Characterization of serine/threonine kinase-signaling systems of *Vibrio parahaemolyticus* that regulate polymyxin resistance

vorgelegt von

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München, 2023

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Dissertation zum Erwerb des Doktorgrades der Naturwissenschaften
an der Fakultät für Biologie Ludwig-Maximilians-Universität München

vorgelegt von

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aus Teheran

München, 2023

Erstgutachter: Dr. Simon Ringgaard

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Tag der Abgabe: 31.Juli.2023

Tag der mündlichen Prüfung: 14.December.2023

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Contents

Abbreviations

µ- Micro

µL- Microliter

aa- Amino acid

ATP- Adenosine triphosphate

CCW- Counterclockwise

CW- Clockwise

DNA- Deoxyribonucleic acid

FDR- False discovery rate

GO- Gene ontology

h- Hour

LB- Luria-Bertani medium

SEM- Standard Error of the Mean

mL- Mililiter

OD- Optical density

GFP -Green fluorescent protein

sfGFP -Superfolded GFP

LC-MS- Liquid chromatography mass spectrometry

MCP- Methyl-accepting chemotaxis protein

Xgal- 5-Bromo-4-chlor-3indoxyl-β-D-Galactopyranosid

DNA- Deoxyribonucleic acid

RNA- Ribonucleic acid

RNAP- RNA polymerase

KEGG- Kyoto Encyclopedia of Genes and Genomes

ECF- Extracytoplasmic function

STK- Serine/Threonine kinase

PBP- Penicillin-binding protein

PASTA- PBP and STK associated domain

EcfP- ECF σ factor activated by Phosphorylation

PknT- Protein Kinase of ECF Threonine

Co-IP- Co-immunoprecipitation

Thr- Threonine

Ser- Serine

T3SS- Type III secretion system

Pol B- Polymyxin B

PTM- Post-translational modification

OM- Outer membrane

DIC- differential interference contrast

FC- Fold change

Summary

In nature, bacteria constantly experience changes in their environment and often have to adapt to stressful surroundings. One type of stress that they encounter is antibiotic stress. Several bacteria have developed mechanisms to sense the presence of distinct antibiotics and survive in their presence. Since treatment with antibiotics is essential in modern human medicine, it is essential to understand the mechanisms utilized by bacteria to detect and survive antibiotic treatment. A general mechanism utilized by bacteria to adapt to changing or stressful environments is to use signal transduction systems and change their protein expression profile. Of particular interest here are resistance-associated signaling genes.

In this work, we have identified a so far uncharacterized system that is required for proper polymyxin B resistance in the human pathogen *Vibrio parahaemolyticus* – particularly focused on VPA1044, a predicted Ser/Thr kinase, VPA1045, a response regulator and VPA1046, a histidine phosphotransferase protein encoding a two-component system.

Wild-type *V. parahaemolyticus* is resistant to polymyxin B antibiotic; however, in the absence of the *vpa1044-vpa1045-vpa1046* system, the pathogen becomes highly sensitive to treatment with this particular antibiotic. Our results suggest that the *vpa1044-vpa1045-vpa1046* system confers polymyxin B resistance by regulating the proteomic profile of *V. parahaemolyticus* and particularly by regulating the expression of the polymyxin B resistance determinant VPA0879. Lastly, our results suggest that *V. parahaemolyticus* has evolved several distinct pathways to respond to and survive polymyxin B treatment.

Zusammenfassung

In der Natur sind Bakterien ständig Veränderungen in ihrer Umgebung ausgesetzt und müssen sich oft an verschiedenste Stressbedingungen anpassen. Eine Art von Stress, dem Bakterien ausgesetzt sind, ist der Antibiotikastress, und viele Bakterien haben Mechanismen entwickelt, um die Präsenz bestimmter Antibiotika zu erkennen und in deren Gegenwart zu überleben. Da die Behandlung mit Antibiotika in der modernen Humanmedizin unverzichtbar ist, ist es wichtig, die Mechanismen zu verstehen, mit denen Bakterien eine Antibiotikabehandlung erkennen und überleben können. Ein allgemeiner Mechanismus, den Bakterien nutzen, um sich an eine veränderte oder stressige Umgebung anzupassen, ist die Nutzung von Signaltransduktion Systemen und die Änderung ihres Proteinexpressionsprofils. Von besonderem Interesse sind dabei Resistenz-assoziierte Signalisierungsgene.

In dieser Arbeit haben wir ein bislang uncharakterisiertes System identifiziert, das für eine angemessene Polymyxin-B-Resistenz im humanen Erreger *Vibrio parahaemolyticus* erforderlich ist - insbesondere VPA1044, eine vorausgesagte Ser/Thr-Kinase, VPA1045, ein Reaktionsregulator, und VPA1046, eine Histidin-Phosphotransferase, welche für ein Zwei-Komponenten-System kodiert.

Der Wildtyp von *V. parahaemolyticus* ist resistent gegen Polymyxin-B-Antibiotika, reagiert jedoch in Abwesenheit des *vpa1044-vpa1045-vpa1046*-Systems sehr empfindlich auf die Behandlung mit diesem speziellen Antibiotikum. Unsere Ergebnisse deuten darauf hin, dass das *vpa1044-vpa1045-vpa1046*-System die Polymyxin-B-Resistenz durch Regulierung des proteomischen Profils von *V. parahaemolyticus* und insbesondere durch Regulierung der Expression der Polymyxin-B-Resistenzdeterminante VPA0879 vermittelt. Schließlich deuten unsere Ergebnisse darauf hin, dass *V. parahaemolyticus* mehrere unterschiedliche Wege entwickelt hat, um auf die Behandlung mit Polymyxin B zu reagieren und zu überleben.

1. Introduction

1.1. Signal transduction in bacteria and stimulus perception

Bacteria constantly experience changes in their external milieu and have developed several strategies to adapt their behavior accordingly. Indeed, it is essential for cells to be able to sense their surroundings and respond to extracellular stresses to survive in changing environments(Logue et al., 2015). The transmission of external data necessitates the active involvement of membrane proteins such as enzymes, channels, and receptors embedded in the membrane(Krämer and Jung, 2010). The major types of transmembrane signaling systems in bacteria include: One-component signaling system, which is made up of a sensor and DNA-binding domain, two-component system, and extracytoplasmic function (ECF) sigma factors (σ), which will be explained in more details in the following sections(Jung et al., 2018). Below are some examples that showcase notable bacterial stimulus perception:

1. Chemotaxis: A phenomenon whereby cells migrate toward or away from attractants/repellents. A well-understood case is the chemotaxis pathway in *E. coli* which demonstrates exceptional sensitivity, capable of detecting even subtle changes in ligand binding to a single receptor, regardless of significant variations in background concentrations. Chemotactic signals are detected by specific transmembrane receptors called Methyl-Accepting Chemotaxis Proteins (MCPs) as well as several other proteins such as CheA, CheW, CheY, CheB, and CheR. Phosphorylated CheY (response regulator) binds to the switch protein FliM on the flagellar motor, causing a reversal in motor rotation, leading to changes in direction. CheB, a two-domain protein, acts as a methylesterase, controlling the adaptation of the MCPs. Phosphorylated CheA (histidine kinase) also phosphorylates CheB, allowing it to compete with constitutive methyltransferase CheR, thus regulating the methylation state of MCPs and resetting their signaling state. *E. coli* chemotaxis pathway enables it to perceive and respond to minute changes in its environment with remarkable sensitivity and adaptability(Wadhams and Armitage, 2004, Eisenbach, 1991).

2. Quarom sensing: A communication mechanism within bacterial communities to deliver information about population density and adjust their collective behavior which involves releasing and detecting signaling molecules called autoinducers. For instance, *Vibrio fischeri* employs quorum sensing to regulate bioluminescence which is the control of N-acylated l-

homoserine lactones (AHLs) and their cognate receptors (LuxR-type proteins). As the cell density grows, autoinducer levels increase, resulting in the activation of gene expression for bioluminescence(Landron, 1981).

3. Osmoregulation: Osmotic stress leads to water flux across the cell envelope, resulting in shrinkage (osmotic upshift) or swelling of the cell (osmotic downshift). However, bacteria have developed osmosensing and osmoregulation mechanisms to retain internal osmolarity, comprising a complex network that includes the regulation of transport systems for ions and compatible solutes such as proline, betaine, as well as genes encoding outer membrane proteins(Da Costa et al., 1998, Krämer, 2010). One extensively studied system is the KdpD/KdpE system, which plays a role during K+ limitation and salt stress(Sugiura et al., 1994, Freeman et al., 2013). Other genes, such as the proU operon in *E. coli*, are regulated by changes in DNA supercoiling in response to osmolality. Mechanosensitive channels (MscL, MscS, and MscK) respond to hypo-osmotic conditions by opening and releasing solutes, preventing cell bursting(Booth and Blount, 2012).

4. c-di-GMP regulation mode: Cyclic diguanylate monophosphate (c-di-GMP) second messenger stimulates several prokaryotic processes. Diguanylate cyclases (DGCs), enzymes characterized by conserved GGDEF domains, are responsible for the synthesis of c-di-GMP. Conversely, phosphodiesterases (PDEs) containing EAL or HD-GYP domains degrade c-di-GMP. The core of this system is the intricate interplay between DGCs and PDEs that maintains a precise equilibrium of intracellular c-di-GMP levels, thereby governing subsequent signaling cascades. In many bacteria, high c-di-GMP levels inhibit flagellar biosynthesis, while low c-di-GMP levels favor flagellar expression and motility(Guttenplan and Kearns, 2013). C-di-GMP influences virulence and pathogenesis in several bacterial pathogens. In the case of *Salmonella enterica*, c-di-GMP controls the expression of type III and Type VI secretion systems, which are essential for host invasion and intracellular survival(Moscoso et al., 2011).

Signal transduction is not a linear pathway and has increased complexity with several interacting elements that eventually affect gene expression to optimize bacterial cells for adaptation; therefore, there is no ‘gold standard’ to categorize them. Comparative genomics has proven to be a valuable tool for studying these intricate signaling systems and certain common trends have been identified such as the ability of several bacteria to activate

multiple regulatory circuits in response to a single environmental signal, the structure of signaling proteins as well as their arrangement to maintain N-terminal to C-terminal flow of information. The microbial signal transduction (MiST 3.0) database is an up-to-date library for microbial signaling machinery(Galperin, 2004).

1.2. Two-component Signaling Pathways

Two-component system (TCS) serves as the predominant mechanism through which bacteria adapt to the environment. By using this mechanism for the detection of physical and/or chemical changes, bacteria transfer the signal to nucleoid, where gene expression is altered. In the classical form, the membrane-bound sensor kinase is phosphorylated at a conserved histidine residue in H1 domain(Hirakawa et al., 2020). Subsequently, the response regulator gets phosphorylated at the conserved aspartic residue in the receiver domain (D1), leading to conformational changes and binding to a target DNA region(Capra and Laub, 2012).

Some two-component systems deviate from the traditional structure and exhibit additional phosphate-binding domains, resulting in what is known as unorthodox TCSs. These systems involve histidine (H1)–aspartate (D1)–histidine (H2) domains in the sensor kinases (HKs) and a single aspartate domain (D2) in the response regulators (RRs). Unorthodox TCSs are characterized by extended phosphorelays, which incorporate extra proteins or domains between the HK and RR components and are a result of evolutionary adaptation. In another multistep variant, which is called the hybrid form, autophosphorylation takes place at the H1 domain in response to a specific stimulus, and the phosphorylated H1 transfers the phosphate group consecutively to D1 and H2, ultimately reaching D2 and the only difference is that H2 is histidine-containing phosphotransfer (HPt) domain can be attached to the C-terminal of the hybrid histidine kinase or also be present as a soluble protein, meaning it can function individually as well and shuttle the phosphate to the response regulator (Fig 1.) (Wolanin et al., 2002, Liu et al., 2019). It is noteworthy that approximately 25% of histidine kinases are hybrids(Cock and Whitworth, 2007), meaning they contain an additional C-terminal domain, indicating the prevalence of phosphorelay mechanisms in two-component systems(Schaechter, 2009).

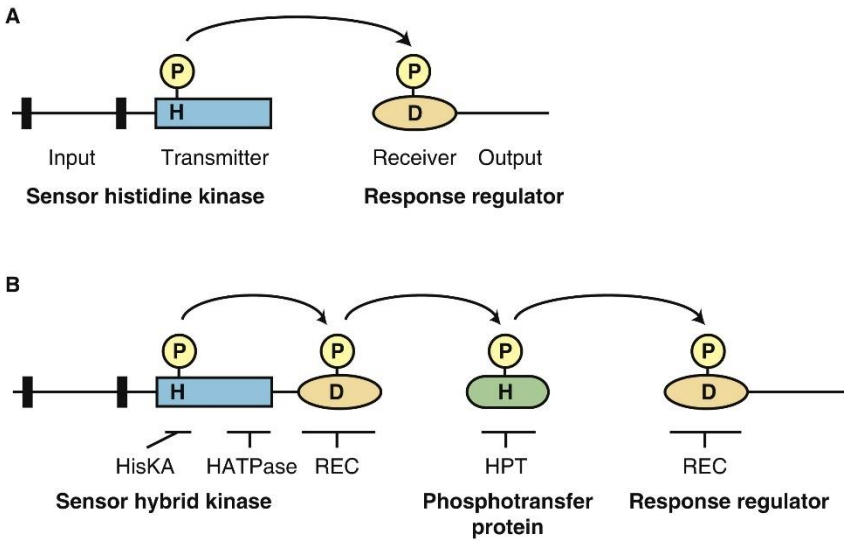

Figure 1. Two-component signal transduction. (A) The classical version with a histidine kinase and a response regulator. (B) The hybrid version in which (Hpt) domain is an external phosphotransfer module that can act individually. Adapted from (Schaller et al., 2011)

1.2.3. Biological roles of two-component systems

Two-component signaling systems play a crucial role in regulating various basic aspects of bacterial life, controlling global responses to stress and other features ranging from growth decisions to pathogenicity(Schaller et al., 2011). There is currently ample evidence that TCS can be found in all kingdoms of life, except animals(Alvarez et al., 2016). Since they regulate diverse activities, each individual bacterial cell has multiple HK/RR sets; for instance, *Pseudomonas aeruginosa* harbors at least 64 distinct response regulator genes and 63 different histidine kinase genes and *E. coli* possesses a minimum of 29 sets of two-component systems(Wang et al., 2021, Choudhary et al., 2020). Given the widespread occurrence of TCSs, it would be advantageous to deepen our understanding of these systems, since they may offer promising targets for controlling bacterial infections and potentially serve as alternatives to antibiotics.

1.2.3.1. Role in Virulence

Two-component systems sense environmental cues such as pH variation, availability of amino acid metabolites or metal ions, and also temperature changes, among other signals, to recognize and respond to them and promote bacterial virulence(Shaw et al., 2022). They exert their effect via gene regulation, adaptive responses, and intracellular communication and coordination among bacterial populations(Pasqua et al., 2022, Zheng et al., 2018).

P. aeruginosa, an environmental pathogen, is a well-studied organism in this field and it has been established that more than half of the TCSs that are identified contribute to the expression of virulence factors(Francis et al., 2017). Several modes of action of TCSs that have been demonstrated to be involved in virulence are described below in two main categories with examples from *P. aeruginosa*(Sultan et al., 2021).

1. Motility: The motility system in bacteria is an important virulence factor to enhance the overall pathogenicity by enabling them to evade immune responses, colonize and invade host cells, and establish persistent infections(Josenhans and Suerbaum, 2002). Swimming, swarming, twitching, and gliding help bacteria to navigate in the environment of the host(Shrout, 2015). TCSs are contributing factors in modulating the expression of flagellar assembly and chemotaxis-associated genes as well as up-regulation and down-regulation of pilus-related genes. The presence of both PilSR and FleSR two-component systems is necessary for *P.aeruginosa* to have its full pathogenic potential. The former regulates the expression of type IV pilus (T4P) major subunit PilA and it was shown that *pilSR* deletion mutants had defects in surface sensing behaviors(Kilmury and Burrows, 2018); the latter is involved with biosynthesis of the single polar flagellum and the response regulator (FleR) directly interact with the promoter region of motility-related genes such as *flgBCDE*, *flgFGHIJKL*, and *filC(Zhou et al., 2021)*.

2. Secretion systems and their substrates: Up to date, there are multiple secretion systems identified in bacteria, categorized as Type I to Type XI secretion systems, each having distinct mechanisms of action(Arechaga Iturregui and Cascales, 2022). T2SS of *P. aeruginosa* is located in the outer membrane and transports proteins such as exotoxin A (ToxA), lipases, and alkaline phosphatases into the extracellular environment(Alhazmi, 2015). ToxA blocks protein synthesis by ADP ribosylation of elongation factor 2, thereby triggering cell death. A study revealed that GtrS and GltR form a two-component system and regulate the expression of *toxA* gene(Daddaoua et al., 2014). T3SSs are needle and syringe-like apparatuses that secret effector proteins across both inner and outer membrane (Green and Mecsas, 2016). Upon sensing contact with host cells, T3SS of *P. aeruginosa* translocates four major toxins into the host cell: ExoS(GTPase-activating protein activity), ExoT(N-terminal GAP activity), ExoU(phospholipase), and ExoY (adenylate cyclase)(Hauser, 2009). Different elements of TCS

have been identified to influence each of these secretory proteins. For instance, *RocS1* is a sensor kinase, *RocA1* is a DNA binding response regulator that regulates the transcription of *exoY* and *exoT*(Shao et al., 2022). The *cbrAB* two-component system has also been shown to indirectly impact the expression of the type III regulon(Rietsch et al., 2004).

1.2.3.2. Role in antibiotic resistance

TCS-driven global regulation can be triggered directly by the presence of antibiotics or indirectly by environmental stressors that would eventually lead to the resistance phenotype. To elaborate on this point, three different examples of how TCS renders bacterial resistance are described below.

1. Modification of cell surface: In this mechanism, the direct targets are either the outer membrane (LPS modification) and peptidoglycan or the biogenesis of these elements that would bring about death to bacterial cells. A good example is polymyxin B resistance that is associated with the sensor histidine kinases (PmrB, PhoP) and their cognate response regulators (PmrA, PhoQ) in *P. aeruginosa, Salmonella enterica, Yersinia pestis*, and several other species. In the case of *P. aeruginosa,* when grown under Mg^{2+}-limiting conditions or the presence of polycationic peptides, either of these two TCSs causes the upregulation of the *arnBCADTEF-pmrE* operon, followed by the addition of phosphoethanolamine (pEtN) and 4-amino-4-deoxy-l-arabinose (l-Ara4N) to the lipid A component. This path leads to the reduction of the negative charge of the outer membrane as well as the binding of polycationic polymyxin molecules (Hussein et al., 2019, McPhee et al., 2003, Gellatly et al., 2012).

2. Regulation of efflux drug pumps: One of the major mechanisms of removal of antibacterial agents in bacteria is to use efflux pumps, which are membrane transporter proteins, to export toxic substrates out of the cell(Li et al., 2015). There have been several reports on how TCSs act as regulatory proteins for efflux pumps. For instance, BaeSR TCS in *A. baumannii* positively regulates the resistance-nodulation-division efflux pump genes *adeA* and *adeb*, and subsequently, it influences the susceptibility of tigecycline(Lin et al., 2014). Furthermore, the response regulator (CpxR) in the CpxAR system of *E. coli* binds and activates the *mar* promoter, which in turn overexpresses AcrAB/TolC efflux pump, contributing to resistance phenotype(Weatherspoon-Griffin et al., 2014, Raivio et al., 2013). Similarly, deletion of a

homolog of CpxAR TCS in *K.pneumonia* decreased the expression of three efflux genes as well as repression of the KpnO porin, and the loss of this porin lead to increased resistance to antibiotics such as nalidixic acid, streptomycin, and tobramycin(Srinivasan et al., 2012a, Srinivasan et al., 2012b).

3. Induction of biofilm formation: The exopolysaccharide matrix of biofilms creates a good structure to limit the penetration of drug molecules as well as a sphere for persister cells to be safe among the population and go through the process of horizontal gene transfer(Stewart, 2002, Bhagirath et al., 2019). It has been long established that GacS/GacA has a regulatory role in biofilm formation. In an interesting example from *P. aeruginosa,* the sensor kinase (GacS) is initially not able to autophosphorylate. Therefore, it is first activated by an orphan kinase, Rets. Through cascade regulation, GasA gets phosphorylated, having a direct effect on its small RNA targets, namely Rsmy and RsmZ, which ultimately leads to RsmA to restore biofilm production(Mikkelsen et al., 2011, Tierney and Rather, 2019).

1.2.4. TCS as targets for antimicrobial therapy

There are several features that make TCSs attractive targets for the development of innovative antimicrobial strategies. Firstly, TCSs regulate pathways that are crucial to bacterial survival, therefore drugs targeting TCSs can disrupt upstream regulatory functions which interfere with the pathogen's physiology and several other downstream functions(Gotoh et al., 2010a). Secondly, humans (mammals) lack direct homologues of bacterial histidine kinases and response regulators(Thomason and Kay, 2000); therefore, the design of selective TCS inhibitors makes them less toxic to mammalian cells. Thirdly, since both Gram-positive and Gram-negative bacteria harbor TCS-encoding genes, this system has broad-spectrum potential(Chen et al., 2022). As mentioned previously, TCSs regulate the expression of antibiotic resistance determinants in bacteria and by targeting them we can also pursue combination therapy and enhance the efficacy of the current antibiotics to achieve a synergistic effect(Worthington and Melander, 2013).

Inhibitors can be categorized based on the specific component of the TCS they target. This includes inhibitors that target histidine kinases, response regulators, or other components involved in TCS signaling(Tiwari et al., 2017).

Catalytic ATP-binding domain (CA) and dimerization and histidine phosphorylation (DHp) domains are part of the core region of all histidine kinases and key residues have a high level of sequence homology. Since the catalytic ATP-binding domain (CA) of HKs is highly conserved, inhibitors targeted at this site can be novel broad-spectrum antimicrobial agents(Bem et al., 2015). WalK/WalR is one of the most noteworthy examples of TCS that is essential for the survival of numerous Gram-positive pathogens, such as *Streptococcus pneumoniae, S. aureus*, and *Streptococcus pyogenes.* Walkmycin B (di-anthracenone) specifically targets WalK in *B. subtilis* and *S. aureus* and exerts its effect by inhibition of sensor kinase auto-phosphorylation(Okada et al., 2010, Chen et al., 2022). In addition, this compound is capable of inhibiting the growth of methicillin-resistant *S. aureus* (MRSA)(Hirakawa et al., 2020). Several “non-specific” inhibitors such as unsaturated fatty acids, halogenated phenyl-thiazoles, benzamin, and naphthalene derivatives target sensor kinase and inhibit the auto-phosphorylation function(Qin et al., 2006, Cai et al., 2011, Ulijasz and Weisblum, 1999). There are other “non-specific” inhibitors such as salicylanilides that bind to HK at the four-helix bundle, causing a conformational change and ultimately protein aggregation(Stephenson et al., 2000).

The most plausible modes of action for inhibitors targeting response regulators are either through inhibition of DNA binding or inhibition of being phosphorylated at its conserved aspartate residue by its cognate HK. There are several cases that the RR can be phosphorylated by non-cognate HKs and in this scenario, inhibitors targeting the RR are a better choice(Laub and Goulian, 2007, Hirakawa et al., 2020). Lactoferricin B, Nitrophenyl derivative, and Rhein are among the components that inhibit phosphorylation of BasR(*E. coli*), NarL (*M. tuberculosis*), and PhoP (*C. pseudotuberculosis*), respectively(Tiwari et al., 2014, Shivakumar et al., 2014, Ho et al., 2012). Interestingly, walrycin A and walrycin B were identified as WalR inhibitors causing dimerization and reduced binding affinity to target DNA(Gotoh et al., 2010b). In summary, the understanding of the association between two-component systems (TCSs) and the enhancement of virulence in bacterial pathogens has led to significant investments by the pharmaceutical industry in the quest for appropriate inhibitors targeting bacterial signal transduction pathways.

1.3. Post-translational modifications

The number and variety of protein post-translational modifications (PTMs) detected and characterized in bacteria has rapidly increased in the past decade. As the name implies, in PTM, proteins go through a vital biochemical change to multiple residues during translation and also in response to environmental cues, leading to increased protein versatility. Protein phosphorylation involves modifying residues such as His, Asp, Arg, Ser, Thr, and Tyr. Additionally, acetylation occurs at Lys and protein amino termini(Ree et al., 2018), while glycosylation is seen in Asn, Arg, Ser, and Thr(Latousakis and Juge, 2018). Lipidation occurs in Cys, while acylation and thiolation occur in Lys and Cys, respectively(Loi et al., 2015, Nguyen et al., 2014). Finally, oxidation of Met is another common post-translational modification seen in proteins(Vincent and Ezraty, 2023). The majority of PTMs are dynamic and reversible, meaning that for instance specialized enzymes, such as kinases and phosphatases for phosphorylation and acetyltransferases and deacetylases for acetylation, can add or remove them from the polypeptide chain(Macek et al., 2019). However, certain PTMs, such as N-terminal acetylation, are irreversible. In addition, lysine acetylation and S-thiolation may occur non-enzymatically. Research into PTMS shows us that covalently attached modifications are substoichiometric; meaning that it is not present in all of the fractions of the proteome(Zhang et al., 2015). This challenges researchers to improve more sophisticated instrumentations and robust databases that can be utilized for the detection of PTMs. Mass spectrometry (MS) is a leading technique in the analysis of PTMs owing to its capability of detecting a vast number of modified proteins with high sensitivity and specificity. Moreover, due to the availability of high-quality modification-specific antibodies, immunoprecipitation (IP) remains to be popular as well(Dunphy et al., 2021).

PTM machinery alters various properties of proteins like its enzymatic activity, conformation, net charge, and interactions with other proteins, ultimately playing a role in numerous biological processes such as virulence(Michard and Doublet, 2015), drug susceptibility pattern(Grassi and Cabrele, 2019), sporulation(Luu et al., 2022), metabolism(Wang et al., 2010), and cell signaling(Ardito et al., 2017). To highlight the complexity of the involved regulatory networks, it is interesting to mention that certain residues can have multiple modifications simultaneously (e.g., lysine residues can be mono-, di-, or trimethylated)(Greer

and Shi, 2012). Additionally, complex modifications like ubiquitin can also be modified (e.g., phosphorylated)(Koyano et al., 2014). Although not all PTMs are functionally important, this cross-talking among them could cause diverse outcomes(Leutert et al., 2021). Owing to the importance of PTMs, several databases and tools have been developed over the decades to help researchers study PTMs in the proteome(de Brevern and Rebehmed, 2022).

1.3.1. Regulatory phosphorylation in bacteria

Of all the diverse types of protein modifications, phosphorylation is one of the most widespread types among bacteria, archaea, and eukarya and is also better documented(Walsh et al., 2005, Cain et al., 2014). This modification involves the reversible addition of a phosphate group to specific amino acid residues. Notably, phosphorylations on His, Asp, and Arg residues are relatively unstable under acidic pH and sensitive to heat, while phosphorylations on Ser, Thr, and Tyr residues are more stable. Phosphorylation of the hydroxyl group (-OH) on serine residues is a highly abundant and functionally versatile post-translational modification in proteins. Executed by phosphotransferases or protein kinases, phosphorylation transfers the phosphate group from ATP to the target protein, while protein phosphatases remove it, creating a finely tuned signaling system. Kinases that undergo autophosphorylation on His or Arg residue can be regarded as high-energy phosphoenzyme intermediates because it leads to the formation of a phosphoramidate bond, which possesses sufficient standard free energy (ΔG) to convert ADP to ATP. In contrast, phosphorylation of proteins on Ser, Thr, or Tyr residues results in the generation of phosphoesters(Stock et al., 1989). Phosphorylation events directly impact protein activity, stability, subcellular localization, and protein-protein interactions(Zhang et al., 2021). This enables bacteria to detect and respond to changes in their environment, such as alterations in nutrient availability, stress conditions, and host interactions.

Protein phosphorylation serves as a dynamic regulatory mechanism in bacteria, involving signal transduction, gene expression, and cellular adaptation. Recent proteomics studies have significantly expanded the understanding of protein phosphorylation in bacteria, particularly in *Escherichia coli*. Over 2000 phosphorylation sites have been identified, challenging the notion that bacterial protein phosphorylation is less prevalent compared to eukaryotes. Through systematic characterization of S/T/Y phosphorylation in *E. coli*

metabolism, a study conducted by Schastnaya E *et.al* identified 44 out of 52 mutated phosphosites to be functional based on growth phenotypes and intracellular metabolome profiles. This finding effectively doubles the number of known functional phosphosites in *E. coli* and also elucidates the mechanisms through which individual phosphosites modulate enzymatic activity in specific pathways. These mechanisms include shielding the substrate binding site, limiting structural dynamics, and disrupting interactions that are relevant for activity *in vivo(Schastnaya et al., 2021)*. Evidence suggests the existence of checkpoint regulations similar to eukaryotes in bacteria, particularly for phosphorylated proteins involved in DNA-related processes and cell cycle events. As an example, the DNA-binding protein RacA plays a role in anchoring the segregating chromosome during sporulation in *B. subtilis*. It is connected to the Ser/Thr kinase YabT and phosphatase SpoIIE, suggesting potential phosphorylation regulation by both enzymes(Ben-Yehuda et al., 2003). Findings such as this contribute to the understanding that phosphoregulation has a role in coordinating cellular responses to DNA damage and maintaining replicating chromosome integrity(Garcia-Garcia et al., 2016).

1.4. An overview of the protein kinase landscape in bacteria

Bacteria exhibit a versatile array of protein kinases, encompassing diverse classes such as histidine kinases, hanks type serine/threonine kinases, and, more recently, tyrosine and arginine kinases (Fig 2.) (Sharma and Tiwari, 2022). Dual specificity protein kinases are a class of enzymes that have the ability to phosphorylate both tyrosine and serine/threonine residues on protein substrates, unlike other kinases that are specific to either tyrosine or serine/threonine phosphorylation(Thiriet and Thiriet, 2013). Each of these kinases orchestrates different cellular responses and forms complex circuit models; there have also been several studies indicating the cross-talk between different regulatory phosphorylation in bacterial systems, which will be explained later. The focus of this thesis primarily revolves around serine/threonine kinases and two-component systems.

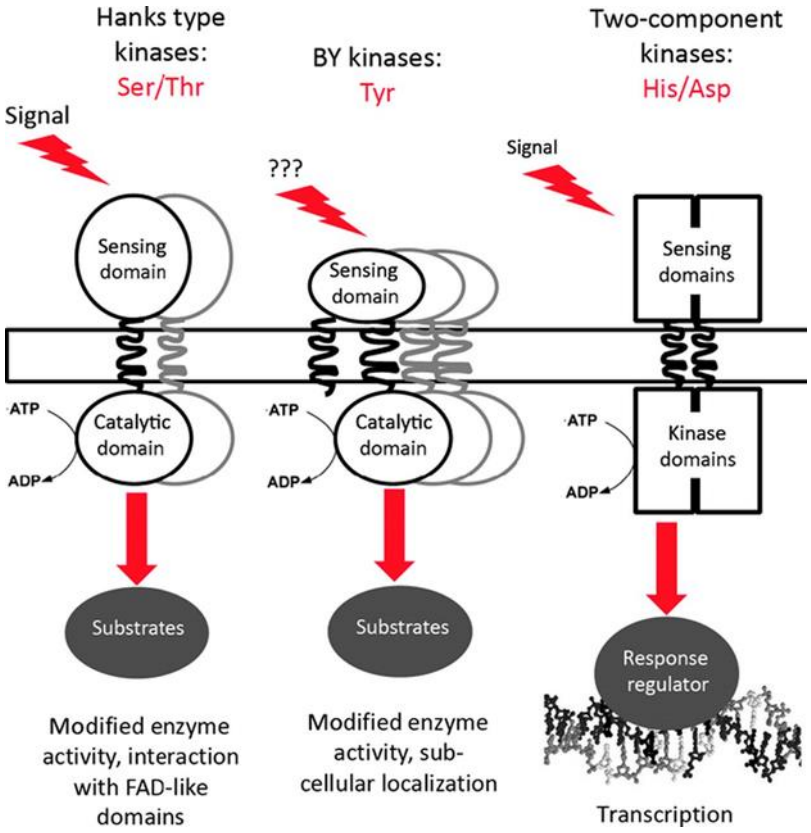

Figure 2. Major bacterial protein kinases. Source of phosphate is indicated next to each kinase and its respective signaling pathway. The gray shadow indicates putative oligomerization of kinase. Adapted from (Mijakovic and Macek, 2012)

1.4.1. Histidine kinases and two-component systems

Histidine kinases are typically homodimeric membrane-anchored receptors that can be found in the Archaea and Eukarya domains of life as well as Prokaryotes. HK is a multi-functional enzyme that has autokinase, phosphotransfer, and phosphatase activities. They are the key elements of the well-known two-component systems, the hallmark of bacterial signaling, and the typical HK consists of an N-terminal sensor domain, transmembrane segment (helical bundle), and a catalytic region near the C-terminal (forming an α-β sandwich). Given the fact that HKs respond to different signals from the environment, the sequence of the sensing domains is varied. On the other hand, the kinase core is more conserved which is made up of conserved ATP-binding catalytic (CA) domain together with the dimerization and histidine phosphotransfer (DHp) domain(Wolanin et al., 2002). There are five conserved primary sequence motifs, namely, H, N, G1, F, and G2 boxes. H-box contains conserved His residue and is the site of autophosphorylation and the other boxes define the nucleotide binding cleft. The high-energy phosphoryl group is then transferred to the aspartate of the

response regulator(Bhate et al., 2015). The receiver domain of the response regulator contains a conserved aspartate residue that undergoes phosphorylation in response to a specific signal. This phosphorylation event triggers conformational changes in the protein and the effector domain, also known as the output domain, is responsible for carrying out the response of the cell to the signal received by the receiver domain. The effector domain can have various functional activities, such as DNA binding, protein-protein interactions, enzymatic activity, or other specific biochemical activities(Gao et al., 2007). In certain examples of two-component systems, when the hybrid version of HK lacks the histidine-containing phosphotransfer (Hpt) domain, it needs another protein to act as an external phosphotransfer module. For example, it was suggested that in *E. coli* YojN acts as an Hpt protein within a phosphorelay system involving the histidine kinase RcsC and the response regulator RstB. In this system, YojN functions as an intermediary in the transfer of phosphoryl groups between the histidine kinase and the response regulator(Takeda et al., 2001). While HPts (are not true histidine kinases (HKs), the region responsible for the transfer of the phosphoryl group between histidine and aspartate is remarkably similar in HPt-response regulator (RR) complexes and in genuine two-component system (TCS) HK-RR complexes(Casino et al., 2010).

1.4.2. Serine/threonine kinases (STKs)

The influential work by Hanks *et al.* in 1988 defined and characterized the principal family of serine/threonine/tyrosine protein kinases predominantly found in eukarya(Hanks et al., 1988). Initially, it was believed that these kinases were absent in bacteria; however, extensive genome sequencing efforts have unveiled their presence in numerous bacterial species. Subsequently, the application of mass spectrometric techniques to investigate bacterial phosphoproteomes has provided compelling evidence that hanks type serine/threonine kinases (also commonly named eukaryotic-like STKs) and their cognate Ser/Thr phosphatases (STPs) play pivotal roles in bacterial signal transduction systems. In a study conducted by stancik et al. in 2018, they reconstructed the phylogenetic relationship between bacterial Hanks-type kinases to their eukaryal counterparts, among other phylostratigraphic analysis and they claimed that they are ubiquitous among eukarya, archaea, and bacteria and it is

strongly likely that they have a monophyletic origin, therefore, it is better to avoid naming them eukaryotic-like STKs(Stancik et al., 2018).

1.4.3. Structure of bacterial STKs

Bacterial STKs typically have modular organizations, consisting of various domains, motifs, and residues that contribute to their overall structure and function. These kinases can be found in both membrane-associated and cytoplasmic forms within cells. Here, the focus is membrane STKs. The key feature of Hanks-type STKs is the presence of a catalytic domain that harbors 12 specific signatures clarified by Hanks(Hanks and Hunter, 1995). These signatures are conserved amino acid sequences or motifs that are crucial for the catalytic activity and proper functioning of the kinase. The activation of STK of at least one Ser/Thr residue is through autophosphorylation or transphosphorylation by another kinase. PknB (Protein Kinase B) from *M.tuberculosis* is the first example of a well-studied crystal structure of bacterial STK, which was revealed to be very similar to the structure of mouse cyclic AMP-dependent protein kinase (PKA). PknB comprises an intracellular kinase domain, connected through a transmembrane domain to an extracellular region (Chawla et al., 2014, Young et al., 2003).

Domains:

The highly conserved intracellular protein kinase domain (also referred to as catalytic domain) typically has a fold characteristic, comprising a central β-sheet surrounded by α-helices. It has a bi-lobed structure and the active site is located in the deep cleft between these two lobes. The N-terminal lobe is involved in binding the phospho-donor ATP molecule and has a glycine-rich loop (also known as the P-loop) that helps in the exchange of the phosphate group of ATP. The C-terminal lobe is involved in substrate recognition and binding. The C-lobe undergoes conformational changes during the catalytic cycle, enabling the proper positioning of the substrate for phosphorylation. The cleft between the N-lobe and the C-lobe is the active site of the STK. This is where ATP binds to the N-lobe and the substrate protein binds to the C-lobe. The interaction between the ATP and the substrate within the active site facilitates the transfer of the phosphate group from ATP to the substrate, resulting in phosphorylation(Janczarek et al., 2018, Krupa and Srinivasan, 2005).

The transmembrane domain of serine/threonine kinases serves as a critical structural component that anchors the kinase to the cell membrane and typically has hydrophobic amino acid residues to form a helical structure. Furthermore, the transmembrane domain of some STKs is often connected to the intracellular kinase domain through a cytoplasmic linker region. In the case of PknB, a juxtamembrane region, consisting of 52 residues, links the kinase domain to the C-terminus of the transmembrane helix(Chawla et al., 2014).

The extracellular domain of serine/threonine kinases is the sensor domain which collects environmental cues. A unique feature found in certain STKs is the presence of extracellular PASTA (Penicillin-binding protein and Serine/Threonine kinase Associated) domain, which is absent in Eukarya. It was first identified in the penicillin-binding protein PBP2x of *Streptococcus pneumoniae* (Dessen et al., 2001)and structural analysis showed that it is a small globular fold consisting of three β strands and an α helix with high structural conservation(Ogawara, 2016). The function of PASTA domain has been studied for several STKs, showing it mediates growth and cell division, and cell wall homeostasis. The extracellular region of pknB contains four PASTA domains that are organized in a linear form. Upon recognition and interaction of PASTA repeats with d-alanyl-d-alanine dipeptides that are used to build the peptidoglycan layers, the kinase is activated through dimerization. This means that PASTA-STKs detect alterations in cell wall integrity caused by environmental cues or antibiotic exposure and subsequently it affects the regulation of the synthesis and remodeling of cell wall components, such as peptidoglycan(Mir et al., 2011).

Loop motifs:

The most important regulatory element is the activation segment (or the catalytic cleft) which consists of several loop motifs: catalytic, magnesium-positioning, activation, and P+1 loop. The catalytic loop has the catalytic Asp residue and subsequently at the N-terminus of the activation loop is the highly conserved DFG motif which is also known as magnesium positioning loop. Active kinases exhibit an inward conformation of DFG motif and the Asp residue is oriented toward the bound ATP, facilitating the coordination of magnesium and ATP, while inactive kinases display an outward-flipped conformation, disrupting this coordination(Peng et al., 2013). The activation loop gets phosphorylated on either Thr or Ser residue and its conformation changes to an extended form to allow the binding of substrate.

This is the most variable region of this segment, which contributes to the specificity of substrate binding(Pereira et al., 2011). Immediately downstream of the activation loop is a small motif, namely p+1 loop, which recognizes the residue immediately following the target residue in the peptide substrate (Fig. 3) (Taylor and Radzio-Andzelm, 1994).

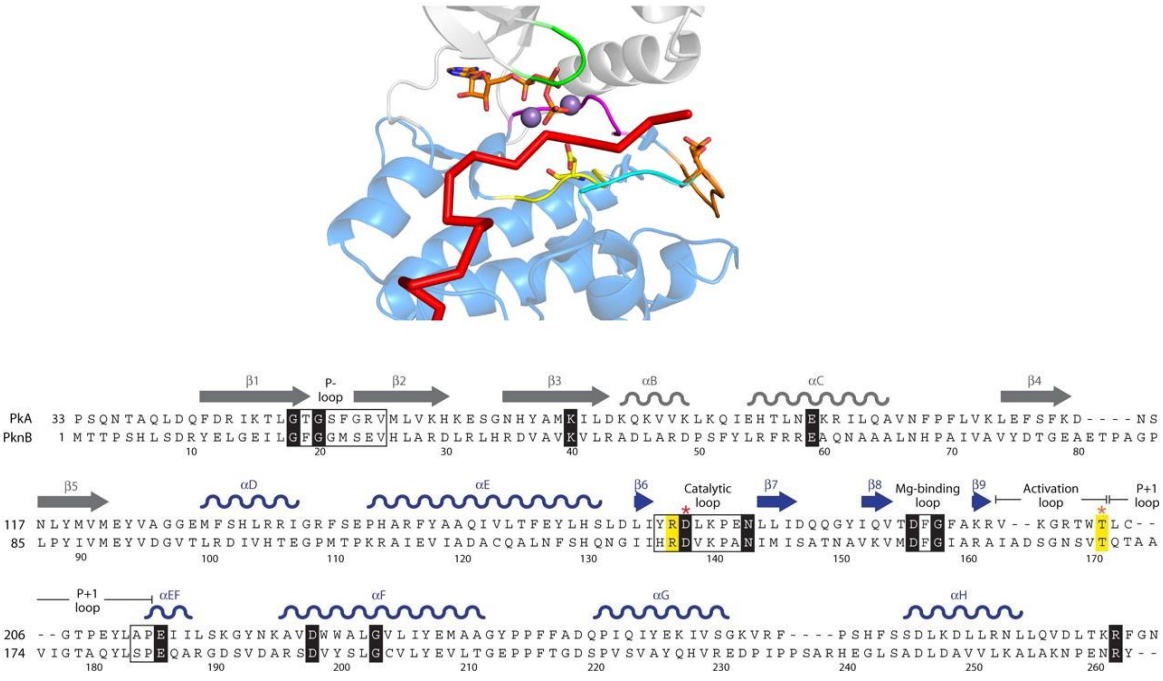

Figure 3. Ser/Thr kinase catalytic domain. A) green, P loop; yellow, catalytic loop with the catalytic Asp residue; magenta, magnesium-binding loop; orange, activation loop with the phosphorylated Thr residue; and cyan, P+1 loop. B) Sequence alignment between *M. tuberculosis* PknB and mouse cyclic AMP (cAMP)-dependent protein kinase (PKA). The N- and C-terminal lobes of PKA are shown in gray and blue, respectively. Boxes represent conserved motifs and black refers to invariant residues. The catalytic Asp and phosphorylated Thr residues are depicted with red and orange asterisk, respectively. Adapted from (Pereira et al., 2011)

1.4.4. Exploring the role of bacterial Serine/Threonine Kinases

In cell division and morphogenesis

Membrane-bound serine/threonine kinases with PASTA motifs (PASTA-STKs) have emerged as key regulators of bacterial cell division and morphogenesis. It is important to note that observations differ between species, indicating the presence of species-specific mechanisms for regulating cell division. PASTA motifs (Penicillin-binding proteins and Serine/Threonine

kinase associated) in the extracellular domain bind β-lactam antibiotics and are also found in the C-terminus of some class B penicillin-binding proteins (PBPs).

S. pneumoniae StkP plays a vital role in the process of cell division. It phosphorylates several targets, including DivIVA, MapZ, and FtsZ. Phosphorylation of DivIVA regulates peptidoglycan synthesis, which is crucial for cell elongation or constriction during division. Additionally, StkP-mediated phosphorylation of MapZ controls the positioning of FtsZ at mid-cell and indirectly influences the closure of the FtsZ ring, a critical step in bacterial cytokinesis. GpsB is an essential protein that facilitates the proper localization of StkP at the division site and subsequent activation. This interaction between GpsB and StkP is necessary for the precise positioning and functioning of StkP during the cell division process(Manuse et al., 2016).

PrkC in *B. subtilis* has a PASTA motif and is shown to be involved in spore germination by responding to muropeptides, which act as spore germinants. Upon phosphorylation, YocH enzyme can degrade peptidoglycan, releasing muropeptides that act as spore germinants. Therefore, Cells lacking PrkC are unable to germinate, indicating its crucial role in signal transduction that triggers spore exit from dormancy. Several other proteins including CpgA, YvcK, GpsB, and WalR are also phosphorylated by PrcK. CpgA is a small ribosome-associated GTPase and upon phosphorylation on Thr166, its interaction with the 30S ribosomal subunit is modulated, ensuring the proper coordination of peptidoglycan synthesis and cell growth processes(Cladière et al., 2006). When YvcK is phosphorylated by PrkC, it affects cell morphogenesis and the synthesis of cell wall precursors. The phosphorylation of YvcK by PrkC has been shown to play a role in the absence of MreB, an actin-like cytoskeleton protein involved in organizing peptidoglycan synthesis. Overexpression of YvcK can restore cell morphology in *B. subtilis* cells deficient in MreB(Foulquier et al., 2011). PrkC-mediated phosphorylation of WalR affects the expression of genes involved in peptidoglycan metabolism(Fig 4.) (Libby et al., 2015).

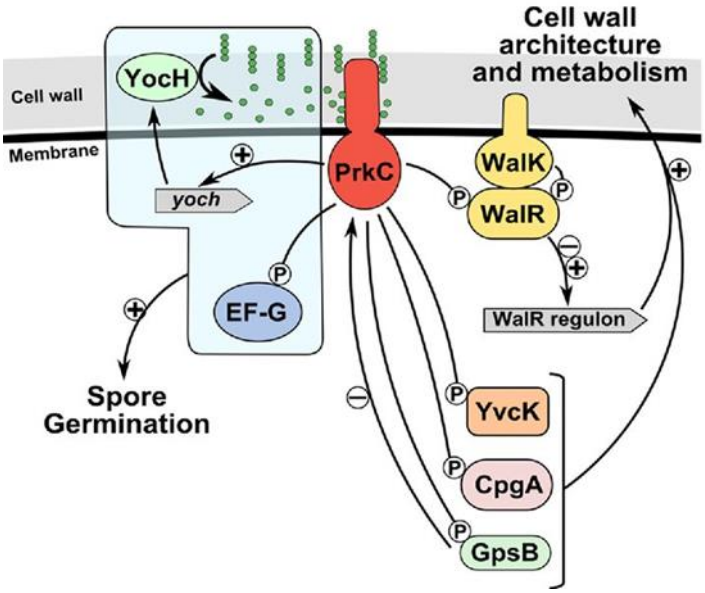

Figure 4. Function of PrkC in *B. subtilis*. In a vegetative state, PrkC can add phosphate groups to multiple targets (CpgA, YvcK, WalR, and GpsB) and have a role in the structure of cell wall. During the process of spore germination, the presence of YocH promotes the production of muropeptides, leading to the activation of PrkC, thus facilitating spore germination. Adapted from (Manuse et al., 2016).

Other STKs also have potential involvement in regulatory circuits. A good example would be AfsK, an STK lacking PASTA motifs, found in *Streptomyces coelicolor* and associated with controlling polar growth. When cell polarity determinant DivIVA is phosphorylated by AfsK, disassembly of the apical polarisome occurs, leading to altered cell wall synthesis. High AfsK activity leads to significant changes in DivIVA localization, apical growth, and hyphal branching. Since the crystal structures of DivIVA suggested its interaction with the membrane, phosphorylation may impact its interaction with other proteins or the membrane (Fig 5.) (Hempel et al., 2012).

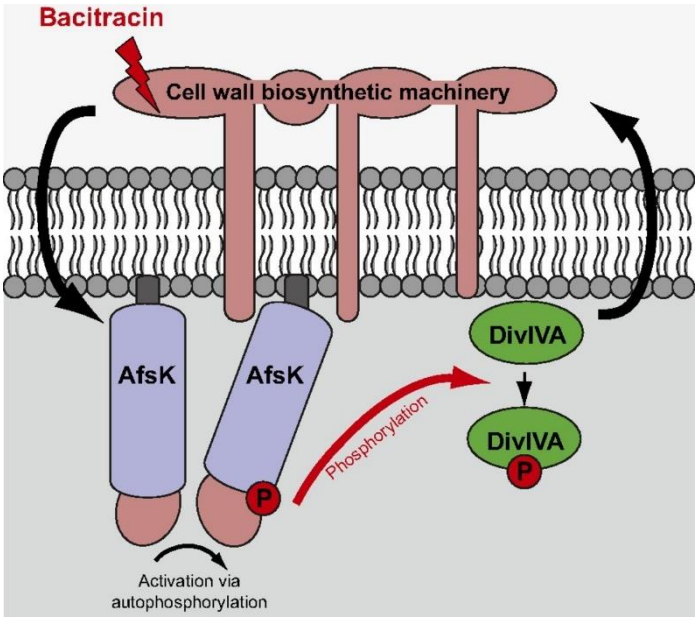

Figure 5. The function of AfsK in *Streptomyces.* Bacitracin induces arrest of cell wall synthesis by the addition of bacitracin, AfsK activity is increased and the kinase subsequently phosphorylates the polarity determinant DivIVA. As a result of this communication apical growth and branching is modulated. Adapted from (Hempel et al., 2012)

In driving antibiotic resistance

One crucial aspect of bacterial adaptation is the ability to respond to stress conditions, such as exposure to antibiotics and STKs are key players in stress response pathways. The contribution of STKs to antibiotic resistance can be via regulation of drug efflux pumps, modulation of antibiotic target sites, and activation of resistance pathways. Specifically, membrane-bound Hanks kinases containing PASTA domains have been observed to confer resistance to cell wall-inhibiting β-lactam-type antibiotics in different Gram-positive bacterial species. This finding aligns with their known involvement in cell division and maintenance of cell wall integrity, as explained previously. Response to the β-lactam drugs can be seen in *S. pneumoniae* StkP, *S.aureus* Stk1, and *Enterococcus faecalis* IreK against penicillin, vancomycin, and ceftriaxone, respectively.

Other antibiotic classes can also be influenced by STK kinases. NorA and NorB are multidrug-resistance efflux pumps associated with quinolone drugs in *S. aureus*. Upon phosphorylation by PknB, MgrA (a global transcriptional regulator) preferentially induces the expression of NorA or NorB, resulting in the differential upregulation of these efflux pumps(Truong-Bolduc and Hooper, 2010).

PknF serine/threonine kinase, identified in mycobacteria, acts as a crucial regulator of EthR, a transcriptional repressor involved in the activation of the antitubercular drug ETH. ETH is a product that relies on the bioactivation by EthA enzyme to exhibit antimycobacterial activity, and PknF phosphorylates EthR on Thr2, Thr3, Ser4, and Ser7 residues, indicating the targeted interaction between the kinase and its substrate. Ultimately, the ability of EthR to bind DNA is negatively affected and this suggests a potential connection between phosphorylation and the modulation of ETH resistance levels in *M. tuberculosis*(Leiba et al., 2014).

In virulence and infection

Ser/Thr phosphorylation plays a vital role in bacterial pathogenesis by regulating various aspects of the infection process from host entry to immune evasion and ultimately persistence. The host-pathogen interaction that is directed via STKs includes cases in which either the bacterial STK activity disrupts host defense mechanisms or phosphorylates host substrates. One example for each of these two scenarios is explained below:

NF-κB is a key transcription factor involved in the proinflammatory signaling pathway. Bacterial infection can trigger the activation of NF-κB signaling pathways. Upon activation, the NF-κB complex is released from its inhibitory proteins (IκBs). The activated NF-κB complex then translocates from the cytoplasm to the nucleus where it binds to the κB sites present in the promoter regions of target genes. This binding initiates the transcriptional activation of a wide range of genes involved in the immune response. These genes include pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6), chemokines, and other immune-related factors(Lawrence, 2009). NleH1 is a Ser/Thr kinase in enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC), which are two pathogenic strains of *E. coli* that cause gastrointestinal infections in humans. NleH1 interacts with RPS3, which is a host protein that enhances the transcriptional activity of NF-κB by stabilizing the p65 subunit; therefore, this interaction

reduces its nuclear localization and impairs its recruitment to target gene promoters. Ultimately, NF-κB-dependent transcriptional activity is inhibited. This mechanism shows us that STKs as bacterial effector proteins have the ability to alter host cell immune mechanisms(Gao et al., 2009).

YpkA (Yersinia protein kinase A) is a serine/threonine kinase that is produced by the bacterial pathogen *Yersinia* species, including (*Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*) and interacts with host proteins and subsequent phosphorylation events. Upon contact with host cells, YpkA is translocated into the cytoplasm of macrophages via T3SS, and is localized at the inner surface of the cytoplasmic membrane. Two of its primary targets are the heterotrimeric G-protein $G\alpha_q$ and vasodilator-stimulated phosphoprotein (VASP), which leads to impaired phagocytosis. The key Ser-47 residue in the binding loop of the G-protein is phosphorylated by YpkA and then the GTP binding function is inhibited and subsequently the rest of the signaling pathway leading to actin cytoskeleton regulation is also inhibited. Similarly, VASP is known to regulate actin polymerization, which is crucial for various cellular functions such as cell motility and shape changes. Phosphorylation of VASP by YpkA can modulate its ability to bind and regulate actin filaments. This may result in altered actin dynamics and cytoskeletal rearrangements of host macrophages (Fig 6.) (Bonne Køhler et al., 2020).

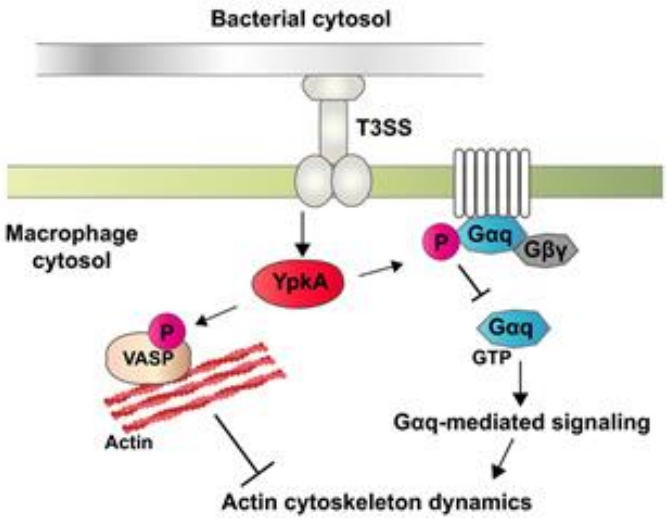

Figure 6. Schematic illustrations of protein phosphorylation by *Yersinia* kinase YpkA in host macrophage cells. The target host proteins are heterotrimeric Gαq protein and the VASP and the phosphorylation events lead to actin cytoskeleton regulation. Adapted from (Bonne Køhler et al., 2020).

1.5. Cross-talk between Ser/Thr kinases and TCS

TCS and STK pathways may converge on common downstream targets, leading to cooperative regulation of gene expression or cellular processes. Both TCS response regulators and histidine kinases can be either direct targets for STKs or influence their activity with indirect interactions. In total, the interaction between TCS and STK systems can result in signal amplification or attenuation. Here, the focus is on some examples of such an interaction:

1. Serine/threonine phosphorylation of TCS HK: The DegS/DegU TCS of *B. subtilis* regulates multiple cellular processes such as motility, production of extracellular proteases, and competence development. The sensory kinase DegS can get phosphorylated by at least two STks: PrkD and YabT. The study by Jers *et al*., revealed that the phosphorylation occurs on a serine residue (S76), and upon such a modification, the kinase activity of DegS is stimulated in addition to the stimulation of phosphate transfer to DegU (Jers et al., 2011).

2. Serine/threonine phosphorylation of orphan RR: An orphan response regulator refers to a response regulator protein that does not have a cognate histidine kinase partner within the same two-component system (TCS), suggesting the presence of an alternative signaling mechanism or crosstalk between different signaling pathways. There is an orphan RR in *Streptococcus pneumoniae* called RitR (repressor of iron transport regulator). It is required for lung pathogenicity and is associated with iron uptake and oxidative stress response. In the receiving domain of RitR, the Asp residue is lacking and is being replaced by asparagine residue. The *in vitro* assay has shown that its DNA-binding domain can be phosphorylated by the serine/threonine kinase, StkP, in addition to being dephosphorylated by phospho-serine/threonine protein phosphatase PhpC. This STK-dependent form of regulation impacts the expression of RitR target genes *in vivo*, i.e. the Piu haem transporter (Nováková et al., 2010).

3. Serine/threonine phosphorylation of TCS RRs: The *Staphylococcus aureus* VraS/VraR system plays a crucial role in the adaptive response to cell wall stress and resistance to antibiotics that target cell wall such as vancomycin. The response regulator, VraR, can be phosphorylated not only by its cognate HK bu also by a STK known as Stk1. Four threonine residues, Thr106, Thr119, Thr175, and Thr178 were identified as phosphoacceptors by site-

directed mutagenesis assays. Subsequently, it was revealed through electrophoretic mobility shift assays (EMSA) that the hyperphosphorylated VraR lost its ability to bind to the *vraRS* promoter; therefore, phosphorylation by Stk1 negatively regulates VraR DNA-binding properties (Canova et al., 2014).

A synergistic effect is observed in the *Mycobacterium tuberculosis* DosR response regulator, which is necessary for adaptation to hypoxia, carbon monoxide (CO), and nitric oxide (NO). In addition to being able to be phosphorylated by its two cognate histidine kinases, DosS and DosT, it can also be phosphorylated by a STK known as PknH. Once DosR is phosphorylated on aspartate and threonine residue, the binding the target promoter region is enhanced(Kalantari et al., 2015).

The crosstalk between RR and STK can also be independent of the cognate HK. For instance, the *S. pneumoniae* response regulator ComE is involved in the regulation of acidic stress-induced lysis, and phosphorylation at Thr128 residue by StkP is followed by ComE dimerization, which is a prerequisite for its activation. ComE conformation in the DNA-binding domain is then altered which increases its DNA-binding affinity to target promoter region in a pH-dependent manner. In acidic conditions, this pathway which is now known as StkP/ComE operates independently of the histidine kinase-dependent ComD/ComE system, regulating the activity of 104 genes associated with various cellular processes, including H2O2 production and oxidative stress tolerance (Pinas et al., 2018).

1.6. Comparison of Ser/Thr kinases and TCS

There are some key aspects to consider when comparing the two-component systems with the serine/threonine kinases in signaling. In the two-component system, the HK primarily acts as the phosphoryl group donor to the response regulator protein rather than phosphorylating other proteins using ATP as in typical STKs. TCS responds to one particular environmental cue; this means that histidine kinase is very specific for its response regulator and discriminates against all others. Hence, TCS has a more linear structure and can respond more quickly to stimuli and this quick response is generally advantageous for prokaryotic survival; however, it's also important to note that slow response can enhance system robustness to noises and false signals (Choi et al., 2008).

The need for phosphatases in the context of two-component systems and phosphorelay signal transduction was initially underestimated in prokaryotes due to the relatively rapid hydrolysis of phosphohistidine and aspartyl-phosphate residues. In contrast, phosphorylated serine, threonine, and tyrosine residues are more stable, requiring cognate phosphatases to regulate signaling cascades(Pereira et al., 2011). As said before, TCS mostly have specific changes in gene expression and STKs have an impact on overall gene expression. However, since STKs lack a distinct transcription factor in their systems, the question arises that how these transcriptional effects are mediated. In some cases, crosstalk can occur between TCS and STK signaling pathways, allowing for the integration and coordination of multiple signaling inputs, which will be explained in more detail in the next section.

1.7. Bacterial Extracytoplasmic Function (ECF) Sigma Factors:

The active RNA polymerase (RNAP) holoenzyme, consisting of catalytic subunits (2α, β, β′, ω) together with promoter-specificity sigma (σ)-factor, starts transcription initiation. Sigma factor 70 (σ^{70}), also known as the primary sigma factor, recognizes −10/−35 promoter elements and also stabilizes the open promoter complex (RPo). These proteins usually have four conserved protein domains σ_1, σ_2, σ_3 and σ_4. Extracytoplasmic function (ECF) proteins belong to the group IV of σ^{70} family and are minimalistic because they contain only the σ_2 and σ_4 domains. Domain σ_2 facilitates promoter melting at the -10 site and domain σ_4 recognizes the −35 motif. ECFs are a vastly versatile and abundant tool among bacterial signal transduction mechanisms. It has been discovered that while they primarily react to external cues, they also exhibit responsiveness to intracellular signals(de Dios et al., 2021). ECfs as signal transducing proteins are key players in bacterial signal transduction pathways. A high-resolution analysis conducted by Casas-Pastor D *et.al* re-classified ECF σ factor family and clustered them in 157 groups according to their genetic context conservation as well as putative regulatory behavior. They also argued that since their scope of analysis was limited to several bacterial genomes, the diversity of ECF subgroups is underestimated and is probably more abundant(Casas-Pastor et al., 2021).

1.7.1. Regulatory strategies of ECF sigma factors:

It is crucial to maintain low ECF activity during normal growth circumstances to prevent the activation of target genes that may lead to undesired results. The ECF regulatory mechanisms are also very diverse and will be explained briefly below:

The most common mode of regulation is via membrane or cytoplasmic proteins called anti-σ factors. In the absence of a specific environmental stimulus, anti-σ factors bind its cognate σ factor directly to sequester it from core RNAP to remain in an inactive state. In the presence of an activating signal, anti-σ factor undergoes targeted proteolysis or conformational changes, or its function is abrogated by the action of an anti-anti-σ factor(Brooks and Buchanan, 2008). Regulation of *B. subtilis* σ^W is a good example of regulated intramembrane proteolysis (RIP) and the key players for the cleavage of transmembrane domains of substrate proteins are called intramembrane cleaving proteases (I-CLiPs). In response to stressors such as phage infection or antimicrobial peptides, the anti-sigma factor Rsiw is completely degraded in a proteolytic cascade leading to the release of σ^W (Heinrich and Wiegert, 2009).

The partner switching system, also known as sigma factor mimicry, involves a cognate anti-sigma factor and an anti-anti-sigma factor. A good example to understand this mechanism is in the methylotrophic plant colonizer *Methylobacterium extorquens*. PhyR is involved in the activation of several stress-related genes and is comprised of a sigma factor-like domain of the extracytoplasmic function (ECF) subfamily linked to a receiver domain of a response regulator. In this scenario, Nepr is an anti-sigma factor that sequesters σ^{EcfG1} and PhyR is the antagonist sigma factor which is inactive under normal conditions. Upon the presence of a stressor, Phyr is phosphorylated and interacts with NepR, thus the interaction with σ^{EcfG1} is released, and the sigma factor can bind RNA polymerase to regulate stress genes (Francez-Charlot et al., 2009).

There can also be a regulatory mechanism in which the ECF sigma factor is not associated with an anti-sigma factor. In a recent study conducted by Iyer *et.al*, a novel mechanism was discovered that included direct phosphorylation of ECF σ factors by serine/threonine protein kinases in *Vibrio parahaemolyticus.* They reported a previously uncharacterized mechanism of transcriptional regulation in which the ECF σ factor is intrinsically inactive and through

phosphorylation by a predicted STK, namely PknT, it gets activated and binds the β'-subunit of RNAP. In *Vibrio parahaemolyticus*, the threonine kinase PknT phosphorylates the σ factor EcfP. Since EcfP lacks a negatively charged DAED motif in region σ2.2, it is unable to bind the β'-subunit of RNAP; however, when it gets phosphorylated on Threonine 63, which resides in this region, the negative charge gets restored and RNAP holoenzyme can be formed and this leads to the expression of an essential polymyxin-resistant regulon (Iyer et al., 2020).

In other cases, in which an obvious anti-σ factor is lacking, Ecf sigma factor can also be transcriptionally regulated and this could happen under the control of another signal-transduction mechanism such as one-component or two-component systems. One well-known example is the alternative sigma factor HrpL in *Pseudomonas syringae,* which recognizes the promoter of TTSS genes needed for plant infection. The induction of these σ factors is under the control of the two-component system HrpRS as well as another alternative sigma factor RpoN (σ^{54}). By sensing changes in environmental signals, HrpRS activates the expression of the *hrp* regulon with the assistance of RpoN-RNA polymerase(Xie et al., 2019).

Although not very typical, C-terminal or N-terminal protein extensions fused to some ECF σ factors could also confer them with enhanced regulatory capabilities and modulate their activity. An ECF sigma factor that has been experimentally studied is SigJ from *M. tuberculosis*, which has a C-terminal extension and is involved in resistance to hydrogen peroxide. It was observed that this extension interferes with β subunit of core RNA polymerase and when this is truncated or deleted, the formation of RNAP holoenzyme is promoted (Mallick Gupta and Mandal, 2020).

1.7.2. Functional significance of ECF sigma factors:

Extracytoplasmic function (ECF) σ factors transfer signals for the transcription of bacterial genes in response to environmental change, usually stress. This section highlights some distinct functions and biological roles of ECF sigma factors across various bacterial species, showcasing their significance in diverse ecological niches.

ECF sigma factors play a significant role in bacterial environmental adaptation. One well-studied example is σ^{E} (RpoE) in *E. coli*, which responds to misfolded proteins and envelope

stress. In response to unfolded porins, the anti-sigma factor RseA is degraded by inner membrane proteases and the free σ^E helps to maintain the integrity of the cell envelope. The σ^E regulon consists of genes encoding for enzymes required for proper outer membrane protein folding and synthesis of phospholipid and LPS as well as complexes involved in LPS assembly in the outer membrane, all of which are necessary for cell envelope homeostasis(Hayden and Ades, 2008). In the case of *Salmonella* RpoE, which is a homolog of *E. coli* RpoE, it has been shown that the sigma factor has a major role in protecting *Salmonella enterica* serovar Typhimurium against polymyxin B, P2 (a derivative of bactericidal and permeability increasing protein produced by neutrophils), and the murine defensin, cryptdin-4. Also, antibiotic susceptibility testing in Δ*rpoE* mutants showed increased resistance to β-lactams, aminoglycosides, and quinolones, suggesting that this alternative sigma factor can be a potential antimicrobial regulator(Crouch et al., 2005).

Bacteria release siderophores (i.e. pyoverdine) to chelate iron and uptake it within their cytoplasm. Most *Pseudomonas* IS σ^{ECF} factors are key in iron homeostasis and are o-transcribed with a transmembrane anti-σ factor under iron starvation conditions. Pseudomonas species have a cell-surface signaling (CSS) regulatory mechanism which involves an outer membrane receptor that transports the CSS-inducing signal, such as an iron carrier. In this model, the receptors of iron-starved cells recognize pyoverdine as an inducing signal, leading to the interaction between the signaling domain and the periplasmic C-domain of the anti-σ factor. Subsequently, proteolytic cascade involving the RseP protease is triggered and anti-σ factor gets degraded. The free σ^{ECF} factor promotes the transcription of regulatory genes such as *pvdS*(Otero-Asman et al., 2019).

*M. tuberculosis i*s a facultative intracellular pathogen that can survive inside macrophages during infection and evade innate immunity by stimulating necrosis of the host macrophage. A series of studies have investigated *Mycobacterium tuberculosis* sigma factors and identified 10 proteins (σ^C, σ^D, σ^E, σ^G, σ^H, σ^I, σ^J, σ^K, σ^L, and σ^M) belonging to ECF σ factor group. Each of them is triggered by the presence of specific inducing stimuli(Rodrigue et al., 2006). *sigB*, *sigE,* and *sigH* respond to heat shock and several of them such as *sigA*, *sigB*, *sigE*, *sigH* and *sigJ* respond to growth in macrophages. It was experimentally shown that the growth ability

of the *sigE* mutant infecting macrophages derived from the human monocytic cell line THP-1 was impaired (Manganelli et al., 2001).

In summary, ECF sigma factors orchestrate the expression of genes involved in a wide array of functions and biological roles, playing critical roles in stress response, environmental adaptation, iron homeostasis, antibiotic resistance, biofilm formation, virulence, and osmotic stress response. Understanding the mechanisms by which ECF sigma factors regulate gene expression in response to environmental cues provides insights into adaptation strategies.

1.8. *Vibrio parahaemolyticus* as a model organism

V. parahaemolyticus is a Gram-negative, rod-shaped, halophilic bacterium. This organism, with its versatile manifestations to respond to environmental cues, has attracted attention because it has two distinct life cycles: short rod-shaped swimmer cells in a liquid environment and elongated swarmer cells in viscous or solid surfaces. *V. parahaemolyticus* has two flagellar systems and it is monotrichous when swimming, however in response to surface contact, it turns into peritrichously flagellated cells(Heering and Ringgaard, 2016). It is naturally present in marine ecosystems and has been frequently isolated from seafood, including shrimp, oysters, fish, lobster, and shellfish. It is a pathogen and has an effect not only on human health but also on aquatic animals (Letchumanan et al., 2014). It was previously mainly in southeast Asia; however, currently, it is also considered one of the leading causes of seafood-borne diseases in India, North America, and the Mediterranean Sea, mainly through the consumption of raw or undercooked food(Parthasarathy et al., 2016, Lopez-Joven et al., 2015). This pathogen is responsible for gastroenteritis; however, the symptoms tend to be mild with a self-limiting disease. It can also cause infections in open wounds and sometimes lead to death. Multiple virulence factors have been identified in this organism, of which type III and VI secretion system, hemolysin (TDH) and adhesion factors are very important (Li et al., 2019). In severe cases, antibiotics are the main choice of treatment and several studies have shown the emergence of multi-drug resistant strains among clinical isolates.

1.9. Aim and scope:

Insights into essential pathways required for proper polymyxin B resistance in the human pathogen *Vibrio parahaemolyticus:*

Vibrio parahaemolyticus occurs naturally in marine environments and can cause gastrointestinal illness in humans as well as significant economic losses in aquaculture. This pathogen exists as swimmer and swarmer cells, specialized for growth in liquid and solid environments, respectively. When exposed to stress, the organism's perception of its physical environment enables it to adapt by regulating the level of gene transcription. A general mechanism utilized by bacteria to adapt to changing or stressful environments is to change their protein expression profile (Tollerson and Ibba, 2020). We recently showed that *V. parahaemolyticus* adapts to distinct environments by inducing specific proteomic profiles (Freitas et al., 2020). Importantly, this proteomic adaptation includes significant changes to the expression profile of essential proteins depending on the specific environment the bacterium experiences.

Previously, in our research group, a novel mechanism of transcriptional regulation was reported (Iyer et al., 2020). This mechanism relies on an intrinsically inactive ECF σ factor that needs to be phosphorylated in order to interact with the RNA polymerase. Particularly, it was shown that upon polymyxin stress, the threonine kinase PknT phosphorylates the σ factor EcfP, resulting in EcfP activation and expression of an essential polymyxin resistance regulon. Having demonstrated that serine/threonine kinase-signaling pathways play a role in antibiotic resistance behavior *in V. parahaemolyticus*, the question arose to further characterize the systems related to polymyxin B resistance in this bacterium. This finding led to the motivation to research further for this thesis.

The main aim of this study was to investigate if there are other signal transduction pathways regulating polymyxin B resistance in *V. parahaemolyticus*. To this end, we aimed to determine the proteome that is specific to mutant strains of a novel signaling system. Furthermore, we attempted to identify potential proteins involved in resistance as well as analyze their effect on the virulence capacity of this pathogen during its vegetative life style.

2. Materials and methods

2.1. Materials

Table 1. List of media and general buffer

Name	Composition
50x TAE	50mM EDTA, 2M Tris base, 1M acetic acid, adjust pH to 8.7
10X Phosphate buffered saline (PBS)	25.6 g Na2HPO4·7H2O, 80 g NaCl 2 g KCl 2 g KH2PO4 Bring to 1 liter with H2O. Autoclave for 40 minutes at 121°C
Luria Bertani (LB)	1% (w/v) tryptone; 0.5% (w/v) yeast extract; 1% (w/v) NaCl
HNN lysis buffer	50 mM HEPES pH 7.5, 150 mM NaCl, and 5 mM EDTA

Table 2. Concentrations of antibiotics used

Antibiotics Carl Roth GmbH + Co KG (Karlsruhe)	***E. coli***	***V. parahaemolyticus***
Ampicillin	100µg/µL	100µg/µL
Kanamycin	50µg/µL	50µg/µL
Chloramphenicol	20µg/µL	20µg/µL

Table 3. List of reagents, commercial kits, and equipment

Material	Supplier
Restriction enzymes	New England Biolabs (NEB)
2-Log DNA Ladder (0.1-10.0KB)	New England Biolabs (NEB)
Color Pre-stained Protein Standard Broad Range (11-245 KDA)	New England Biolabs (NEB)
T4 Ligase	New England Biolabs (NEB)
10X Buffer for T4 DNA Ligase with 10mM ATP	New England Biolabs (NEB)
Q5 Hot Start High FidelityDNA Polymerase	New England Biolabs (NEB)
Desoxyribonucleotide (dNTP)Solution Mix	New England Biolabs (NEB)
Alkaline Phosphatase CalfIntestinal (CIP)	New England Biolabs (NEB)

Isopropyl β-D-1 thiogalactopyranoside (IPTG)	Peqlab (Erlangen)
Red-gal	Sigma-Aldrich (Steinheim)
Ethidium bromide	Carl Roth GmbH + Co KG (Karlsruhe)
LB-Medium (Luria/Miller)	Carl Roth GmbH + Co KG (Karlsruhe)
L (+)-Arabinose and D (+) Saccharose	Carl Roth GmbH + Co KG (Karlsruhe)
GFP-Trap® Agarose	ChromoTek (Planegg)
cOmplete, EDTA-free protease inhibitor cocktail	Roche diagnostics (Mannheim)
Roswell Park Memorial Institute (RPMI) medium	Gibco
Newborn calf serum (NBCS)	Sigma-aldrich (Steinheim)
Kits	
NucleoSpin Gel and PCRClean-up kit	Macherey-Nagel (Düren)
NucleoSpin Plasmid Kit	Macherey-Nagel (Düren)
Equipment	
Electroporation: MicroPulser electroporator	Bio-rad (München)
PCR: Mastercycler nexus PCRSystem	Eppendorf (Hamburg)
Thermomixing: Thermomixer compact	Eppendorf (Hamburg)
DNA documentation: E-BOX VX2 imaging system	PeqLab (Eberhardzell)
DNA illumination: UVT_20 LE	Herolab (Wiesloch)
Stereomicroscopy: Nikon H600L	Nikon (Düsseldorf)
Imaging system: Deltavision Elite Widefield fluorescence microscope	IMSOL (UK)

2.2 Microbiological methods

2.2.1 Growth conditions and Media

In most experiments, strains were grown in LB medium at 37°C, containing antibiotics at the following concentrations: ampicillin, 100 μg/ml; streptomycin, 200 μg/ml; kanamycin, 50 μg/ml, and chloramphenicol, 5 μg/ml. For *E. coli*, we used chloramphenicol, 20 μg/ml. When appropriate, the medium was supplemented with of 0.2% w/v L-arabinose and 500 μM IPTG for induction. All media were sterilized by autoclaving at 121 °C for 20 min. Antibiotics and carbohydrates were filter-sterilized (pore size 0.2 μm or 0.45 μm; Sarstedt, Germany) and added to the medium (at ~60 °C).

2.2.2 Strains, plasmids, and primers

Escherichia coli DH5αλpir was used for cloning purposes, and each plasmid was introduced to *V. parahaemolyticus* by *E. coli* strain SM10λpir as the donor strain to deliver allelic exchange vectors. The wild-type strain used in this study was *V. parahaemolyticus* RIMD 2210633, and all mutants are derivatives of this strain. All strains, plasmids, and primers used are listed in Tables 4–6.

Table 4. Primers used in this study

Primer name	Sequence (5’ -> 3’)
vpa1044-del-a	ccccctctagaactggcgtcttaacctctgg
vpa1044-del-b	gctggtggtctttctcttattcaattccatcacaaggactccatataa
vpa1044-del-c	ttgaataagagaaagaccaccagc
vpa1044-del-d	ccccctctagaacgcggtcgcacatactttac
vpa1044-del-chk	taaaccaaaatcttccatctctgg
vpa1045-del-a	cccccgcatgcactctactctactttctattgctc
vpa1045-del-b	attcgaagttgatcattttcactcagtctatcttccttactacagcta
vpa1045-del-c	gagtgaaaatgatcaacttcgaat
vpa1045-del-d	cccccgtcgacagaggggcgtctaacaaccaag
vpa1045-del-chk	actcacataaaatcgaagttcttgc
vpa1046-del-a	cccccgcatgctggttctcgttttctctcagc
vpa1046-del-b	ctaagccttaaggtgttttatttttttcactctctatcggttatgtg

vpa1046-del-c	aaataaaacaccttaaggcttag
vpa1046-del-d	ccccctctagaagggtgaggatgattgattacgag
vpa1046-del-chk	acgacatgcgctctgtttggc
vpa1044-sfgfp-a	cccccggtaccgtgatggaagaaaaaaataaagaca
vpa1044-sfgfp-b	gaaaagttcttctcctttgctcatagatgggccctggacactttgctcggaatcttgctc
vpa1044-sfgfp-c	atgagcaaaggagaagaacttttc
vpa1044-sfgfp-d	cccccgcatgcttatttgtagagctcatccatgcc
Sfgfp-vpa1045-a	cccccgagctcgtccagggcccatctttggccgagggaagagtcg
Sfgfp-vpa1045-b	cccccgcatgctcactctctatcggttatgtgat
Sfgfp-vpa1046-a	cccccgagctcgtccagggcccatctgtgaaaatgatcaacttcgaattgc
Sfgfp-vpa1046-b	cccccgcatgcttattttaagttttctaagtaagcgaa
vpa1044 in pSRKKM-a	ccccccatatgatgatggaagaaaaaaataaagacaaatc
vpa1044 in pSRKKM-b	cccccccgcggttaactttgctcggaatcttgctc
vpa1044-pKNT25cw	cccccgcatgcatgatggaagaaaaaaataaagacaa
vpa1044-pKNT25ccw	cccccggtacccgactttgctcggaatcttgctcaa
vpa1044-pKT25cw	ccccccctgcagggatgatggaagaaaaaaataaagac
vpa1044-pKT25ccw	cccccggtaccttaactttgctcggaatcttgct

Table 5. Strains used in this study

Strain name	**Genotype**	**Reference**
Vibrio parahaemolyticus RIMD 2210633	Clinical isolate, wild type	(Makino et al., 2003)
Vibrio parahaemolyticus SI 205	*Δvpa1044*	This work
Vibrio parahaemolyticus CF52	*Δvpa1045*	This work
Vibrio parahaemolyticus CF53	*Δvpa1045*	This work
Vibrio parahaemolyticus ΔVSCN1	*ΔVSCN1*	Ringgaard lab
Vibrio parahaemolyticus SI1	*Δvp0057*	(Iyer et al., 2020)
Vibrio parahaemolyticus SI12	*Δvp0055*	(Iyer et al., 2020)

Escherichia coli DH5αλpir	*sup E44, ΔlacU169 (ΦlacZΔM15), recA1, endA1, hsdR17, thi-1, gyrA96, relA1*, λpir	
Escherichia coli SM10λpir	KmR, *thi-1*, *thr, leu, tonA, lacY, supE, recA*::RP4-2-Tc::Mu, λpir	

Table 6. Plasmids used in this study

Plasmid Name	**Description**	**Reference**
pDM4	Suicide vector for construction of deletion mutants	(Milton et al., 1996)
pJH036	Pbad::sfGFP	(Iyer et al., 2020)
pSI009	For deletion of *vpa1044*	This work
pSI044	For deletion of *vpa1045*	This work
pSI045	For deletion of *vpa1046*	This work
pSN001	pBAD::sfGFP-*vpa1045*	This work
pSN002	pSRKKm::*vpa1044*	This work
pSN003	pBAD::sfGFP-*vpa1046*	This work
pSN004	pBAD::*vpa1044*-sfGFP	This work
pSI053	Plac::vpa1044-t25	This work
pSI055	Plac::t25-vpa1044	This work
pSI056	Plac::t18-vpa1044	This work
pSI025	Plac::vp0057-t18	(Iyer et al., 2020)
pSI027	Plac::t18-vp0057	(Iyer et al., 2020)

2.2.3. Plasmid construction

Plasmid pSI009. The flanking DNA regions of *vpa1044* were PCR amplified using the primers vpa1044-del-a/b (upstream region) and vpa1044-del-c/d (downstream region). The resulting fragments were fused together in a third reaction using primers vpa1044-del-a/d. The end product was digested with XbaI for ligation into the corresponding site of pDM4.

Plasmid pSI044. The flanking DNA regions of *vpa1045* were PCR amplified using the primers vpa1045-del-a/b (upstream region) and vpa1045-del-c/d (downstream region). The resulting fragments were fused together in a third reaction using primers vpa1045-del-a/d. The end product was digested with SphI and SalI for ligation into the corresponding site of pDM4.

Plasmid pSI045. The flanking DNA regions of *vpa1046*were PCR amplified using the primers vpa1046-del-a/b (upstream region) and vpa1046-del-c/d (downstream region). The resulting fragments were fused together in a third reaction using primers vpa1046-del-a/d. The end product was digested with SphI and XbaI for ligation into the corresponding site of pDM4.

Plasmid pSN001. The gene *vpa1045* was amplified from *V. parahaemolyticus* RIMD 2210633 using the primers Sfgfp-vpa1045-a / Sfgfp-vpa1045-b. The DNA fragment obtained was restricted with the enzymes SacI and SphI and fused into the corresponding site of the plasmid pJH036.

Plasmid pSN002. The gene *vpa1044* was amplified from *V. parahaemolyticus* RIMD 2210633 using the primers vpa1044 in pSRKKM-a / vpa1044 in pSRKKM-b. The DNA fragment obtained was restricted with the enzymes NdeI and SacII and fused into the corresponding site of the plasmid pSRKKm.

Plasmid pSN003. The gene v*pa1046* was amplified from *V. parahaemolyticus* RIMD 2210633 using the primers Sfgfp-vpa1046-a / Sfgfp-vpa1046-b. The DNA fragment obtained was restricted with the enzymes SacI and SphI and fused into the corresponding site of the plasmid pJH036.

Plasmid pSN004. The gene v*pa1044* was amplified from *V. parahaemolyticus* RIMD 2210633 using the primers vpa1044-sfgfp-a/vpa1044-sfgfp-b. sfGFP was amplified from the pJH036 plasmid using the primers vpa1044-sfgfp-c/vpa1044-sfgfp-d. The resulting fragments were fused together in a third reaction using primers vpa1044-sfgfp a/d. The end product was restricted with the enzymes KpnI and SphI and fused into the corresponding site of the plasmid pBAD.

Plasmid pSI053/ pSI056. The gene *vpa1044* was amplified from *V. parahaemolyticus* RIMD 2210633 using the primers vpa1044-pKNT25 cw / vpa1044-pKNT25 ccw. The DNA fragment produced from the PCR was digested with the enzymes KpnI and SphI and ligated into the corresponding sites of the plasmids pKNT25 and pUT18, resulting in the plasmids pSI053 and pSI056 respectively.

Plasmid pSI055. The gene *vpa1044* was amplified from *V. parahaemolyticus* RIMD 2210633 using the primers vpa1044-pKT25 cw / vpa1044-pKT25 ccw. The DNA fragment produced from the PCR was digested with the enzymes PstI and KpnI and ligated into the corresponding sites of the plasmids pKT25.

2.2.4. Growth curves

In this study, *V. parahaemolyticus* bacterial colonies were cultured and grown until they reached the stationary phase ($OD6_{00}$ ≥3.0) in LB liquid media. Then 10 µl were taken and transferred to 1 ml of new LB medium and 1-2 µl was inoculated into microtiter plates containing 200 µl LB medium. The microtiter plates were placed in a TECAN Microplate Reader (Infinite 200 PRO) and incubated at 37 °C with shaking. The absorbance (OD_{600}) values were measured every 15 minutes for a duration of 18 hours. Lastly, the average values of the replicates were plotted against time.

2.2.5. Swimming assay

For swimming assay, a liquid culture of *V. parahaemolyticus* was grown in LB to an OD_{600} ≈ 1 and then using a sterile toothpick, cells were spotted on LB agar 0.3%. The plates were incubated overnight at 30°C for 15h. To plot a bar graph, the diameter of the swimming colony was then manually measured. Calculations were made with respect to the wild-type strain.

2.2.6. Swarming assay

To perform swarming assay, a liquid culture of *V. parahaemolyticus* was grown from a plate into 5 ml LB to an OD_{600} between 0.1-0.5, and subsequently 1 µl of the culture was spotted on swarm agar HI agar supplemented with 4 mM CaCl2 and 50 mM 2,2'-bipyridyl (Sigma Aldrich). The swarm agar plates were dried for 10 min at 37°C before spotting the liquid

culture. The plates were sealed with clear plastic tape and incubated overnight at 24°C. The diameter of the swarming colony was then manually measured the next day.

2.2.7. Bacterial two-hybrid assay

The bacterial adenylate cyclase two-hybrid system (BACTH) is a relatively quick method to detect *in vivo* protein–protein interactions in *E. coli*. The key element in this test is *Bordetella pertussis* adenylate cyclase toxin (CyaA) and is performed in *ΔcyaA E. coli* mutant, BTH101 strain in our experiments. It takes advantage of the fact that when the two catalytic domains of CyaA, termed T25 and T18, are brought close to each other by proteins that interact, then adenylate cyclase activity is reconstituted and cyclic adenosine monophosphate (cAMP) is produced. cAMP is a diffusible intracellular second messenger and binds to the catabolite activator protein (CAP). AMP/CAP complex controls the expression of several genes such as lac operon and the consequent production of β-galactosidase. X-Gal is a chromogenic substrate for this enzyme and its cleavage permits the screening of blue-white colonies.
For transformation in all cases, 20 to 25 ng of plasmid DNA was used to transform into BTH101 and plated on selective agar plates containing 100 µg/ml of ampicillin and 50 µg/ml of. For blue-white screening, plates also contained 0.5 mM IPTG and 40 µg/ml X-gal. Subsequently, plates were incubated at 30° for a period no longer than 48 h. Plates, where colonies turned blue, were kept at 4° C. Next, three colonies were taken per plate, grown in LB added with the respective antibiotics, and plated in three indicator plates, which were also incubated at 30° for no longer than 48 h. Pictures were taken of the three replicates at different intervals during the incubation time. As a negative control in all cases, cells were transformed with empty vectors, and as a positive control, cells were transformed with plasmid pUT18C-zip and pKNT25-zip (Euromedex, Soouddelweyersheim, France).

2.2.8. Spot assay

The effect of polymyxin B as a stressor was tested on *V. parahaemolyticus* wild-type and mutants with three biological replicates. Bacterial strains were grown at optimal conditions overnight. After the population reached the stationary phase, serial dilutions of cells were then spotted onto the LB agar plates containing 0,5 and 10 µg/ml of polymyxin B and incubated at 37 °C overnight.

2.3. Molecular Methods

2.3.1. Genomic DNA/Plasmid isolation

For DNA extraction by the boiling method, suspended cells were incubated at 99°C in a Thermomixer (Eppendorf Thermomixer C). The samples were centrifuged at 10000 rpm for 10 min. The genomic DNA in the supernatant was transferred to a fresh Eppendorf tube for further use. For isolation of plasmid DNA from *E. coli*, 5 ml of LB (Plasmid-containing strain with the relevant antibiotic) was shaken at 37 °C to reach $OD6_{00}$ ≥3.0. Then, the NucleoSpin Plasmid kit (Macherey-Nagel) was used to obtain plasmids. The concentration of the DNA was then measured using a Nanodrop spectrophotometer.

2.3.2. Polymerase chain reaction

gDNA, or plasmid DNA was used as a template for PCR reactions. Q5 Hot Start High-Fidelity DNA Polymerase or Phusion High-Fidelity DNA Polymerase was used for cloning purposes. Annealing temperature and elongation time were adapted respectively depending on the length of the PCR product and its properties. If necessary, PCR products were extracted from the agarose gel and purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) according to the manufacturer's instructions. PCR conditions for each polymerase are summarized in Table 7.

Table 7: PCR conditions according to polymerase. (*) sign indicates the variables depending on the primer and product properties.

Phusion Polymerase (NEB)		**Reaction Conditions**		
Template	1 µl	98°C	05:00	
Forward Primer (0.5 µM)	2 µl	98°C	00:30	x35
Reverse Primer (0.5 µM)	2 µl	Annealing *	00:30	
10x Reaction Buffer	5 µl	72°C	Extension*	
Phusion Polymerase	1 µl	72°C	05:00	
dNTPs (10 mM each)	1 µl	4°C	∞	
Nuclease-free water	Up to 50 µl			

Q5 Polymerase (NEB)		**Reaction Conditions**		
Template	1 µl	98°C	05:00	
Forward Primer (0.5 µM)	2 µl	98°C	00:30	x35
Reverse Primer (0.5 µM)	2 µl	Annealing *	00:30	
5X Q5 reaction buffer	10 µl	72°C	Extension*	
5X Q5 High GC enhancer	10 µl	72°C	02:00	
dNTPs (10 mM each)	1 µl	4°C	∞	
Polymerase	0.5 µl			
Nuclease-free water	Up to 50 µl			

2.3.3. Preparation of competent cells

In order to prepare chemically competent *E. coli* cells, culture from an initial OD_{600} of approximately 0.05 was grown at 37 °C with shaking until the cells reached an $OD6_{00}$ of 0.5-0.7. Then, the pellet was obtained by centrifugation of the samples at 4700 rpm for 10 minutes at 4 °C and then washed twice with 50mM ice-cold calcium chloride. Lastly, the pellet obtained after the second wash was resuspended in a solution of 50 mM (ice-cold) calcium chloride.

In order to prepare electro-competent *Vibrio parahaemolyticus,* cultures were grown in LB media at 37 °C with shaking until the cells reached an OD_{600} of 1.0. Then, the pellet was

obtained by centrifugation of the samples at 4700 rpm for 10 minutes at 4 °C and then washed twice with 273 mM ice-cold sucrose solution (that is buffered with KOH to a pH of 7.2-7.4). Lastly, the pellet obtained after the second wash was resuspended in a solution of 273 mM ice-cold sucrose solution.

2.3.4. Transformation of competent cells

In order to introduce plasmid DNA into *E. coli* cells, 50- 100 ng of the corresponding plasmid was added to 50 µl of the chemically competent cells. The resulting mixture was gently mixed and placed on ice for approximately 30 minutes. Then the cells were heat shocked in the water bath at 42°C for 30 seconds and immediately placed on ice. They were then resuspended in 500 µl of LB medium and shaken for 45 minutes at 37°C followed by centrifugation. After removing most parts of the supernatant, approximately 50-100 µl of the cells were plated onto LB-agar plates containing the relevant antibiotic.

In order to introduce plasmid DNA into electro-competent *V. parahaemolyticus* cells, 100-1000 ng of the corresponding plasmid was added to 100 µl of the cells. After being placed on ice for about 1 hour, the cells were transferred into a pre-chilled electroporation cuvette and MicroPulser electroporator (Bio-Rad) was used to perform electroporation at the following conditions: voltage 2200, µF 25, and 200 Ω. They were immediately transferred back to ice and resuspended in 1 ml of LB medium and into a 1.5 ml Eppendorf tube and shaken for at least 2 hours at 37 °C, followed by centrifugation. After decanting most of the supernatant, approximately 50-100 µl of the cells were plated onto LB-agar plates containing the relevant antibiotic.

2.3.5. Cloning strategy

The gene of interest for the respective cloning module was amplified with the primers listed in Table 4). Restriction of DNA fragments was performed using the corresponding restriction endonuclease unless otherwise specified by the enzyme manufacturer, the restriction reaction was carried out at 37° C for 1.5h. In order to prevent self-ligation, 1 µl of calf intestine alkaline phosphatase (CIP) to the vector. This was followed by running a gel and then eluting the band (using the NucleoSpin Gel and PCR Clean-up kit (Machenery-Nagel) corresponding to the desired fragment size.

Ligation reactions were performed using the T4 DNA ligase (New England Biolabs). Approximately 50 ng of vector DNA and 3 to 5-fold molar excess of insert DNA were mixed (1 µl of T4 DNA ligase was added to the mix with a total volume of 20 µl) and kept at room temperature for 1 h minimum. Ligation mixtures were then used to transform *E. coli* strains DH5αλpir, SM10λpir. Lastly, approximately 100-200 ng of plasmid were sent to the sequencing services to confirm the desired sequence via Sanger sequencing with appropriate primers.

2.4. Proteomics methods

2.4.1. Proteomic analysis of total cell lysates by LC-MS

Sample preparation and tryptic digestion for a label-free experiment were performed according to our previous report(Iyer et al., 2020). This experiment was done in triplicate for each cell type and all replicates were from one condition. Briefly, cells were collected at the approximate OD of 0.45 by centrifugation at 4,700 r.p.m for 10 min at 4 °C. After one step of washing, the pellets were lysed with 1% sodium laureth sulfate (SLS) and boiled for 5min. Then, the samples were sonicated on ice, followed by another boiling step at 90 °C for 15min. Protein concentration was measured by bicinchoninic acid (BCA) assay. After reduction with TCEP and alkylation with iodoacetamide, overnight digestion was performed with sequencing grade modified trypsin (Promega) at 37°C. To quench the digestion, trifluoroacetic acid (TFA) was added to a final pH of ~2, followed by C18 desalting. Desalted peptides were aliquoted, dried using a vacuum concentrator, and used for MS analysis. This experiment was done in triplicate for each cell type. The procedure for LC–MS analysis of total cell lysates was extensively described elsewhere(Glatter et al., 2015).

For performing proteomics upon polymyxin treatment, overnight cultures (5 ml) of *V. parahaemolyticus* were sub-cultured to an initial OD_{600} of approximately 0.05 in 40 ml of LB and were grown until they reach an OD_{600} of approximately 0.39-0.41. The cultures were then divided into two flasks of 20 ml each: to the experimental flask, polymyxin B was added to a final concentration of 10 µg/ ml, whereas nothing was added to the other “control” flask. The two were then shaken under standard laboratory conditions for five minutes. This was proceeded by harvesting and subsequent sample treatment as described in the above section

2.4.3. Co-immunoprecipitation assays followed by LC-MS analysis

The experimental procedure was carried out as previously described in detail, with minor modifications(Iyer et al., 2020). Briefly, overnight bacterial cultures were resuspended in 100 ml of LB medium containing the antibiotics and inducers to an initial OD of 0.05 and they were grown until they reached OD of 0.9. Subsequently, cell pellets were resuspended in 10mL cold Lysis Buffer (50mM HEPES buffer, 150mM NaCl and 5mM EDTA) pre-mixed with protease inhibitors (complete EDTA-free, Sigma-Aldrich) as well as NP-40 to a final concentration of 0.5%. After two steps of ultrasonication followed by centrifugation for 10 min, the supernatant was incubated with anti-GFP Nanobody (ChromoTek) while rotating at 4 °C. The beads were then washed four times with 700 µl of 100 mM ammoniumbicarbonate (Sigma-Aldrich). For elution, an on-bead digestion was performed by adding 200 µl trypsin-containing elution buffer 1 (1.0 M urea, 100 mM ammoniumbicarbonate, 1 µg trypsin (Promega)) to each sample. After 30 min shaking incubation (1400 rpm) at 30 °C, the supernatant containing digested proteins was collected. Beads were then washed twice with 80 µL of elution buffer 2 (1.0 M urea, 100 mM ammoniumbicarbonate, 5 mM Tris(2-carboxyethyl) phosphine (TCEP)) (Thermo Scientific) and the supernatant was added to the first elution fraction. Digestion was allowed to proceed overnight at 30°C. Following digestion, the peptides were incubated with 10 mM iodoacetamide (IAA, Sigma-Aldrich) for 30 min at 25°C in the dark. The samples were handed to the proteomics facility in MPI_Marburg for the peptides to be further processed and analyzed for LC-MS on a Q-Exactive Plus instrument connected to an Ultimate 3000 RSLC nano and a nanospray flex ion source (Thermo Scientific). The procedure forLC–MS analysis of total cell lysates was extensively described elsewhere(Glatter et al., 2015).

2.5. Stereo microscopy

In order to perform stereomicroscopy imaging of swarm colony architecture, Nikon H600L stereomicroscope is equipped with a Q2 digital camera. The samples were prepared from section “2.2.6. Swarming assay” and images were analyzed and captured using the NIS Elements AR (v 5.02.00) tool. The magnification of the lens used is indicated in the respective pictures.

2.6. HEp-2 cell infection assay

HEp-2 cells were maintained in Roswell Park Memorial Institute (RPMI) medium (Gibco) supplemented with 7.5% newborn calf serum (Sigma-Aldrich) in 5% CO2 at 37°C. HEp-2 cells were seeded into NuncIon Delta Surface 96-well flat plates (Thermo Scientific) at a cell density of 2.0×10^4 cells per well. Bacterial strains were inoculated in 5 ml LB and grew for 90 min at 37°C. OD_{600} was measured and let 1 ml of LB culture to spin for 5 min 5000xg 4°C, the supernatant was discarded and the pellet was resuspended in 500 µl 1xPBS. Then, the OD was measured again to reach a dilution of 1:10, followed by calculation of the dilution of cells for final OD=0.2 to infect HEp-2 cells, 11.2 ul of bacterial suspension was added to the 96 well plate. It is important to pipette twice to mix and then allow the bacteria to sediment. The plates were placed in the 37C incubator with 5% CO2 for 1.5 and 6h. For dead cell control, the cells were treated with 0.3% Triton 100/PBS for 10 minutes. After incubation, the wells were washed 2 times (100 µl) with warmed RPMI. Lastly, 100 µl of fresh media was added and checked under the microscope to make sure all the bacteria were removed. Uninfected cells and cell-free well plates were used as negative and background controls, respectively Cells were then imaged using Deltavision Elite microscope (×20 objective). The cell shape of HEp-2 cells was manually classified by blinded observers. At least three independent experiments were conducted.

2.7. Software

Table 8. Software and on-line tools

Name	Source/Reference	Additional information
CLC Main Workbench software V20	Qiagen, USA	
SeqMan Pro v12.3.1	DNASTAR Software for Life Scientists (Madison, WI)	
ImageJ-Fiji	(Schindelin et al., 2012)	http://rsbweb.nih.gov/ij
R studio version 2022.12.0		http://www.rstudio.com/
GraphPad Prism v9.5.1	GraphPad Software (La Jolla CA)	https://www.graphpad.com/
Scaffold (v4.6.2)	(Portland, OR, USA)	
Maxquant/Perseus (v1.6.12.0)		https://maxquant.net/perseus/
SMART: EMBL-Heidelberg	(Letunic & Bork, 2018)	http://smart.embl-heidelberg.de/
Oligo Calc: Oligonucleotide	(Kibbe, 2007)	http://biotools.nubic.northwestern.edu/OligoCalc.html
STRING-known and predicted protein- protein interactions	(Jensen et al., 2009)	http://string-db.org/

3. Results

3.1. Investigation of known Ser/Thr kinases in *V. parahaemolyticus*

We recently identified the threonine kinase, VP0057, namely PknT, as a regulator of antibiotic resistance in the human pathogen *V. parahaemolyticus (Iyer et al., 2020).* With the help of *in-silico* analysis, two additional genes, *vp1400* and *vpa1044,* that encode for a predicted STK protein in *V. parahaemolyticus* were selected. To understand how similar are the candidate kinases in our model organism to other species, we performed multiple sequence alignments using the MUSCLE alignment tool. The gene encoding for VP1400 is located on the first chromosome and shares some of the key features of several other eukaryotic-like serine/threonine kinases (Fig 7). We next took a look at the predicted motifs of the kinase using the InterPro web tool. VP1400 is predicted to be comprised of two non-cytoplasmic domains of varying lengths and one cytoplasmic domain between them. The cytoplasmic domain is separated from the other two domains by a transmembrane helical domain. Another student in our lab did some phenotypic tests by performing swimming, swarming, biofilm, and growth curve assays on its mutant strain and compared it to the wild type and saw no significant differences in any of the experiments (unpublished data). Upon alignment of the other predicted kinases using NCBI's MUSCLE alignment tool with eukaryotic-like serine/threonine kinases from other bacterial species, it was found that VPA1044 possessed the highly conserved ATP-binding and catalytic domains, characteristic of these kinases (Fig 1). Henceforth, we decided to proceed with the characterization of VPA1044, as available in the following sections.

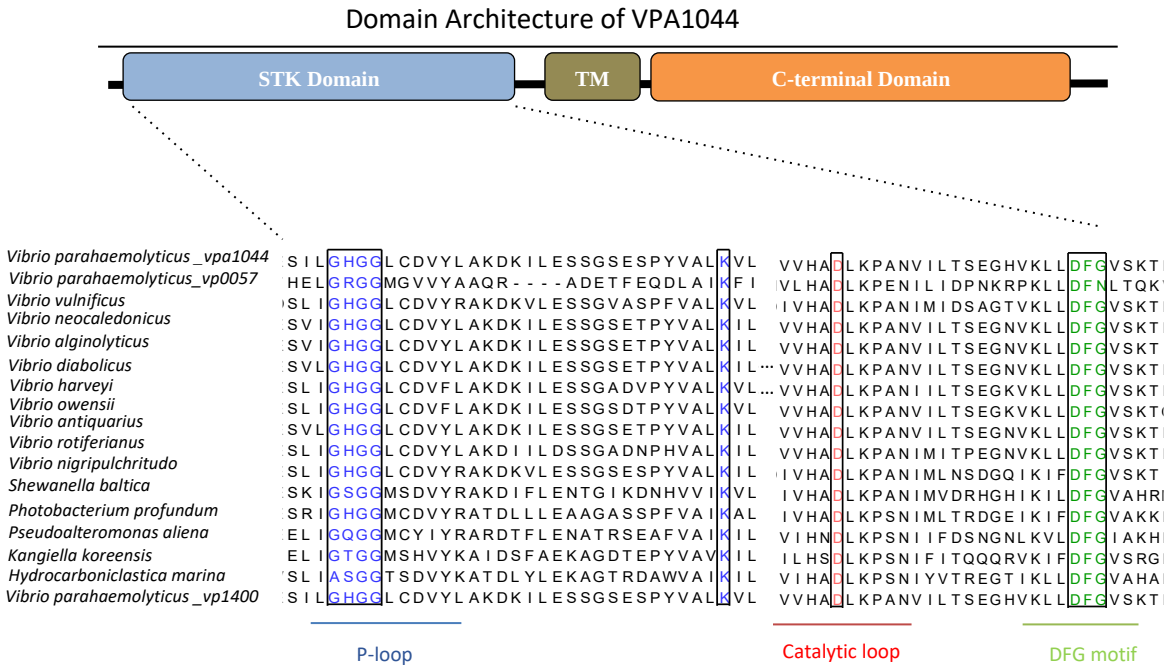

Figure 7. Multiple sequence alignment of the kinase domains of the candidate serine/threonine kinases in our model organism to other species. The regions corresponding to the P-Loop, the catalytic Loop, and the magnesium-binding Loop are highlighted. The schematic domain architecture of VPA1044 is also shown on top.

3.2. VPA1044 appears to be a functional serine/threonine kinase

VPA1044 is a membrane-bound protein with a size of ~80 kDa (obtained using ProtParam (ExPASy)). *In silico* analysis predicted VPA1044 to consist of an N-terminal STK domain (amino acids (aa) 1-360) and a C-terminal domain of unknown function (aa 383-714), separated by a single transmembrane region (aa 361- 383). The predicted structure of VPA1044 (through Protter) is comprised of a large cytoplasmic domain connected to the extracellular region by a short transmembrane helix. A cartoon representation of the structure is shown in Figure 8.

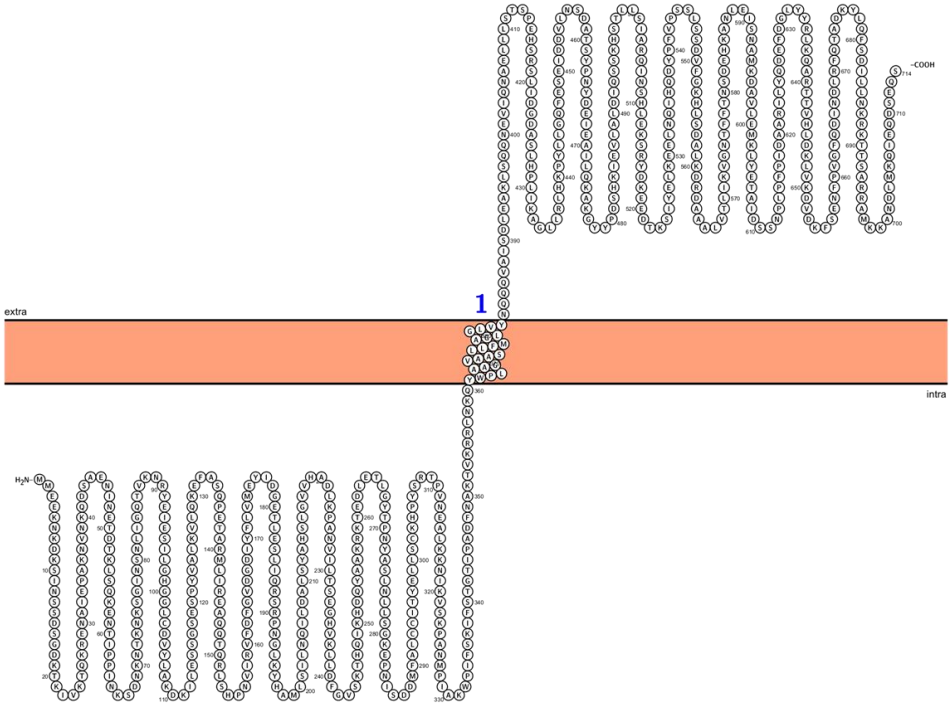

Figure 8: *in silico* secondary structure prediction of VPA1044. The predicted structure shows only one transmembrane domain. Analysis performed by the Protter tool (http://wlab.ethz.ch/protter/start).

3.3. Deletion of *vpa1044* causes a significant swarming defect

To test the function and importance of *vpa1044*, we constructed an in-frame deletion of the gene in *V. parahaemolyticus* (strain Δ*vpa1044*) to characterize its mutant phenotypes. The typical phenotypic tests, namely growth, swimming, and swarming were carried out on the deletion mutant and compared with the wild type. There were no significant differences between the *vpa1044* mutant and wild type in growth and swimming assays (Fig 9. A-C). However, there was a highly significant swarming defect in the *vpa1044* mutant with respect to the wild type, amounting to almost a 50% reduction in the size of the swarm colony. We also found morphological differences in the swarm colony (Fig 9D) between the deletion mutant and wild type. The flares in the peripheral area of the swarm colony were more distinct and spiked when compared to those in the mutant.

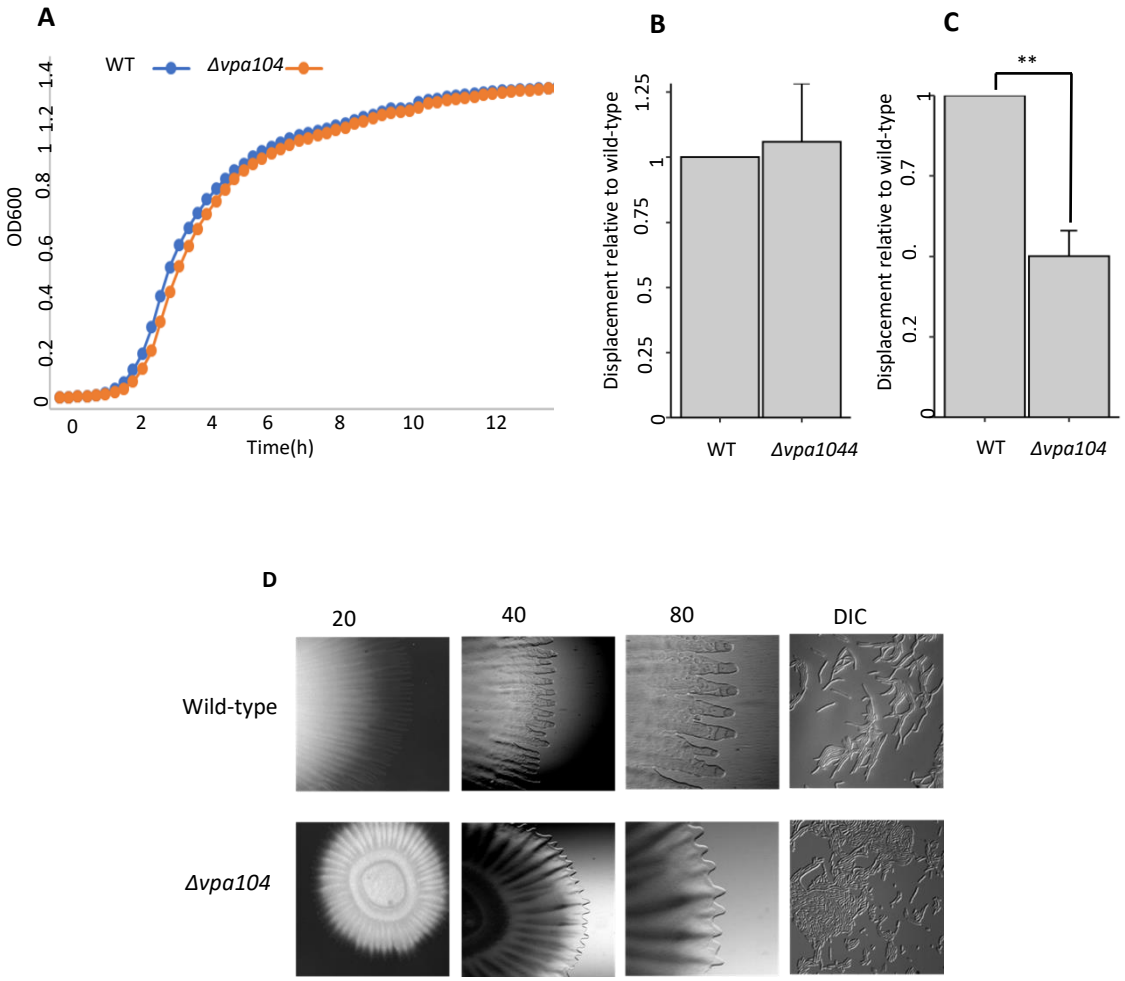

Figure 9: Phenotypes of the Δ*vpa1044* mutant with respect to wild type: (A) Growth curve experiment, (B) Swimming assay, (C) Swarming assay (OD: 1.0-1.3). (D)Stereo-microscopy images of the swarm colony showing clear morphological changes between Δvpa1044 compared to the wild type

3.4. VPA1044 is essential for polymyxin B resistance in *V.parahaemolyticus*

Wild-type *V. parahaemolyticus* is highly resistant to polymyxin B antibiotics (Donovan and Van Netten, 1995). We hypothesized that *V. parahaemolyticus* could encode an additional pathway for regulating polymyxin B resistance. However, the genetic neighborhood of *vp1400* and *vpa1044* vary significantly from each other and that of the *pknT*/*ecfP* system. The gene *vp1400* is encoded at the upstream of one the type VI secretion system putative operon and is likely to be involved in regulating the function of T6SS1 in *V. parahaemolyticus* (Ma et al., 2012, Makino et al., 2003). Thus, we focused our interest on the gene *vpa1044*. Particularly, as this gene was so far uncharacterized and because our *in-silico* analyses suggested that based on its specific presence in *V. parahaemolyticus*, *vpa1044* might play a regulatory role in the life of this particular bacterium. Using the Δ*vpa1044* strain, we tested the effect of the absence of the gene on the ability of *V. parahaemolyticus* to survive treatment with polymyxin B antibiotics. As previously published (Iyer et al., 2020), a wild-type strain was resistant to polymyxin B treatment; however, a strain deleted for *vpa1044* was highly sensitive to polymyxin B treatment (Fig 10). Altogether, our data suggest that the gene *vpa1044* is required for polymyxin resistance of *V. parahaemolyticus*.

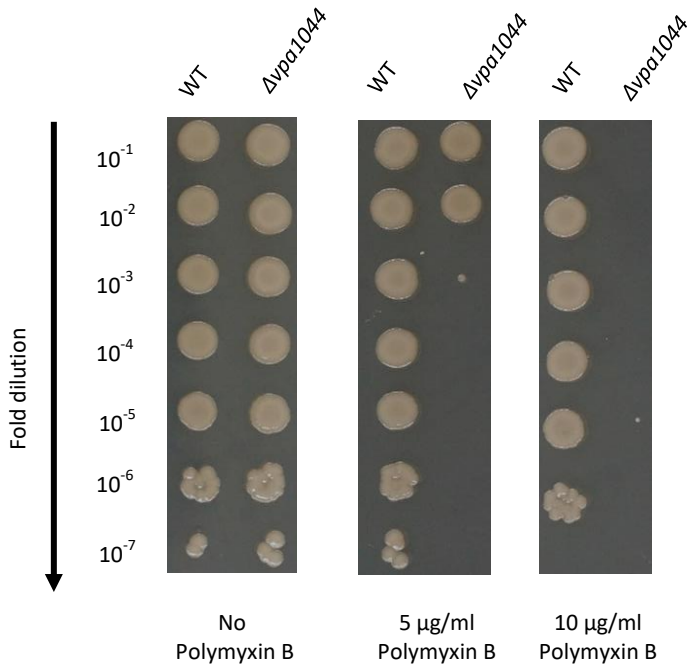

Figure 10. VPA1044 is essential for conferring resistance to polymyxin antibiotics. Spot dilution assay (10-fold dilutions) of wild-type and *Δvpa1044* on LB growth medium in the presence and absence of 5 and 10 µg/ml Polymyxin B.

3.5. The genes *vpa1044*, *vpa1045*, and *vpa1046* are individually required for polymyxin B resistance of *V. parahaemolyticus*

In prokaryotes, genes that are involved in the same pathway are more likely to be in the same cluster (Osterman and Overbeek, 2003). Hence, in order to better understand the functional network associated with *vpa1044,* we took a closer look at its genomic neighborhood. We found that this gene is downstream of one of the operons of type VI secretion system. Interestingly, the KEGG database indicates that *vpa1044* is located in in the upstream region of two proteins that are normally co-transcribed as part of a two-component system. These proteins are VPA1045, which is a putative response regulator, and VPA1046, a histidine phosphotransferase protein (Fig. 11A). *In silico* analysis predicted VPA1045 to possess an N-terminal CheY-like receiver (phosphoacceptor) domain (amino acids (aa) 10-1150), which is a common regulatory module among response regulators (Galperin, 2006). VPA1046 is also predicted to be part of the phosphorelay signal transduction system, where an HpT (His-containing phosphotransfer) domain is used. In multistep phosphorelay pathways, HpT can be a module that acts as a single-domain protein. As mentioned earlier, there have been numerous studies indicating that STKs can be part of different interacting networks and that there is cross-talk between STKs and TCSs. Subsequently, we constructed *V. parahaemolyticus* strains with individual in-frame deletions of the genes *vpa1045* (Δ*vpa1045*) and *vpa1046* (Δ*vpa1046*) and subsequently analyzed the effect of polymyxin B on *V. parahaemolyticus* in the absence of *vpa1045* and *vpa1046*, respectively. Strikingly, cells lacking either *vpa1045* or *vpa1046* were highly sensitive to polymyxin B treatment when compared to wild-type cells (Fig. 11B). Indeed, cells lacking *vpa1045* or *vpa1046* showed identical phenotypes and were 10^4-fold more sensitive to polymyxin B treatment than wild-type cells. Interestingly, however, cells lacking *vpa1044* always showed a stronger phenotype and were generally more sensitive to polymyxin B treatment than cells lacking *vpa1045* or *vpa1046* (Fig. 11B). Altogether, these data indicate that each of the genes *vpa1044*, *vpa1045* and *vpa1046* is individually required for polymyxin B resistance of *V. parahaemolyticus*.

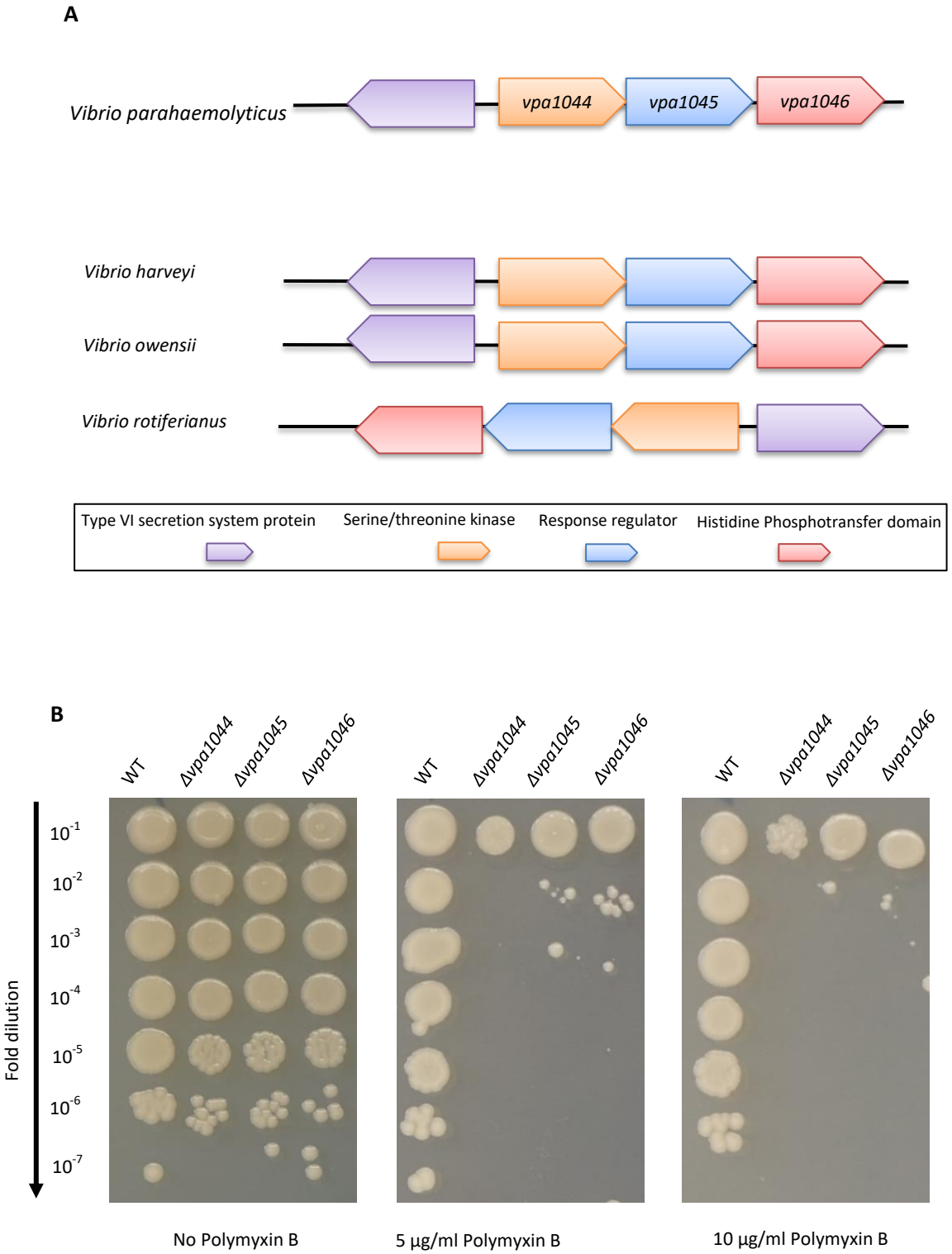

Figure 11. VPA1044, VPA1045, and VPA1046 are essential for conferring resistance to polymyxin antibiotics. (A) The genetic context surrounding VPA1044 is used as a reference and compared with several other *Vibrio* species (B) Spot assay (10-fold dilutions) of wild-type and mutant variants on LB growth medium in the presence and absence of 5 and 10 µg/ml Polymyxin B

3.6. The *vpa1044-vpa1045-vpa1046* system regulates the protein expression profile in *V. parahaemolyticus*

We next employed LC/MS global proteomics analysis to elucidate the cellular output of the *vpa1044-vpa1045-vpa1046* system in the absence of polymyxin B on exponentially grown wild-type, Δ*vpa1044*, Δ*vpa1045,* and Δ*vpa1046* swimmer cells. A total of 2300 proteins were significantly detected with at least two peptides, which represents approximately 47.5% of the 4832 predicted open reading frames in the *V. parahaemolyticus* RIMD2210633 genome. Many abundant proteins such as subunits of bacterial RNA polymerase were detected in higher peptide counts for example, rpoA=33, rpoB=95, and rpoC=105. Additionally, ribosomal proteins of the 30S and 50S subunits were detected with an average of 8 and 11 peptides, respectively. Moreover, we were able to detect the σ factors RpoH, RpoE, and RpoD with 5, 15, and 30 counts, respectively. Lastly, it is worth noting that many low-abundant transcription factors such as, among others, arcA (response regulator), flhD (flagellar transcriptional activator), as well as cell division proteins (e.g. FtsA), were detected (Fang et al., 2017). Therefore, LC-MS/MS detection of high and low-abundant proteins enabled us to proceed with profiling of protein expression in our *V. parahaemolyticus* strains. Next, we compared the proteome of cells from the three different mutant strains and scored protein abundance changes of more than twofold as being significantly differentially regulated between the cell groups. In the absence of all of the genes individually, there were significant changes in the proteomic profile when compared to wild-type cells (Fig. 3A), suggesting all three genes individually are required for correct protein expression in *V. parahaemolyticus*. Particularly, 129 proteins were significantly downregulated and 87 were significantly upregulated in the absence of *vpa1044*, 68 proteins were significantly downregulated and 102 were significantly upregulated in the absence of *vpa1045* and 151 proteins were significantly downregulated and 89 were significantly upregulated in the absence of *vpa1046* (Fig. 12B-D).

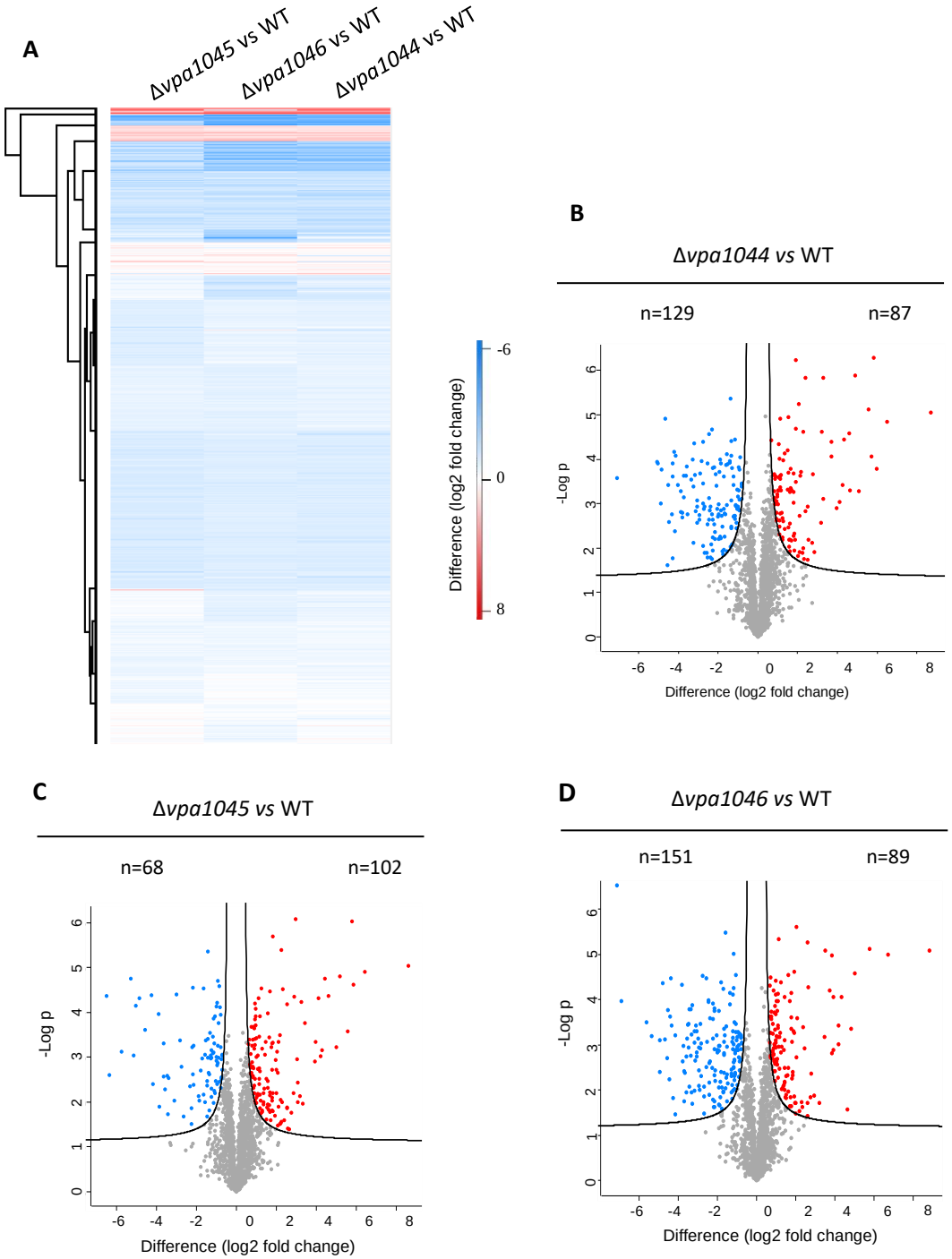

Figure 12: Proteomic profile of *V. parahaemolyticus* WT and Δ*vpa1044*, Δ*vpa1045* and Δ*vpa1046* mutants. (A) Hierarchical cluster heat map in comparison groups. The ratios of differential proteins in replicates were clustered. Blue indicates gradually downregulated proteins, whereas red indicates gradually upregulated proteins. (B) Volcano plots of proteins in the proteome. Y-axis indicates the -log of the p-value and "n" indicates the number of proteins significantly down- or upregulated between the two conditions compared. Grey points represent unchanged proteins, red and blue represent the upregulated and downregulated proteins respectively.

Interestingly, there was an overlap of 47 proteins that were significantly upregulated and 46 proteins that were significantly downregulated between Δ*vpa1044*, Δ*vpa1045,* and Δ*vpa1046* cells when compared to wild-type *V. parahaemolyticus* (Fig. 13A-B), thus suggesting that *vpa1044*, *vpa1045* and *vpa1046* work in the same pathway when influencing the protein expression profile of *V. parahaemolyticus*. The pie chart representing the commonly and significantly up- and down-regulated proteins among the aforementioned three comparisons is shown in Figure 13. D-C. The complete list of genes that were significantly downregulated or upregulated in a mutant background when compared to the wild-type can be found in supplementary tables 9-11. Interestingly, many of the proteins whose expression decreased in all three mutants were proteins of the Type III secretion system 1 (T3SS) (Fig. 13C). As *V. parahaemolyticus* T3SS triggers virulence, our results suggest that the *vpa1044-vpa1045-vpa1046* system seems to be important for infection. Proteins involved in metabolism comprised the largest fraction of both down- and up-regulated proteins in all the comparisons. Proteins with transport activity, transcriptional regulators, as well as those involved in chemotaxis, constituted a large fraction of regulated proteins. Some groups were found to be exclusive to either the up or downregulated set of proteins. Particularly, proteins involved in the cell division were found only among the up-regulated targets, whereas flagellar and T3SS proteins were specific to the down-regulated group (Fig. 14). Some of the other categories displayed here will be discussed in detail in Section 4.

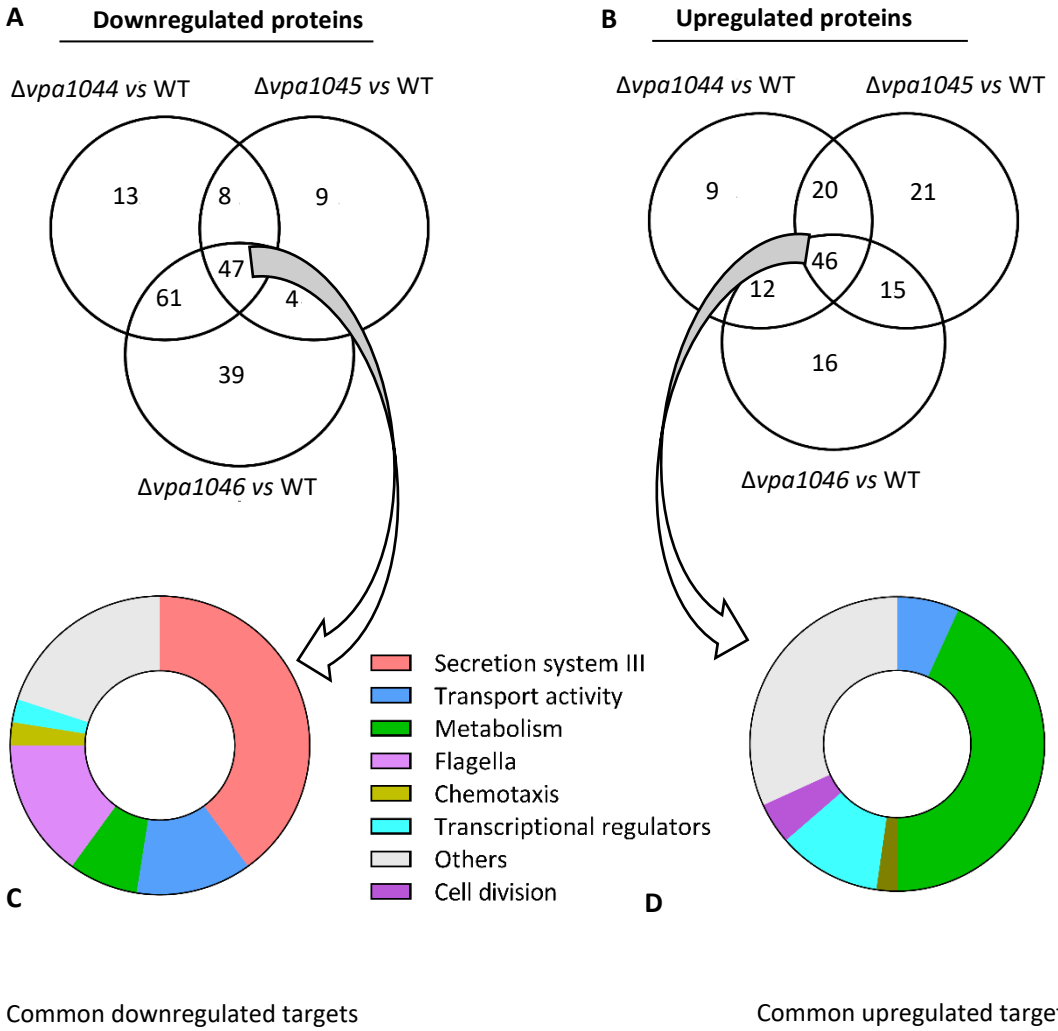

Figure 13: General characterization of regulated proteins. (A, B) Venn diagrams showing the number of shared proteins differentially upregulated and downregulated and (C, D) the pie chart showing categories of the overlap of identified proteins in all three mutants when compared to WT.

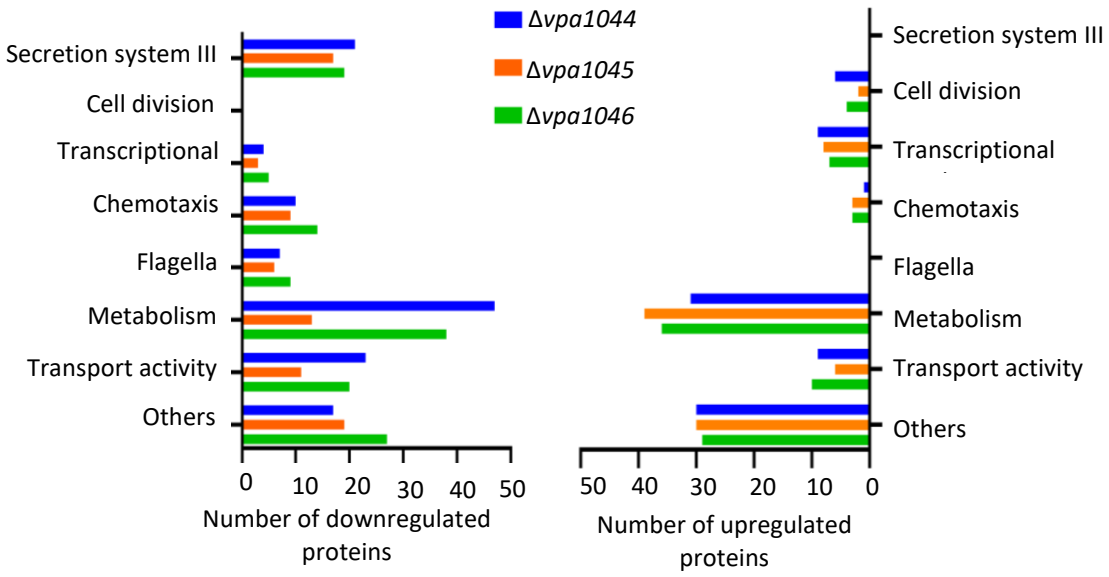

Figure 14: The most present functional categories of proteins differentially regulated specifically in each comparison are shown in the bar graph with the number of detected proteins. Classification of the molecular function and categories of the identified proteins were based on UniProt KB search and gene ontology (GO) annotations.

3.7. The *vpa1044-vpa1045-vpa1046* system is required for the expression of the polymyxin B resistance determinant VPA0879

We recently identified the protein VPA0879 as a major polymyxin B resistance determinant in *V. parahaemolyticus* (Iyer et al., 2020). Polymyxin antibiotics belong to a group of cyclic non-ribosomal polypeptides that bind to the lipopolysaccharide (LPS) layer of Gram-negative bacteria and disrupt the outer and inner membrane, which ultimately leads to cell lysis and death. They are clinically used for the treatment of a range of infections. To understand polymyxin resistance in *V. parahaemolyticus*, we analyzed for any effect of deleting *vpa1044*, *vpa1045,* and *vpa1046* on VPA0879 expression in our LC-MS proteomics data. Interestingly, the expression of VPA0879 was downregulated in the absence of *vpa1044*, *vpa1045*, and *vpa1046* (Fig. 3B). The results also showed that the level of expression of VPA0879 was similar in all three mutants (Fig. 15B), thus indicating that the *vpa1044-vpa1045-vpa1046* system is important for the expression of the polymyxin B resistance determinant VPA0879. In addition to VPA0879, the absence of these genes also resulted in a significant reduction in the expression of the predicted heme transport protein HutA (Fig. 3A and B). Importantly, a Δvpa0879 strain was highly sensitive to polymyxins, to a level similar to *Δvpa1044*, *Δvpa1045,* and *Δvpa1046* cells (Figure 26), indicating that the *vpa1044-vpa1045-vpa1046* system confers polymyxin resistance by regulating the expression of the essential polymyxin resistance determinant vpa0879.

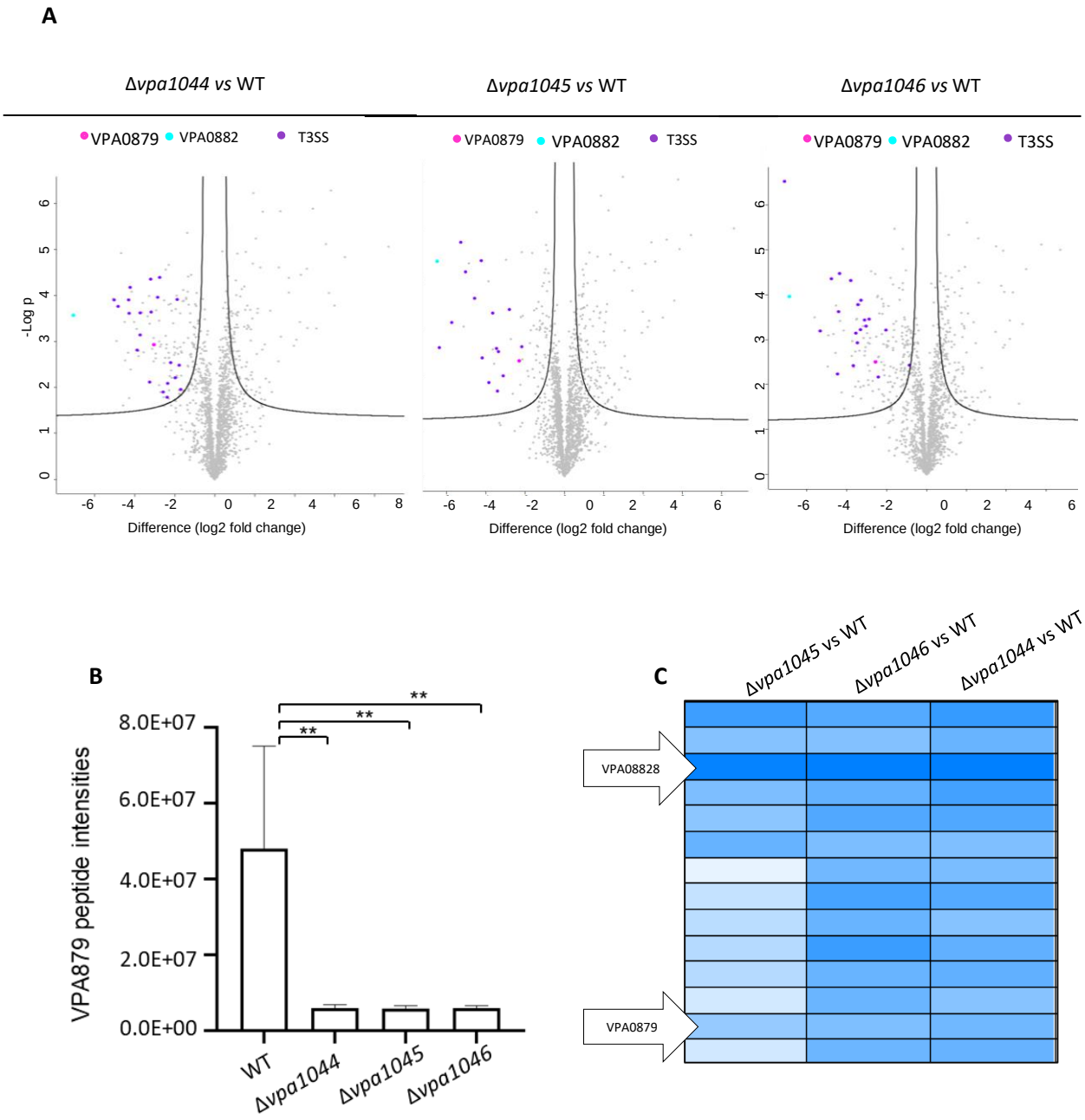

Figure 15: The *vpa1044-vpa1045-vpa1046* system regulates expression of VPA0879. (A) Volcano plot identical to those presented in fig 12B-D, here highlighting in color, the distinct functional protein groups that were significantly regulated in mutants when compared to WT. (B) Bar graphs showing the peptide intensities of VPA0879 in wild-type and mutant backgrounds, as obtained through global LC-MS proteomics analysis. Error bars indicate the standard error of the 743 mean (SEM). Statistical significance was calculated using the two-sided unpaired t-test (** indicates a $p < 0.001$). (c) Zoom-in of the clustering map showing the changes in expression levels of VPA0879 and VPA0882.

3.8. Investigating the role of the system in the virulence of *V.parahaemolyticus*

The T3SS genes have been commonly used as indicators of virulence in pathogenic bacteria. The type III secretion system (T3SS) utilizes a syringe-like transmembrane structure to deliver various effectors into eukaryotic cells of the host organism and enable the expression of virulence. There have been many reports on cytotoxicity to eukaryotic cells of *V. parahaemolyticus* to date(Ono et al., 2006). The proteomics results show that many genes involved in Type III secretion system 1 (TTSS1) in *V. parahaemolyticus* were significantly downregulated in all of the *Δvpa1044, Δvpa1045,* and *Δvpa1046* mutants when compared to wild type. Our previous transcriptomics and proteomics study on *ΔpknT* and *ΔecfP* showed the same results and T3SS genes are among the common targets of these two systems. This led us to investigate these mutants on infection of mammalian cells to shed more insights into the biological importance of the system. *vscN* is a gene that encodes a type III secretion system ATPase and we observed its level was significantly decreased in the proteomics; therefore, the assay was performed using the *ΔvscN* strain as well. Morphological changes in Hep-2 cells (detectable as rounded cells) were recorded.

As predicted, cells infected with the wild-type strain showed round morphology, including a decrease in cytoplasm and shrunken nuclei cells at 1.5h post-infection and the number of round cells increased at 3 h post-infection. The untreated Hep-2 cells were shaped like a leaf and firmly attached to the surface; similarly, cells infected with *ΔvscN* strain were not significantly different from that of uninfected cells at 1.5 and 3h post-infection. Hep-2 morphology of the mutant group contained only a few rounded cells after 1.5h compared with the WT-infected group. Following an extended incubation time (3h), the three strains (*Δvpa1044*, *Δvpa1045,* and *Δvpa1046*) displayed more but equivalent levels of morphological changes, indicating cell death when compared to the reference strain and together with *ΔpknT*, they showed a moderately more effect on Hep-2 cells than that of *ΔecfP* (as evidenced by ~20% more rounding up). Altogether, it can be inferred from the results that the mutants have some importance for virulence, as they showed a moderately more pronounced effect on HEp-2 cells compared to *ΔecfP*.

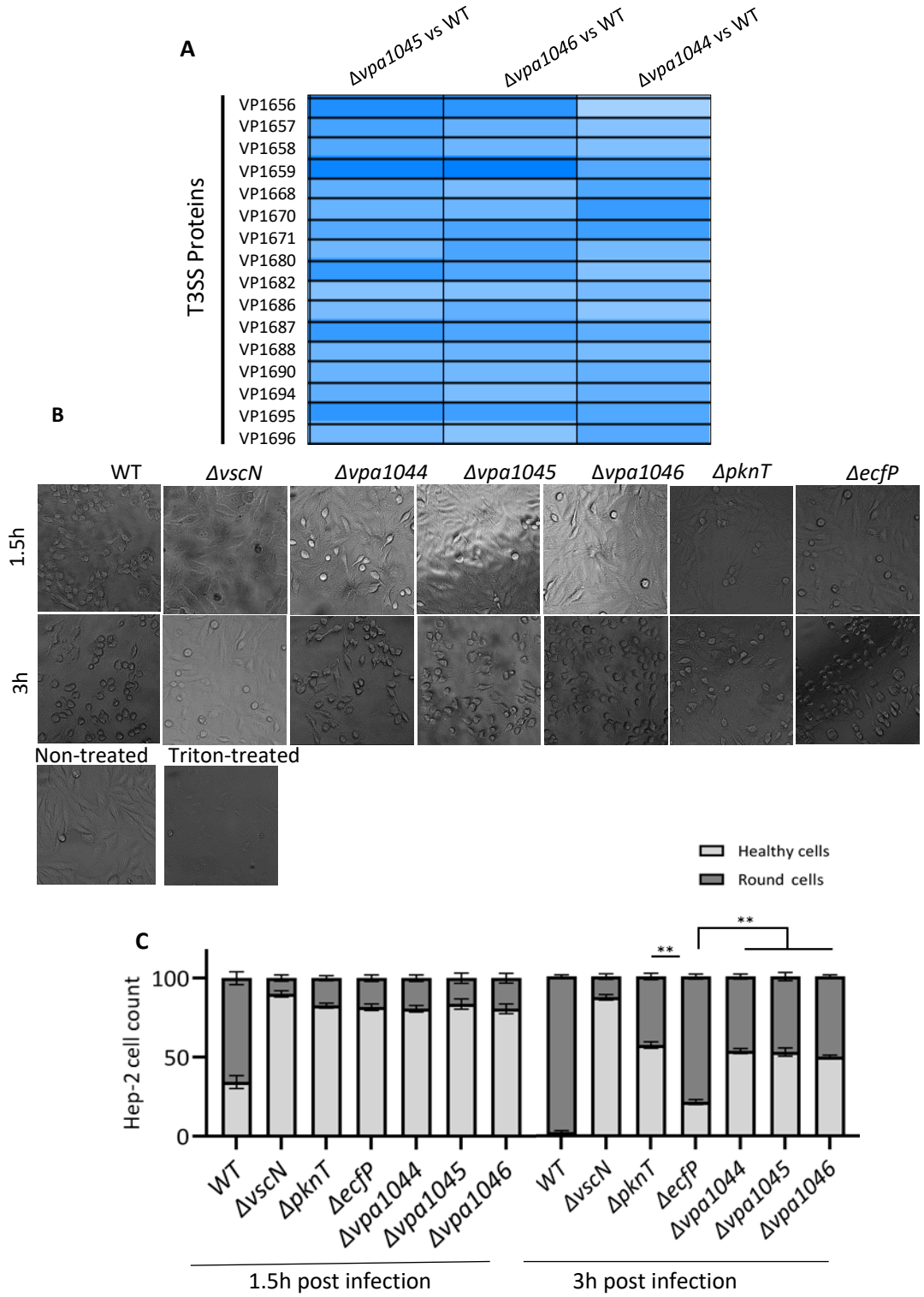

Figure 16. Infection of HEp-2 cells with V. *parahaemolyticus* strains. HEp-2 cells were infected at MOI of 10. (A) Zoom-in of the clustering map showing the changes in expression levels of T3SS proteins in mutants compared to wild type. The color blue shows downregulation of the targets. (B) Micrographs depicting cultured HEp-2 cells that have been incubated with the indicated strains for 1.5 and 3h. Under certain treatments, the healthy cells with cellular protrusions change morphology to round with less volume. (C) Bar graph showing healthy/round Hep-2 cell count after 1.5 and 3h. Statistical differences were marked with (**) in a two-tailed t-test, which represents p-value <0.001. All results are presented as mean values ± standard deviation.

3.9. *vpa1044* is required for proper polymyxin B-induced expression of VPA0879

We performed global LC-MS proteomic analysis on *V. parahaemolyticus* swimmer cells as well as Δ*vpa1044* strain in the absence or presence of polymyxin B. The liquid culture of cells was divided in the mid-exponential growth phase with one half being treated with polymyxin B while the other half remained untreated. After five minutes, the samples under both conditions were harvested and analyzed by LC-MS. A total of 1997 proteins were significantly detected with at least 2 peptides, which represents approximately 41% of the 4832 predicted Open Reading Frames in the *V. parahaemolyticus* RIMD2210633 genome. Of these, 417 were significantly regulated in the presence of polymyxin B compared to non-treated cells. This constitutes 20% of the detected proteome. Importantly, as previously shown, treatment with polymyxin B significantly stimulated the expression of VPA0879 in planktonic cells (Fig. 17A, Orange).

To test if the *vpa1044-vpa1045-vpa1046* system is required for the upregulation of VPA0879 expression upon polymyxin treatment, we performed global LC-MS proteomics in wild-type and Δ*vpa1044* cells after addition of polymyxin B (Fig. 4). A total of 311 proteins were significantly regulated, belonging to several functional groups which are depicted in a pie chart with their total occurrence with the significantly regulated detected proteins, 276 were upregulated and 35 were downregulated in polymyxin B treated cells compared to non-treated cells (Fig. 18, Supplementary Table S12). Importantly, there was a significant reduction in VPA0879 expression in the absence of *vpa1044* after polymyxin B treatment when compared to wild-type *V. parahaemolyticus* (Fig. 17B), thus indicating that *vpa1044* is required for the proper polymyxin B-induced expression of VPA0879.

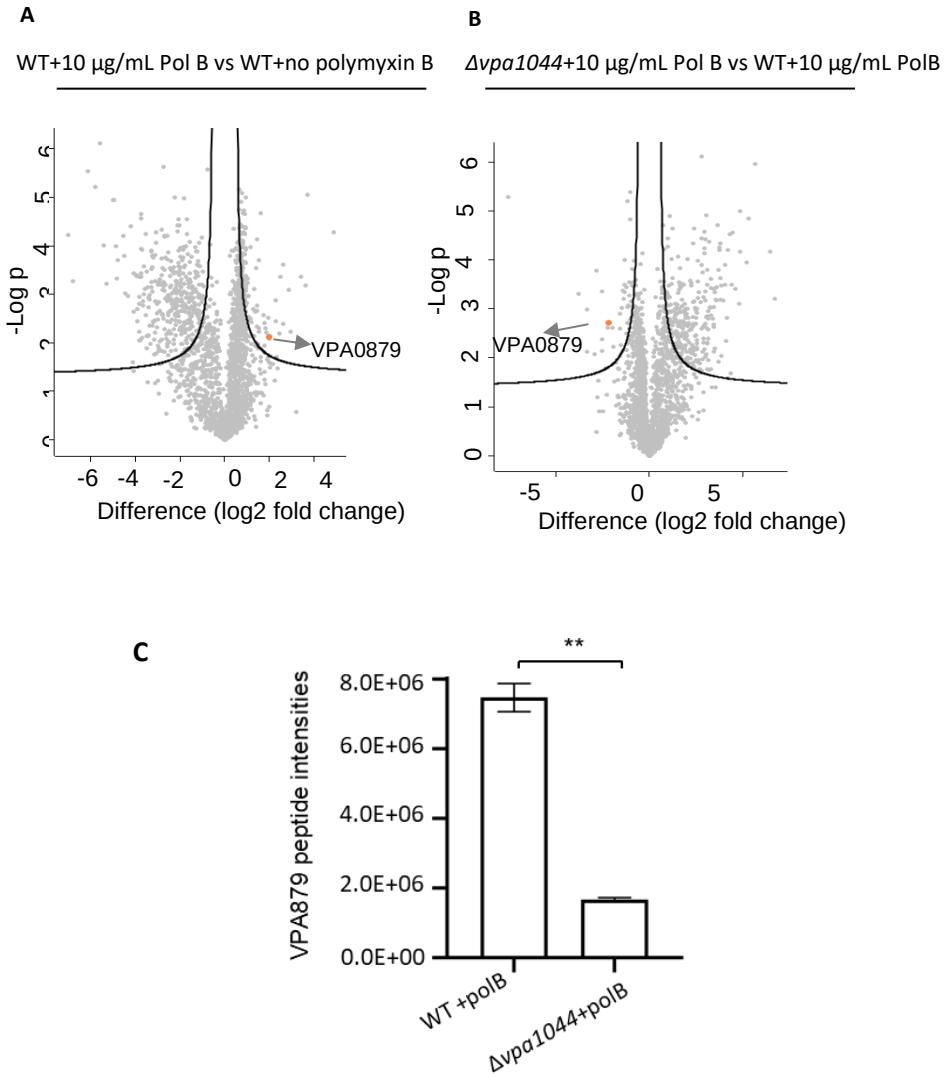

Figure 17. Adaptive responses of *V. parahaemolyticus* WT and *Δvpa1044* strain to polymyxin B treatment. (A) Volcano plot showing the fold changes of protein levels of cells from wild-type V. parahaemolyticus and swimmer cells before and after treatment with 10 µg/ml of polymyxin B. (B) Volcano plot showing the fold changes of protein levels of cells from *Δvpa1044* strain swimmer cells compared to WT after treatment with 10 µg/ml of polymyxin B. The Y axis indicates the -log of the p-value and "n" indicates the number of proteins significantly down- or upregulated between the two conditions compared. (c) Bar graphs showing the peptide intensities of VPA0879 in WT and *Δvpa1044* swimmer cells in the presence of 10 µg/ml of polymyxin B, as obtained through global LC-MS proteomics analysis. Error bars indicate standard error of the mean (SEM). Statistical significance was calculated using the two-sided unpaired t-test (** indicates a $p < 0.001$).

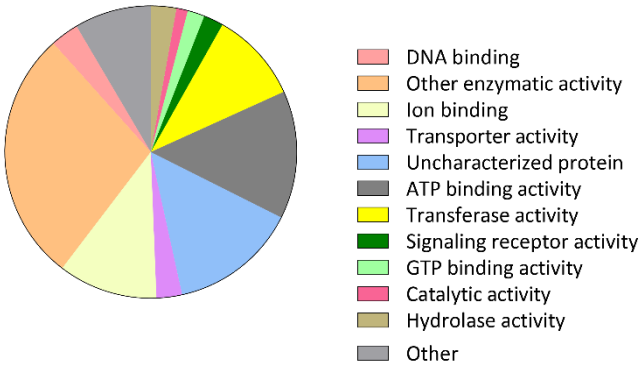

Figure 18. Pie chart showing functional groups of the proteins significantly regulated by *Δvpa1044* strain swimmer cells when treated with 10 µg/ml of polymyxin B. The significantly regulated targets of *Δvpa1044* mutant, when compared to wild type in the presence of pol B obtained by global LC-MS proteomics studies were categorized into different functional groups based on the gene ontologies of the targets.

4. Discussion and future perspectives:

Utilization of antimicrobials by the fisheries and aquaculture sector can lead to building a route for exposing antimicrobial-resistant bacteria to humans and animals (Schar et al., 2020). A series of studies have examined detection frequencies and concentrations of antibiotics (ranging from ng/L to µg/L) in aquatic environments such as seawater, lakes, and rivers(Zhang et al., 2013, Liu et al., 2018). For example, Danner *et al.* reported that the concentration of antibiotics in surface fresh water can be detected up to 50 µg/L with a mixture of different antibiotics (Danner et al., 2019). Multidrug Resistance (MDR), as a critical and widespread phenomenon, is a good example of the consequences of antibiotic pollution in aquatic environments. Several studies have highlighted increased rate of drug resistance and MDR strains of *Vibrio parahaemolyticus* isolated from marine environments compared to clinical isolates (Xie et al., 2017). The multiple antibiotic resistance index (MARI) is a valid method to track antibiotic-resistant organisms and a value higher than 0.2 indicates a high-risk source of contamination where antibiotics are often used (Davis and Brown, 2016). The MAR indices of *V. parahaemolyticus* isolates from seafood samples such as freshwater fish and prawns ranged from 0.4 to 0.7 and more than 70% of the isolates were found resistant to more than one antibiotic (Silvester et al., 2015). Therefore, to reduce the potential impacts of antibiotics on the ecological surroundings, it is essential to understand how marine bacteria adapt or develop resistance in their natural habitats in response to stressors such as antibiotics.

In this work, the main focus is on antibiotics as a specific environmental stimulus, especially to classes that target the cell envelope. Polymyxin B is a polypeptide bactericidal antibiotic that targets lipopolysaccharide (LPS), leading to the disruption of the cell membrane. One of the prominent regulatory features of bacteria is the different signal transduction systems by which they can respond to environmental stimuli and elicit cellular responses. This adaptive response can be exerted via two-component systems (TCS), which are mainly composed of a sensor histidine kinase (HK) to receive external inputs and a corresponding response regulator (RR) to activate proper change in cellular physiological processes (Stock et al., 2000). Several studies have characterized the TCS signaling pathway of polymyxin resistance

in gram-negative bacteria, such as ParS/ParR and CprR/CprS in *Pseudomonas aeruginosa* (Fernández et al., 2012).

Our findings reveal a new role for a previously uncharacterized serine/threonine kinase and a putative two-component system in facilitating resistance against polymyxin B in *V. parahaemolyticus*, which is a significant human pathogen. We have recently demonstrated that polymyxin antibiotic stress can activate PknT, a Ser/The kinase in *Vibrio parahaemolyticus*, which then phosphorylates inactive ECF σ factors, namely EcfP. This mode of transcriptional regulation by σ factor phosphorylation leads to the expression of polymyxin resistance-related genes (Iyer et al., 2020). In this work, we expanded the characterization of predicted STKs in *V. parahaemolyticus* and particularly focused on the predicted STK, VPA1044. Subsequent analysis of its genetic context showed a genetic link with VPA1045, which is a response regulator, and VPA1046, a histidine phosphotransferase protein encoding a two-component system. These proteins are typically co-transcribed as a two-component system. Interestingly, we show that deleting respective genes encoding these regulatory proteins results in the same phenotype, namely resistance to polymyxin B.

We also recently showed that the bacterial human pathogen *Vibrio parahaemolyticus* adapts to distinct environments by inducing specific proteomic profiles. Importantly, this proteomic adaptation includes significant changes to the expression profile of essential proteins depending on the specific environment that the bacterium experiences (Freitas et al., 2020). To build upon this knowledge and gain a comprehensive view of the proteins that could potentially promote polymyxin resistance, we studied the contribution of the Ser/The kinase (VPA1044) and the two-component system (VPA1045, VPA1046) on the dynamic behavior of cellular networks induced by antibiotic stress via proteomics approach when the individual genes were deleted. When investigating the adaptive response of *V. parahaemolyticus* in different mutant strains via global LC-MS based proteomics, we detected differential expression of VPA0879 (a polymyxin-resistant determinant), iron uptake systems, as well as virulence factors, prominently T3SS1 proteins.

In this work, we showed that the expression level of VPA0879 was significantly downregulated in *Δvpa1044, Δvpa1045, and Δvpa1046* mutants when compared to wild-type. This is in line with the transcriptomics data of PknT/EcfP system in which it was revealed

that in both *ΔecfP* and *ΔpknT* strains, the most significantly down-regulated gene was *vpa0879* and it was also previously shown that the deletion of *vpa0879* results in high susceptibility to polymyxin B. Based on bioinformatics analyses, it is predicted that VPA0879 is a UDP-glucose 4-epimerase of unknown function. Specifically, VPA0879 shares homology with the UDP-glucose 4-epimerase protein GalE, which plays a role in the reversible conversion of UDP-galactose to UDP-glucose during galactose metabolism. Considering that galactose is among the sugars present in the O-antigen oligosaccharide layer of *V. parahaemolyticus* LPS (Hisatsune et al., 1980), any disruption in the enzyme responsible for synthesizing UDP-galactose and its subsequent integration into the LPS could potentially compromise the integrity of this layer. Future studies could investigate the LPS composition of the *vpa0879* mutants to explore the influence of this gene on susceptibility to polypeptide bactericidal antibiotics.

VP1912, which is MarR family transcriptional regulator, was among the commonly downregulated proteins in all three mutants of the *vpa1044-vpa1045-vpa1046* system when compared to WT. The MarR transcription factor is named after its involvement in the multiple antibiotic resistance (Mar) system. The Mar system is a global regulatory system that protects bacteria against a broad range of antibiotics and other stress-inducing compounds. It is particularly well-studied in *Escherichia coli* and it has been shown that when the MarR transcription factor dissociates from the DNA, it results in the derepression of the target genes. These target genes include efflux pump components, such as AcrAB-TolC, which are involved in the active extrusion of antibiotics from the bacterial cell, as well as other genes involved in antibiotic resistance (Perera and Grove, 2010). Interestingly, VP0425, which is an outer membrane protein TolC in *V. parahaemolyticus* is among the commonly upregulated proteins in *Δvpa1044, Δvpa1045, and Δvpa1046* mutants when compared to wild-type. It is worth noting that the MarR system is just one of many regulatory systems involved in the response to antibiotic stress, and its activation is often part of a broader network of interconnected pathways.

Additionally, our proteomics data show that genes involved in the TTSS1 (Type III secretion system 1) of *V. parahaemolyticus* are significantly downregulated in *Δvpa1044, Δvpa1045, and Δvpa1046* mutants. This finding is also similar to that found in PknT/EcfP system. Bacteria

use T3SSs to secrete effector proteins into the host cell through a syringe-like delivery apparatus, resulting in cell death. *V. parahaemolyticus* possesses two TTSS-encoding gene clusters: TTSS1, which is located on the large chromosome of all *V. parahaemolyticus* strains and is involved in host cell cytotoxicity; on the other hand, TTSS2 is on the small chromosome and is present in Kanagawa-positive strains and it is involved in enterotoxicity in animal models (Ono et al., 2006). There are several T3SS proteins among the common significantly downregulated targets in our proteomics data. VP1686 (also known as VopS) is a T3SS effector protein and is implicated in cell rounding. VP1657 (VopB1) and VP1656 (VopD1) are essential for the translocation of T3SS1 effectors into host cells. VP1658 is a low calcium response locus protein H. It has been previously reported that low calcium conditions induce the expression of type III secretion system effectors and proteins in several pathogens (Gode-Potratz et al., 2010). VP1694 (similar to YscF in *Yersinia pestis*) makes up the needle structure used to inject effector proteins. Chaperons help with the assembly of injectosomes, and VP1682 is a protein similar to CesT, which is a multi-effector chaperone found in *E. coli (Thomas et al., 2005)*. VscN is a single ATPase located in the basal cytoplasm of the T3SS assembly. This ATPase plays a vital role in separating the effector-chaperone complexes and providing the energy required for protein secretion. Previous research showed a significant reduction of virulence in *ΔvscN* strain (Zhuang et al., 2021). The T3SS ATPases in *V. parahaemolyticus* is VP1668 and is among the common downregulated targets. To investigate more about the role of mutant strains on virulence, we conducted an infection assay on mammalian cells. As expected, Hep-2 cells rapidly rounded up when treated with the wild-type, and a significant reduction in morphological changes to Hep-2 cells when treated with the *ΔvscN* strain. There was little difference in the levels of morphological change observed with mutant strains compared to WT when incubations were performed for 1.5 h. However, 3h post-incubation, more cells rounded up. It was interesting to see that the total number of round cells was about 20% less in *ΔpknT, Δvpa1044, Δvpa1045, and Δvpa1046* compared to *ΔecfP* in extended incubation time. It can be concluded that the mutants could be of importance to the development of the virulence ability of this pathogen. However, future work with prolonged incubation time needs to be done to further investigate the mechanism of these proteins in the observed delayed cytotoxicity.

Interestingly, VP2258 (FlaA), VP2258 (FlaB), and VP0769 (FlgP, essential for flagellar assembly) are among the common significantly downregulated proteins. Several studies have shown that the highly conserved proteins of the Type III Secretion System (T3SS) exhibit similarities to the proteins found in the bacterial flagellar structure (Aizawa, 2001). As a result, it has been proposed that the T3SS and bacterial flagella share a common evolutionary origin (Saier, 2004). The flagellar system is classically important for bacterial-host cell contact and upon contact, T3SS is triggered, followed by the injection of effectors into eukaryotic host cells.

One of the notable proteins that was significantly downregulated in mutant cells compared to WT cells was VPA0882, which is a putative heme transport protein (HutA). Interestingly, it is also among the top significantly downregulated proteins in *Δvpa1044* swimmer cells after treatment with polymyxin B. Iron is essential for many cellular processes such as swarming motility. For instance, it has been established that disruption of iron scavenging systems leads to iron limitation and the induction of swarming phenotype (Lin et al., 2016). Bacteria can either utilize their specialized iron acquisition systems or directly use iron-containing proteins in the host as sources of iron. They secrete siderophores, which are iron-chelating compounds, to sequester iron or in a siderophore-independent fashion(Henderson and Payne, 1994). In order to facilitate iron uptake into the cell, bacteria use iron-regulated outer membrane receptors. These receptors require energy from the TonB protein, which forms a complex with the inner membrane proteins ExbB and ExbD. This complex forms a proton channel that spans the periplasmic space between the inner and outer membranes and acts as an energy transducer, utilizing the proton motive force to energize the transport of iron complexes (Held and Postle, 2002). Interestingly, in our proteomics data, VPA0154 (TonB system transport protein ExbD2) is also significantly downregulated. Similarly, we discovered that two other iron-regulated proteins were significantly downregulated in *Δvpa1044* swimmer cells when compared to WT upon polymyxin B treatment, namely VPA1656 (ferric vibrioferrin receptor) and VPA1657 (ferric siderophore receptor). Additionally, iron can influence the production and activity of enzymes involved in the modification of lipid A, a component of LPS that directly interacts with polymyxins. Iron limitation can lead to changes in the lipid A structure, which can impact the binding affinity of polymyxins and reduce their effectiveness. Based on this data, we hypothesized that VPA1044 could be involved in

sensing iron in the environment; however, more experiments need to be carried out to unravel the mechanism of iron uptake at different concentrations and how the expression of iron metabolism genes is regulated post-transcriptionally.

Interaction of response regulators and transcription factors with Ser/Thr kinases have been elucidated in several prokaryotes, allowing them to control their expression profile (Dworkin, 2015). This crosstalk has been shown in different bacteria such as *M. tuberculosis*, *Bacillus subtilis*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* (Molle et al., 2003, Libby et al., 2015, Ulijasz et al., 2009). Co-immunoprecipitation LC/MS and bacterial two-hybrid assays can be used to determine protein-protein interactions under varying conditions. In this work, we performed co-IPs using ectopic expression of sfGFP-VPA1044 and VPA1045 in *E. coli* cells. The proteins were successfully enriched and pulled down suggesting that they may be interaction partners and form a complex (Supplementary Figure. 21). Another striking result using the BTH assay was the interaction between PknT (VP0057) and VPA1044, suggesting that these two proteins interact with each other (Supplementary Figure. 20). Furthermore, in some of our preliminary phosphoproteomics results, we showed that the enriched peptide sequence corresponding to the VPA1044 kinase is only detected in wild type, but not in the *vp0057* deletion mutant. This also leads us to speculate about a possible interaction between these two kinases, which is typical for this family of kinases. The similarity observed in the polymyxin B stress response, shared targets in proteomics data, swarming phenotype between the *vpa1044* and *vp0057* deletion mutants and morphological identity of the swarmer cells could also be indications of potential crosstalk between these two kinases. A schematic presentation of our proposed model for predicting the interaction between the two systems is presented in Figure 19. However, these results warrant further investigation. For example, our observations do not address the identification of kinase-specific phosphorylation sequence motifs. We also have not investigated the binding sites on proteins of the VPA1044/45/46 system, or whether the downstream targets are directly or indirectly regulated. Thus, proximity labeling proteomics and a phosphorylation enrichment-based workflow are recommended for future work. Additionally, the gel shift assay of response regulator binding to the promoter region of *vpa0879* and *vpa0882* is another interesting area for research.

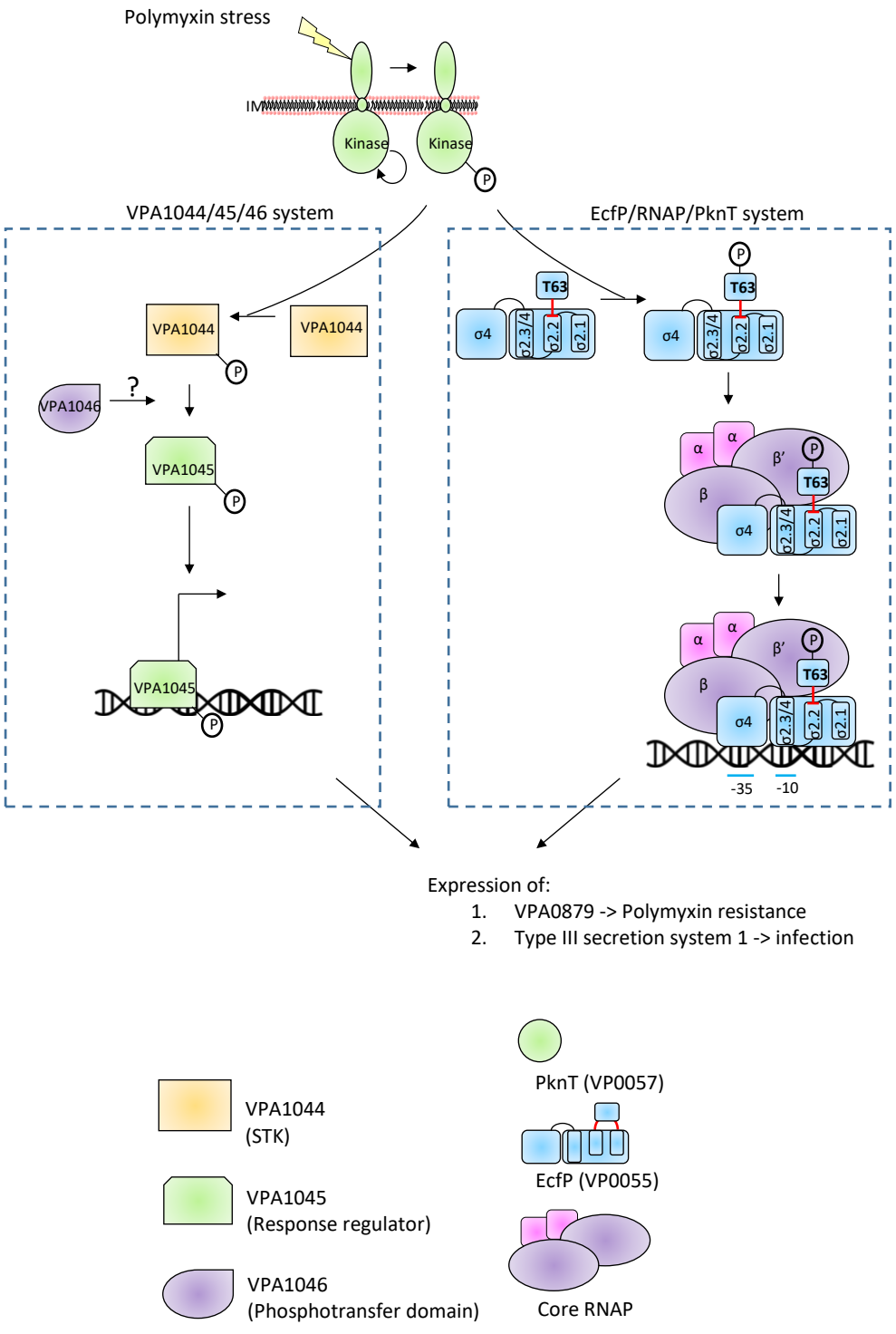

Figure 19: Schematic illustration exhibiting our proposed model of two signal transduction systems regulating polymyxin B resistance in *V. parahaemolyticus*.

Our results show that deletion of *vpa1044* (Ser/Thr kinase) significantly impacts swarm motility. Firstly, it is interesting to check if the deletion of the other two genes, *vpa1045* and *vpa1046*, produces the same phenotype. Secondly, further studies are still required to investigate the direct or indirect mechanism of these proteins in swarming phenotype. Additionally, it is necessary to determine if the cell division proteins are upregulated in swarming cells in the same manner as shown in our proteomics data of non-swarmer cells. Lastly, a comparison between resistance to polymyxin B in swarmer vs swimmer cells and checking if VPA0879 is also a relevant player as a regulator of polymyxin resistance in swarmer cells would be an interesting topic for future work. Moreover, investigating the expression profile of *Δvpa1045* and *Δvpa1046* upon polymyxin B stress can provide us with more insight into the system.

Biochemical characterization of the elements and the mechanism of how the sensing of polymyxin stress occurs, as well as its impact on the regulation of kinase and response regulator activity is still unknown. A combination of transcriptomics and metabolomics is a valuable approach to further investigate significant changes in the abundance and activities of key players in the transport system, iron acquisition, and lipid A modification pathways. This approach can also explore how the elements of the *vpa1044-vpa1045-vpa1046* system affect the permeability barrier of the outer membrane to protect the cell envelope from antibiotic stress.

In conclusion, bacteria have evolved intricate signal transduction systems that allow them to sense the presence of distinct antibiotics and respond appropriately to ensure their survival. These systems enable the coordination of cellular processes, including the activation or repression of specific genes, which in turn leads to altered phenotypic outcomes. Even though some of these pathways are well characterized, there is still much to be understood about the many convergent signaling pathways and the mechanism behind their synergistic or antagonistic effects on downstream cellular responses. This study's findings emphasize the need to further explore resistance-associated signaling genes and their role in mediating antibiotic stress responses. The knowledge gained from such investigations will provide a foundation for developing novel strategies to combat antibiotic resistance and preserve the efficacy of antibiotic therapies in modern medicine.

5. Supplementary material:

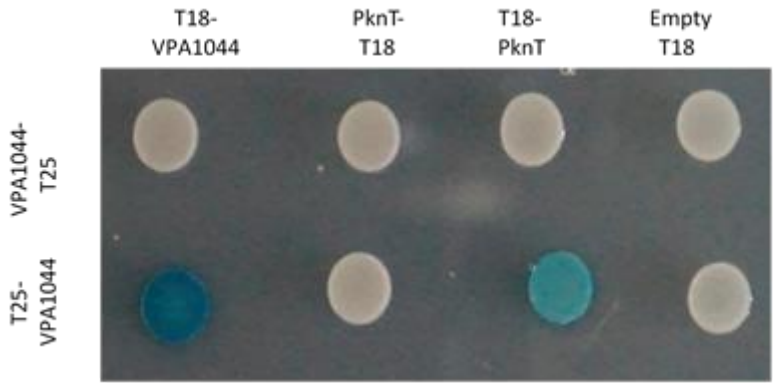

Figure 20: PknT and VPA1044 form an interaction complex. Bacterial two-hybrid assay (BACTH), testing for protein-protein interaction between PknT and VPA1044. Blue colony formation suggests that a direct interaction occurs.

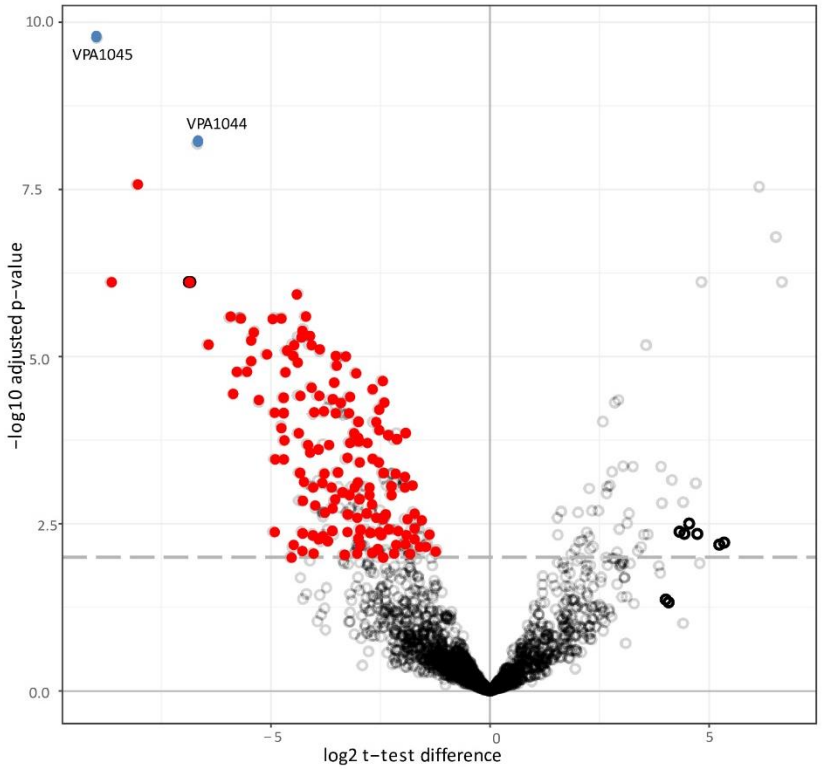

Figure 21: VPA1044 interacts with VPA1045 in IP-MS/MS experiment. Volcano plot of co-immunoprecipitation analysis in *E. coli* cells ectopically expressing sfGFP-VPA1044 (bait) in the presence or absence of ectopic expression VPA1045, followed by immunoprecipitation of sfGFP-VPA1044. The proteins in the upper left corner represent the bait and its interactors. Dashed horizontal line shows the p-value cutoff. The pull-down was performed using GFP-Trap® Agarose.

Table 9. Proteins differentially expressed (log2 fold change (FC ≥1 or ≤-1) and statistically significant (FDR=0.01 S0=0.2) between *Δvpa1044* strain vs. wild-type planktonic cells. The targets were obtained through LC/MS analyses.

***Δvpa1044* Strain vs Wild type planktonic cells**

Upregulated Protein names		Downregulated Protein names	
	- log P-value		*- log P-value*
cdd	2.99	**VPA0882**	3.57
VP0327	3.30	**VPA0450**	3.93
VP0904	3.30	**VP1671**	3.91
recA	4.33	**hisD**	3.01
VP0876	3.59	**VP1680**	3.76
mpl	4.01	**VPA1449**	4.92
VPA0812	2.48	**VPA0851**	1.61
VPA0427	2.61	**VPA0986**	3.42
VPA0577	2.31	**VPA0298**	2.59
fabH2	2.78	**nhaB**	2.76
VPA1157	3.37	**VP1668**	3.91
nhaP2	3.57	**VPA1638**	1.77
murC	3.63	**VP1698**	3.61
murA	4.91	**VP1659**	4.17
argD	3.30	**VP1501**	2.39
VP2598	3.64	**VP2864**	4.08
katG2	2.79	**VPA0297**	3.43
VP0426	2.23	**VPA0984**	3.28
VPA1527	2.68	**VP1690**	2.81
dapA	2.19	**VP1514**	2.68
pncB	2.60	**VP1513**	2.79
VPA0097	3.98	**VP1695**	3.63
VPA0655	2.43	**VP1696**	3.14
VP0764	4.14	**VP2445**	3.63
VP1335	3.32	**VPA0451**	3.84
VP1057	4.20	**uppS**	2.77
VP1544	3.69	**VP2159**	3.54
msrA	2.46	**VPA0154**	3.99
VP1267	4.95	**VPA0706**	3.39
VPA1042	2.19	**VP1682**	2.11
VP0536	2.03	**VP1694**	4.36
coaE	1.93	**VP1687**	3.64
cysC	3.27	**VPA0985**	2.84
VPA0919	2.54	**VPA0253**	3.08
prmC	2.19	**bioD**	2.56
VP2126	3.78	**VPA0879**	2.94
VP2970	3.35	**lamB**	2.74
VP2567	2.16	**VP2447**	2.63
rpoH	3.31	**VPA1604**	3.67
VPA0477	3.68	**VP1658**	3.96

VP1991	3.26	**malF**	3.00
VP2092	2.27	**vopS**	3.45
VP0949	3.02	**VPA0127**	2.05
VP2913	1.95	**VP1657**	4.39
VP0751	2.33	**VP1510**	2.88
tadA	2.18	**VPA1401**	1.90
VP0552	3.50	**VP2014**	2.93
VP0425	4.69	**VP1688**	1.90
VP0151	6.23	**VPA0128**	1.78
VP2074	1.83	**VP2448**	2.53
thrB	5.24	**VP0768**	3.90
VP2494	1.90	**VPA1424**	2.64
VP2630	2.73	**VPA0987**	3.12
VP1145	3.62	**flaA**	4.56
VPA0099	3.71	**malK**	2.85
VPA1361	1.88	**VP1088**	2.93
VP0585	2.00	**VP1665**	1.78
VPA1035	4.61	**VP1667**	2.09
trpC	1.75	**VP1512**	1.75
VPA0740	2.82	**ompK**	4.66
VP2594	5.83	**VP2266**	2.75
VPA1437	2.94	**VPA1613**	3.80
VPA1126	1.73	**VP1511**	2.64
VPA0320	2.19	**VP0118**	1.93
VP2671	2.12	**VP2142**	2.64
VP0950	3.67	**VPA1612**	2.24
murE	1.90	**VP1662**	2.53
VP2462	2.56	**VPA1651**	3.82
VPA1034	4.61	**VP0940**	2.37
VP2443	5.83	**VPA1648**	2.21
VPA1500	3.10	**flaB**	3.67
fadJ	4.38	**VPA1279**	3.39
VP2044	4.06	**VP2013**	2.88
VP0080	2.90	**VP0994**	2.71
VP2967	3.03	**VPA1486**	2.99
VP0375	3.42	**VP1670**	2.21
VP0648	4.45	**VP2841**	2.76
murF	4.58	**VP0995**	3.71
VP0628	3.29	**VPA1620**	2.22
fadI	5.89	**VPA1724**	2.26
atpE	3.29	**VP1851**	4.00
VPA0312	5.12	**pepT**	2.85
VP0795	4.05	**VPA1619**	2.22
VPA1211	6.28	**VP1656**	3.91
VP2309	3.78	**VPA1000**	2.73
VPA0808	4.84	**VP1697**	2.49
VPA0807	5.06	**VP2516**	1.93

	grcA	1.86
	VP2827	3.61
	VP2011	2.88
	VP1669	1.96
	VP2121	2.78
	VP1348	2.26
	VP2840	2.76
	flgH1	3.87
	VP1633	4.12
	lnt	1.93
	VP1634	2.98
	VP0996	3.89
	flaC	3.95
	VP0319	3.93
	VP1091	5.36
	VPA1268	2.02
	VP2799	2.47
	VP1092	4.08
	VP0710	3.07
	VP0769	4.41
	VPA0941	2.45
	VP0051	2.09
	VP2706	3.80
	VP0244	3.61
	VP2229	3.41
	VPA1280	2.71
	VP0066	2.72
	cheB	4.45
	VPA1277	2.73
	VP2707	2.66
	VP0121	2.97
	VP1628	2.95
	VP2046	2.56
	VPA0318	2.57
	VP0584	2.45
	VP1207	2.93
	VP1736	2.92
	btuB	2.87
	VP1912	3.32
	VP0183	2.87
	VPA1466	3.15
	VP1009	2.94

Table 10. Proteins differentially expressed (log2 fold change (FC ≥1 or ≤-1) and statistically significant (FDR=0.01 S0=0.2) between *Δvpa1045* strain vs Wild type planktonic cells. The targets were obtained through LC/MS analyses

***Δvpa1045* Strain vs Wild type planktonic cells**

Upregulated Protein names		Downregulated Protein names	
	- log P-value		*- log P-value*
VPA0257	3.43	**VPA0882**	4.37
VPA0097	3.00	**VP1659**	2.61
mdh	1.98	**VP1656**	3.12
ubiC	2.15	**VP1698**	4.75
fabH2	2.74	**vopS**	3.04
VP2588	2.18	**VP1690**	4.16
VP1614	3.05	**VPA0450**	4.32
pncB	2.15	**VP1657**	3.62
VPA0829	4.32	**VP1658**	4.38
VP0327	3.47	**VP1680**	2.40
VPA1035	2.52	**VP1696**	3.96
katG2	2.76	**VP1668**	1.91
hisH	2.41	**VP1671**	3.32
argD	3.92	**VPA1604**	2.57
VP1481	1.86	**VP1695**	2.28
VPA1527	2.68	**VP1694**	2.59
VP2612	2.17	**VP1682**	1.73
dapA	2.03	**VP1662**	2.53
recR	1.75	**VP1688**	2.04
VP1267	4.54	**VPA1449**	4.40
VPA1343	1.79	**VP1687**	3.38
VP2126	3.19	**VPA0451**	2.78
VPA0088	1.82	**VP0948**	1.67
VPA1027	1.95	**VPA0879**	2.35
VPA0577	2.00	**VP2159**	1.52
VP0426	2.20	**VP1088**	1.86
VPA1339	2.30	**VPA1000**	3.23
rpoH	3.05	**VP1670**	2.63
VP1335	3.35	**ompK**	4.55
VP0949	2.53	**VPA1465**	2.38
VPA1419	1.62	**VPA1279**	3.26
VP0700	1.89	**VP0865**	2.14
VPA0919	2.32	**VPA1648**	2.03
metE	2.47	**VPA1464**	2.70
VP1544	3.97	**flaA**	2.97
VP2432	1.95	**VP1634**	4.54

VPA0648	1.84	**VP2142**	2.17
VP2970	3.45	**VP0584**	3.06
VPA0460	1.93	**VP2516**	2.00
VP2913	1.79	**flgH1**	3.37
VP0751	2.21	**VP0768**	1.67
VP0425	4.46	**VPA1602**	2.36
VPA0746	2.15	**VPA1651**	2.47
VPA1216	1.60	**VP2376**	2.99
VP0552	3.22	**VP1633**	5.37
prmC	2.11	**VP1851**	3.41
ispD1	1.82	**VP2827**	2.65
cysC	3.31	**VP0712**	1.82
VPA0477	2.72	**VP0018**	2.63
VP2567	2.04	**VP1091**	3.55
VP1991	3.33	**VP1009**	2.97
VP0151	5.70	**flaB**	3.03
VPA0320	1.80	**VPA0979**	2.30
msrA	2.20	**VP2799**	2.11
VP2207	1.69	**btuB**	3.16
tadA	2.94	**VPA1280**	3.00
VP2630	2.72	**VP1736**	3.08
VP2092	2.20	**VP0996**	3.46
thrB	3.12	**VPA1482**	1.99
VPA0740	2.65	**VP1092**	3.86
ccmA	1.48	**VP1912**	3.11
coaE	1.89	**VP0881**	1.98
VP1145	3.34	**VP2229**	3.31
VP2631	3.50	**VPA1189**	2.25
VP2781	1.51	**VPA1278**	2.20
VP0536	2.04	**VP0769**	3.92
VP0153	1.54	**VP2707**	2.47
glpE	1.55	**cheB**	4.13
VP2594	5.40		
VP0585	2.00		
VPA0099	4.51		
VPA1126	1.60		
trpC	1.86		
VPA1034	4.18		
VP2860	1.88		
VP2835	1.42		
VP2339	1.39		
VP0755	1.76		
VP2671	2.20		
VP0053	4.35		
VP2443	6.08		
VPA1500	2.96		
VP2462	2.44		

murE	2.00
VPA1469	2.13
fadJ	4.23
VPA1361	1.97
VP2044	3.77
VP0080	2.91
VP0375	3.34
VP0648	4.32
VP1203	3.03
VP0628	3.16
fadI	4.76
murF	4.36
atpE	3.22
VPA0312	4.81
VP2309	3.57
VPA1211	6.03
VP0795	4.62
VPA0808	4.90
VPA0807	5.03

Table 11. Proteins differentially expressed (log2 fold change (FC ≥1 or ≤-1) and statistically significant (FDR=0.01 S0=0.2) between *Δvpa1046* strain vs Wild type planktonic cells. The targets were obtained through LC/MS analyses.

***Δvpa1046* Strain vs Wild type planktonic cells**

Upregulated Protein names		Downregulated Protein names	
	- log P-value		*- log P-value*
VPA1684	2.58	**VP1659**	6.53
cysJ	3.19	**VPA0882**	3.97
ppk	2.63	**VPA0297**	3.50
VPA0829	3.97	**VP1656**	3.19
fabH2	2.68	**VPA0985**	2.40
argD	2.56	**VP1513**	3.10
VPA0257	3.75	**VP1698**	4.36
cysD	3.17	**VPA0298**	3.12
VP0904	4.12	**VP1501**	2.71
VP1057	3.37	**VPA0040**	3.76
VPA0961	3.96	**VP1682**	2.24
VP2598	3.43	**VP1680**	3.63
VPA1157	2.27	**VP1690**	4.47
VPA0427	2.96	**vopS**	3.42
murA	5.34	**VPA1638**	1.82
VPA1096	3.18	**VPA0450**	3.20
nhaP2	2.46	**VPA0851**	1.46
cysN	3.26	**VP1688**	4.32
VP0876	4.38	**VPA1449**	2.64

katG2	2.84	**VPA0984**	2.97
VP2497	2.96	**VPA0986**	1.74
VPA1120	2.03	**VP1657**	2.42
iscR	2.08	**VPA0127**	2.39
VP2970	2.89	**VP1510**	1.96
VPA0919	2.11	**VP1514**	2.73
VP0764	4.05	**VP1694**	3.15
VP0949	2.16	**VP2014**	3.45
VP1614	3.11	**VP2445**	3.47
VPA1683	2.18	**VP1671**	2.93
VPA0655	2.49	**VP1658**	3.78
VPA1418	2.00	**VP0963**	2.07
VPA0746	1.84	**VPA0253**	3.78
msrA	1.82	**lamB**	2.75
VPA1121	2.32	**bioD**	1.95
VP2432	1.90	**VP1695**	3.22
cysC	2.33	**VP1670**	3.88
VP0700	1.97	**pepT**	1.69
VP1267	4.54	**VPA0987**	3.31
VP0536	2.49	**flgH1**	1.97
VPA0097	3.97	**malF**	3.04
VP2207	1.65	**VP2266**	2.65
VPA1476	1.85	**VP1668**	3.43
VP1544	3.60	**VPA1424**	2.74
VP2126	4.15	**VP2582**	2.83
VPA1216	1.64	**VP1696**	3.30
thrB	3.35	**VPA1604**	3.14
rpoH	3.06	**VP2159**	2.43
VP2092	2.24	**VPA1401**	2.10
VP1257	2.40	**VP2447**	2.93
VP1991	2.33	**VPA0706**	1.99
VP2276	3.30	**VP1687**	3.46
VPA0477	3.26	**VP2618**	2.92
VP0425	4.62	**VPA1613**	2.30
VPA0320	1.85	**malK**	3.04
VP0153	1.71	**VPA0451**	4.52
VPA1361	1.48	**VPA0128**	1.86
VP0585	1.71	**VPA0102**	2.08
VP2630	2.71	**VP2864**	1.48
VP0151	5.61	**VP1348**	2.51
VP0552	3.58	**ompK**	4.47
ccmA	1.54	**VP0994**	3.07
trpC	1.72	**flaB**	3.95
VP2781	1.62	**VPA1651**	3.98
VPA1437	3.39	**VP1665**	2.17
VPA0740	2.92	**VP2142**	2.60
VP2339	1.43	**VPA1648**	1.89

murE	1.71	**VPA1620**	2.63
VP2594	5.26	**VP2011**	3.58
VPA1034	4.27	**VPA1619**	2.50
VPA1126	1.84	**flaA**	3.91
VP2462	2.37	**VP2121**	3.13
VP0755	1.87	**mtlD**	1.79
VPA1469	1.72	**VP2013**	3.57
VPA1500	3.17	**VPA0592**	1.62
VP2443	5.08	**VP1851**	3.74
VP2044	4.20	**VPA1486**	2.32
fadJ	4.98	**VP0995**	3.80
VP0080	2.82	**VP2841**	2.91
VP0648	4.06	**VP1512**	1.75
VP2967	2.88	**VP1511**	1.62
VP1203	3.01	**grcA**	2.21
VP0375	3.43	**VPA0310**	2.43
murF	4.06	**VP1667**	3.21
atpE	1.58	**VP2448**	2.88
VP0628	3.35	**VPA1279**	2.83
fadI	4.58	**VP2840**	2.97
VPA0312	5.12	**VP0066**	2.86
VPA0808	5.00	**VP2718**	2.05
VPA0807	5.08	**VPA1724**	1.67
		VP2827	3.75
		VPA1000	2.97
		VP0948	1.65
		VPA0652	2.18
		VPA1693	1.84
		flaC	4.37
		secF	2.40
		glgA	2.06
		VP1163	1.59
		VPA1612	1.96
		VP0768	1.94
		VP0244	4.23
		VP0996	3.06
		VP2799	2.81
		VP0051	2.77
		VP0769	3.35
		VP0118	2.37
		VP0710	3.21
		glgB	2.03
		VP1633	5.48
		VP1683	2.26
		VP0319	3.41
		VPA0941	2.96
		purL	2.34

	VPA1200	1.74
	VP1376	2.15
	VP0121	4.15
	VP2229	3.89
	VPA1611	1.95
	VP0899	2.45
	VPA1189	2.31
	VP0018	2.36
	VP2430	2.11
	VP0711	1.81
	VPA0318	3.07
	VP0920	3.15
	VP1634	3.31
	htpG	4.12
	VP1092	2.91
	VP2890	1.98
	VP0990	2.50
	VPA1321	2.48
	VPA1276	5.02
	VPA1280	3.01
	VP1091	4.26
	VP1072	2.62
	VPA1643	2.31
	VP2046	2.66
	VP2158	2.01
	VP2629	2.67
	napA	3.50
	VP2960	2.22
	VP2376	2.82
	VP0616	2.34
	VP1628	2.87
	VP1009	2.37
	VP0778	3.45
	VP0183	2.88
	VP1912	3.27
	cheB	4.55
	VP0252	3.09
	guaA	2.96

Table 12. Proteins differentially expressed (log2 fold change (FC ≥1 or ≤-1) and statistically significant (FDR=0.01 S0=0.2) between *Δvpa1044* strain vs Wild type planktonic cells when treated with 10 µg/mL Pol B. The targets were obtained through LC/MS analyses.

***Δvpa1044*+10 µg/mL Pol B vs WT+10 µg/mL Pol B**			
Upregulated Protein names	*- log P-value*	**Downregulated Protein names**	*- log P-value*
VP0236	3.29	**VPA0882**	5.28
VP0433	4.45	**VPA0154**	3.30
VP2714	3.07	**VPA1464**	2.12
VP0234	2.74	**VP1075**	2.98
tufA	2.96	**VPA0810**	2.07
VP0797	2.67	**VP1635**	3.77
VP1011	3.47	**VP1634**	2.14
VP0597	3.24	**VP1007**	3.35
rpsL	2.80	**VP0193**	2.38
sucC	4.89	**VPA1482**	1.79
VP1945	2.58	**VPA0979**	2.61
VP0551	2.94	**VPA0879**	2.72
carA	3.09	**VPA1462**	2.62
VP0205	2.75	**VP0533**	2.23
VP2324	2.44	**VPA1449**	2.91
fusA2	3.50	**VP1454**	2.59
VP0873	4.02	**VP3056**	2.26
VP0730	2.95	**norM**	3.02
fbp	3.62	**flaB**	3.38
VP0327	2.95	**VP1167**	3.21
VP2052	2.71	**VP2476**	3.33
VP0511	2.67	**VPA1657**	3.49
VP0850	4.63	**btuB**	3.99
VP2279	2.75	**hmuV**	2.50
VP0237	2.50	**VP1736**	3.45
carB	2.52	**VPA1000**	5.20
VPA0246	2.29	**VPA1648**	3.04
mdh	2.81	**VP0878**	2.72
serS	2.20	**VPA1650**	3.06
prfC	3.10	**VP2707**	3.55
tkt1	2.79	**VPA1492**	2.60
VPA0169	2.94	**VPA0256**	3.27
argG	3.00	**VPA1656**	4.82
proA	2.24	**VP0996**	5.38
ackA1	3.00	**VP1609**	2.78
minE	2.87		
VP2491	2.49		

tyrS1	2.29
VP0598	3.02
hslO	2.17
VPA0083	2.77
fusA1	2.94
pgk	2.51
VP3054	2.47
gshB	2.54
purA	2.92
mgsA	2.51
tal	2.23
hscA	2.31
VP2048	2.11
VP0749	2.90
VP1349	2.71
murA	3.09
VP1604	2.41
VP0229	2.40
gltX	2.41
efp	2.34
VP2711	3.32
tdh	2.64
leuS	2.31
rpiA	4.07
VP0671	2.27
VPA1216	2.50
VP2157	2.66
VP2194	2.43
ribB	3.54
nfuA	3.00
VP2599	2.32
VP0076	2.47
glyA1	3.40
pfkA	2.94
VPA0231	2.74
uvrB	3.84
pgi	2.22
VP1900	3.48
VPA0144	2.72
deoD2	2.05
menC	2.94
VP0223	2.63
VP0580	3.44
VP2767	2.27
leuB	2.44

VP2783	2.72
glyA2	3.51
VPA1011	2.22
kbl	3.18
ackA2	1.98
gpt	2.24
orn	1.96
ribA	3.05
ndk	3.19
cdd	2.10
VP0794	2.74
VP2487	2.17
VP1906	2.75
VP0290	2.60
luxS	2.95
acpP	2.52
aroA	2.48
VP1616	3.49
tsf	2.48
VP1272	2.74
adk	3.10
VP0444	2.97
VP0857	2.19
dksA	1.94
VP0012	2.39
VP2153	2.67
VP2515	2.70
VP2920	2.82
gpmI	2.47
VP0197	2.88
VPA0011	2.91
VP1165	2.52
VP1253	3.00
fabV1	1.97
hscB	2.86
VP1251	2.26
greA	2.91
coaE	1.96
VPA1470	2.12
VP0070	2.49
groS1	2.45
murE	2.65
hisI	4.01
nagZ	3.00
VP2198	1.91

nadA	2.00
VP0192	2.37
tsaB	2.66
VPA0574	1.86
VP1991	1.99
VP1233	2.71
ung	1.84
VP2957	2.82
VP0019	1.89
VP2976	2.30
miaA	2.57
VP1940	2.11
VP2355	3.58
VP2468	2.17
VP2033	1.99
VP0835	2.60
VP2684	3.53
VPA0071	2.59
rsmG	3.48
VP0793	2.16
dxr	2.25
VPA0535	1.86
VPA1500	2.09
deoA	3.14
ilvD	2.84
sthA	2.22
VPA0139	4.33
VP0028	2.13
VP1709	2.65
VP0960	2.69
VP3021	2.21
minC	2.70
ruvA	2.65
leuA	1.91
VP2241	3.15
thyA	1.82
VP2197	4.65
prmA	2.17
VP0206	2.45
prfA	2.06
dusA	3.02
serC	2.47
menB	2.70
VPA1289	1.96
VP2299	2.82

hemE	2.97
frr	2.17
lolA	3.01
VP0569	2.92
VP1526	1.92
purH	2.21
VPA1085	2.29
VP2118	2.36
VP2616	3.22
pdxA	3.56
ppa	3.99
deoC	3.35
astE	1.95
glgB	1.89
VP0945	1.78
VP0601	3.14
VP2055	1.71
VP0512	1.72
gmk	2.61
VPA0577	1.74
VP2676	6.11
VPA0005	3.90
VP0426	4.43
gshA	2.13
dapA	1.97
VPA1281	3.74
VP1948	2.31
pyrC	3.24
VP1278	2.65
apt	2.39
VPA0138	2.17
VP0876	4.13
purD	4.57
DST	4.23
mpl	3.18
VP2995	4.43
VP2443	3.36
gcvH	2.70
VP2590	2.02
VP2794	2.08
trpB1	2.84
VP2821	2.62
VP0080	4.92
VP0068	3.49
pyrE	1.77

VPA0466	2.30
surE	2.32
cmk	2.85
VP2656	3.80
pdxB	1.96
VPA0258	2.39
VP2580	2.80
VP2514	2.58
folD	2.96
VP1313	1.76
argH	4.36
VP2741	3.01
VPA0805	3.03
queF	3.62
VP2975	2.87
VP0949	1.77
VP0829	3.42
purM	2.81
VPA1123	3.42
gmhA	3.04
VP2167	2.12
VP1149	2.68
katG2	2.63
prmB	3.04
VPA1084	2.19
ispH	3.34
VP1535	3.19
mtnN	3.44
queA	3.18
VP1744	4.71
VP0964	3.79
VP3001	1.92
VP1898	4.21
pyrF	4.26
hisG	4.33
VP0711	3.62
VP2627	3.18
VP3018	2.05
grcA	3.47
murF	3.18
VP2262	3.49
VPA1401	3.90
VP0510	2.35
VP0406	2.81
VPA0475	4.21

VPA0230	1.59
VP0755	4.52
VPA1620	1.68
asd	3.49
cysS	4.55
add	3.40
VPA1496	3.11
panC	4.99
deoB	4.18
VP0795	3.95
tpiA	4.85
VP0556	5.96
infA	4.18
groS2	3.21

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Acknowledgment

I would like to express my sincere gratitude to all the members of the Ringgaard lab. Your encouragement and guidance made this achievement possible. Thanks to Erick, with you, I re-shaped myself as a researcher. To Shankar and Carolina, my exceptional teachers and mentors. To Petra, for your constant support and warm spirit. To Patrick, my buddy who was there in the darkest days. And all the colleagues that I had the honor of working with: Karin, Nathalie, Hyeyoung, Nenci, Sarah, Walter, and Lukas

I would like to extend my appreciation to my TAC Committee members, Dr. Andreas Diepold and Dr. Jürgen Lassak, for their support in this journey. To Dr. Timo Glatter and the Mass Spectrometry Unit at the MPI, whose expertise refined this project. To Prof Dr. Jung, whose guidance helped me learn more about the biochemical aspect of this project.

To my very special friend Mina, thank you for your friendship and for wiping my tears away.

To Marie-Paule and Ossie, the kindest family that I have in Munich.

Finally and most importantly, to my amazing family in Tehran: Maman and Baba, doosetun daram, kheili ziad.