600} were measured using a TECAN Spark 20 M plate reader during a 3-hour incubation. RFU, the Relative Fluorescence Unit / OD_{600}. The experiments were done in triplicate. Mean ± SD is shown. SD, standard deviation.

A



B

The fold change of the *gadA* promoter activity after acid stress compared to Non-inducing condition			
P_{gadA}*:mcherry* (fold-change)	WT-pCA24N	*mnmG::kan*-pCA24N-*mnmG*	*mnmG::kan*-pCA24N
Acetic acid	27.67	27.43	2.86
propionic acid	60.14	36.98	3.79
Butyric acid	42.24	23.15	3.49
Hydrocloric acid	12.44	11.90	8.52
Non-inducing	1.00	1.00	1.00

Figure 3.16. MnmG is required for the expression of *gadA* in response to SCFAs. (A) The dynamics of *gadA* promoter activity. **(B)** The fold-change of *gadA* promoter activity in *E. coli* strains after 140 min treatment with different acids treatment compared to the non-inducing condition. The *mnmG* mutant was complemented with plasmid pCA24N-*mnmG* under the control of the $P_{T5\text{-}lac}$ promoter. The wild-type strain and *mnmG* transformed with pCA24N empty plasmid were used as the positive and negative control, respectively. All of the strains transformed with additional reporter plasmid pPBBR1MCS4-P_{gadA}:*mcherry* were used to analyze *gadA* promoter activity. Acids were added to the exponential growth phase cells, cultivated in glucose glutamate minimal medium with a start OD_{600} 0.05 for 3 hours. The

fluorescence and OD_{600} were measured using a TECAN Spark 20 M plate reader during a 3-hour incubation. RFU, the Relative Fluorescence Unit / OD_{600}. The experiments were done in triplicate. Mean ± SD is shown. SD, standard deviation.

To test the pCA24N plasmid, the *gadE* mutant was complemented with pCA24N-*gadE*. Strains transformed with pCA24N plasmid and the reporter plasmid pPBBR1MCS4-P_{gadB}:*mcherry*-P_{gadA}:*egfp* were used to measure *gadB* promoter activation in response to different acids. *gadB* promoter activity was diminished in the absence of the *gadE*, and partly restored in the strain complemented with the pCA24N-*gadE* plasmid (Figure 3.17), confirming the requirement of *gadE* in the expression of the *gadB* in response to the SCFAs. The activation of *gadB* requires the overexpression of the *gadE*, the basic expression level of *gadE* is not enough. This result is consistent with the previous research that GadE is the central regulator of the GDAR system (Hommais et al., 2004). The successful complementation of *gadE* with the pCA24N-*gadE* also proves the compatibility of the two plasmids we used during our measurement.

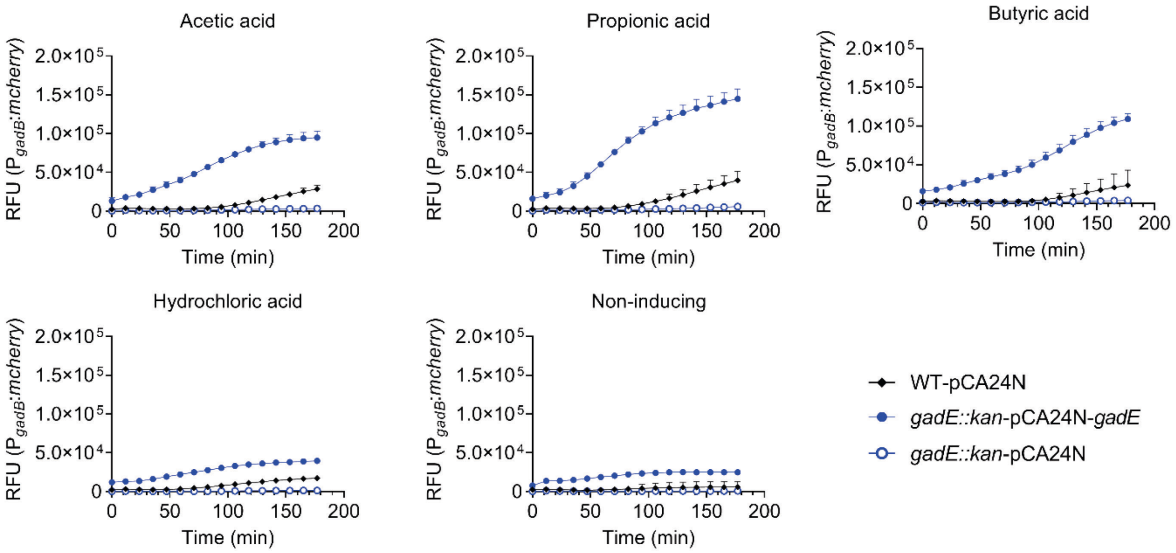


Figure 3.17. *gadE* is required for the expression of *gadB* in response to SCFAs. The *gadE::kan* was transformed with pCA24N-*gadE* plasmid expressing *gadE* for the complementation. The wild-type strain and the *gadE::kan* transformed with empty plasmid pCA24N were used as the positive and

negative control, respectively. The reporter plasmid pPBBR1MCS4-P_{gadB}:*mcherry*-P_{gadA}:*egfp* was used to measure *gadB* promoter activity. Acids were added to the exponential growth phase cells, which were cultivated in glucose glutamate minimal medium with a start OD_{600} 0.05 for 3 hours. The fluorescence and OD_{600} were measured using a TECAN Spark 20 M plate reader during a 3-hour incubation. RFU, the Relative Fluorescence Unit / OD_{600}. The experiments were done in triplicate. Mean ± SD is shown. SD, standard deviation.

The central regulator GadE is required for the *gadB* expression in response to the SCFAs. To investigate whether the MnmG regulatory circuit via *gadE*, we measured the *gadE* promoter activity in wild-type, *mnmG* mutant and the control *gadE* mutant strains using the reporter plasmid pPBBR1MCS4-P_{gadE}:*mcherry*. The absence of *mnmG* caused a decrease of *gadE* promoter activity in response to SCFAs (Figure 3.18), indicating that the MnmG regulates the GDAR system via the central regulator GadE. There was no defect in the *gadE* promoter activity in the *mnmG* mutant in response to the hydrochloric acid and no-inducing conditions (Figure 3.18). This is consistent with the result that the *mnmG* mutant did not show the defect in the *gadB* or *gadA* promoter activity under the stress of hydrochloric acid, confirming the *mnmG* circuit is particularly responsive to the SCFAs. In addition, the increase of *gadE* promoter activity in *gadE* mutant strain proved that the *gadE* reporter plasmid is functional and the *gadE* is involved in the regulatory network of the GDAR system in response to the SCFAs in *E. coli*.

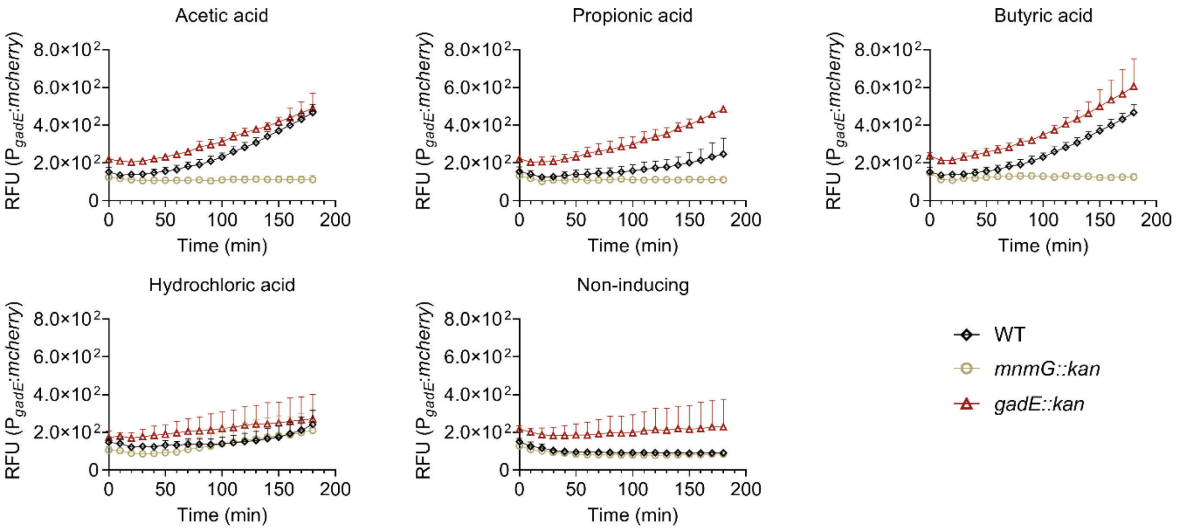


Figure 3.18. MnmG regulates the GDAR system in *E. coli* in response to the SCFAs via the central regulator GadE. The reporter plasmid pPBBR1MCS4-P_{gadE}:*mcherry* was transformed into *E. coli* BW25113 wild-type, *mnmG* mutant and *gadE* mutant. 50 mM acid was added to the cultures after 5-hour incubation (exponential growth phase) in a minimal glycerol medium (pH 7.6). mCherry fluorescence and OD_{600} were measured by using an infinite 200Pro plate reader during 3-hour incubation on *E. coli* cells in the 96-well plate with shaking. RFU, the Relative Fluorescence Unit / OD_{600}. The experiments were done in triplicate. Mean ± SD is shown. SD, standard deviation.

As described in the introduction, the regulation of the GDAR system contains the RpoS-dependent and RpoS-independent pathways (Castanie-Cornet et al., 1999; Waterman & Small, 2003). We analyzed the RpoS expression using a translational fusion plasmid pPBBR1MCS4-RpoS:mCherry in *E. coli* in response to the SCFAs to reveal the related pathway. The expression of the RpoS was not activated in response to the SCFAs, and there was no difference between the wild-type strain and the *mnmG* mutant (Figure 3.19), indicating that the expression of the GDAR system in *E. coli* in response to the SCFAs is the *rpoS*-independent pathway.

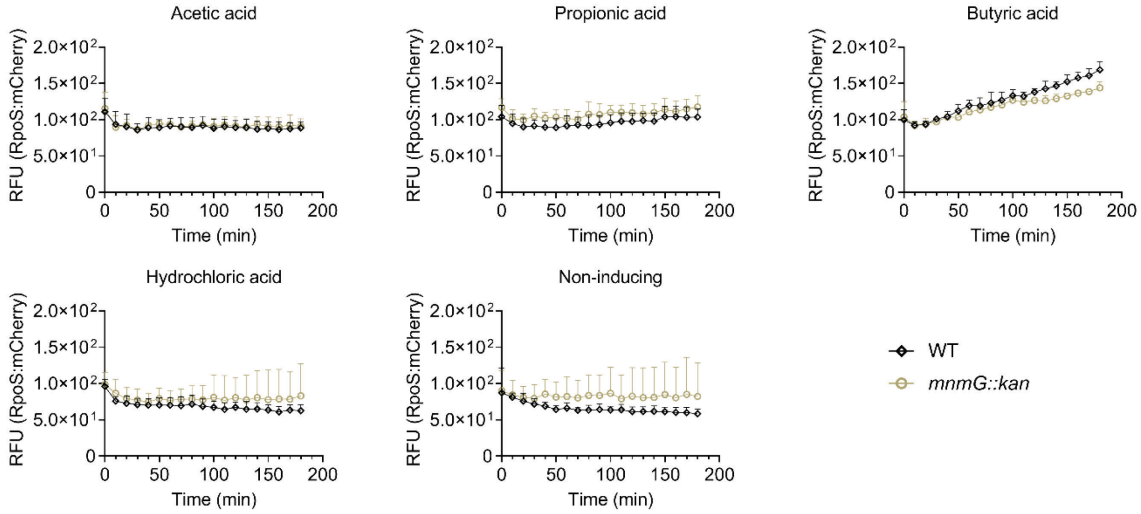


Figure 3.19. MnmG regulates the *gadB* and *gadA* expression via a RpoS-independent pathway. *E. coli* BW25113 wild-type strain and *mnmG::kan* were transformed with the translational reporter plasmid pPBBR1MCS4-RpoS-mCherry. 50 mM acid was added to the cultures after 5-hour incubation (exponential growth phase) in a minimal glycerol medium (pH 7.6). mCherry fluorescence and OD_{600} were measured by using an infinite 200Pro plate reader during 3-hour incubation on *E. coli* cells in the 96-well plate with shaking. RFU, the Relative Fluorescence Unit / OD_{600}. The experiments were done in triplicate. Mean ± SD is shown. SD, standard deviation.

This study proved that the MnmG-GadE activation circuit responds to the SCFAs, and this activation circuit is RpoS-independent. The *mnmE* mutant also showed a defect in the expression of the *gadB* and *gadA* in *E. coli* in response to the SCFAs. However, the *gadB* and *gadA* promoter activities in the *mnmE* mutant could not be complemented with the pCA24N-*mnmE* plasmid. As mentioned above, the G protein MnmE and its partner protein MnmG always form a functional heterotetrameric complex MnmE/MnmG (Yim et al., 2006). The MnmE is critical for expressing the *gadE-mdtEF* multidrug efflux operon in *E. coli* cells cultivated in glucose medium under anaerobic conditions (Deng et al., 2013). So, here we still propose MnmG/MnmE-GadE circuit is the central regulatory circuit responding to the SCFAs.

3.3 The extended regulatory network of the GDAR system

3.3.1 Candidate gene screening of the GDAR system regulatory network

This study has proved that the MnmG/MnmE-GadE circuit activates the GDAR system in *E. coli* in response to the SCFAs. However, neither MnmG or MnmE is a membrane-bound sensor or transporter, which can sense the external stimuli. So, we proposed the hypothesis that other genes might be involved in the regulatory network of the GDAR system in *E. coli*. So, we performed the candidate gene screening by measuring *gadB* and *gadA* promoter activity using the reporter fusion in different mutants from the Keio collection (Baba et al., 2006). In bacteria, two-component systems, consisting of a histidine kinase protein and its cognate response regulator, are the predominant means for sensing and responding to the environment (Capra & Laub, 2012). This study mainly focused on TCSs: the anoxic redox control ArcB/ArcA TCS, which is involved in mediating the switch fermentation under oxygen-limitation (Brown, Anderson, Bachman, & Mobley, 2022; Iuchi, Cameron, & Lin, 1989; Iuchi & Lin, 1988); the osmotic stress-related EnvZ/OmpR TCS (Mizuno & Mizushima, 1990); the SCFAs related BarA/UvrY TCS (Chavez, Alvarez, Romeo, & Georgellis, 2010; Pernestig et al., 2003; Pernestig, Melefors, & Georgellis, 2001; Vazquez-Ciros, Alvarez, & Georgellis, 2020); and the pyruvate related YpdA/YpdB TCS (Fried, Behr, & Jung, 2013; Miyake et al., 2019). In addition, we also performed the candidate gene screening among the following genes, encoding the membrane transporter DucA, the low pH sensor CadC of the LDAR system, the soluble transhydrogenase SthA, the leucine biosynthesis related LeuO and Lrp (Takao, Yen, & Tobe, 2014). It is reported that the response regulator RcsB of the Rcs TCS is absolutely required for the *gadB* and *gadA* expression (Castanie-Cornet et al., 2010; Castanié-Cornet et al., 2007). So, here the *rcsB::kan* was used as the negative control.

The results indicated that the absence of *cadC* caused an increase of *gadB* and *gadA* promoter activities under the induction of SCFAs, hydrochloric acid, and even non-

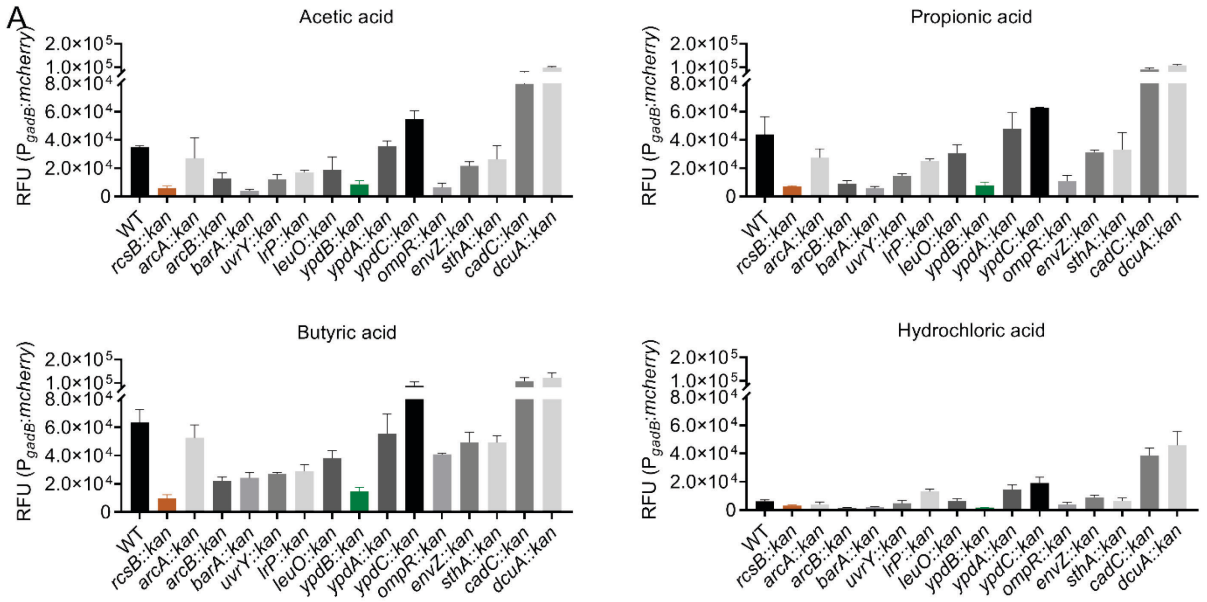
inducing condition (Figures 3.20A & 3.21A). It seems that the inactivation of the LDAR system is compensated by the GDAR system, which is consistent with the previous study (Brameyer, Schumacher, Kuppermann, & Jung, 2022). In addition, the absence of the L-aspartate transporter gene *ducA* also caused an increase of *gadB* and *gadA* promoter activities (Figures 3.20A & 3.21A). The soluble transhydrogenase gene *sthA* was proven to be required for the *gad* genes expression in *E. coli* under acetate stress (H. Zhao et al., 2018). However, there was no significant effect on the *gadB* and *gadA* promoter activities in the *sthA* mutant compared to the wild type strain in response to SCFAs (Figures 3.20A & 3.21A).

The *arcB* mutant displayed a decrease of *gadB* and *gadA* promoter activities as well as the fold-change value of P_{gadB}:*mcherry* in response to all tested acids normalized to the non-inducing condition (Figures 3.20AB & 3.21AB), indicating that the ArcB plays a role in the activation of the GDAR system in response to low pH. However, the *arcA* mutant only caused the decrease of the *gadA* promoter activity, but not *gadB* promoter activity in response to propionic acid and acetic acid (Figures 3.20 & 3.21), indicating that ArcA is not as crucial as ArcB for the *gad* genes expression. The absence of *envZ* caused a decrease of *gadA* promoter activity in response to SCFAs, but not *gadB* (Figures 3.20 & 3.21). The *ompR*, the *leuO* and the *lrp* mutants caused the decrease of the *gadB* and *gadA* promoter activity in response to SCFAs, but not hydrochloric acid (Figures 3.20 & 3.21), indicating they also plays a role in the *gad* genes expression in *E. coli* in response to SCFAs.

The *barA* and *uvrY* mutants displayed a decrease in *gadB* and *gadA* promoter activities in response to the SCFAs (Figures 3.20 & 3.21), indicating they contribute to the expression of the GDAR system in *E. coli* in response to SCFAs. The absence of *ypdA* or *ypdC* did not down-regulate the *gadB* and *gadA* expression. Still, the lack of *ypdB* caused a substantial defect in the *gadB* and *gadA* promoter activities in response to the SCFAs and hydrochloric acid, which is in the same range as the negative control

rcsB::kan (Figures 3.20 & 3.21), indicating that YpdB is required for the activation of the GDAR system, not only in response to the SCFAs, but also in response to the low pH.

In summary, these results indicated that there might be cross-regulation between the GDAR system and the TCSs in *E. coli*. The histidine kinases (ArcB), response regulators (OmpR, YpdB and RcsB), or both members (BarA and UvrY) of TCSs are involved in the regulatory network of the GDAR system. Our data extended the regulatory network of the GDAR system. Among all tested candidates, the *rcsB::kan* and the *ypdB::kan* displayed the lowest *gadB* and *gadA* promoter activities. Therefore, we analyzed the role of the RcsB and YpdB on the regulatory network of the GDAR system in more detail in this study.

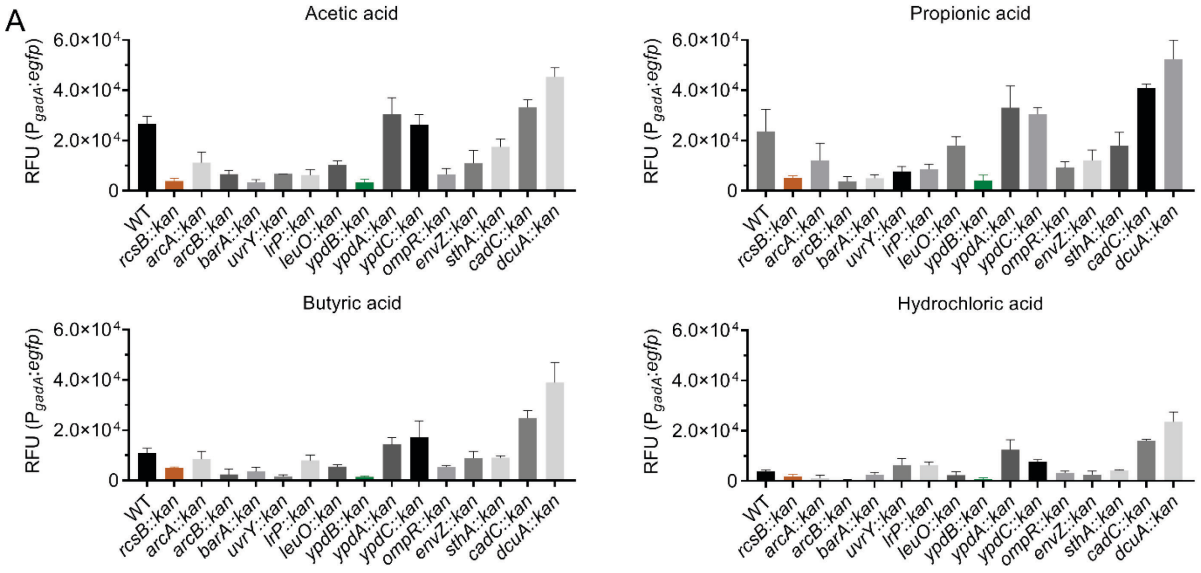


B

The fold change of the *gadB* promoter activity after acid stress compared to Non-inducing condition

P_{gadB}:*mcherry* (fold-change)	WT	*rcsB::kan*	*arcA::kan*	*arcB::kan*	*barA::kan*	*uvrY::kan*	*lrP::kan*	*leuO::kan*	*ypdB::kan*	*ypdA::kan*	*ypdC::kan*	*ompR::kan*	*envZ::kan*	*sthA::kan*	*cadC::kan*	*dcuA::kan*
Acetic acid	10.65	2.71	8.06	8.03	2.76	4.43	1.99	4.10	6.84	4.66	4.70	3.02	6.79	7.22	4.95	4.41
Propionic acid	13.07	3.19	8.74	5.60	3.73	5.27	2.93	6.86	6.26	6.20	5.31	4.88	9.78	9.53	5.60	4.84
Butyric acid	19.09	4.36	18.32	13.84	16.17	9.88	3.40	8.63	11.27	7.36	7.60	20.31	15.16	14.79	6.60	5.43
Hydrochoric acid	1.85	1.40	1.25	0.92	1.23	1.76	1.55	1.46	1.39	1.85	1.56	1.69	2.76	1.77	2.44	2.00
Non-inducing	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Figure 3.20. *gadB* promoter activity in various mutants of *E. coli* BW25113 exposed to the SCFAs. **(A)** The dynamics of *gadB* promoter activity. **(B)** The fold-change of *gadB* promoter activity in *E. coli* strains after a 3-hour treatment with different acids treatment compared to the non-inducing condition. The wild-type strain and different mutants of *E. coli* BW25113 were transformed with reporter plasmid pPBBR1MCS4-P_{gadB}:*mcherry*-P_{gadA}:*egfp*. 50 mM acid was added to the cultures after 5-hour incubation (exponential growth phase) in a minimal glycerol medium (pH 7.6). The *gadB* and promoter activity was tested by measuring the fluorescence and OD_{600} using a TECAN Spark 20 M plate reader after 3-hours of incubation. RFU, the Relative Fluorescence Unite / OD_{600}. The experiments were performed in triplicate. Mean ± SD is shown. SD, standard deviation.



B

The fold change of the *gadA* promoter activity after acid stress compared to Non-inducing condition

P_{gadA}:*egfp* (fold-change)	WT	*rcsB::kan*	*arcA::kan*	*arcB::kan*	*barA::kan*	*uvrY::kan*	*lrP::kan*	*leuO::kan*	*ypdB::kan*	*ypdA::kan*	*ypdC::kan*	*ompR::kan*	*envZ::kan*	*sthA::kan*	*cadC::kan*	*dcuA::kan*
Acetic acid	12.82	2.28	-	-	1.32	3.63	2.70	5.40	1.99	6.61	7.33	4.02	20.64	11.43	5.38	4.79
Propionic acid	11.04	3.00	-	-	1.91	4.31	3.67	9.24	2.71	7.07	8.56	5.98	23.77	11.37	6.56	5.54
Butyric acid	5.31	2.97	-	-	1.38	0.81	3.46	2.84	0.79	3.28	4.70	3.70	17.57	5.53	3.95	4.07
Hydrochoric acid	1.89	0.99	-	-	0.99	3.39	2.61	1.17	0.36	2.60	2.16	2.15	4.21	2.66	2.58	2.47
Non-inducing	1.00	1.00	-	-	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00

Figure 3.21. *gadA* promoter activity in various mutants of *E. coli* BW25113 exposed to SCFAs. **(A)** The dynamics of *gadA* promoter activity. **(B)** The fold-change of *gadA* promoter activity in *E. coli* strains after a 3-hour treatment with different acids treatment compared to the non-inducing condition. The wild-type strain and different mutants of *E. coli* BW25113 were transformed with reporter plasmid pPBBR1MCS4-P_{gadB}:*mcherry*-P_{gadA}:*egfp*. 50 mM acid was added to the cultures after 5-hour incubation (exponential growth phase) in a minimal glycerol medium (pH 7.6). The *gadA* promoter activity was tested by measuring the fluorescence and OD_{600} using a TECAN Spark 20 M plate reader after 3-hours of incubation. RFU, the Relative Fluorescence Unite / OD_{600}. The experiments were performed in triplicate. Mean ± SD is shown. SD, standard deviation.

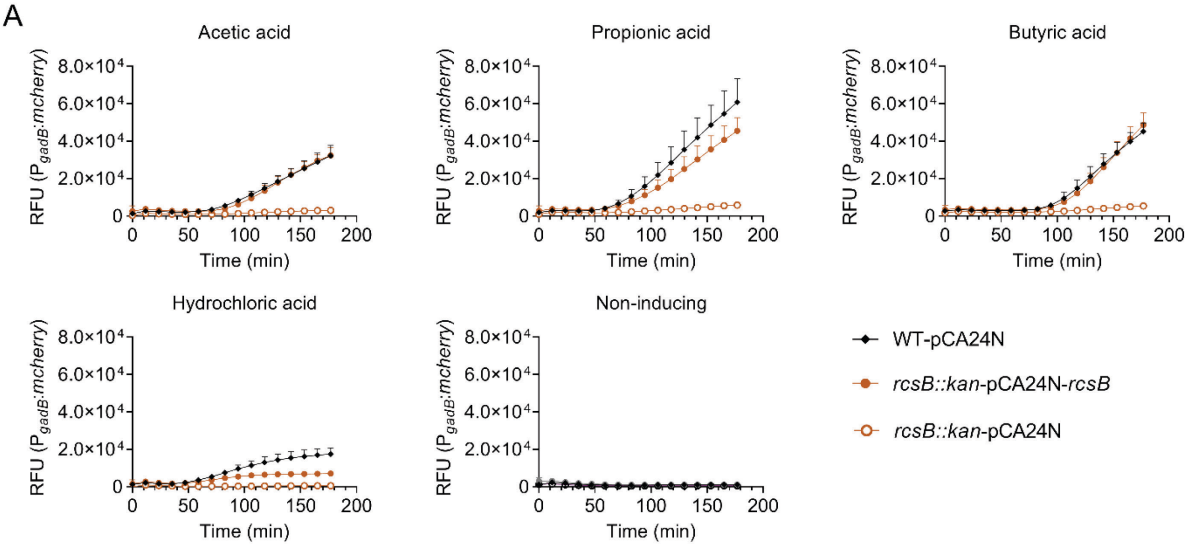
3.3.2 The cross-regulation between the GDAR system and the Rcs TCS

As mentioned above, the absence of the *rcsB* caused the strongest defect in *gadB* and *gadA* promoter activities in *E. coli* in response to the SCFAs. Next, the *rcsB::kan* was complemented with plasmid pCA24N-*rcsB*. The results showed that *gadB* promoter

activity was diminished in *rcsB::kan*, and completely restored with the pCA24N-*rcsB* plasmid (Figure 3.22), confirming the requirement of RcsB in the expression of *gadB* in *E. coli* in response to the SCFAs. Notably, the *gadB* activity can only be restored with the low expression level of *rcsB* in the *rcsB::kan* complemented with the pCA24N-*rcsB* plasmid. Still, it cannot be restored under the overexpression of the *rcsB* induced with IPTG (data not shown). These results are supported by the previous reports from other labs that the basal level of RcsB is required and sufficient for the expression of the GDAR system, while the overexpression of the *rcsB* will repress the expression of the GDAR system (Castanie-Cornet et al., 2010; Castanié-Cornet et al., 2007).

The complex non-canonical TCS Rcs system belongs to His-Asp phosphorelays that use more than one phosphorylation step to transduce signals, and the proteins RcsA, RcsC, RcsD and RcsF are involved in the phosphorylation pathway of the response regulator RcsB (Figure 3.23A) (Castanié-Cornet et al., 2007; Pannen, Fabisch, Gausling, & Schnetz, 2016; Shiba et al., 2012; Zhang & Shi, 2005). To investigate the contribution of the Rcs components to the RcsB-dependent activation of *gadB* in response to the SCFAs, we measured the *gadB* promoter activity in different *rcs* mutants, and the wild-type strain. The absence of *rcsD* or *rcsF* did not cause a significant effect on the *gadB* and *gadA* promoter activities; the lack of *rcsC* caused a decrease in the promoter activity of *gadA* but not *gadB* (Figures 3.23B & C). Combined the effects of the absence of *rcsD*, *rcsF* and *rcsC* on the expression of the *gad* genes, we get the conclusion that the RcsB phosphorylation from the RcsFCD phosphorelay is not required for the activation of the GDAR system in *E. coli* in response to the SCFAs, which is consistent with the previous research from the other group (Castanié-Cornet et al., 2007). The absence of *rcsA* caused a decrease in both *gadB* and *gadA* promoter activities in *E. coli* in response to butyric acid and acetic acid. In contrast, it did not cause a significant effect in response to hydrochloric acid or non-inducing condition (Figures 3.23B & C), indicating the RcsA has a positive impact on the activation of *gadB* and *gadA* expression in *E. coli* in response to butyric acid and acetic acid.

A



B

The fold change of the *gadB* promoter activity after acid stress compared to Non-inducing condition			
P_{gadB}:*mcherry* (fold-change)	WT-pCA24N	*rcsB*::*kan*-pCA24N-*rcsB*	*rcsB*::*kan*-pCA24N
Acetic acid	27.22	31.10	6.19
Propionic acid	39.80	30.19	9.22
Butyric acid	26.87	25.73	8.49
Hydrochoric acid	14.63	6.54	0.92
Non-inducing	1.00	1.00	1.00

Figure 3.22. RcsB is required for the expression of *gadB* in *E. coli.* (A) The dynamics of *gadB* promoter activity. **(B)** The fold-change of *gadB* promoter activity in *E. coli* strains after a 3-hour treatment with different acids treatment compared to the non-inducing condition. The *rcsB::kan* was complemented with plasmid pCA24N-*rcsB* under the control of the $P_{T5\text{-}lac}$ promoter. The wild-type strain and *rcsB* transformed with pCA24N empty plasmid were used as the positive and negative control, respectively. All the strains transformed with additional reporter plasmid pPBBR1MCS4-P_{gadB}:*mcherry*-P_{gadA}:*egfp* were used to analyze *gadB* promoter activity. Acids were added to the exponential growth phase cells, which were cultivated in glucose glutamate minimal medium with a start OD_{600} 0.05 for 3 hours. The fluorescence and OD_{600} were measured using a TECAN Spark 20 M plate reader during a 3-hour of incubation. RFU, the Relative Fluorescence Unit / OD_{600}. The experiments were performed in triplicate. Mean ± SD is shown. SD, standard deviation.

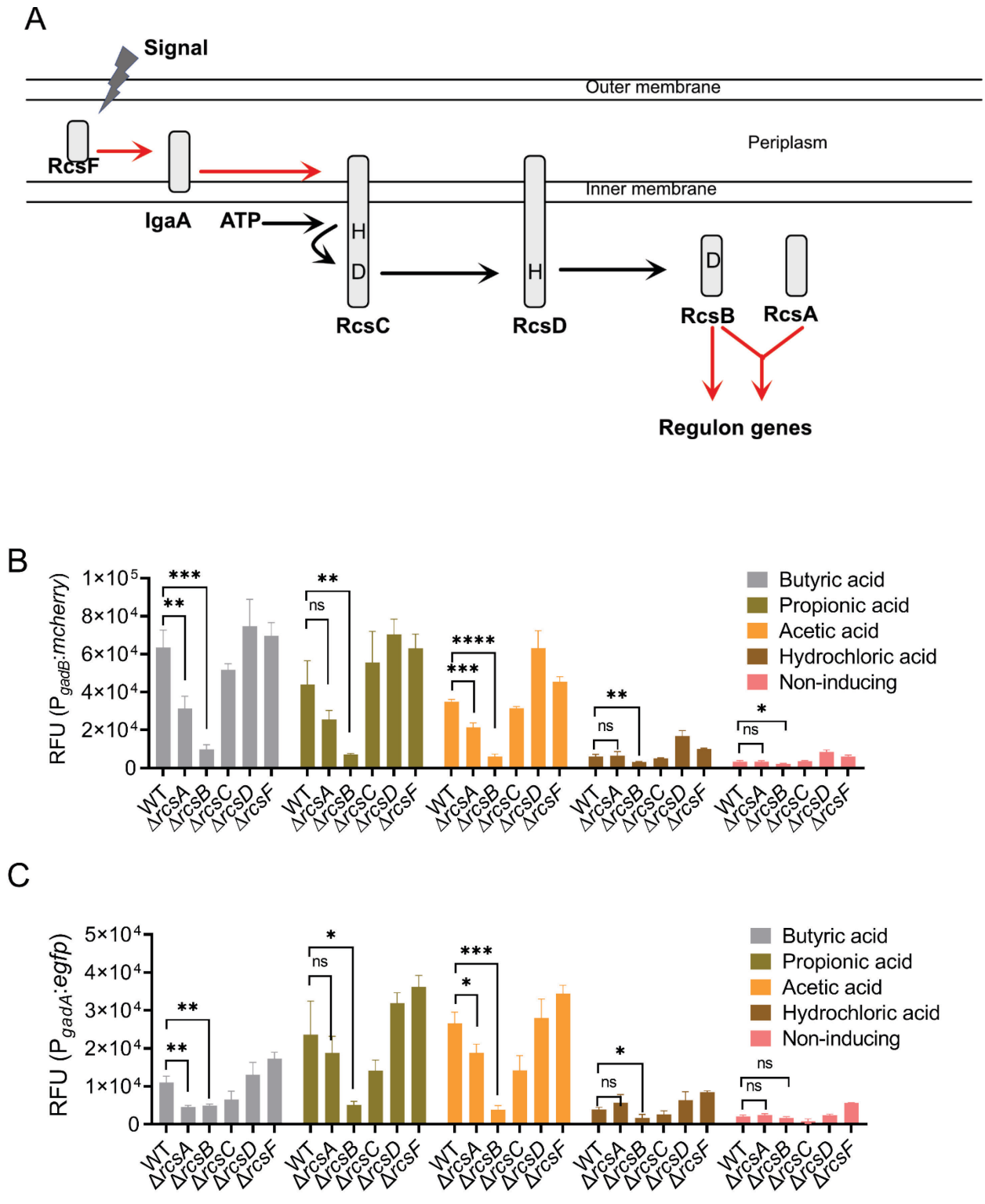


Figure 3.23. The *gadB* and *gadA* promoter activities in various mutants involved in the Rcs TCS of *E. coli* BW25113 exposed to SCFAs. (A) Schematic diagram of the Rcs TCS. RcsF activates the histidine kinase RcsC via IgaA. Black arrows indicate autophosphorylation of the histidine residue (H) in

the sensor kinase domain of RcsC, and phosphotransfer through the aspartic acid (D) residue in the receiver domain of RcsC and histidine in the phosphotransmitter domain of RcsD to aspartic acid in the receiver domain of RcsB. Red arrows indicate the transcription regulator function. Adapted from Shiba et al (2012) (Shiba et al., 2012). **(B)** *gadB* promoter activity. **(C)** *gadA* promoter activity. Wild-type strain and different mutants of *E. coli* BW25113 were transformed with the reporter plasmid pPBBR1MCS4P$_{gadB}$:*mcherry*-P$_{gadA}$:*egfp*. 50 mM acid was added to the cultures after 5-hour incubation (exponential growth phase) in a minimal glycerol medium (pH 7.6). The *gadB* and *gadA* promoter activities were tested by measuring the fluorescence and OD_{600} using a TECAN Spark 20 M plate reader after 3-hours incubation. RFU, the Relative Fluorescence Unite / OD_{600}. The experiments were done in triplicate. Statistical analysis was done using a two-tailed unpaired t-test. ****p<0.0001. Mean ± SD is shown. SD, standard deviation.

To test whether the RcsB phosphorylation is required for stimulating the expression of *gadB* in response to the SCFAs, we compared the *gadB* promoter activity in MG1655 wild-type strain, the constitutively activated mutant *rcsB*-D56E, and the non-phosphorylatable mutant *rcsB*-D56S. The *gadB* was not activated in the non-phosphorylatable mutant *rcsB*-D56S in response to the SCFAs, butyric acid, propionic acid and acetic acid. In contrast, it was induced in the constitutively activated mutant *rcsB*-D56E (Figure 3.24), indicating that the phosphorylation of RcsB is required to activate the *gadB* expression.

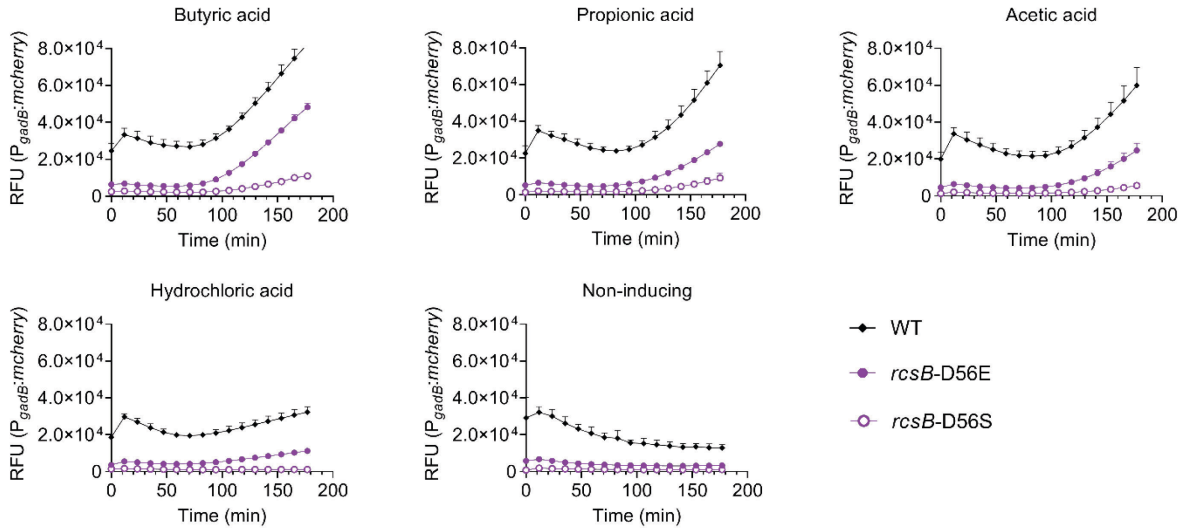


Figure 3.24. *gadB* transcriptional activation in *E. coli* requires RcsB phosphorylation. The *E. coli* MG1655 wild-type strain, the constitutively activated mutant *rcsB*-D56E, and the non-phosphorylatable mutant *rcsB*-D56S were transformed with the reporter plasmid pPBBR1MCS4-P_{gadB}:*mcherry*-P_{gadA}:*egfp*. 50 mM acid was added to the cultures after 5-hour incubation (exponential growth phase) in a minimal glycerol medium (pH 7.6). The *gadB* promoter activity was tested by measuring the fluorescence and OD_{600} using a TECAN Spark 20 M plate reader during a 3-hours of incubation. RFU, the Relative Fluorescence Unit / OD_{600}. The experiments were performed in triplicate. Mean ± SD is shown. SD, standard deviation.

To investigate whether the RcsB regulates *gad* genes via *gadE*, we measured the *gadE* promoter activity in the wild-type strain and *rcsB::kan* by the reporter plasmid pPBBR1MCS4-P_{gadE}:*mcherry*. The absence of *rcsB* did not cause a decrease in *gadE* promoter activity in response to SCFAs (Figure 3.25), indicating that the regulation of the RcsB on the *gadB* expression doesn't work via the transcriptional expression of the central regulator gene *gadE*. This result also can be supported by the fact that RcsB forms a heterodimer with GadE, which is required for the *gad* expression (McClements & Li, 2010).

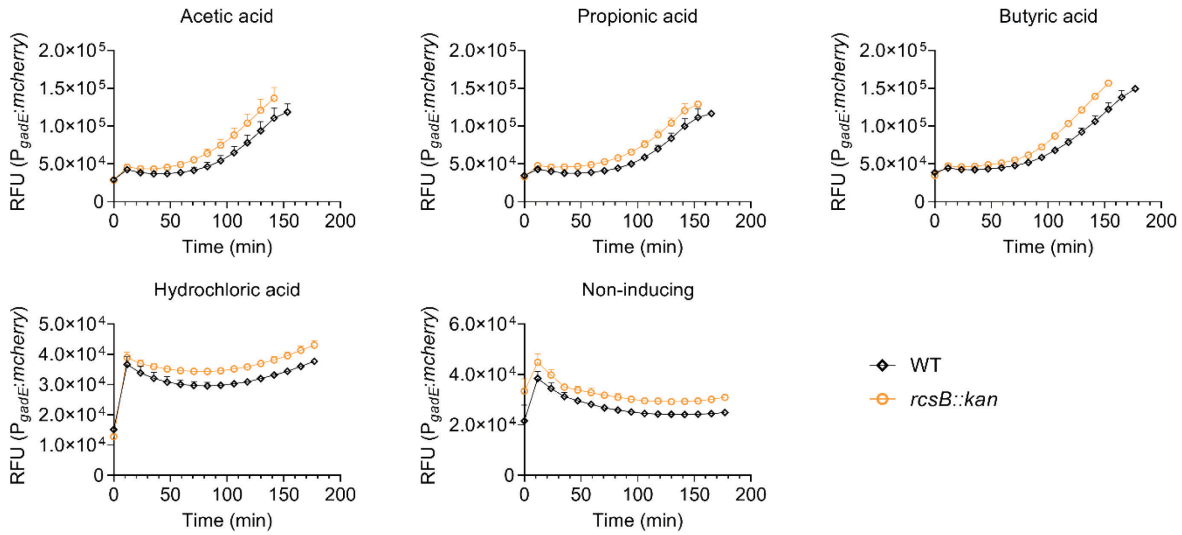


Figure 3.25. RcsB does not affect the *gadE* transcriptional expression. The *E. coli* BW25113 wild-type and *rcsB* mutant strains were transformed with reporter plasmid pPBBR1MCS4-P_{gadE}:*mcherry*. 50 mM acid was added to the cultures after 5-hour incubation (exponential growth phase) in a minimal glycerol medium (pH 7.6). mCherry fluorescence and OD_{600} were measured using TECAN Spark 20 M plate reader during a 3-hour of incubation on *E. coli* cells in the 96-well plate with shaking. RFU, the Relative Fluorescence Unit / OD_{600}. The experiments were performed in triplicate. Mean ± SD is shown. SD, standard deviation.

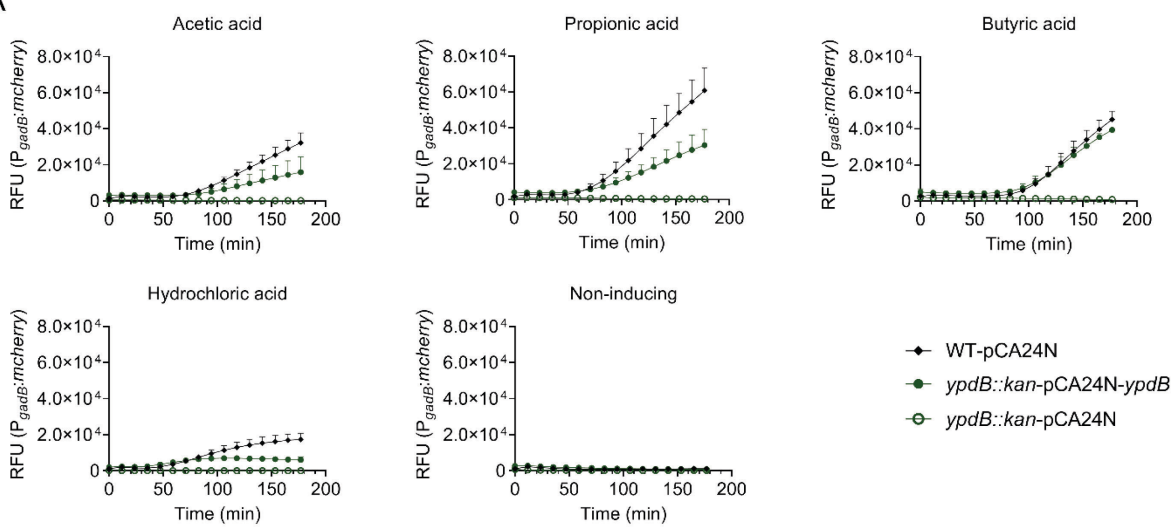
In summary, the activation of *gad* genes in exponential growth phase *E. coli* cells in response to the SCFAs requires the phosphorylation of RcsB and the auxiliary regulatory protein RcsA. Still, the phosphorylation is not via the RcsFCD phosphorelay. The RcsB does not affect the transcriptional expression of *gadE*, which only regulates the *gad* gene expression together with GadE.

3.3.3 The YpdB is required for the expression of the GDAR system in *E. coli*

The absence of *ypdB* significantly down-regulated both *gadB* and *gadA* transcriptional expression in *E. coli* BW25113 in response to the SCFAs and hydrochloric acid at the range of *rcsB* (Figures 3.20 & 21). The *gadB* promoter activity was diminished in the absence of the *ypdB*, and partially restored in the *ypdB::kan* complemented with the

pCA24N-*ypdB* plasmid (Figure 3.26), confirming the requirement of *ypdB* in the expression of *gadB* in *E. coli*. YpdB is required to activate the *gadB* expression, which can only be restored with the low expression level of *ypdB* in *ypdB::kan* complemented with the pCA24N-*ypdB* plasmid. Still, it cannot be restored under the overexpression of the *ypdB* induced with IPTG (data not shown). This is the first discovery that YpdB is required for the expression of the GDAR system.

A



B

The fold change of the *gadB* promoter activity after acid stress compared to Non-inducing condition			
P_{gadB}:mcherry (fold-change)	WT-pCA24N	*ypdB::kan*-pCA24N-*ypdB*	*ypdB::kan*-pCA24N
Acetic acid	27.22	17.32	1.29
Propionic acid	51.11	33.61	3.08
Butyric acid	38.03	44.18	5.55
Hydrochoric acid	14.58	6.77	0.21
Non-inducing	1.00	1.00	1.00

Figure 3.26. YpdB is required for the expression of *gadB* in *E. coli* BW25113. (A) The dynamics of *gadB* promoter activity. **(B)** The fold-change of *gadB* promoter activity in *E. coli* strains after a 3-hour of treatment with different acids compared to the non-inducing conditions. The *ypdB::kan* was complemented with plasmid pCA24N-*ypdB* under the control of the $P_{T5\text{-}lac}$ promoter. The *E. coli* BW25113 wild-type strain and *ypdB::kan* transformed with pCA24N empty plasmid were used as the positive and negative control, respectively. All the strains transformed with additional reporter plasmid

pPBBR1MCS4-P_{gadB}:*mcherry*-P_{gadA}:*egfp* were used to analyze *gadB* promoter activity. Acids were added to the exponential growth phase cells, cultivated in glucose-glutamate minimal medium with a start OD_{600} 0.05 for 3 hours. The fluorescence and OD_{600} were measured using a TECAN Spark 20 M plate reader during a 3-hour of incubation. RFU, the Relative Fluorescence Unit / OD_{600}. The experiments were performed in triplicate. Mean ± SD is shown. SD, standard deviation.

The pyruvate sensing YpdA/YpdB TCS consists of the histidine kinase YpdA and the cognate response regulator YpdB. *ypdA* and *ypdB* form an operon together with *ypdC* (Fried et al., 2013). However, the absence of *ypdA* or *ypdC* did not show the defect in the *gadB* and *gadA* expression (Figures 3.20 & 21), indicating that only the regulator YpdB is required for the *gad* genes expression. Because the YpdA/YpdB TCS is a pyruvate sensing system, then this study also tested whether pyruvate can induce the expression of the GDAR system. The external pyruvate did not induce *gadB* and *gadA* expression (Figure 3.27), which confirmed that the pyruvate histidine kinase YpdA is not required for the *gad* genes expression in *E. coli* (Figures 3.20 & 21).

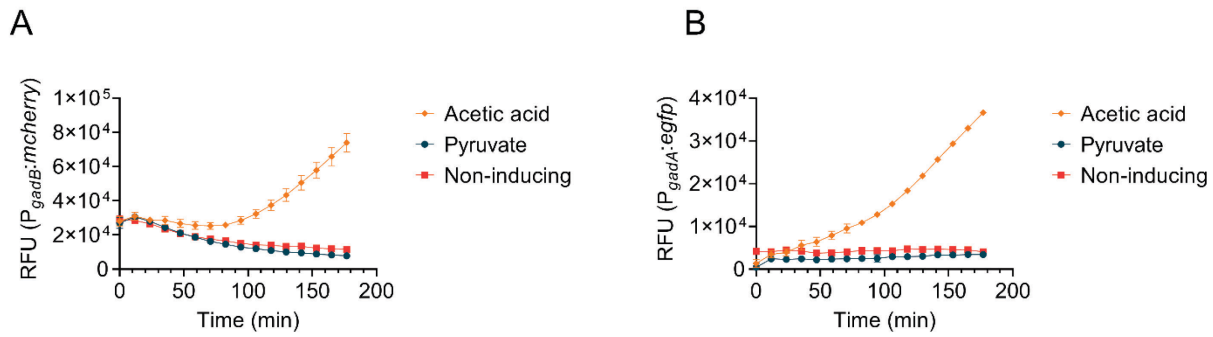


Figure 3.27. Pyruvate is not an external stimulus of the GDAR system in *E. coli*. The *E. coli* MG1655 wild-type strain was transformed with the reporter plasmid pPBBR1MCS4-P_{gadB}:*mcherry*-P_{gadA}:*egfp*. 50 mM pyruvate was added to the cultures after a 5-hour incubation (exponential growth phase) in a minimal glycerol medium (pH 7.6). 50 mM acetic acid and non-inducing conditions were used as a positive and negative control, respectively. The *gadB* and *gadA* promoter activities were tested by measuring the fluorescence and OD_{600} using a TECAN Spark 20 M plate reader for 3 hours of

incubation. RFU, the Relative Fluorescence Unit / OD_{600}. The experiments were performed in triplicate. Mean ± SD is shown. SD, standard deviation.

Furthermore, this study also tested whether the phosphorylation of YpdB is required for the expression of the GDAR system. For this purpose, this study compared the *gadB* promoter activity in MG1655 wild-type strain, the constitutively activated mutant *ypdB*-D53E, and the non-phosphorylatable mutant *ypdB*-D53N in response to different acids. There was no difference in the *gadB* promoter activity between the two mutants (Figure 3.28), indicating that the phosphorylation of the YpdB is not required to activate the expression of *gadB* in *E. coli*. This result also supports that the histidine kinase YpdA was not required for expressing the GDAR system.

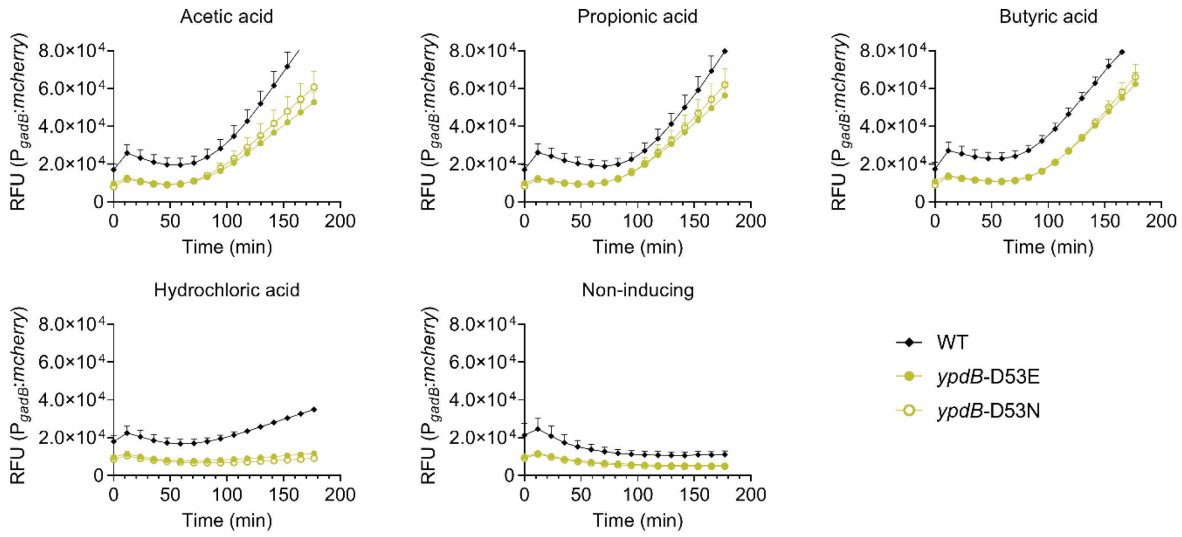


Figure 3.28. *gadB* transcriptional activation in *E. coli* does not require the phosphorylation of YpdB. The *E. coli* MG1655 wild-type strain, the constitutively activated mutant *ypdB*-D53E, and the non-phosphorylatable mutant *ypdB*-D53N were transformed with the reporter plasmid pPBBR1MCS4-P_{gadB}:*mcherry*-P_{gadA}:*egfp*. 50 mM acid was added to the cultures after 5-hour incubation (exponential growth phase) in a minimal glycerol medium (pH 7.6). The *gadB* promoter activities were tested by measuring the fluorescence and OD_{600} using a TECAN Spark 20 M plate reader during a 3-hour of incubation. RFU, the Relative Fluorescence Density/OD_{600}. The experiments were performed in triplicate. Mean ± SD is shown. SD, standard deviation.

GadE is the central regulator of the GDAR system (Hommais et al., 2004). To investigate whether the YpdB activates the *gadB* expression via *gadE*, this study measured the *gadE* promoter activity in wild-type strain and *ypdB* mutant using the reporter plasmid pPBBR1MCS4-P_{gadE}:*mcherry*. Surprisingly, the absence of *ypdB* did not cause a decrease in *gadE* promoter activity (Figure 3.29), indicating that YpdB regulates *gadB* but not *gadE* expression. It seems like that YpdB regulates *gadB* directly, which needs the further investigation.

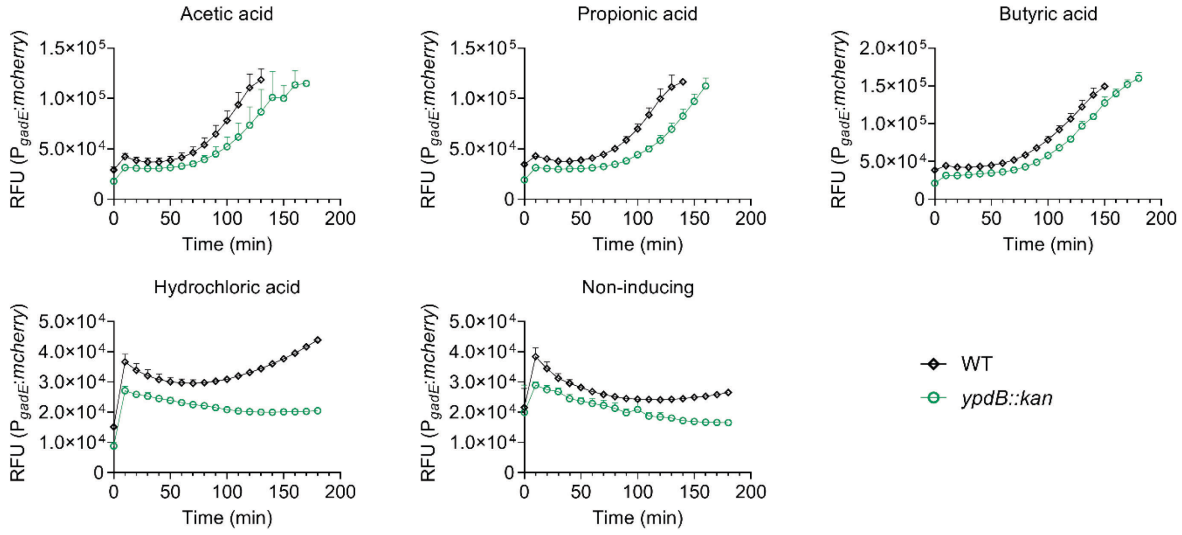


Figure 3.29. YpdB does not affect the *gadE* expression in *E. coli*. The *E. coli* BW25113 wild-type strain and *ypdB* mutant were transformed with the reporter plasmid pPBBR1MCS4-P_{gadE}:*mcherry*. 50 mM acid was added to the cultures after 5-hour incubation (exponential growth phase) in a minimal glycerol medium (pH 7.6). mCherry fluorescence and OD_{600} were measured using TECAN Spark 20 M plate reader during 3 hours of incubation on *E. coli* cells in the 96-well plate with shaking. RFU, the Relative Fluorescence Unit / OD_{600}. The experiments were performed in triplicate. Mean ± SD is shown. SD, standard deviation.

In summary, this study revealed that the expression of the GDAR system in response to the SCFAs and hydrochloric acid in *E. coli* requires the response regulator YpdB of the pyruvate TCS YpdA/YpdB, but not the histidine kinase YpdA. Still, the

phosphorylation of YpdB is not required, and the external pyruvate is not a stimulus of the GDAR system. Interestingly, the regulation of *gadB* on the expression of the GDAR system was proved not through regulating the expression of the central regulator gene *gadE*.

3.4 The effect of the mRNA m^{6}A modification on the expression of the GDAR system

m^{6}A, as the most abundant internal RNA epigenetic modification, affects the translation and stability of the modified transcripts, thus providing a mechanism to coordinate the regulation of groups of transcripts during cell state maintenance and transition in eukaryotes (Frye, Harada, Behm, & He, 2018; Fu, Dominissini, Rechavi, & He, 2014). The mechanisms of m^{6}A modification including the methyltransferases, demethyltransferases and m^{6}A reader proteins have been characterized in eukaryotes (Frye et al., 2018). The high-resolution transcriptome-wide m^{6}A profiling in *E. coli* and *P. aeruginosa* revealed a conserved m^{6}A pattern that is distinct from those in eukaryotes (X. Deng et al., 2015). The determined m^{6}A/A ratios (> 0.2%) were found to be comparable to eukaryotes (0.1% – 0.4%). The unique GCCA*G consensus motif (*-marks the modification sites) is different from the rRNA and tRNA (X. Deng et al., 2015). However, the mechanisms of m^{6}A modification in prokaryotes are still unrevealed. Our group has proved that no mRNA m^{6}A modification was detected in the double deletion strain Δ*rlmF*Δ*rlmJ* of *E. coli* MG1655, indicating that the rRNA methyltransferases RlmF and RlmJ are also mRNA methyltransferases and play essential role in the mRNA m^{6}A modification in *E. coli* (D.P Petrov, unpublished). mRNAs of *gad* genes *gadA, gadB and gadC* have been proven to contain m^{6}A modification sites (Deng et al., 2015).

To analyze the function of the m^{6}A modification on the expression of *gad* genes, we analyzed the *gadA* expression using the transcriptional reporter pPBBR1MCS4-P$_{gadA}$:*mcherry* and translational reporter pPBBR1MCS4-GadA:mCherry, respectively.

The results indicated that the *gadA* promoter activity and the GadA protein product were both significantly increased in the double deletion strain Δ*rlmF*Δ*rlmJ* compared to the wild-type strain in response to SCFAs, as well as in response to the hydrochloric acid and the non-inducing condition (Figure 3.30). We speculated that the lack of mRNA m^6A methyltransferases RlmF and RlmJ might increase the stability of mRNA, subsequently, it increases the protein production. This speculation also can be supported by an RNA-seq data where the absence of *rlmF* and *rlmJ* genes downregulates the genes related to synthesis of UDP, resulting the RNA synthesis (Petrov et al, unpublished).

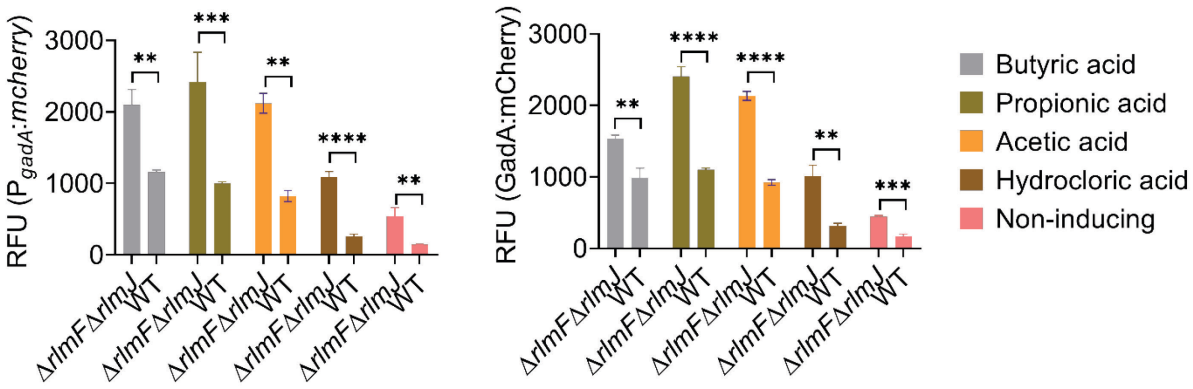


Figure 3.30. The effect of the m^6A methylation on the expression of *gadA* at transcriptional and translational levels in *E. coli* MG1655 in response to different acids. The *E. coli* MG1655 wild-type strain and the double deletion mutant strain Δ*rlmF*Δ*rlmJ* were transformed with the transcriptional reporter pPBBR1MCS4-P_{gadA}:*mcherry* or translational reporter pPBBR1MCS4-GadA:mCherry. 50 mM acid was added to the cultures after 5-hour incubation (exponential growth phase) with a start OD_{600} of 0.05 in glycerol glutamate minimal medium (pH 7.6). The fluorescence and OD_{600} were measured by using an infinite 200Pro plate reader after 3 hours of incubation. RFU refers to the Relative Fluorescence Unit / OD_{600}. The experiments were performed in triplicate. Statistical analysis was done by using a two-tailed unpaired t-test. ****p<0.0001. Mean ± SD is shown. SD, standard deviation.

Next, we analyzed the effect of the deletion of *rlmF* and *rlmJ* on the activation of *gadA* in stationary phase *E. coli* cells cultivated under different cultivation conditions with different carbon sources and pH. The *gadA* promoter activity was increased in all conditions in the double deletion strain Δ*rlmF*Δ*rlmJ* compared to the wild-type strain. The most significant difference occurred in the glucose pH 5.8 condition, in which the *gadA* promoter activity achieved the highest level (Figure 3.31). In summary, RlmF and RlmJ negatively regulate the *gadA* expression in *E. coli* under different cultivation conditions at transcriptional and translational levels. We hypothesize that the transcript stability in the absence of the methyltransferases RlmF and RlmJ and the m^6A modification resulting in an elevated level of GadA.

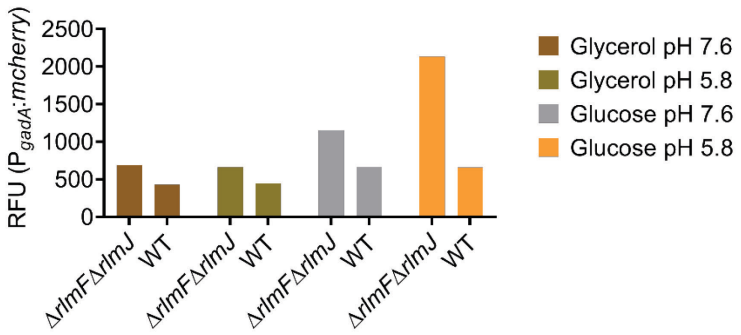


Figure 3.31. The effect of the m^6A methylation on the expression of *gadA* at the transcriptional level in *E. coli* MG1655 cultivated in different conditions. *E. coli* MG1655 wild-type strain and Δ*rlmF*Δ*rlmJ* were transformed with reporter plasmid pPBBR1MCS4-P_{gadA}:*mcherry*. Strains were cultivated to stationary phase in KE minimal medium with carbon source glucose or glycerol at pH 7.6 or 5.8. The fluorescence and OD_{600} were measured by using an infinite 200Pro. RFU refers to the Relative Fluorescence Unit / OD_{600}. This experiment was performed in single measurement.

The unique consensus motif of UGCCAG was found in the m^6A-containing peak regions (Deng et al., 2015). There is one identical UGCCAG motif and one variant GGCCAG motif in *gadC*. To investigate the function of the m^6A modification on the GDAR system, we generated point-mutant strain P(CCA)325P(CCT) and

P(CCA)121P(CCT) by replacing the 325th and 121th proline amino acid code CCA with CCT, respectively. There was no significant effect on the survival of *E. coli* MG1655 at pH 2.5 in the mutant strain P(CCA)121P(CCT) compared to the wild type strain (Figure 3.32), the adenine in the variant motif GGCCAG might be not a real m^{6}A modification site. The survival of *E. coli* MG1655 at pH 2.5 was significantly increased in the mutant strain P(CCA)325P(CCT) compared to the wild-type strain (Figure 3.32), indicating that the m^{6}A modification might affect the *gadC* mRNA stability. The lack of m^{6}A modification might increase the *gadC* mRNA stability, resulting in the increase of the survival of *E. coli* under the extreme acid stress. Notably, this is a predicted m^{6}A modification site by the consensus motif. The modification site needs to be tested to confirm the functional role of the m^{6}A modification of the GDAR system.

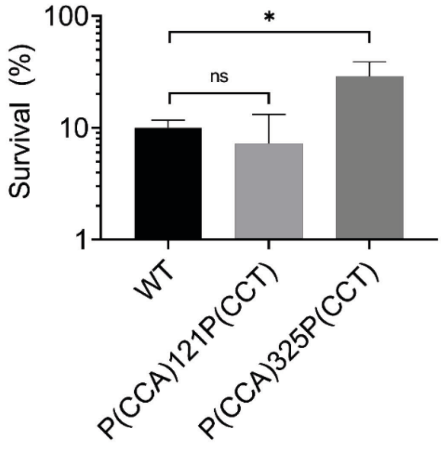


Figure 3.32. The effect of different proline amino acid codes in *gadC* on the survival of *E. coli* MG1655 at pH 2.5. Overnight cultures were grown in 100 mL-baffled flasks in KE glucose glutamate minimal medium (pH 5.8). They were diluted into fresh KE glucose glutamate minimal medium (pH 2.5) with a start OD_{600} of 0.05. Viable cells were measured by plating serially diluted cultures onto LB agar after 0 and 2 hours of pH 2.5 acid stress. The percentage survival was calculated using time 0 as the reference. This experiment was performed in triplicate. Statistical analysis was done by using a two-tailed unpaired t-test. ****p<0.0001. Mean ± SD is shown. SD, standard deviation.

In summary, this study proved that the deletion of two mRNA and rRNA m^6A methyltransferases genes *rlmF* and *rlmJ* increased the *gadA* promoter activity of *E. coli* MG1655, subsequently, it increased the GadA protein production. The replacement of the 325^{th} proline code of CCA with CCT in the predicted m^6A modification site of *gadC* increased the survival of *E. coli* MG1655 at pH 2.5. Based on these two results, we propose that the m^6A modification might affect the transcript stability of *gadA* and *gadC*. To reveal the role and molecular mechanism of m^6A modification in the GDAR system, further studies are needed in the future

4. Discussion

4.1 Activation conditions of the GDAR system

The GDAR system provides the most robust protection for the majority of the commensal and pathogenic *E. coli* strains in extremely acidic environments such as the human stomach where the pH is lower than 2.5 (Bearson et al., 1997; Bhagwat et al., 2005; Castanie-Cornet et al., 1999; De Biase & Pennacchietti, 2012). The regulatory network of the GDAR system is extremely complicated, depending on the growth phases, media, and cultivation conditions. A systematic analysis of the activation conditions is still lacking despite numerous studies about the GDAR system, mainly focusing on the regulatory network and the molecular mechanism.

In this study, we systematically analyzed the GDAR system expression profile, focusing on the individual contribution of the particular stimuli such as pH, growth phase, relative dissolved oxygen and external metabolites. By monitoring the growth and external factors during bioreactor cultivations of *E. coli* cells, our data confirmed that both acid stress and the onset of the stationary phase induce the *gadA*/*gadB* expression (Figures 3.3 – 3.5) which is consistent with previous studies (Castanie-Cornet et al., 1999; De Biase et al., 1999; Weber, Polen, Heuveling, Wendisch, & Hengge, 2005). Previous work revealed that oxygen-limitation induces the *gadA*/*gadB* expression in buffered potassium-modified LB (Hayes et al., 2006). This study showed that oxygen-limitation induces *gadA*/*gadB* expression in a minimal glucose medium, adding new evidence for the positive effect of the oxygen-limitation on the expression of the GDAR system (Figures 3.4 & 3.6). Notably, this study revealed that oxygen was depleted during the transition into the stationary phase, while at the same time *gadA/gadB* was induced (Figure 3.3). Further studies confirmed the positive effect of the oxygen limitation on the activation of the GDAR system. In this study, *E. coli* performed the mixed fermentation and produced acetate when the glucose was used

as the carbon source under oxygen-limitation condition. The GDAR expression profile in *E. coli* cultivated in a KE minimal glucose medium revealed a transient, fine-tuned activation profile with the highest inducibility by oxygen-limitation and acetate, followed by stationary phase and acid stress (Figure 4.1). The highest inducibility by oxygen-limitation added new insights into the role of the GDAR system in the anaerobic growth of *E. coli* in the human gut.

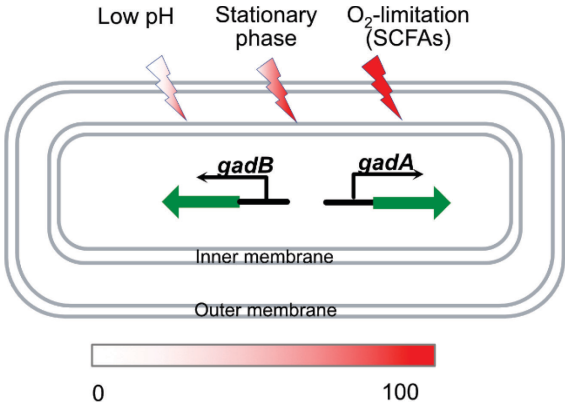


Figure 4.1. Schematic diagram of the activation model of the GDAR system in *E. coli*. Oxygen limitation, stationary phase and low pH all induce the expression of the GDAR system. The oxygen-limitation, which is mainly related to acetic acid produced by *E. coli*, displaced highest inducibility, followed by stationary phase and acid stress. The gradient of the color represents the activation strength.

4.2 The SCFAs and the GDAR system

The facultative anaerobic bacteria *E. coli* derive energy via electron transport-linked phosphorylation reaction under aerobic and anaerobic conditions in the present of alternative electron acceptors (Gunsalus, 1992; Gunsalus & Park, 1994). Upon oxygen depletion, *E. coli* processes glucose via mixed acid fermentation in the absence alternative electron acceptor, producing acetate, formate, succinate, lactate, and ethanol (Clark, 1989; Förster & Gescher, 2014; Stewart, 1993). In this study, there was no alternative electron receptor available. So, *E. coli* cells performed mixed fermentation when the oxygen was depleted. The external metabolite profile proved that acetate, formate and a small amount of succinate are the majority of the

fermentation products during the growth of the *E. coli* MG1655 during the transition into the stationary phase (in KE minimal glucose glutamate medium in the bioreactor under air-aeration conditions) (Figure 3.3F). Further investigation proved that only the acetic acid, but not other acids of the mixed fermentation products (formic acid, succinic acid, lactic acid) strongly induce the *gadA* and *gadB* expression in the exponential growth phase of *E. coli* MG1655. Acetic acid also plays a major role for the survival at pH 2.5 in the presence of glutamate, compared to the control hydrochloric acid. This study proved that acetic acid is mainly an external stimulus of the GDAR system in the exponential growth phase. These results support previous findings where it was shown that acetate increased the transcript levels of *gadA*, *gadB*, *gadX* and *gadW* in a global analysis of gene expression in pathogen *E. coli* O157:H7 cultivated in a different glucose minimal medium supplied with amino acids (Arnold et al., 2001).

The SCFAs refers to the organic acids with six or fewer carbon atoms and are the primary end products of fermentation of non-digestible carbohydrates by the gut microbiota (Morrison & Preston, 2016). Acetic acid and propionic acid, mainly produced by Bacteroidetes (also known as Bacteroidota), and butyric acid, mainly produced by Firmicutes (also known as Bacillota), are the most abundant SCFAs in the human colon with the approximal molar ratio of 60:20:20, which depends on the substrate, microbiota composition and the gut transit time (Morrison & Preston, 2016; Portincasa et al., 2022). The total amount of the SCFAs in the proximal colon is 70 – 140 mM, and falls to 20 – 70 mM in the distal colon (Topping & Clifton, 2001; Wong, de Souza, Kendall, Emam, & Jenkins, 2006). The SCFAs play essential roles in maintaining colonic health, serving as energy source, and decreasing the pH levels, and thereby inhibiting the growth of some acid-sensitive pathogens (Morrison & Preston, 2016). Addition of 50 mM of different SCFAs into the media caused stronger growth defects compared to hydrochloric acid. Growth defect (from strongest to weakest effect) was follows: butyric acid > propionic acid > acetic acid (data not shown). The primary characteristic contributing to the toxicity of the SCFAs is their diffusion ability to across

the cellular membranes (Stewart, 1993; Walter & Gutknecht, 1984; Warnecke & Gill, 2005). The undissociated SCFAs can freely diffuse into the cytoplasm, where the pH is maintained at around 7.5, which is most often higher than the pH of external media and pKas of the SCFAs (Goulbourne, Matin, Zychlinsky, & Matin, 1986; Maurer et al., 2005). As a result, the SCFAs dissociate into protons and anions in cytoplasm, disrupting the cytoplasm's internal pH, anion pool and the membrane potential (Warnecke & Gill, 2005). This study proved that the SCFAs (including acetic acid produced by *E. coli* and the other gut bacteria, and the propionic acid and butyric acid produced by other gut bacteria) are stimuli of the GDAR system during exponential growth of *E. coli*. These findings added new insights into the role of the GDAR system in *E. coli*, which is not only resistant to the extreme acid stress in the stomach (≤ pH 2.5) but also resistant to the organic acid stress caused by the SCFAs in the colon.

As mentioned above, the regulatory network of the GDAR system is remarkably complex and mainly regulated by EvgS/EvgA, MnmE, and GadX/GadW three circuits (Foster, 2004). This study extended the MnmE circuit with its partner protein MnmG and revealed the MnmE/MnmG is the primary circuit responding to SCFAs in the exponential growth phase of *E. coli* cells. Among all the three regulatory circuits related genes, the absence of *mnmE* or *mnmG* caused the strongest defect on *gadA* and *gadB* promoter activities in *E. coli* in response to the SCFAs, comparable to the negative control *gadE* mutant (Figure 3.13). Further experiments proved that MnmE/MnmG regulates *gadA* and *gadB* expression by affecting *gadE* promoter activity (Figure 3.18). The diminished *gadA* and *gadB* promoter activities in the *mnmG* mutant were fully restored when complemented with *mnmG* in trans, confirming the role of the MnmG in the GDAR system in response to SCFAs. This is the first time that the role of MnmG is studied in relation to the GDAR system.

On the other hand, the diminished *gadA* and *gadB* promoter activities in the *mnmE* mutant could not be restored by complementation under the stress caused by SCFAs.

This might be due to a dysregulation caused by an increased copy number of MnmE generated by plasmidic expression. MnmE was reported to play an essential role in the GDAR system in *E. coli* in the presence of glucose or under anaerobic conditions (Deng et al., 2013; Gong et al., 2004; Vivijs et al., 2016), in which *E. coli* produces acetate. In addition, MnmE and MnmG always form a heterotetrameric $\alpha_2\beta_2$ complex MnmE/MnmG (Yim et al., 2006). MnmE is highly likely to be involved in responding to SCFAs together with MnmG in *E. coli*, although this was not confirmed in this work.

The MnmE/MnmG plays a role in tRNA cmnm modification (Böhme et al., 2010; Fislage et al., 2014; Shi et al., 2009; Yim et al., 2006). This study, together with the previous studies (Deng et al., 2013; Gong et al., 2004; Vivijs et al., 2016), adds a link between the tRNA modification and the regulation of the GDAR system. Hence, in the future, it would be fascinating to investigate the exact molecular mechanism of acid sensing and the following regulation by the MnmE/MnmG circuit.

4.3. RcsB and YpdB are required to activate the GDAR system in *E. coli*

The complex non-canonical Rcs TCS (Regulator of Capsule Synthesis) attracted our attention because its response regulator RcsB is essential for the expression of *gadB* and *gadA* genes. RcsB forms functional heterodimers with GadE (Castanie-Cornet et al., 2010; Castanié-Cornet et al., 2007). This study proved that RcsB is required to activate the expression of *gadB* and *gadA* in *E. coli* in response to the SCFAs and hydrochloric acid (Figures 3.21 & 3.22). RcsB directly affects the expression of the *gadB* and *gadA*, instead of affecting the expression of *gadE* (Figure 3.25). These results go in hand with previously described findings showing that the Gad Box and RcsB Box are in the promoter region of *gadB* and *gadA* (Castanie-Cornet et al., 2010).

Pyruvate sensing YpdA/YpdB TCS consists of a LytS-like histidine kinase YpdA and a LytTR-like response regulator YpdB (Fried et al., 2013; Miyake et al., 2019). Eight regulatory target genes (*yhjX*, *pbpC*, *yghW*, *yhcC*, *xthA*, *gltBDF* and *astCADBE*) of the

YpdA/YpdB TCS have been identified, which are involved in the modulation of the structure and function of the membrane, in response of environmental stress and glutamate biosynthesis and arginine degradation (Miyake et al., 2019). Among the eight targets, the major target is the transporter gene *yhjX*. This study revealed that YpdB is required for the expression of *gadA* and *gadB* in *E. coli* in response to the SCFAs and the hydrochloric acid (Figures 3.21 & 3.26), adding a new regulator to the regulatory network of the GDAR system. Surprisingly, the absence of *ypdB* did not cause a decrease of promoter activity of central regulator gene *gadE* of the GDAR system (Figure 3.29), indicating that the regulation of YpdB on *gadA* and *gadB* expression is not linked to the expression of *gadE.* There are different possibilities about this regulatory model. First, YpdB might directly regulate the expression of *gadA* and *gadB* by directly binding to their promoter regions. Second, YpdB might perform the same model as RcsB, which regulates the *gadA* and *gadB* expression by forming a functional heterodimer with GadE. Third, YpdB might indirectly regulate *gadA* and *gadB* by regulating the expression of other target genes.

In contrast, the histidine kinase YpdA of the YpdA/YpdB TCS was not necessary to activate the expression of *gadA* and *gadB* (Figure 3.21). This conclusion was confirmed by further studies, where pyruvate did not induce the expression of *gadA* and *gadB* (Figure 3.27) and the phosphorylation of the YpdB was not required to activate the expression of the *gad* genes (Figure 3.28).

This study extended the regulatory network of the GDAR system by adding the regulator YpdB. YpdB and RcsB displayed a similar effect on activating the GDAR system: they are both required to activate the *gadA* and *gadB* expression, which is not achieved through the expression of *gadE*. The YpdB and RcsB are both required for the expression of the GDAR system not only in response to the SCFAs but also to the hydrochloric acid. It seems like that the YpdB and RcsB are required for the general expression of the GDAR system. Neither the histidine kinase of YpdA/YpdB TCS nor

Rcs TCS are necessary for the expression of the GDAR system. Therefore, we hypothesized that YpdB and RcsB might display the similar mechanism on regulating the GDAR system. This hypothesis needs to be further investigated.

4.4 The extended regulatory network of the GDAR system

This study further extended the regulatory network of the GDAR system with the MnmG/MnmE-GadE circuit and the new regulatory protein YpdB (Figure 4.2). Firstly, this study mainly focused on the MnmG/MnmE-GadE circuit, which is the primary circuit in *E. coli* in response to the SCFAs (Figure 4.2). This circuit does not respond to the hydrochloric acid. MnmG/MnmE regulates the *gadA* and *gadB* expression through the transcription of the central regulator gene *gadE*. Then this study revealed that YpdB is necessary to activate the expression of *gadA* and *gadB*, but not to *gadE* (Figure 4.2). The requirement of YpdB for the expression of *gadA* and *gadB* is not only under the induction of the SCFAs, but also under the hydrochloric acid.

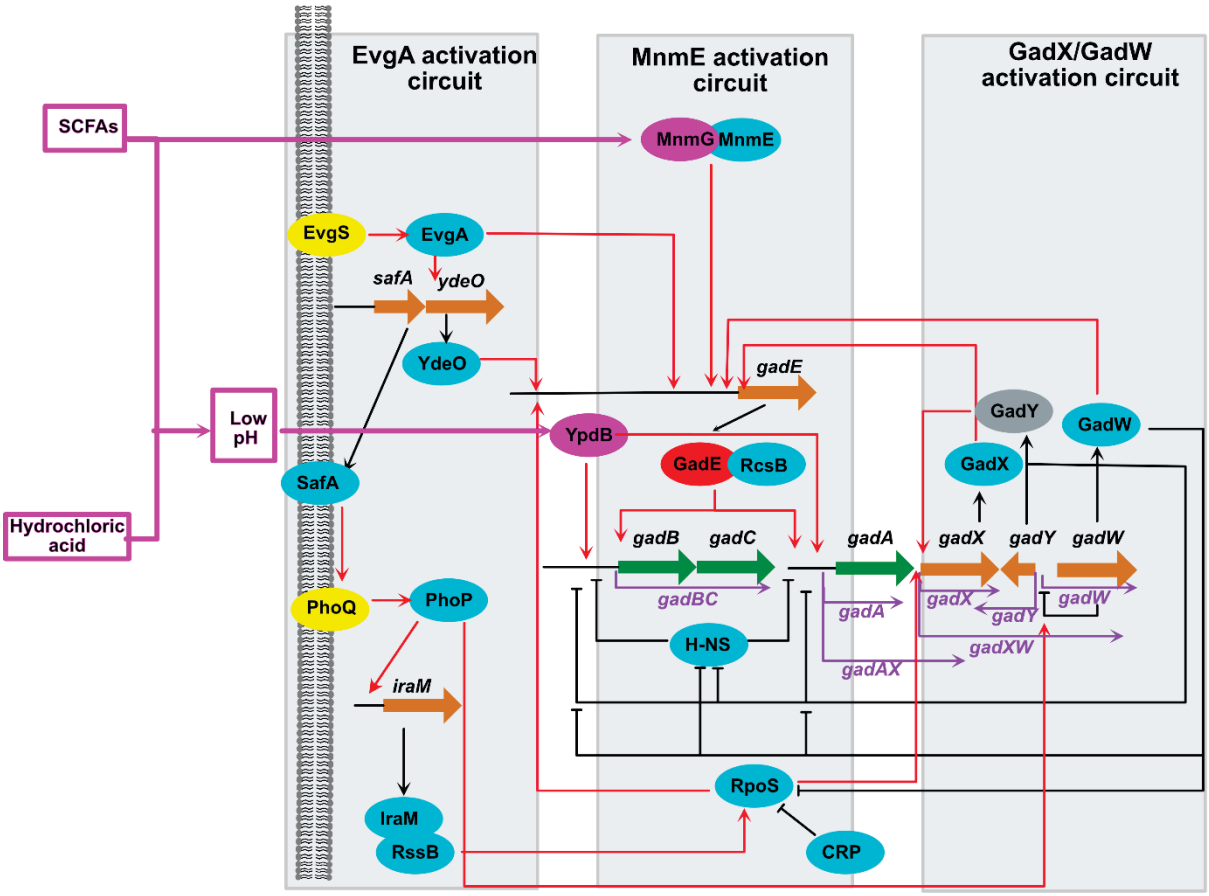


Figure 4.2. Schematic diagram of the extended regulatory network of the GDAR system in *E. coli*. Thick arrows represent genes, *gad* genes are colored in green, while the regulatory genes are colored in brown. Ellipses colored in purple represents new extended regulatory proteins, ellipses in blue represent well-known regulatory proteins, ellipses colored in yellow represent sensor histidine kinases, ellipse in red represents the central regulator, ellipse in grey represents small RNA. Thin bent arrows in purple represent transcription units, thin bent arrows in blue indicate the positive regulation which might be indirect, small bent arrows in red indicate positive regulation, black bars indicate negative regulation. This figure is adapted from (Foster, 2004) and (Tramonti et al., 2008).

4.5 Outlook

In this thesis, the activation profile of the GDAR system in *E. coli* was systematically analyzed. The activation profile led us to investigate the effect of the oxygen-limitation

on the expression of the GDAR system. Further research revealed that the SCFAs are external stimuli of the GDAR system. SCFAs are produced by intestinal bacteria in the large intestine, and play important roles in maintaining colonic health, especially butyrate (Macfarlane & Gibson, 1997; Yap et al., 2021). Understanding the regulatory network of the GDAR system can help us to reveal its mechanism of action, which has important implications for controlling the microbes in the food chain for prevention and clinical treatment (Arcari et al., 2020; Seo, Kim, O'Brien, Szubin, & Palsson, 2015).

This study revealed the YpdB is necessary for activating the expression of the *gadB* and *gadA* in *E. coli* in response to SCFAs and hydrochloric acid. YpdB displayed a similar pattern to RcsB on activating the GDAR system. Interestingly, this regulation is independent from the signaling cascade typical of two component systems. The fact that the phosphorylation state of YpdB does not change expression of *gadA* and *gadB* and that YpdA or pyruvate do not play a role, indicates that YpdB acts as a regulatory protein without being activated by its cognate kinase. The mechanism by which YpdB might regulate the expression of *gadA* and *gadB* needs to be further investigated.

In eukaryotes, m^6A modification affects the translation and stability of modified transcripts, thus providing a mechanism to coordinate the regulation of groups of transcripts during cell state maintenance and transition (Frye et al., 2018; Fu et al., 2014). However, the function and mechanism of m^6A modification are relatively poorly understood in prokaryotes. This study opened a new door for investigating the role of m^6A modification on the expression of *gad* genes. Our data indicated that the transcript stability in the absence of the methyltransferases RlmF and RlmJ and the m^6A modification resulting in an elevated level of GadA and GadC. Further studies are needed in order to properly elucidate the function of m6A modification in prokaryote and its relevance in regulation of the GDAR system.

5. Summary

Acid resistance is an important feature for all human enterobacteria to survive extreme acid stress in the stomach (pH < 2.5) and to adapt to mild acid stress in the colon (pH 6.0). The glutamate-dependent acid resistance (GDAR) system provides the most robust protection for *Escherichia coli* strains in extremely acidic environments. The GDAR system consists of the two homologous glutamate decarboxylases GadA/GadB and the glutamate/γ-aminobutyric acid antiporter GadC. Induction of the GDAR system is complex and controlled by many regulatory proteins, but the primary stimuli for its activation are poorly understood. In this work, the dynamics of *gadA/gadB* expression and GadA/GadB production were systematically analyzed in correlation with alterations of pH, oxygen and several metabolites during the growth of *E. coli* MG1655 in a bioreactor. The expression profile and further research revealed a transient, fine-tuned activation profile, in which oxygen limitation plays a major role, followed by stationary phase and acid stress.

Under oxygen limitation, *E. coli* switches to mixed acid fermentation. Therefore, in this study, the effect of fermentation products, as well as short-chain fatty acids (SCFAs) on the expression of the *gadB* and *gadA* was investigated. The SCFAs acetic acid, propionic acid, and butyric acid were found to be the most important external stimuli of the GDAR system. The MnmG/MnmE-GadE circuit was identified to respond to these SCFAs in *E. coli*.

In addition, a screening of regulators that sense the low pH caused by SCFAs and hydrochloric acid revealed the response regulator of the pyruvate-sensing YpdA/YpdB histidine kinase/response regulator system is important for the expression of *gadA* and *gadB* in *E. coli*. This activation is independent of the expression of the expression of the central regulator gene *gadE* and does not require the phosphorylation of YpdB.

In summary, a systematic analysis of the expression profile of the GDAR system has revealed SCFAs as external stimuli for the MnmG/MnmE-GadE regulatory circuit, as well as a new regulatory component, the response regulator YpdB in this complex regulatory network.

6. Zusammenfassung

Säureresistenz ist eine wichtige Eigenschaft für humane Enterobakterien um die extrem sauren Bedingungen des Magens (pH < 2.5) zu überleben und um sich an den leichten Säurestress des Dickdarms (pH 6.0) anzupassen. Das Glutamat-abhängige Säureresistenzsystem (GDAR) bietet den robustesten Schutz für *Escherichia coli* gegenüber extrem sauren Bedingungen. Das GDAR System besteht aus zwei homologen Glutamatdecarboxylasen GadA/GadB und dem Glutamat/γ-Aminobuttersäure Antiporter GadC. Die Induktion des GDAR Systems ist komplex und wird von mehreren regulatorischen Proteinen kontrolliert, wobei der primäre Stimulus für die Aktivierung nicht genau bekannt ist. In dieser Arbeit werden die Dynamiken der *gadA/gadB* Expression und GadA/GadB Produktion systematisch analysiert und korreliert mit verschiedenen pH Werten, der Sauerstoffverfügbarkeit und verschiedenen Metaboliten während des Wachstums von *E. coli* MG1655 in einem Bioreaktor. Das Expressionsprofil und zusätzliche Experimente zeigten ein transientes, fein abgestimmtes Aktivierungsprofil, in welchem Sauerstoffverfügbarkeit die wichtigste Rolle spielt, gefolgt von der stationären Wachstumsphase und Säurestress.

Unter Sauerstofflimitation wechselt *E. coli* zur gemischten Säuregärung. Deshalb wurden in dieser Arbeit der Effekt von Fermentationsprodukten sowie kurzkettiger Fettsäuren auf die Expression von *gadB* und *gadA* untersucht. Propansäure und Buttersäure wurden dabei als die wichtigsten externen Stimuli für das GDAR System aufgezeigt. Es wurde ebenfalls demonstriert, dass der MnmG/MnmE-GadE Kreislauf in *E. coli* auch auf kurzkettige Fettsäuren reagiert.

Zusätzlich konnte über ein Screening von potentiellen Regulatoren, welche den niedrigen pH Wert versucht durch kurzkettigen Fettsäuren oder Salzsäure wahrnehmen, gezeigt werden, dass der Antwortregulator des Pyruvatsensorik YpdA/YpdB Histidinkinase/Antwortregulator Systems wichtig für die Expression von

gadA und *gadB* in *E. coli* ist. Diese Aktivierung ist unabhängig von der Expression des Gens des zentralen Regulators *gadE* und benötigt auch keine Phosphorylierung von YpdB.

Zusammengefasst konnte durch die systematische Analyse des Expressionsprofils des GDAR Systems bewiesen werden, dass kurzkettige Fettsäuren ein externer Stimulus für den MnmG/MnmE-GadE regulatorischen Schaltkreis sind, sowie dass der Antwortregulator YpdB ein neues regulatorisches Element in diesem komplexen regulatorischen Netzwerk darstellt.

7. References

Agris, P. F., Vendeix, F. A., & Graham, W. D. (2007). tRNA's wobble decoding of the genome: 40 years of modification. *J Mol Biol, 366*(1), 1-13. doi:10.1016/j.jmb.2006.11.046

Arcari, T., Feger, M. L., Guerreiro, D. N., Wu, J., & O'Byrne, C. P. (2020). Comparative Review of the Responses of *Listeria monocytogenes* and *Escherichia coli* to Low pH Stress. *Genes (Basel), 11*(11). doi:10.3390/genes11111330

Ares, M. (2012). Bacterial RNA isolation. *Cold Spring Harb Protoc, 2012*(9), 1024-1027. doi:10.1101/pdb.prot071068

Arnold, C. N., McElhanon, J., Lee, A., Leonhart, R., & Siegele, D. A. (2001). Global analysis of *Escherichia coli* gene expression during the acetate-induced acid tolerance response. *J Bacteriol, 183*(7), 2178-2186. doi:10.1128/JB.183.7.2178-2186.2001

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., . . . Mori, H. (2006). Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol, 2*, 2006 0008. doi:10.1038/msb4100050

Bearson, S., Bearson, B., & Foster, J. W. (1997). Acid stress responses in enterobacteria. *FEMS Microbiol Lett, 147*(2), 173-180. doi:10.1111/j.1574-6968.1997.tb10238.x

Bergholz, T. M., Tarr, C. L., Christensen, L. M., Betting, D. J., & Whittam, T. S. (2007). Recent gene conversions between duplicated glutamate decarboxylase genes (*gadA* and *gadB*) in pathogenic *Escherichia coli*. *Mol Biol Evol, 24*(10), 2323-2333. doi:10.1093/molbev/msm163

Bhagwat, A. A., Chan, L., Han, R., Tan, J., Kothary, M., Jean-Gilles, J., & Tall, B. D. (2005). Characterization of enterohemorrhagic *Escherichia coli* strains based on acid resistance phenotypes. *Infect Immun, 73*(8), 4993-5003. doi:10.1128/iai.73.8.4993-5003.2005

Böhme, S., Meyer, S., Krüger, A., Steinhoff, H. J., Wittinghofer, A., & Klare, J. P. (2010). Stabilization of G domain conformations in the tRNA-modifying MnmE-GidA complex observed with double electron electron resonance spectroscopy. *J Biol Chem, 285*(22), 16991-17000. doi:10.1074/jbc.M109.096131

Brameyer, S., Hoyer, E., Bibinger, S., Burdack, K., Lassak, J., & Jung, K. (2020). Molecular design of a signaling system influences noise in protein abundance under acid stress in different γ-Proteobacteria. *J Bacteriol*. doi:10.1128/jb.00121-20

Brameyer, S., Schumacher, K., Kuppermann, S., & Jung, K. (2022). Division of labor and collective functionality in *Escherichia coli* under acid stress. *Commun Biol, 5*(1), 327. doi:10.1038/s42003-022-03281-4

Brown, A. N., Anderson, M. T., Bachman, M. A., & Mobley, H. L. T. (2022). The ArcAB Two-Component System: Function in Metabolism, Redox Control, and Infection. *Microbiol Mol Biol Rev, 86*(2), e0011021. doi:10.1128/mmbr.00110-21

Capitani, G., De Biase, D., Aurizi, C., Gut, H., Bossa, F., & Grütter, M. G. (2003). Crystal structure and functional analysis of *Escherichia coli* glutamate decarboxylase. *Embo j, 22*(16), 4027-4037. doi:10.1093/emboj/cdg403

Capra, E. J., & Laub, M. T. (2012). Evolution of two-component signal transduction systems. *Annu Rev Microbiol, 66*, 325-347. doi:10.1146/annurev-micro-092611-150039

Castanie-Cornet, M. P., Cam, K., Bastiat, B., Cros, A., Bordes, P., & Gutierrez, C. (2010). Acid stress response in *Escherichia coli*: mechanism of regulation of *gadA* transcription by RcsB and GadE. *Nucleic Acids Res, 38*(11), 3546-3554. doi:10.1093/nar/gkq097

Castanie-Cornet, M. P., Penfound, T. A., Smith, D., Elliott, J. F., & Foster, J. W. (1999). *Control of acid* resistance in *Escherichia coli*. *J Bacteriol, 181*, 3525–3535.

Castanié-Cornet, M. P., Treffandier, H., Francez-Charlot, A., Gutierrez, C., & Cam, K. (2007). The glutamate-dependent acid resistance system in *Escherichia coli*: essential and dual role of the His-Asp phosphorelay RcsCDB/AF. *Microbiology (Reading), 153*(Pt 1), 238-246. doi:10.1099/mic.0.29278-0

Chavez, R. G., Alvarez, A. F., Romeo, T., & Georgellis, D. (2010). The physiological stimulus for the BarA sensor kinase. *J Bacteriol, 192*(7), 2009-2012. doi:10.1128/JB.01685-09

Clark, D. P. (1989). The fermentation pathways of *Escherichia coli*. *FEMS Microbiol Rev, 5*(3), 223-234. doi:10.1016/0168-6445(89)90033-8

De Biase, D., & Pennacchietti, E. (2012). Glutamate decarboxylase-dependent acid resistance in orally acquired bacteria: function, distribution and biomedical

implications of the *gadBC* operon. *Mol Microbiol, 86*(4), 770-786. doi:10.1111/mmi.12020

De Biase, D., Tramonti, A., Bossa, F., & Visca, P. (1999). The response *to* stationary-phase stress conditions in Escherichia coli: role and regulation of the glutamic acid decarboxylase system. *Mol Microbiol, 32*, 1198–1211.

Deng, X., Chen, K., Luo, G. Z., Weng, X., Ji, Q., Zhou, T., & He, C. (2015). Widespread occurrence of N6-methyladenosine in bacterial mRNA. *Nucleic Acids Res, 43*(13), 6557-6567. doi:10.1093/nar/gkv596

Deng, Z., Shan, Y., Pan, Q., Gao, X., & Yan, A. (2013). Anaerobic expression of the *gadE-mdtEF* multidrug efflux operon is primarily regulated by the two-component system ArcBA through antagonizing the H-NS mediated repression. *Front Microbiol, 4*, 194. doi:10.3389/fmicb.2013.00194

Ducret, A., Quardokus, E. M., & Brun, Y. V. (2016). MicrobeJ, a tool for high throughput bacterial cell detection and quantitative analysis. *Nat Microbiol, 1*(7), 16077. doi:10.1038/nmicrobiol.2016.77

Dutyshev, D. I., Darii, E. L., Fomenkova, N. P., Pechik, I. V., Polyakov, K. M., Nikonov, S. V., . . . Sukhareva, B. S. (2005). Structure of *Escherichia coli* glutamate decarboxylase (GADalpha) in complex with glutarate at 2.05 angstroms resolution. *Acta Crystallogr D Biol Crystallogr, 61*(Pt 3), 230-235. doi:10.1107/s0907444904032147

Eguchi, Y., Ishii, E., Hata, K., & Utsumi, R. (2011). Regulation of acid resistance by connectors of two-component signal transduction systems in *Escherichia coli*. *J Bacteriol, 193*(5), 1222-1228. doi:10.1128/JB.01124-10

Eguchi, Y., Itou, J., Yamane, M., Demizu, R., Yamato, F., Okada, A., . . . Utsumi, R. (2007). B1500, a small membrane protein, connects the two-component systems EvgS/EvgA and PhoQ/PhoP in *Escherichia coli*. *Proc Natl Acad Sci U S A, 104*(47), 18712-18717. doi:10.1073/pnas.0705768104

Eguchi, Y., Okada, T., Minagawa, S., Oshima, T., Mori, H., Yamamoto, K., . . . Utsumi, R. (2004). Signal transduction cascade between EvgA/EvgS and PhoP/PhoQ two-component systems of *Escherichia coli*. *J Bacteriol, 186*(10), 3006-3014. doi:10.1128/JB.186.10.3006-3014.2004

Eguchi, Y., & Utsumi, R. (2014). Alkali metals in addition to acidic pH activate the EvgS histidine kinase sensor in *Escherichia coli*. *J Bacteriol, 196*(17), 3140-3149. doi:10.1128/JB.01742-14

Epstein, W., & Kim, B. S. (1971). *Potassium transpo*rt loci in *Escherichia coli* K-12. *J Bacteriol, 108*, 639–644.

Fislage, M., Brosens, E., Deyaert, E., Spilotros, A., Pardon, E., Loris, R., . . . Versées, W. (2014). SAXS analysis of the tRNA-modifying enzyme complex MnmE/MnmG reveals a novel interaction mode and GTP-induced oligomerization. *Nucleic Acids Res, 42*(9), 5978-5992. doi:10.1093/nar/gku213

Förster, A. H., & Gescher, J. (2014). Metabolic Engineering of *Escherichia coli* for Production of Mixed-Acid Fermentation End Products. *Front Bioeng Biotechnol, 2*, 16. doi:10.3389/fbioe.2014.00016

Foster, J. W. (2004). *Escherichia coli* acid resistance: tales of an amateur acidophile. *Nat Rev Microbiol, 2*(11), 898-907. doi:10.1038/nrmicro1021

Fried, L., Behr, S., & Jung, K. (2013). Identification of a target gene and activating stimulus for the YpdA/YpdB histidine kinase/response regulator system in *Escherichia coli*. *J Bacteriol, 195*(4), 807-815. doi:10.1128/jb.02051-12

Fritz, G., Koller, C., Burdack, K., Tetsch, L., Haneburger, I., Jung, K., & Gerland, U. (2009). Induction kinetics of a conditional pH stress response system in *Escherichia coli*. *J Mol Biol, 393*(2), 272-286. doi:10.1016/j.jmb.2009.08.037

Frye, M., Harada, B. T., Behm, M., & He, C. (2018). RNA modifications modulate gene expression during development. *Science, 361*(6409), 1346-1349. doi:10.1126/science.aau1646

Fu, Y., Dominissini, D., Rechavi, G., & He, C. (2014). Gene expression regulation mediated through reversible m⁶A RNA methylation. *Nat Rev Genet, 15*(5), 293-306. doi:10.1038/nrg3724

Gajiwala, K. S., & Burley, S. K. (2000). HDEA, a periplasmic protein that supports acid resistance in pathogenic enteric bacteria. *J Mol Biol, 295*(3), 605-612. doi:10.1006/jmbi.1999.3347

Giangrossi, M., Zattoni, S., Tramonti, A., De Biase, D., & Falconi, M. (2005). Antagonistic role of H-NS and GadX in the regulation of the glutamate decarboxylase-dependent acid resistance system in *Escherichia coli*. *J Biol Chem, 280*(22), 21498-21505. doi:10.1074/jbc.M413255200

Gong, S., Ma, Z., & Foster, J. W. (2004). The Era-like GTPase TrmE conditionally activates *gadE* and glutamate-dependent acid resistance in *Escherichia coli*. *Mol Microbiol, 54*(4), 948-961. doi:10.1111/j.1365-2958.2004.04312.x

Goulbourne, E., Jr., Matin, M., Zychlinsky, E., & Matin, A. (1986). Mechanism of delta pH maintenance in active and inactive cells of an obligately acidophilic bacterium. *J Bacteriol, 166*(1), 59-65. doi:10.1128/jb.166.1.59-65.1986

Gunsalus, R. P. (1992). Control of electron flow in *Escherichia coli*: coordinated transcription of respiratory pathway genes. *J Bacteriol, 174*(22), 7069-7074. doi:10.1128/jb.174.22.7069-7074.1992

Gunsalus, R. P., & Park, S. J. (1994). Aerobic-anaerobic gene regulation in *Escherichia coli*: control by the ArcAB and Fnr regulons. *Res Microbiol, 145*(5-6), 437-450. doi:10.1016/0923-2508(94)90092-2

Hayes, E. T., Wilks, J. C., Sanfilippo, P., Yohannes, E., Tate, D. P., Jones, B. D., . . . Slonczewski, J. L. (2006). Oxygen limitation modulates pH regulation of catabolism and hydrogenases, multidrug transporters, and envelope composition in *Escherichia coli* K-12. *BMC Microbiol, 6*, 89. doi:10.1186/1471-2180-6-89

Heux, S., Philippe, B., & Portais, J. C. (2011). High-throughput workflow for monitoring and mining bioprocess data and its application to inferring the physiological response of *Escherichia coli* to perturbations. *Appl Environ Microbiol, 77*(19), 7040-7049. doi:10.1128/aem.05838-11

Hommais, F., Krin, E., Coppee, J. Y., Lacroix, C., Yeramian, E., Danchin, A., & Bertin, P. (2004). GadE (YhiE): a novel activator involved in the response to acid environment in *Escherichia coli*. *Microbiology (Reading), 150*(Pt 1), 61-72. doi:10.1099/mic.0.26659-0

Itou, J., Eguchi, Y., & Utsumi, R. (2009). Molecular mechanism of transcriptional cascade initiated by the EvgS/EvgA system in *Escherichia coli* K-12. *Biosci Biotechnol Biochem, 73*(4), 870-878. doi:10.1271/bbb.80795

Iuchi, S., Cameron, D. C., & Lin, E. C. (1989). A second global regulator gene (*arcB*) mediating repression of enzymes in aerobic pathways of *Escherichia coli*. *J Bacteriol, 171*(2), 868-873. doi:10.1128/jb.171.2.868-873.1989

Iuchi, S., & Lin, E. C. (1988). *arcA* (dye), a global regulatory gene in *Escherichia coli* mediating repression of enzymes in aerobic pathways. *Proc Natl Acad Sci U S A, 85*(6), 1888-1892. doi:10.1073/pnas.85.6.1888

Johnson, M. D., Burton, N. A., Gutiérrez, B., Painter, K., & Lund, P. A. (2011). RcsB is required for inducible acid resistance in *Escherichia coli* and acts at *gadE*-dependent and -independent promoters. *J Bacteriol, 193*(14), 3653-3656. doi:10.1128/jb.05040-11

Jung, I. L., & Kim, I. G. (2003). Polyamines and glutamate decarboxylase-based acid resistance in *Escherichia coli*. *J Biol Chem, 278*(25), 22846-22852. doi:10.1074/jbc.M212055200

Kanjee, U., Gutsche, I., Ramachandran, S., & Houry, W. A. (2011). The enzymatic activities of the *Escherichia coli* basic aliphatic amino acid decarboxylases exhibit a pH zone of inhibition. *Biochemistry, 50*(43), 9388-9398. doi:10.1021/bi201161k

Kanjee, U., & Houry, W. A. (2013). Mechanisms of acid resistance in *Escherichia coli*. *Annu Rev Microbiol, 67*, 65-81. doi:10.1146/annurev-micro-092412-155708

Kashiwagi, K., Miyamoto, S., Suzuki, F., Kobayashi, H., & Igarashi, K. (1992). Excretion of putrescine by the putrescine-ornithine antiporter encoded by the *potE* gene of *Escherichia coli*. *Proc Natl Acad Sci U S A, 89*(10), 4529-4533. doi:10.1073/pnas.89.10.4529

Kashiwagi, K., Suzuki, T., Suzuki, F., Furuchi, T., Kobayashi, H., & Igarashi, K. (1991). Coexistence of the genes for putrescine transport protein and ornithine decarboxylase at 16 min on *Escherichia coli* chromosome. *J Biol Chem, 266*(31), 20922-20927.

Kern, R., Malki, A., Abdallah, J., Tagourti, J., & Richarme, G. (2007). *Escherichia coli* HdeB is an acid stress chaperone. *J Bacteriol, 189*(2), 603-610. doi:10.1128/jb.01522-06

Kitagawa, M., Ara, T., Arifuzzaman, M., Ioka-Nakamichi, T., Inamoto, E., Toyonaga, H., & Mori, H. (2005). Complete set of ORF clones of *Escherichia coli* ASKA library (a complete set of *E. coli* K-12 ORF archive): unique resources for biological research. *DNA Res, 12*(5), 291-299. doi:10.1093/dnares/dsi012

Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., 2nd, & Peterson, K. M. (1995). Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene, 166*(1), 175-176. doi:10.1016/0378-1119(95)00584-1

Krishna, V. N., Karthika, D., Surya, D. M., Rubini, M., Vishalini, M., & Pradeepa, Y. (2010). Analysis of Monosodium l-Glutamate in Food Products by High-Performance Thin Layer Chromatography. *J Young Pharm, 2*(3), 297-300. doi:10.4103/0975-1483.66795

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680-685. doi:10.1038/227680a0

Lange, R., & Hengge-Aronis, R. (1994). The cellular concentration of the sigma S subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation, and protein stability. *Genes Dev, 8*(13), 1600-1612. doi:10.1101/gad.8.13.1600

Lund, P., Tramonti, A., & De Biase, D. (2014). Coping with low pH: molecular strategies in neutralophilic bacteria. *FEMS Microbiol Rev, 38*(6), 1091-1125. doi:10.1111/1574-6976.12076

Ma, D., Lu, P., & Shi, Y. (2013). Substrate selectivity of the acid-activated glutamate/gamma-aminobutyric acid (GABA) antiporter GadC from *Escherichia coli*. *J Biol Chem, 288*(21), 15148-15153. doi:10.1074/jbc.M113.474502

Ma, Z., Gong, S., Richard, H., Tucker, D. L., Conway, T., & Foster, J. W. (2003). GadE (YhiE) activates glutamate decarboxylase-dependent acid resistance in *Escherichia coli* K-12. *Mol Microbiol, 49*(5), 1309-1320. doi:10.1046/j.1365-2958.2003.03633.x

Ma, Z., Masuda, N., & Foster, J. W. (2004). Characterization of EvgAS-YdeO-GadE branched regulatory circuit governing glutamate-dependent acid resistance in *Escherichia coli*. *J Bacteriol, 186*(21), 7378-7389. doi:10.1128/JB.186.21.7378-7389.2004

Ma, Z., Richard, H., Tucker, D. L., Conway, T., & Foster, J. W. (2002). Collaborative regulation of *Escherichia coli* glutamate-dependent acid resistance by two AraC-like regulators, GadX and GadW (YhiW). *J Bacteriol, 184*(24), 7001-7012. doi:10.1128/jb.184.24.7001-7012.2002

Macfarlane, G. T., & Gibson, G. R. (1997). Carbohydrate Fermentation, Energy Transduction and Gas Metabolism in the Human Large Intestine. In R. I. Mackie & B. A. White (Eds.), *Gastrointestinal Microbiology: Volume 1 Gastrointestinal Ecosystems and Fermentations* (pp. 269-318). Boston, MA: Springer US.

Masuda, N., & Church, G. M. (2002). *Escherichia coli* gene expression responsive to levels of the response regulator EvgA. *J Bacteriol, 184*(22), 6225-6234. doi:10.1128/jb.184.22.6225-6234.2002

Masuda, N., & Church, G. M. (2003). Regulatory network of acid resistance genes in *Escherichia coli*. *Mol Microbiol, 48*(3), 699-712. doi:10.1046/j.1365-2958.2003.03477.x

Maurer, L. M., Yohannes, E., Bondurant, S. S., Radmacher, M., & Slonczewski, J. L. (2005). pH regulates genes for flagellar motility, catabolism, and oxidative

stress in *Escherichia coli* K-12. *J Bacteriol, 187*(1), 304-319. doi:10.1128/jb.187.1.304-319.2005

McClements, D. J., & Li, Y. (2010). Review of in vitro digestion models for rapid screening of emulsion-based systems. *Food Funct, 1*(1), 32-59. doi:10.1039/c0fo00111b

Meyer, S., Wittinghofer, A., & Versées, W. (2009). G-domain dimerization orchestrates the tRNA wobble modification reaction in the MnmE/GidA complex. *J Mol Biol, 392*(4), 910-922. doi:10.1016/j.jmb.2009.07.004

Mihalcescu, I., Van-Melle Gateau, M., Chelli, B., Pinel, C., & Ravanat, J. L. (2015). Green autofluorescence, a double edged monitoring tool for bacterial growth and activity in micro-plates. *Phys Biol, 12*(6), 066016. doi:10.1088/1478-3975/12/6/066016

Miyake, Y., Inaba, T., Watanabe, H., Teramoto, J., Yamamoto, K., & Ishihama, A. (2019). Regulatory roles of pyruvate-sensing two-component system PyrSR (YpdAB) in *Escherichia coli* K-12. *FEMS Microbiol Lett, 366*(2). doi:10.1093/femsle/fnz009

Mizuno, T., & Mizushima, S. (1990). Signal transduction and gene regulation through the phosphorylation of two regulatory components: the molecular basis for the osmotic regulation of the porin genes. *Mol Microbiol, 4*(7), 1077-1082. doi:10.1111/j.1365-2958.1990.tb00681.x

Morrison, D. J., & Preston, T. (2016). Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microbes, 7*(3), 189-200. doi:10.1080/19490976.2015.1134082

Opdyke, J. A., Fozo, E. M., Hemm, M. R., & Storz, G. (2011). RNase III participates in GadY-dependent cleavage of the *gadX-gadW* mRNA. *J Mol Biol, 406*(1), 29-43. doi:10.1016/j.jmb.2010.12.009

Opdyke, J. A., Kang, J. G., & Storz, G. (2004). GadY, a small-RNA regulator of acid response genes in *Escherichia coli*. *J Bacteriol, 186*(20), 6698-6705. doi:10.1128/JB.186.20.6698-6705.2004

Paczia, N., Nilgen, A., Lehmann, T., Gätgens, J., Wiechert, W., & Noack, S. (2012). Extensive exometabolome analysis reveals extended overflow metabolism in various microorganisms. *Microb Cell Fact, 11*, 122. doi:10.1186/1475-2859-11-122

Pannen, D., Fabisch, M., Gausling, L., & Schnetz, K. (2016). Interaction of the RcsB Response Regulator with Auxiliary Transcription Regulators in *Escherichia coli*. *J Biol Chem, 291*(5), 2357-2370. doi:10.1074/jbc.M115.696815

Pernestig, A. K., Georgellis, D., Romeo, T., Suzuki, K., Tomenius, H., Normark, S., & Melefors, O. (2003). The *Escherichia coli* BarA-UvrY two-component system is needed for efficient switching between glycolytic and gluconeogenic carbon sources. *J Bacteriol, 185*(3), 843-853. doi:10.1128/JB.185.3.843-853.2003

Pernestig, A. K., Melefors, O., & Georgellis, D. (2001). Identification of UvrY as the cognate response regulator for the BarA sensor kinase in *Escherichia coli. J Biol Chem, 276*(1), 225-231. doi:10.1074/jbc.M001550200

Portincasa, P., Bonfrate, L., Vacca, M., De Angelis, M., Farella, I., Lanza, E., . . . Di Ciaula, A. (2022). Gut Microbiota and Short Chain Fatty Acids: Implications in Glucose Homeostasis. *Int J Mol Sci, 23*(3). doi:10.3390/ijms23031105

Sayed, A. K., Odom, C., & Foster, J. W. (2007). The *Escherichia coli* AraC-family regulators GadX and GadW activate *gadE*, the central activator of glutamate-dependent acid resistance. *Microbiology (Reading), 153*(Pt 8), 2584-2592. doi:10.1099/mic.0.2007/007005-0

Schlüter, J. P., Czuppon, P., Schauer, O., Pfaffelhuber, P., McIntosh, M., & Becker, A. (2015). Classification of phenotypic subpopulations in isogenic bacterial cultures by triple promoter probing at single cell level. *J Biotechnol, 198*, 3-14. doi:10.1016/j.jbiotec.2015.01.021

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc, 3*(6), 1101-1108. doi:10.1038/nprot.2008.73

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nat Methods, 9*(7), 671-675. doi:10.1038/nmeth.2089

Seo, S. W., Kim, D., O'Brien, E. J., Szubin, R., & Palsson, B. O. (2015). Decoding genome-wide GadEWX-transcriptional regulatory networks reveals multifaceted cellular responses to acid stress in *Escherichia coli*. *Nat Commun, 6*, 7970. doi:10.1038/ncomms8970

Shi, R., Villarroya, M., Ruiz-Partida, R., Li, Y., Proteau, A., Prado, S., . . . Cygler, M. (2009). Structure-function analysis of *Escherichia coli* MnmG (GidA), a highly conserved tRNA-modifying enzyme. *J Bacteriol, 191*(24), 7614-7619. doi:10.1128/jb.00650-09

Shiba, Y., Miyagawa, H., Nagahama, H., Matsumoto, K., Kondo, D., Matsuoka, S., . . . Hara, H. (2012). Exploring the relationship between lipoprotein mislocalization and activation of the Rcs signal transduction system in *Escherichia coli*. *Microbiology (Reading), 158*(Pt 5), 1238-1248. doi:10.1099/mic.0.056945-0

Shin, S., Castanie-Cornet, M. P., Foster, J. W., Crawford, J. A., Brinkley, C., & Kaper, J. B. (2001). An activator of glutamate decarboxylase genes regulates the expression of enteropathogenic *Escherichia coli* virulence genes through control of the plasmid-encoded regulator, Per. *Mol. Microbiol., 41*, 1133–1150.

Stewart, V. (1993). Nitrate regulation of anaerobic respiratory gene expression in *Escherichia coli*. *Mol Microbiol, 9*(3), 425-434. doi:10.1111/j.1365-2958.1993.tb01704.x

Stim-Herndon, K. P., Flores, T. M., & Bennett, G. N. (1996). Molecular characterization of *adiY*, a regulatory gene which affects expression of the biodegradative acid-induced arginine decarboxylase gene (*adiA*) of *Escherichia coli*. *Microbiology (Reading), 142 (Pt 5)*, 1311-1320. doi:10.1099/13500872-142-5-1311

Takao, M., Yen, H., & Tobe, T. (2014). LeuO enhances butyrate-induced virulence expression through a positive regulatory loop in enterohaemorrhagic *Escherichia coli*. *Mol Microbiol, 93*(6), 1302-1313. doi:10.1111/mmi.12737

Tobe, T., Nakanishi, N., & Sugimoto, N. (2011). Activation of motility by sensing short-chain fatty acids via two steps in a flagellar gene regulatory cascade in enterohemorrhagic *Escherichia coli*. *Infect Immun, 79*(3), 1016-1024. doi:10.1128/IAI.00927-10

Topping, D. L., & Clifton, P. M. (2001). Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiol Rev, 81*(3), 1031-1064. doi:10.1152/physrev.2001.81.3.1031

Tramonti, A., De Canio, M., & De Biase, D. (2008). GadX/GadW-dependent regulation of the *Escherichia coli* acid fitness island: transcriptional control at the *gadY-gadW* divergent promoters and identification of four novel 42 bp GadX/GadW-specific binding sites. *Mol Microbiol, 70*(4), 965-982. doi:10.1111/j.1365-2958.2008.06458.x

Tramonti, A., De Canio, M., Delany, I., Scarlato, V., & De Biase, D. (2006). Mechanisms of transcription activation exerted by GadX and GadW at the *gadA* and *gadBC* gene promoters of the glutamate-based acid resistance

system in *Escherichia coli*. *J Bacteriol, 188*(23), 8118-8127. doi:10.1128/JB.01044-06

Tramonti, A., Visca, P., De Canio, M., Falconi, M., & De Biase, D. (2002). Functional characterization and regulation of *gadX*, a gene encoding an AraC/XylS-like transcriptional activator of the *Escherichia coli* glutamic acid decarboxylase system. *J Bacteriol, 184*(10), 2603-2613. doi:10.1128/jb.184.10.2603-2613.2002

Tsai, M. F., McCarthy, P., & Miller, C. (2013). Substrate selectivity in glutamate-dependent acid resistance in enteric bacteria. *Proc Natl Acad Sci U S A, 110*(15), 5898-5902. doi:10.1073/pnas.1301444110

Vazquez-Ciros, O. J., Alvarez, A. F., & Georgellis, D. (2020). Identification of Z nucleotides as an ancient signal for two-component system activation in bacteria. *Proc Natl Acad Sci U S A, 117*(52), 33530-33539. doi:10.1073/pnas.2006209117

Vivijs, B., Aertsen, A., & Michiels, C. W. (2016). Identification of Genes Required for Growth of *Escherichia coli* MG1655 at Moderately Low pH. *Front Microbiol, 7*, 1672. doi:10.3389/fmicb.2016.01672

Walter, A., & Gutknecht, J. (1984). Monocarboxylic acid permeation through lipid bilayer membranes. *J Membr Biol, 77*(3), 255-264. doi:10.1007/bf01870573

Warnecke, T., & Gill, R. T. (2005). Organic acid toxicity, tolerance, and production in *Escherichia coli* biorefining applications. *Microb Cell Fact, 4*, 25. doi:10.1186/1475-2859-4-25

Waterman, S. R., & Small, P. L. (2003). Transcriptional expression of *Escherichia coli* glutamate-dependent acid resistance genes *gadA* and *gadBC* in an *hns rpoS* mutant. *J Bacteriol, 185*(15), 4644-4647. doi:10.1128/jb.185.15.4644-4647.2003

Weber, H., Polen, T., Heuveling, J., Wendisch, V. F., & Hengge, R. (2005). Genome-wide analysis of the general stress response network in *Escherichia coli*: sigmaS-dependent genes, promoters, and sigma factor selectivity. *J Bacteriol, 187*(5), 1591-1603. doi:10.1128/jb.187.5.1591-1603.2005

Wolfe, A. J. (2005). The acetate switch. *Microbiol Mol Biol Rev, 69*(1), 12-50. doi:10.1128/mmbr.69.1.12-50.2005

Wong, J. M., de Souza, R., Kendall, C. W., Emam, A., & Jenkins, D. J. (2006). Colonic health: fermentation and short chain fatty acids. *J Clin Gastroenterol, 40*(3), 235-243. doi:10.1097/00004836-200603000-00015

Yarian, C., Townsend, H., Czestkowski, W., Sochacka, E., Malkiewicz, A. J., Guenther, R., . . . Agris, P. F. (2002). Accurate translation of the genetic code depends on tRNA modified nucleosides. *J Biol Chem, 277*(19), 16391-16395. doi:10.1074/jbc.M200253200

Yim, L., Moukadiri, I., Björk, G. R., & Armengod, M. E. (2006). Further insights into the tRNA modification process controlled by proteins MnmE and GidA of *Escherichia coli*. *Nucleic Acids Res, 34*(20), 5892-5905. doi:10.1093/nar/gkl752

Zhang, W., & Shi, L. (2005). Distribution and evolution of multiple-step phosphorelay in prokaryotes: lateral domain recruitment involved in the formation of hybrid-type histidine kinases. *Microbiology (Reading), 151*(Pt 7), 2159-2173. doi:10.1099/mic.0.27987-0

Zhao, B., & Houry, W. A. (2010). Acid stress response in enteropathogenic gammaproteobacteria: an aptitude for survival. *Biochem Cell Biol, 88*(2), 301-314. doi:10.1139/o09-182

Zhao, H., Zhou, F., Xing, Q., Cao, Z., Liu, J., & Zhu, G. (2018). The soluble transhydrogenase UdhA affecting the glutamate-dependent acid resistance system of *Escherichia coli* under acetate stress. *Biol Open, 7*(9). doi:10.1242/bio.031856

Zwir, I., Shin, D., Kato, A., Nishino, K., Latifi, T., Solomon, F., . . . Groisman, E. A. (2005). Dissecting the PhoP regulatory network of *Escherichia coli* and *Salmonella enterica*. *Proc Natl Acad Sci U S A, 102*(8), 2862-2867. doi:10.1073/pnas.0408238102

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