REGULATORY NETWORKS IN BILE SALT AND FERRIC IRON INDUCED RESISTANCE OF ENTEROHEMORRHAGIC *ESCHERICHIA COLI* TO CATIONIC ANTIMICROBIAL PEPTIDES

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Regulatory Networks in Bile Salt and Ferric Iron Induced Resistance of Enterohemorrhagic *Escherichia coli* to Cationic Antimicrobial Peptides

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Abstract

Enterohemorrhagic *Escherichia coli* (EHEC) possess the ability to respond to its surroundings though two-component systems. We hypothesized that gastrointestinal cues such as bile, iron and pH promote EHEC resistance to cationic antimicrobial peptides (CAMPs) during infection. Killing assays reveal that exposure to low pH, high iron or bile salt mixture results in increased CAMP resistance that is dependent on *pmrB* but independent of *pmrD*. Low Mg^{+2}-induced CAMP resistance is dependent on *pmrD*. *pmrD* promoter analysis indicate that EHEC responds to PhoPQ-inducing conditions by increasing *pmrD* expression. However, *pmrD* expression is repressed upon exposure to low acid, bile salt mix and iron. This study suggests that a complex interplay of PhoPQ, PmrAB and PmrD is involved in EHEC's response to various microenvironmental signals and in the promotion of EHEC's resistance to CAMPS. The results also provide intriguing evidence of both cooperation and redundancy in the mediation of CAMP resistance by these molecular players.

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DEDICATION

In memory of my mother, Bishnu

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List of Abbreviations

AmpR	Ampicillin Resistance
Ara4N	4-Amino-4-deoxy-L-arabinose
ArnT	enzyme that modifies lipid A with aminoarabinose
BSM	Bile Salt Mixture
CAMPs	Cationic Antimicrobial Peptides
CFU	Colony Forming Units
EHEC	Enterohaemorrhagic *Escherichia coli*
FIC	Fractional inhibitory concentration
HBD	human Beta-Defensin
hCAP-18	human Cationic Antimicrobial Protein, 18 kDa
HD-5	Human Defensin-5
HNP	Human Neutrophil Peptide
HUS	Hemolytic Uremic Syndrome
IM	Inner Membrane
KanR	Kanamycin Resistant
L-Ara4N	4-amino-4-deoxy-L-arabinose
LB	Lysogeny Broth
LEE	Locus of enterocyte effacement
LPS	Lipopolysaccharide
OD_{600}	Optical Density 600 nm
OM	Outer Membrane

PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEtN	Phosphoethanolamine
PMB	Polymyxin B
PmrAB	Two-component regulatory system (response and sensor regulator)
PmrD	Polymyxin resistant protein D
rpm	revolutions per minute
Stx	Shiga toxin
Stx1	Shiga toxin 1
Stx2	Shiga toxin 2
T3SS	Type III secretion system
TCRS	Two-Component Regulatory System
Tir	Translocated intimin receptor
TLR	Toll-Like Receptor
VT	Verotoxin
WT	Wild-Type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

1 Introduction

1.1 Overview of enterohemorrhagic *Escherichia coli* (EHEC)

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a member of the large family of *Enterobacteriaceae*. It is a gram-negative, non-spore-forming, rod-shaped bacterium. *Escherichia coli* exists as part of the natural flora of gastrointestinal tract of warm-blooded animals, including humans and considered non-pathogenic. However, there are specific strains of *E coli* that are associated with both intestinal and extraintestinal diseases. The strains that are associated with human diarrheal diseases are usually classified into five pathotypes: enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) (Croxen et al. 2013). Among these classes, EHEC is usually the most important pathogen from public health point of view (Lim et al. 2010).

Escherichia coli O157:H7 is the predominant serotype representing the EHEC group. Serotype identification is based on surface structures such as the somatic antigen O and flagellar antigen H. EHEC O157:H7 is the cause of foodborne outbreaks around the world, mainly affecting developed parts of the world such as USA, Canada and Europe. The other serotypes that are common in food borne outbreaks after O157 are O26, O111, O103, O121, O45 and O145 (Smith et al. 2014).

EHEC O157:H7 was first identified as a human pathogen in 1982 after food-borne outbreak investigation in USA. This pathogen is a major concern for scientists and healthcare professionals, due to its low infectious dose (10-100 microorganisms) (Kiranmayi et al. 2010) and high capacity to cause severe systemic complications such as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (Page and Liles 2013). Treatment options for this infection are limited to supportive care to control the symptoms. Because of the risk of release of Shiga toxin, a potent cytotoxin of EHEC by the use of conventional antibiotics, effective intervention options are therefore very limited (Safdar et al. 2002).

1.2 EHEC outbreaks and public health significance

Cattle are the primary reservoir of EHEC. When shed from the reservoir, they can survive for a month in the environment such as food and water (Mayer et al. 2012). EHEC outbreaks occur in different parts of the world including North America, Europe and Asia, sometimes with simple diarrhea but occasionally involving HUS and sometimes leading to death.

There are multiple ways by which humans can contract EHEC infection. The majority of human infections have been associated to consumption of meat and raw food as evidenced by outbreak investigations. The consumption of contaminated food or water is the main route of transmission of EHEC infection. Both O157 and non-O157 *E. coli* cause large numbers of cases in the United States and Canada (Crim et al., 2013, Sockett et al., 2014).

The Centers for Disease Control and Prevention estimates that over 265,000 cases of foodborne illnesses due to *E. coli* O157:H7 occurs every year in the United States alone resulting in more than 3,600 hospitalizations and 30 deaths (CDC 2016). Some of the large foodborne outbreaks like one in Germany where entirely new strain, O104:H4 with more incidence of HUS emerged, makes this pathogen even more dangerous foodborne pathogen. Furthermore, the economic burden due to *E. coli* O157:H7 associated infections is very large. The total estimate from medical expenses and productivity is estimated to be $405 million (Kiranmayi et al. 2010). According to the recent data, the annual cost associated with *E. coli* O157:H7 illness in Canada alone is estimated to be over 377 million dollars (Sockett et al. 2014). The effects are not only confined in human health and medical costs. The food industry, particularly the meat and vegetables production and distribution system may suffer huge economic losses from recalls or removal of *E. coli* O157:H7-contaminated food items from the markets. With the large number of outbreaks both locally and globally and the high costs associated with the EHEC-related illnesses, there is a need to invest in these fields. Since foodborne EHEC outbreaks continue to be a problem worldwide, improved intervention in the food industry and improved treatment methods are essential.

1.3 Virulence factors

EHEC is equipped with multiple virulence factors that enables it to become a successful enteric pathogen in human. Two of the most important virulence factors are the Shiga

toxins (Stx). Besides Stx, EHEC harbors the highly conserved locus of enterocyte effacement (LEE) pathogenicity island, which encodes a number of proteins, including type 3 secretion system (T3SS), the primary adhesin intimin, the major adhesins and other effector proteins required for initiating pathogenesis.

EHEC is a non-invasive mucosal pathogen which possesses a unique mechanism of colonization and pathogenesis. The main virulence factor that contributes to the initiation of colonization is the main adhesin, intimin. When bacteria transit though intestine, intimin mediates the attachment to human colonic epithelial cells. After colonization and effector protein secretion, EHEC destroy the brush border microvilli and induce cytoskeletal rearrangements underneath itself (Nguyen and Sperandio 2012). This results in characteristic lesions known as attaching and effacing (A/E) lesions which are a classic feature of EHEC and EPEC infection.

Shiga-toxins are potent cytotoxins that can specifically interact with their receptors, mainly globotriaosylceramide (Gb3) or globotetraosylceramide (Gb4) which are found extensively on colonic, vascular epithelial, and kidney cells (Johannes and Römer 2010). Shiga toxins 1(Stx1) and 2 (Stx2) are proteins encoded by chromosomally located phage-encoded genes (Page and Liles 2013). Intestinal epithelial cells are sensitive to Stx, which can cause damage by inhibiting the cellular protein synthesis, leading to bloody diarrhea, hemorrhagic colitis and hemolytic uremic syndrome (HUS). In the same way, kidney cells

are also highly susceptible to Stx damage due to presence of Gb3 on this plasma membrane. When Stx reaches the kidneys via the bloodstream it might become a cause of localized thrombotic microangiopathy, it may progress to HUS (Vanaja et al. 2013). Children and the elderly are the most susceptible groups to EHEC-induced HUS (Mayer et al. 2012).

Additional virulence factors include EHEC hemolysin, catalase peroxidase, serine protease, type 1 fimbriae, p fimbriae and α-hemolysin (Nguyen and Sperandio 2012). These factors contribute to the pathogenesis ranging from subverting host defenses to hijacking the host cell signaling which ultimately enables the bacteria to colonize, multiply, and cause disease.

1.4 Host factors and modulation of virulence

The human gastrointestinal tract is not a favorable environment for external invaders. Some of these bactericidal environments include gastric secretions and hydrochloric acid in the stomach, bile in the small intestine and antimicrobial peptides along the entire intestine.

The gastric acidic environment which can reach a pH 2.0 because of the presence of hydrochloric acid, destroys a majority of pathogenic microorganisms that transit though the stomach (Gorden and Small 1993). However, EHEC O157:H7 is unusually resistant to acid and bile salt (Gunn 2000). The duodenum and jejunum are the sites where a very high concentration of bile exists which serves as another checkpoint for potentially pathogenic microorganisms. Bile is produced in the liver and stored in the gallbladder. In addition to

aiding the digestion of fatty foods, bile has bactericidal properties (Begley et al. 2005). Human bile is a mixture of variety of components, such as proteins, ions, pigments, lipids and various bile salts (Begley et al. 2005). Cholate, deoxycholate, taurocholate, glycocholate, chenodeoxycholate, lithocholate are the main bile salts present in the bile. The intestinal microbial flora deconjugates the tauryl and glycyl group from cholate and chenodeoxycholate. The deconjugated primary bile salts can further be metabolized by the microbial flora to deoxycholate and lithocholate, respectively as shown in Fig 1 (Sorg and Sonenshein 2008). Bile salts are bactericidal by virtue of their DNA damaging and membrane disrupting properties. EHEC has the ability to resist bile salts and easily survive in the intestine (Prouty et al. 2004). This unique characteristic is achieved through efflux pumps that expel bile from the bacterial cell as well as the modification of membrane structures that reduce bile permeability. The bile salt concentration generally varies from 0.2–2.0% in the small intestine (Begley et al. 2005). The concentration decreases as bile salts are passively reabsorbed along the small intestine.

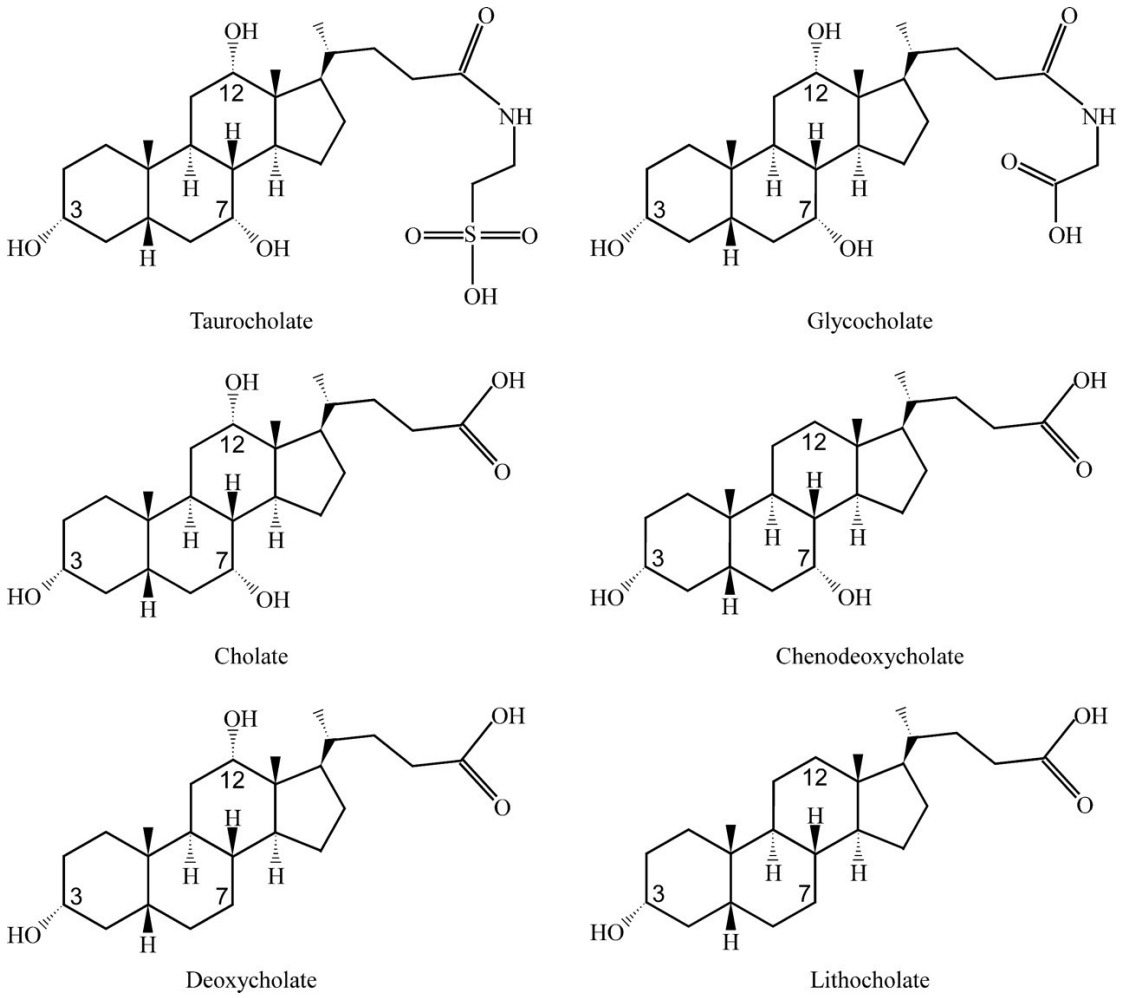


Figure 1 Structures of common primary and secondary bile acids: The primary bile salts cholate and chenodeoxycholate typically are conjugated with taurine or glycine. The secondary bile salts deoxycholate and lithocholate (Sorg and Sonenshein 2008).

Another major challenge to the pathogenic microorganism is the presence of a wide array of cationic antimicrobial peptides (CAMPs) on the epithelial surfaces of the entire gastrointestinal tract. CAMPs, mainly human alpha and beta-defensins and cathelicidins,

are found as components of the innate immune system and serve as nonspecific defenses against microorganisms.

Responding to the environment is critically important for pathogens to colonize and establish infection within the host. They utilize environment-specific signals to directly regulate the expression of their unique virulence genes, thus giving them a competitive advantage over commensal flora. A successful pathogen like EHEC must overcome various gastrointestinal barriers encountered throughout the GI tract. EHEC has been shown to possess necessary mechanisms to deal with the above-mentioned challenges. Interestingly, EHEC not only survives these host challenges but also exploits them as the chemical signals to enhance expression of genes involved in survival and virulence. This is a subject of great curiosity for investigators working with enteric pathogens. In recent years there has been an explosion of interest in understanding the impact of gastrointestinal signals such as low acid, bile salts and short chain fatty acids in pathogenesis of these enteric bacteria. Exposure to low pH mimicking the stomach environment enhances host cell adhesiveness and motility in EHEC (House et al. 2009). In another study, Kus et al show that EHEC treatment with a physiologically relevant bile salt mixture promotes expression of many virulence associated genes including *arn (pbgP)* operon which assists EHEC to achieve increased resistance to cationic antimicrobial peptides (Kus et al. 2011). Analysis of bile salt mixture-induced EHEC gene expression revealed significant changes in the expression of numerous genes whose protein products are associated with modification of the Lipid A moiety of lipopolysaccharide. Modifications of lipopolysaccharides (LPS) in the cell envelope are recognized mechanisms that trigger resistance to cationic

antimicrobial peptides (CAMP). CAMPs, acidic pH, high ferric iron (Fe^{3+}), high aluminum (Al^{3+}) or low magnesium levels are the principal signals that stimulate the expression of lipid-modifying enzymes in gram-negative bacteria, resulting in increased CAMP resistance (Kim et al. 2006, Chen and Groisman 2013). These findings suggest that various sections of human gastrointestinal tract can serve as an important environmental cue for EHEC by triggering protective modifications of the bacterial outer membrane, thereby increasing resistance to human CAMPs.

Thus, EHEC has developed mechanisms to counteract many of the host innate defense mechanisms encountered along the entire gastrointestinal tract. When ingested by the human host, EHEC senses the changing environment inside the host though a number of two-component signaling systems. Instead of being killed in the intestine, EHEC undergoes an extensive remodeling of its outer membrane which enhance its resistance to host antimicrobial peptides.

1.5 Cationic antimicrobial peptides

Cationic antimicrobial peptides (CAMPs) are a structurally diverse group of molecules that are found virtually in all eukaryotes examined to date (Hancock and Diamond 2000). CAMPs are small molecular weight peptides usually made up of a few to hundred amino acids. They are ubiquitous in biological systems which are evolutionarily thought to be a component of the first line of defense against infectious agents. Because of the presence of

high content of positively charged amino acids such as arginine, histidine and lysine, CAMPs are cationic molecules (Omardien et al. 2016). The cationic characteristic is an important feature of CAMPs, which initiates an electrostatic interaction of CAMPs with negatively charged phospholipid membrane of bacteria. These cationic peptides are involved in host defense through both direct bactericidal and immunomodulatory properties. They are critical components of the innate immune system by exhibiting broad-spectrum activity against both gram-positive and gram-negative bacteria (Pazgier et al. 2013). Although thousands of CAMPs exist in nature, mammalian CAMPs can broadly be classified into three major classes: the α- and β-defensins and the cathelicidin as outlined in table 1.

Table 1 Major human cationic antimicrobial peptides: defensins and cathelicidins

CAMP family	Subgroups	Tissue expression	Activity
Defensins α-Defensins	HNP-1-4	Phagocytic cells, bone marrow, respiratory tract	Antimicrobial and immunomodulatory
	HD-5 and HD-6	Paneth cell, HD-5 also found in kidney and reproductive tract	Antimicrobial and immunomodulatory
β-Defensins	HBD-1, HBD-2, HBD-3, HBD-4	GI tract, airway and genitourinary epithelium	Antimicrobial and immunomodulatory
Cathelicidins	LL-37	Small intestine, colon, airway epithelium, bone marrow	Antimicrobial and immunomodulatory

1.5.1 Defensins

Defensins constitute one of the largest groups of CAMPs. Mammalian defensins contain six cysteine residues that form three characteristic disulphide bridges and can be divided into three classes (α-, β-and θ-defensins). The three subfamilies mostly differ in their peptide length, structure of precursors and the sites of their expression (Schneider et al. 2005). Human α-defensins are mainly expressed in neutrophils and the mucosal epithelial cells, including cells of gastrointestinal system where their primary function is to protect against microbial invasion (Sankaran-Walters et al. 2017). The α-defensins, first identified in rabbit neutrophils and found exclusively in mammals. They are commonly known as classic defensins which contain 29–35 amino acids (Schneider et al. 2005) . β-defensins, as the name suggests, form β-sheets consisting of 30–50 amino acid residues. θ-defensins are phylogenetically the youngest defensins which most probably appeared through mutation of a pre-existing α-defensin gene. Active θ-defensins are cyclic peptides found exclusively in non-human primates.

1.5.2 Cathelicidins

In humans, LL-37 is the most important cathelicidin which is produced by many cell types. The cathelicidins are a family of at least 30 structurally divergent antimicrobial peptides from various mammalian species (Fabisiak et al. 2016). As the name implies, they are characterized by having an evolutionarily conserved N-terminal domain called the cathelin domain. The C-terminal domain, which is released by cleavage of proteases, has both

antimicrobial and immunomodulatory properties (Doss et al. 2010). Cathelicidin peptides include protegrins from pigs, CAP-18 from rabbits, bactenecin and indolicidin from cows and more recently RL-37 from monkeys (Xhindoli et al. 2016). In humans, only one cathelicidin antimicrobial has been found to date, the α-helical peptide LL-37 (also known as hCAP18, human cationic antimicrobial protein) (Xhindoli et al. 2016). Like defensins, cathelicidins are also stored as inactive propeptide precursors which, upon activation, are processed and released. Human cathelicidins are of major importance in enteric pathogen infection, particularly in EHEC infections (Yi et al. 2017).

As part of the defense system of the body, multiple classes of CAMPs are present throughout the various anatomical niches which are prone to attack by bacteria. The human HD5 and HD6, HBD1-6 and cathelicidin LL-37 establish a protective barrier against enteric pathogens and commensals in the GI tract (Le et al. 2017). Due to structural dissimilarity between prokaryotic and eukaryotic cells, most CAMPs preferentially target bacterial cells. Electrostatic interaction between the positively charged CAMPs with the negatively charged membrane surface of the microorganism is the main molecular mechanism underlying the binding (De Smet and Contreras 2005). After successful binding with the target organism, the CAMPs are reported to create pores within the microbial membrane which then cause leakage of ions, metabolites as illustrated in Figure 2 (Bevins and Salzman 2011). Successful enteric pathogens like EHEC have mechanisms to resist the antimicrobial action of CAMPS encountered in the gastrointestinal tract.

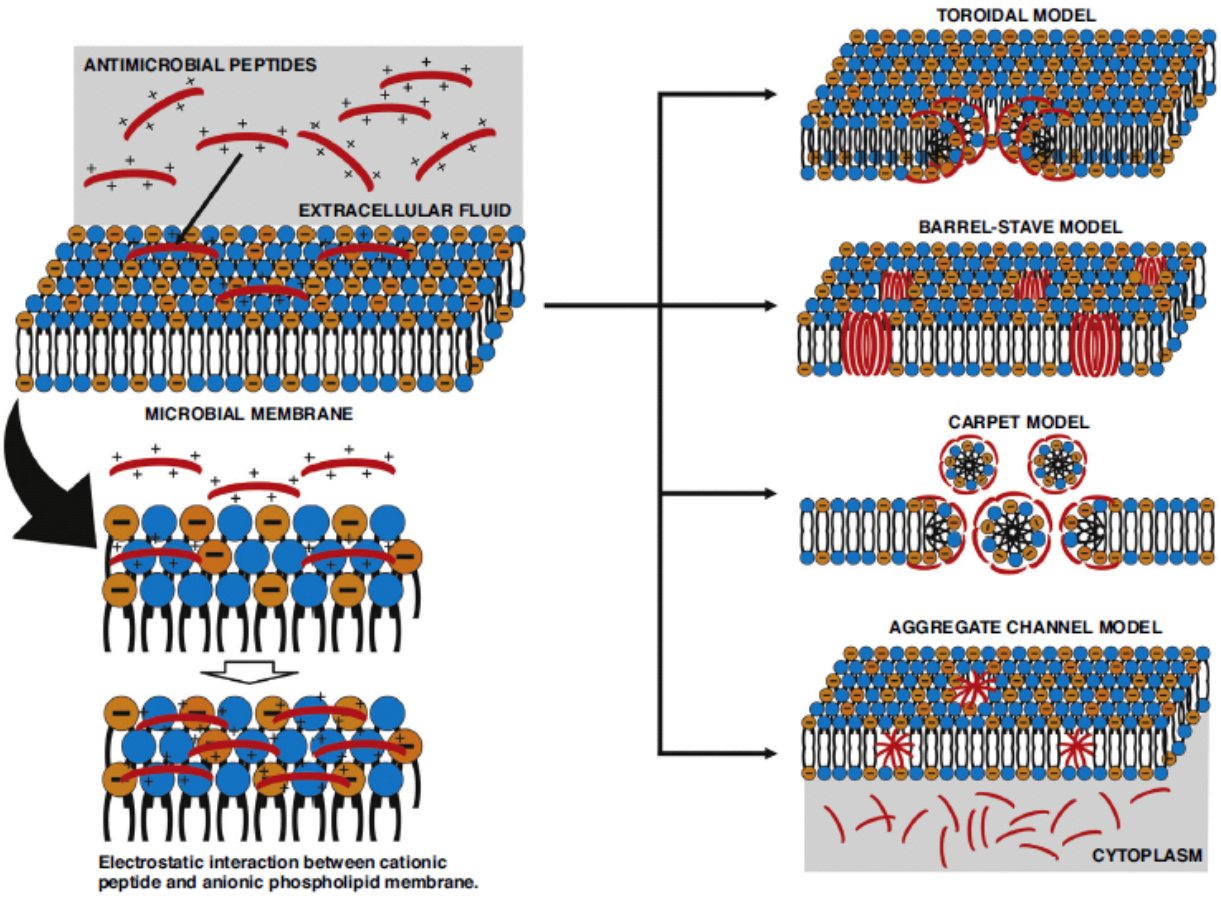


Figure 2 Proposed mechanisms of action of cationic antimicrobial peptides: Among the proposed mechanisms, the most cited are the toroidal model, barrel-stave model, and carpet model, all ultimately leading to pore formation and leakage of cellular content and cell death. (Sivieri et al. 2017)

1.6 Two-component regulatory systems and cross talk

Enteric pathogens like EHEC encounter large variety of stressors mandating an urgent response inside and outside of the host whether it is food products or the human gastrointestinal tract. These can include stresses such as nutrient limitations, high acidity, presence of high iron and cationic antimicrobial peptides and bile salts. As a result, EHEC must manage to sense and appropriately respond to these stresses in order to survive. The two component regulatory system is a predominant signal transduction system present in prokaryotes and is responsible for sensing and responding to the external stimuli (Chen and Groisman 2013).

Typical two-component regulatory systems, as the name suggests, consist of two components, a transmembrane sensor (histidine) kinase that recognizes the external signal and a cytosolic response regulator that determines the response action of the organism in response to environmental changes (Jung et al. 2012). The sensor kinase gets activated by autophosphorylation when it encounters the appropriate signal. Activation of a sensor kinase leads to the transfer of the phosphoryl group to a conserved aspartate residue on its cognate response regulator (Jung et al. 2012). The response regulator is also a transcription factor whose DNA binding affinity for its promoters is modulated by phosphorylation which controls gene expression. PhoPQ and PmrAB systems are the major two-component systems involved in LPS modification in enterobacteriaceae.

1.6.1 PhoP/PhoQ

The PhoPQ two-component system is composed of the sensor kinase PhoQ and its cognate response regulator PhoP. PhoQ's kinase activity is activated by low concentrations of Mg^{2+}, acidic pH (H^{+} ions) and the presence of cationic antimicrobial peptides but inactivated by high levels of Mg^{2+} ions (Omardien et al. 2016). In *Salmonella,* low Mg^{2+} concentration signals to the cell a transition from an extracellular environment to an intracellular location, increasing the phosphorylation of PhoP (PhoP-P) (Choi and Groisman 2017). This is a great advantage for survival to intracellular pathogens like *Salmonella*. Activation of PhoP-dependent genes leads to up-regulation of many virulence factors in enteric pathogens. Some of these include functions that remodel the bacterial outer membrane to help improve its barrier function.

1.6.2 PmrA/PmrB

Like PhoPQ, PmrAB is composed of the sensor kinase PmrB and its cognate regulator PmrA. The PmrAB two-component system is capable of sensing environmental cues such as acidic pH, high Fe^{3+} ions, and the presence of CAMPs (Chen and Groisman 2013a). Exactly like PhoP-P, PmrA-P strongly bind to its target promoter and regulates gene expression that mediate lipid A modifications in bacterial outer membrane (Chen and Groisman 2013b). PmrAB is required for resistance to antimicrobial peptide PMB resistance in gram negative bacteria (Chen and Groisman 2013b). PmrAB-regulated LPS modifications can also reduce susceptibility to intestinal CAMPs present on the epithelial

niches of intestine (Chen and Groisman 2013a). The products of PmrA-activated genes mediate the additions of compounds such as 4-aminoarabinose and phosphoethanolamine to lipid A which reduces the net negative charge of the gram negative outer membrane (Chen and Groisman 2013). The PmrAB is intimately connected with PhoPQ through a small protein, PmrD.

1.6.3 PmrD

The PMB Resistant Protein D (PmrD) is a connector protein thought to connect the bacterial two-component systems-PhoPQ to the PmrAB system. The PmrA/PmrB two-component system is the major regulator of gene products that modifies the LPS in a wide variety of pathogenic bacteria (Luo et al. 2013). In *S. typhimurium* and *K. pneumoniae*, activation of the PmrA/PmrB system occurs when the bacteria experience low pH, or high Fe^{3+} environments, which are detected by the PmrB sensor kinase (Luo, et al 2013). In response to these inducing signals, PmrB autophosphorylates and then transfers the phosphoryl group to its response regulator PmrA. Phosphorylated PmrA is the active form of the protein that binds to the promoter region of DNA, promoting expression of variety of PmrA-dependent genes which are responsible for LPS modification (Zhou et al. 2001; Rubin et al. 2015). The expression of these PmrA-dependent genes mediates resistance to cationic antimicrobial peptides including PMB.

Signals received by PhoP/PhoQ and PmrA/PmrB can be integrated to generate the appropriate cellular response in *Salmonella* (Kato and Groisman 2004). PmrD acts as a connector protein between these two signal transduction pathways. Early work suggested that the communication between these two pathways was not connected in *E. coli* (Winfield and Groisman, 2004). However, more recently, a study in *E. coli*, has demonstrated that the *pmrA*-dependent genes can also become activated indirectly through the cross-talk with PhoPQ under low magnesium environment via a small connector protein, PmrD, that stabilizes the activated state of the response regulator PmrA (Rubin et al. 2015) (Figure3).

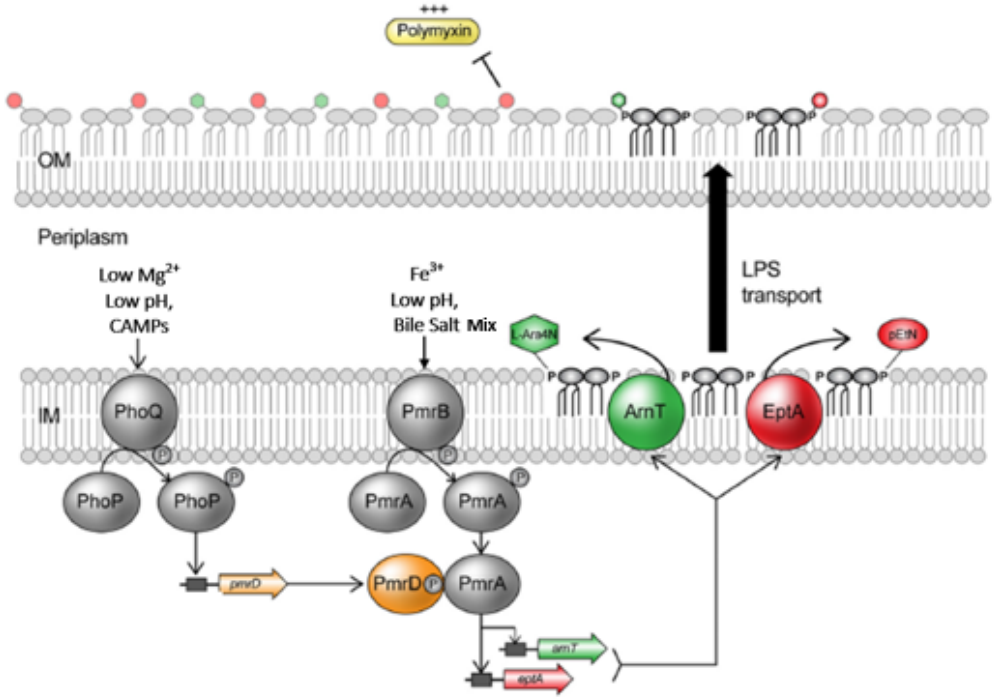


Figure 3 PhoP/PhoQ and PmrA/PmrB two-component systems in *S. typhimurium* and *E. coli.* PhoP/PhoQ two component system gets activated when sensor kinase PhoQ is activated by signals like low Mg^{2+}, CAMPs and low pH in the periplasm. PhoP, a response

regulator of PhoP/PhoQ promotes transcription of the *pmrD* gene. The PmrD protein binds to phospho-PmrA, a response regulator of PmrA/PmrB system. PmrD mechanically inhibits dephosphorylation of PmrA by PmrB, allowing continued transcription of PmrA-dependent genes, including *eptA* and *arnT*. In the same way, sensor kinase PmrB when activated by signals like high Fe^{+3}, low pH and bile salts, leads to phosphorylation of the response regulator, PmrA and promotes the same pathway (Rubin et al. 2015). Major lipid A modifying genes *arnT* and *eptA*, with their respective products are denoted by green and red. (adapted from Rubin et al., 2015)

A number of studies of gram-negative bacteria have indicated that lipid A modifications including addition of aminoarabinose and phosphoethanolamine which are associated with increased resistance to CAMPS are dependent on *pmrD* under low magnesium conditions (Kato, Latifi, & Groisman, 2003, Luo et al., 2010, Rubin et al., 2015). However, interestingly, expression of *pmrD* was only partially dependent on *phoP* under low magnesium conditions, suggesting that another as yet unidentified system was responsible for regulating expression of *pmrD* under low magnesium conditions (Rubin et al. 2015). Under those conditions, *pmrD* expression was also not dependent on the presence of *pmrA* or *pmrB*. Nevertheless, under limiting magnesium conditions, expression of *pmrA*, the *arn* operon which governs amino-arabinose addition and *eptA* which is responsible for phosphoethanolamine modification were all dependent on the protein PmrD. However, resistance to PMB was dependent on *pmrD* only under low magnesium conditions but not

under mild acid inducing conditions. Therefore, although previous studies have indicated that mild acid induces CAMP resistance that is dependent on both PmrAB and PhoPQ, these recent findings suggest that the mild acid autonomously induces PmrAB and downstream activation of lipidA modifications, thereby bypassing *pmrD*. This would suggest that expression of *pmrA*, the *arn* operon and *eptA* would all be independent of *pmrD* under conditions such as mild acid that supposedly bypass *pmrD*. However, no evidence of this has been provided yet.

Physiological bile salt mixture and ferric iron are also known to induce CAMP resistance in a *pmrAB* dependent manner in EHEC (Kus et al. 2011). Based on the findings from the Rubin study, it is expected that bile salt or ferric iron induction should also bypass PmrD, thereby not requiring it for CAMP resistance nor affecting its expression. What is also not clear is how limiting magnesium or mild acid conditions would affect bile salt mixture or ferric iron-mediated effects. A recent study has indicated that mild acid induces significant EHEC resistance to PMB regardless of the presence of limiting magnesium (Francis A. 2017). This study also suggests that limiting magnesium enhances the induction of resistance by each of bile salt mixture and ferric iron, suggesting that PhoPQ plays at least a partial role in the induction of resistance by ferric iron and bile salts. Finally, interestingly, it appears that bile salt mixtures and ferric iron both stimulate PmrB-mediated changes through the same iron binding site on PmrB and so it is possible that the bile mixture may be enhancing the solubility or delivery of iron to PmrB (Francis A. Thesis 2017).

Taken together, these data reveal a sophisticated, sensitive bacterial defense system for responding to stressful environmental conditions in the intestinal lumen, one that creates a more fortified, CAMP resistant membrane. Understanding how these various signals mediate CAMP resistance in EHEC and the specific roles of PmrD, PmrAB and PhoPQ in this resistance will provide valuable insight into how these host environmental signals affect EHEC fitness and virulence.

2 Thesis rationale

EHEC is an important food and water borne pathogen. It continues to be a main cause of foodborne outbreaks as evidenced by frequent news and scientific articles, improved intervention in food industry or the treatment methods are essential. Due to the fact that EHEC's ability to survive in hostile environments such as gastrointestinal tract inside the host and outside the host in environments such as soil, food products and water, it poses a huge threat to human health and food industry (Avery et al. 2008). Lipopolysaccharide modification and subsequent host defense peptide resistance is the central mechanism by which enteric pathogens survives in GI tract and establish disease. This mechanism have been reported on many gram-negative pathogens of the enterobacteriaceae family that has the orthologs of the PmrA/PmrB two-component system, including *Y. pestis* (Lee et al. 2004), *K. pneumoniae* (Mitrophanov et al. 2008), and *E. coli* (Winfield and Groisman 2004). However, the two-component regulatory system that is being used to sense the local environment and enhance the resistance to CAMP, display considerable differences among the related species.

EHEC experiences various environments in the anatomical niches of GI tract during infection. The major microenvironments include low pH, high iron and bile salt mixture. Earlier work in the Foster lab provided the evidence that physiological mix of bile salt enhances EHEC resistance to CAMPs (Kus et al. 2011)**.** We hypothesize that EHEC may use these microenvironments as the signals to enhance CAMP resistance and that PhoPQ,

PmrAB and PmrD all play varying roles in this resistance. The role of *pmrD* in EHEC resistance to CAMPs is still poorly understood and more specifically with respect to pH, bile salt mixture and ferric iron-induced conditions. This research project focuses on how these gastrointestinal cues are sensed and what strategies EHEC pursues to survive in the human host. The hypothesis and specific research objectives of this thesis are listed below.

3 Hypotheses and objectives

3.1 Central Hypothesis

PmrD is critical for enterohemorrhagic *Escherichia coli* (EHEC) resistance to cationic antimicrobial peptides (CAMPs) induced by limiting magnesium but is irrelevant in each of bile salt mixture and ferric iron-induced resistance to CAMPS.

3.2 Hypothesis 1

In EHEC, PmrD plays a key role in low magnesium-induced resistance to PMB, but only a minor role in mild acid-induced resistance to PMB.

3.2.1 Objective 1

Determine the PMB resistance of WT EHEC and the isogenic *pmrD* mutant under varying combinations of low (10 uM)/high (10 mM) magnesium and low (5.8)/neutral (7.0) pH.

3.3 Hypothesis 2

In EHEC, PmrD plays no role in each of bile salt mixture and ferric-iron induced resistance to PMB.

3.3.1 Objective 2.1

Determine the PMB resistance of WT EHEC and its isogenic *pmrD* mutant under each of bile salt mixture-inducing and ferric iron-inducing conditions.

3.3.2 Objective 2.2

Assess the role of low magnesium in the induction of PMB resistance by each of bile salt mixture and ferric iron-induced resistance in both the WT and the isogenic *pmrD* mutant.

3.4 Hypothesis 3

In EHEC, *pmrD* expression is sensitive to low magnesium but is insensitive to bile salt mixture or ferric iron or mild acid stimulation. Expression is partially dependent on *phoP* but is independent of *pmrA* and *pmrB*.

3.4.1 Objective 3.1

Construct a translational GFP-*pmrD* reporter translational fusion in EHEC

3.4.2 Objective 3.2

Assess GFP levels in the *GFP-pmrD* reporter translational fusion in each of the strains after treatment with each of bile salt mixture, ferric iron and mild acid. Examine the effect of introducing low magnesium into the treatments.

4 Materials and methods

4.1 Bacterial strains, growth conditions and reagents

Lysogeny broth (LB) with glycerol was used to store all strains at – 80°C. Primary cultures of all strains were grown from glycerol stock on LB agar medium. Bacterial strains and plasmids used in this study can be found in Table 2. EHEC O156:H7 strain 86-24 was the wild type strain used in this study and all subsequent strains and mutants were derived from this strain.

Bacterial strains were grown in either LB broth (1 % tryptone, 0.5% yeast extract and 0.5 % NaCl) or modified N-minimal media (NM) supplemented with 0.1% casamino acids, 38 mM glycerol, 10 μM or 10 mM $MgCl_2$ and 100 μM $FeSO_4$ as indicated in the experiment. N-minimal media base contains 5 mM KCI, 7.5 mM $(NH^4)SO_4$, 0.5 mM K_2SO_4, 1 mM KH_2PO_4. The pH of the medium was buffered with either 100 mM Bis-Tris buffer pH 7.0 or pH 5.8. When appropriate, LB broth or agar was supplemented with the appropriate antibiotic (Sigma) at the following concentrations: ampicillin 100 μg/mL, kanarnycin 50 μg/mL, gentamicin 30 μg/mL. N-minimal medium was used in all polymyxin B resistance and *pmrD* promoter activity assays with appropriate high/low Mg^{+2} and pH conditions. N-minimal was supplemented with 1.5 % bile salt mixture (Sigma-B-3426) and 100 μM $FeSO_4$ (BDH) wherever indicated in the experiment.

A physiological bile salt mixture was purchased from Sigma (Burlington ON). The "Bile Salts Mixture" with Catalog Number B3426 was prepared from fresh bile, which was repeatedly extracted to purify and retain its inhibitory properties according to the manufacturer's data sheet.

Table 2 Bacterial strains used in the study

Bacterial Strains		
Strain	Description	Reference/ Source
EHEC 86-24	Clinical isolate of O157:H7 serotype of enterohemorrhagic *Escherichia coli*	Dr. Jorge Giron, University of Florida
S17-1 λpir : pRE112	S17-1 λpir (conjugative donor strain for *E coli*) with pRE112-GmR with Δ*pmrD* deletion construct	McPhee Lab
EHEC 86-24Δ*pmrD*	*pmrD* deletion mutant	This study
S17-1 λpir : pRE112	S17-1 λpir (conjugative donor strain for *E coli*) with pRE112-GmR with Δ*phoP* deletion construct	McPhee Lab
EHEC 86-24Δ*phoP*	*phoP* deletion mutant	This study

EHEC 86-24 Δ*pmrD*:pBADGr:: *pmrD*	86-24 transformed with vector pBADGr containing full *pmrD* coding sequence	This study
EHEC 86-24Δ*pmrA*	*pmrA* deletion mutant	Kus et al., 2011
EHEC 86-24Δ*pmrB*	*pmrB* deletion mutant	Kus et al., 2011
Plasmids		
pKD4	FRT–flanked; KanR	(Datsenko and Wanner 2000)
pKD46	Lambda-Red helper plasmid; AmpR	(Datsenko and Wanner 2000)
pRE112	Suicide vector for allelic exchange with *Sac B* gene	McPhee Lab
pBADGr	expression vector with L-arabinose inducible promoter	Kus et al., 2011
pCR2.1 TOPO	Commercially available TA Cloning vector, Invitrogen	Catalog # K450002

4.2 Bacterial strain construction

4.2.1 Construction of *pmrD* and *phoP* isogenic mutants

Each of *pmrD* and *phoP* targeting allelic exchange construct cloned in pRE112-Gm (GmR) vector were generously provided by McPhee lab in *E. coli* S17-1 λ pir strain. pRE112 is a suicide plasmid which can only replicate in bacterial strains containing lambda pir (conjugative strain able to host lambda pir-dependent plasmids). The recipient (EHEC 86-24 [pKD46]) was then prepared. The donor pRE112 (GmR) containing the target construct and the recipient were allowed to mate, selected, the selective markers were removed. Selected colonies were screened by colony PCR. The isogenic *pmrD* and *phoP* mutant were verified using PCR and DNA sequencing results (The Centre for Applied Genomics, Hospital for Sick Children, Toronto, Ontario, Canada). When grown in LB, the growth characteristics of *pmrD and phoP* mutant were not very different than that of WT EHEC 86-24.

4.2.2 Construction of Δ*pmrD* complementation strain

A complemented Δ*pmrD* strain was constructed by using pBADGr expression vector. *pmrD* coding sequences were placed under the L-arabinose inducible pBAD promoter. The coding sequence of *pmrD* was amplified from WT 86-24 using specially designed primers, Comp1 and Comp2 from Table 3. Restriction digestion of this PCR product and pBADGr vector were carried out with *XbaI* and *HindIII*. This PCR product and *pBAD* digested with the same restriction enzymes were ligated using DNA ligase and transformed into

chemically competent *pmrD* mutant strain. As the final step, the sequence was verified by DNA sequencing, using pBAD sequencing primer Seq F and Seq R as mentioned in Table 3.

4.2.3 Construction of reporter plasmid

The 500bp region upstream of *pmrD* (this region contains the promoter sequence of *pmrD*) and the 500bp region downstream of *pmrD* regions were amplified from WT EHEC (EHEC 86-24) with the oligonucleotides P1/ P2 and P5/P6 in a BIO-RAD C1000-Touch thermal Cycler. Similarly, the gfp_{mut3} coding sequences were amplified from GFP plasmid (p3174) with oligonucleotides P3/P4 where P3 and P4 are chimeric oligonucleotides that possess the complementary sequences of upstream and downstream regions of *pmrD* as well as the sequences that are designed to amplify the coding sequences of *gfp* from p3174. The sequences of the oligonucleotides used in this work are described in Table 3.0. All the three PCR reactions were carried out with high fidelity DNA polymerase (Phusion HF polymerase). Once all three fragments were amplified, they were verified on agarose gel for their appropriate sizes. Then all the three fragments were stitched together by SOE PCR and the resulting 1700 bp fragment was confirmed by agarose gel. The *pmrD*-gfp-*pmrD* linear construct was then cloned into pCR2.1 TOPO vector (Invitrogen) as per the kit guideline. The cloned fragment was confirmed by DNA sequencing result. The *pmrD-gfp* reporter plasmid was miniprepped and transformed into *pmrD* mutant strain to generate the *pmrD-gfp* reporter strain.

In order to measure the background auto-fluorescence, a control strain lacking the gfp fluorescence reporter was also constructed. The control strains (harboring TOPO plasmid but not *pmrD-gfp* construct) were prepared by transforming the empty TOPO into *pmrD* mutant strain.

Table 3 List of oligonucleotides used in the study

Name	Sequence	Source
P1	GTTTCACTGGTTAATGACGAAGGCTGGTACG	This study
P2	GAAAAGTTCTTCTCCTTTACGCATTGTATTATCCTGTTTGCTAAGAGTTTTC	This study
P3	GAAAACTCTTAGCAAACAGGATAATACAATGCGTAAAGGAGAAGAACTTTTC	This study
P4	CCTGCCCACGACAAAACAACGTTATTTGTATAGTTCATCCATGCCATG	This study
P5	CATGGCATGGATGAACTATACAAATAACGTTGTTTTGTCGTGGGCAGG	This study
P6	GAATTTTGTCTGGGTGACGCTGGCTGCAG	This study
Comp F	GCGCGC**TCTAGA**GAATGGCTGGTCAAAAAA	This study
Comp R	GCGCGC**AAGCTT**TTACTGAGTTTTCCCTGC	This study
Seq F	AAGTGTCTATAATCACGGCAGA	Burrows et al.
Seq R	TCACTTCTGAGTTCGGCATGG	Burrows et al.

Underlined sequences are the targets of restriction enzymes used in the primers

4.3 Polymyxin B (PMB) resistance assay

Bacterial cells were grown overnight at 37°C in N-minimal medium with or without treatment with BSM (bile salt Sigma-B-3426) or/ Fe^{3+} (BDH $FeSO_4$) as stated in the individual experiment under varying conditions of high/low pH and /or Mg^{+2}. The next day, cultures were diluted 1:100 in respective medium again and incubated for 3 to 6 hours at 37°C with shaking until they reach mid-log phase (OD_{600} 0.4 - 0.6). Cells were spun down, washed with PBS (pH 7.2) three times to remove the pretreatment agent and resuspended in N-minimal salts buffered at pH 7.0. OD_{600} was again recorded. The live cell density was then adjusted to 1×10^8 CFU/mL with N-minimal salts buffered at pH 7.0 for both pretreatment group and untreated groups. A 1/100 dilution (1×10^6 CFU/mL) was then subjected to killing by freshly prepared cationic antimicrobial peptide polymyxin B (PMB). 100µL of each preparation were mixed with 100µL of appropriate concentration of polymyxin B (Sigma) and the other 100µL mixed with PBS. All killing assays were incubated at 37°C for 1 h under static condition. Serial dilutions of each culture were prepared in PBS and plated on LB agar plates for CFU enumeration. Survival values were calculated by dividing the number of CFU following treatment with PMB relative to those incubated in the presence of PBS and then multiplied by 100 (protocol details provided in the Appendix). For each result, three independent experiments were performed with three technical replicates.

4.4 GFP reporter assays

Overnight cultures of *pmrD* reporter strains (carrying *gfp* under *pmrD* promoter in the TOPO plasmid) and *pmrD* control strain (carrying only TOPO plasmid) were centrifuged, washed, and resuspended in respective medium (low/high Mg^{+2} with or without BSM or Fe^{3+}) and adjusted to 0.1 OD_{600}. 200 µl of each cell suspension was immediately transferred into a 96-well plate and the cultures were grown in a Synergy HTX fluorescent plate reader (Biotek) at 37°C under continuous shaking mode. The cell density ($OD_{600\ nm}$) and fluorescence (using a 485/20 nm excitation filter and a 528/20 nm emission filter) were monitored every 15 min for 16 h. The GFP fluorescence and the corresponding OD_{600} readings were retrieved from the machine and divided to generate the normalized ratio value of relative fluorescence units (RFU)/OD_{600}. Values were then adjusted for background by subtracting the normalized ratio value obtained for the corresponding control strain (protocol details provided in the Appendix). The fluorescence was calculated as relative fluorescent units (RFU) per OD600 of each well by using the following formula.

$$\left(\frac{GFP_{reporter} - GFP_{medium}}{OD_{reporter} - OD_{medium}}\right) - \left(\frac{GFP_{control} - GFP_{medium}}{OD_{control} - OD_{medium}}\right)$$

4.5 Synergy assays

Synergy between BSM and PMB was tested using the checkerboard method as described by CLSI (Clinical Laboratory Standard Institute, 2007). The total fractional inhibitory concentration (FIC) for interactions between BSM and PMB was determined. Double

dilutions of appropriate concentrations of the BSM and PMB were prepared in respective medium low Mg^{+2}-neutral pH and high Mg^{+2}-neutral pH. 50 µl of double dilutions of BSM and PMB and the 50 µl of 2×10^5 CFU/mL log-phase bacterial suspension prepared in low Mg^{+2}-neutral pH and high Mg^{+2}-neutral pH medium were mixed and incubated for 18 hours at 37°C under static conditions. Plate readings were done with HTX Microplate Reader (Biotek) after 18 hours. The following calculation techniques were used to estimate the individual and the final FIC of BSM and PMB as described in conventional antimicrobial susceptibility testing methods (Jenkins and Schuetz 2012).

$$\text{FIC of BSM} = \frac{\text{BSM MIC when tested in combination with PMB}}{\text{BSM MIC alone}}$$

$$\text{FIC of PMB} = \frac{\text{PMB MIC when tested in combination with BSM}}{\text{PMB MIC alone}}$$

Total FIC = FIC of BSM + FIC of PMB

The total FIC values were interpreted as follows:

Synergistic	≤ 0.5
Indifferent	>1.0 – 4.0
Antagonistic	>4.0

5 Statistical Analysis

Results were presented as means ± standard error of mean from at least three biological replicates. To test statistical significance among multiple groups, a one-way ANOVA with post-hoc comparisons with Tukey's method were used where appropriate, assuming standard level of significance (< 0.05). The data were analyzed using GraphPad Prism 5.0 software (GraphPad Software Inc. San Diego, CA). Level of significance was indicated on Figures using asterisk (*) mark.

6 Results

6.1 Low Mg^{+2} and mild acid signal promotes PMB resistance in EHEC through specific two-component systems

One hour killing assays were used to assess PMB resistance of each strain after pre-treatment with selected media. Initial PMB resistance assays examined EHEC 86-24 WT and *pmrD* mutant without any specific inducing signals in the medium. Figure 4A shows the results of the experiment carried out in N-minimal medium without any known PmrAB or PhoPQ activating signals. The survival rates of both WT and *ΔpmrD* strains decline significantly after exposure to 2μg/ml PMB compared to untreated strains. Only 28% of WT and 18 % of *pmrD* survived the PMB treatment. There is no significant difference in survival of WT and *pmrD* mutant after PMB challenge.

Figure 4B shows results for the same experiment as Figure 4A except that it was done under mild acidic condition (pH 5.8). Mildly low pH is a signal that is thought to be sensed by both two component systems as demonstrated in *Salmonella typhimurium* and *Shigella flexneri* (Prost et al., 2007, Lin et al., 2018) . However, it is still unclear how EHEC responds to mildly low pH. The PMB survival rate of WT and *pmrD* mutant strains grown at pH 5.8 and high Mg^{+2} (10 mM $MgCl_2$) increased compared to strains grown at non-activating (high Mg^{+2} and neutral pH) condition. However, no significant difference observed between WT and *pmrD* mutant. Growth at pH 5.8 did not significantly affect the PMB survival of *pmrD* mutant relative to the WT. As no sign of *pmrD* involvement appears

here, these results suggest that PhoPQ signal transduction pathway is not the main low pH sensing strategy in EHEC 86-24. Further experiment with *ΔpmrB* shows that PMB resistance of *ΔpmrB* significantly decreases with low pH as shown in Figure 4D. This is an interesting finding showing how EHEC responds to the low pH condition as opposed to *Salmonella typhimurium* where PhoQ has also been shown to be activated by mildly low pH for survival adaptation. From the same experiment, it appears that low pH induced PMB resistance is also dependent on *phoP*.

Figure 4C shows a significant difference in PMB survival between WT and *pmrD* mutant grown in limiting Mg^{+2} and neutral pH conditions. Survival rate of PMB-treated *pmrD* mutant significantly declines in low Mg^{+2} and neutral pH condition. This means *pmrD* inactivation significantly impairs PMB resistance when grown in Mg^{+2}-limiting environment Moreover, complementation of *pmrD* restores the PMB survival phenotype to WT levels. These results confirm that *pmrD* plays a key role in low Mg^{+2}-induced resistance to PMB.

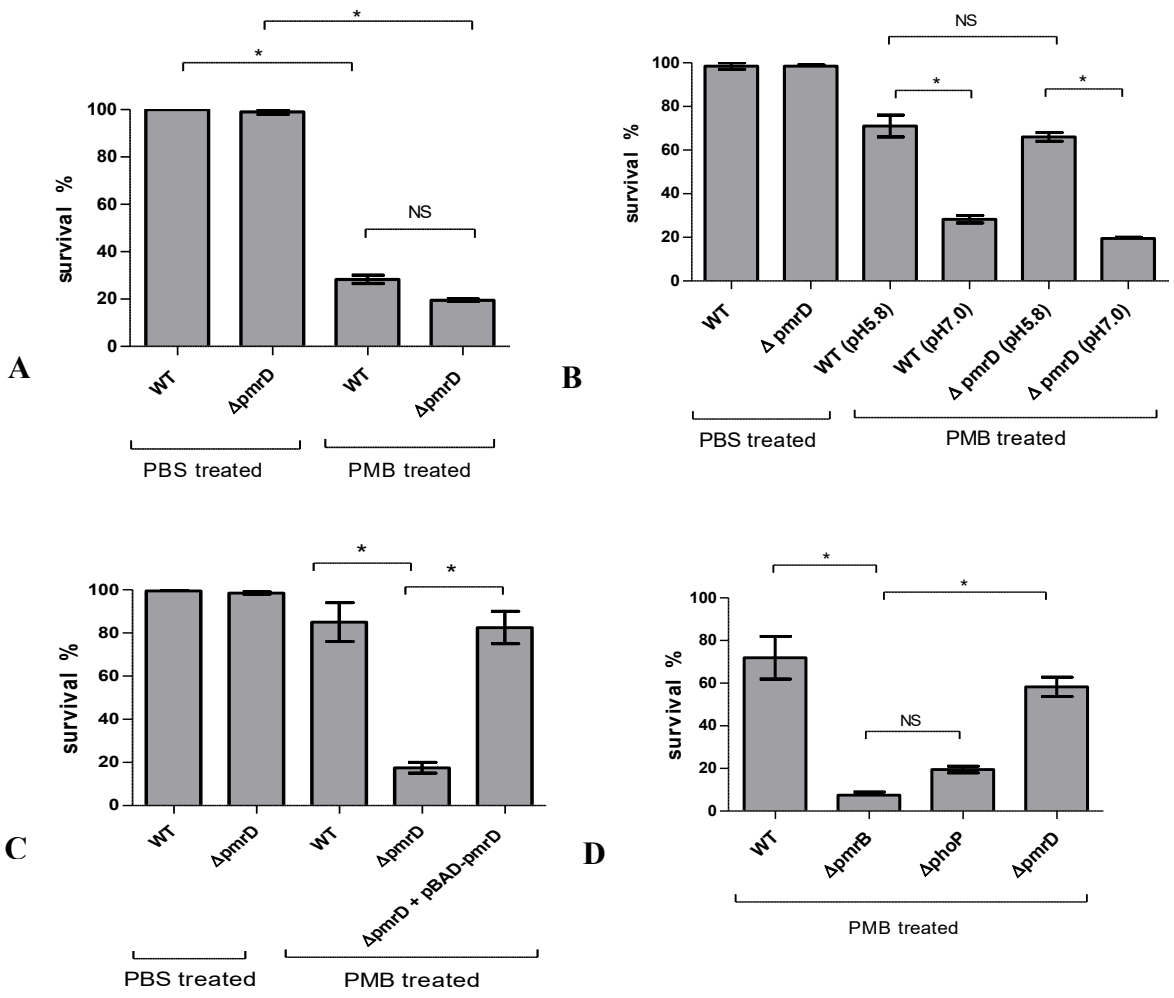


Figure 4 Low Mg^{+2} and mild acid both promote PMB resistance in EHEC 86-24 differently: A) PMB resistance minimal without activating signals in both WT EHEC and *ΔpmrD*. **B)** Survival rate of EHEC 86-24 WT and *pmrD* mutant after PMB treatment cultured in N-minimal medium supplemented with high Mg^{+2} (10 mM $MgCl_2$) and buffered at low pH (5.8) and neutral pH 7.0 **C)** Survival rate of EHEC 86-24 WT and *pmrD* mutant after PMB treatment cultured under low Mg^{+2} (10 µM $MgCl_2$) and neutral pH (7.0). pBADGr induction carried out with 0.2 % L-Arabinose. **D)** Survival rate of EHEC 86-24 WT, *pmrB, phoP and pmrD* mutant after PMB treatment cultured in N-minimal medium

supplemented with high Mg^{+2} (10 mM $MgCl_2$) and buffered at low pH (5.8). Data bars represent means ± SEMs, $N = 3$. One-way ANOVA followed by Tukey's post-hoc test. * $P < 0.05$.

6.2 Exposure to physiological relevant bile salt mixture induces PMB resistance in EHEC 86-24

Figure 5A shows that survival rates significantly decline in both strains when exposed to PMB for 1 hour for indicated strains grown in high Mg^{+2} and neutral pH medium. However, pre-treatment with 1.5% BSM significantly increases the survival ability in both strains. *pmrD* inactivation did not affect this resistance phenotype. This suggests that *pmrD* does not play an important role in bile salt mixture-induced PMB resistance in high Mg^{+2} and neutral pH condition.

Figure 5B shows that under low Mg^{+2} and neutral pH conditions WT strain has a significantly higher PMB survival rate than the *pmrD* mutant. Introducing BSM (as a signal) did not change the PMB survival of WT or mutant. These PMB survival levels indicate that under low Mg^{+2} condition, BSM signal did not enhance survival possibly because the low Mg^{+2} induction swamps out the BSM induction of resistance. Secondly, the results indicate that *pmrD* is required for PMB survival under low Mg^{+2} conditions and may be necessary for BSM induction of resistance under these conditions.

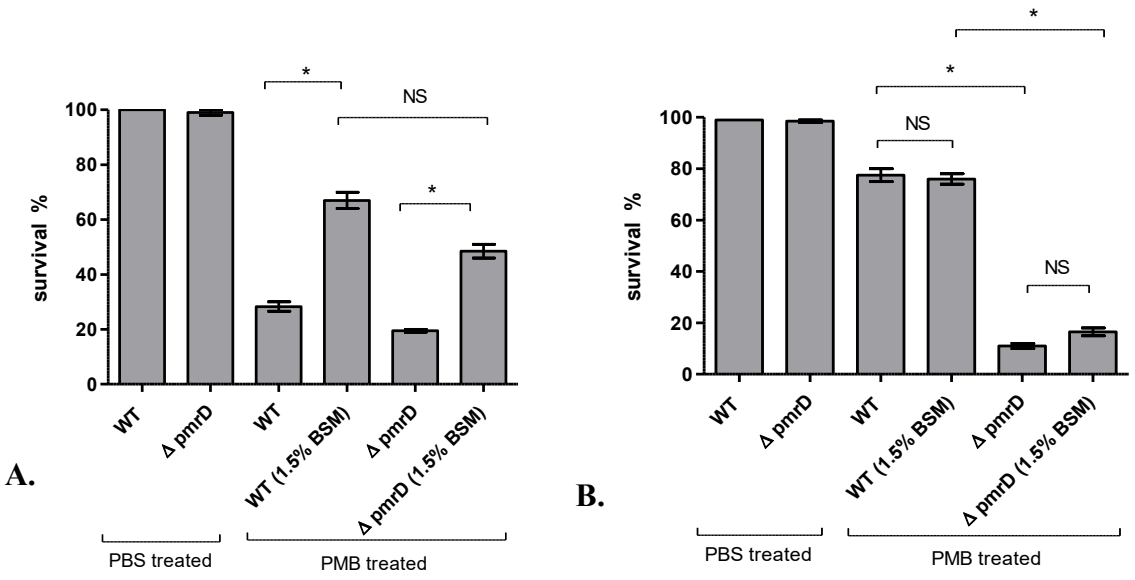


Figure 5 Bile salt mixture treatment increases the PMB resistance in WT and *pmrD* mutant under high Mg^{+2} at neutral pH but not under low Mg^{+2} at neutral pH: A) Survival rate of EHEC 86-24 WT and *pmrD* mutant after PMB treatment cultured under high Mg^{+2} (10 mM $MgCl_2$) and neutral pH (7.0) and with or without BSM as pre-treatment. **B)** Survival rate of EHEC 86-24 WT and *pmrD* mutant after PMB treatment cultured under low Mg^{+2} (10 µM MgCl2) and neutral pH (7.0) and with or without BSM as pre-treatment. Data shown are the means ± SEMs, N=3, One-way ANOVA followed by Tukey's post-hoc test. * $P < 0.05$.

6.3 Exposure to high iron induces PMB resistance in EHEC 86-24

High iron (100µM $FeSO_4$) treatment increases the PMB resistance in WT and *pmrD* mutant

in high Mg^{+2} at neutral pH as well as in low Mg^{+2} at neutral pH. Figure 6A shows results of the experiment conducted at high Mg^{+2} (10 mM $MgCl_2$) and neutral pH with Fe^{3+} (100µM $FeSO_4$) as pre-treatment. Survival rates significantly declines in both strains when exposed to PMB. However, pre-treatment with high iron significantly increases the PMB survival ability in both strains. *pmrD* inactivation did not affect this resistance phenotype. It suggests *pmrD* does not play important role in high iron-induced PMB resistance under high Mg^{+2} and neutral pH condition.

Figure 6B shows results for the same experiment performed under low Mg^{+2} at neutral pH. Introducing Fe^{3+} as a signal into the low Mg^{+2} medium significantly increased the survival rate of *pmrD* mutant whereas no significant change was observed in WT strain. These results indicate that EHEC does not require *pmrD* for sensing Fe^{3+} as an environmental signal even under low Mg^{+2} conditions.

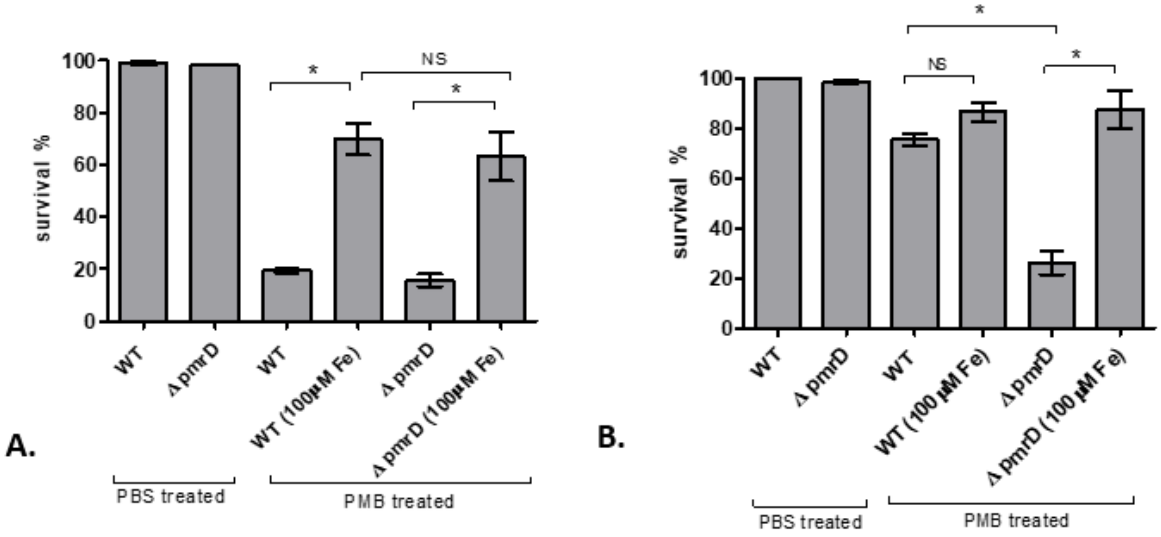


Figure 6 Iron-induced PMB resistance is independent of PmrD: **A)** Survival rate of EHEC 86-24 WT and *pmrD* mutant after PMB treatment cultured under high Mg^{+2} (10 mM $MgCl_2$) and neutral pH (7.0) and with or without Fe^{3+} as pre-treatment. **B)** Survival rate of EHEC 86-24 WT and *pmrD* mutant after PMB treatment cultured under low Mg^{+2} (10 μM $MgCl_2$) and neutral pH (7.0) and with or without Fe^{3+} as pre-treatment. Data shown are the means ± SEMs, N=3, One-way ANOVA followed by Tukey's post-hoc test. * $P < 0.05$.

6.4 BSM and Fe^{3+}-induced PMB resistance is dose-dependent

Previous experiments have shown that the two signals - BSM and Fe^{3+} have the ability to induce CAMP resistance in EHEC. PmrA/PmrB signal transduction pathway is thought to be involved in sensing these signals in EHEC. To determine whether these signals could have additive effects, we repeated the experiment by growing WT and *pmrD* mutant in 10 mM $MgCl_2$ with low concentrations of BSM or Fe^{3+} first and then increasing the concentration of each signal- BSM and Fe^{3+} separately as indicated in the Figure 7 A and B. With increasing concentration of either BSM or Fe^{3+}, the rate of PMB survival increases in both WT and *pmrD* mutant under high Mg^{+2} and neutral pH conditions.

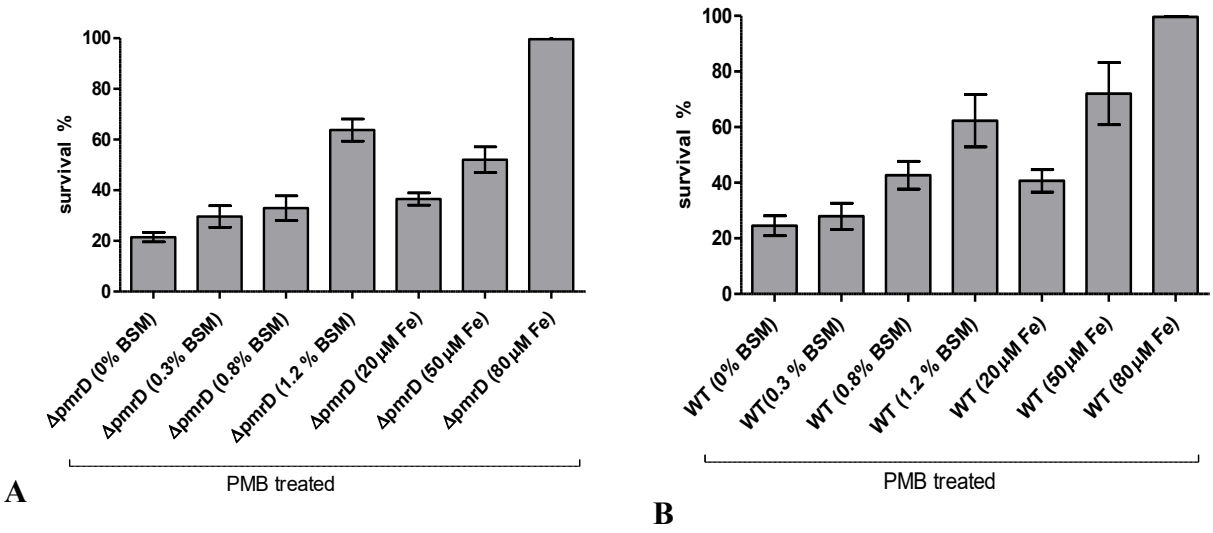


Figure 7 Pre-treatment of WT and *pmrD* mutants with increasing concentration of BSM and Fe^{3+} induce correspondingly increased survival: Survival after PMB treatment of EHEC 86-24 WT and *pmrD* mutants cultured in high Mg+2 (10 mM $MgCl_2$) and neutral pH with increasing concentration of BSM and Fe3+. **A)** *pmrD* mutant **B)** WT. Data bars represent means ± SEMs, N = 3.

6.5 BSM and Fe^{3+} have no additive effect on PMB resistance

When the two signals, 0.8% BSM and 50 μM $FeSO_4$ shown to induce partial PMB resistance (Figure 8 A and B). When these inducing conditions were used in combination as the pre-treatment in another experiment, the survival rates still appear the same (Figure 8). In the absence of other activating signals, BSM and Fe^{3+} triggers PMB resistance but when combined, they fail to produce either an additive or synergistic effect. This could be explained that if BSM and Fe^{3+} compete for the same PmrB binding site. Previous work

in our lab has shown that BSM-induced resistance is mediated through the iron-binding site on PmrB and therefore these results provide support for this interpretation. Consequently, it can be assumed when one signal is already activating PmrB, the other one is unable to bind or activate PmrB.

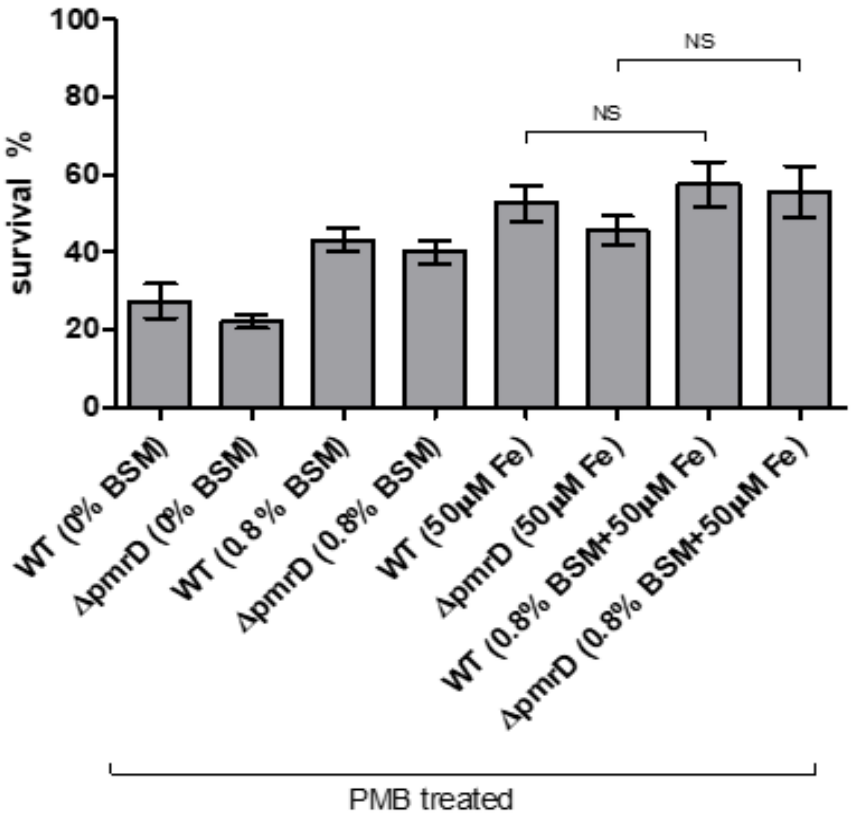


Figure 8 Combined pre-treatment with 0.8 % BSM and 50μM Fe^{3+} signals has no additive effect on survival rate: Survival after PMB treatment of EHEC 86-24 WT and *pmrD* mutants cultured in high Mg^{+2} (10 mM $MgCl_2$) and neutral pH with different

concentration of BSM and Fe^{3+}. Data bars represent means ± SEMs, N = 3. One-way ANOVA followed by Tukey's post-hoc test. * $P < 0.05$

6.6 BSM and Fe^{3+} signal activate PMB resistance in a PmrB dependent fashion

BSM- and Fe^{3+}-induced PMB resistance is fully dependent on *pmrB* as shown in Figure 9A and B. Under high Mg and neutral pH (non-PhoPQ activating condition), PMB survival of WT grown in medium supplemented with 1.5% BSM and 100 μM Fe is significantly higher than the WT strain grown in non-supplemented medium. However, the survival of the *pmrB* mutant grown in medium supplemented with 1.5% BSM and 100μM Fe is significantly decreased. BSM-mediated PMB resistance also appears to be dependent on *phoP*. PMB survival of *phoP* mutant grown in 1.5% BSM is significantly lower than the WT strain grown in the same condition whereas survival rate of *phoP* mutant grown in100μM Fe displays significantly higher survival but still lower than the WT strain grown in the same condition. These results suggest that under high Mg^{2+} and neutral pH conditions, BSM and Ferric iron-induced PMB resistance is fully dependent on *pmrB* and at least partially dependent on *phoP*.

Under low Mg^{2+} neutral pH (PhoPQ activating condition), PMB survival rate of *pmrB* mutant is maintained but lower than the WT strain as shown in Figure 9 B. The survival

rate of *phoP* mutant is significantly lower than the WT strain. Interestingly, survival rate of *pmrB* mutant grown under low Mg^{+2} and high iron condition is significantly decreased than the survival rate of *pmrB* mutant without 100µM iron signal. These results suggest that under low Mg^{+2} and neutral pH conditions, Ferric iron-induced resistance is still fully dependent on *pmrB* and again partially dependent on *phoP*. But interestingly, *pmrB* is not required for PMB resistance in the absence of iron.

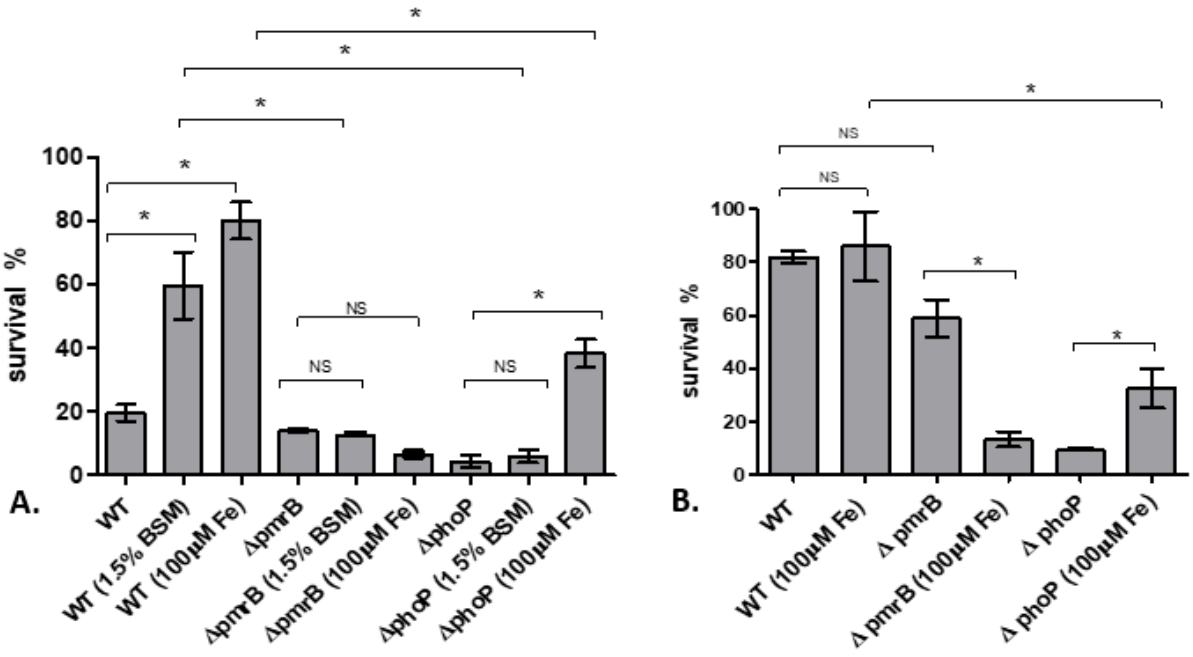


Figure 9 Survival rate after PMB treatment of EHEC WT, Δ*pmrB* and Δ*phoP:* A) Under high Mg^{+2} (10 mM $MgCl_2$) and pH 7.0, **B)** Under low Mg^{+2} (10 µM $MgCl_2$) and neutral pH (7.0). Data bars represent means ± SEMs, $N = 3$. One-way ANOVA followed by Tukey's post-hoc test. *$P < 0.05$.

6.7 The activity of the *pmrD* promoter is influenced by numerous signals

The *pmrD-gfp* translational reporter plasmid was constructed, verified by DNA sequencing results and then transformed in *pmrD* mutant EHEC 86-24. GFP expression dynamics were tested by growing the fusion construct under both *pmrD*-activating and non-activating conditions as suggested by the previous experiments. In these experiments, GFP expression is considered a measure of *pmrD* promoter activity. The *pmrD-gfp* reporter and control strain were grown in Synergy HTX fluorescent plate reader (Biotek) at 37°C under continuous shaking mode and monitored for OD_{600} and fluorescence for 14 hours in various conditions as indicated in the graph. Fluorescence activities (*pmrD* promoter activities) were expressed as a ratio of relative fluorescent unit (RFU) per number of cells (RFU/OD_{600}). Figure 10 shows the GFP expression as a measure of *pmrD* promoter activity under various conditions. In order to understand the promoter activities, we compared the growth kinetics (Figure 10A) and *gfp* fluorescence activities under defined media treatments (Figure 10 B and C).

6.7.1 The *pmrD* promoter is highly active under low Magnesium

Promoter activity under high Mg^{+2} did not increase any time during 14 hours of GFP fluorescence monitoring, suggesting that high Mg^{+2} does not promotes *pmrD* expression regardless of the presence of signals either BSM or Fe^{+3}present in the growth medium.

pmrD activity is maximal in low Mg^{+2} and neutral pH. When fluorescence was monitored for 14 hours, we observed that *pmrD* promoter activity has a noticeable delay in GFP fluorescence. It takes approximately 4 hours to achieve the persistent rise in fluorescence. After 4 hours, promoter activity under low Mg^{+2} and neutral pH increases dramatically. When *gfp* expression patterns were compared, the *pmrD* promoter activity appears to be significantly higher under low Mg^{+2} at pH 7.0 than under low Mg^{+2} at low pH, or under low Mg^{+2}, neutral pH with BSM and Fe^{3+} (Figure. 10 B/ C)

6.7.2 The *pmrD* promoter activity repressed by BSM and Fe^{3+}

The *pmrD-gfp* reporter and control strains were grown in Synergy HTX fluorescent plate reader (Biotek) at 37°C under continuous shaking mode and monitored for OD_{600} and fluorescence for 14 hours in various conditions. Promoter activity under BSM and Fe^{3+} signals appears to plateau out after 4 hours, suggesting *gfp* expression is inhibited by these signals. The *pmrD* promoter activity seems significantly repressed by high Fe^{3+} and BSM signal (Figure. 10 B/ C). These signals which all activate PmrA/PmrB likely suppress the expression of *pmrD* through the phospho-PmrA.

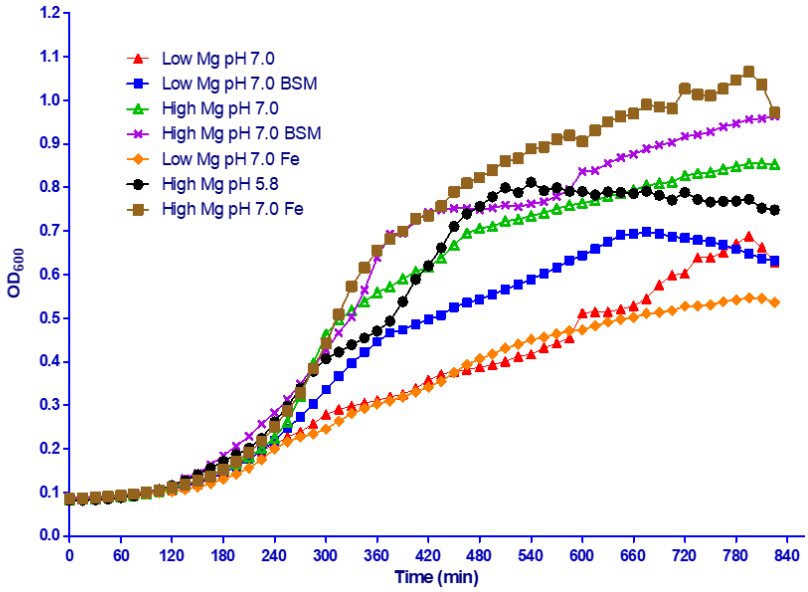
When *gfp* expression patterns were compared, the *pmrD* promoter activity appears to be significantly higher under low Mg^{+2} at pH 7.0 but *pmrD* promoter activity seems significantly repressed by high Fe^{3+} and BSM signal (Figure 10 B/ C). The fact that *pmrD*

is not expressed under high Mg^{+2} and in the presence of BSM (Figure 10B/ C) supports the finding that BSM-induced PMB resistance is independent of *pmrD*.

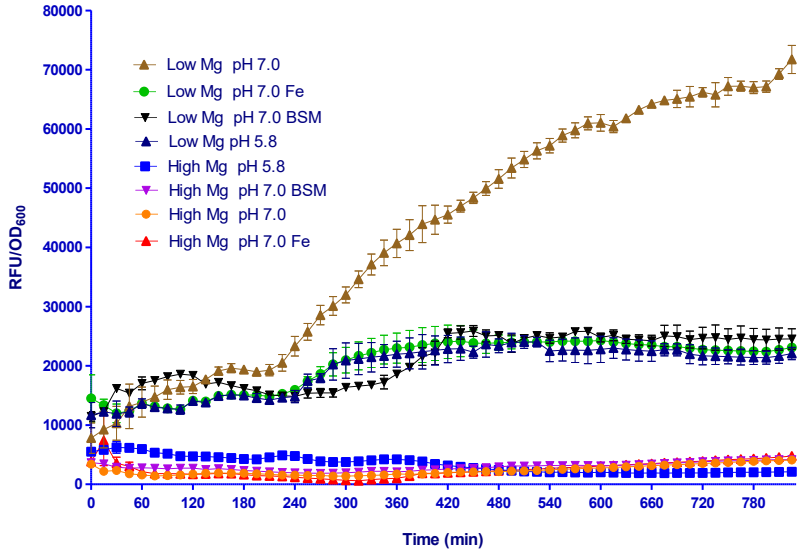
6.7.3 The *pmrD* promoter is repressed by low pH

The *pmrD* promoter activity seems significantly repressed by mildly low pH (pH 5.8) as well (Figure 10 B/ C). These signals which all activate PmrA/PmrB likely suppress the expression of *pmrD* through the phospho-PmrA. The fact that *pmrD* is not expressed under high Mg^{+2} and mildly low pH (Figure 10 B/ C) supports the finding that low pH-induced PMB resistance is independent of *pmrD*.

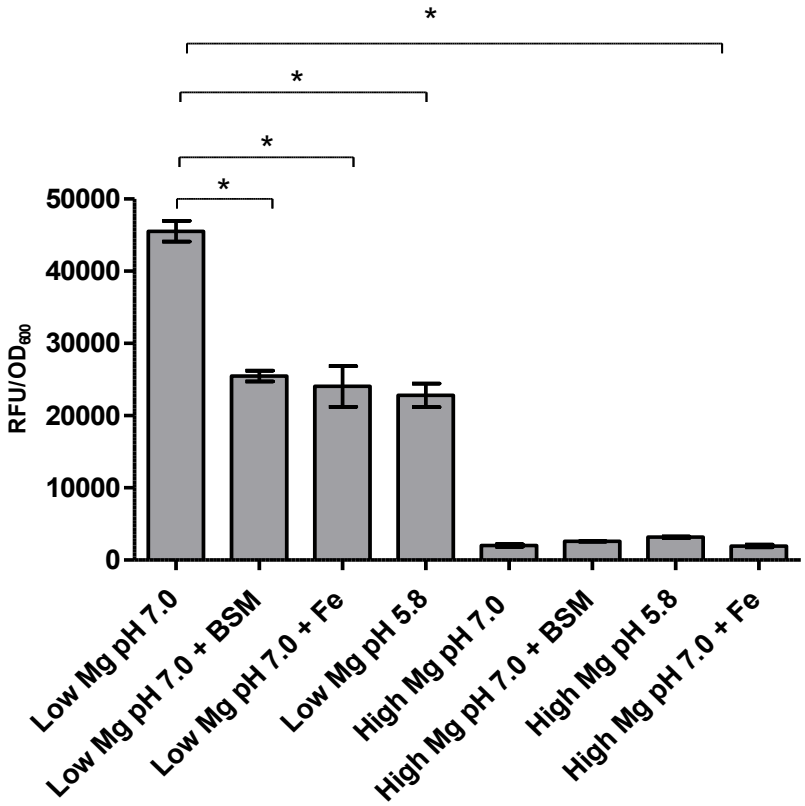
A.



B.


80000
70000
60000
50000
40000
30000
20000
10000
0
RFU/OD600
Low Mg pH 7.0
Low Mg pH 7.0 Fe
Low Mg pH 7.0 BSM
Low Mg pH 5.8
High Mg pH 5.8
High Mg pH 7.0 BSM
High Mg pH 7.0
High Mg pH 7.0 Fe
0
60
120
180
240
300
360
420
480
540
600
660
720
780
Time (min)

C.


*
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50000
40000
30000
20000
10000
0
RFU/OD600
Low Mg pH 7.0
Low Mg pH 7.0 + BSM
Low Mg pH 7.0 + Fe
Low Mg pH 5.8
High Mg pH 7.0
High Mg pH 7.0 + BSM
High Mg pH 5.8
High Mg pH 7.0 + Fe

Figure 10 *pmrD* promoter activity monitored under low Mg^{+2} at neutral pH and measured the effect of signals such as low pH, 1.5% BSM and high Fe^{3+}: A) Growth kinetics of *pmrD-gfp* reporter strain (86-24*ΔpmrD* harboring TOPO plasmid with *gfp* coding sequence under *pmrD* native promoter) grown in high/ low Mg^{+2} with or without BSM or Fe^{3+}. The OD_{600} of *gfp* reporter strains grown in defined (N-minimal) medium supplemented with 1.5% BSM or 100 μM $FeSO_4$ was recorded at the indicated time points and plotted over time for 14 hours. **B)** GFP expression presented in a relative fluorescent unit (RFU) as a function of growth (OD_{600}) over time. Fluorescent activities (*pmrD* promoter activities) were expressed as a ratio of relative fluorescent unit (RFU) per number of cells (RFU/OD_{600}). **C)** GFP fluorescence (*pmrD* promoter activity) compared at the 7 hours' time point in defined conditions as mentioned in the Figure. Error bar represents the upper and lower limit of sd. N=3, One-way ANOVA followed by Tukey's post-hoc test. *$P < 0.05$.

6.8 No interaction exists between BSM and PMB

In order to detect potential synergistic or antagonistic interactions between the inducing signal, BSM and the killing cationic antimicrobial peptide, PMB used in the PMB resistance assay, we ran the synergy test between these two compounds in both high Mg^{+2}-neutral pH and low Mg^{+2}-neutral pH N-minimal media.

Synergy between BSM and PMB was tested using the checkerboard method as described by CLSI (Clinical Laboratory Standard Institute, 2007). The fractional inhibitory

concentrations for interactions between BSM and PMB were determined. No synergistic or antagonistic interaction was detected between BSM and PMB in both high Mg^{+2}- neutral pH and low Mg^{+2}-neutral pH N-minimal media as shown in Table 4. Therefore, we can conclude that PMB killing carried out with 2, 4, 8 µg/ml PMB does not seem to be influenced positively or negatively by the antimicrobial properties of 1.5% BSM. Additionally, in order to remove any kind of effect associated with the use of 1.5 % BSM during the pre-treatment stage of the culture, cells were washed three times before the killing assays.

The total FIC values were interpreted as follows:

Synergistic	≤ 0.5
Indifferent	>1.0 – 4.0
Antagonistic	>4.0

Checkerboard assay were performed as described by CLSI on 96 microwell plate on log-phase bacterial suspension prepared in respective medium as indicated in the result table. Plate readings were done with HTX Microplate Reader (Biotek) after 18 hours' incubation at 37°C.

Table 4 Checkerboard assay results

Checkerboard assay condition	Calculated TFIC (FIC of BSM + FIC of PMB)	Interpretation
N-minimal low Mg^{+2} pH 7	= 1.06	Indifferent
N-minimal high Mg^{+2} pH 7	= 1.25	Indifferent

7 Discussion

The gram-negative bacterial outer membrane is an asymmetric bilayer composed of an inner leaflet of phospholipids and an outer leaflet of lipopolysaccharide. Lipid A appears to be highly conserved structure of pathogenic bacteria and therefor serves as the critical component if LPS structure (Raetz et al. 2007). It not only serves as the toxic component of bacterial cell to animal host but also serves as means of survival tool. Lipid A is readily modifiable at its 1'- and 4' positions, where covalent modifications can be made. These modifications have been shown to enhance bacterial fitness and survival in harsh host or non-host environmental conditions and to escape killing by host defenses systems (Matsuura 2013).

A comprehensive understanding of survival strategies and virulence mechanisms of EHEC could help design an effective control strategy for EHEC infection. In order to establish itself as a successful pathogen, EHEC has the ability to evade host defense mechanisms and survive within the human gastrointestinal tract. The gastrointestinal tract which is a naturally inhospitable environment to most of the microorganisms due to the presence of high acidity, digestive enzymes and bile salt mixture in various anatomical niches within the GI tract. EHEC are exposed to these conditions during their transit through the GI tract. Instead of being killed by these environments, EHEC utilizes these environments as the signals for upregulation of mechanisms that help them to evade the attack by host defenses including cationic antimicrobial peptides likely to be encountered in the subsequent part of

the intestine. It is still not fully understood how EHEC manages to overcome the effect of these environments and shows increased CAMP resistance. In this study, we attempted to understand EHEC responses to selected chemical and biochemical cues likely present in the gastrointestinal tract environment in terms of resistance to a classical cationic antimicrobial peptide, PMB. Our purpose was to look at role of signal transduction pathways, particularly the PmrD connector protein, and how the main host environmental conditions such as high acidity, high iron and bile salt mixtures are used as signals to mount the CAMP resistance ability.

To investigate the effect of limiting Mg^{2+} as a signal for lipopolysaccharide modification, we determined the PMB resistance after growth in the presence of low Mg^{2+}. The two-component regulatory system PhoP/PhoQ has been shown to be responsible for low Mg^{2+} sensing in many gram-negative bacteria such as in *Salmonella* and several other enteric bacteria (Luo et al., 2010, Kato, Latifi, & Groisman, 2003). In high Mg^{2+} condition PhoQ kinase activity remains repressed due to divalent cation salt-bridge formation between periplasmic domain of PhoQ and inner membrane. Under low Mg^{2+} condition, this salt-bridge is disrupted causing conformational change that leads to PhoQ activation. Activated PhoQ then promotes phosphorylation of PhoP and subsequently results in increased expression of the target genes. PhoP-regulated gene products are involved in lipopolysaccharide modifications and promote growth in low Mg^{2+} environments. We used this low Mg^{+2} condition, a well-established PhoQ-activating condition for *Salmonella* to study the role of EHEC PmrD in LPS modification and CAMP resistance. While the role

of *Salmonella* PmrD in LPS modification is already clear, there is still uncertainty about whether EHEC PmrD can transduce the low Mg^{+2}-induced signal to the master regulators of LPS modification, PmrA, as many *pmrA*-regulated gene products are LPS modifying enzymes (Gunn 2001). We found the exposure of EHEC to the low Mg^{2+} condition enhances PMB resistance in a *pmrD*- and *phoP*-dependent manner. Secondly, *pmrD* promoter activity was also dramatically increased in the low Mg^{+2} condition. GFP fluorescence starts to rise after 4 hours of growth in this medium. This type of delayed response is normal in the connector-mediated pathway since it may take extra time to process the signals (Kato et al. 2007). Another reason for this delayed response could be explained by the fact that bacterial response through protein expression is low in lag phase and high in exponential phase. This observation tells us that even though during the evolutionary race *Salmonella* and EHEC diverged from a common ancestor some 100 million years ago and there is only 55% identity in *pmrD* sequence (Winfield and Groisman 2004), EHEC still retains the functionally equivalent PmrD.

EHEC has the extraordinary tolerance ability to low pH as demonstrated by its survival at pH levels as low as 2·5 for 2h (House et al. 2009). To investigate whether low pH is related to the ability to resist cationic antimicrobial peptide PMB, we determined the PMB resistance after culturing EHEC 86-24 in the presence of low pH. We found that both WT and *pmrD* mutant strains were able to grow and showed increased resistance to PMB in the presence of low pH. The PMB survival of *pmrD* mutant was not impaired in the presence of mildly low pH. This observation suggests that EHEC can easily adapt to this stressful

environment of low pH and not only resume growth in this condition but also uses the low pH as the signal to improve its CAMP resistance without the PmrD-mediated PhoPQ two component signal transduction pathway. Using the *pmrD* promoter analysis assay, we were able to observe the genetic basis of this phenotype. Exposure to low pH resulted in minimal *pmrD* promoter activity. This result substantiates the results of the killing assay which showed that pH-induced PMB resistance in EHEC was independent of *pmrD*. Similar research in *Salmonella typhimurium* has shown that mildly low pH (pH5.8) is sensed by the sensor domain located in PhoQ (Ohl and Miller 2001) as well as in PmrB (Perez & Groisman, 2007). Based on our data that inactivation of *pmrB* gene significantly decrease the PMB survival rate of EHEC grown in low pH condition suggest that EHEC prefers to use the PmrA/PmrB two component system for low pH sensing, in contrast to *Salmonella typhimurium* which can use both two-component system. Additionally, our study also revealed that exposure to low pH represses the *pmrD* expression under low Mg^{+2} conditions. *pmrD* expression is usually very high under low $Mg^{2+.}$ However, GFP fluorescence is significantly decreased when grown in the presence of pH 5.8. Due to the fact that GFPmut3 fluorescence intensity does not decrease significantly at this pH (Cheng et al., 2017), suggests that *pmrD* expression may be repressed by low pH signal under low Mg^{2+} condition. EHEC may adopt this strategy when it has enough phosphorylated PmrA to promote lipid A modification and confer CAMP resistance. Some food products, especially ones with acidic preservatives and some fermented food products may therefore support the survival of EHEC and may promote infection.

Bile is a complex mixture secreted in the duodenum which has membrane disrupting and DNA damaging effects on microorganism. To establish infection an enteric pathogen must resist the effect of bile salt mixture and be able to grow in physiologically relevant concentrations of BSM. Even though it has been known that many gram-negative bacteria can resist bile and salt mixture to some extent, but it is still unclear whether exposure to it induces PMB resistance in enteric pathogens. In our experiments, we found that EHEC 86-24 grew with some level of inhibition at 1.5 % BSM. In the PMB resistance assays, EHEC 86-24 grown at 1.5% BSM until log phase had significantly higher PMB resistance than the untreated strain. Wild type EHEC and *pmrD* mutant when exposed to the same concentration of BSM, showed similar PMB resistance pattern under neutral pH and non-limiting Mg^{+2}. This observation shows that the BSM-mediated resistance does not require PmrD protein, suggesting that BSM signal is directly sensed by PmrAB two-component system. The promoter activity analysis showed no or very minimal *pmrD* promoter activities for EHEC grown under high Mg^{+2}, neutral pH in 1.5 % BSM. This supports the conclusion drawn from the PMB killing assay data that BSM-mediated cationic antimicrobial resistance does not require PmrD. Interestingly, exposure to BSM represses the *pmrD* expression when 1.5% BSM is introduced into the low Mg^{+2} condition which suggests that BSM signal is sensed directly by PmrA/PmrB two-component system. Bile salts have been shown to trigger virulence factor expression in many gram-negative bacteria like *Salmonella*, *Vibrio*s and *Campylobacter* (Merritt and Donaldson 2009). We previously reported that *pmrAB* and the *pmrA*-activated operon *pmrHFIJKLM* and *ugd* are all upregulated in EHEC when exposed to BSM (Kus et al. 2011). The PMB resistance and promoter analysis results from this study clearly suggest that the bile salt mixture is sensed

though the PmrAB two component system and that the PMB resistance is *pmrB*-dependent. This agrees with the findings of the microarray analysis (Kus et al. 2011). The interaction between bile salt mixture and a bacterial sensor kinase may be physiologically most efficient for activating protective mechanisms like lipid A modifications on the outer surface. Transducing the signal through connector mediated pathways appears to be less efficient than the direct interaction of the BSM with PmrB sensor kinase.

We also investigated the ability of Fe^{3+}cations to activate CAMP resistance in EHEC. For people on a standard diet, the stomach and initial part of intestine may provide a relatively iron rich environment, typically in the range of 29 to 733 μM (Simpson and Peters 1990). Our results showed higher PMB survival rates for iron-treated EHEC compared to untreated EHEC. Inactivating the *pmrD* gene did not affect the overall PMB survival rates of EHEC under high as well as under low Mg^{2+} condition when exposed to high iron condition. These results suggest that an iron rich environment triggers a protective response in EHEC where Fe^{3+}-induced CAMP resistance is mediated though PmrA/PmrB two-component system. This result was confirmed by the findings that very minimal *pmrD* promoter activity was observed in iron treated EHEC. The experimental finding that introducing iron in low Mg^{+2} conditions inhibits the *pmrD* promoter activity provides evidence for a feedback control loop between the PmrAB and PhoPQ. These findings are consistent with previous findings that the periplasmic domain of PmrB has an iron binding motif in *Salmonella typhimurium, Escherichia coli* (K-12) and *Klebsiella pneumoniae* (Chen and Groisman 2013). Furthermore, all of these bacteria also have functional PmrD.

PmrAB has been reported to serve as an important regulator of LPS modification and is critical for Fe^{3+} induced resistance in *Salmonella*. This suggests that the iron sensing ability is highly conserved among enterobacteriaceae and serves as the regulator of virulence in enteric pathogens within the host. This response helps bacteria survive where iron concentrations are high.

In order to understand the details how BSM and high iron signals are sensed, we performed PMB killing on WT, *pmrB* mutant and *phoP* mutant in various conditions. The fact that BSM and Fe^{3+}-induced PMB resistance increases on WT strains but is lost in *pmrB* mutant strongly suggests that BSM and Fe^{3+} is sensed mainly though PmrB. Under high Mg^{+2} (10 mM $MgCl_2$) and pH 7.0 condition, BSM mediated PMB resistance is dependent on *pmrB* as well as on *phoP*. It appears that for the PMB resistance due to BSM signal under high Mg^{+2} and neutral pH *phoP*-activated genes are necessary. PhoP-activated gene products have been reported in mediating the bile resistance in *Salmonella typhimurium* (Van Velkinburgh and Gunn 1999). But this would be the first observation that BSM-mediated PMB resistance in EHEC is also dependent on *phoP* gene.

Iron mediated PMB resistance is fully dependent on *pmrB* and partially dependent on *phoP*. Interestingly, inactivation of *pmrB* gene severely impairs the PMB survival rate of EHEC under low Mg^{+2} (10 μM $MgCl_2$) and neutral pH (7.0) when supplemented with extra iron. It appears that *pmrB* mutant under this condition fails to regulate the modifications in the

outer membrane which is supposed to protect EHEC from toxic metal ions as well as from the PMB.

Taken together, the results from this work demonstrate that EHEC uses PmrAB and PhoPQ to monitor its surroundings, including pH, divalent cation, iron and BSM to direct its survival and virulence. Under PhoPQ activating condition (low Mg^{+2} condition) where *pmrA*-dependent lipid A modifying enzymes are already activated, PmrB-activating signals do not act additively. Instead, BSM, Fe^{+3} and low pH repress the excessive activation of lipid A modifying enzymes. This is how EHEC PhoPQ and PmrAB two-component system stimulate the optimal level of lipid A modifications but prevent the potential detrimental effects of excessive LPS modification. In future studies, it would be important to look at another relevant signal the CAMP itself. It can serve as activation signals for PhoQ for LPS modification in enteric pathogens (Bader, Navarre et al. 2003; Bader, Sanowar et al. 2005). In summary, exposure to low Mg^{+2} promotes CAMP resistance by a mechanism that requires the PhoPQ two-component system and PmrD. However, the PhoPQ system and PmrD are not necessary for the low pH, bile salt mixture and iron-mediated CAMP resistance in EHEC, at least under nonlimiting Mg^{2+} conditions. However, under limiting Mg^{2+} conditions, bile salt mixture and iron-induced CAMP resistance have a more complex molecular mechanism.

8 Summary and significance

Gram-negative bacteria use two-component regulatory systems, including PmrAB and PhoPQ to sense and respond to environmental signals by modifying their outmost layer to adapt to the diverse environments that they encounter. Studies on the regulatory architecture of two-component regulatory systems in the related pathogen, *Salmonella*, have revealed that PmrA, the response regulator of PmrAB system can be activated either by its own sensor domain, PmrB or by a non-cognate response regulator, PhoP, the response regulator of another two-component regulatory system, PhoPQ. The PMB resistant protein D (PmrD), is believed to establish the connection between PhoPQ and PmrAB system. This connectivity appears to serve as a critical step in sensing and integrating multiple signals for pathogen's own increased fitness and survival in some enteric pathogens (Winfield and Groisman 2004).

This project aimed to investigate the role of EHEC *pmrD* in CAMP resistance induced in a variety of environmental signals. Our results indicate that low Mg^{2+} triggers the *pmrD* expression and subsequently increases the resistance to CAMPS, as tested by PMB resistance assay. When *pmrD* is inactivated, EHEC resistance to CAMP is significantly reduced. This result suggests that like *Salmonella typhimurium*, EHEC PmrD connects the signal sensed by PhoPQ and passed to PmrAB system for coordinated protective modifications in bacterial outer layer.

To understand how EHEC responds to low pH, bile salt mixture and iron. Some of the most common environmental signals likely to be encountered during the transit through human gastro-intestinal tract, we used N-minimal medium supplemented with appropriate signals representative of the enteric environments. For example, 1.5 % BSM pre-treatment condition mimics duodenum and small intestine environment, high iron and low pH pretreatment is representative of gastric environment. High iron condition may also represent the natural surface water and soil as well as some food products since iron is a very abundant element in the nature. Our result indicates that BSM and iron serve as potent intestinal cues for EHEC survival against CAMPs. 1.5 % BSM and 100 uM $FeSO_4$-induced PMB resistance do not require PmrD. Like *Salmonella typhimurium,* our result indicates that mildly low pH is also an intestinal cue for EHEC survival to PMB but unlike *Salmonella typhimurium* it does not appear to be sensed by PhoPQ but rather is dependent on PmrB and PhoP. The presence of either 1.5 % BSM or 100 uM Fe^{3+} appear to suppress *pmrD* expression which suggest that iron and BSM-mediated pathway inhibits the expression of *pmrD* through feedback mechanism as depicted in the model of regulation of PMB resistance in EHEC 86-24 in Figure 11.

We assessed the EHEC phenotypic responses to various signals in various in-vitro conditions by PMB resistance assays. These results are logically backed up by *pmrD* promoter activity measurements. We constructed the *pmrD-gfp* reporter strains and carried

out *gfp* expression analysis in those conditions in which we conducted PMB resistance assays.

By our results, we noted that EHEC not only survives the challenges of these host factors but also exploits them to manage protective outer layer and evade killing by host cationic antimicrobial peptides (CAMPs). It helps us to understand how efficiently engineered their signaling network in enteric pathogen. The absence of *pmrD* gene in pathogens like *C. rodentium* and *Y. pestis* still permits these pathogens to sense and respond to these types of signals. In general, our study contributes to our understanding how various signals are sensed and used by enteric pathogens to survive and enhance virulence.

TCRSs are signal transduction pathways unique to prokaryotes. Targeting these TCRS proteins may emerge as an effective strategy to control EHEC and related foodborne pathogens. The knowledge obtained during this study will contribute to the understanding of the action of TCSs in *enterobacteriaceae*, as well as their contribution in the survival mechanisms adopted by these types of microorganism in food or/and food processing and storage environment.

9 Model of regulation of PMB resistance in EHEC 86-24

Figure 11 and 12 represent mechanistic models how EHEC 86-24 could regulate PMB resistance.

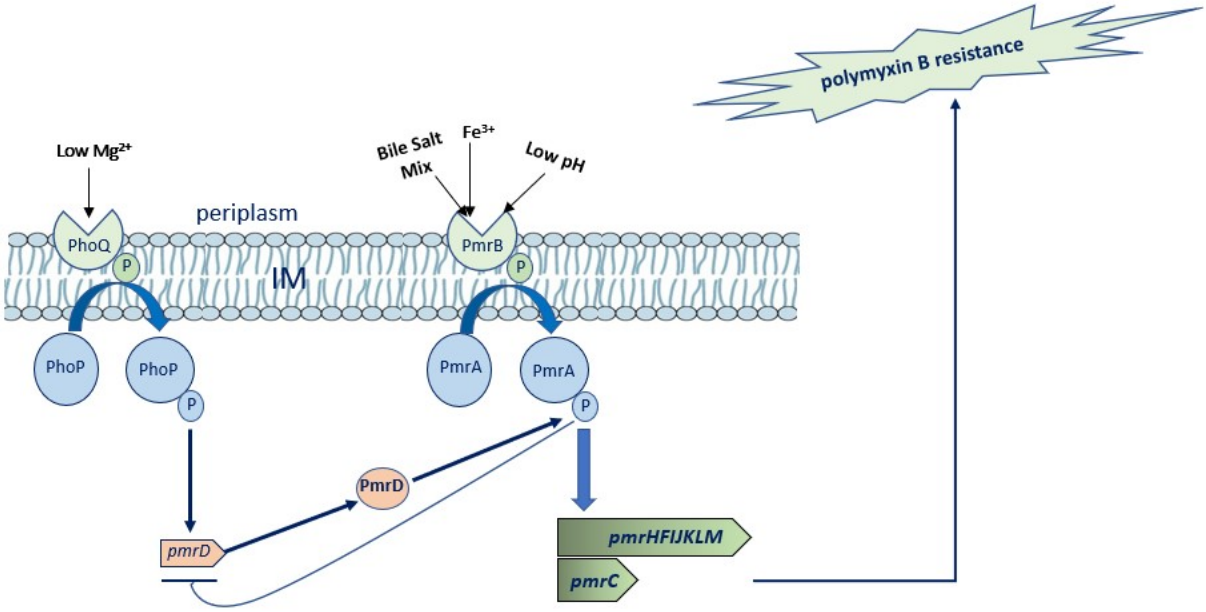

Figure 11 Model of regulation of PMB resistance under low Mg^{2+} in EHEC 86-24: Low Mg^{2+} (10 µM $MgCl_2$) condition is sensed by inner membrane bound sensor kinase PhoQ which autophosphorylates and then transfers the phosphoryl group to PhoP, PhoP-P, an activated transcription factor which promotes transcription of the *pmrD* gene. The PmrD protein then binds to phospho-PmrA, a response regulator of another two-component system, PmrA/PmrB system. PmrD blocks dephosphorylation of PmrA by sensor kinase PmrB, allowing continued transcription of *pmrHFIJKLM* and *pmrC, a* major regulator of lipid A modification system. In the same way, sensor kinase PmrB when activated directly by high Fe^{+3} (100 µM $FeSO_4$) or low pH (5.8) promotes the phosphorylated state of the

response regulator, PmrA which leads to the transcription of *pmrHFIJKLM* and *pmrC*. Excessive amount of PmrA-P represses transcription of the *pmrD* gene, thus establishing a negative feedback loop regulating *pmrD* expression.

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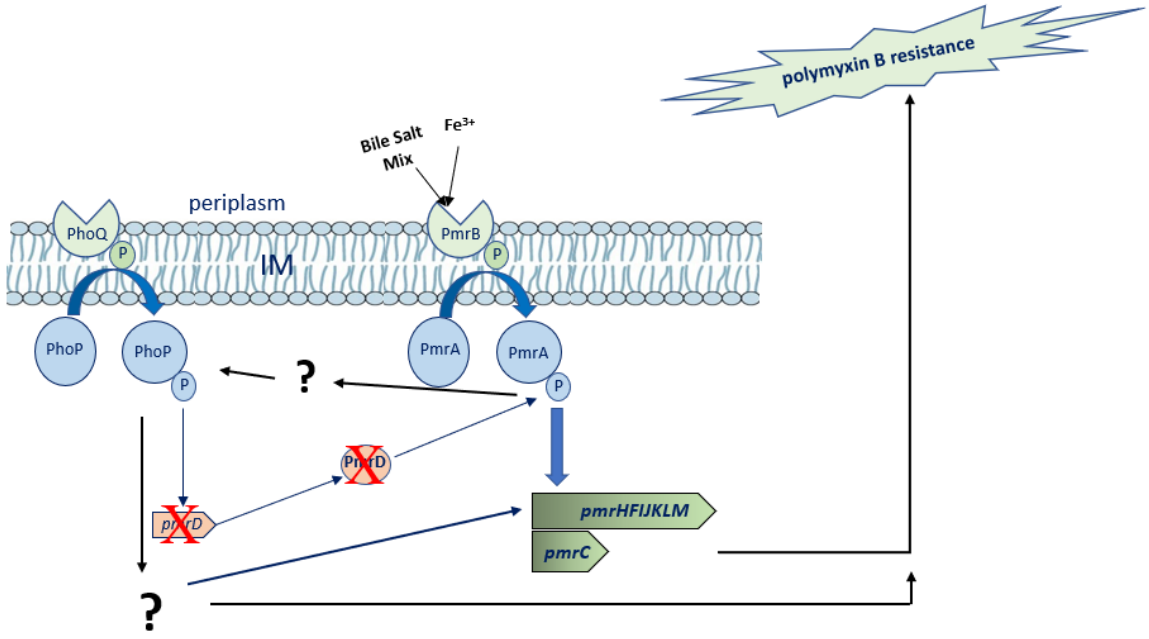

Figure 12 Model of regulation of PMB resistance under high Mg^{2+} and neutral pH in EHEC 86-24: Inner membrane bound sensor kinase PmrB when activated by high Fe^{+3} (100 µM $FeSO_4$) or BSM, promotes the phosphorylated state of the response regulator, PmrA, a DNA binding transcription factor. Activated PmrA (PmrA-P) then leads to transcription of *pmrHFIJKLM* and *pmrC*. Also, BSM-mediated PMB resistance is fully dependent on *phoP* by unknown connector proteins. Iron-mediated PMB resistance is also partially dependent on phoP.

Bacterial two-component connector proteins may promote activation of both response regulators and sensor kinases. For example, EHEC PmrD connects the response regulator PhoP to response regulators of another two-component system, PmrA as demonstrated in this work and by many others in other members of enterobacteriaceae family. In the same way, SafA a connector protein from *E. coli* connects the response regulator (EvgA) of EvgA/EvgS system to the sensor kinase (PhoQ) of PhoP/PhoQ system (Eguchi et al. 2012). Therefore, we propose additional layer of interconnection between PmrAB and PhoPQ via small connector proteins might exist. The question marks illustrate potential spot for unknown connector protein which connects the signals activated by BSM and iron to PhoP and then to the appropriate points which then leads to expression of genes involved in outer membrane modification in EHEC.

10 Future directions

We studied the phenotypic characteristics PMB resistance in response to selected individual and combined intestinal cues. It is clear that low Mg^{+2} and low pH induced conditions trigger the *pmrA*-regulated genes in many gram-negative bacteria by lipid A modification. The major cationic residues produced as a result of this type of signals are L-aminoarabinose and phosphoethanolamine. When added to the lipid A moiety of LPS, they neutralize the negative charge on the bacterial surface and thus prevent the binding of positively charged human CAMPs.

The BSM-mediated EHEC PMB resistance mechanism is gradually unfolding in our lab. We know it is *pmrAB* and *arnT* dependent and now we know that at high Mg^{+2} and neutral pH, it is independent of *pmrD* but dependent on *phoP*. We also know that it is swamped by low Mg^{+2} induction. We do not know which individual bile salts are the most effective in job. Therefore, the immediate further step is to perform killing assay with individual bile salts.

We know that lipid A is highly modifiable structure in gram-negative bacteria. To explore the molecular basis behind the PMB resistance due to BSM, analyze lipid A chemical profile by matrix assisted lase desorption/ionization-time of flight (MALDI-TOF) on

BSM-induced EHEC cells. This assay would reveal the chemical moiety added to lipid A structure as a result of BSM exposure.

In this study we focused on *pmrD*, which connects the two major two-component system pathways. We also discovered BSM-mediated PMB resistance still appears to be dependent on *phoP.* To get the detailed insight into EHEC response to BSM and cross talk between these pathways or potentially others, it is important to further investigate interconnectivity between the response regulators and sensor kinase or vice versa. We can use genomic and proteomic approach to investigate this question. Briefly, we need to identify two-component proteins most impacted in BSM-treated EHEC, trace down the genes, generate mutants and look PMB resistance phenotype or gene expression responses.

Finally, PMB used in this study as a classical CAMP is not a human cationic antimicrobial peptide. It would be more relevant if we perform the killing assays with CAMPs of mammalian origin such as HD-5, HBD-1, HBD-2 and LL-37.

Appendices

Additional details of protocols used in the work.

A) Killing assay protocol for 1.5% BSM condition

To assess the % survival of EHEC 86-24 to Polymyxin B (2 ug/ml) induced under N-minimal medium (NM) with high Mg and Neutral pH + 0 % bile salt mix (BSM) and NM with high Mg and Neutral pH + 1.5% BSM.

Day 1

O/N culture

1. Prepare 20% BSM solution and filter sterilize using 0.22μm membrane filter and syringe.
2. Prepare overnight samples of untreated and 1.5% bile salt mixture (BSM) treated cultures using a total volume of 5 mL in 10 mL round bottom culture tubes
    a. Untreated → Prepare 5 ml NM with high Mg buffered at pH 7.0 (No BSM)
    b. 1.5% BSM treated → Prepare 5 ml 1.5% BSM in NM with high Mg buffered at neutral pH and mix well (4.625 ml NM and 0.375 ml 20% BSM)
3. Inoculate a single colony from recent EHEC 86-24 plate.
4. Incubate at 37℃ O/N on a shaker.

Day 2

5. Read OD_{600} of overnight cultures.
6. Repeat #1, 2 to prepare media for subcultures with a total volume of 5 mL in 10 mL round bottom culture tubes.
7. Inoculate 0 % BSM-NM and 1.5 % BSM-NM tubes with respective cultures 60μL and 120μL respectively from the O/N culture.

8. Incubate at 37℃ on a shaker for 3-4 hours and monitor OD so that it does not reach more than 0.8 (~ 0.6 preferable)
9. Record OD_{600} for all (both OD should look between 0.5 and 0.6)
10. Centrifuge using the big benchtop centrifuge at 3000 xg for 10 minutes
11. Remove the supernatant and add 5 ml cold PBS. Vortex mix.
12. Repeat step 12 two more times and make sure the cells were washed properly so that no traces of BSM left in the 1.5% NM-BSM tube. If you still see frothing, wash one more time.
13. Re-suspend cells in 700µL N-minimal salts buffered at pH 7.0 (No Mg added, or just salt mixture buffered at pH7.0).
14. Read the OD of both again and adjust the OD of 0 % NM-BSM OD to 0.600 and 1.5 % NM-BSM OD to 0.800 respectively with N-minimal salts buffered at pH 7.0
15. Transfer 30µL of each into 2ml of N-minimal salts buffered at pH 7.0
16. Mix 100µL of each with 100µL of PBS.
17. Prepare 10-fold dilution in PBS up to 10^{-3} and plate out on LB agar to count bacteria the next day to confirm CFU/ml. (by mixing 20µL + 180µL)
18. In a micro-titre plate, mix 100µL of each from step 16 and with 100µL of desired 2X concentration of PMB
19. Incubate at 37℃ in 5% CO_2 incubator for 1 hour.
20. Prepare 10-fold dilution in PBS up to 10^{-3} of each and plate out on LB agar to count the cells (CFU) the next day.
21. Use the already surface-dried LB plates, transfer 10µL onto LB plate and do track plating. Make sure all of them dry on the surface immediately. This is critical for PMB treated preparations. If remains unabsorbed into the media, PMB keeps acting on the cells. (If you use spot culture. Use dried plates and make sure the 10 µL transferred onto LB dries out immediately, you can keep lid open in hood)
22. Incubate all the plates at 37℃ O/N

Day 3

23. Count the CFU, express in CFU/ml and calculate survival %
24. The percent survival is calculated as follows: (CFU of polymyxin B-treated count/CFU of PBS-treated count)

B) Killing assay protocol for 100 uM Fe^{3+} condition

To assess the % survival of EHEC 86-24 to polymyxin B (2 ug/ml) induced under N-minimal medium (NM) with high Fe^{3+} (100 uM $FeSO_4$) and neutral pH + 0 Fe^{3+} and NM with high Mg and neutral pH.

Day 1

O/N culture

1. Prepare 1mM $FeSO_4$ solution and filter sterilize using 0.22µm membrane filter and syringe.
2. Prepare overnight samples of untreated and 100 uM $FeSO_4$ treated cultures using a total volume of 5 mL in 10 mL round bottom culture tubes
    a. Untreated → Prepare 5 ml NM with high Mg buffered at pH 7.0.
    b. 100 uM $FeSO_4$ treated → Prepare 5 ml 100 uM $FeSO_4$. 100 uM $FeSO_4$ in NM with high Mg buffered at neutral pH and mix well (4.5 ml NM and 0.5 ml 1mM $FeSO_4$)
3. Inoculate a single colony from recent EHEC 86-24 plate.
4. Incubate at 37℃ O/N on a shaker.

Day 2

5. Read OD_{600} of overnight cultures.
6. Repeat #1, 2 to prepare media for subcultures with a total volume of 5 mL in 10 mL round bottom culture tubes.

7. Inoculate 0 mM $FeSO_4$-NM and 100 uM $FeSO_4$-NM tubes with respective cultures 60µL from the O/N culture.
8. Incubate at 37℃ on a shaker for 3-4 hours and monitor OD so that it does not reach more than 0.8 (~ 0.6 preferable)
9. Record OD_{600} for all (both OD should look between 0.5 and 0.6)
10. Centrifuge using the big benchtop centrifuge at 3000 xg for 10 minutes
11. Remove the supernatant and add 5 ml cold PBS. Vortex mix.
12. Repeat step 12 two more times and make sure the cells were washed properly so that no traces of Fe left in the NM-Fe tube. If you still see brown precipitates, wash one more time.
13. Re-suspend cells in 700µL N-minimal salts buffered at pH 7.0 (No Mg added, or just salt mixture buffered at pH7.0).
14. Read the OD of both again and adjust the OD of both 0.600 with N-minimal salts buffered at pH 7.0
15. Transfer 30µL of each into 2ml of N-minimal salts buffered at pH 7.0
16. Mix 100µL of each with 100µL of PBS.
17. Prepare 10-fold dilution in PBS up to 10^{-3} and plate out on LB agar to count bacteria the next day to confirm CFU/ml. (by mixing 20µL + 180µL)
18. In a micro-titre plate, mix 100µL of each from step 16 and with 100µL of desired 2X concentration of PMB
19. Incubate at 37℃ in 5% CO_2 incubator for 1 hour.
20. Prepare 10-fold dilution in PBS up to 10^{-3} of each and plate out on LB agar to count the cells (CFU) the next day.
21. Use the already surface-dried LB plates, transfer 10µL onto LB plate and do track plating. Make sure all of them dry on the surface immediately. This is critical for PMB treated preparations. If remains unabsorbed into the media, PMB keeps acting on the cells. (If you use spot culture. Use dried plates and make sure the 10 µL transferred onto LB dries out immediately, you can keep lid open in hood)
22. Incubate all the plates at 37℃ O/N

Day 3

23. Count the CFU, express in CFU/ml and calculate survival %
24. The percent survival is calculated as follows: (CFU of polymyxin B-treated count/CFU of PBS-treated count)

C) Killing assay protocol for mildly low pH (pH 5.8) condition

To assess the % survival of EHEC 86-24 to polymyxin B (2 ug/ml) induced under N-minimal medium (NM) high Mg with low pH (pH 5.8) and NM neutral pH with high Mg.

Day 1

O/N culture

1. Prepare overnight samples of neutral pH and low pH cultures using a total volume of 5 mL in 10 mL round bottom culture tubes
    a. Neutral pH → Prepare 5 ml NM with high Mg buffered at pH 7.0
    b. Mildly low pH → Prepare 5 ml low pH (pH 5.8).
2. Inoculate a single colony from recent EHEC 86-24 plate.
3. Incubate at 37℃ O/N on a shaker.

Day 2

4. Read OD_{600} of overnight cultures.
5. Repeat #1, 2 to prepare media for subcultures with a total volume of 5 mL in 10 mL round bottom culture tubes.
6. Inoculate neutral pH NM and low pH NM tubes with respective cultures 60μL from the O/N culture.
7. Incubate at 37℃ on a shaker for 3-4 hours and monitor OD so that it does not reach more than 0.8 (~ 0.6 preferable)

8. Record OD_{600} for all (both OD should look between 0.5 and 0.6)
9. Centrifuge using the big benchtop centrifuge at 3000 xg for 10 minutes
10. Remove the supernatant and add 5 ml cold PBS. Vortex mix.
11. Repeat step 12 two more times and make sure the cells were washed properly.
12. Re-suspend cells in 700µL N-minimal salts buffered at pH 7.0 (No Mg added, or just salt mixture buffered at pH7.0).
13. Read the OD of both again and adjust the OD of both 0.600 with N-minimal salts buffered at pH 7.0
14. Transfer 30µL of each into 2ml of N-minimal salts buffered at pH 7.0
15. Mix 100µL of each with 100µL of PBS.
16. Prepare 10-fold dilution in PBS up to 10^{-3} and plate out on LB agar to count bacteria the next day to confirm CFU/ml. (by mixing 20µL + 180µL)
17. In a micro-titre plate, mix 100µL of each from step 16 and with 100µL of desired 2X concentration of PMB
18. Incubate at 37℃ in 5% CO_2 incubator for 1 hour.
19. Prepare 10-fold dilution in PBS up to 10^{-3} of each and plate out on LB agar to count the cells (CFU) the next day.
20. Use the already surface-dried LB plates, transfer 10µL onto LB plate and do track plating. Make sure all of them dry on the surface immediately. This is critical for PMB treated preparations. If remains unabsorbed into the media, PMB keeps acting on the cells. (If you use spot culture. Use dried plates and make sure the 10 µL transferred onto LB dries out immediately, you can keep lid open in hood)
21. Incubate all the plates at 37℃ O/N

Day 3

22. Count the CFU, express in CFU/ml and calculate survival %
23. The percent survival is calculated as follows: (CFU of polymyxin B-treated count/CFU of PBS-treated count)

D) *pmrD-gfp* promotor reporter assays

1. Overnight cultures of *pmrD* reporter strains (carrying *gfp* under *pmrD* promoter in the TOPO plasmid) and *pmrD* control strain (carrying only TOPO plasmid) were centrifuged, washed, and resuspended in respective medium (low/high Mg^{+2} with or without BSM or Fe^{3+}) and adjusted to 0.1 OD600.
2. 200 µl of each cell suspension was immediately transferred into a 96-well plate and the cultures were grown in a Synergy HTX fluorescent plate reader (Biotek) at 37°C under continuous shaking mode.
3. Cultures were grown in a Synergy HTX Multi-Mode Plate Reader (Biotek) at 37°C under continuous shaking.
4. Measure absorbency ($OD_{600\ nm}$) and fluorescence (using a 485/20 nm excitation filter and a 528/20 nm emission filter) were monitored every 15 min for 16 h.
5. The GFP fluorescence and the corresponding OD_{600} readings were retrieved from the machine and divided to generate the normalized ratio value of relative fluorescence units (RFU)/OD_{600} as mentioned in methodology section.
6. The GFP fluorescence values were then adjusted for background by subtracting the normalized ratio value obtained for the corresponding control strain.
7. Fluorescence data were calculated as relative fluorescent units (RFU) per OD_{600} of each well as mentioned in methodology section.
8. Experiments were performed with three independent cultures, and the average fluorescence and standard deviations were calculated on these triplicates.

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