Identification of Novel Genetic Mechanisms

Required for Bacterial Resistance to Antimicrobial Peptides

by

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ABSTRACT

The study of bacterial resistance to antimicrobial peptides (AMPs) is a significant area of interest as these peptides have the potential to be developed into alternative drug therapies to combat microbial pathogens. AMPs represent a class of host-mediated factors that function to prevent microbial infection of their host and serve as a first line of defense. To date, over 1,000 AMPs of various natures have been predicted or experimentally characterized. Their potent bactericidal activities and broad-based target repertoire make them a promising next-generation pharmaceutical therapy to combat bacterial pathogens.

It is important to understand the molecular mechanisms, both genetic and physiological, that bacteria employ to circumvent the bactericidal activities of AMPs. These understandings will allow researchers to overcome challenges posed with the development of new drug therapies; as well as identify, at a fundamental level, how bacteria are able to adapt and survive within varied host environments.

Here, results are presented from the first reported large scale, systematic screen in which the Keio collection of ~4,000 *Escherichia coli* deletion mutants were challenged against physiologically significant AMPs to identify genes required for resistance. Less than 3% of the total number of genes on the *E. coli* chromosome was determined to contribute to bacterial resistance to at least one AMP analyzed in the screen. Further, the screen implicated a single cellular component (enterobacterial common antigen, ECA) and a single transporter system (twin-arginine transporter, Tat) as being required for resistance to each AMP class.

Using antimicrobial resistance as a tool to identify novel genetic mechanisms, subsequent analyses were able to identify a two-component system, CpxR/CpxA, as a global regulator in bacterial resistance to AMPs. Multiple previously characterized CpxR/A members, as well as members found in this study, were identified in the screen. Notably, CpxR/A was found to transcriptionally regulate the gene cluster responsible for the biosynthesis of the ECA. Thus, a novel genetic mechanism was uncovered that directly correlates with a physiologically significant cellular component that appears to globally contribute to bacterial resistance to AMPs.

DEDICATION

This body of work is dedicated to my family who continues to believe in me and inspires me to do great things. I truly can do *all* things through Him that strengthens me.

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TABLE OF CONTENTS

Page

APPENDIX Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

INTRODUCTION

S. enterica serovar Typhimurium and *Escherichia coli* are classic representatives of the *Enterobacteriaceae* family of Gram-negative, rod-shaped bacteria. While most enterics remain harmless to their host, i.e., maintain a synergistic rather than an antagonistic relationship and likely persist as part of the normal flora (e.g., *E. coli*), others (e.g., *S. typhimurium*) can invade host cells and cause disease. Regardless of the relationship, these microbes encounter an array of toxic host-mediated factors, such as antimicrobial peptides, and a variety of intracellular environments that threaten their livelihoods. Nevertheless, bacteria have developed mechanisms in which various chromosomally encoded systems are employed to evade these host factors and conditions, allowing for survival and adaptation in host environments.

Importance of two-component systems

Microbial mechanisms of intracellular survival and adaptation are often mediated by chromosomally encoded two-component systems (TCS). These phosphotransfer systems consist of a histidine kinase sensor which, upon activation by a specific signal molecule(s), will phosphorylate its cognate response regulator. The active regulator can then bind to target promoters, with a higher affinity than the non-active form, to activate or repress gene transcription (1). Sensors are often bifunctional and contain phosphatase activity so that when the signal level is brought to non-activating concentrations, the phosphatase can inactivate its regulator by dephosphorylation. In this case, the abundance of the signaling molecule modulates the sensor kinase and phosphatase activities which will determine the phosphorylation level of the regulator (2). Additionally, an intrinsic positive feedback mechanism is found with most of these systems to modulate the

amount of phosphorylated regulator in the absence of the signal, as seen with the classic TCS PhoP/PhoQ (3). In the presence of the signal, low Mg^{2+}, PhoQ will phosphorylate PhoP which in turn binds to target promoters, including its own to generate more PhoQ and PhoP, thus establishing autoregulation via positive feedback. The autoregulatory capabilities have been shown to allow for temporal increase in gene expression, as in *Bacillus subtilis* sporulation (4), but also to cause an instant abundance in phosphorylation of the response regulator, as in *Salmonella* PhoP/PhoQ (5). Of course TCS can be more extensive and complex than just described, including: (i) the use of multiple phosphotransfers in a single system (referred to as phosphorelays) which is predicted to provide control points to avoid activation of regulators during inopportune times; (ii) the presence of branched phosphorylation pathways that can have multiple phosphoryl sources or targets which allows for a more integrated approach to signal transduction; and (iii) an additional ability to integrate signals in multiple TCS pathways via cross-phosphorylation (referred to as cross-talk) (reviewed in (6)). Thus, it is apparent that sophisticated systems are in place to mediate bacterial survival in host environments.

The PhoP/PhoQ two-component system

The *Salmonella* and *E. coli* PhoP/PhoQ TCS has been well studied and characterized as a model system (reviewed in (7)). PhoP/Q mediates virulence and host adaptation by responding to components of the host environment, such as low extracellular Mg^{2+}, Mn^{2+}, and Ca^{2+}, as well as low pH, and host-secreted antimicrobial peptides.

PhoP/Q can also activate another TCS, PmrA/PmrB (8), by stimulating expression of *pmrD* whose gene product post-transcriptionally activates PmrA/B. Thus, several PhoP-dependent genes are also regulated by PmrA/B in response to extracytoplasmic Fe^{3+} (9).

Both PhoP/Q and PmrA/B have been shown to mediate *Salmonella* and *E. coli* resistance to antimicrobial peptides (discussed below) further demonstrating their contributions to survival and adaptation in host environments.

The CpxR/CpxA two-component system

The *E. coli* CpxR/CpxA TCS represents one of three characterized stress response systems, including BaeRS and σ^E, that work coordinately and individually to maintain cell envelope integrity under various harsh extracytoplasmic conditions (reviewed in (10)). The *cpxRA* operon encodes a sensor histidine kinase/phosphatase, CpxA, and its cognate response regulator, CpxR, that globally regulate a vast number of promoters in response to periplasmic stress caused by misfolded proteins, inner membrane disruptions, alkaline pH, starvation, and high osmolarity (reviewed in (10)). CpxR/A has been experimentally demonstrated or proposed to regulate transcription of over 150 loci (**APPENDIX E**), including *cpxP* which encodes a negative regulator of the CpxR/A system (11). Upon the generation of misfolded proteins, CpxP is predicted to become unbound to the CpxA sensor to bind misfolded proteins and mediate DegP-facilitated degradation of the CpxP-misfolded protein complex (12). Thus, CpxR/A becomes activated due to the lack of CpxP binding to CpxA. As the CpxR/A regulon continues to expand, the vastness of its circuitries become evident as it continues to be implicated in virulence (reviewed in (10)), biofilm formation (13), and chemotaxis (14) therefore having a global effect in signal transduction pathways pertaining to bacterial survival under stress conditions. Recently, CpxR/A has been demonstrated to facilitate bacterial resistance to various classes of antimicrobial substances, including antimicrobial peptides (15) and antibiotics (16-20) by upregulating target promoters; meanwhile facilitating the

bactericidal effects of peptidoglycan recognition proteins (PGRPs) (21) and certain classes of antibiotics, such as aminoglycosides (22). This suggests a dual role of the CpxR/A system regarding bactericidal substances: a protector and a facilitator, depending on the signal (antimicrobial substance) and magnitude of activation.

The dual response of CpxR/A to antimicrobials

Antibiotics represent a major group of antimicrobials, in which there are several classes with diverse activities and cellular targets. Aminoglycosides are a class of antibiotics that target the 30S ribosomal subunit resulting in mistranslation of proteins due to mismatching of tRNAs during translation (23). It is believed that the incorporation of mistranslated proteins into the inner membrane ultimately leads to bacterial cell death (24).

CpxR/A-mediated protection against antibiotics. The accumulation of mistranslated proteins, and subsequent misfolded proteins caused by aminoglycosides, should generate a signal for the sensor kinase, CpxA, which will in turn phosphorylate the response regulator, CpxR, to upregulate expression of key target genes. Such genes include *degP* which encodes a periplasmic protease that functions to alleviate the stress of misfolded proteins by degrading the protein (25). Transcription of *degP* is also regulated by an additional stress response system, σ^E, that is generally activated upon perturbations in the outer membrane (26), suggesting multiple lines of extracytoplasmic defense mediated by DegP. This theory is supported because expression of *degP* was significantly increased in the presence of an aminoglycoside, gentamicin (22), suggesting that this antibiotic activates the CpxR/A system and thus its downstream target, *degP*, which functions to (presumably) reduce the load of mistranslated proteins. However, when the load

becomes too great, the CpxR/A system is unable to alleviate the stress resulting in cell death. Along these lines, constitutive activation of CpxR/A was recently shown to provide *E. coli* protection against aminoglycosides, likely due to the constitutive expression of DegP, but not other classes of antibiotics (20) including fluoroquinolones (which inhibit DNA replication) or β-lactams (which inhibit cell wall biosynthesis) because they do not perpetuate the production of misfolded outer membrane proteins.

CpxR/A-facilitated killing by antimicrobials. An alternative explanation regarding the bactericidal nature of aminoglycosides suggests that the antibiotics alter the overall cellular physiology, disrupting essential processes such as respiration, which ultimately leads to the formation of reactive oxygen species (27). While the generation of reactive oxygen species has recently been shown to not contribute to antibiotic-dependent killing (28, 29), the theory is supported by evidence which demonstrates that aminoglycosides stimulate the formation of hydroxyl radicals, however (surprisingly) in a CpxR/A-dependent manner. Deletion of *cpxR* or *cpxA* abolished the formation of hydroxyl radicals and reduced antibiotic-induced killing; meanwhile, deletion of *degP* significantly reduced radical formation (22). This was surprising since DegP had been previously shown to combat disruptions caused by reactive oxygen species (30). These findings, however, are in agreement with the observation that deletion of *cpxA* could increase bacterial resistance to antibiotics (22); although it was presumed to be due to the lack of CpxA phosphatase activity, resulting in increased phosphorylated CpxR. In this case, constant phosphorylation of CpxR by alternative phosphor donors should activate downstream targets to counteract the bactericidal activities of the antibiotic. Similarly, peptidoglycan recognition proteins (PGRPs) have been shown to activate the CpxR/A

system to promote their bactericidal effect (21). PGRPs bind the outer membrane of *E. coli* to activate CpxR/A which kills the bacterium by inducing depolarization of the membrane (21). Furthermore, Δ*cpxA* and Δ*cpxR* mutants were more resistant to PGRPs than their isogenic wild-type counterparts (21) providing additional evidence of a role for CpxR/A to facilitate antimicrobial killing.

On the other hand, a deletion mutant of *cpxR* and *cpxA* (Δ*cpxAR*) was more susceptible than the wild-type hyper-virulent K1 serotype *Klebsiella pneumoniae* NTUH-K2044 to bile salts, chloramphenicol, and β-lactams (19), further suggesting a protective role of CpxR/A in resistance to antimicrobials. Cumulatively, these data suggest a dual role of the CpxR/A system regarding bactericidal substances depending on the signal (antimicrobial substance) and magnitude of activation.

The Mar system

The multidrug-resistant operon, *marRAB*, encodes a repressor, MarR, an activator, MarA, and a protein of unknown function, MarB, which coordinately regulate the *mar* promoter, in concert with global regulators Rob and SoxS, to maintain intrinsic resistance to antimicrobial substances.

The Mar response. The *marRAB* promoter has been extensively studied and well-characterized (31-34). In the presence of specific antibiotics, bile salts, and reactive oxygen species, MarA (35) (36), 50), Rob (37, 38), and SoxS (39, 40), respectively, will bind to a 20 nucleotide sequence designated as the marbox (33) to activate transcription of the operon. In contrast, MarR is a strong repressor that binds at the operator region whose effect is only alleviated when a compound, such as salicylate, binds the protein to result in a disassociation to the promoter allowing for transcription (31, 32, 41). Recently

several salicylate-like aromatic metabolites including 2,3-dihydroxybenzoate (DHB) and anthranilate (ANT) in an *E. coli* wild-type background, and 4-hydroxybenzoate (HBA) in a Δ*tolC* background were shown to activate the *mar* promoter (42). In addition, DHB was found to activate the promoter by directly binding to MarR with a similar affinity as salicylate, while ANT and HBA could not, indicating that their efforts are independent of MarR.

Role of Mar in resistance to AMPs. The *marRAB* operon was shown to be activated by sub-lethal concentrations of AMPs via Rob (which is required for polymyxin B-induced upregulation of *micF* (43)) and could facilitate resistance, at least in part, by overexpressing the AcrAB/TolC efflux pump (44). While deletion of *marA* had no apparent phenotype regarding susceptibility to AMPs, constitutive expression of *marA* (here, termed *marA**) by either a point mutation in *marR* or a plasmid harboring a wild-type copy of *marA*, decreased bacterial susceptibility to multiple AMPs representing various classes, including: cathelicidin LL-37, α-defensin human neutrophil peptide-2 (HNP-2), human β-defensin-1 (HBD-1), and the cyclic lipopeptide polymyxin B (44). This effect was dependent on the AcrAB/TolC efflux system since deletion of *acrAB* or *tolC* abolished the decreased susceptibility to LL-37, HBD-1, and polymyxin B (44). Noteworthy, deletion of *tolC* in the *marA** background resulted in higher susceptibility to AMPs than deletion of *acrAB* (44) indicating that an additional TolC-dependent system contributes to resistance. Importantly, deletion of *tolC* resulted in increased susceptibility to each AMP analyzed, however, susceptibility to polymyxin B could be reduced in a *marA** background (44) suggesting that additional MarA-dependent genes contribute to bacteria resistance to polymyxin B. Conversely, *marA** in a Δ*tolC* background increased

bacterial susceptibility to defensins HNP-2 and HBD-2 when compared to the original Δ*tolC* mutant (44). Thus, MarA can play a protective role as well as a facilitator role, depending on the signal, similar to CpxR/A (discussed above).

The twin-arginine transporter (Tat) system

The twin-arginine transporter (Tat) system is a Sec-independent system responsible for the transport of globular proteins across the inner membrane (45). The Tat system is encoded by the chromosomal loci *tatA, tatB,* and *tatC*. Although *tatD* is located within the operon, its function with the Tat system remains to be determined (46). Additionally, translocation via Tat is facilitated by an accessory protein, TatE, which is not part of the *tatABCD* operon, but appears to be a functional ortholog of TatA. The Tat system has many characterized substrates with diverse cellular functions, including ion transport, energy metabolism, cell wall biosynthesis, and virulence (reviewed in (47)). As a requirement, folded proteins transported by this system must have an N-terminal signal peptide that is recognized by the Tat apparatus and cleaved prior to translocation. The signal sequence has a polar N-terminal region, an uncharged and hydrophobic region, and a C-terminal signal peptidase region that is cleaved before the protein is transported. The hallmark of Tat-dependent substrates is the presence of a twin-arginine motif that carries two conserved arginine residues within the signal sequence recognized by Tat, (S/T)RRxFLK (48), although a couple of Tat-dependent substrates have been characterized without the twin-arginine motif (49, 50).

Deletions of *tat* genes have pleiotropic effects. In *E. coli*, such effects include mislocalization of peptidoglycan amidases AmiA and AmiC that cause a cell chaining phenotype due to the inability to cleave the septum, filamentation, inability to grow

anaerobically with certain electron acceptors, and increased membrane permeability (51-53). Thus, the Tat system has an important role in cell metabolism and survival.

The enterobacterial common antigen

Members of the *rfe-rff* gene cluster encode enzymes required for the biosynthesis of the enterobacterial common antigen (ECA), a glycolipid composed of a trisaccharide repeat located on the outer leaflet of the outer membrane in all species of the *Enterobacteriaceae* family (reviewed in (54)). The first step in the synthesis of the trisaccharide repeat is catalyzed by Rfe in which GlcNAc-1-phosphate is transferred from UDP-GlcNAc to undecaprenyl monophosphate to generate lipid I. Next, ManNAcA is incorporated to generate lipid II followed by incorporation of Fuc4NAc, via RffC and RffT, to generate lipid III. Finally, sequential elongation, via WzzE and WzyE, followed by presentation to the outer leaflet of the outer membrane, via WzxE, completes the ECA biosynthesis process. ECA has been reported to exist in three forms (54): ECA_{CYC}, a water-soluble cyclic form that consists of four repeating units; ECA_{LPS}, in which the trisaccharide repeat is linked to the core region of LPS; and, ECA_{PG}, in which the trisaccharide repeat is linked to the outer membrane phosphoglyceride.

Phenotypic observation of ECA mutants. While the physiological function of ECA remains to be determined, it has been implicated in bacterial resistance to bile salts and organic acids (55-57), swarming motility (58), and virulence (57). Sensitivity to bile salts: Deletion of *E. coli rffA* (*wecE*), *rffT* (*wecF*), or *rffH* (*rmlA*), each of which encodes an enzyme involved in the conversion of lipid II to lipid III, resulted in increased sensitivity to bile salts as the mutants were unable to grow on MacConkey agar, presumably due to the accumulation of the lipid II intermediate (55). Further studies

determined that deletion of *Salmonella rfe* (*wecA*) and *rffC* (*wecD*) caused sensitivity to the bile salt deoxycholate (57). This proved that accumulation of lipid II could not be the sole cause of increased susceptibility to bile salts and proposed that biosynthesis of ECA is required for bacterial resistance to bile salts. Sensitivity to organic acids: Deletion of *rfe* (*wecA*) and *rffE* (*wecB*) in Shiga Toxin-producing *E. coli* (STEC) O157:H7 NGY9 rendered cells sensitive to acetic acid (56), suggesting that ECA is required for bacterial resistance to acetic acid. Defect in swarming motility: Deletion of *E. coli rfe (wecA), rffE (wecB), rffD (wecC), rffG, rffH, rffC (wecD), rffA (wecE), wzx (wzxE), rffT (wecF),* or *rffM (wecG)* caused a defect in cell swarming, but not swimming motility (58), indicating that ECA plays a significant role in swarming motility. Decrease in virulence: *Salmonella rfe* and *rffC* mutants were highly attenuated in a mouse model when orally inoculated (57) suggesting a protective role of ECA in virulence.

Peptidoglycan amidases, AmiA and AmiC

The peptidoglycan matrix, or cell wall, of the bacterial cell envelope is a complex structure that ultimately provides support for the accompanying phospholipid membrane. Thus, it gives the cell its overall shape and rigidity, as well as provides protection against changes in osmotic pressure. The matrix is composed of (i) a backbone that consists of polysaccharide strands, *N*-acetylglucosamine and *N*-acetylmuramic acid, that are cross linked by a β-1,4 glycosidic bond and (ii) a meshwork of peptides that are covalently connected to the *N*-acetylmuramic acid residues by cross-linked peptides to form a matrix around the cytoplasmic membrane (59). During bacterial growth and cell division, the peptidoglycan is constantly remodeled by synthases and hydrolases that cooperatively work to generate and cleave peptide bonds. AmiA and AmiC are *N*-acetylmuramoyl-L-

alanine amidases that cleave the bond between the L-alanine amino acid on the peptide chain and the *N*-acetylmuramic acid residue of the peptidoglycan matrix (51, 60). They are transported by the Tat system (discussed above, (61)) to the periplasm in which they carry out their enzymatic activities. Cells lacking these enzymes form chains as the septum is unable to be completely cleaved during daughter cell separation (51). Additionally, cells that inhibit the activity of these enzymes such as (i) absence of LytM factors EnvC and NlpD that activate the amidases (62) or (ii) absence of the FtsEX cell-wall hydrolysis regulator (63) exhibit a chaining phenotype, further demonstrating the significance of these amidases in cell wall biogenesis.

Cumulatively, bacteria have developed strategies to evade host-mediated defenses in efforts to promote survival and adaptation. The use of multiple, integrated systems is a key approach in such defenses. In the upcoming chapters of this dissertation study, the importance of the aforementioned systems and cellular components will be further discussed in regards to bacterial resistance to antimicrobial peptides.

Characterization of antimicrobial peptides

Characteristics of AMPs. Antimicrobial peptides (AMPs) represent a class of host-mediated factors that function to prevent microbial infection of their host (64). They are short peptides (12-100 amino acids) with a net charge between +2 and +7 (65, 66) produced by the innate immune system in a variety of organisms, including animal, plant and bacterial species, and serve as a first line of defense (67). As such, AMPs exert their activities at epithelial surfaces and within specialized phagocytic cells, such as macrophages. Due to their amphipathic nature, in which they contain an abundance of hydrophobic residues, they are thought to interact with bacterial membranes to

subsequently kill cells (reviewed in (68)). To date, over one thousand AMPs have been experimentally validated or predicted and can be found in several on-line databases (The Antimicrobial Peptide Database, http://aps.unmc.edu/AP/main.php (64); CAMP: Collection of Anti-Microbial Peptides, http://www.bicnirrh.res.in/antimicrobial/ (69); DAMPD: Dragon Antimicrobial Peptide Database, http://apps.sanbi.ac.za/dampd/).

Classification of AMPs. Based on their secondary structure, AMPs can be grouped into four classes (70), summarized in **Table 1**: (i) amphipathic peptides that form α-helix structures upon initial contact with the bacterial membrane, (ii) β-sheet peptides that are joined by 2-4 disulfide bridges, (iii) extended peptides that generally consist of an abundance one or more amino acids, and (iv) loop peptides that are formed by a single disulfide bond. β-sheet peptides, which include α-, β- and θ-defensins (characterized from mammals), and amphipathic α-helix peptides, which include cathelicidins (primarily characterized from mammals), cecropins (characterized from insects), magainins (characterized from amphibians) and melittin (characterized from insects), make up the majority of the AMPs. In many cases, different classes of peptides and different variants within the classes can be found within a single host. In fact, it is rare for the same AMP sequence to be characterized from two different hosts even though the amino acid sequences may be well conserved in the precursor molecule from which the mature AMP is derived.

α-helix structured AMPs. α-helix structured AMPs are one of the largest and most extensively studied groups of AMPs. Upon interaction with phospholipid membranes, these disordered peptides fold into an α-helical conformation to either absorb or insert into the membrane to exert their activities.

Table 1. Four classes of AMPs based on their structure.

AMP type	Characteristics	Examples	Sequence	Ref
α-helix structured peptides		**Cecropin A** (*Hyalophora cecropia*, moth)	KWKLFKKIEKVGQNIRDG IKAGPAVAVVGQATQIAK	(71)
		Magainin 2 (*Xenopus laevis*, toad skin)	GIGKFLHSAKKFGKAFVG EIMNS	(72)
		SMAP29 (sheep myeloid)	RGLRRLGRKIAHGVKKYG PTVLRIIRIAG	(73)
		LL-37 (humans, leukocytes, epithelia)	LLGDFFRKSKEKIGKEFKR IVQRIKDFLRNLVPRTES	(74)
		***OH-CATH30** (King cobra)	KFFKKLKNSVKKRAKKFF KKPRVIGVSIPF	(75)
		***OH-CM6** (King cobra)	KFFKKLKKAVKKGFKKF AKV	(75)
β-sheet structured peptides	Peptides with 2 disulphide bonds	**Protegrin-1** (porcine leukocytes)	RGGRLC1YC2RRRFC1VC2 VGR	(76)
		Tachyplesin-1 (*Tachypleus gigas*, crab)	KWC1FRVC2YRGIC2YRR C1R	(77)
	Peptides with 3 disulphide bonds	**human θ defensins-1** (human tissue)	DHYNC1VSSGGQC2LYSA C3PIFTKIQGTC2YRGKAK C1C3K	(78)
		Rhesus θ defensin-1 (Rhesus monkey)	GFCRCLCRRGVCRCICTR	(79)
		***Rhesus θ defensin-2** (Rhesus monkey)	GVCRCLCRRGVCRCLCRR	(80)
Extended peptides	Rich in His	**Histatin-5** (human saliva)	DSHAKRHHGYKRKFHEK HHSHRGY	(81, 82)
	Rich in Trp	**Indolicidin** (bovine neutrophils)	ILPWKWPWWPWRR	(83, 84)
	Rich in Arg and Pro	**Bactenecin-5** (bovine neutrophils)	RERPPIRRPPIRPPFYPPFRP PIRPPIFPPIRPPFRPPLRFP	(85, 86)
	Rich in Arg and Pro	**PR-39** (porcine neutrophils)	RRRPRPPYLPRPRPPPFFPP RLPPRIPPGFPPRFPPRFP	(87)
	Rich in Phe and Pro	**Prophenin-1** (porcine neutrophils)	RGGRLCYCRRRFCVCVGR	(88, 89)
	Rich in Arg	***Protamine** (Salmon sperm)	MPRRRRSSSRPVRRRRRP RVSRRRRRRGGRRRR	(90)
Loop structured peptides		**Lactoferricin** (cow and human milk)	FKC1RRWQWRMKKLGAP SITC1VRRAF	(91)
		Bactenecin (bovine neutrophils)	RLCRIVVIRVCR	(92)
		Nisin A (*Lactococcus lactis*)	ITSISLCTPGCKTGALMGC NMKTATCHCSIVHSK	(93)

* Analyzed in this study

β-sheet structured AMPs. Another extensively studied group of AMPs are β-sheet structured AMPs. These cyclic peptides are connected by disulfide bonds which contribute to their antimicrobial activity (94). While they exist as β-sheets, their conformations are stabilized upon interaction with phospholipids. It has been proposed that these AMPs exert their activities by disrupting the phospholipid membranes or by forming channels (95, 96).

Extended AMPs. The class of extended AMPs is characterized by their unusual amino acid composition in which there is an abundance of one or more amino acids in the peptide sequence. The nature of the abundant amino acid(s) will ultimately determine the mechanism in which the AMP will behave. For example, indolicidin, which is abundant in tryptophan residues, is proposed to have a turn conformation to increase activity toward the membrane (83) as well as permeabilize the outer membrane to form channels (84, 97). Meanwhile, the role of the multiple tryptophan residues remains undetermined.

Loop structured AMPs. The proline-arginine-rich loop structured AMPs require adoption to a polyproline helical type-II structure (98, 99) due to the inability to form amphipathic conformations because of the excessive proline residues.These looped peptides gather and form tubular structures that increase the permeability of the membrane. This class of AMPs is thought to be the most promising to be developed into antimicrobial therapeutics due to the short peptide length and ease of synthesis.

Therapeutic application of antimicrobial peptides

Salmonella and serotypes of *E. coli* are Gram-negative enteric bacterial pathogens, responsible for a variety of infectious diseases. In the United States, *Salmonella* was estimated to have an incidence of about 14 cases per 100,000 people, which roughly

equates to 30,000 cases in 2006 (100). Moreover, in 2011, *Salmonella* was implicated in over 1 million cases of infection – 20,000 of which resulted in hospitalization, while 378 were fatal (100). These data made *Salmonella* the cause of ~30% of all foodborne illness-related deaths in the U.S. last year (100). Similarly, in 2003, *E. coli* infections were estimated to have caused more than 2,000 hospitalizations in the U.S. resulting in about 60 fatalities (101). In that year, pathogenic *E. coli* infections were estimated to cost $405 million leaving a large impact and burden on the health care system (102). Although there has been a decline in the number of reported cases over the decades due to various treatment regimens, including multiple generations of antibiotics, lack of or improper treatment continues to lead to millions of deaths worldwide each year (103). To make matters worse, the past few decades have seen the emergence of multidrug-resistant (MDR) bacteria, which has led to increased disease and fatality incidence (104-106). As the dangers of MDR bacteria begin to surface due to the rising prevalence of these strains, the fight to prevent and treat these infections becomes increasingly difficult. With millions of cases leading to thousands of hospitalizations and excessive costs in the U.S. each year, it is vital to develop alternative treatments to combat these quickly evolving pathogens.

Antimicrobial peptides (AMPs) can be considered a promising next-generation pharmaceutical therapy to combat bacterial pathogens because they exert their effects in broad and different ways than conventional antibiotics. Due to their amphipathic nature, in which they contain an abundance of hydrophobic residues (~50%), they are thought to interact with bacterial membranes without specific receptors which would reduce the possibility of spontaneous resistance (reviewed in (68)). This is unlike antibiotics which

act on proteins generally involved in specific cellular processes and are commonly modified in drug resistant strains (reviewed in (68)). Additionally, AMPs can broadly exert their bactericidal activity against Gram-positive and/or Gram-negative bacteria, as well as fungi, parasites, protozoa, viruses, and cancer cells (65, 107). They are currently used in the food industry; for example, nisin and pediocin PA-1, bacteriocins produced from lactic acid bacteria, are commonly used due to their potent activity against foodborne pathogens and fungal microbes that can spoil food (108). While AMPs present a promising alternative to conventional antibiotics, the sensitivity of their nature presents challenges, including limit in drug delivery due to the inability to be taken orally. While this issue is somewhat minor (topical and injection delivery methods have provided solutions), the major challenge of relative high production costs remains unresolved (reviewed in (68)). Therefore, it will be necessary to design novel AMPs that can exert their effects broadly and potently, while being simple in structure to allow for high production at minimum costs (68). The design of such peptides is limited, however, due to the incomplete understanding of the mechanisms in which AMPs exert their activities and how bacteria evade these host-secreted peptides.

Physiological mechanisms of action of antimicrobial peptides

It is no surprise that the diversity of the characterized AMPs would yield diversity in their mechanisms of action. As such, several physiological models regarding the bactericidal activity of AMPs have been proposed (summarized in (109)).

Outer membrane disruption. One commonality amongst several AMPs characterized is their ability to disrupt the outer membrane. Several models of the disruption have been discussed, such as neutralization of membrane charge in a small area or binding to the

negatively charged lipopolysaccharide (LPS). Such disruptions allow the AMP to form voltage-dependent ion channels or transmembrane pores to permeabilize or penetrate the membrane to exert their activities (66). In fact, in *E. coli* β-sheet defensins and the amphipathic melittin have been shown to permeabilize the phospholipid membrane and generate blebs (110), while α-helix structured AMPs such as cecropin penetrate the membrane to form voltage-dependent ion channels (111, 112). Others, such as magainin 2, have been shown to penetrate the membrane which results in the loss of intracellular metabolites ultimately disrupting energy-transducing processes which lead to cell lysis (113, 114).

Intracellular targets of AMPs. It was determined that the permeabilization of the outer membrane is not the primary cause of bacterial death when challenged with AMPs (66, 115). For pore-forming AMPs, it can be stipulated disruption of the proton motive force is the ultimate cause of cell death. However, not all AMPs form pores in the membrane suggesting they may target other components of the cell. Along these lines, proline-rich Buforin II does not damage the bacterial membrane; instead, it accumulates in the cytoplasm to carry out its bactericidal activity against nucleic acids (116). Other AMPs have also been shown to traverse the outer and inner membranes to inhibit synthesis of biologically important molecules and cellular pathways (117). For example, the arginine-rich peptide indolicin completely inhibits nucleic acid synthesis. Meanwhile, the α-helix structured peptides, pleurocidin and dermaseptin; proline-rich peptide, PR-39; and *α*-defensin human neutrophil peptides, HNP-1 and HNP-2, interfere with nucleic acid and protein synthesis. It is difficult to identify a specific target or mechanism due to the breadth of the cellular pathways inhibited by AMPs. It can be posited that an

accumulation of hydroxyl radicals or other reactive oxygen species can be the cause of the mass inhibition of cellular pathways (118).

Commonality amongst mechanisms. Regardless of the mechanism of action, one common feature amongst the models is the initial interaction between the positively charged AMP and the negatively charged membrane. A hallmark of AMPs is the specificity to microbial cells while being relatively ineffective against eukaryotic membranes. This is due to the difference in membrane composition (77) (119); bacteria have an abundance of negatively charged phospholipids that comprise the outer and inner membranes of Gram-negative bacteria and the cytoplasmic membrane of Gram-positive bacteria. For example, the *E. coli* membrane contains approximately 5% cardiolipin and 20-25% phosphatidylglycerol (120). Additionally, the presence of the negatively charged LPS on the outer membranes of Gram-negative bacteria or the teichoic acids that are present in the cell walls of Gram-positive bacteria makes the cell envelope an attractive target for positively charged AMPs. On the other hand, the membrane composition of normal mammalian cells is remarkably different as it is mostly composed of zwitterionic phospholipids phosphatidylcholine and sphingomyelin (121, 122), making the membranes inappropriate targets for AMPs. Thus, the cell envelope, specifically the cell membranes and respective cell wall components, play a vital role in the activity of AMPs.

Physiological characteristics that affect the bactericidal activity of antimicrobial peptides

Role of LPS in resistance to AMPs. Studies with *Salmonella* and magainin 2 have been carried out to ascertain the role of LPS in AMP-mediated killing. Increased loss of resistance was found in LPS mutants in which the length of the LPS moiety was

sequentially reduced (123) suggesting that LPS can have a protective role and prevent AMPs from reaching the membrane. Further, overall charge and abundance of LPS molecules on the cell envelope were found to be contributing factors in determining the effect of LPS on AMP activity. This suggests that LPS can serve as a protector by inhibiting the AMP from reaching the outer membrane.

Role of phospholipid membranes in resistance to AMPs. The composition of the phospholipid bilayer (discussed above) can influence the efficacy with which AMPs exert their activities. Specifically, the net charge, fluidity, and curvature strain of the membranes are major contributing factors. Charge: Cationic AMPs have been shown to have greater electrostatic interactions with phospholipid membranes containing the negatively charged phosphatidylglycerol (114). Fluidity: In general, bilayers with increased fluidity have been shown to have less resistance to AMPs (124) because cholesterol, which reduces bilayer fluidity, was able to reduce AMP activity (125) Curvature strain: Phospholipids possessing small hydrophilic head groups such as phosphatidylethanolamine cause a concave bending of the membrane monolayer resulting in negative curvature and inhibition of magainin-induced pore formation (114). Addition of palmitoyllysophosphatidylcholine, which reduces the negative curvature, facilitated the magainin-induced permeabilization (114). Further, it can be postulated that AMPs target cellular locations with the largest cell wall curvature, i.e., the poles and septum, which is primarily composed of the acidic phospholipid, cardiolipin (126, 127). This is supported by the observation that the human derived cationic AMP, LL-37, preferentially targets the septa of dividing cells (128).

Genetic mechanisms of action to evade bactericidal effects of antimicrobial peptides

Bacterial development of a complete resistance to AMPs is thought to be impossible (66); however, enteric bacteria have developed mechanisms to offset the bactericidal activities of AMPs. Many loci representing several independent pathways have been implicated in resistance to various AMPs (summarized in **Table 2**); however, the *Salmonella typhimurium* PhoP/PhoQ two-component system has been demonstrated as a global regulator of AMP resistance.

PhoP/PhoQ-mediated resistance to AMPs. Deletion of *Salmonella phoP* is pleiotropic as it has many phenotypes associated with it, including increased susceptibility to AMPs due to the lack of various PhoP-dependent genes that mediate resistance (**Table 2**). There are multiple PhoP-mediated strategies reported to confer resistance to AMPs (discussed in (7)) that make the PhoP/Q system a global contributor in resistance to AMPs, including (i) modification of lipopolysaccharide (LPS) and (ii) synthesis of extracytoplasmic proteases that degrade the AMPs. Modification of the outer membrane: PhoP-dependent modification of the outer membrane is a mechanism *Salmonella* employ to evade AMP killing. Resistance is facilitated by modification of the lipid A moiety of LPS to reduce electrostatic interactions between the positively charged AMPs and the negatively charged outer membrane (129-131) as well as alter the membrane fluidity to reduce the effectiveness of the AMP to peremabilize the membrane (discussed above; (132)). Specifically, the outer membrane protein, PagP, modifies LPS via addition of palmitate to lipid A (133) to increase *Salmonella* resistance to α-helical AMPs (115). Also regulated by the PmrA/PmrB TCS, *ugd* and *pmrF* encode enzymes required for the synthesis and incorporation of 4-aminoarabinose into LPS. As such, resistance to

Table 2. Genes previously determined to be required for AMP resistance.

Peptide(s)	**Bacteria**	**Gene(s)**	**Reg. System(s)**	**Ref**
Protamine, Magainin 2, Melittin	*E. coli* *Salmonella*	*amiA* *amiC*	CpxR/A	(15)
Lactoferricin B	*E. coli*	*degP*	CpxR/A	(134)
Polymyxin B, Protamine, Protegrin-1	*Salmonella*	*mig-14*	PhoP/Q SlyA	(135)
Protamine	*E. coli*	*ompT*		(136)
Polymyxin, Azurocidin, BPI, Protamine, Polylysine	*Salmonella*	*pagB*	PhoP/Q PmrA/B	(137)
C18G	*Salmonella*	*pagP*	PhoP/Q	(115)
Protamine, C18G	*Salmonella*	*pgtE*	PhoP/Q SlyA	(138)
Cecropin P1, Magainin 2, Mastoparan, Melittin, Defensin NP-1, Polymyxin B, Protamine	*E. coli* *Salmonella*	*phoP* *phoQ*	PhoP/Q	(139-142)
Polymyxin, Polylysine, Protamine, CAP37, CAP57	*Salmonella*	*pmrA* *pmrB*	PhoP/Q PmrA/B	(137, 143-146)
Polymyxin B	*Salmonella*	*pmrE* *pmrF*	PmrA/B	(129)
Polymyxin B	*Salmonella*	*rcsC*	RcsB/C	(147)
Crp4, P2 (BPI)	*Salmonella*	*rpoE*	σ^E	(148)
Protamine	*Salmonella*	*sapABCDF*		(149)
Protamine	*Salmonella*	*sapG* *sapJ*		(150)
Magainin 2, Polymyxin B	*Salmonella*	*slyA*	PhoP/Q SlyA	(140, 151)
Polymyxin B	*Salmonella*	*somA*	PhoP/Q	(147)
Protamine, Magainin 2, Melittin	*E. coli* *Salmonella*	*tatA* *tatB* *tatC*		(15)
Magainin 2, Polymyxin B	*Salmonella*	*ugtL*	PhoP/Q SlyA	(140)
Polymyxin B	*Salmonella*	*virK*	PhoP/Q SlyA	(147)
Protamine, Melittin, Polymyxin B, Human Defensins	*Salmonella*	*yejABEF*		(152)
Protamine, Magainin 2, Melittin	*Salmonella*	*yqjA*	CpxR/A PhoP/Q	(140)

polymyxin B is also facilitated by LPS modification to lipid A (130, 153).

Protease-mediated degradation of AMPs: Protease-specific degradation of AMPs is another strategy employed to evade AMP activity. PhoP-dependent *pgtE* encodes an outer membrane protease that, when expressed at high levels, will contribute to resistance to the C18G AMP by cleaving C18G (138). Similar protease-facilitated cleavage of AMPs is observed by the OmpT protease in *E. coli* (136).

PhoP-independent resistance to AMPs. While PhoP/Q has been demonstrated as a global regulator in bacterial resistance to AMPs, it should be noted that several PhoP-independent loci have been demonstrated to contribute to bacterial resistance to AMPs.

The Sap transporter: Deletion of members of the *sapABCDF* operon, which encodes a periplasmic oligopeptide-binding protein and its inner membrane transporter, rendered *Salmonella* cells susceptible to the model AMP, protamine (150). It is thought that this system mediates resistance by transporting protamine from the periplasm to the cytoplasm where it is degraded by cytoplasmic proteases. The Yej Transporter: Deletion of *yefF*, which encodes the ATPase component of the ATP-binding cassette transporter encoded by the *yejABEF* operon, rendered *Salmonella* cells susceptible to multiple classes of AMPs, represented by protamine, melittin, polymyxin B, and human β-defensins HBD-1 and HBD-2 (152). Additionally, Δ*yejF* had reduced virulence when inoculated intragastrically (152) suggesting that the YefABEF transporter contributes to *Salmonella* virulence by facilitating resistance to AMPs.

Rationale of the dissertation studies

Over 1,000 AMPs of various natures have been characterized (64) and shown to exert their effects in ways different from modern day antibiotics, making them a promising

next-generation pharmaceutical therapy to combat bacterial pathogens. Therefore, it is important to understand the molecular mechanisms, both genetic and physiological, behind bacterial resistance to AMPs to overcome the challenges faced with the development of new drug therapies and vaccines. Research regarding the genetic circuitries required for bacterial resistance to AMPs has been challenging due to the sensitivity of AMPs to various laboratory conditions (salts, buffers, media, etc.) and a lack of adequate and efficient genetic tools. Although there have been a few mechanisms proposed to mediate bacterial resistance to AMPs (discussed above), including (i) lipidA modification of the LPS (115, 129), (ii) cleavage of AMPs by outer membrane proteases (136, 138) and (iii) membrane bilayer rearrangements to inhibit lipid-peptide pore formation (113, 114), the lack of genetic determinants limits the identification of additional physiological mechanisms. While the PhoP/Q system has been demonstrated as a major contributor in resistance to AMPs, I believe that *additional PhoP/Q-independent mechanisms must exist to further contribute to resistance.*

In this study, data and analysis are presented from a large scale, systematic screen in which I used the entire Keio collection (154) of ~4,000 *E. coli* in-frame, single gene deletion mutants to identify loci required for resistance to AMPs. The identification of these loci served as a tool for me to identify novel genetic circuitries required for resistance and likely other physiologically significant processes; and thus, has provided a basis for the work described in this dissertation study.

In Chapter 1, I discuss the results of the systematic analysis in which *E. coli* mutants were challenged against physiologically significant peptides representing α-helix and β-sheet classes of AMPs, as well as a model AMP. Notably, this chapter identifies the

significance of the Tat system in bacterial resistance to AMPs which is further characterized in Chapter 2. I also provide evidence of the importance of enterobacterial common antigen biosynthesis in resistance to antimicrobial substances. Cumulatively, the analysis of this chapter served as a foundation for the work described in Chapters 2-4.

In Chapter 2, I build on a hypothesis generated from data in Chapter 1 in which I identify Tat-dependent substrates required for bacterial resistance to protamine. Two of these substrates, AmiA and AmiC, are further characterized in Chapter 3.

Chapter 3 discusses the biochemical and genetic aspects of this study in which the CpxR/CpxA two-component system is found to be a major regulator for several loci required for resistance to AMPs. Specifically, I determined that the *amiA, amiC,* and *marRAB* loci, as well as the *rfe-rff* gene cluster are regulated by CpxR.

Chapter 4 wraps up the study by collectively describing a new regulatory system, CpxR/A, as a major contributor to bacterial resistance to AMPs. Multiple CpxR/A-dependent loci, either characterized previously or in this study, were found to contribute to resistance. The identification of this system proves the original hypothesis of this study in which additional PhoP-independent systems are required for bacterial resistance to AMPs.

Disclaimer. This project was initially carried out using a collection of *Salmonella* mutants to identify genes required for resistance to AMPs. However, the collection was never completed by collaborating laboratories and as such, never received in its entirety. Thus, to continue the project, the Keio collection of *Escherichia coli* mutants was used and represents a significant proportion of the strains discussed in this work.

MATERIALS AND METHODS

Bacterial strains and growth conditions

All bacterial strains used in this study are provided in **Appendix A**. *Escherichia coli* strains were obtained or derived from mutant strains in the Keio Collection (154) or derived from the wild-type strain BW25113 using the one-step gene deletion method (155) with primers listed in **Appendix C**. All *Salmonella enterica* serovar Typhimurium strains were derived from the wild-type strain using the one-step gene deletion method (155). Bacteria were grown at 37°C in Luria-Bertani broth (LB). When necessary, antibiotics were added at final concentrations of 50 μg ml^{-1} for ampicillin, 20 μg ml^{-1} for chloramphenicol, or 50 μg ml^{-1} for kanamycin. *E. coli* DH5α was used as host for the preparation of plasmid DNA. *E. coli* BL21-Gold (Stratagene, Inc.) was used for protein expression.

Construction of strains with chromosomal mutations harboring *lac* fusions

Strains harboring deletions constructed using the one-step gene deletion method (155) were used to construct chromosomal *lac* fusions.The kanamycin- or chloramphenicol-resistant cassette was removed using plasmid pCP20 (155) and the appropriate *lac* transcriptional fusion plasmid pCE36 or pCE37 (156) was integrated into the FLP recombination target sequence in the deleted locus.

Plasmid construction

All plasmids used in this study are described in **Appendix B**. Primers used to generate DNA fragments cloned into each plasmid are listed in **Appendix C**.

Plasmid pUHE-*nlpE* was constructed by cloning the *nlpE* gene fragment (synthesized using 14028s or BW25113 chromosomal DNA and primers 1421 and 1422) into the

*Hin*dIII site of pUHE21-2*lacI*^{q} (157).

Plasmid pUHE-*yaeJ* was constructed by cloning the *yaeJ* gene fragment (synthesized using 14028s chromosomal DNA and primers 1413 and 1414) into the *Hin*dIII site of pUHE21-2*lacI*^{q} (157).

Plasmid pUHE-*amiA* was constructed by cloning the *amiA* gene fragment (synthesized using 14028s chromosomal DNA and primers 1327 and 1328) into the *Hin*dIII site of pUHE21-2*lacI*^{q} (157).

Plasmid pUHE-*amiC* was constructed by cloning the *amiC* gene fragment (synthesized using 14028s chromosomal DNA and primers 1402 and 1403) into the *Hin*dIII site of pUHE21-2*lacI*^{q} (157).

Plasmid pBAD-*tatC* was constructed by cloning the *tatC* gene (synthesized using 14028s chromosomal DNA and primers *tatC*-forward and *tatC*-reverse) into pBAD TOPO (Invitrogen).

Plasmid pYS2135 was constructed by cloning the *cpxR* coding region (synthesized using 14028s chromosomal DNA and primers 1512 and 1513) into into the Nde*I* and Sal*I* site of pET28a (Novagen).

Plasmid pYS1734 was constructed by cloning 570 bp of the *marR* promoter region (synthesized using BW25113 chromosomal DNA and primers 1731 and 1734) into the Sal*I* and Xho*I* site of pYS1000 (158).

Plasmid pYS1736 was constructed by cloning 120 bp of the *marR* promoter region (synthesized using BW25113 chromosomal DNA and primers 1731 and 1736) into the Sal*I* and Xho*I* site of pYS1000 (158).

Selection for genes required for resistance to θ-defensin peptide

The Keio collection of *E. coli* mutants, containing nearly 4,000 single mutant strains (154) was used to screen for genes required for resistance to the θ-defensin peptide, RTD-2 (79). Strains were cultured overnight, re-inoculated (1:100) in LB broth, and grown for 4 h at 37°C. Cultures were diluted in a challenge medium (10 mM PIPES, 0.5% LB) and approximately 10^5 cells were challenged in a 96 well microtiter plate with 1.5 μg mL^{-1} peptide in the challenge medium for 2 hours in 37°C. Samples were then diluted (1:10) in LB broth and spotted onto LB agar plates for overnight growth at 37°C.

Selection for genes required for resistance to α-helix structured peptides

The Keio collection (154) was used to screen for genes required for resistance to the α-helix structured peptides, OH-CATH30 and OH-CM6 (75). Strains were cultured overnight, re-inoculated (1:100) in LB broth, and grown for 4 h at 37°C. Cultures were diluted in a challenge medium (10 mM PIPES, 0.5% LB) and approximately 10^5 cells were challenged in a 96 well microtiter plate with 2 - 4 μg mL^{-1} peptide in the challenge medium for 2 hours in 37°C. Samples were then diluted (1:10) in LB broth and spotted onto LB agar plates for overnight growth at 37°C.

Selection for genes required for resistance to protamine

The Keio collection (154) was used to screen for genes required for resistance to protamine (90). Strains were cultured overnight, re-inoculated (1:100) in LB broth, and grown for 4 h at 37°C. Strains were diluted and approximately 10^2 - 10^3 cells were dropped onto LB agar plates containing varying concentrations (0.6 - 1.2 mg mL^{-1}) of protamine sulfate (MP Biomedicals) and incubated overnight at 37°C to screen for sensitivity.

Vancomycin killing Assay

Salmonella and *E. coli* survival after a vancomycin challenge was determined as follows. Overnight cultures were inoculated 1:5 in fresh LB broth. An appropriate inducer (0.25 mM IPTG or 10 mM L-arabinose) was added when necessary. Strains were shaken for 4 h at 37°C to allow for induction. Cells were diluted to ~10^5 bacteria mL^{-1} and added to microtiter wells containing 0 and 0.5 mg mL^{-1} (final concentration) vancomycin (Sigma). Strains were challenged overnight with aeration at 37°C, and survival was determined by measuring the optical density. Survival percentage was calculated as described previously (*i.e.* $A_{600\ nm}$ of culture with vancomycin/$A_{600\ nm}$ of culture without vancomycin)×100 (53).

SDS killing Assay

Salmonella and *E. coli* survival after a sodium dodecyl sulfate (SDS) challenge was determined as follows. Overnight cultures were inoculated 1:5 in fresh LB broth and shaken for 4 h at 37°C. Cells were diluted to ~10^5 bacteria mL^{-1} and added to microtiter wells containing 0, 0.01, 0.02 and 0.04 % (final concentration) SDS. Strains were challenged overnight with aeration at 37°C, and survival was determined by measuring the optical density. Survival percentage was calculated as described previously (*i.e.* $A_{600\ nm}$ of culture with SDS/$A_{600\ nm}$ of cultures without SDS) × 100 (53).

Deoxycholate killing assay

Strains were cultured overnight, re-inoculated (1:100) in LB broth, and grown for 4 h at 37°C. Strains were diluted and approximately 10^2 - 10^3 cells were dropped onto LB agar plates containing 0 and 1% deoxycholate and incubated overnight growth at 37°C to screen for sensitivity.

Screening for a regulator that upregulates *amiA* and *amiC* transcription

Chromosomal DNA prepared from wild-type strain 14028s was digested with Sau3AI (1 unit; New England Biolabs) for 15, 30, or 45 min. The digested DNA was separated on 0.8% agarose gel, and 2–5-kb fragments were recovered and ligated to BamHI-digested pUC19 plasmid DNA. The ligation mixture was transformed into *E. coli* DH5α selecting for ampicillin-resistant transformants. Plasmid DNA was isolated from a pool of about 20,000 transformants (~95% of which carried an inserted chromosomal fragment) and introduced into strains, YS13637 and YS13640, which harbored a chromosomal *lac* transcriptional fusion at the *amiA* and *amiC* loci, respectively. Ampicillin-resistant transformants were selected on LB ampicillin agar plates containing X-Gal (40 μg mL^{-1}). Plasmid DNA was purified from those colonies that were darker blue than others and reintroduced into YS13637 and YS13640 by electroporation. The resulting strains were used to measure β-galactosidase activity and to compare with those that received a control plasmid pUC19. To determine the inserted fragments, the plasmids were sequenced using primers 232 and 233.

β-galactosidase assay

β-galactosidase assays were carried out in triplicate (159) and the activity (Miller Unit) was determined using a VERSAmax plate reader (Molecular Device). Data correspond to three independent assays conducted in duplicate, and all values are mean ± standard deviation.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Bacterial cells were grown for 8 h in LB medium. Total RNA was isolated from bacterial culture using TRIzol LS reagent (Invitrogen) according to the manufacturer's instructions.

RNA concentration was determined by spectrophotometry at 260 nm and quality determined by agarose gel electrophoresis. cDNA was synthesized using murine leukemia virus reverse transcriptase and random primers (BioLabs). DNA was amplified with primers indicated in the text and resolved on an agarose gel.

Purification of His_6-CpxR protein

The His_6-CpxR protein was purified from pYS2135 expressed in *E. coli* BL21-Gold with His-Select Nickel Affinity Gel (Sigma) according to the manufacturer's instructions. After purification, the fractions containing His_6-CpxR protein were desalted and concentrated using Amicon Ultra centrifugal filter (Millipore).

Primer Extension

The primer extension assay was performed using primers 1472 for *amiA* and 1482 for *amiC* as described previously (160). Total RNA was isolated from bacterial cells grown in 5 ml of LB medium containing IPTG (0.25 mM) to $A_{600\ nm}$ 0.6 with RNAzol (Molecular Research Center) by following the manufacturer's instructions. Samples were analyzed by 6% denaturing polyacrylamide electrophoresis by comparison with DNA sequences amplified from *Salmonella* chromosome with primers ^{32}P-1472 and 1567 for *amiA*, or ^{32}P-1482 and 1484 for *amiC* and generated using Maxam and Gilbert A + G reactions.

Electrophoretic Mobility Shift Assay (EMSA)

Primers were labeled using T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P] ATP (PerkinElmer Life Sciences). Approximately 10 nmol of ^{32}P-labeled DNA fragments described in the text were incubated at room temperature for 30 min with increasing amounts of His_6-CpxR protein in 20 μl of an EMSA buffer consisting of 10

mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM DTT, 10 mMNaCl, 1 mM $MgCl_2$, and 5% glycerol. After the addition of the DNA dye solution (40% glycerol, 0.05% bromphenol blue, 0.05% xylene cyanol), the mixture was directly subjected to 4% polyacrylamide electrophoresis. Signals were detected by autoradiography.

DNase I footprinting assay

DNase I footprinting assays were carried out using DNA fragments amplified by PCR using BW25113 or 14028s chromosomal DNA as template. Before PCR, one primer for each set was labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P] ATP (Perkin Elmer) to yield a labeled coding strand and a labeled non-coding strand. Approximately 25 pmol of labeled DNA and increasing amounts of His_6-CpxR protein were mixed in a 100 µl reaction containing 2 mM HEPES pH8.0, 10 mM KCl, 20 µM EDTA, 0.5 mg ml^{-1} BSA, 20 µg ml^{-1} poly(dI-dC), 2% glycerol (161). The reaction mixture was incubated at room temperature for 20 min. Then, a DNaseI solution (10 mM $CaCl_2$, 10 mM $MgCl_2$, and 0.01 units of DNase I (Fermentas)) was added and the mixture was incubated at room temperature for 3 min. The DNase I digestion was stopped by phenol treatment and the DNA was precipitated. Samples were analyzed by 6% polyacrylamide electrophoresis by comparison with a DNA sequence ladder generated with the same primers using a Maxam and Gilbert A+G reaction. The site-directed mutagenesis of the *amiA* and *amiC* DNA fragments was performed by following a two-step PCR method described previously (162). The first step used the mutagenic primers and the reverse universal primer 1472 or 1482 flanking the 3′ end of the *amiA* or *amiC* promoter region. The mutagenic primers for CpxR box 1 mutation of *amiA* was 1595; while the CpxR box mutation of *amiC* was 1598. The second step used the product of the

first PCR as a primer and the forward universal primer 1567 or 1484 to yield the whole promoter region with the desired mutation. DNase I footprinting assay was then carried out as described above.

CHAPTER 1

IDENTIFICATION OF GENETIC LOCI REQUIRED FOR BACTERIAL RESISTANCE TO ANTIMICROBIAL PEPTIDES

Introduction

Antimicrobial peptides (AMPs) are products of the host innate immune system serving as a first line of defense for protection against microbial invaders (67). They exert their potent bactericidal activities against multiple pathogens, making them a promising alternative to conventional antimicrobial treatments. While there have been some reports describing the genetic basis for AMP resistance, research in the area has been a challenge due to the inability and unfeasibility to conduct large scale screens with sensitive, physiologically significant AMPs, as well as a lack of adequate genetic tools. In fact, previous screening efforts to identify genetic determinants required for resistance employed transposon-mediated mutant libraries in which transposons were randomly inserted into the chromosome (141). Otherwise, plasmid-mediated screens were used in which a plasmid library, constructed by randomly cleaving the chromosome and inserting fragments into an appropriate vector, was introduced into a Δ*phoP* background to identify DNA fragments that could rescue the AMP-susceptible phenotype (140). While feasible, both strategies were, however, limited by the likelihood of an incomplete library due to the possibility of uneven distribution of transposon insertion across the chromosome or insufficient DNA fragments inserted into the vector. As such, research in this area has remained limited. In fact, only a single two-component regulatory system, PhoP/PhoQ, had been implicated as a global regulator for bacterial resistance to AMPs, as well as the

PmrA/PmrB two-component system which can be activated by PhoP/PhoQ (139, 163, 164).

The availability of the Keio collection (154) containing ~4,000 defined *Escherichia coli* single gene deletion mutants provided an opportunity for a large scale, systematic screen. As such, this collection has successfully been used as a tool to identify loci required for resistance to various antibiotics (165-167). Therefore, the Keio collection was used in this study to individually challenge mutant strains against four AMPs representing three classes (summarized in **Table 3)**. The first, protamine, is a 32-amino acid AMP isolated from salmon sperm (90). Although it is not physiologically significant (i.e., its natural function is not to protect its host from invading pathogens), it is commonly used as a model AMP because it has been shown to kill *Salmonella* cells harboring mutations of virulence determinants, such as *phoP* (164). In lieu of peptides isolated from biologically significant organisms, such as defensins and magainins, protamine is commonly used amongst the scientific community because it allows for large-scale use due in part to its availability and relatively low cost, as well as its integrity under harsh laboratory conditions. It has been argued that analyses to understand bacterial resistance to AMPs using an AMP that is not naturally occurring are insignificant; therefore, it was necessary to analyze naturally occurring, and thus physiologically significant peptides. The second peptide used in the screen, RTD-2, is a θ-defensin isolated from Rhesus monkey, representing the β-sheet class of AMPs (79, 80). The high potency and insensitivity to physiological NaCl (79) compared to other characterized AMPs make RTD-2 a physiologically significant peptide and thus relevant for this study. The last class of

Table 3. Description of AMPs analyzed in this study.

AMP Name	AMP Type	AMP Class	AMP Sequence	Ref
Protamine	Arg-rich	extended	MPRRRRSSSRPVRRRRRPRVSRRRRRRGGRRRR	(90)
RTD-2	θ-defensin	β-sheet	GVCRCLCRRGVCRCLCRR	(79)
OH-CM6	cathelicidin	α-helix	KFFKKLKKAVKKGFKKFAKV	(75)
OH-CATH30	cathelicidin	α-helix	KFFKKLKNSVKKRAKKFFKKPRVIGVSIPF	(75)

AMPs used in the screen, α-helix structured peptides, are represented by two peptides: OH-CATH30, a cathelicidin characterized from king cobra, and its short-form analog, OH-CM6 (75). The potent efficacy and low host toxicity of these peptides (75) make them ideal candidates for therapeutic use and physiologically significant for this study.

Systematic screen to identify genes required for bacterial resistance to AMPs

Method for identifying genes required for bacterial resistance to AMPs. Systematic screens were carried out in parallel to identify gene loci required for resistance to 3 classes of AMPs, which allowed for identification of trends or common mechanisms amongst the AMPs. The premise was based on the notion that if a gene product is required for resistance to an AMP, then deletion of the gene should result in increased susceptibility to the AMP when compared to the isogenic wild-type. Nearly 4,000 *E. coli* single gene deletion mutant strains from the Keio collection (154) were challenged against each AMP to determine if deletion of individual loci could result in increased susceptibility. Challenges were carried out using standard laboratory conditions, i.e., cells cultured in LB broth with aeration at 37°C, spotted onto LB agar plates and incubated at 37°C overnight. These enriched conditions were used as opposed to defined media to avoid inadvertent bias towards a specific group of loci (e.g., PhoP-activating conditions in low Mg^{2+} conditions). Overall, the screen identified 112 mutants that were susceptible to at least one screened peptide (**APPENDIX D**): 11 mutants susceptible to RTD-2; 19 mutants susceptible to OH-CM6; 79 mutants susceptible to OH-CATH30; and, 32 mutants susceptible to protamine.

Table 4. Gene mutants susceptible to θ-defensin, RTD-2.

Functional Group	Gene Function	Gene Name
Enterobacterial Common Antigen (ECA) Synthesis	TDP-fucosamine acetyltransferase	*rffC*
	TDP-Fuc4NAc:lipidIIFuc4NAc transferase	*rffT*
DNA Repair	DNA strand exchange and recombination protein	*recA*
Membrane Protein	outer membrane protein A	*ompA*
	predicted peptidase, outer membrane lipoprotein	*spr*
Regulator	DNA-binding transcriptional dual regulator	*argR*
	DNA-binding regulator in TCS with CpxA	*cpxR*
	DNA-binding transcriptional dual regulator	*crp*
	predicted folate-dependent regulatory protein	*ygfZ*
Transporter	TatABCE protein translocation system subunit	*tatC*
Unknown Function	conserved protein	*yajD*

Systematic screen to identify genetic loci required for bacterial resistance to β-sheet structured θ-defensin, RTD-2. It has been argued that genetic screens using an AMP that is not naturally occurring are insignificant; therefore, a screen was carried out using a synthetic form of the naturally occurring θ–defensin, RTD-2, isolated from circulating leukocytes of the Rhesus monkey (79, 80). The synthetic form was found to be biochemically and functionally indistinguishable from the natural isolate; thus, it exhibits potent bactericidal and fungicidal activities as well as insensitivity to physiological NaCl compared to other physiologically significant AMPs (79). To identify genetic loci required for resistance to RTD-2, mutant strains of the Keio collection (154) were individually cultured overnight in LB broth medium in a 96-well format, re-inoculated (1:100) into fresh LB broth and grown with shaking at 37°C to log phase (approximately four hours). Approximately 10^3 cells were challenged with RTD-2 for 2 h with shaking at 37°C. Samples were diluted 1:10 and spotted onto LB agar plates and incubated overnight at 37°C. Mutant strains in which growth was inhibited, compared to wild-type, were selected for further analysis. The screen identified 11 sensitive mutant strains which represented an array of cellular functions, including enterobacterial common antigen biosynthesis, DNA repair, gene regulation, and protein transport (**Table 4)**. Notably, 4 out of the 11 loci encode a regulator (i.e., ArgR, CpxR, Crp, and YgfZ), suggesting an integrated regulatory approach to mediate bacterial resistance to the θ–defensin. Additionally, 9 of the 11 mutant strains were susceptible to at least one cathelicidin screened (**Figure 7**), suggesting a global contribution of their gene products to AMP resistance.

Systematic screen to identify genetic loci required for bacterial resistance to α-helix cathelicidins, OH-CATH30 and OH-CM6. An additional systematic screen was conducted to identify genetic loci required for resistance to a naturally occurring α-helix cathelicidin peptide, OH-CATH30, characterized from king cobra, and its short-form analog, OH-CM6 (75). The potent efficacy and low toxicity of these peptides make them suitable candidates for therapeutic use and therefore, physiologically significant for this study.The screen was carried out as described above using various concentrations of OH-CATH30 and OH-CM6. The short-form AMP, OH-CM6, was expected to have less susceptible candidates due to the higher minimum inhibitory concentration (MIC) values reported compared to OH-CATH30 when used against clinical bacterial isolates (75), perhaps due to the loss of residues that contribute to the bactericidal effect of the AMP. As predicted, the screen identified 19 OH-CM6-sensitive mutant strains and 79 OH-CATH30-sensitive mutant strains (**APPENDIX D)**.

Since OH-CM6 is a short-form isomer derived from OH-CATH30 (75), a significant overlap in the mutants susceptible to these cathelicidins was predicted. Indeed, 13 candidates (68% of OH-CM6 candidates and 16% of OH-CATH30 candidates) were sensitive to both cathelicidins (**Figure 7**, double line) and represent diverse cellular functions, including cell wall/envelope biogenesis, gene regulation, ion transport, and DNA/RNA synthesis/repair (**Table 5**). Cumulatively, this provides evidence that OH-CM6 contains a region of OH-CATH30 that allows it to exert its bactericidal activity because a significant overlap of OH-CM6-sensitive mutants (~68%) that was also sensitive to OH-CATH30 (**Figure 7**, double line). Conversely, these data also suggest that OH-CM6 lacks specific residues required for bactericidal activity because very few

Table 5. Gene mutants susceptible to both cathelicidins.

Functional Group	Gene Function	Gene Name
Cell Wall/ LPS/Fatty Acid Biosynthesis	PLP-dependent alanine racemase 2	*dadX*
	fatty acid oxidation complex subunit alpha	*fadB*
	TDP-fucosamine acetyltransferase	*rffC*
	TDP-Fuc4NAc:lipidIIFuc4NAc transferase	*rffT*
DNA/RNA/ Protein Biosynthesis/ Degradation	endonuclease IV	*nfo*
	DNA strand exchange/recombination protein	*recA*
	ssDNA exonuclease	*recJ*
	50S ribosomal subunit protein L32	*rpmF*
	endonuclease/exonuclease/phosphatase family	*xthA*
Regulator	DNA-binding transcriptional dual regulator	*feaR*
	manno(fructo)kinase	*mak*
	predicted DNA-binding transcriptional regulator	*yijO*
Respiration	predicted iron-sulfur protein in electron transport	*rsxB*
Transporter	citrate:succinate antiporter	*citT*
	gluconate transporter	*ddpX*
	hexuronate transporter	*exuT*
	potassium translocating ATPase, subunit A	*kdpA*
	manganese/divalent cation transporter	*mntH*
Unknown Function	predicted protein	*yehM*

(13 of the 79 mutants, 16%) OH-CATH30-sensitive mutants were also sensitive to OH-CM6 (**Figure 7**, double line). To further characterize the bactericidal actions of these AMPs, a concentration-dependent challenge against wild-type, BW25113, was carried out to determine the MIC of each AMP. In agreement with previous reports (75), OH-CATH30 had a lower MIC of 1 μg mL^{-1} than OH-CM6 at 2 μg mL^{-1} (**Figure 1**).

Systematic screen to identify genetic loci required for bacterial resistance to model AMP, protamine. Although protamine is not considered physiologically significant, it is commonly used as a model AMP because it has been shown to kill *Salmonella* cells harboring a *phoP* muatant (164) and allows for large-scale use due in part to its availability and relative low cost, as well as its integrity under harsh laboratory conditions. Once again, mutants of the Keio collection (154) were individually cultured overnight in LB broth in a 96-well format, re-inoculated (1:100) into fresh LB broth and grown with shaking at 37°C to log phase (approximately four hours). Samples were diluted 10^5 times and spotted onto LB agar plates supplemented with no or various amounts of protamine and incubated overnight at 37°C. Mutant strains in which growth was inhibited, compared to wild-type, were selected for further analysis. A total of 32 protamine sensitive mutant strains were identified which represented a multitude of cellular functions, including respiration, biosynthesis and metabolism, cell wall/envelope biogenesis, DNA/RNA synthesis/repair, and stress response (**Table** 6). Notably, 7 out of the 32 (~28%) protamine sensitive candidates had overlapping sensitivities to other AMP classes (**Figure 7**), suggesting that protamine can be used as a tool to ascertain the bactericidal effects of AMPs. Therefore, a series of assays was conducted using

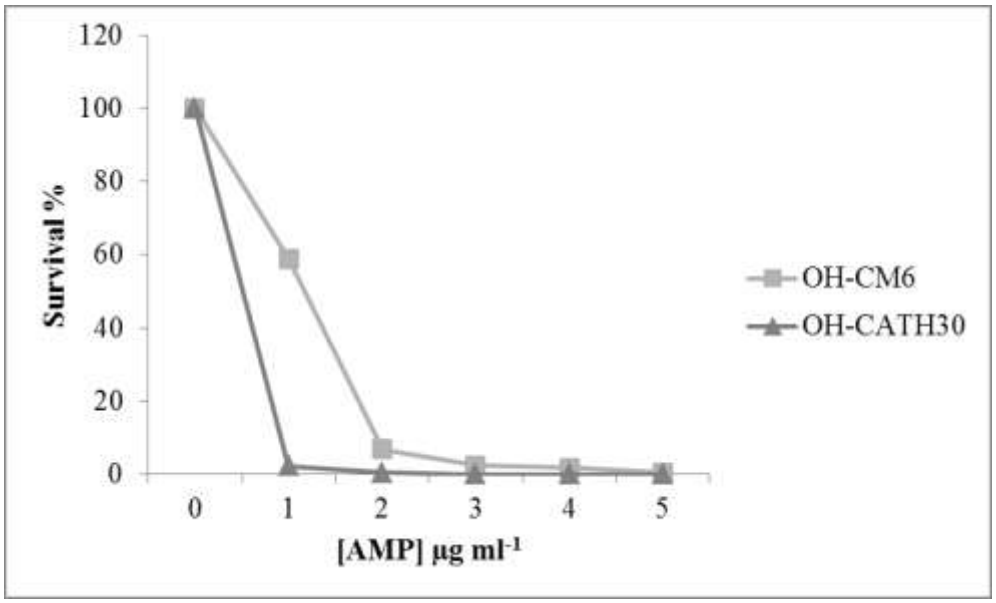


Figure 1. Dose-dependent killing by cathelicidins.
Dose-dependent killing of *E. coli* wild-type (BW25113) cells by cathelicidins OH-CM6 and OH-CATH30 demonstrate that OH-CATH30 has higher bactericidal activity due to the lower MIC, 1 µg ml^{-1}, compared to the short-form isoform OH-CM6 with a MIC of 2 µg ml^{-1}.

Table 6. Gene mutants susceptible to protamine.

Functional Group	Gene Function	Gene Name
ATP Synthase Subunits	F0 sector of membrane-bound ATP synthase, subunit a	*atpB*
	F1 sector of membrane-bound ATP synthase, epsilon subunit	*atpC*
	F1 sector of membrane-bound ATP synthase, gamma subunit	*atpG*
Biosynthesis/ Metabolism	shikimate kinase I; catalyzes step in chorismate biosynthesis	*aroK*
	phosphomannomutase/phosphoglucomutase	*cpsG*
	alkaline phosphatase homolog	*gpmI*
Cell Wall/ LPS Biosynthesis	N-acetylmuramoyl-L-alanine amidase	*amiC*
	protease with a role in cell division	*envC*
	heat shock protein acyltransferase	*lpxL*
	myristoyl-acyl carrier protein-dependent acyltransferase; *htrB* suppressor	*lpxM*
	ADP-L-glycero-D-manno-heptose-6-epimerase	*rfaD*
	Inner membrane protein of unknown function	*yqjA*
Enterobacterial Common Antigen (ECA) Synthesis	UDP-N-acetylglucosamine:undecaprenyl-phosphate GlcNAc-1-phosphate transferase	*rfe*
	TDP-fucosamine acetyltransferase	*rffC*
	glucose-1-phosphate thymidylyltransferase	*rffH*
	TDP-Fuc4NAc:lipidIIFuc4NAc transferase	*rffT*
PLP-dependent	pyridoxine 5'-phosphate (PLP) oxidase	*pdxH*
	predicted enzyme that binds PLP	*yggS*
	predicted diguanylate cyclase	*yhjK*
Protein Folding	monomeric thiol disulfide oxidoreductase	*dsbA*
	oxidoreductase for reoxidation of DsbA	*dsbB*
RNA/DNA Synthesis/ Degradation	AMP nucleosidase activated by PhoB	*amn*
	ATP-dependent RNA helicase	*deaD*
	chaperone Hsp70; DNA biosynthesis	*dnaK*
Stress Response	Polynucleotide phosphorylase that protects against damage	*pnp*
	Heat shock response chaperone; protease	*degP*
	SoxRS-regulated glucose-6-phosphate dehydrogenase	*zwf*
Transporters/ Pores	cytoplasmic component of protein translocase	*secB*
	Sec-independent protein translocase protein	*tatC*
	outer membrane channel protein	*tolC*
	betaine-choline-carnitine-transporter	*yeaV*
	putative outer membrane protein	*yedS*

protamine as the model AMP to characterize mutant strains identified in the screen and explore the mechanisms in which specific loci contribute to resistance.

Characterization of select loci required for bacterial resistance to protamine

The role of ATP production in resistance to protamine. Among the mutant strains susceptible to protamine were *atpB, atpC,* and *atpG,* that encode the a, ε, and γ subunits of the F_1F_0-ATP synthase, respectively. Subsequent analysis demonstrated that deletion of any ATP synthase-encoding loci, except *atpE* and *atpA* (not tested), resulted in increased susceptibility to protamine when compared to the wild-type (**Figure 2, left**). This observation generated the hypothesis that the F_1F_0-ATP synthase is required for *E. coli* resistance to protamine which is contrary to reports that microcin H47 requires ATP synthase for its activity (168) or that various cationic AMPs can bind to the ATP synthase and inhibit its activity (169).

The F_1 sector of the ATP synthase catalyzes the reversible processes of ATP hydrolysis and synthesis while the F_0 sector facilitates proton transport through the inner membrane. Therefore, it was necessary to determine whether the synthesis/hydrolysis of ATP or the transport of protons through the membrane was required for resistance to protamine. Wild-type *E. coli* cells were challenged with sodium azide (a well-established inhibitor of the electron transport chain (ETC) which inhibits cytochrome oxidase activity), protamine, and both sodium azide and protamine to determine if inhibition of the ETC would increase susceptibility to protamine. On the contrary, bacteria were able to survive when challenged with protamine in the presence of sodium azide, similarly as the non-treated cells (**Figure 2, right**), indicating that inhibition of ETC does not influence susceptibility to protamine and perhaps the generation of ATP was required. It was

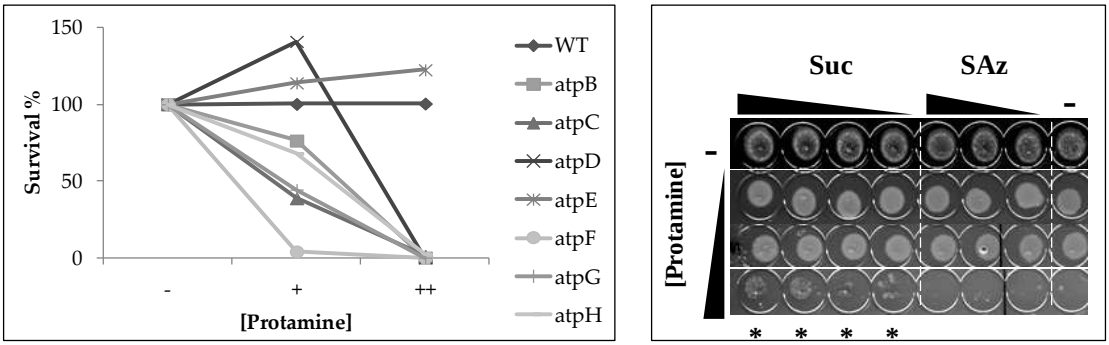


Figure 2. Effect of ATP synthesis on resistance to protamine.
(**left**) Dose-dependent killing of *atp* mutants show that each mutant assayed, except for *atpE*, was susceptible to protamine. (**right**) Succinic acid (Suc) is able to increase *E. coli* wild-type resistance to protamine (indicated with *) while sodium azide (SAz) has no effect, compared to the untreated cells (-).

therefore postulated that protamine could impede, however not abolish, cellular respiration at the glycolysis level. This notion is supported by the following observations: (i) Deletion of *zwf*, which encodes the glucose-6-phosphate dehydrogenase (G6PD), results in increased susceptibility to protamine (**APPENDIX D**). G6PD initiates the first step in the pentose phosphate pathway (PPP), an alternative pathway used instead of glycolysis to generate NADPH and ATP from a glucose substrate. This suggests that protamine can target any step in glycolysis between the first step, conversion of glucose to glucose-6-phosphate, and an intermediate step, conversion of fructose-6-phosphate to fructose 1,6-bisphosphate because the PPP shunt provides an alternative route to produce fructose-6-phosphate, after which, the PPP reconvenes with the glycolysis pathway (illustrated **Figure 3**). Thus, protamine should impede the traditional glycolysis pathway and inhibit the production of NADH and pyruvate which feed into the next step of respiration, the tricarboxylic acid (TCA) cycle. However, cells are able to bypass this putative impedance of protamine by using the PPP which serves as a shunt to utilize glucose and produce NADPH and ATP. Thus, in the absence of *zwf*, the shunt is not available resulting in the inability to generate ATP, as well as NADH and pyruvate. These byproducts are required to continue the process of cellular respiration, thus deletion of *zwf* in the presence of protamine would ultimately inhibit production of ATP by oxidative and substrate-level phosphorylation methods. (ii) Deletion of the F_1F_0-ATPase (which abolishes the major contributor to ATP production via oxidative phosphorylation) increases *E. coli* susceptibility to protamine, presumably due to the inefficient production of ATP from substrate-level phosphorylation. In other words, deletion of the major ATP-generating system would be detrimental in the presence of

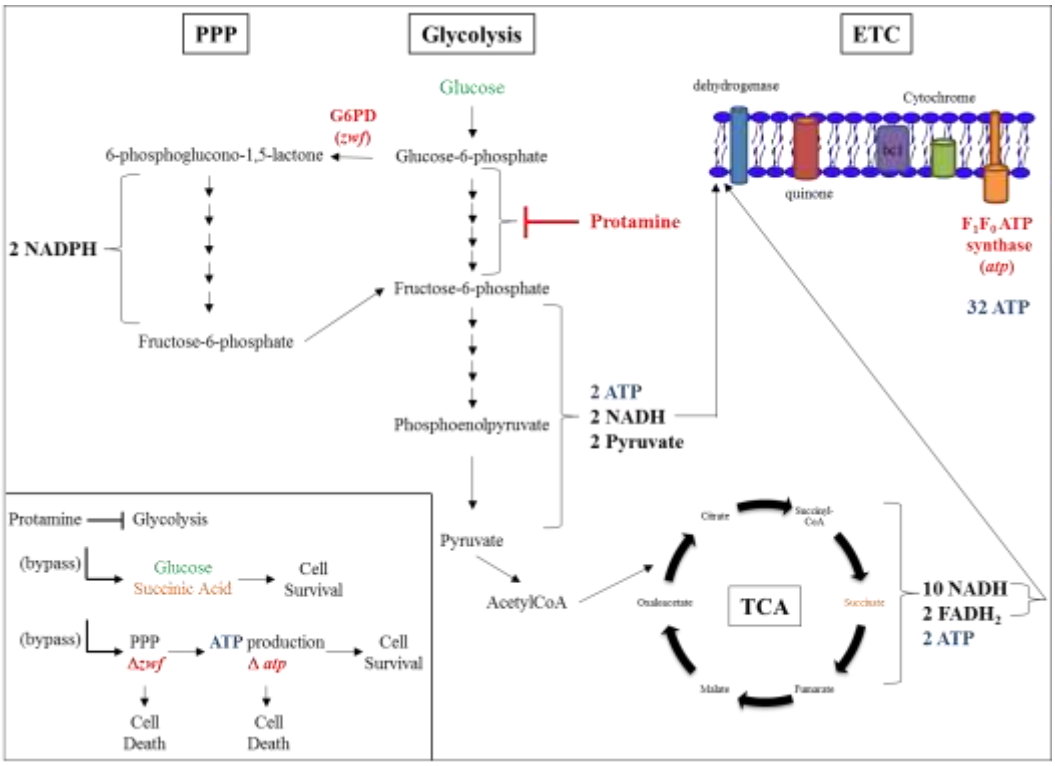


Figure 3. Illustration of the glycolysis and pentose phosphate pathways.
Protamine is hypothesized to impede the glycolysis pathway between the glucose-6-phosphate and fructose-6-phosphate intermediate steps. Cells are able to bypass this impedance by the pentose phosphate pathway (PPP) which provides an alternative route to produce fructose-6-phosphate. Thus, cellular respiration is allowed to continue through the tricarboxylic acid cycle (TCA) and electron transfer chain (ETC) to generate ATP. In the absence of the glucose-6-phosphate dehydrogenase (G6PD), encoded by *zwf*, cells become susceptible to protamine likely due to the inability to generate sufficient amounts of ATP. Likewise, in the absence of the F_1F_0-ATP synthase, encoded by *atp* loci, cells become susceptible to protamine likely due to the inability to generate sufficient amounts of ATP. Additionally, cells can bypass the impedance of protamine by increasing substrate amounts, i.e., supplementing glucose or succinic acid, to presumably increase the production of ATP.

protamine which is impeding the relatively low production of ATP via glycolysis/PPP. This analysis is supported by the ability of glucose supplementation to rescue the susceptibility of *atp* mutant cells to protamine (data not shown), which would presumably increase the production of ATP via substrate-level phosphorylation by glycolysis/PPP. (iii) Supplementation of formate or succinate, organic acids oxidized in the TCA cycle (which would increase the production of ATP via substrate-level phosphorylation), is able to protect bacterial cells from the oxygen consumption inhibitory effects of human bactericidal/permeability-increasing protein (BPI) and increase bacterial survival in the presence of BPI (170) and protamine (**Figure 2, right**). Cumulatively, these data support the hypothesis in which protamine, and likely other AMPs, can impede cellular respiration which can ultimately lead to cell death due to the inhibition of ATP production (illustrated in **Figure 3**).

Protamine susceptible mutants with defective outer membranes. To continue analysis of the loci identified in the primary screen, the susceptibilities of the mutants to various antibiotics and compounds were determined in order to ascertain the physiological condition of the mutant cells. Sodium dodecyl sulfate (SDS) and vancomycin (VAN) were used as tools to detect any deficiencies in the outer membrane. Deletion of *envC, pnp, amiC, lpxL,* and *tatC* were more susceptible to VAN than the isogenic wild-type (**Figure 4**). Similarly, deletion of *envC, pnp, amiC, rfaD,* and *tolC* were more susceptible to SDS than the isogenic wild-type (**Figure 4**). While a *tolC* deletion mutant is known to be sensitive to SDS due to the inability to pump the drug out of the cell (171), it is likely that the other mutant strains have defects in their outer membranes which likely increases permeability allowing certain compounds to enter the cell that would normally be

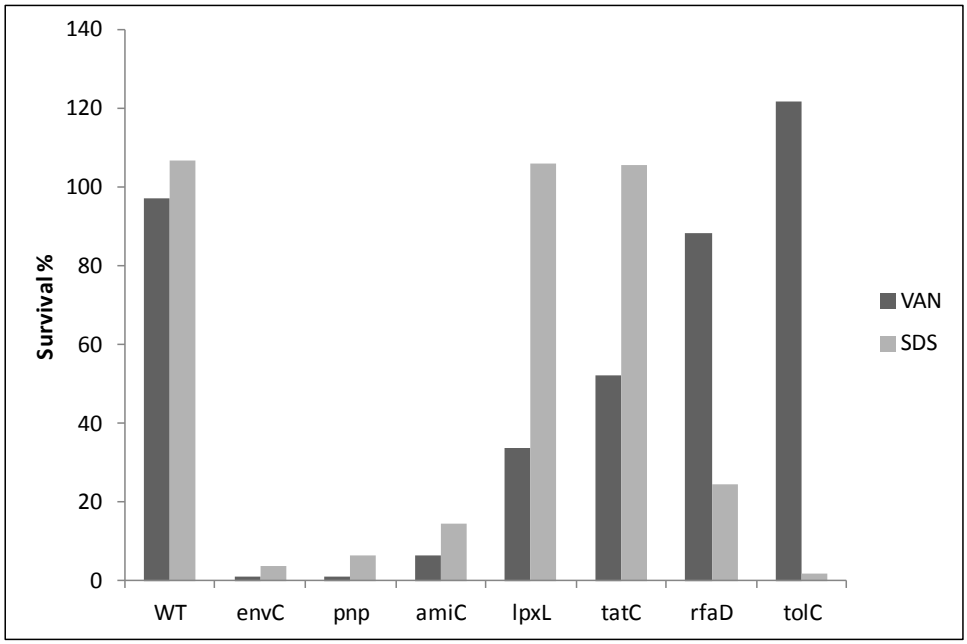


Figure 4. Gene mutant strains with increased drug sensitivity.
Several protamine sensitive mutants are sensitive to vancomycin (VAN) and sodium dodecyl sulfate (SDS) inferring an increase in membrane permeability.

prevented by an intact outer membrane. Notably, deletion of *tatC* has previously been shown to have a defective outer membrane and thus increased susceptibility to VAN (15, 52, 53).

Analysis of TolC-dependent resistance to protamine. Deletion of *tolC*, which encodes an outer membrane protein involved in drug efflux, was found to result in increased susceptibility to protamine (**APPENDIX D**), suggesting a role of drug efflux systems in bacterial resistance to AMPs. TolC represents the outer membrane component of the classic AcrAB-TolC tripartite resistance-nodulation-division (RND) efflux pump in *E. coli* which facilitates the efflux of various classes of compounds out of the bacterial cell (discussed in (172)). It is therefore not surprising that deletion of *tolC* renders cells sensitive to AMPs since previous efforts have demonstrated the role of RND-type efflux pumps in bacterial resistance to AMPs in *E. coli* (44), *Neisseria gonorrhoeae* (173), *Neisseria meningitidis* (174), *Campylobacter* (175), *Pseudomonas* (176, 177), *Yersinia* (178), and *Helicobacter* (179). While deletion of *tolC* resulted in increased susceptibility to protamine, deletion of *acrA* or *acrB* also increased susceptibility (**Figure 5**), compared to the isogenic wild-type, suggesting that an additional TolC-dependent efflux system could mediate bacterial resistance to protamine.

TolC is often considered a promiscuous protein as it has many binding partners with other pumps, including partners belonging to other classes of efflux pumps (reviewed in (180)). Therefore, I sought to identify the partner(s) wtih which TolC coordinates to confer resistance to protamine on *E. coli*. Mutants of each family of drug transporter systems including *acrA, acrB, acrD, acrE, acrF, mdtE,* and *mdtF* (which belong to the

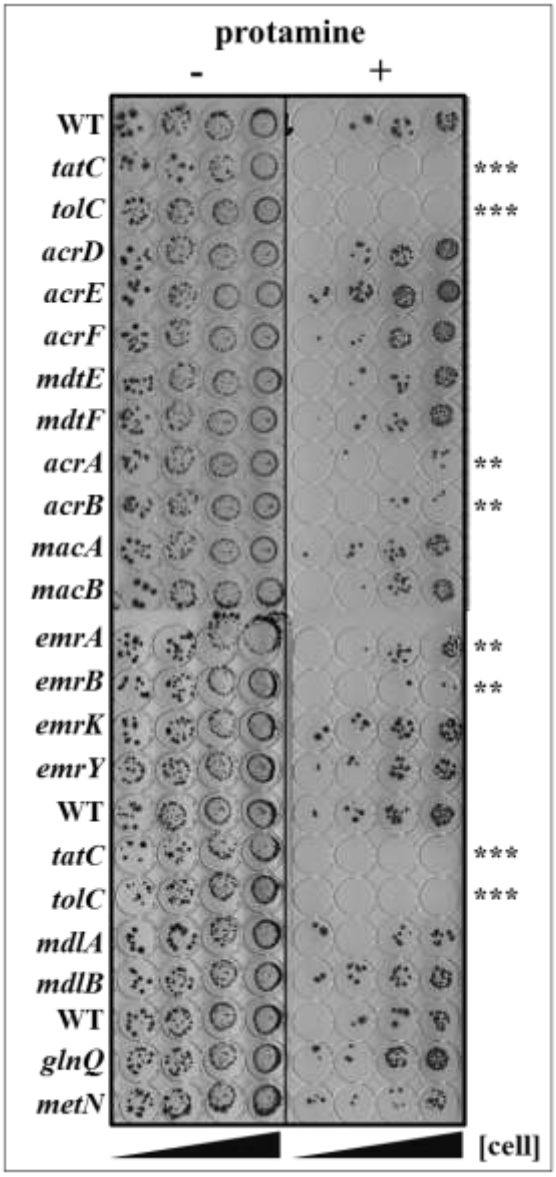


Figure 5. Protamine sensitivity assay of TolC-dependent mutants.
Protamine sensitivity assay of mutant strains of genes encoding TolC-dependent efflux pumps. Deletion of *acrA, acrB, emrA,* and *emrB* render cells more susceptible (**) to protamine when compared to the isogenic wild-type, but not as susceptible as Δ*tolC* or Δ*tatC* controls (***).

RND family of drug transporter systems), *emrA*, *emrB*, *emrK*, and *emrY* (which belong to the major facilitator superfamily (MFS) of drug transporter systems), *macA* and *macB* (which belong to the ATP-binding cassette (ABC) family of drug transporter systems), and *msbA*, *mdlA*, *mdlB*, *cydC*, *glnQ*, and *metN* (which encode HlyB homologs of 34%, 31%, 28%, 32%, 36%, and 34% sequence similarity, respectively, and belong to the ABC family of drug transporter systems) were individually challenged against protamine to determine if deletion of any gene could result in increased susceptibility to protamine when compared to wild-type. Indeed, deletion of *acrA*, *acrB*, *emrA*, and *emrB* resulted in increased susceptibility to protamine when compared to the isogenic wild-type; however, these mutants lower susceptibility when compared to the *tolC* deletion mutant (**Figure 5**). Thus, double mutants were constructed to determine if an additive effect was observed due to the contribution of multiple efflux systems in resistance to protamine. Double mutants containing either *acrB* or *emrB* along with a representative of an additional efflux system were constructed and challenged against protamine. As expected, deletion of *acrB* and *emrB* resulted in increased susceptibility compared to either individual mutant, however, lower susceptibility when compared to the *tolC* deletion mutant (**Figure 6**). Additionally, deletion of *acrB* along with any other efflux representative (i.e., *acrE*, *emrY*, *glnQ*, *macB*, *mdtF*, or *metN*), but not *mdlB*, resulted in increased susceptibility to protamine compared to any single mutant; however, none of these was as susceptible as the *tolC* mutant (**Figure 6**).

A final set of mutants were constructed in which three genes (two of which were *acrB* and *emrB*) representing independent efflux systems were deleted in a single strain to determine if a cumulative effect could be observed when challenged against protamine.

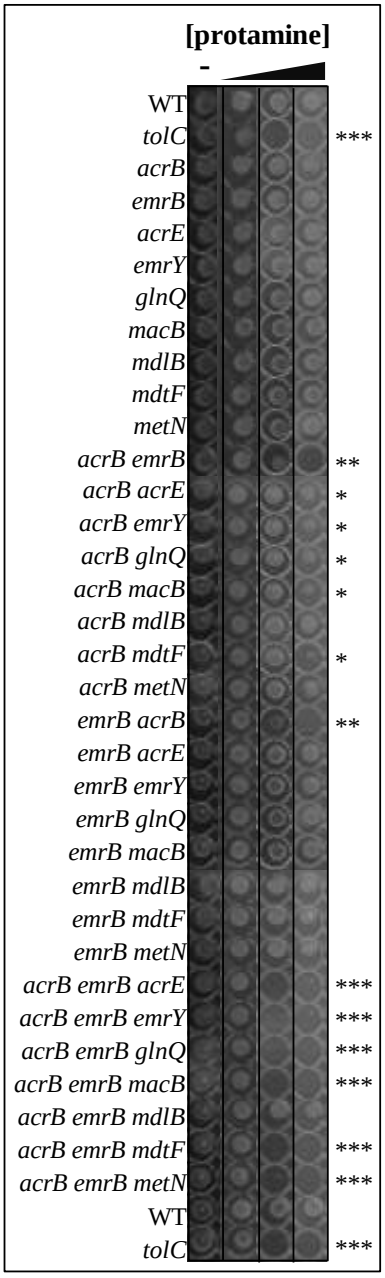


Figure 6. Protamine sensitivity assay of TolC-dependent double mutants.
Protamine sensitivity assay of double and triple mutant strains of genes encoding TolC-dependent efflux pumps. An *acrB emrB* double deletion renders cells more susceptible to protamine when compared to either single deletion, however, not as susceptible as a *tolC* deletion. Deletion of a third TolC-dependent system (i.e., *acrE, emrY, glnQ, macB, mdtF,* or *metN)* renders triple mutant cells as sensitive to protamine as a *tolC* deletion, except for simultaneous deletion of *mdlB* in an *acrB emrB* background.

Not surprisingly, deletion of any other efflux system representative, except for *mdlB*, along with simultaneous deletion of *acrB* and *emrB* resulted in increased susceptibility to protamine similar to the *tolC* deletion (**Figure 6**), suggesting a global role of TolC-dependent efflux systems in resistance to protamine. Cumulatively, these data suggest that the AcrA/AcrB and EmrA/EmrB systems are major efflux contributors to bacterial resistance to protamine, while the other systems, except for MdlB, play a modest role only in the absence of AcrA/B or EmrA/B. Although protamine has a relatively large molecular weight (~4,000 Da) compared to characterized substrates of TolC-dependent efflux systems, it is possible that TolC mediates the transport of protamine out of the cell as it is able to do for other large compounds such as haemolysin and colicins (181) and since AMPs have been demonstrated to be substrates of RND pumps in various bacterial species (44, 175-179).

Characterization of loci required for bacterial resistance to multiple antimicrobial peptides

Analysis of candidates susceptible to two or more AMPs. Surprisingly, a majority of the mutant strains identified in each screen were sensitive to only a single AMP. This suggests that (i) the gene indirectly contributes to resistance or (ii) each AMP exerts its bactericidal activity via different mechanisms. Overall, the screen identified 112 mutants that were susceptible to at least one screened peptide (**APPENDIX D**); 24 (~21%) of those mutants were found to be susceptible to two or more screened AMPs (**Figure 7**), while 13 (~12%) were susceptible to two or more classes of AMPs (**Figure 7**, solid line) suggesting a global contribution of their gene products to bacteria resistance to AMPs.

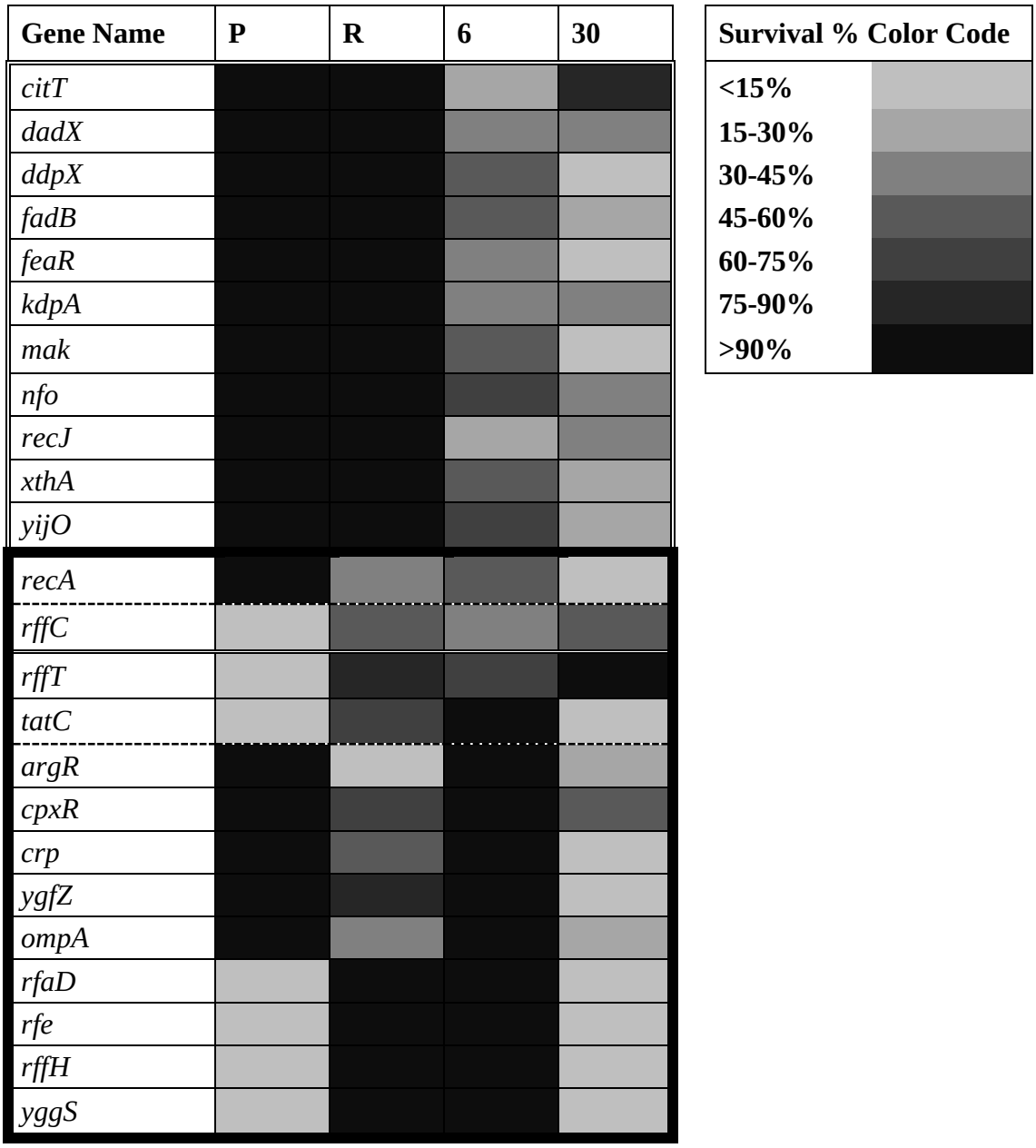


Figure 7. Gene mutants found to be susceptible to 2 or more AMPs.
Heat map illustrating gene mutants found to be susceptible to 2 or more AMPs in the screen.Thick line, mutants susceptible to 2 or more classes of AMPs; double line, mutants susceptible to both cathelicidins; dashed line, mutants susceptible to all classes of AMPs analyzed; *P*, protamine; *R*, RTD-2; *6*, OH-CM6; *30*, OH-CATH30.

Moreover, only three mutants (Δ*rffC*, Δ*rffT*, and Δ*tatC*) were susceptible to all three classes screened (**Figure 7**, dashed line); thus, these genes were further analyzed.

Analysis of candidates susceptible to each AMP class. Deletion of *tatC* rendered *E. coli* cells susceptible to each class of AMPs analyzed in this study. Specifically, cells were susceptible to protamine, θ-defensin RTD-2, and cathelicidin OH-CATH30 (**Figure 7**). The *tatC* gene encodes a member of the twin arginine transport (Tat) system that transports globular proteins across the cytoplasmic membrane (45). Since *tatC* encodes an integral component of a transport system, it was hypothesized that a Tat-dependent substrate, and not the Tat system itself, is directly responsible for bacterial resistance to AMPs. Indeed, the transport of peptidoglycan amidases AmiA and AmiC to the periplasm was determined to be required for bacterial resistance to protamine (demonstrated in Chapter 2, (15)).

Deletion of *rffC* and *rffT* rendered *E. coli* cells susceptible to each class of AMPs analyzed in this study. Specifically, the Δ*rffC* mutant was susceptible to each AMP while the Δ*rffT* mutant was susceptible to protamine, θ-defensin RTD-2, and cathelicidin OH-CAM6 (**Figure 7**). The *rffC* and *rffT* genes encode enzymes involved in the conversion of lipid II to lipid III in biosynthesis of the enterobacterial common antigen (ECA) (182). It was previously reported that deletion of *rffA* (*wecE*), *rffT* (*wecF*), and *rffH* (*rmlA*), which encode enzymes involved in the conversion of lipid II to lipid III, increased *E. coli* sensitivity to bile salts as they were unable to grow on MacConkey agar plates (55). The authors postulated that the accumulation of the lipid II intermediate was directly responsible for the increased susceptibility since deletion of *rfe* could rescue

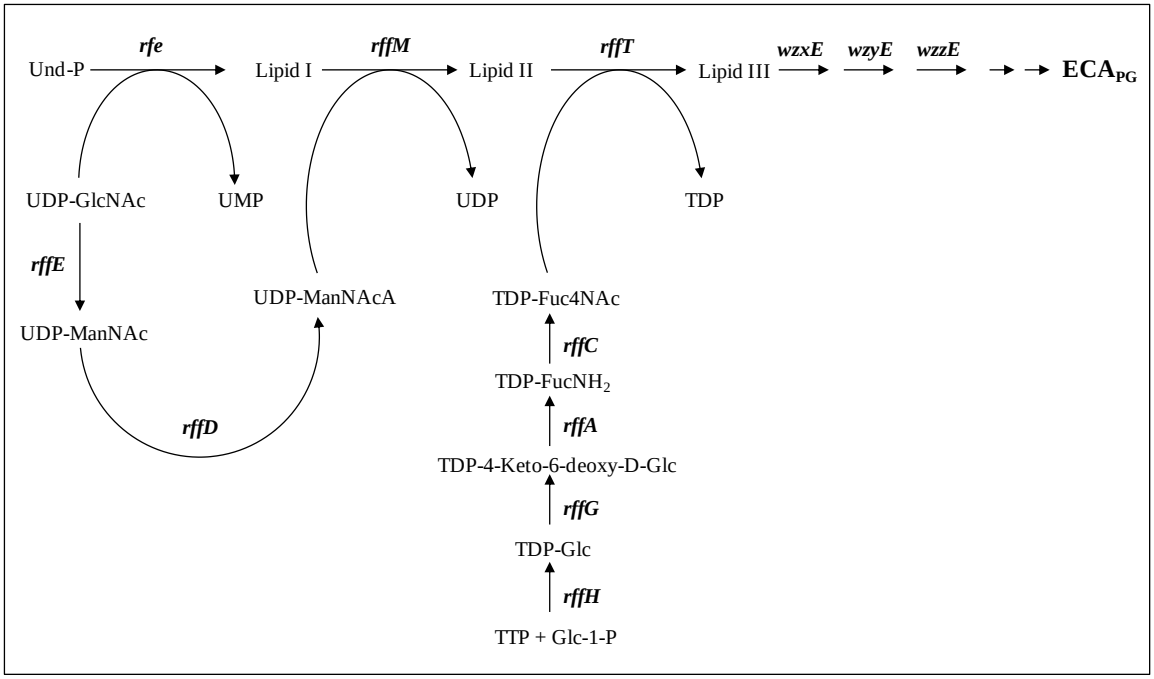


Figure 8. Enterobacterial common antigen biosynthesis pathway.
Illustration of the biosynthesis of the enterobacterial common antigen (adapted from (55)).

susceptibility (55). Therefore, it was hypothesized that accumulation of lipid II in the *rffC* and *rffT* mutants is responsible for the susceptibility to AMPs.

Characterization of the role of the *rfe-rff* gene cluster in resistance to antimicrobial peptides

Analysis of the rfe-rff gene cluster in resistance to AMPs. Members of the *rfe-rff* operon encode enzymes required for the biosynthesis of ECA (illustrated in **Figure 8**). If accumulation of lipid II was the cause of the increased susceptibility to AMPs, then deletion of other loci in the *rfe-rff* gene cluster should not be sensitive to AMPs as their gene products function elsewhere in the biosynthesis pathway. A more stringent AMP killing assay was conducted in which deletion of individual members of the *rfe-rff* gene cluster were challenged with the model AMP, protamine. Deletion at any locus, except *rffG* and *wzzE*, resulted in increased susceptibility to protamine when compared to wild-type (**Figure 9**, **left**). This suggested the following: (i) lack of ECA, and not the accumulation of lipid II or any other intermediate, is responsible for the increased susceptibility to AMPs; and, (ii) RffG and WzzE likely have functional homologs that can rescue the ECA biosynthesis when these enzymes are absent. Indeed, RffG and RfbB (also a dTDP-glucose 4,6-dehydratase) have a 74% amino acid similarity, while WzzE has a 24% and 23% amino acid similarity with Cld and FepE (both also polysaccharide chain length modulation proteins), respectively, suggesting possible redundant or overlapping functions. Indeed, a Δ*rffG* Δ*rfbB* double mutant resulted in increased susceptibility to protamine, while Δ*wzzE* Δ*cld* and Δ*wzzE* Δ*fepE* exhibited no increase in susceptibility (**Figure 9, right**).

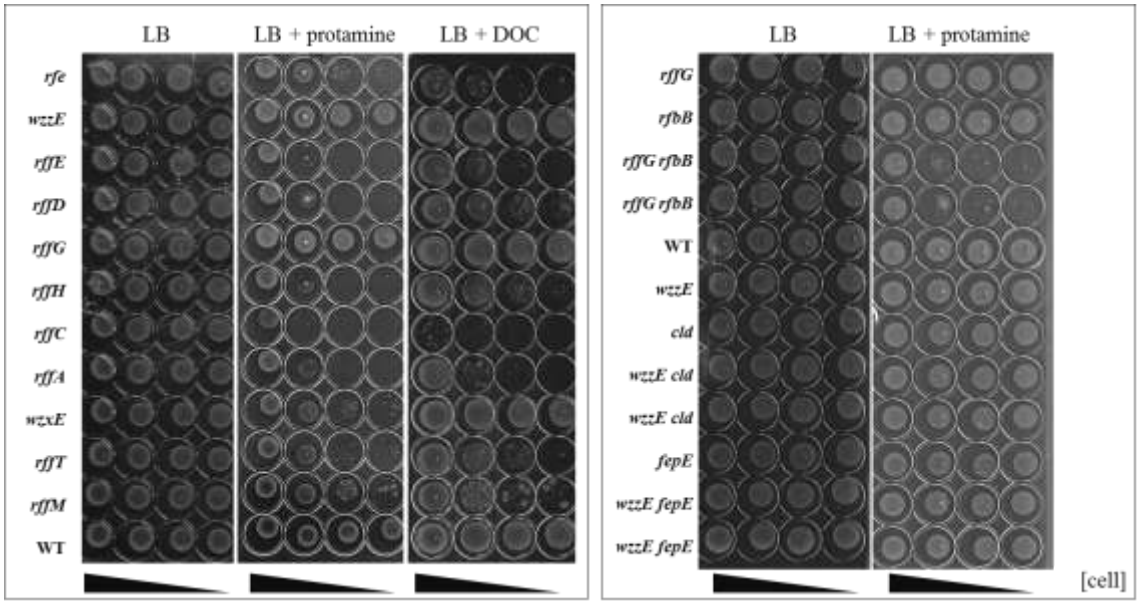


Figure 9. Susceptibility profile of mutants of the *rfe-rff* gene cluster.
(left) Susceptibility profile of the *rfe-rff* gene cluster demonstrates that ECA is required for resistance to protamine and deoxycholate (DOC). (**right**) Double deletion of *rffG* and *rfbB* (*rffG rfbB*) makes cells susceptible to protamine.

Analysis of the rfe-rff gene cluster in resistance to bile salts. The observation that deletion of genes encoding enzymes required at any step of the ECA biosynthesis pathway resulted in increased susceptibility to protamine prompted a re-investigation into the role of ECA in resistance to bile salts. Again, it was previously determined that deletion of *rffA* (*wecE*), *rffT* (*wecF*), and *rffH* (*rmlA*) resulted in increased sensitivity to bile salt presumably due to the accumulation of the lipid II intermediate (55). However, another group demonstrated that deletion of *Salmonella rfe* (*wecA*) and *rffC* (*wecD*) caused sensitivity to the bile salt deoxycholate (57), thus dismissing the notion that lipid II accumulation caused sensitivity to bile salt since deletion of *rfe* abolishes the initiation of ECA biosynthesis. To support these findings, deletion of individual members of the *rfe-rff* gene cluster were challenged with deoxycholate. Deletion at any locus, except *rffG*, *wzzE*, and *wzxE* resulted in increased susceptibility to deoxycholate when compared to wild-type (**Figure 9, left**), further demonstrating that the lack of ECA, and not the accumulation of intermediates, is responsible for the susceptibility to bile salts.

Deletion of ECA does not alter membrane permeability. The possibility that deletion of ECA could indirectly influence the susceptibility of bacteria to antimicrobials such as AMPs and bile salt was acknowledged. It is documented that alterations to cell wall and cell envelope components can increase membrane permeability (183) making cells more susceptible to various compounds, including sodium dodecyl sulfate (SDS) and vancomycin (VAN). With this in mind, survival assays were conducted with individual mutants of the *rfe-rff* gene cluster to determine if mutation would affect the sensitivity to SDS or vancomycin which would suggest a defect in the outer membrane. Notably, there was no increase in susceptibility to either compound compared to wild-type (**Figure 10**

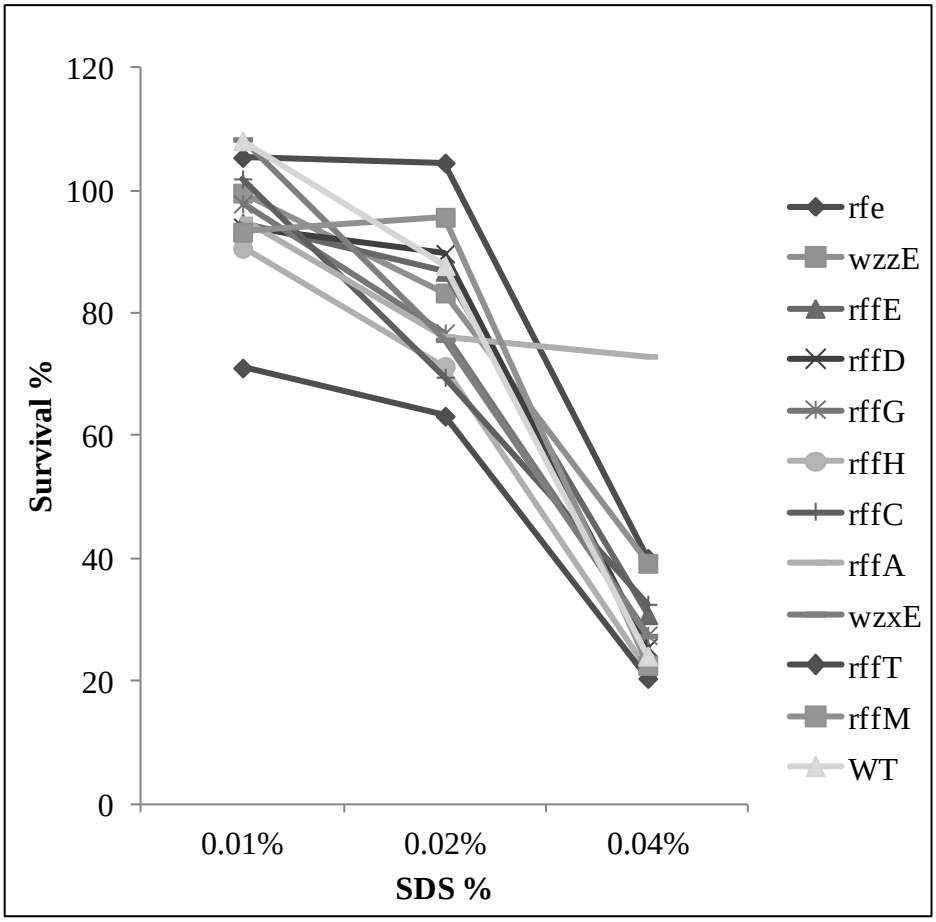


Figure 10. SDS survival assay of *rfe-rff* gene cluster mutants.
SDS survival assay of gene mutants of the *rfe-rff* gene cluster.

and data not shown), suggesting that deletion of ECA does not affect membrane permeability, thus inferring that it is the lack of ECA that is directly responsible for the increased susceptibility to AMPs and bile salts.

The physiological role of ECA remains to be elucidated. Interestingly, Δ*rfe* and Δ*rffC* mutants were highly attenuated in a mouse model when orally inoculated, suggesting a protective role of ECA in virulence; and also intraceullular survival by protecting bacteria from bile salts (57) and AMPs (**Figure 9**). Therefore, understanding the means by which inhibition of ECA biosynthesis, or accumulation of its intermediates, confers sensitivity to bile salts and AMPs remain elusive. It has been postulated that the incomplete trisaccharide may impede the biosynthesis of the cell envelope near the site of ECA biosynthesis which may make the outer membrane more permeable (55). However, the data presented suggest otherwise due to the lack of sensitivity to SDS and vancomycin (**Figure 10** and data not shown). Another theory suggests that protamine can act on *E. coli* similar to the way the AMP nisin acts on Gram-positive bacteria by binding to lipid II. It is possible that the accumulation of the ECA intermediates can increase the negative charge of the outer membrane resulting in increased electrostatic attraction between the AMP and cell membrane. While this theory may be correct, it does not explain why inhibition of ECA biosynthesis altogether by deletion of *rfe* results in increased susceptibility to protamine or deoxycholate (**Figure 9**, (57)).

It is plausible that the susceptibility to AMPs caused by deletion of *rfe* is due in part to the absence of ECA, but also due to the absence of the LPS O-antigen since Rfe is also required for biosynthesis of the O-antigen (182, 184)**.**

Conclusion

Overall, the systematic screens were successful in identifying genetic loci required for bacterial resistance to multiple AMPs. The identification of these loci can now serve as a tool to identify novel genetic circuitries required for resistance and likely other physiologically significant processes. Interestingly, data from the screen support postulations that AMPs work by different mechanisms while also describing some overlapping features. Specifically, the identification of the enterobacterial common antigen as a protector for antimicrobial substances is an important finding as it further posits a physiological role for ECA in survival and adaptation in host environments. Furthermore, the identification of these loci has served as a basis for the work described in the upcoming chapters.

CHAPTER 2

IDENTIFICATION OF TAT-DEPENDENT SUBSTRATES REQUIRED FOR BACTERIAL RESISTANCE TO ANTIMICROBIAL PEPTIDES

Introduction

The phenotypic screens carried out in Chapter 1 indicated that the *tatC* locus is required for bacterial resistance to antimicrobial peptides (AMPs) since deletion of *tatC* resulted in susceptibility to each AMP class analyzed (**Figure 7**). Wild-type resistance could be restored by expression of a plasmid (pBAD-*tatC*) harboring a wild-type copy of *tatC* and not a vector only (pBAD) control (**Figure 11)**. This suggests that *tatC*, which encodes an integral component of the twin-arginine transporter (Tat) system, a Sec-independent system responsible for the transport of globular proteins across the inner membrane (45), has a global role in bacterial resistance to AMPs. The Tat system, encoded by the chromosomal loci *tatA*, *tatB*, and *tatC*, has many characterized substrates with diverse cellular functions, including ion acquisition, energy metabolism, cell wall biosynthesis, and virulence (reviewed in (47)). Since *tat* encodes a transporter, it was hypothesized that a Tat-dependent substrate(s) is directly required for bacterial resistance to AMPs.

Identification of Tat-dependent substrates required for bacterial resistance to AMPs

To identify the possible Tat-dependent substrate(s) required for resistance to AMPs, a more stringent screen was conducted using *Escherichia coli* mutants whose gene products encode one of the approximately 40 proteins that have been experimentally proven or hypothesized to be transported by the Tat system (**Table 7**). Deletion of either *amiA* or *amiC* resulted in an increase in protamine susceptibility when compared to wild-type, but was not as susceptible as Δ*tatC*. Δ*amiA* and Δ*amiC* mutants were constructed in

Table 7. Analysis of Tat-dependent substrates in resistance to protamine.

Ref.	Gene Product	Function	Protamine (R/S)
(45, 185)	AmiA	N-acetylmuramoyl-L- alanine amidase I	susceptible
(45, 185)	AmiC	N-acetylmuramoyl-L- alanine amidase	susceptible
(185)	CitE	citrate lyase beta chain	resistant
(45)(185)	CueO	multicopper oxidase	resistant
(45, 185)	DmsA	dimethyl sulfoxide reductase, A	resistant
(45, 185)	FdnG	formate dehydrogenase	resistant
(45, 185)	FdoG	formate dehydrogenase	resistant
(45, 185)	FhuD	hydroxamate dependent iron uptake	resistant
(185)	HolD	DNA polymerase III, psi subunit	resistant
(45, 185)	HyaA	hydrogenase 1, small subunit	resistant
(45, 185)	HybA	putative hydrogenase	resistant
(45, 185)	HybO	putative hydrogenase	resistant
(45, 185)	MdoD	glucans biosynthesis	resistant
(45, 185)	NapA	periplasmic nitrate reductase	resistant
(45, 185)	NapG	ferredoxin, electron transfer	resistant
(45, 185)	NrfC	formate dependent nitrate reductase	resistant
(185)	PepE	alpha-aspartyl dipeptidase	resistant
(185)	PepP	proline aminopeptidase II	resistant
(185)	PgpB	phosphatidylglycerophosphate phosphatase	resistant
(45, 185)	SufI	suppressor of ftsI	resistant
(185)	ThiP	thiamine transporter, ABC family	resistant
(45, 185)	TorA	TMAO reductase	resistant
(45, 185)	TorZ	biotin sulfoxide reductase 2	resistant
(45, 185)	WcaM	putative colanic acid biosynthesis	resistant
(45)	YaeI	putative phosphodiesterase	resistant
(45, 185)	YagT	unknown	resistant
(45, 185)	YahJ	putative deaminase	resistant
(185)	YbfL	pseudogene	resistant
(185)	YbiP	putative integral membrane protein	resistant
(45, 185)	YcbK	putative outer membrane protein	resistant
(45, 185)	YcdB	putative iron dependent peroxidase	resistant
(45)	YcdO	unknown	resistant
(185)	YcgF	putative phosphodiesterase	resistant
(185)	YdcG/MdoG	glucan biosynthesis	resistant
(45, 185)	YdhX	putative oxidoreductase	resistant
(45, 185)	YedY	putative nitrate reductase	resistant
(185)	YidJ	putative sulfatase	resistant
(185)	YkfF	putative prophage protein	resistant
(45, 185)	YnfE	oxidoreductase	resistant
(45, 185)	YnfF	putative dimethyl sulfoxide reductase	resistant

in *S. typhimurium* and also found to be susceptible to protamine when compared to wild-type, but not as susceptible as the Δ*tatC* and Δ*phoP* controls (**Figure 11,** (15)).

To confirm that deletion of the *amiA* and *amiC* loci was solely responsible for the increased susceptibility to protamine, plasmids for each gene were constructed in which the coding region for *amiA* and *amiC* was inserted into the IPTG-inducible pUHE21-2*lacI*^{q} vector (157) to construct pUHE-*amiA* (p*amiA*) and pUHE-*amiC* (p*amiC*), respectively. Introduction of the plasmids into their respective mutants restored resistance of each mutant to wild-type levels (**Figure 11**, (15)).

Both *amiA* and *amiC* encode *N*-acetylmuramoyl-L-alanine amidases that cleave the bond between the L-alanine and *N*-acetylmuramoyl residues of the peptidoglycan matrix (51, 60) and are transported to the periplasm by the Tat system (51, 61). The redundant function of these amidases suggested that there could be an additive effect when both loci were deleted in a single strain. Data supported this hypothesis because deletion of both *amiA* and *amiC* genes (*amiA amiC*) in a single strain was more susceptible to protamine than either single mutant, which could be rescued to single deletion-mutant levels when a plasmid harboring either gene was introduced (**Figure 11**, (15)). Interestingly, the *amiA amiC* double mutant was not as susceptible to protamine as the *tatC* mutant suggesting that there may be an additional Tat- dependent substrate(s) required for resistance to protamine. This prompted an investigation to identify the potential substrates.

Analysis of Tat double and triple mutant susceptibility to protamine

It is plausible that AmiA and AmiC are not the only Tat-dependent substrates responsible for bacterial resistance to protamine. It is possible that other substrates contribute to resistance to a lesser extent than AmiA and AmiC; therefore, the individual deletion may

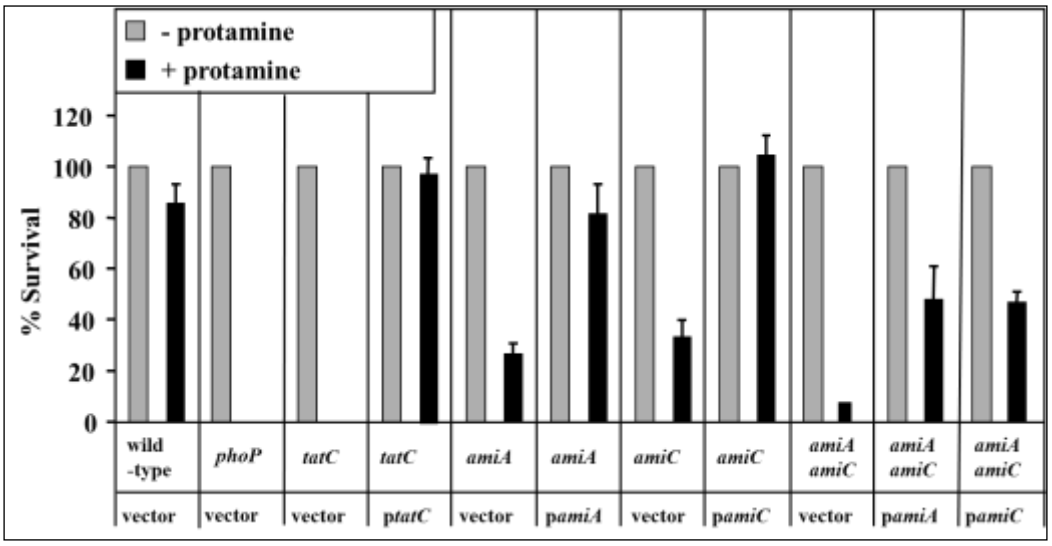
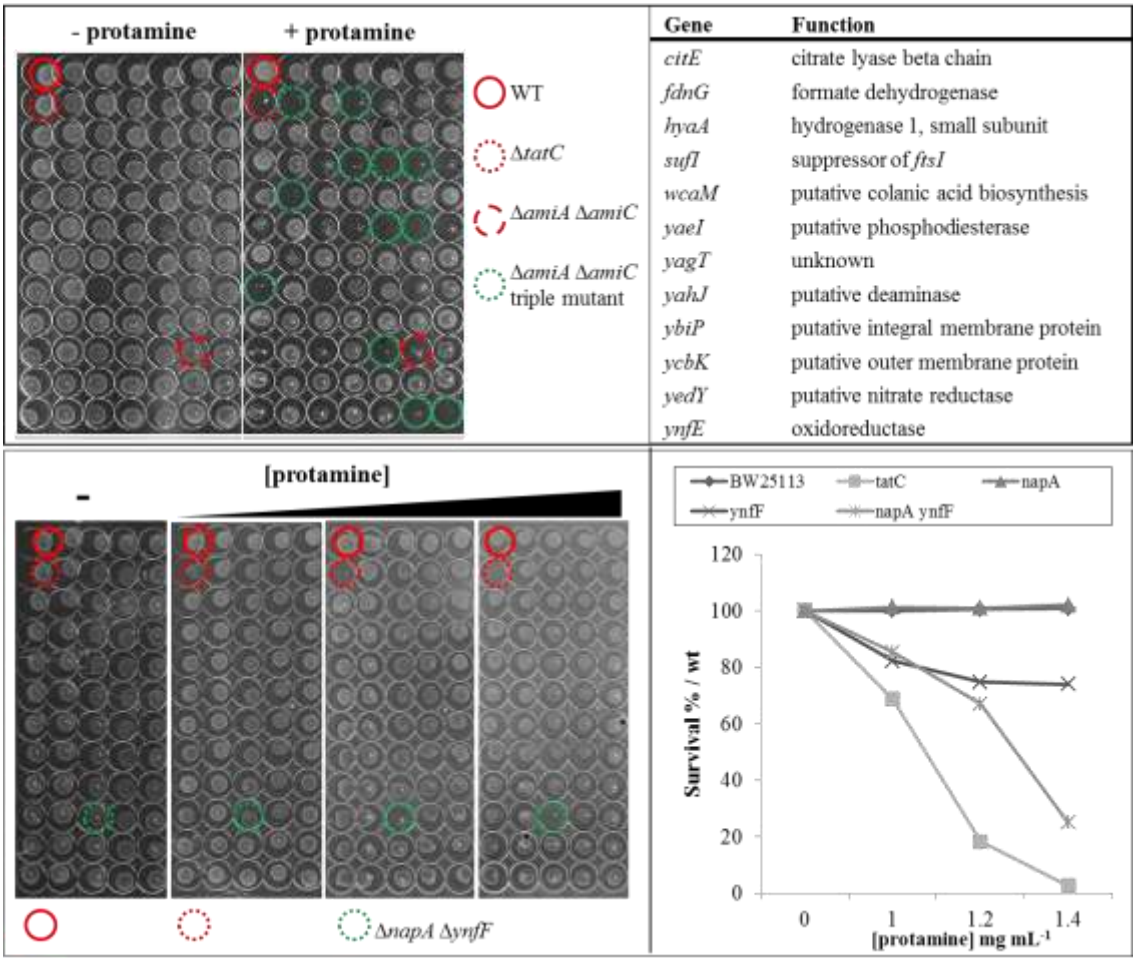


Figure 11. Complementation of protamine sensitivity.
Susceptibility of *Salmonella* Δ*phoP*, Δ *tatC*, Δ *amuA*, Δ *amiC*, andΔ *amiA* Δ*amiC* mutants to protamine can be rescued by a plasmid harboring a wild-type copy of the respective gene.

not have an obvious phenotype. To explore this possibility, individual deletion of *E. coli* genes encoding Tat-dependent substrates were introduced into an Δ*amiA* Δ*amiC* double mutant. Triple mutants were then challenged against protamine to determine if the triple mutant would render cells more susceptible to the AMP than the Δ*amiA* Δ*amiC* double mutant. Indeed, deletion of *citE, fdnG, hyaA, sufI, wcaM, yaeI, yagT, yahJ, ybiP, ycbK, yedY,* or *ynfE* in the Δ*amiA* Δ*amiC* strain increased susceptibility to protamine compared to Δ*amiA* Δ*amiC* double mutant (**Figure 12, top**). Notably, several of these loci encode products that function in cellular respiration. Therefore, it was postulated that deletion of *tatC* has a negative effect on cellular respiration, which makes cells susceptible to AMPs. A systematic analysis was conducted in which double mutants of genes encoding Tat-dependent substrates involved in cellular respiration were constructed. The double mutants were then challenged against protamine to determine if cells were susceptible compared to the isogenic wild-type. Deletion of both *napA* and *ynfF* (Δ*napA* Δ*ynfF*) in a single strain rendered cells more susceptible to protamine, however, not as susceptible as the Δ*tatC* mutant strain (**Figure 12, bottom**). It remains to be determined how *napA*, which encodes a periplasmic nitrate reductase, and *ynfF*, which encodes a paralog of the DmsA dimethyl sulfoxide reductase (186), together contribute to resistance to protamine. These enzymes are similar in that they require molybdenum cofactors for reduction of nitrate (187, 188). However, nitrate is generally reduced in anaerobic conditions suggesting that these enzymes can function in aerobic processes or the presence of protamine mimics an anaerobic environment, i.e., protamine targets cellular respiration (discussed in Chapter 1) and, as a result, may impede the use of oxygen as an acceptor in the electron transfer chain. This hypothesis is supported by the following observations:



Gene	Function
citE	citrate lyase beta chain
fdnG	formate dehydrogenase
hyaA	hydrogenase 1, small subunit
sufI	suppressor of *ftsI*
wcaM	putative colanic acid biosynthesis
yaeI	putative phosphodiesterase
yagT	unknown
yahJ	putative deaminase
ybiP	putative integral membrane protein
ycbK	putative outer membrane protein
yedY	putative nitrate reductase
ynfE	oxidoreductase

Figure 12. Protamine sensitivity assay of Tat-dependent mutants.
(**top**) Protamine susceptibility assay of Δ*amiA* Δ*amiC* triple mutants. Deletion of *citE, fdnG, hyaA, sufI, wcaM, yaeI, yagT, yahJ, ybiP, ycbK, yedY,* or *ynfE* in a Δ*amiA* Δ*amiC* background increases susceptibility to protamine, similarly as Δ*tatC* mutant. Solid red circle, WT; dashed red circle, Δ*tatC* mutant; long dashed red circle, Δ*amiA* Δ*amiC* mutant; dashed green circle, Δ*amiA* Δ*amiC* triple mutant. (**bottom**) Protamine susceptibility assay using double mutants of genes encoding Tat-dependent substrates involved in cellular respiration demonstrates that deletion of both *napA* and *ynfF* results in increased susceptibility to protamine. (**bottom, left**) Double mutants were spotted onto LB agar plates with various concentrations of protamine. Solid red circle, WT; dashed red circle, Δ*tatC* mutant; dashed green circle,Δ*napA* Δ*ynfF* mutant. (**bottom, right**) Determination of cfu survival of Δ*tatC*, Δ*napA*, Δ*ynfF* and Δ*napA* Δ*ynfF*.

(i) protamine (at a concentration within the range tested in this study) has been previously demonstrated to inhibit oxygen consumption (189); (ii) deletion of the F_1F_0-ATPase (which abolishes the production of ATP via oxidative phosphorylation) increases *E. coli* susceptibility to protamine (**Figure 2**) presumably due to the inefficient production of ATP from substrate-level phosphorylation; and, (iii) supplementation of formate or succinate, organic acids oxidized in the tricarboxylic acid cycle, (which should increase the production of ATP via substrate-level phosphorylation) is able to protect bacterial cells from the oxygen consumption inhibitory effects of human bactericidal/ permeability-increasing protein (BPI) and increase bacterial survival in the presence of BPI (170) and protamine (**Figure 2**). Nevertheless, it remains to be determined how these nitrate-reducing enzymes facilitate protamine resistance since exogenous nitrate was not added to the bacteria-AMP challenge systems.

Conclusion

Cumulatively, the data presented here demonstrate the importance of the Tat system in bacterial resistance to AMPs. The Tat system transports proteins involved in various cellular functions (reviewed in (47)); therefore, it stands to reason that the absence of the Tat system as a whole, and not just individual substrates, is detrimental to bacterial cells in the presence of AMPs. Nevertheless, it is clear that peptidoglycan amidase, AmiA and AmiC, as well as two nitrate-reducing enzymes, NapA and YnfF, contribute to the Tat-dependent resistance since deletion of genes encoding these enzymes increases bacterial susceptibility to protamine.

CHAPTER 3

GENETIC AND BIOCHEMICAL ANALYSIS OF NOVEL CPXR/A-DEPENDENT LOCI REQUIRED FOR BACTERIAL RESISTANCE TO ANTIMICROBIAL PEPTIDES

Introduction

The *Escherichia coli cpxRA* operon encodes a two-component system comprised of a sensor kinase, CpxA, and its cognate response regulator, CpxR, that controls a specific set of genes in response to cell envelope stress caused by several factors, including alkaline pH, high osmolarity, inner membrane disruptions, misfolded proteins, and starvation (reviewed in (10)). CpxR/A represents one of three characterized stress response systems, including BaeRS and σ^E, that work coordinately and individually to maintain cell envelope integrity under various harsh extracytoplasmic conditions. Specifically, CpxR/A has been suggested and experimentally demonstrated to contribute to bacterial virulence discussed in (190)), biofilm formation (13), and chemotaxis (14), by upregulating a multitude of genes in response to their respective signals. Recently, CpxR/A has been demonstrated to facilitate bacterial resistance to various classes of antimicrobial substances, including antimicrobial peptides ((15) and this chapter) and antibiotics (17-20) by upregulating target promoters; meanwhile facilitating the bactericidal effects of peptidoglycan recognition proteins (PGRPs) (21) and certain classes of antibiotics (22).

CpxR-dependent regulation of *amiA* and *amiC* promoters

Genetic screen to identify transcriptional regulators of amiA and amiC. As described in Chapter 2, two N-acetylmuramoyl-L-alanine amidases, encoded by *amiA* and *amiC*, are

required for bacterial resistance to AMPs because deletion of *amiA* and *amiC* resulted in increased susceptibility to protamine (**Figure 11)**. To gain insight regarding the regulatory mechanism in which these amidases work to confer bacterial resistance to AMPs, regulatory factors were sought to determine how these genes were regulated. To identify putative transcriptional regulators of *amiA* and *amiC*, a blue/white genetic screen was carried out using chromosomal *lac* fusions (156) constructed at both *Salmonella typhimurium* loci (to generate Δ*amiA-lac* and Δ*amiC-lac*) and a plasmid library (constructed with the multi-copy number plasmid, pUC19) introduced into each strain. The premise of the screen (illustrated in **Figure 13**) was that if an activator was overexpressed, it would increase transcription of its target genes. Thus, the *lac* fusion would produce more β-galactosidase which could be visualized by cells with an intense blue color when plated on media containing X-gal, an analog of the natural substrate of β-galactosidase, lactose. On the other hand, if a repressor was overexpressed, the *lac* fusion would produce less β-galactosidase which could be visualized by a white color when plated on media containing X-gal. It was unlikely that a repressor would be identified due to the relatively low level of transcription from the *lac* fusions (<15 Miller Units); therefore, efforts were focused on identifying putative activators.

Characterization of chromosomal regions that increase transcription of amiA and amiC. To identify the activator(s) of *amiA* and *amiC*, plasmids of dark blue colonies were isolated and sequenced to identify the DNA sequence that was overexpressed. Multiple plasmids selected from dark blue colonies contained a *Salmonella typhimurium* LT2 chromosomal region from 281130 to 283006 in the Δ*amiA-lac* strain (**Figure 14**). This region contains two open reading frames, *yaeJ* and *nlpE*, which forms an operon with

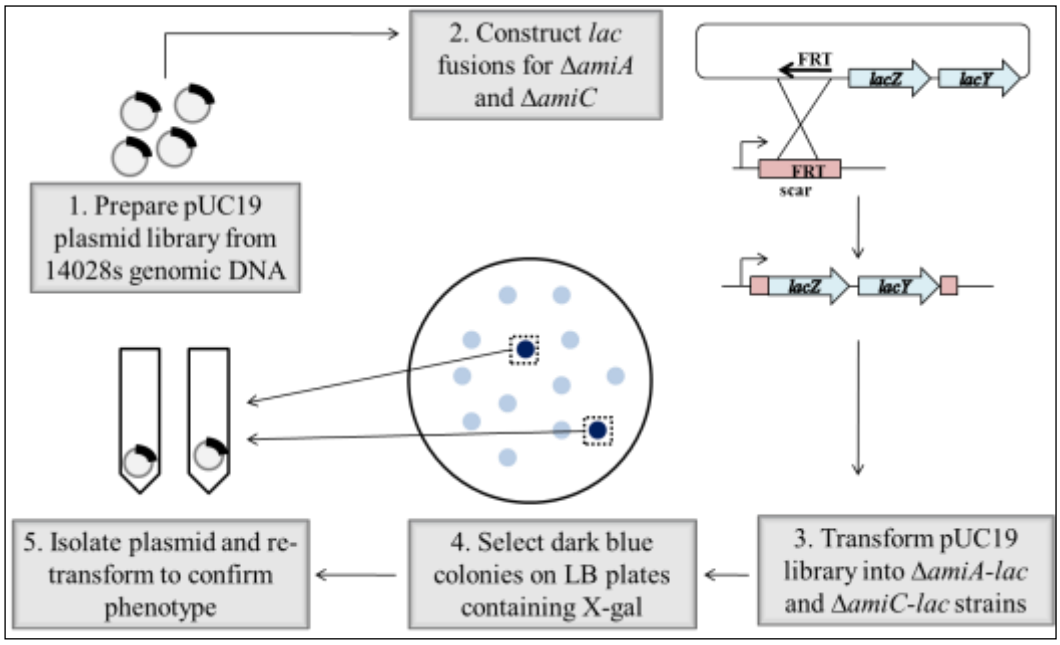


Figure 13. Schematic of screen to identify *amiA* and *amiC* activators.
Illustration of the blue/white screen conducted to identify potential transcriptional activators of *amiA* and *amiC*.

yaeQ on the chromosome (191). To determine which gene was responsible for the activation, *yaeJ* and *nlpE* were each cloned into the IPTG-inducible pUHE21-2*lacI*^{q} vector (157) to obtain pUHE-*yaeJ* (p*yaeJ*) and pUHE-*nlpE* (p*nlpE*). The resulting plasmids were introduced into the Δ*amiA-lac* strain and β-galactosidase activity was determined.

Overexpression of *nlpE*, but not *yaeJ*, was able to activate Δ*amiA-lac* transcription because β-galactosidase activity was detected only when *nlpE* was overexpressed (**Figure 14,** (15)). Interestingly, chromosomal fragments representing the same chromosomal region were isolated and characterized from dark blue colonies in the Δ*amiC-lac* library. As expected, overexpression of *nlpE,* but not *yaeJ*, was able to increase Δ*amiC-lac* transcription because β-galactosidase activity increased 5- to 6-fold when compared to the vector only control (**Figure 14,** (15)). NlpE is a lipoprotein involved in copper homeostasis and adhesion (192, 193) and has been reported to stimulate the regulatory activity of the CpxR/A system (194). Therefore, it was hypothesized that induction of *amiA* and *amiC* transcription by *nlpE* overexpression was dependent on CpxR. To determine this, the *cpxR* locus was deleted in both Δ*amiA-lac* and Δ*amiC-lac* strains containing p*nlpE*. As expected, deletion of *cpxR* prevented induction of both loci by *nlpE* (**Figure 15,** (15)), further suggesting that the Cpx system transcriptionally regulates these genes.

Analysis of CpxR-dependent regulation of amiA and amiC. To further determine the role of CpxR in the regulation of *amiA* and *amiC, in vitro* assays were performed to ascertain the specificity of CpxR-dependent activation of *amiA* and *amiC.* First, transcription start sites for each gene were identified via primer extension. To further establish CpxR-

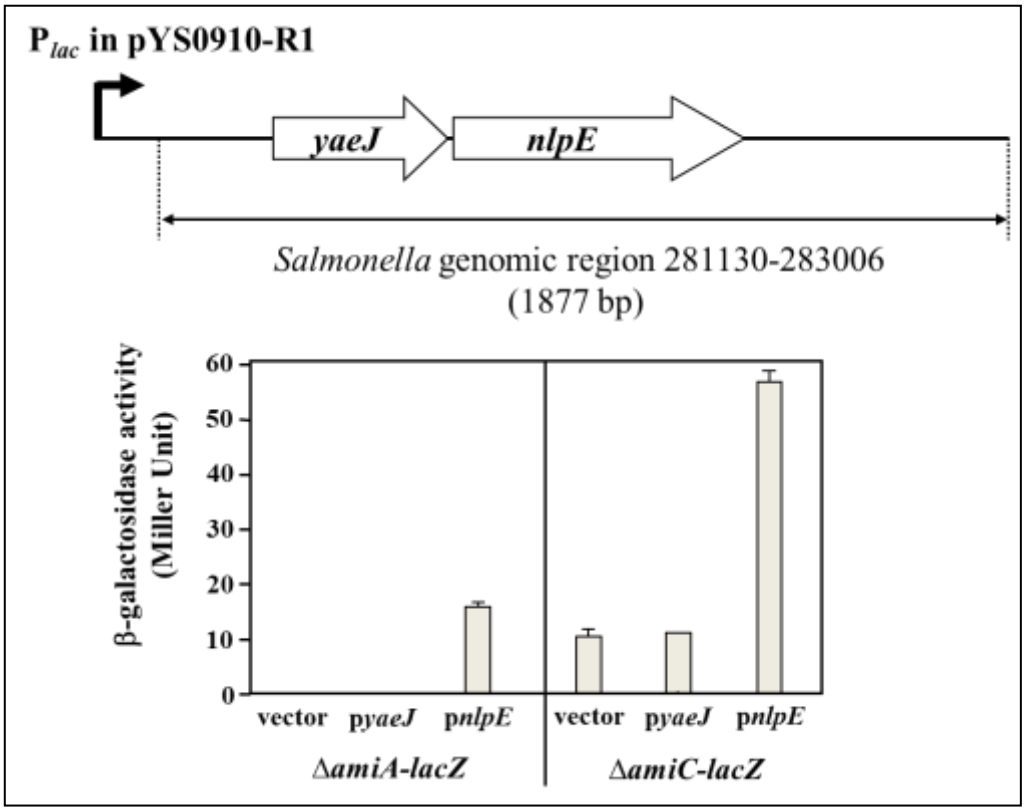


Figure 14. Chromosomal region that activates *amiA* and *amiC*.
(**top**) Chromosomal region contained in a plasmid identified in the screen that activates Δ*amiA-lac* contains two genes, *yaeJ* and *nlpE*. (**bottom**) β-galactosidase activity of Δ*amiA-lac* and Δ*amiC-lac* harboring a vector or plasmids containing *yaeJ* (p*yaeJ*) or *nlpE* (p*nlpE*) determines that *nlpE* is responsible for the activation of Δ*amiA-lac* and Δ*amiC-lac* (15).

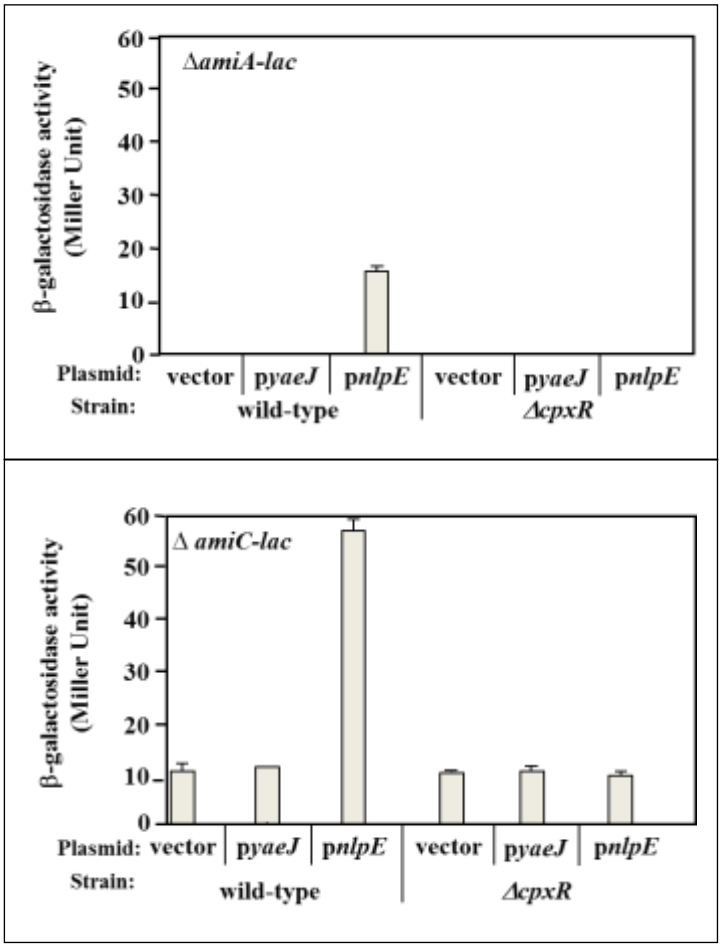


Figure 15. CpxR/A-dependent induction of *amiA* and *amiC.*
β-galactosidase activity of Δ*amiA-lac* (**top**) and Δ*amiC-lac* (**bottom**) harboring a control plasmid (vector) or plasmids containing *yaeJ* (p*yaeJ*) or *nlpE* (p*nlpE*) in a wild-type and Δ*cpxR* background. Overexpression of *nlpE*, but not others, can activate transcription in a wild-type but not Δ*cpxR* background (15).

dependent expression, RNA was isolated from wild-type vector, wild-type p*nlpE*, and Δ*cpxR* p*nlpE* strains and reverse transcribed using ^{32}P-labeled primer 1472 to generate ^{32}P-cDNA of the putative *amiA* promoter. Samples were analyzed on a 6% acrylamide-urea gel in which cDNA fragments ran according to their size in length (representing the transcription start site) and compared to a ladder generated from a Maxam and Gilbert A+G reaction. The *amiA* promoter generated two transcript products located 71 and 62 bp upstream of the start codon (**Figure 16,** (15)) suggesting that transcription could be initiated from two locations. Additionally, the cDNA level of both transcripts increased when *nlpE* was overexpressed in wild-type, but not in Δ*cpxR*, further demonstrating CpxR-dependent activation of *amiA* transcription. Meanwhile, a single transcript located 220 bp upstream of the *amiC* start codon was characterized using ^{32}P-labeled primer 1482 (**Figure 16,** (15)). Likewise, the cDNA level was significantly increased in a CpxR-dependent manner when *nlpE* was overexpressed.

Further analysis of the putative *amiA* and *amiC* promoter regions established by the primer extension analysis were carried out. *In silico* analysis revealed the presence of two imperfect direct repeat sequences (**Figure 17, top**), 5'-GAAAT-N_5-GTAAA-3' (solid box) and 5'-GTATT-N_5-GAAAA-3' (dashed box) located 96 bp and 101 bp, respectively, upstream of the *amiA* start codon. These sequences are similar to the well-established CpxR consensus sequence, 5'-GTAAA-N_5-GTAAA-3' (195), suggesting that CpxR could directly bind to these sequences. To determine the specific nucleotide sequence in which CpxR binds, DNase I footprinting assays were carried out using purified His_6-CpxR protein and DNA generated with primers ^{32}P-1567 and 1472 for the coding strand and ^{32}P-1472 and 1567 for the noncoding strand that represent the putative

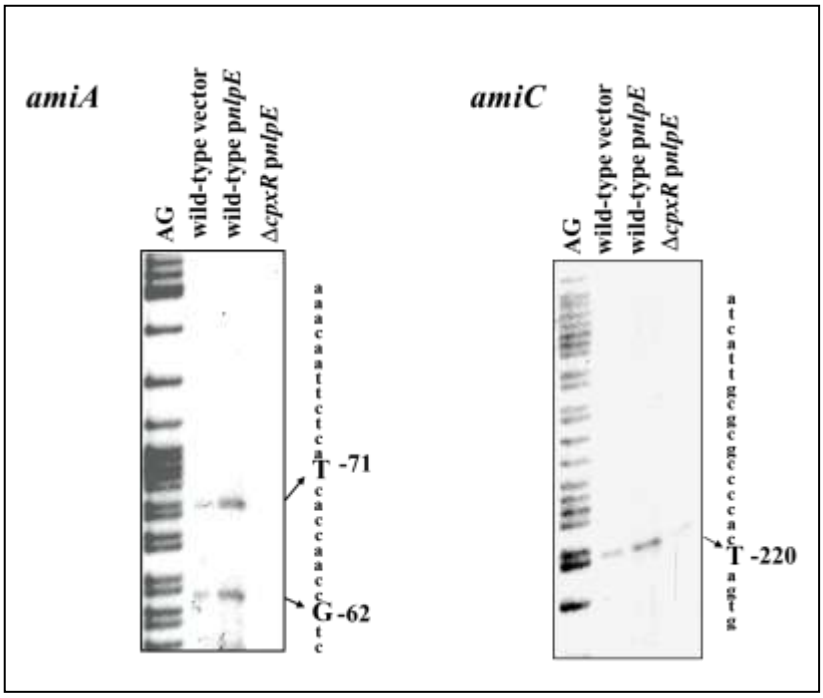


Figure 16. Primer extension analyses of *amiA* and *amiC* promoters.
Primer extension analysis of *amiA* (**left**) and *amiC* (**right**) transcripts. mRNA was isolated from wild-type vector, wild-type p*nlpE*, and *cpxR* p*nlpE* backgrounds and reverse transcribed using ^{32}P-labeled primers. cDNA samples were ran against a ladder (AG) prepared from DNA synthesized from the same ^{32}P-labeled primers. (**left**) Analysis of the *amiA* promoter provides evidence of two transcription start sites located 71 and 62 bp upstream of the start codon. (**right**) Analysis of the *amiC* promoter provides evidence of a transcription start site located 220 bp upstream of the start codon (15).

amiA promoter region. As predicted, the His_6-CpxR protein was able to protect the *amiA* promoter region from -118 to -74 (numbering from the *amiA* start codon) in the coding strand, and the -106 to -80 region in the noncoding strand (RI, **Figure 17**), which includes the predicted CpxR binding sites. The predicted CpxR box located from -96 to -82 in the *amiA* promoter is located upstream of the putative -10 and partly overlaps with the putative -35 regions for σ^{70} to start transcription from -62 (nucleotide G, **Figure 17**). The other predicted CpxR box located from -101 to -87 is located between the alternative putative -10 and -35 regions and remains to be determined if it is involved in transcription initiated from -71 (nucleotide T, **Figure 17**). It is clear, however, that the sequence representing RI is required for CpxR binding because substitution of RI abolishes His_6-CpxR protection from DNase I (**Figure 17**). An additional region (RII, **Figure 17**), was weakly protected by CpxR. However, due to its location downstream of the transcription start site and lack of a homologous CpxR box, it is unlikely to have a CpxR-dependent regulatory function.

Likewise, an *in silica* analysis revealed a putative CpxR box, 5'-ATAAA-N_5-GTAAA-3', located 250 bp upstream of the *amiC* start codon and on the opposite strand (**Figure 18**, **top**). DNase I footprinting analysis was conducted using DNA generated with primers ^{32}P-1484 and 1482 for the coding strand and ^{32}P-1482 and 1484 for the noncoding strand. The analysis demonstrated that His_6-CpxR protein could protect the *amiC* promoter region from -257 to -232 of the coding strand and from -257 to -232 of the noncoding strand (R, **Figure 18**) which includes the putative CpxR box. Thus, the CpxR box is located on the opposite strand and between the -35 and -10 regions relative to the identified transcription start site at -220 (nucleotide T, **Figure 18**). Additionally, CpxR

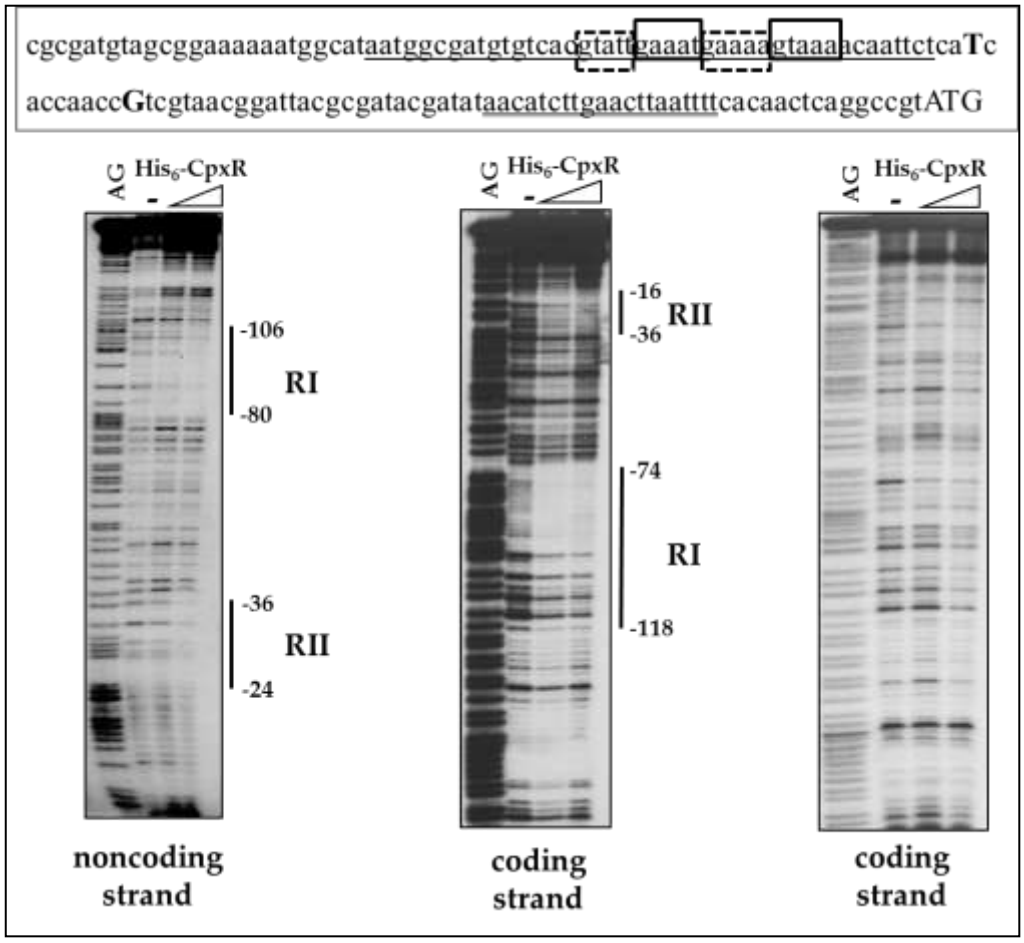


Figure 17. Analysis of the *amiA* promoter.
(**top**) *In silico* analysis of the *amiA* promoter reveals the presence of two putative CpxR binding sites (boxed) relative to the two identified transcription start sites (uppercase and bold nucleotides) and the start codon (uppercase, ATG). The underlined region represents the sequence protected by CpxR after DNase I digestion (single underline, protected region I (RI); double underline, protected region II (RII)). (**bottom**) DNase I footprinting assay reveals two regions protected by CpxR protein, RI and RII on the noncoding (left panel) and coding (middle panel) strands. Binding by CpxR is specific to the sequence since substitution of RI abolishes protection (right panel) (15).

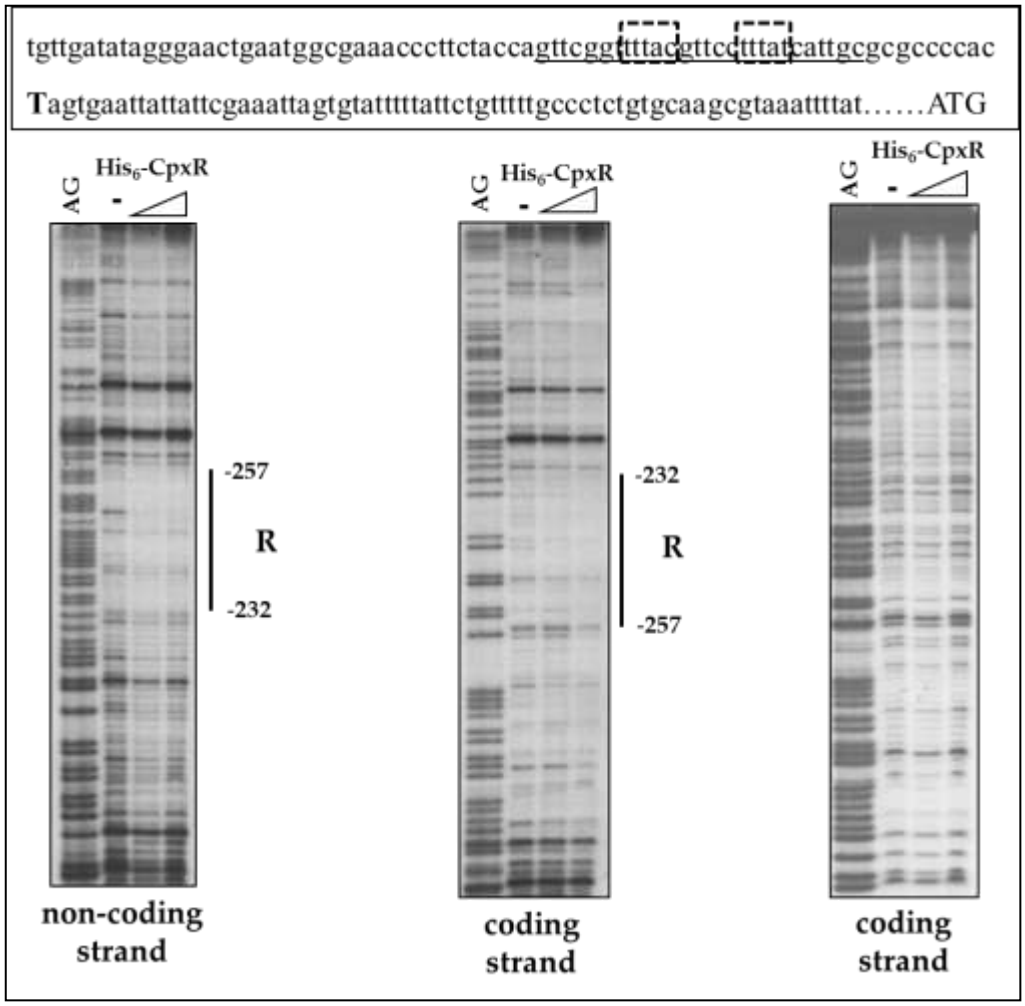


Figure 18. Analysis of the *amiC* promoter.
(**top**) *In silico* analysis of the *amiC* promoter reveals the presence of a putative CpxR binding site (boxed) relative to the identified transcription start site (uppercase and bold nucleotide) and the start codon (uppercase, ATG). The underlined region represents the sequence protected by CpxR after DNase I digestion. (**bottom**) DNase I footprinting assay reveals a region (R) protected by CpxR protein on the noncoding (left panel) and coding (middle panel) strands. Binding by CpxR is specific to the sequence since substitution of R abolishes protection (right panel) (15).

binding to the sequence represented by R is specific because substitution of R abolished His_6-CpxR protection from DNase I.

Cumulatively, these data provide evidence of CpxR-dependent regulation of the *amiA* and *amiC* promoters in which CpxR directly binds to a homologous CpxR box residing in the promoter regions to exert its regulatory activity.

CpxR-dependent regulation of the *rfe-rff* gene cluster

Rationale to study regulation of the rfe-rff gene cluster. The *rfe-rff* gene cluster encodes enzymes involved in the biosynthesis of the enterobacterial common antigen (ECA), a glycolipid composed of a trisaccharide repeat located on the outer leaflet of the outer membrane in all bacteria species of the *Enterobacteriaceae* family (reviewed in (54)). Although the physiological function of ECA remains to be determined, it has been implicated in bacterial resistance to bile salts (57), organic acids (56), and AMPs (Chapter 1) as well as virulence (57). Deletion of loci residing in the *rfe-rff* gene cluster was found to increase susceptibility to all peptide classes analyzed in this study (**Figure 7**), suggesting a global contribution of their gene products to AMP resistance. Further analysis (discussed in Chapter 1) determined that the increased susceptibility is the result of the absence of ECA and not accumulation of intermediates of the ECA biosynthesis pathway.

Since the biosynthesis of ECA was proposed to be required for resistance to AMPs, experiments were carried out to determine how the *rfe-rff* gene cluster is regulated since it had not been previously reported. An *in silico* analysis of the *rfe* promoter, the first gene of the cluster, revealed a putative CpxR binding site (5'-GAAAA-N_5-GGAAT-3') located 154 bp upstream of the start codon (**Figure 19, top**) suggesting that the *rfe*

promoter (and by extension, the entire *rfe-rff* gene cluster), may be regulated by CpxR/A, further demonstrating a global role for CpxR/A in resistance to antimicrobials.

Activation of CpxR increases transcriptional expression of the rfe promoter. To determine if CpxR could regulate transcription of the *rfe* promoter, and possible downstream genes, chromosomal *lac* fusions were constructed at the *rfe, rffT,* and *rffM* loci to construct Δ*rfe-lac*, Δ*rffT-lac*, Δ*rffM-lac*, respectively. Plasmid p*nlpE* was introduced into each *lac* fusion strain to stimulate the regulatory activity of the CpxR/A system (194). Overexpression of *nlpE* increased β-galactosidase activity of Δ*rfe-lac* 4-fold, Δ*rffT-lac* 2-fold, and Δ*rffM-lac* 3-fold (**Figure 19, bottom left**). This observation is consistent with previous reports in which overexpression of CpxR could increase expression of *rffA* (another member of the *rfe-rff* gene cluster) 1.9-fold after analysis by qRT-PCR (196). The increase in β-galactosidase activity was dependent on CpxR as deletion of *cpxR* in each strain abolished the transcriptional increase (**Figure 19, bottom left**). Cumulatively, this demonstrates that CpxR can regulate transcription activity of members of the *rfe-rff* gene cluster (and likely the entire cluster) and postulates that the *rfe-rff* gene cluster comprises a single transcriptional operon.

The rfe-rff gene cluster likely comprises a single transcriptional unit. To address the possibility that the *rfe-rff* gene cluster is localized in a single operon, a series of reverse transcription-polymerase chain reactions (RT-PCR) were conducted. Total RNA, extracted from wild-type, was reverse transcribed and the cDNA product was used in a series of PCR reactions in combination with sets of defined primers to demonstrate the singularity of the operon (illustrated in **Figure 19, top**). Specifically, primer pairs 1912/1915, 1914/1917, 1916/1804, and 1803/1919 were used to generate products to

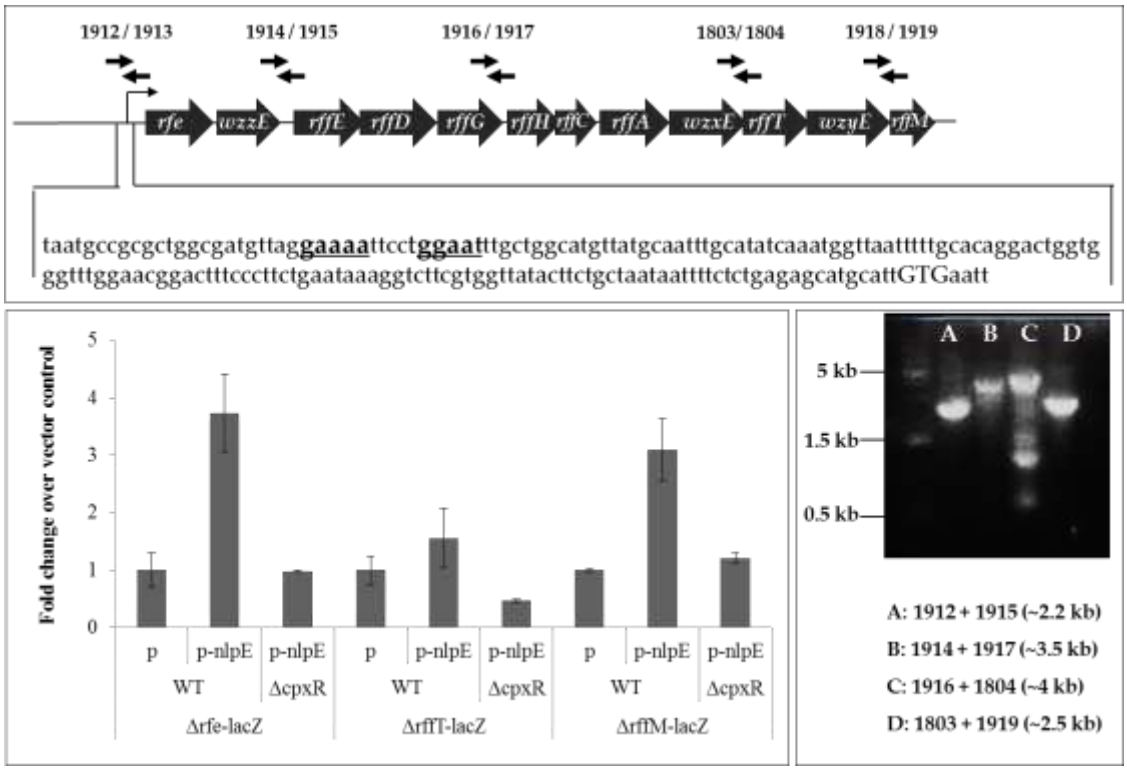


Figure 19. Cpx-dependent activation of the *rfe-rff* gene cluster.
(**top**) *In silico* analysis of the *rfe* promoter (the first gene in the cluster) reveals a putative CpxR-binding site. Right-facing arrow, 5' primer; left-facing arrow, 3' primer. Numbers correspond to the primer number. (**bottom, left**) Overexpression of *nlpE* increases β-galactosidase activity of chromosomal *lac* constructs at the *rfe, rffT,* and *rffM* loci. (**bottom, right**) RT-PCR reveals that the *rfe-rff* gene cluster may comprise a single transcriptional unit.

represent the initial operon transcript, the *rfe/wzzE/rffE* chromosomal region (~2.2 kb, set A), the *wzzE/rffE/rffD/rffG/rffH* chromosomal region (~3.5 kb, set B), the *rffG/rffH/rffC/ rffA/wzxE/rffT* chromosomal region (~4 kb, set C), and the *wzxE/rffT/wzyE/rffM* chromosomal region (~2.5 kb, set D), respectively. Single positive bands were identified for sets A, B, and D (**Figure 19, bottom right**), suggesting that the transcripts represented in the primer set region comprise a single RNA transcript. Lane C generated multiple bands (**Figure 19, bottom right**); however, the longest DNA amplified migrated to the estimated size of a single transcript, suggesting that there may be multiple levels of regulation in this region of the gene cluster. Cumulatively, these data suggest that the *rfe-rff* gene cluster could comprise a single transcriptional unit; however, there may be an additional promoter region between the *rffG* and *rffT* loci that can initiate transcription of a single gene or multiple genes between the loci.

CpxR directly binds to the rfe promoter. Previous transcriptional data demonstrated that CpxR could activate transcription of loci within the *rfe-rff* gene cluster when induced by *nlpE* overexpression (**Figure 19, bottom left**). To determine if CpxR directly regulated these loci by interacting with the promoter region, an electromobility shift assay was performed using purified His_6-CpxR protein and a 420-bp DNA fragment corresponding to the upstream and overlapping region of the *rfe* start codon (representing the putative promoter region). The CpxR protein was able to shift the DNA fragment (**Figure 20, bottom left**), indicating the presence of CpxR-binding sites in the *rfe* promoter region. As the amount of CpxR increased to 150 pmol, two distinct shifts were observed confirming direct interaction of the CpxR protein with the promoter DNA. Next, DNase I footprinting analysis was conducted to determine the specific nucleotide sequence

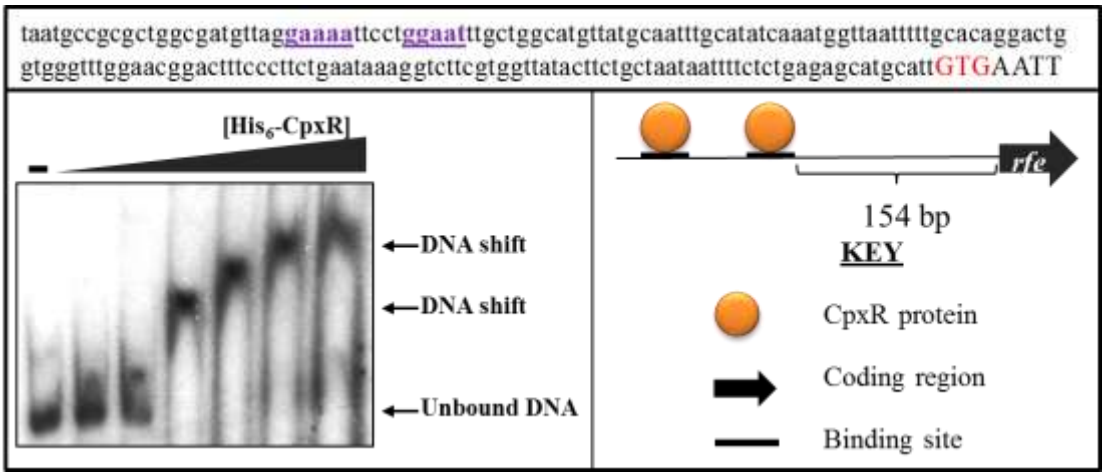


Figure 20. Analysis of CpxR binding to the *rfe* promoter.
(**top**) DNA sequence of putative *rfe* promoter highlighting (in purple) the putative CpxR binding site. (**bottom, left**) EMSA shows that CpxR can bind to at least two locations within the *rfe* promoter. (**bottom, right**) Illustration of CpxR binding to the *rfe* promoter 154 bp upstream of the *rfe* start codon, GTG.

recognized by CpxR. Using purified His_6-CpxR protein and DNA representing the *rfe* promoter generated with primers ^{32}P-1921 and 1913 for the coding strand, protection was observed upstream of the *rfe* start codon (illustrated in **Figure 20, bottom right**). The region protected by CpxR contains an imperfect direct repeat sequence, 5'-GAAAA-N_5-GGAAT-3', 154 bp upstream of the start codon (**Figure 20, top**) which is similar to the homologous CpxR box described previously (195). This surmounting evidence confirms that CpxR binds to the *rfe* promoter to activate transcription of the *rfe* locus, and likely the *rfe-rff* gene cluster.

CpxR-dependent regulation of the *marRAB* operon

Rationale to study the regulation of mar. The multiple-antibiotic resistant operon, *marRAB*, encodes a repressor, MarR, and activator, MarA, which coordinately regulate the *mar* promoter, in concert with the global regulators Rob and SoxS. It was recently reported that the *marRAB* operon could be activated by sub-lethal concentrations of AMPs via Rob (which is required for polymyxin B-induced upregulation of *micF* (43)) and could facilitate resistance, at least in part, by overexpressing the AcrAB/TolC efflux pump (44). While deletion of *marA* had no apparent phenotype regarding susceptibility to AMPs, constitutive expression of *marA* decreased bacterial susceptibility to AMPs in an AcrAB/TolC-dependent manner (discussed in Introduction). Importantly, deletion of *tolC* resulted in increased susceptibility to each AMP analyzed, however, susceptibility to polymyxin B could be reduced or increased in a constitutive *marA* background depending on the AMP (44). This evidence suggested that MarA-dependent genes contribute to bacterial resistance and susceptibility to AMPs. Cumulatively, these data launched an investigation to further analyze the *marRAB* operon for additional regulatory activities in

regards to bacterial resistance to antimicrobial peptides as well as the role of a downstream target.

Analysis of CpxR-dependent regulation of the mar operon. The *marRAB* promoter has been extensively studied and is well characterized (31-33) (illustrated in **Figure 21, top**). In the presence of specific antibiotics, bile salts, and reactive oxygen species, MarA (35, 36), Rob (37, 38) and SoxS (39, 40), respectively, will bind to a 20 nucleotide sequence designated as the marbox (33) to activate transcription of the operon. In contrast, MarR is a strong repressor that binds at the operator region (dashed rectangle, **Figure 21, top**) whose effect is only alleviated when a compound, such as salicylate, binds the protein to result in its disassociation from the promoter to allow for transcription (31) (32, 41). Further examination of the promoter sequence revealed a putative CpxR-binding site approximately 50 nucleotides upstream of the marbox (**Figure 21, top**). The putative CpxR-box contains the sequence 5'-GTAAA-N_5-ATAAA-3' which is similar to the well established CpxR consensus sequence (195), making it a good candidate sequence for CpxR to bind.

To determine whether CpxR can regulate the *marRAB* operon, a series of chromosomally- and plasmid-encoded *lac* fusions were constructed to ascertain the activity of the *mar* promoter (illustrated in **Figure 21, bottom**). Chromosomal *lac* fusions (156) were constructed at each of the three loci of the *mar* operon to yield Δ*marR-lac* (in which the MarR repressor is absent), Δ*marA-lac* (in which the MarA activator is absent), Δ*marB-lac* (in which MarB is absent), and Δ*marRA-lac, (*in which both the activator and repressor are absent). It was first necessary to determine whether these constructs could serve as suitable reporters for the *mar* promoter. Therefore, β-

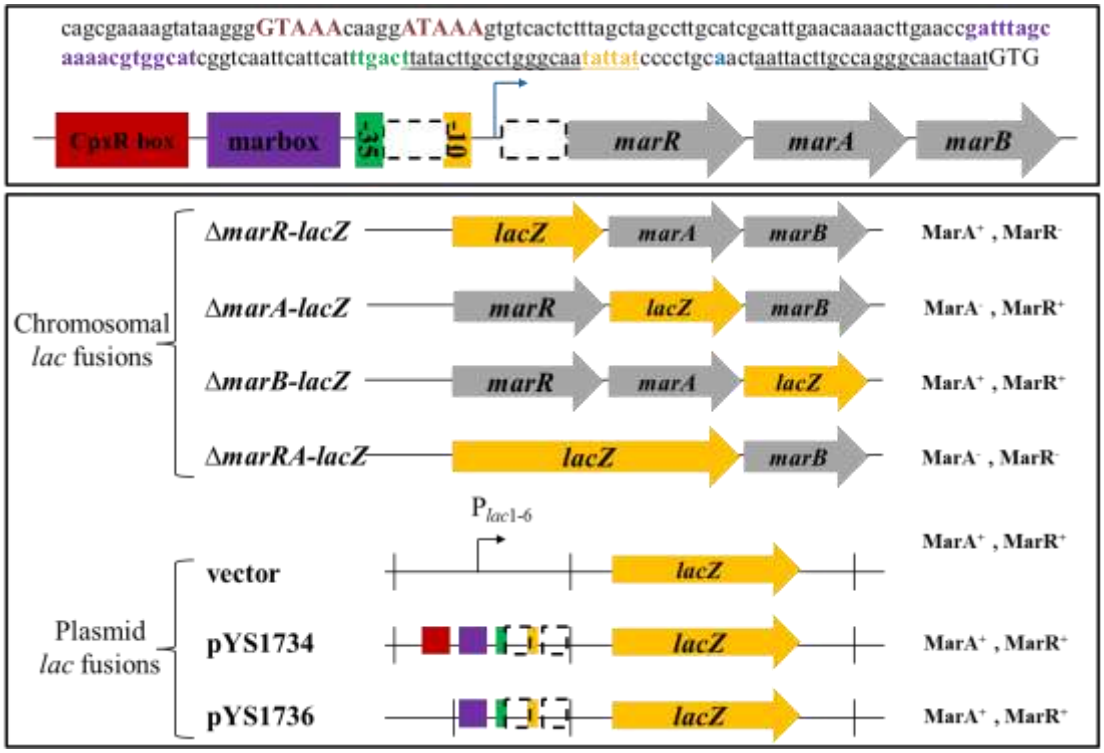


Figure 21. Analysis of the *marRAB* promoter.
(**top**) *In silico* analysis of the *marRAB* promoter region reveals a putative CpxR box upstream of the previously characterized marbox (purple), -35 (green), -10 (yellow), transcription start site (blue), operator region (underlined/dashed box), and start codon (GTG). (**bottom**) Illustration of the chromosomally- and plasmid-encoded *lac* fusions constructed to analyze the transcriptional activity of the *mar* promoter.

galactosidase activity was determined in the presence of salicylate, a gratuitous inducer of the *mar* promoter (32), to confirm that expression could be increased. Expression increased 28-fold in the Δ*marA-lac* strain when saliciylate was present; however, no significant increase in expression of the Δ*marR-lac*, Δ*marB-lac* or Δ*marRA-lac* constructs was observed (**Figure 22, top**). While it was concluded that Δ*marB-lac* could not serve as a suitable reporter for the *mar* promoter, it was deduced that the lack of induction by salicylate in Δ*marR-lac* and Δ*marRA-lac* was due to constitutive expression of the promoter because of the lack of the MarR repressor. Therefore, Δ*marA-lac*, and perhaps Δ*marR-lac* and Δ*marRA-lac*, were determined to be adequate reporters to measure *mar* promoter expression levels.

Next, p*nlpE* was introduced into each chromosomal *lac* fusion strain to determine if CpxR could activate transcription of these reporters. Overexpression of *nlpE* increased β-galactosidase activity of Δ*marR-lac* 3- fold and Δ*marRA-lac* 3.8- fold when compared to the vector only control (**Figure 22, bottom**), suggesting that CpxR could activate transcription of this operon. This increase in expression was abolished when *cpxR* was deleted, confirming CpxR-dependent activation of this operon via *nlpE* overexpression. Interestingly, p*nlpE* had no effect on Δ*marA-lac* transcription (nor Δ*marB-lac* which was previously demonstrated to be an inadequate reporter for the *mar* promoter) (**Figure 22, top**). These data posit two regulatory mechanisms: (i) CpxR-dependent activation of the *mar* promoter facilitated by MarA since deletion of *marA* abolishes the transcriptional induction by *nlpE* overexpression; (ii) CpxR-MarA and MarR participation in an antagonistic relationship because in the absence of *marA*, CpxR cannot exert its regulatory activity unless MarR is also absent (illustrated in **Figure 22, bottom**).

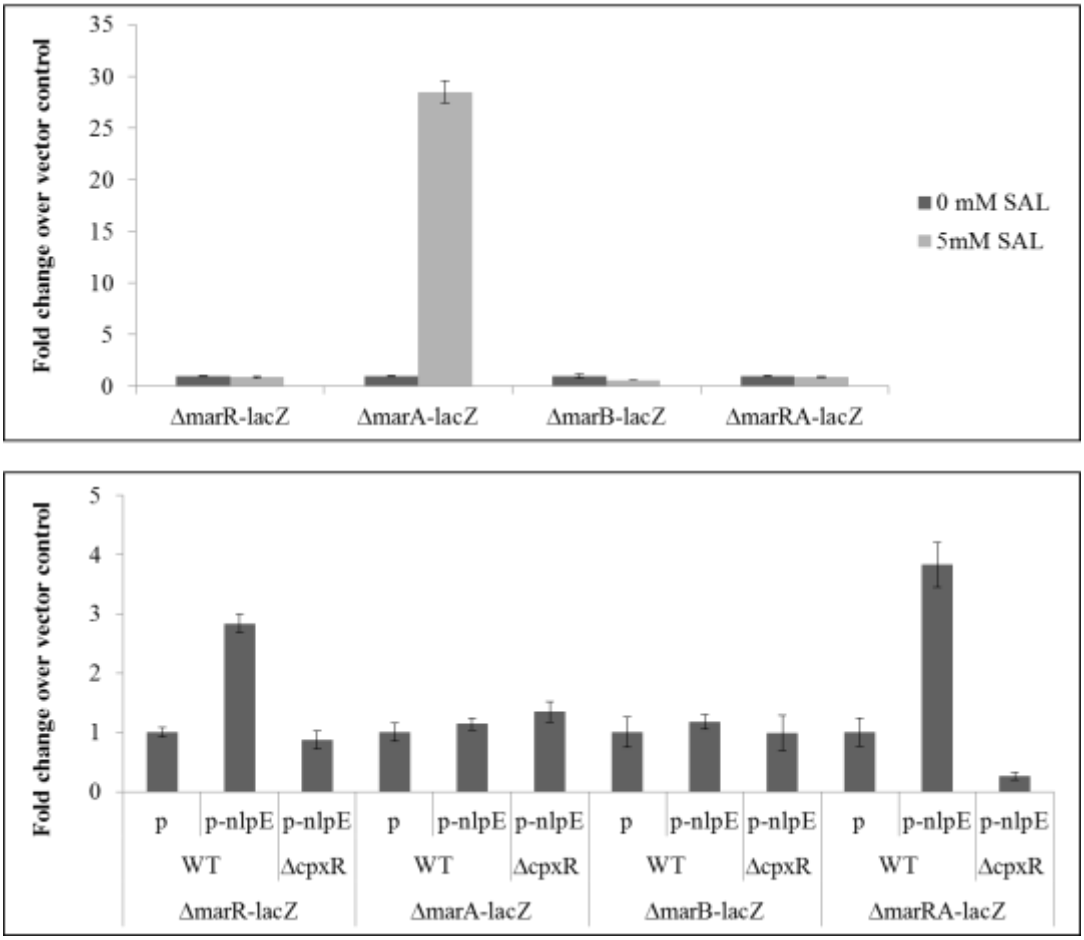


Figure 22. β-galactosidase activity of chromosomal *mar* promoter constructs. (**top**) Expression of Δ*marA-lac* increased when saliciylate, a well-known inducer of the *mar* promoter, was supplemented. However, no significant increase in expression of Δ*marR-lac*, Δ*marB-lac* or Δ*marRA-lac* was observed. (**bottom**) Overexpression of *nlpE* (p*nlpE*) is able to increase transcription of the *mar* promoter in Δ*marR-lac* and Δ*marRA-lac* in a CpxR-dependent manner, however, is unable to increase transcription of Δ*marA-lac* or Δ*marB-lac*.

To further analyze the effect of CpxR on transcriptional activity of the *mar* promoter, plasmid-encoded *lac* fusions (pYS1734 and pYS1736) were constructed which contain different lengths of the *mar* promoter followed by the *lac* coding region (illustrated in **Figure 21, bottom**). The longest promoter region represented in pYS1734 contains an extension of the previously characterized *mar* promoter (32) as it contains 540 bp upstream of the transcription start site and thus carries the marbox and the putative CpxR-binding site. Meanwhile, plasmid pYS1736 contains only 120 bp upstream of the transcription start site and therefore contains the marbox, but not the putative CpxR-binding site. To ascertain whether these constructs could serve as suitable reporters for the *mar* promoter, β-galactosidase activity was determined in the presence of salicylate to confirm that expression could be increased. Indeed, the presence of salicylate increased expression of pYS1734 2.6-fold and expression of pYS1736 2.2-fold (**Figure 23, top**), which is less than previous reports of 5-10-fold induction by salicylate (32, 42); however, these data were determined to be significant via Student's *t*-Test ($p = 0.0035$ and 0.0023, respectively). Next, p*nlpE* was introduced into strains harboring either plasmid and β-galactosidase activity determined. Overexpression of *nlpE* increased expression of pYS1734 2.8-fold and pYS1736 2.6-fold, when compared to the vector only control (**Figure 23, bottom**), suggesting that CpxR could activate transcription of this operon. This increase in expression was abolished when *cpxR* was deleted, confirming CpxR-dependent activation of the *mar* promoter via *nlpE* overexpression. It was unexpected that *nlpE* overexpression could increase transcriptional activity of pYS1736 since this plasmid doesn't contain the putative CpxR box. Therefore, it was hypothesized that CpxR could bind the *mar* promoter region downstream of the predicted CpxR box, within the

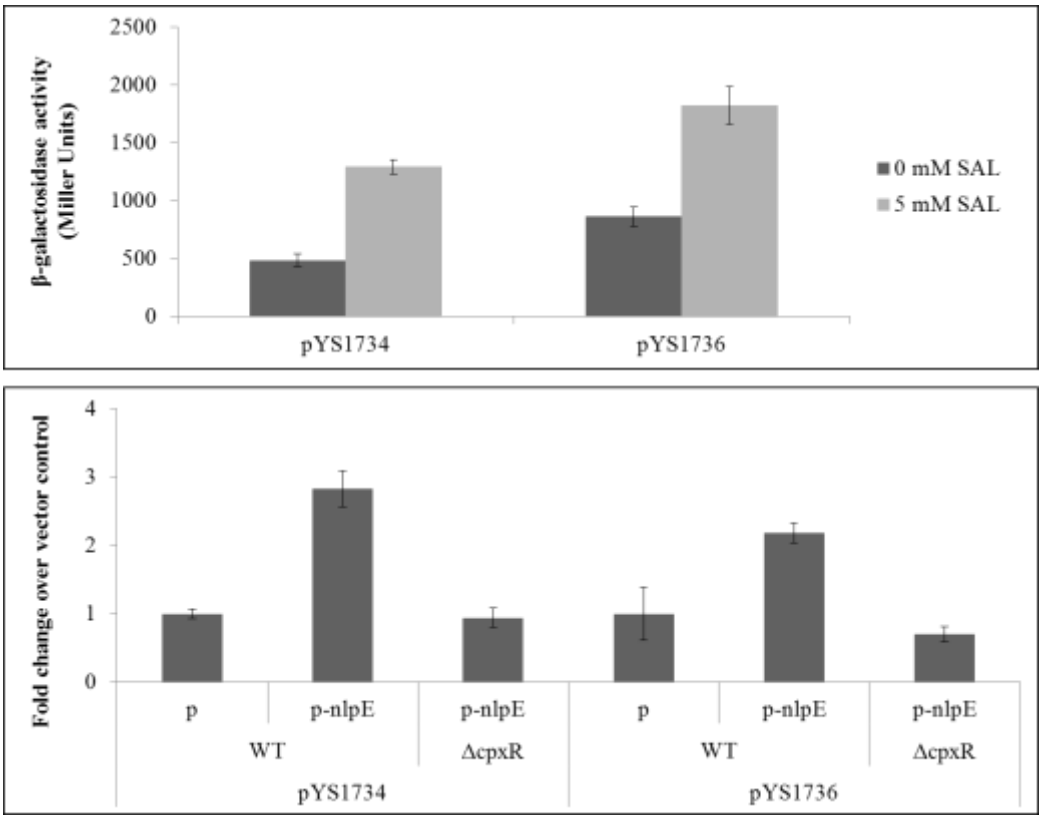


Figure 23. β-galactosidase activity of plasmid *mar* promoter constructs.
(**top**) Plasmids pYS734 and pYS1736 are able to respond to salicylate, a well-known inducer of the *mar* promoter. (**bottom**) Overexpression of *nlpE* (p*nlpE*) is able to increase transcription of the *mar* promoter encoded in pYS1734 and pYS1736 in a CpxR-dependent manner.

chromosomal region represented in pYS1736.

It is noteworthy to mention that while MarA has been shown to activate the *marRAB* promoter in the presence of specific signaling molecules, it is unclear whether MarA is required for activation of the promoter in the absence of the MarR repressor. In other words, it is unclear whether the absence of MarR is sufficient to activate transcription of the operon, or if activators, such as MarA are still required. The data presented suggest that MarA plays a role, but is not absolutely required, in MarR-depleted conditions to activate transcription of the promoter. This is demonstrated in both the chromosomal- and plasmid-encoded *lac* fusions. When comparing Δ*marR-lac* with Δ*marRA-lac* in non-inducing conditions, β-galactosidase activity is reduced ~2-fold in non-inducing conditions when *marA* is absent (**Figure 24**). Additionally, when measuring β-galactosidase activity of pYS1734 in Δ*marR* and Δ*marRA* backgrounds, expression is reduced ~ 2.5-fold in non-inducing conditions when *marA* is deleted (**Figure 24**). Thus, MarA likely plays a role, but is not required for transcription of *marRAB* in the absence of the MarR repressor because transcription still occurred in the absence of MarA.

It was previously mentioned that MarA may facilitate CpxR binding to the *mar* promoter to exert its regulatory effect in the presence of the MarR repressor. CpxR has been previously shown to facilitate BaeR-dependent regulation of *acrD* and *mdtABC* promoters which encode drug efflux systems (17, 18). Therefore, to ascertain the CpxR-dependent activation in relation to the known regulators of the *mar* operon, expression of each plasmid-encoded fusion was determined in wild-type, Δ*marA*, Δ*marR*, Δ*marRA*, Δ*rob* and Δ*soxS* backgrounds, each overexpressing *nlpE,* to determine if CpxR could directly influence the *mar* promoter activity or if the activation is facilitated by other *mar*

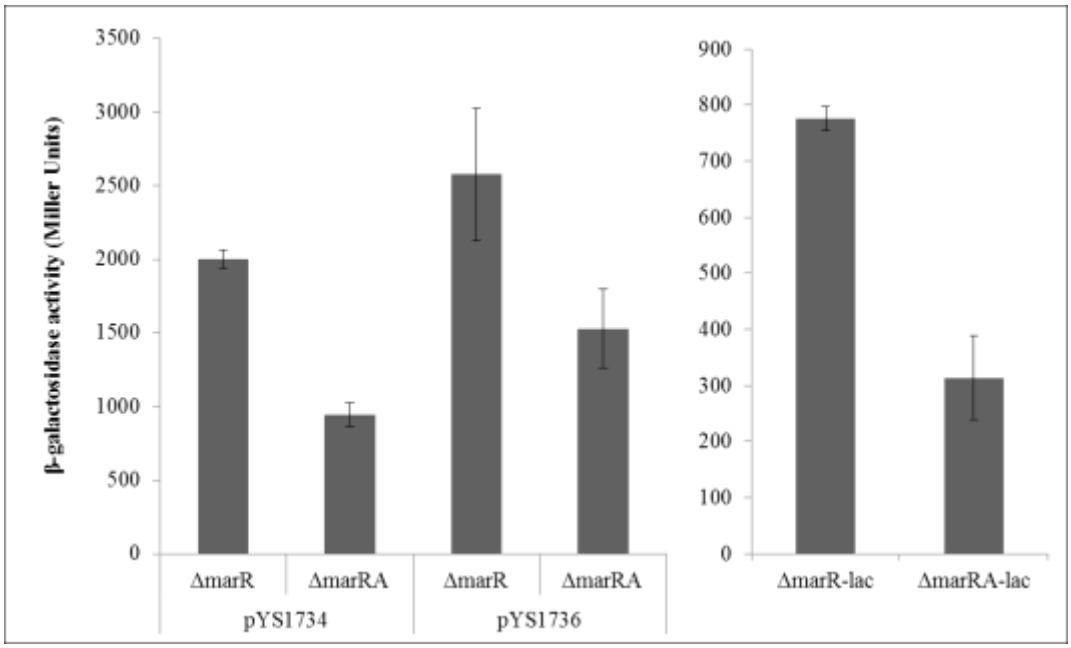


Figure 24. β-galactosidase activity comparing Δ*marR* and Δ*marRA*.
β-galactosidase activity comparing Δ*marR* and Δ*marRA* backgrounds demonstrate that MarA plays a role in transcription of the *marRAB* promoter in non-inducing conditions in the absence of MarR, but is not solely required.

regulators. Deletion of *marA* and *rob* reduced the transcription induction by p*nlpE* to 1.38-fold and 1.27-fold, respectively in pYS1734 and 1.3-fold and 2.0-fold, respectively in pYS1736 (**Figure 25**), suggesting that CpxR regulation of the *mar* promoter is dependent on either MarA and/or Rob activators. Meanwhile, deletion of *marR* or *soxS* maintained CpxR-dependent induction by *nlpE* overexpression (**Figure 25**). Interestingly, deletion of *marRA* (in which both MarR and MarA is absent) did not abolish induction by *nlpE* (**Figure 25**) demonstrating that in the presence of MarR, MarA is required for CpxR-dependent induction, however, MarA is not required in the absence of MarR (illustrated in **Figure 26**).

Biochemical evidence of CpxR-dependent regulation of mar. As previously mentioned, an *in silico* analysis of the *mar* promoter revealed a putative CpxR binding site, 5'-GTAAA-N_5-ATAAA-3', located 50 bp upstream of the marbox. An electromobility shift assay (EMSA) was carried out to determine if purified CpxR protein could directly bind to a DNA fragment, generated with primers 1723 and 1725, representing the *mar* promoter. Indeed, two DNA shifts were observed (**Figure 27, bottom left**) when increasing amounts of CpxR protein were added to the system, indicating the likelihood of two (or more) CpxR binding sites within this sequence. To determine the nucleotide sequence to which CpxR binds within the *mar* promoter, a DNase I footprinting assay was carried out using purified His_6-CpxR protein and DNA generated from primers ^{32}P-1723 and 1725 for the coding strand and ^{32}P-1725 and 1723 for the noncoding strand. CpxR was able to protect DNA from DNase I cleavage in three regions (numbering from the start codon): -96 to -115 (RI), -140 to -164 (RII), and -215 to -239 (RIII) on the coding strand; and -56 to -65 (RI), -110 to -117 (RII), and -145 to -165 (RIII) on the noncoding strand (**Figure**

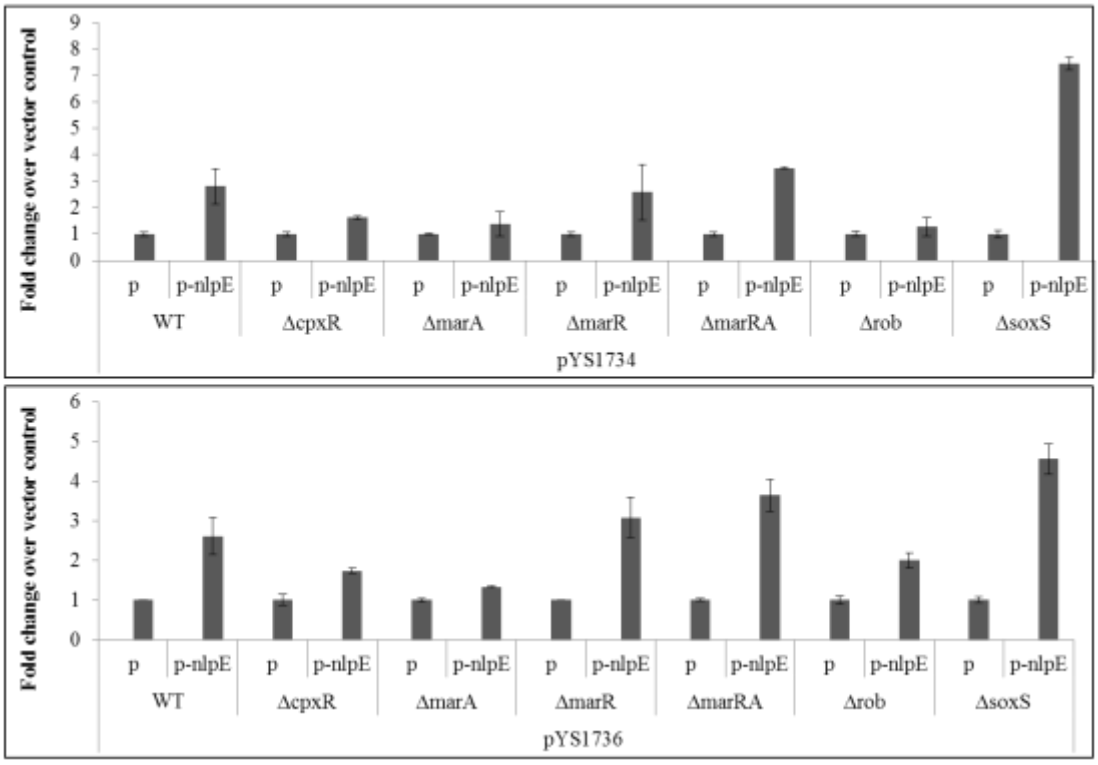


Figure 25. β-galactosidase activity of *mar* promoter with deleted regulators. β-galactosidase assays to determine the effect of *mar* promoter regulators on CpxR-dependent induction when *nlpE* is overexpressed (p*nlpE*) using the plasmid-encoded *lac* constructs pYS1734 (**top**) and pYS1736 (**bottom**).

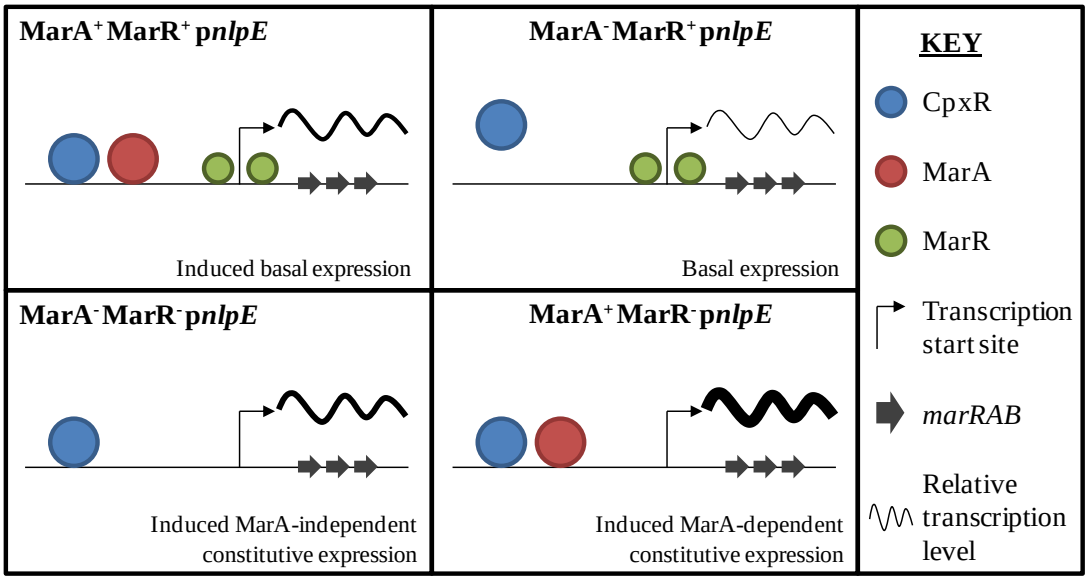


Figure 26. Illustration of CpxR regulation of the *marRAB* promoter.
In the presence of both MarA and MarR, CpxR can induce basal-level expression (**top, left**). In the presence of MarR but absence of MarA, CpxR is not sufficient to induce expression, therefore, transcription remains at basal levels (**top, right**). In the absence of MarR, CpxR is able to induce constitutive expression regardless of the absence or presence of MarA (**bottom**).

28). Each region is located upstream of the marbox, and thus, upstream of the reported transcription start site indicating that these regions can serve as regulatory elements for CpxR. Moreover, regions RII and RIII contain sequences similar to the consensus CpxR box. RII contains 5'- CTTGA-N_5-TTTAG-3' located 107 bp upstream of the start codon, overlapping the marbox, and on the opposite strand; while RIII contains 5'-GTAAA-N_5-ATAAA-3' located 162 bp upstream of the start codon. Thus, these two sequences likely contribute to CpxR-dependent regulation of *mar*. Notably, the RII protected sequence is present in pYS1736 and thus explains why overexpression of *nlpE* is able to increase transcription of the *mar* promoter construct. Cumulatively, these data demonstrate that CpxR can directly bind to the *mar* promoter to activate transcription of the *marRAB* operon.

Identification of a CpxR/A signal

Rationale to identify the physiological signal for CpxR/A. Microbial two-component systems consist of a sensor histidine kinase/phosphatase which, upon activation by a signal molecule, will activate its cognate response regulator by phosphorylation and inactivate its regulator by dephosphorylation when the signal is removed (reviewed in (1)). A common challenge amongst researchers, however, is to identify the signal molecules that have a global effect regarding cell survival and maintenance. The *cpxRA* operon encodes a sensor, CpxA, and its cognate response regulator, CpxR, which globally regulates a vast number of promoters in response to periplasmic stress, including misfolded proteins, inner membrane disruptions, alkaline pH, starvation, and high osmolarity (reviewed in (10)). While the physiological signal for CpxA has not been identified, Cpx-dependent regulation continues to be characterized as its regulon

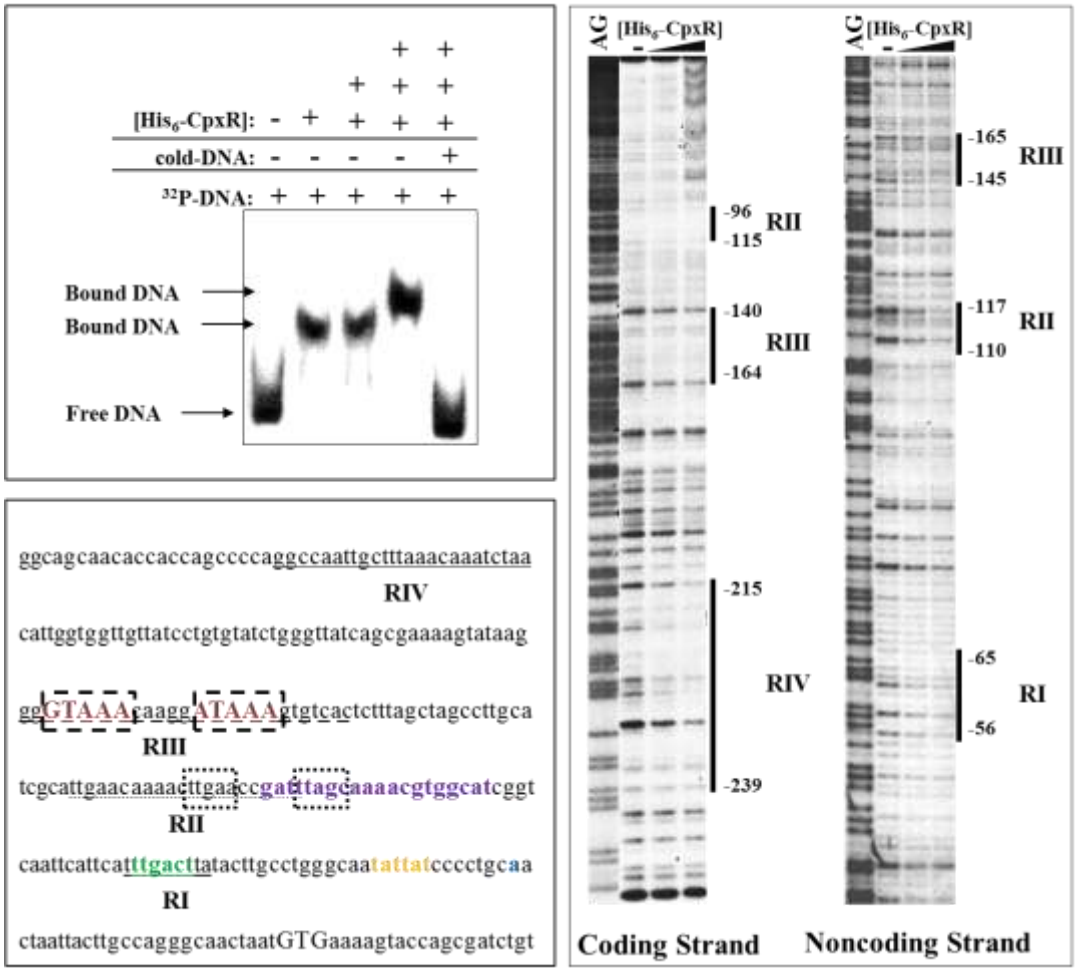


Figure 27. Biochemical analysis of CpxR binding to the *mar* promoter. (**top, left**) EMSA shows that CpxR can bind to at least two locations within the *mar* promoter. Binding is specific because competition with unlabeled DNA (cold-DNA) abolishes the shift. (**bottom, left**) Analysis of CpxR binding to the *marRAB* promoter relative to the previously characterized marbox (purple), -35 (green), -10 (yellow), transcription start site (blue), operator region (underlined), and start codon (GTG). RI, RII, RIII, RIV represent the regions protected by CpxR in the DNase I footprinting analysis. Long dashed box, putative CpxR binding site originally hypothesized. Short dashed box, putative CpxR binding site identified after the analysis. (**right**) DNase I footprinting analysis reveals multiple sites of CpxR protection; RII and RIII protected regions are found on both the coding and noncoding strands.

continues to grow. Additionally, the natural signal for the *marRAB* operon has not been identified as it remains to be investigated whether plant-derived napthoquinones are natural inducers (197). Compounds such as salicylate (SAL), chloramphenicol and tetracycline, acetaminophen, sodium benzoate, 2,4-dinitrophenol, cinnamate, and carbonyl cyanide m-chlorophenylhydazone, menadione and plumbagin have been shown to activate the operon (198). Recently, several aromatic amino acid metabolites were characterized and shown to activate the *mar* promoter (42). Specifically, salicylate-like compounds including 2,3-dihydroxybenzoate (DHB) and anthranilate (ANT) could activate the *mar* promoter (42). In addition, DHB was found to activate the promoter by directly binding to MarR with a similar affinity as SAL, while ANT could not, indicating that the efforts by ANT are independent of MarR (42). ANT is an early intermediate product of the tryptophan biosynthesis pathway (**Figure 28**) and was reported to inhibit growth of an *E. coli* Δ*cpxRA* mutant (199).

Cumulatively, several intermediates of the tryptophan biosynthesis pathway have been shown to induce multidrug resistance in *E. coli*. As previously mentioned, ANT activates the *mar* promoter to increase intrinsic multidrug resistance (42). Additionally, indole (IND), a late intermediate product of the tryptophan biosynthesis pathway (**Figure 28**), can activate the *mdtE* promoter, which encodes a xenobiotic exporter, independently of the EvgA regulator (17). Likewise, IND can activate the BaeR/BaeS two component system which specifically binds to and activates target promoters including other xenobiotic exporters, *acrD* and *mdtABC*, interestingly mediated by CpxR (17).

Chorismate serves as a precursor for aromatic amino acids, phenylalanine, tyrosine, and tryptophan; as well as IND, ANT, DHB and SAL. Deletion of the CpxR-dependent *aroK*

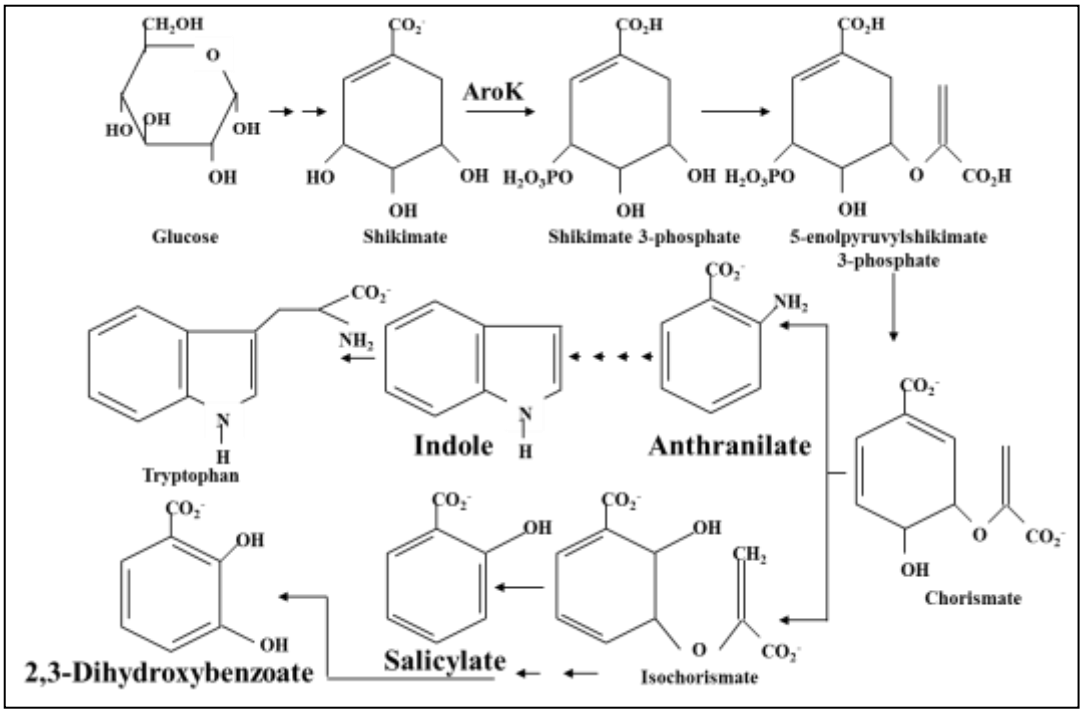


Figure 28. Tryptophan biosynthesis pathway.
The tryptophan biosynthesis pathway generates intermediates previously demonstrated to activate the *mar* operon and confer multidrug and antimicrobial resistance.

gene (195) (which encodes an secondary kinase that catalyzes an early step in chorismate biosynthesis: shikimate to shikimate 3-phosphate, **Figure 28**), resulted in increased susceptibility to protamine (**APPENDIX D**). Taken together, these reports suggested a novel, physiological signal for the CpxR/CpxA system to mediate resistance to antimicrobial substances because (i) aromatic metabolites could activate the *mar* locus, (ii) CpxR regulates an enzyme (AroK) involved in the biosynthesis of aromatic metabolites but suggested to have an additional physiological role, (iii) deletion of *cpxRA* results in increased susceptibility to an aromatic metabolite (ANT), and (iv) an aromatic metabolite (IND) was found to mediate multidrug resistance with CpxR.

Investigation of aromatic metabolites as signals for CpxR/A. To ascertain if aromatic metabolites could serve as an activation signal for the CpxR/A, chromosomal *lac* fusions of the Cpx-dependent loci *cpxP* (*cpxP-lac*, (200, 201)) and *degP* (*degP-lac*, (55, 201)) were used to determine if transcription could be increased in the presence of aromatic metabolites, ANT, IND, and SAL. β-galactosidase activity of *cpxP-lac* was increased 3.9-fold, 2.9-fold, and 2.2-fold in the presence of ANT, IND and SAL, respectively (**Figure 19, top**). Induction by IND was shown to be significant with a Student's *t*-Test in which the $p = 0.001$; however, not significant for ANT and SAL ($p = 0.22$ and 0.04, respectively) indicating that IND can serve as a signal for CpxR/A. Likewise, IND significantly increased β-galactosidase activity of *degP-lac* 3.4-fold ($p = 0.0099$), while ANT and SAL were unable to increase activity (**Figure 29, top**), providing additional evidence that IND can serve as a signaling cue for the Cpx system. As a positive control, Δ*marA-lac* and pYS1734 were used since SAL and ANT have previously been determined to induce expression of the *mar* operon (32, 42). Indeed, the presence of SAL

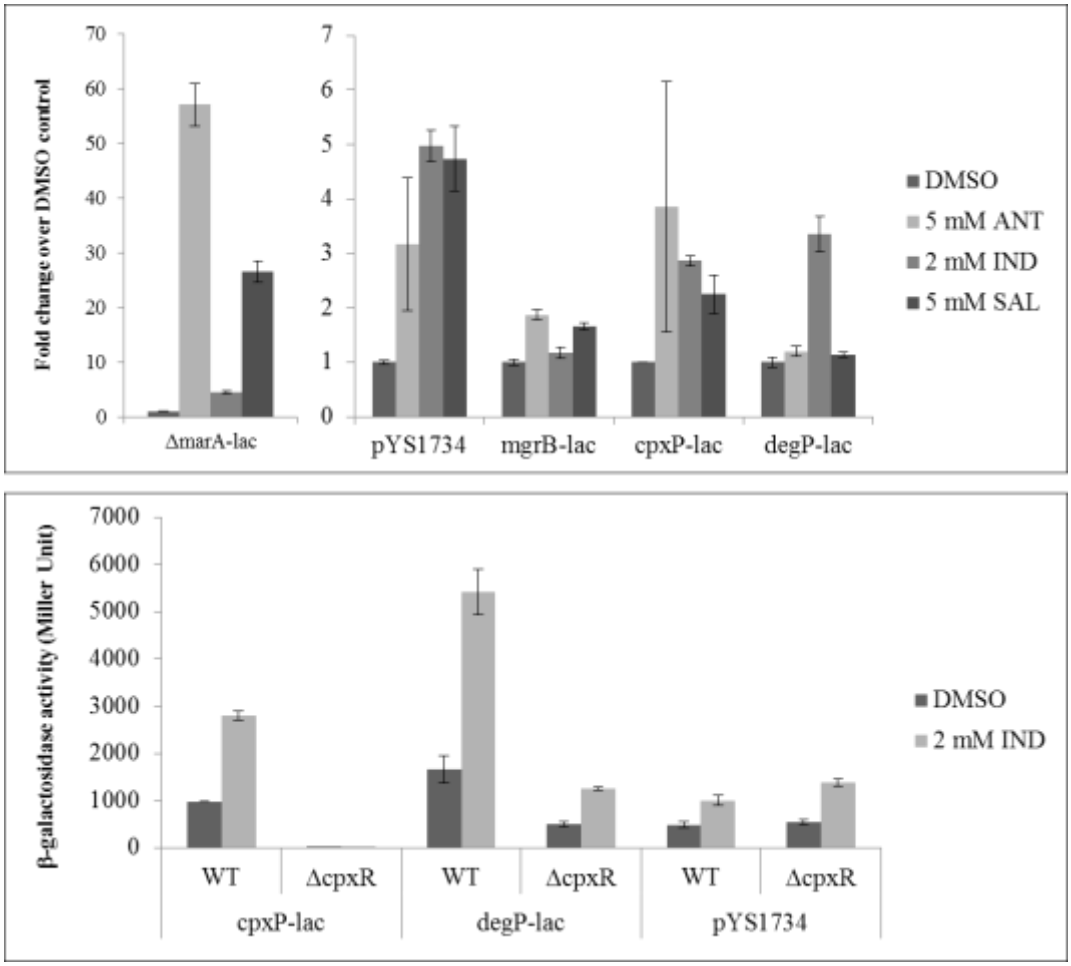


Figure 29. Induction of β-galactosidase activity by aromatic metabolites.
(**top**) IND is able to significantly induce expression of *cpxP-lac* and *degP-lac* reporters, but not ANT or SAL. IND, ANT, and SAL are all able to induce expression of the *mar-lac* consructs, Δ*marA-lac* and pYS1734 controls, but not *mgrB-lac*. (**bottom**) Induction by IND is independent of CpxR since deletion of *cpxR* is not sufficient to abolish IND-dependent induction. DMSO, Dimethyl sulfoxide ; ANT, anthranillate; IND, indole; SAL, salicylate.

increased transcription of Δ*marA-lac* and pYS1734 26.5-fold and 4.7-fold, respectively, while ANT increased transcription 57.1-fold and 3.2-fold, respectively (**Figure 29, top**). Interestingly, IND was also able to significantly increase transcription of both Δ*marA-lac* and pYS1734 4-fold and 5-fold, respectively (**Figure 29, top**), thus providing evidence of IND as an additional inducer of the *mar* operon, as well as the CpxR/A system. Notably, *mgrB-lac*, a PhoP-dependent (202) construct served as a negative control. None of the aromatic metabolites were able to significantly increase β-galactosidase activity suggesting that the IND induction is specific to the CpxR/A and Mar systems and not a general transcriptional phenomenon.

Since IND was demonstrated to be a signal for the *mar* promoter and the CpxR/A system, an investigation was carried out to determine if the induction was dependent on the CpxR/A system directly. Δ*cpxR* was introduced into each strain to yield *cpxP-lac* Δ*cpxR*, *degP-lac* Δ*cpxR*, and Δ*cpxR* pYS1734; β-galactosidase activity was once again determined in the presence of IND. Except in *cpxP-lac* Δ*cpxR* in which deletion of *cpxR* completely abolishes transcription of *cpxP*, each *lac* construct was able to be induced in the presence of IND (**Figure 29, bottom**) suggesting that the induction by IND is independent of CpxR/A. While CpxR/A was not the direct system mediating the response to IND, it is noteworthy to determine that IND can activate both the CpxR/A system and the *mar* operon. The mechanism by which IND induces expression of these systems remains to be investigated. An analysis was carried out in which simultaneous deletion of *cpxR* and a gene encoding a regulator of a known two-component system in the *degP-lac* strain was assayed to determine if transcription induction by IND could be eliminated. Independent deletion of known two-component system regulators in *degP-lac* Δ*cpxR* was

not sufficient to abolish IND-dependent induction (data not shown) suggesting that another regulatory system mediates induction.

Conclusion

The *E. coli* CpxR/CpxA two-component system regulates a set of genes in response to general periplasmic stress (reviewed in (10)). As the Cpx regulon continues to be expanded (to date, there are over 150 demonstrated or putative members (**APPENDIX E**)), the vastness of its circuitries becomes evident as it continues to be implicated in virulence (reviewed in (10)), biofilm formation (13), chemotaxis (14) and recently, resistance to antimicrobials (15, 16, 18, 20), therefore having a global effect in signal transduction pathways and bacterial resistance.

CHAPTER 4

CHARACTERIZATION OF CPXR/A-DEPENDENT LOCI REQUIRED FOR BACTERIAL RESISTANCE TO ANTIMICROBIAL PEPTIDES

Introduction

Many studies have focused on the characterization of the CpxR/CpxA two-component system. CpxR/A globally regulates a vast number of promoters in response to periplasmic stresses caused by misfolded proteins, inner membrane disruptions, alkaline pH, starvation, and high osmolarity (reviewed in (10)). Moreover, CpxR/A appears to contribute globally to *Escherichia coli* resistance to antimicrobial peptides (AMPs) since deletion of *cpxR* or *cpxA* increased bacterial susceptibility to RTD-2 and OH-CATH30 (**APPENDIX D**). Additionally, Chapter 3 characterized new members of the CpxR/A regulon, including *amiA, amiC, marRAB,* and the *rfe-rff* gene cluster. Furthermore, analysis of the systematic screen carried out in Chapter 1 identified six candidates (*aroK, degP, dnaK, dsbA, tolC,* and *yqjA*) that were previously characterized as part of the CpxR/A regulon, further suggesting that CpxR/A could play a global role in bacterial resistance to AMPs.

Characterization of CpxR/A-dependent loci required for bacterial resistance to antimicrobial peptides

The notion that the CpxR/A system could be a major contributor to *E. coli* resistance to AMPs led to further analysis of the gene candidates identified in the systematic screen in Chapter 1. Six of those members, as well as those identified in Chapter 3 (**Table 8**), were found to contribute to bacterial resistance to AMPs because deletion of the genes resulted in increased susceptibility. Although several CpxR/A-dependent loci were identified as

Table 8. CpxR/A-dependent loci required for resistance to protamine

Gene	Function
amiA	*N*-acetylmuramoyl-L-alanine amidases
amiC	*N*-acetylmuramoyl-L-alanine amidase
aroK	shikimate kinase involved in tryptophan biosynthesis
degP	proteinase/chaperone
dnaK	Hsp70 molecular chaperone
dsbA	disulfide isomerase
rfe-rff	enterobacterial common antigen biosynthesis
tolC	outer membrane pore involved in efflux
yqjA	DedA-like predicted inner membrane protein

contributing to bacterial resistance to AMPs, it does not infer that they work together to contribute to resistance.

Identification of multiple CpxR/A-dependent pathways that contribute to bacterial resistance to AMPs. To determine if the CpxR/A-dependent genes (or gene products) contribute to bacterial resistance to AMPs cooperatively or independently of each other, double mutants were systematically constructed in which each mutant, except members of the *rfe-rff* gene cluster, were crossed with one another to generate each combination of double mutants. It was hypothesized that if two genes work independently, then simultaneous deletion of both genes would cause a synthetic phenotype and render cells more sensitive to AMPs than either single deletion (illustrated in **Figure 30**). Conversely, if two genes work coordinately in the same pathway to contribute to resistance, then simultaneous deletion of both genes would not change the sensitivity to AMPs when compared to either single deletion (illustrated in **Figure 30**). Double deletion mutants were challenged against the model AMP, protamine, along side their single mutant counterparts to determine if deletion of both genes would render cells more sensitive to protamine than either single deletion. Notably, the analysis suggested that both TolC and YqjA contribute to bacterial resistance to AMPs independently of the other CpxR/A-dependent loci identified (illustrated in **Figure 30**) because simultaneous deletion of *tolC* or *yqjA* with each of the other mutations rendered cells more susceptible to protamine than either single deletion mutant. On the other hand, data suggest that *amiA, amiC, aroK,* and *dnaK* work coordinately in their contribution to AMP resistance (illustrated in **Figure 30**), since double deletions within this group of genes did not increase susceptibility to protamine. Likewise, the data suggest that *dsbA, degP,* and *aroK*

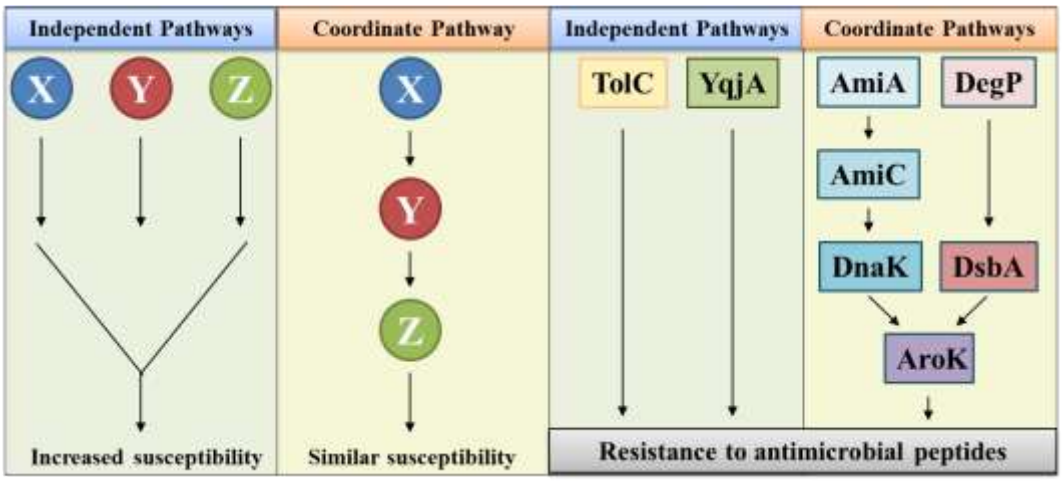


Figure 30. Independent and coordinate CpxR/A-dependent pathways. Illustration of the independent and coordinate pathways of CpxR/A-dependent loci required for bacterial resistance to protamine. TolC and YqjA appear to contribute to bacterial resistance to protamine independently than any of the other Cpx-dependent loci identified in the systematic analysis. On the other hand, AmiA, AmiC, DnaK, and AroK appear to comprise a single pathway, while DegP, DsbA, and AroK comprise an additional pathway. Notably, AroK contributes to resistance in both pathways, suggesting a vital role in the *aroK* gene product in resistance. (NOTE: gene products are listed alphabetically and do not represent the true progression of the pathway to bacterial resistance to AMPs).

contribute to bacterial resistance to protamine on the same pathway (illustrated in **Figure 30**) because simultaneous double deletions did not increase susceptibility to protamine. Interestingly, AroK seems to play a role in both independent pathways suggesting a significant role of the *aroK* gene product.

AroK mediated resistance to AMPs. AroK is a kinase that catalyzes the conversion of shikimate to shikimate-3-phosphate in an early step in chorismate biosynthesis (**Figure 28**). Therefore, it can be speculated that downstream products in the chorismate biosynthesis, or chorismate-dependent biosynthesis pathways (such as tyrosine, phenylalanine, tryptophan, or siderophore biosynthesis), contribute to resistance. To support this claim, shikimate analogs have been synthesized and determined to have antimicrobial activity because they inhibit the production of downstream products of the chorismate biosynthesis pathway (203, 204).

Cooperativity of AmiA, AmiC, and DnaK in resistance to AMPs. The cooperative contribution between the peptidoglycan amidases, AmiA and AmiC, and the molecular chaperone, DnaK, was apparent. AmiA and AmiC are *N*-acetylmuramoyl-L-alanine amidases transported by the twin-arginine transporter (Tat) system (51, 61), a Sec-independent system responsible for the transport of globular proteins across the inner membrane (45). The *E. coli* Tat system transports folded proteins containing a conserved twin arginine motif within the signal peptide sequence at the N-terminal (48). The signal peptide is recognized by the Tat apparatus and is cleaved prior to transport. The Tat signal peptide, however, is vulnerable to proteolytic degradation as it is not folded into the mature protein (205). Therefore, molecular chaperones bind the signal sequence to protect it from proteolysis prior to its recognition by Tat. Specifically, SlyD, GroEL, and

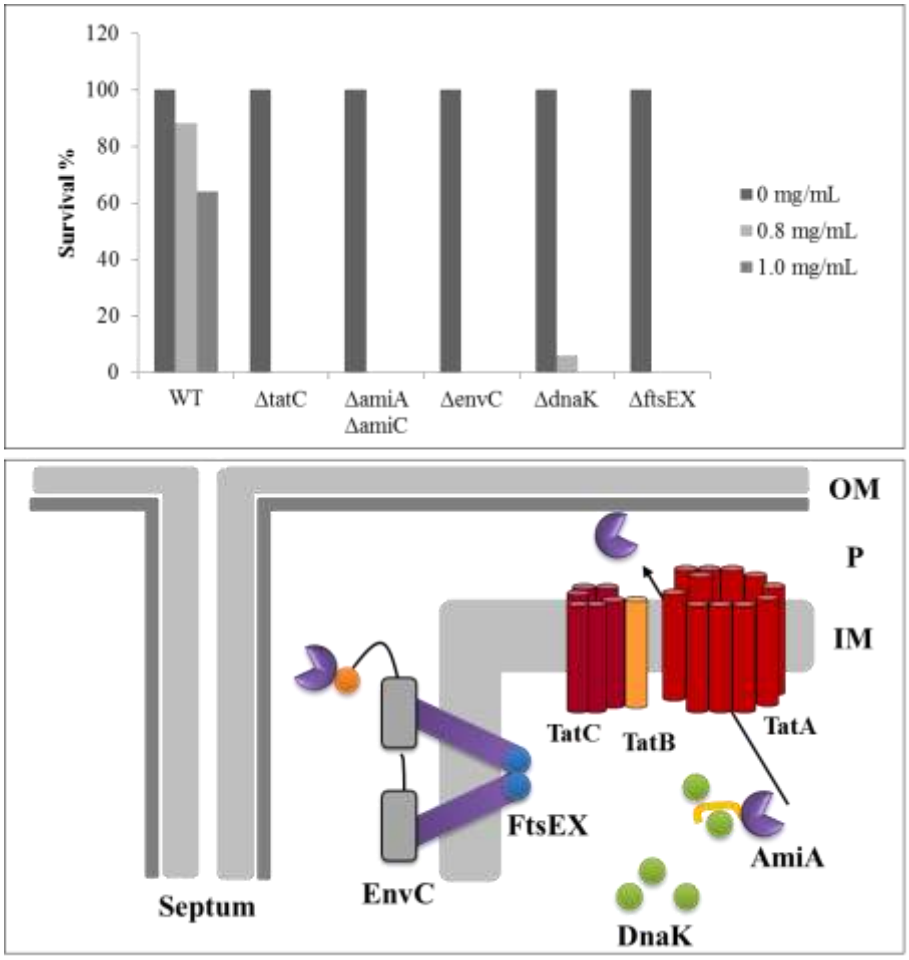


Figure 31. Importance of amidase synthesis, transport and activity.
(**top**) Survival % of *tatC, amiA amiC, envC, dnaK,* and *ftsEX* mutant strains after challenged against protamine. (**bottom**) Schematic illustrating the importance of amidase synthesis, transport, and activity mediated by DnaK, Tat, EnvC, and FtsEX.

DnaK chaperones have all been demonstrated as Tat signal binding chaperones for an array of Tat-dependent substrates (206-208). Therefore, it feasible that DnaK and AmiA/AmiC coordinately contribute to bacterial resistance to protamine via DnaK-mediated protection of AmiA and AmiC transport. Furthermore, it is likely that these amidases have a direct role in resistance since alteration of their stability either prior to transport via DnaK, during transport via Tat, or during their enzymatic activities via EnvC (a functional activator of AmiA (62) or FtsEX (regulators of cell-wall hydrolysis that directly recruit EnvC to the septum (63)) increases bacterial susceptibility to protamine since deletion of *dnaK, tatC, envC,* and *ftsEX* increased bacterial susceptibility to protamine (**Figure 31**).

Cooperativity of DsbA and DegP in resistance to AMPs. The cooperative contribution of DsbA and DegP in the same pathway is obscure. Both DsbA and DegP are involved in bacterial pathogenesis by contributing to virulence and intracellular survival, respectively (reviewed in (10)). It was recently reported that simultaneous deletion of *dsbA* and *degP* results in synthetic phenotypes which render cells sensitive to salt and SDS, likely due to the increase in membrane permeability (209). Additionally, the double mutant is unable to remove unfolded outer membrane proteins from the periplasm resulting in an accumulation of OMPs and subsequent activation of σ^E, a stress-response system that has been reported to activate transcription of *degP* (209). Notably, single deletions of either gene rendered cells sensitive to salt and SDS, although at a lower level than the double mutant. Thus, it is possible that DsbA and DegP do not contribute directly to bacterial resistance to AMPs. Instead, deletion of either gene alters the membrane permeability, which results in an increase in protamine susceptibility. Deletion of both genes simply

increases the sensitive phenotype.

CpxR/A activation increases bacterial resistance to antimicrobial peptides

The data presented in this study suggest a global contribution of the CpxR/A system in resistance to AMPs. Therefore, it was hypothesized that activation of the CpxR/A system would increase bacterial resistance. Survival of wild-type and Δ*cpxR* strains harboring a vector or p*nlpE* plasmid were compared to determine if activation of CpxR/A via *nlpE* overexpression could increase *Salmonella typhimurium* resistance to protamine. Indeed, overexpression of *nlpE* was able to increase bacterial resistance to protamine as determined by a 61% survival rate at a lethal protamine concentration for *Salmonella* wild-type cells, 1.8 mg mL^{-1} (**Figure 32, top**). The increase in resistance was dependent on CpxR as deletion of *cpxR* abolished the increased resistance (**Figure 32, top**). Further, overexpression of *nlpE* could increase resistance in a Δ*tatC* mutant in a CpxR-dependent manner because cells exhibited a 54% survival rate at a lethal protamine concentration for the Δ*tatC* mutant strain, 1.0 mg mL^{-1} (**Figure 32, middle**).

Other work has demonstrated that overexpression of *nlpE* can increase resistance of several *Salmonella* protamine susceptible-mutants, including Δ*phoP* (unpublished data, **Figure 32, bottom**). Therefore, it was not surprising that overexpression of *nlpE* could increase protamine resistance in a Δ*tatC* mutant strain, suggesting that CpxR/A globally contributes to bacterial resistance to protamine. An investigation was initiated to identify the CpxR/A-dependent gene(s) that contributes to the increase in resistance. Δ*tolC* was an obvious candidate as deletion of *tolC* abolished NlpE-mediated multidrug resistance [Nishino *et al*, 2010]. Both a vector control and p*nlpE* were introduced into a Δ*tolC* background to determine if overexpression of *nlpE* could increase resistance to

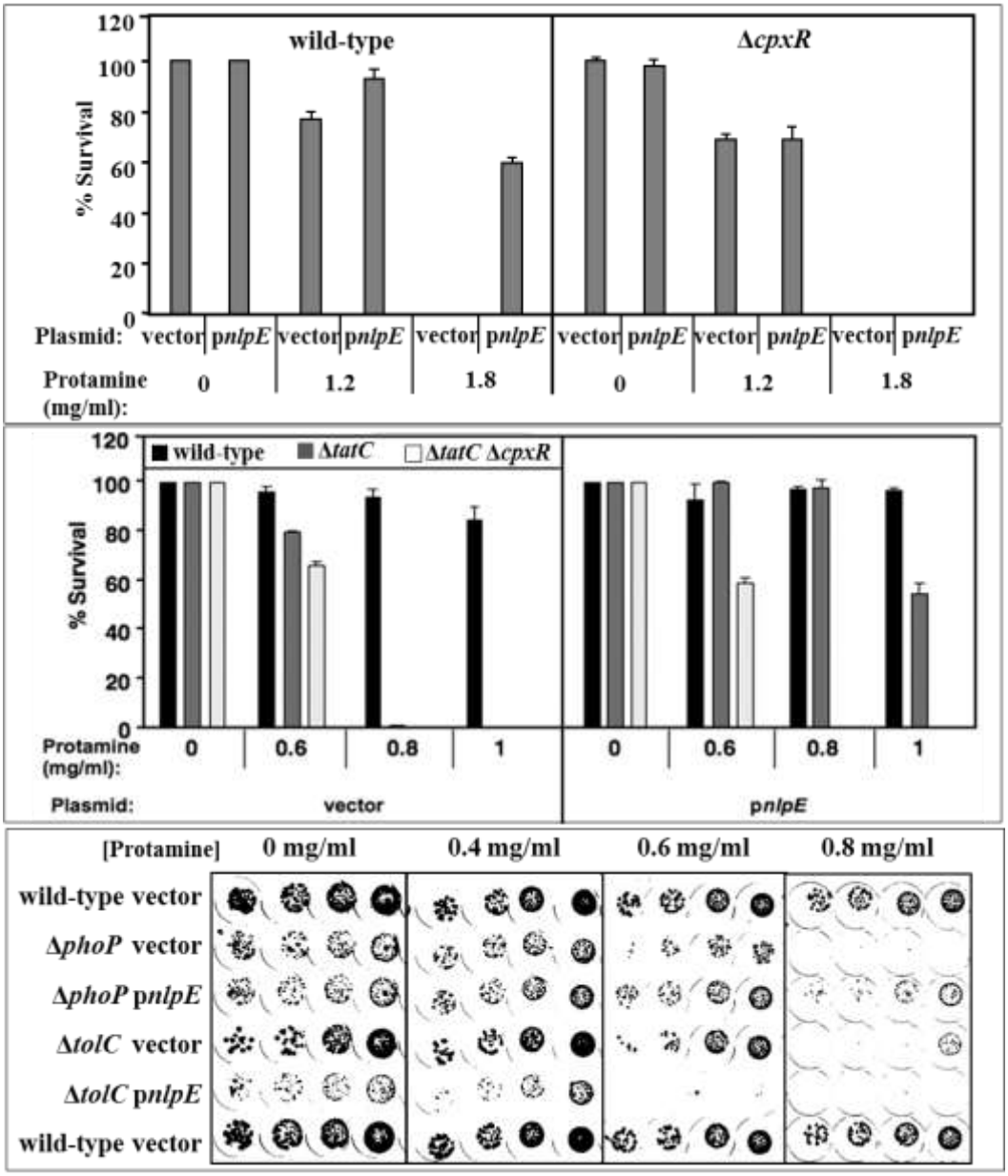


Figure 32. CpxR/A-activation rescues resistance to protamine.
Overexpression of *nlpE* can increase protamine resistance of *Salmonella* (**top**) wild-type, (**middle**) Δ*tatC* and (**bottom**) Δ*phoP* in a CpxR-dependent manner. Resistance of Δ*tolC* cannot be increased by *nlpE* overexpression.

protamine. As predicted, overexpression of *nlpE* could not increase resistance in Δ*tolC*, but could in the Δ*phoP* control (**Figure 32, bottom**) suggesting that the increase in NlpE-mediated bacterial resistance to protamine was dependent, at least in part, on TolC. Interestingly, overexpression of *nlpE* appeared to have a cytotoxic affect in the Δ*tolC* strain since protamine susceptibility was increased (**Figure 32, bottom**). The cytotoxicity is proposed to be due to the accumulation of an unknown toxic protamine-influenced byproduct that is generally expelled by TolC.

Conclusion

The data presented in this study cumulatively identified the CpxR/CpxA two-component system as a global regulator that has a significant contribution to bacterial resistance to AMPs. Through the upregulation of several loci, CpxR/A can mediate bacterial resistance by either actively counteracting the bactericidal effects of AMPs or by playing a protective role against AMPs.

CHAPTER 5

DISCUSSION AND FUTURE WORK

Systematic screen to identify genes required for bacterial resistance to antimicrobial peptides

The data presented here represent the results and subsequent analysis from the first reported large-scale, systematic screen to identify *Escherichia coli* genes that contribute to bacterial resistance to antimicrobial peptides (AMPS). The Keio collection (154) of ~4,000 *E. coli* single gene deletion mutants was challenged against physiologically significant peptides representing α–helix and β–sheet classes of AMPs, as well as a model AMP, to identify mutant strains that were susceptible to the AMPs thus suggesting that the gene (or gene product) contributes to resistance. In total, the screen identified 112 loci (<3% of genes present on the *E. coli* chromosome) that contributed to resistance to at least one analyzed AMP (**APPENDIX D**) and function in a diverse array of cellular processes.

Surprisingly, most of the mutant strains identified in the analyses were sensitive to only one AMP suggesting that the gene indirectly contributes to resistance to that particular AMP or that each AMP exerts its bactericidal activity differently. The latter is confirmed by previous observations in which different classes of AMPs were shown to exert their effects via different mechanisms (as discussed in Introduction). Nevertheless, a commonality regarding the bactericidal activity of the AMP or the mechanism of resistance was expected. Thus, it was not surprising that two gene products, a transporter and an outer membrane component, were found to contribute to bacterial resistance against each AMP analyzed.

Tat-dependent resistance

Deletion of *tatC*, which encodes a major component of the twin-arginine translocation (Tat) system rendered cells susceptible to each AMP class, suggesting a global contribution to bacterial resistance to AMPs. Further analysis revealed that the Tat-dependent amidases, AmiA and AmiC, contribute in part to the Tat-dependent resistance. The contributions of AmiA and AmiC to resistance are likely to be direct as interference in their transport or activity renders cells sensitive to protamine. It remains to be determined how AmiA and AmiC contribute to resistance. It can be postulated that the positively charged AMP binds to the negatively charged pentapeptide units of the peptidoglycan. Thus, AmiA and AmiC facilitate resistance by cleaving the pentapeptide-AMP complex at its cleavage site (between the N-acetylmuramic acid residue and the pentapeptide) to prevent accumulation of the AMP in the periplasm, specifically within the peptidoglycan. Further, a periplasmic peptidase/protease could cleave the pentapeptide-AMP complex to prevent accumulation in the periplasm which could be supported by the observation that a Δ*degP* strain is susceptible to protamine. It is unlikely that DegP functions cooperatively with AmiA and AmiC since a mutant with a deletion of *degP* in a Δ*amiA* Δ*amiC* background was more susceptible to protamine, suggesting that *amiA/amiC* and *degP* contribute to resistance in independent pathways. Alternatively, after being cleaved by the amidases, a transporter could mediate transport of the pentapeptide-AMP complex to the cytoplasm for degradation or processing. This explanation is supported by previous data which demonstrate that the *Salmonella* YejABEF ABC transporter is required for resistance to many classes of AMPs (152). Further studies should be carried out to confirm the functional relationship between the

amidases and YeJABEF transporter.

Further analysis regarding Tat-dependent resistance to AMPs implicated two nitrate reductases, NapA and YnfF, in bacterial resistance to protamine. Simultaneous deletion of *napA* and *ynfF* rendered *E. coli* cells susceptible, indicating that the gene products contribute to resistance in concert. Since nitrate is generally reduced in anaerobic conditions, it was proposed that the presence of protamine can simulate an anaerobic environment by inhibiting the use of oxygen as a final electron acceptor in the electron transfer chain, thus disrupting cellular respiration. This is supported by the following observations: (i) protamine can inhibit oxygen consumption (189); (ii) deletion of the F_1F_0-ATPase (which abolishes the production of ATP via oxidative phosphorylation) increases *E. coli* susceptibility to protamine presumably due to the inefficient production of ATP from substrate-level phosphorylation because supplementation of glucose (which should increase the production of ATP via glycolysis) can restore resistance to wild-type levels (data not shown); and, (iii) supplementation of formate or succinate, organic acids oxidized in the tricarboxylic acid cycle (which should increase the production of ATP via substrate-level phosphorylation) is able to protect bacterial cells from the oxygen consumption inhibitory effects of human bactericidal/permeability-increasing protein (BPI) and increase bacterial survival in the presence of BPI (170) and protamine (**Figure 2, right**). Nevertheless, it remains to be determined how these nitrate-reducing enzymes facilitate protamine resistance since exogenous nitrate was not added to the bacteria-AMP challenge systems.

Enterobacterial common antigen-dependent resistance

Deletion of multiple loci residing in the *rfe-rff* gene cluster was found to cause

susceptibility to all peptide classes analyzed, suggesting a global contribution of their gene products to AMP resistance. The *rfe-rff* gene cluster encodes enzymes involved in the biosynthesis of the enterobacterial common antigen (ECA), a glycolipid composed of a trisaccharide repeat located on the outer leaflet of the outer membrane in all bacteria species of the *Enterobacteriaceae* family (reviewed in (54)). Initial analyses suggested that the periplasmic accumulation of intermediates of the ECA biosynthesis pathway was responsible for the increased susceptibility to AMPs. This idea was not supported as deletion of *rfe,* which encodes the first enzyme in the pathway and thus inhibits the formation of ECA or its intermediates, also rendered cells susceptible to protamine. This evidence led to the re-evaluation of previous studies which postulate that accumulation of the lipid II intermediate caused susceptibility to bile salt (55). Analysis with each gene mutant determined that the absence of ECA and not the accumulation of a specific intermediate was the sole reason for increased susceptibility to bile salt. Thus, it appears that ECA plays a protective role against host-secreted factors, bile salt and AMPs. It remains to be determined how ECA can mediate resistance. Perhaps ECA facilitates resistance similarly to lipopolysaccharide (LPS), which are quite similar in regards to the biosynthesis and structural components. It may serve as a critical barrier to prevent AMPs from reaching the outer membrane to exert their activity. Additional studies should be carried out, however, to resolve this mechanism of resistance since ECA appears to be a global contributor to bacterial resistance to host-secreted antimicrobial substances. In fact, prevous biochemical analyses have shown that protamine can form a complex with purified ECA (210) suggesting that ECA can function in bacterial resistance to AMPs by binding to AMPs to prevent integration into the cell.

CpxR/A: a global regulator in *E. coli* resistance to antimicrobial peptides

Genetic and biochemical analyses were conducted that identified the CpxR/CpxA two-component system as a global regulator in bacterial resistance to antimicrobial peptides. First, several previously characterized CpxR/A-dependent loci were identified in the screen (**Table 8**), providing initial evidence of the contribution of this system. Next, analyses demonstrated that CpxR could activate transcription of *amiA* and *amiC*, the *marRAB* operon, as well as, the *rfe-rff* gene cluster; each of which contributes to *E. coli* resistance to AMPs. It remains to be determined how and why CpxR-dependent regulation of these loci contributes to resistance.

CpxR/A-dependent regulation of amiA and amiC. The CpxR/A system has historically been shown to respond to extracytoplasmic stress. For example, in the presence of misfolded proteins, CpxR will upregulate the production of proteases that act to reduce the load of misfolded proteins. Meanwhile, CpxR will downregulate the production of outer membrane proteins which likely are the source of the misfolded proteins. In the parameter of AMP resistance, it is known that AMPs can traverse the multiple layers of the cell envelope to exert its effect. Certain AMPs, for example LL-37, have been shown to translocate the outer membrane and laterally diffuse throughout the periplasm prior to crossing the inner membrane (128). It is feasible that during the periplasmic diffusion, AMPs disrupt cellular processes carried out in the periplasm and thus cause a stress that triggers activation of the CpxR/A system. In this case, AmiA and AmiC may facilitate removal of the stress by remodeling the peptidoglycan matrix by reducing the accumulation of the AMP bound to the tetrapeptide (as discussed above).

CpxR/A-dependent regulation of the rfe-rff gene cluster. Deletion of *rfe* (*wecA*) or *rffA*

(*wecE*) has been previously shown to activate transcription of the CpxR/A-dependent *degP-lac* due to accumulation of the ECA lipid II intermediate in the periplasm (55). Thus, it was surprising to find that CpxR/A could activate transcription of these genes and the others that comprise the *rfe-rff* gene cluster. The physiological role of the ECA has yet to be defined; therefore, it is difficult to ascertain the significance of CpxR/A-dependent regulation. Since CpxR/A has been shown to mediate bacterial resistance to antimicrobial substances, perhaps that upregulation of the ECA facilitates resistance by increasing the amount of ECA on the outer membrane to prevent the antimicrobial substance from entering the cell.

CpxR/A-dependent regulation of marRAB. It was not surprising that CpxR/A could activate transcription of the *marRAB* operon. CpxR has previously been shown to mediate multridrug resistance by facilitating BaeR-dependent activation of *acrD* and *mdtABC* promoters to activate the production of xenobiotic efflux pumps (17). Additionally, activation of CpxR/A via *nlpE* overexpression has been shown to increase bacterial resistance to antibiotics (18) further positing a significant role for CpxR/A in resistance to antimicrobials. The addition of the Mar system to the arsenal of CpxR/A-mediated resistance mechanisms establishes it as the major regulator in antimicrobial resistance since it regulates the sysem previously thought to be the major contributor (Mar), as well as Mar-independent systems.

Cumulatively, this study has identified a multitude of integrated genetic circuitries required for bacterial resistance to AMPs (illustrated in **Figure 33**). This evidence will allow for further elucidation of bacterial resistance to host-mediated factors, as well as shed insight into the strategies of bacterial survival and adaptation in host environments.

Further, it provided additional information regarding the physiological significance of several cellular components.

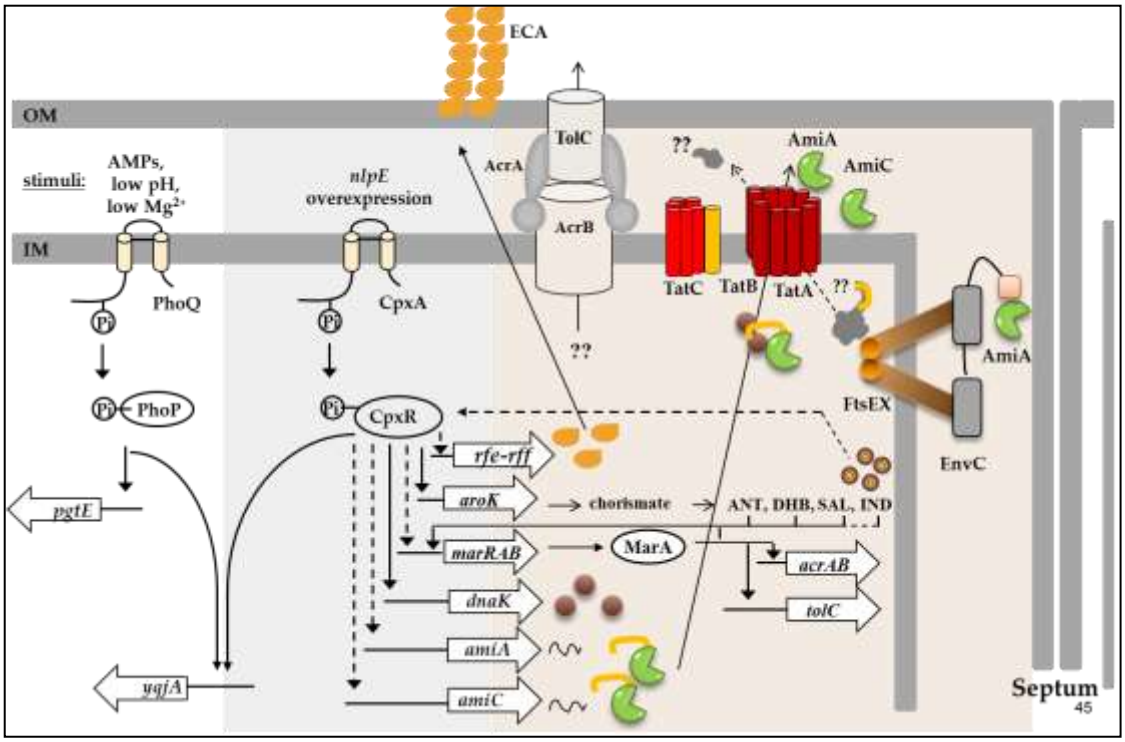


Figure 33. Illustration of novel genetic mechanisms required for bacterial resistance to the model antimicrobial peptide, protamine.
Before this dissertation study, PhoP/PhoQ was the only two-component system determined to be required for resistance to protamine. Now, an extensive CpxR/A-dependent circuitry has been uncovered in which CpxR/A-mediates resistance by regulating several loci. Dashed line, regulation identified in this study. Solid line, regulation previously identified.

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APPENDIX A

LIST OF STRAINS USED IN THIS STUDY

Strain	Description	Reference/Source
Escherichia coli		
BW25113	Δ(*araD-araB*)567 Δ*lac*4787(::*rrnB*-3) λ^- *rph*-1 Δ(*rhaD-rhaB*)568 *hsd*R514	(154)
DH5α	F^- *supE*44 Δ*lacU*169 (φ80 *lac* Δ*M15*) *hsdR*17*recA*1 *endA1* *gyrA*96 *thi*-1 *relA*1	(211)
BL21 (DE3)		Stratagene
Keio collection	Keio collection	(154)
TU190	MG1655 Δ*lacIZYA*::*frt* Δ*ftsEX*::Kan^R	(63)
YS Collection 7	BW25113 Δ*acrB*::*frt* Δ*emrB*::Kan^R	This work
YS Collection 7	BW25113 Δ*acrB*::*frt* Δ*acrE*::Kan^R	This work
YS Collection 7	BW25113 Δ*acrB*::*frt* Δ*emrY*::Kan^R	This work
YS Collection 7	BW25113 Δ*acrB*::*frt* Δ*glnQ*::Kan^R	This work
YS Collection 7	BW25113 Δ*acrB*::*frt* Δ*macB*::Kan^R	This work
YS Collection 7	BW25113 Δ*acrB*::*frt* Δ*mdlB*::Kan^R	This work
YS Collection 7	BW25113 Δ*acrB*::*frt* Δ*mdtF*::Kan^R	This work
YS Collection 7	BW25113 Δ*acrB*::*frt* Δ*metN*::Kan^R	This work
YS Collection 7	BW25113 Δ*emrB*::*frt* Δ*acrB*::Kan^R	This work
YS Collection 7	BW25113 Δ*emrB*::*frt* Δ*acrE*::Kan^R	This work
YS Collection 7	BW25113 Δ*emrB*::*frt* Δ*emrY*::Kan^R	This work
YS Collection 7	BW25113 Δ*emrB*::*frt* Δ*glnQ*::Kan^R	This work
YS Collection 7	BW25113 Δ*emrB*::*frt* Δ*macB*::Kan^R	This work
YS Collection 7	BW25113 Δ*emrB*::*frt* Δ*mdlB*::Kan^R	This work
YS Collection 7	BW25113 Δ*emrB*::*frt* Δ*mdtF*::Kan^R	This work
YS Collection 7	BW25113 Δ*emrB*::*frt* Δ*metN*::Kan^R	This work
YS Collection 7	BW25113 Δ*acrB*::*frt* Δ*emrB*::*frt* Δ*acrE*::Kan^R	This work
YS Collection 7	BW25113 Δ*acrB*::*frt* Δ*emrB*::*frt* Δ*emrY*::Kan^R	This work
YS Collection 7	BW25113 Δ*acrB*::*frt* Δ*emrB*::*frt* Δ*glnQ*::Kan^R	This work
YS Collection 7	BW25113 Δ*acrB*::*frt* Δ*emrB*::*frt* Δ*macB*::Kan^R	This work
YS Collection 7	BW25113 Δ*acrB*::*frt* Δ*emrB*::*frt* Δ*mdlB*::Kan^R	This work
YS Collection 7	BW25113 Δ*acrB*::*frt* Δ*emrB*::*frt* Δ*mdtF*::Kan^R	This work
YS Collection 7	BW25113 Δ*acrB*::*frt* Δ*emrB*::*frt* Δ*metN*::Kan^R	This work
	BW25113 Δ*marA*::Kan^R	(154)
	BW25113 Δ*marR*::Kan^R	(154)
	BW25113 Δ*marB*::Kan^R	(154)
YS14530	BW25113 Δ*marRA*::Cm^R	This work
YS14490	BW25113 Δ*cpxR*::Cm^R	This work
	BW25113 Δ*rob*::Kan^R	(154)
	BW25113 Δ*soxS*::Kan^R	(154)
YS15193	BW25113 Δ*rffG*::*frt* Δ*rfbB*::Kan^R	This work
YS15189	BW25113 Δ*wzzE*::*frt* Δ*cld*::Kan^R	This work
YS15191	BW25113 Δ*wzzE*::*frt* Δ*fepE*::Kan^R	This work
YS13683	BW25113 Δ*amiA*::*frt* Δ*amiC*::*frt*	This work

Strain	Description	Reference/Source
YS Collection 9	BW25113 Δ*amiA::frt* Δ*amiC::frt* Δ*citE* ::Kan^R	This work
YS Collection 9	BW25113 Δ*amiA::frt* Δ*amiC::frt* Δ*fdnG* ::Kan^R	This work
YS Collection 9	BW25113 Δ*amiA::frt* Δ*amiC::frt* Δ*hyaA* ::Kan^R	This work
YS Collection 9	BW25113 Δ*amiA::frt* Δ*amiC::frt* Δ*sufI* ::Kan^R	This work
YS Collection 9	BW25113 Δ*amiA::frt* Δ*amiC::frt* Δ*wcaM* ::Kan^R	This work
YS Collection 9	BW25113 Δ*amiA::frt* Δ*amiC::frt* Δ*yaeI* ::Kan^R	This work
YS Collection 9	BW25113 Δ*amiA::frt* Δ*amiC::frt* Δ*yagT* ::Kan^R	This work
YS Collection 9	BW25113 Δ*amiA::frt* Δ*amiC::frt* Δ*yahJ* ::Kan^R	This work
YS Collection 9	BW25113 Δ*amiA::frt* Δ*amiC::frt* Δ*ybiP* ::Kan^R	This work
YS Collection 9	BW25113 Δ*amiA::frt* Δ*amiC::frt* Δ*ycbK* ::Kan^R	This work
YS Collection 9	BW25113 Δ*amiA::frt* Δ*amiC::frt* Δ*yedY* ::Kan^R	This work
YS Collection 9	BW25113 Δ*amiA::frt* Δ*amiC::frt* Δ*ynfE* ::Kan^R	This work
YS Collection 8	BW25113 Δ*napA::frt* Δ*ynfF* ::Kan^R	This work
YS15226	BW25113 Δ*rfe-lac lacY*$^+$	This work
YS14891	BW25113 Δ*rffT-lac lacY*$^+$	This work
YS15227	BW25113 Δ*rffM-lac lacY*$^+$	This work
YS14331	BW25113 Δ*marR-lac lacY*$^+$	This work
YS14329	BW25113 Δ*marA-lac lacY*$^+$	This work
YS14330	BW25113 Δ*marB-lac lacY*$^+$	This work
YS14534	BW25113 Δ*marRA-lac lacY*$^+$	This work
YS11195	BW25113 Δ*mgrB-lac lacY*$^+$	This work
TR49	MC4100 λRS88[*degP-lac*]	(200, 201)
TR50	MC4100 λRS88[*cpxP-lac*]	(55, 201)
***S. enteric* serovar Typhimurium**		
14028s	wild-type	ATCC
YS13007	14028s Δ*tatC*::Kan^R	This work
YS13629	14028s Δ*amiA*::Cm^R	This work
YS13630	14028s Δ*amiC*::Cm^R	This work
YS13766	14028s Δ*amiA* Δ*amiC*::Cm^R	This work
YS11590	14028s Δ*phoP*	(212)
YS13644	14028s Δ*cpxR*::Cm^R	This work
YS13637	14028s Δ*amiA-lac lacY*$^+$	This work
YS13640	14028s Δ*amiC-lac lacY*$^+$	This work
YS13995	14028s Δ*tatC*::*frtC* Δ*cpxR*::Cm^R	This work

APPENDIX B

LIST OF PLASMIDS USED IN THIS STUDY

Plasmid	Description	Reference/Source
pKD3	$rep_{R6K}\gamma$ Ap^R FRT Cm^R FRT	(155)
pKD46	$rep_{pSC101}{}^{ts}$ Ap^R P_{araBAD} γ β exo	(155)
pCP20	$rep_{pSC101}{}^{ts}$ Ap^R Cm^R cI857 λP_R	(155)
pCE36	$rep_{R6K}\gamma$ Km^R FRT *lacY* t_{his}	(156)
pCE37	$rep_{R6K}\gamma$ Km^R FRT *lacY* t_{his}	(156)
pUC19	rep_{pMB1} Ap^R	(213)
pBAD TOPO	rep_{pBR322} Ap^R araC P_{BAD}	Invitrogen
pBAD-*tatC*	rep_{pBR322} Ap^R araC P_{BAD} *tatC*	This work
pUHE21–2*lacI*^{q}	rep_{pMB1} Ap^R *lacI*^{q}	(157)
pUHE-*amiA*	rep_{pMB1} Ap^R *lacI*^{q} *amiA*	This work
pUHE-*amiC*	rep_{pMB1} Ap^R *lacI*^{q} *amiC*	This work
pUHE-*yaeJ*	rep_{pMB1} Ap^R *lacI*^{q} *yaeJ*	This work
pUHE-*nlpE*	rep_{pMB1} Ap^R *lacI*^{q} *nlpE*	This work
pET28a	rep_{ColE1} Km^R *lacI* P_{T7}	Novagen
pYS2135	rep_{ColE1} Km^R *lacI* P_{T7} His6-*cpxR*	This work
pYS1000	rep_{p15A} Cm^R $p_{lac1\text{-}6}$ *lac* t_{his}	(158)
pYS1734	rep_{p15A} Cm^R $p_{lac1\text{-}6}$ *lac* t_{his} *marR* 5'UTR1-570	This work
pYS1736	rep_{p15A} Cm^R $p_{lac1\text{-}6}$ *lac* t_{his} *marR* 5'UTR1-120	This work

APPENDIX C

LIST OF PRIMERS USED IN THIS STUDY

Number	Sequence
tatC-forward	GGGACCGTAAACATGGCTGTA
tatC-reverse	CGGTTGTGTAAAGTCTTCAGT
232	GAAACAGCTATGACCATG
233	TTCCCAGTCACGACGTTG
1327	CGGGATCCTTTTCACAACTCAGGC
1328	CCCAAGCTTTTACCGTTTCTTCGTG
1369	CTTGAACTTAATTTTCACAACTCAGGCCGTCATATGAATATCCTCCTTAG
1370	CTTTCTGATTATCAAACCAGTGAAAATAACGTGTAGGCTGGAGCTGCTTC
1396	ATCTCTATTTAGTTTTTGCTCGGGAGAAGCCATATGAATATCCTCCTTAG
1397	CCCGCGCAATAAACTCGCCGTCATCTCAGGGTGTAGGCTGGAGCTGCTTC
1402	CCCAAGCTTTTGCTCGGGAGAAGC
1403	CCCAAGCTTAACTTCTTCTCGCCAGCG
1407	CGTAATTTCTGCCTCGGAGGTACGTAAACACATATGAATATCCTCCTTAG
1408	TCCTATCATGAAGCGGAAACCATCAGATAGGTGTAGGCTGGAGCTGCTTC
1413	CGGGATCCTGGCAACAGCCCTCATG
1414	CCCAAGCTTCAATCCAGTGGACGAC
1421	CGGGATCCATTTCATAAGGATTTTATGG
1422	CCCAAGCTTAGTGAGTGCAATCTTTAC
1461	GTTTAGCCGATTAGCTATAAAGGTGGCGGGCATATGAATATCCTCCTTAG
1462	CCAGCGGCGATTTGGTTCGCAAGCTGCGGGGTGTAGGCTGGAGCTGCTTC
1472	TTAGGAGTTTAAAAGTGCTCAT
1482	ATAAAATTTACGCTTGCACAGA
1484	CTTCGCCGCCGAGCAT
1512	GTTTCATATGAATAAAATCCTG
1513	ACGCGTCGACTCATGAAGCGGAAACCAT
1567	CAAGATTATGGCGCAAACATCTG
1595	ATAATGGCGATGTGTCACGTATTCACATGAAAACACATACAATTCTCATCACCAAC
1598	CTTCTACCAGTTCGGTATGTGGTTCCATGTGCATTGCGCGCCCCACTAG
1723	TTGCCTGCCAGGCCA
1725	CAGATCGCTGGTACTTTTCAC
1731	CCGCTCGTTCATTGAACAGATCGCTGGTACTTTTCAC
1734	GCGTCGACGCAATATTGGCACTGGGTTCATCTTCCAGC
1736	GCGTCGACCTTTAGCTAGCCTTGCATCGCATTGAACAA
1803	GACTGTACTGATTCACG
1804	CACTTAAGCCGTCGTC
1912	CTGACAGTGAGTACTGATCTC
1913	GTGACGTTTGCGGAAGTTTG
1914	CATTAACCCGCCGTTGCTCG
1915	CGCGTACCAAATACAGTCAG
1916	GGCAGCTATCAGGGCGAGCG

Number	Sequence
1917	CGCGTAATCGGATGCAATCG
1919	GGCAACCAGCGTTCCCTGC
1921	CTGGCAATGACCAAGACCAATGACG

APPENDIX D

GENE MUTANTS IDENTIFIED IN THE SYSTEMATIC SCREEN

Functional Group	Gene Name	P	R	6	30
ATP Synthase Subunits	*atpB*				
	atpC				
	atpG				
Biosynthesis/ Metabolism	*aroK*				
	cpsG				
	gpmI				
Cell Wall/ Envelope/ Fatty Acid Biosynthesis	*amiC*				
	dacA				
	dadX				
	envC				
	fadB				
	fadR				
	lpp				
	lpxL				
	lpxM				
	prc				
	rfaD				
	yihS				
	yqjA				
DNA/RNA Synthesis/ Degradation/ Repair	*amn*				
	deaD				
	dnaK				
	nfo				
	nrdD				
	recA				
	recJ				
	rep				
	rimK				
	rpmF				
	rpoZ				
	xerC				
	xseA				
	xthA				

Survival % Color Code	
<15%	
15-30%	
30-45%	
45-60%	
60-75%	
75-90%	
>90%	

Susceptibility profile of gene mutants whose gene products function in: ATP synthase complex; biosynthesis/metabolism; cell wall/envelope/fatty acid biosynthesis; and, DNA/RNA biosynthesis/repair. *P*, protamine; *R*, RTD-2; *6*, OH-CM6; *30*, OH-CATH30.

Functional Group	Gene Name	P	R	6	30
Enterobacterial Common Antigen (ECA) Synthesis	*rfe*				
	rffC				
	rffH				
	rffT				
Metabolism/ Catabolism	*cobS*				
	ilvG				
	melA				
	menC				
	pgm				
	ptrB				
	ptsG				
	ycdM				
	yieK				
Regulation	*acnB*				
	argR				
	cpxA				
	cpxR				
	crp				
	feaR				
	gcvA				
	gcvR				
	glnK				
	hflD				
	lldR				
	mak				
	pmrD				
	ygfZ				
	ygiP				
	yijO				
	yrbA				
	rsxB				
Stress Response	*degP*				
	hfq				
	pnp				
	yciM				
	yeaV				
	zwf				

Survival % Color Code	
<15%	
15-30%	
30-45%	
45-60%	
60-75%	
75-90%	
>90%	

Susceptibility profile of gene mutants whose gene products function in: enterobacterial common antigen synthesis; metabolism/catabolism; regulation; and, stress response. *P*, protamine; *R*, RTD-2; *6*, OH-CM6; *30*, OH-CATH30.

Functional Group	Gene Name	P	R	6	30
Transporters/Pores	*alsC*				
	citT				
	cycA				
	ddpX				
	exbB				
	exuT				
	frlA				
	glnQ				
	gntP				
	kdpA				
	mntH				
	putP				
	secB				
	tatA				
	tatC				
	tolC				
	ycjP				
	yedS				
	yggR				
Other/Unknown	*dsbA*				
	dsbB				
	flgN				
	fliI				
	iscU				
	ompA				
	pdxH				
	spr				
	yajD				
	ybhL				
	yddL				
	yedN				
	yehM				
	yfdI				
	yfhM				
	yfjZ				
	ygeY				
	yggS				
	yhjK				
	yigG				
	yiiQ				
	ymgD				
	ypfN				

Survival % Color Code	
<15%	
15-30%	
30-45%	
45-60%	
60-75%	
75-90%	
>90%	

Susceptibility profiles of gene mutants whose gene products function as: transporters/pores and other/unknown functions. *P*, protamine; *R*, RTD-2; *6*, OH-CM6; *30*, OH-CATH30.

APPENDIX E

DEMONSTRATED OR PREDICTED CPXR/A-DEPENDENT LOCI AND THEIR RESISTANCE/SUSCEPTIBILITY TO PROTAMINE

Gene Name	CpxR-Reg	Function	Protamine R/S	Ref
aceE	Positive	Pyruvate dehydrogenase dihydrolipotransacetylase	Resistant	(196)
aceF	Positive	Pyruvate dehydrogenase dihydrolipotransacetylase	Resistant	(196)
acpP	ND	Fatty acid synthesis, acyl carrier protein	Resistant	(195)
acrD	Positive	Component of efflux pump	Resistant	(214)
adhE	Negative	Ethanol oxioreductase	Resistant	(195, 196)
aer	Negative	Aerotaxis	Resistant	(214)
agp	Negative	Periplasmic glucose-1-phosphatase	Resistant	(196)
amiA	Positive	*N*-acetylmuramoyl-L-alanine amidase	Sensitive	(15)
amiC	Positive	*N*-acetylmuramoyl-L-alanine amidase	Sensitive	(15)
argA	ND	*N*-acetylglutamate synthase	Resistant	(195)
ariR (ymgB)	Positive	Regulator of acid resistance influenced by indole	Resistant	(196)
aroG	ND	DAHP synthase	Resistant	(214)
aroK	Positive	Shikimate kinase I	Sensitive	(195, 214)
b2503	ND	Putative cytochrome C-type protein	Resistant	(195)
b2504	ND	Unknown	Resistant	(195)
bacA	ND	Lipid kinase, bacitracin resistance	Resistant	(195)
bssR (yliH)	Positive	Regulator of biofilm formation	Resistant	(196)
chaA	ND	Ca^{2+}/H^{+} and Na^{+}/H^{+} antiporter	Resistant	(195)
chaB	ND	Ca^{2+}/H^{+} and Na^{+}/H^{+} antiporter	Resistant	(195)
cobUST	ND	Cobalamin synthesis	Resistant	(195)
cpxP	Positive	Inhibitor of CpxA activity	Resistant	(195, 196, 214)
cpxRA	Positive	Signal transduction system	Resistant	(195, 214)
csgBAC	Negative	Curlin fimbriae components	Resistant	(195, 214)
csgDEFG	ND	Curlin fimbriae synthesis and regulation	Resistant	(195, 214)
csiR (gabC)	Positive	Regulator of Gab gene expression	ND	(196)
cspD	Negative	DNA replication inhibitor	Resistant	(196)
csrB	Positive	Regulatory RNA/carbon storage regulation	ND	(196)
degP	Positive	Periplasmic serine endoprotease	Sensitive	(195, 214)
deoC	ND	Deoxyribse phosphate aldolase	Resistant	(195)
dnaK	ND	σ^{32}-regulated heat shock chaperone	Sensitive	(195)
dppC	Positive	Component of dipeptide ABC transporter	Resistant	(196)
dppD	Positive	Component of dipeptide ABC transporter	Resistant	(196)
eda	ND	2-Keto-3-deoxy-6-*P*-gluconate aldolase	Resistant	(195)
efeU	Negative	Elemental ferrous iron uptake permease	Resistant	(214)
endA	Negative	DNA-specific endonuclease I	Resistant	(196)
fliA	Positive	SIgmaF, regulation of flagellar regulon	Resistant	(196)
fliY	Positive	Cystine-binding periplasmic protein	Resistant	(196)
flu	Positive	Antigen 43, potential adhesion OMP	Resistant	(196)
fryA (ypdD)	Negative	fused predicted PTS system	Resistant	(196)
ftnB	Positive	Ferritin-like protein	Resistant	(196, 214)
ftsJ-hflB	ND	Regulates σ^{32} and lambda cIII degradation	Resistant	(195)
fucU	Positive	L-fucose mutarotase, fucose catabolisme	Resistant	(196)

Gene Name	CpxR-Reg	Function	Protamine R/S	Ref
galP	ND	Galactose/H+ symporter	Resistant	(195)
gatDCBAZY	Negative	Glactitol specific enzyme component of PTS	Resistant	(196)
gcvTHP	Negative	Glycine-cleavage enzyme system	Resistant	(195, 196)
glpA	Negative	Glycerol-3-phosphate dehydrogenase subunit	Resistant	(196)
glpB	Negative	Glycerol-3-phosphate dehydrogenase subunit	Resistant	(196)
glpC	Negative	Glycerol-3-phosphate dehydrogenase subunit	Resistant	(196)
gltK	ND	Glutamate-aspartate transport	Resistant	(195)
gspE	Positive	General secretory pathway component, cryptic	Resistant	(196)
hdhA	Negative	7-alpha-hydroxysteroid dehydrogenase	Resistant	(196)
hlpA/ompH	ND	σ^E controlled periplasmic chaperone	Resistant	(195)
hns	Positive	Global DNA-binding transcriptional dual regulator	Resistant	(196)
hslTS	ND	σ^{32} regulated heat shock proteins	Resistant	(195)
htpX	Positive	Heat shock protease	Resistant	(214)
intB	ND	Integrase B	Resistant	(195)
lamB	Negative	Outer membrane porin; Phage lambda receptor protein	Resistant	(196)
leuS	ND	Leucyl-tRNA synthetase	Resistant	(195)
lgt (umpA)	Positive	Prolipoprotein diacylglyceryl transferase	Resistant	(196)
ligB (yicF)	Negative	DNA ligase B	Resistant	(196)
lpd	Positive	Lipoamide dehydrogenase	Resistant	(196)
manXYZ	ND	Mannosephosphotransferase system	Resistant	(195)
mdtABC	Positive	Multidrug transporter subunit	Resistant	(214)
mobAB	ND	Guanidine dinucleotide synthesis	Resistant	(195)
motAB/cheAW	Negative	Motility and chemotaxis	Resistant	(195, 214)
mviM	Positive	Virulence factor	Resistant	(195, 214)
nanC	Positive	NAN (*N*-acetylneuraminic acid) channel	Resistant	(214)
ompC	Positive	Outer membrane porin 1	Resistant	(195, 214)
ompF	Negative	Outer membrane porin	Resistant	(195, 214)
pap	Negative	Uropathogenic *E. coli* P pilus subunits	Resistant	(214)
pdhR	Positive	Pyruvate dehydrogenase decarboxylase	Resistant	(196)
pepT	Negative	Peptidase T	Resistant	(196)
pheM	Positive	Phenylalanyl-tRNA synthetase (PheST) leader peptide	Resistant	(196)
ppc	ND	Phosphoenolpyruvate carboxylase	Resistant	
ppiA	Positive	Periplasmic peptidyl/prolyl isomerase A	Resistant	(195, 214)
ppiD	Positive	Periplasmic isomerase D	Resistant	(195, 214)
psd	Positive	Phosphatidyl serine decarboxylase	Resistant	(195, 214)
pspF	Negative	Phage-shock protein	Resistant	(196)
ptsI	ND	Enzyme I of PEP:PTS carbohydrate uptake	Resistant	(195)
purR	ND	Regulator purine synthesis	Resistant	(195)
puuC (aldH)	Positive	γ-glutamyl-γ-aminobutyraldehyde dehydrogenase	Resistant	(196)
pykA	Negative	Pyruvate kinase II/Anaeroic respiration	Resistant	(196)
qseB	Positive	TCS activator of the flagellar regulon	Resistant	(196)

Gene Name	CpxR-Reg	Function	Protamine R/S	Ref
rcsA	ND	Regulator capsular polysaccharide synthesis	Resistant	(195)
rdoA-dsbA	Positive	Disulfide oxidoreductase	Resistant	(214)
rfaY	Positive	Lipopolysaccharide core biosynthesis protein	Resistant	(196)
rffA	Positive	dTDP-4-oxo-6-deoxy-D-glucose transaminase	Sensitive*	(196)
rpoE	Positive	σ^E transcription factor	ND	(195, 214)
rpoH	ND	Heat-shock transcription factor σ^{32}	Resistant	(195)
rpsP	ND	30 S ribosomal subunit protein S16	Resistant	(195)
rpsT	Negative	30S ribosomal protein S20	Resistant	(196)
rseABC	Positive	Regulators of σ^E transcription factor	Resistant	(195, 214)
secA	Positive	Secretion subunit A	Resistant	(214)
slt	ND	Iron regulated Shiga-like toxin	Resistant	(195)
smpA	Positive	Outer membrane lipoprotein	Resistant	(195, 196)
spy	Positive	Periplasmic protein, induced by oxidative stress	Resistant	(195, 196, 214)
sspA	ND	Stringent starvation, adherence and invasion	Resistant	(195)
tap	ND	Taxis toward peptides, methyl-accepting	Resistant	(195)
thyA	Positive	Thymidylate synthetase	Resistant	(196)
tig	ND	Trigger factor with DnaK	Resistant	(195)
tnaA	Negative	Tryptophan transport and utilization	Resistant	(196)
tnaB	Negative	Tryptophan transport and utilization	Resistant	(196)
tnaL (tnaC)	Negative	Tryptophan transport and utilization	Resistant	(196)
tolA	Positive	Component of the Tol-Pal cell envelope complex	Resistant	(196)
tolB	Positive	Component of the Tol-Pal cell envelope complex	Resistant	(196)
tolC	Positive	Outer membrane pore involved in efflux	Sensitive	(18)
tolQ	Positive	Component of the Tol-Pal cell envelope complex	Resistant	(196)
tsr	Negative	Serine chemoreceptor	Resistant	(195, 214)
udk	ND	Uridine/cytidine kinase	Resistant	(195)
ulaR	Positive	DNA-binding transcriptional dual regulator	Resistant	(196)
ung	Positive/ Negative	Uracil-DNA glycosylase	Resistant	(195, 214)
uspA	Negative	Universal stress global response regulator	Resistant	(196)
uxuR	ND	Represses hexuronate degradation	Resistant	(195)
vsr	Positive	DNA mismatch endocnuclease	Resistant	(196)
yafK	Positive	Conserved protein (periplasmic)	Resistant	(196)
ybaJ	Positive	Unknown	Resistant	(214)
ybaQ	Positive	Predicted transcriptional regulator	Resistant	(196)
ybaR	ND	Putative ATPase	Resistant	(195)
ybaS	ND	Putative glutaminase	Resistant	(195)
ybcU (borD)	Negative	Lipoprotein Bor homolog	Resistant	(196)
ybeL	Positive	Conserved protein	Resistant	(196)
ybgC	Positive	Predicted acyl-coA thioesterase	Resistant	(196)
ybgF	Positive	Component of the Tol-Pal cell envelope complex	Resistant	(196)
ybhT	Positive	Predicted protein	Resistant	(196)

Gene Name	CpxR-Reg	Function	Protamine R/S	Ref
ybjS	Positive	Predicted oxidoreductase with NAD(P) binding domain	Resistant	(196)
ycbZ	Negative	Putative ATP-dependent protease	Resistant	(196)
yccA	ND	Transmembrane, degraded by FtsH	Resistant	(195, 196, 214)
yceI	Positive	Periplasmic protein induced at high pH/osmotic shock	Resistant	(196)
yceJ	Positive	Predicted cytochrome	Resistant	(196)
ycfS	Positive	Periplasmic protein with unknown function	Resistant	(214)
ycgZ	Positive	Uncharacterized protein	Resistant	(196)
yciF	Negative	Putative structural protein, osmotically inuced	Resistant	(196)
ydeH	Positive	Unknown	Resistant	(214)
ydeK	Positive	Predicted lipoprotein	Resistant	(196)
ydeN	Negative	Uncharacterized sulfatase	Resistant	(196)
ydjF	Negative	Transcriptional regulator	Resistant	(196)
yebE	Positive	Putative inner membrane protein	Resistant	(214)
yefJ	ND	Putative creatinase	Resistant	(195)
yfiD	ND	Putative formate acetyl-transferase	Resistant	(195)
ygaW	Negative	Predicted inner membrane protein	Resistant	(196)
ygjT	Positive	Putative transport protein	Resistant	(195)
yhaI	Positive	Predicted inner membrane protein	Resistant	(196)
yhdG	ND	Probable oxidoreductase, similar NifR3	Resistant	(195)
yhdJ	ND	Putative methyltransferase	Resistant	(195)
yhfC	ND	Putative transporter	Resistant	(195)
yhjE	ND	Putative transport protein	Resistant	(195)
yhjV	Positive	Inner membrane transport protein	Resistant	(196)
yiaF	Positive	Putative inner membrane lipoprotein	Resistant	(196)
yidZ	Positive	HTH-type transcriptional regulator	Resistant	(196)
yihE-dsbA	Positive	Disulfide oxioreductase	Sensitive	(195)
yjfN	ND	Unknown	Resistant	(195)
yjfP	ND	Unknown	Resistant	(195)
yjiY	ND	Putative carbon starvation protein	Resistant	(195)
ymgA	Positive	Uncharacterized protein	Resistant	(196)
yojN	ND	Putative sensor kinase	Resistant	(195)
ypdF	Negative	aminopeptidase	Resistant	(196)
yqjA	Positive	DedA-like predicted inner membrane protein	Sensitive	(214)
yzgL	Positive	Uncharacterized protein	Resistant	(196)