BILE SALTS DIFFERENTIALLY ENHANCE RESISTANCE OF ENTEROHEMORRHAGIC *ESCHERICHIA COLI* O157:H7 TO HUMAN CATIONIC ANTIMICROBIAL PEPTIDES

by

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Author Declaration

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Abstract

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Crystal Gadishaw-Lue, Master of Science, Molecular Science, Ryerson University, 2016

Enterohemorrhagic *Escherichia coli* (EHEC) causes severe food and water-borne illness associated with diarrhea, hemorrhagic colitis (HC), and hemolytic-uremic syndrome (HUS). Previously, we reported that treatment of EHEC with a physiologically relevant bile salt mixture (BSM) upregulates genes encoding a two-component system (TCS) (*basRS*) and a lipid A modification pathway (*arnBCADTEF*). The current study examines the effect of BSM treatment on EHEC resistance to human cationic antimicrobials, human defensin, HD-5 and cathelicidin, LL-37. Results show a significant increase in resistance to HD-5 when EHEC are pre-treated with BSM as compared to untreated EHEC. The BS-induced resistance phenotype is lost in each of the *arnT* and *basS* mutants. Interestingly, BSM treatment does not affect resistance to LL-37. The results of this study provide evidence that BS serve as an environmental cue by triggering changes via a TCS that result in protective modifications of the bacterial outer membrane, thereby increasing resistance to HD-5.

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"Education is not filling a pail, but the lighting of a fire."

-William Butler Yeats

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List of Abbreviations

AMP	antimicrobial peptide
BSM	bile salt mix
CAMP	cationic antimicrobial peptide
EHEC	enterohemorrhagic
HUS	haemolytic uremic syndrome
L-Ara4N	aminoarabinose
LPS	lipopolysaccharide
MALDI-TOF	matrix assisted laser desorption/ ionization- mass spectrometry
NH_4VO_3	metavanadate
Stx	Shiga toxin
TLR	toll like receptor
TNF-α	Tumor necrosis factor alpha
wt	wild type

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1 Introduction

1.1 Introduction to *Escherichia coli*

First isolated from faeces by German paediatrician Theodore Escherich in 1885 (Cooke 1974), the organism now designated as *Escherichia coli* began its journey to become one of the best known model organisms in biology. *E. coli* is a short, non-sporulating, encapsulated, straight, gram-negative bacterium that uses flagella for motility and fimbriae to promote adherence to host cells and surfaces (Cooke 1974). *E. coli* first colonizes the intestine a few hours after birth of humans and warm-blooded animals and remains indefinitely throughout the life of the host (Drasar and Hill 1975).

Serotyping of *E. coli* began in the 1940's by Kaufman and was extensively pursued up to the early 1990s (Montenegro et al. 1990; Besser 1993). *E. coli* is mainly typed by three main antigens, molecules that produce an immune response when introduced to the body: the O somatic antigen, the K capsular antigen, and the H flagellar antigen(Cooke 1974). The majority of serotypes of *E. coli* survive symbiotically within the intestines of humans and ruminants. However there are some serotypes that are pathogenic to its host through a variety of pathogenic mechanisms.

1.2 Classification of Pathogenic *Escherichia coli*

The *E. coli* strains that cause intestinal illness are categorized into five main groups based on their virulence properties, mechanisms of infection, clinical symptoms and serology: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC),

enteroaggregative *E. coli* (EAEC), enteroinvasive *Escherichia coli* (EIEC), and enterohemorrhagic *E. coli* (EHEC) (Liaqat 2008).

1.3 **EHEC Related Public Health Impact**

EHEC are opportunistic food-borne pathogens that can cause severe infections including bloody diarrhea that can lead to life-threatening disease including hemolytic-uremic syndrome (HUS) (Noris and Remuzzi 2005). The majority of EHEC symptoms are flu-like and can include a mild fever, nausea and vomiting. Abdominal cramping is also a common symptom experienced in EHEC infections. Most EHEC related symptoms resolve within 6-8 days. However, some cases advance to haemolytic uremic syndrome (HUS), which is a life-threatening disease that causes kidney damage (Cooke 1974).

In 1991 in the Northwest Territories, five hundred and twenty-one persons developed diarrhea from an *E. coli* outbreak. There was a 29% recovery rate of the pathogen from fecal specimens, twenty-nine incidences of HUS in children and two mortalities (Orr et al. 2009). More recently, in 2000 in Walkerton, ON, drinking water from wells nearby farms was contaminated with *E. coli* O157:H7, *Campylobacter jejuni,* as well as other pathogens. A large outbreak of gastroenteritis ensued and eventually affected over 2300 inhabitants. Twenty-seven of these cases were reported to have advanced to HUS and included seven fatalities (Marshall et al. 2006).

E. coli O157:H7 is responsible for over 1% of the five million bacterial food-borne illnesses that occur annually in the United States and of all the EHEC serotypes, *E. coli*

O157:H7 has the highest association with HUS worldwide (Kaper and Karmali 2008; Mead et al. 1999).

The majority of cases of EHEC related cases are associated with ingestion of undercooked, contaminated beef or raw milk (Dean-Nystrom et al. 1998) and are transmitted via a fecal/oral route. With a low minimum infectious dose, on the order of 3-5 cell forming units (CFU), (Normanno 2011) this knowledge provides a persuasive evidence for the need for proper sanitary procedures in the food handling industry. Currently, there is no known treatment for HUS in humans (Noris and Remuzzi 2005) and the use of antibiotics is widely debated (Wong et al. 2000; Safdar et al. 2002).

1.4 **EHEC Virulence Factors**

In order for *E. coli* O157:H7 infection to develop, the organism must elude the host's immune response system, colonize the intestine, successfully replicate *in situ*, and produce toxins (Normanno 2011). The two main toxins that *E. coli* O157:H7 produces are Shiga toxins Stx1 and Stx2 (Etienne-Mesmin et al. 2011; Kaper and Karmali 2008) with the latter being more virulent. Stxs are composed of active A and B subunits. The B subunit forms a doughnut-shaped structure with a central pore and binds at the surface of endothelial cells, leading to subsequent internalization of the toxin. The A subunit inhibits elongation of the peptide chain during protein synthesis, resulting in eukaryotic cell death (Noris and Remuzzi 2005).

The genes that encode for Stxs are located next to a pathogenicity island known as Locus of Enterocyte Effacement (LEE) which encode for proteins that cause attaching and effacing lesions (Sperandio et al. 1998).

1.5 **Impact of Ingestional Stresses on Virulence Properties**

During the journey to the colon where adhesion and colonization occurs, EHEC must first overcome a variety of ingestional stresses including salivary enzymes in the mouth, extremely low pH in the stomach, bile salts in the small intestine and short chain fatty acids (SCFA) in the small and large intestine (Chowdhury, Sahu, and Das 1996). In the mouth, immunoglobulin antibodies, IgA1 and IgA2, are specific for microbial antigens located on the lipopolysaccharide (LPS) of several microbes including *Bacteroides gingivalis*, *Bacteroides fragilis*, and *Escherichia coli* (Brown and Mestecky 1985). The stomach provides an acidic challenge to all ingested microorganisms including *Shigella*, *Salmonella enterica* serovar Typhimurium*, Salmonella paratyphi, Salmonella enteritidis,and Serratia marcescens* and even pathogenic strains of *E. coli* (Dare, Magee, and Mathison 1972; Giannella, Broitman, and Zamcheck 1972). EHEC are able to survive exposure to acute acid and research now indicates that acute acid triggers increased adhesion of EHEC to host cells (House et al. 2009). In another study, exposure of EHEC to bile salts resulted in increased resistance to polymyxin B (PMB), a potent broad spectrum cationic antimicrobial peptide as well as the upregulation of an efflux pump and a two component signal transduction system (Kus et al. 2011). In addition, treatment with SCFA in mouse models has been shown to increase S. Typhimurium adhesion in the ileum leading to a higher number of gastroenteritis and typhoid cases (Lawhon et al. 2002).

1.5.1 **Bile and Bile Salts**

1.5.1.1 *Bile Composition and Function*

Bile is a bitter yellowish, blue and green fluid that is secreted by hepatocytes in the liver. It is stored in the gallbladder and discharged into the duodenum (first section of the small intestines) via hepatic ducts that merge to form the common bile duct (Begley, Gahan, and Hill 2005). Bile has various functions in the human hepatic system. It plays a major role in the emulsification and solubilisation of lipids by increasing enzymatic surface area and also as a transporter to excrete antibiotics, metabolites, toxins, phospholipids and inorganic ions no longer needed by the body (A. F. Hofmann 1999; Kristiansen 2004). The major components of human hepatic bile are sodium, chloride and bile salts with a pH ranging from 7.5-8.0 and a high osmolality. Table 1 below lists the various components found in bile (Begley, Gahan, and Hill 2005).

Bile acids account for approximately 50% of the organic components of bile. They are synthesized in the liver but before secretion into the duodenum, they are conjugated with either glycine or taurene (Begley, Gahan, and Hill 2005; A. Hofmann and Mysels 1992). Conjugation of bile acids with amino acids increases their solubility in hepatic bile over a wide range of pH values and also decreases passive absorption of bile acids across the lining of the small intestines which results in a high intraluminal concentration of bile acids (Begley, Gahan, and Hill 2005). These new structures are known interchangeably as bile salts. The major bile salts present in the intestine are glycocholate, deoxycholate, chenodeoxycholate, and ursodeoxycholate (Rampone 1972; Bernstein et al. 1999; Kus et al. 2011). The normal physiological concentration of bile salts in the mammalian small

intestines range from 0.2 to 2% (w/v) but may differ depending on dietary intake (John S. Gunn 2000; Pumbwe et al. 2007). If the intraluminal concentration becomes too high, this may induce enhanced excretion of electrolytes and water, which are common symptoms of diarrhea (A. F. Hofmann 1999). Conversely, if bile salt concentration is decreased, bile may become supersaturated with cholesterol and may cause the formation of gallstones (Paumgartner and Sauerbruch 1991).

Table 1 Components of human hepatic bile. (Begley, Gahan, and Hill 2005)

Component	Amount
Sodium (mmol/L)	145
Potassium (mmol/L)	4
Chloride (mmol/L)	90
Conjugated Bile salts (mmol/L)	40
Cholesterol (mmol/L)	3
Phospholipids (mmol/L)	7
Bile acids(g/L)	3-45

1.5.1.2 *Antimicrobial actions of bile*

Along with excreting fatty acids from the body, bile also has an antimicrobial effect against bacteria and plays a major role in defending the host against pathogens (Bernstein et al. 1999). Bile salts at a concentration of 2-3 mM can disrupt membrane lipids and integral membrane proteins (Coleman, Lowe, and Billington 1980), resulting in leakage of intracellular material confirmed by enzyme assays (Fujisawa and Mori 1997; Noh and Gilliland 1993). As a result, cells that have been exposed to bile have a shrunken and dehydrated appearance, as observed by electron microscopy (de Valdez et al. 1997).

1.5.1.3 *Bacteria Tolerance of Bile*

There are a variety of elements that affect the ability of bacteria to tolerate bile such as external factors during food production and storage, and internal factors such as low pH, minimal oxygen levels and unsaturated fatty acids (Chowdhury, Sahu, and Das 1996). The continuously varying environments may allow bacteria to become either more tolerant or more sensitive. It is well documented that enteric bacteria are able to resist the deleterious effects of bile. A number of bacteria have been isolated from the gall bladder of both humans and animals including *E. coli, Salmonella, Helicobacter, and Campylobacter* (Fox et al. 1995; Flores et al. 2003; Prouty et al. 2004).

1.5.1.4 *Bile Sensing and Response Regulation*

The mechanisms used to sense changes in the concentration of bile are not well understood. The ability of an organism to tolerate bile requires, but is not limited to, the proteins that maintain cell envelope composition and structure, and those that preserve intracellular homeostasis by extruding bile. A microorganism may also utilize enzymatic action to modify or transform salts to a less harmful form (Begley, Gahan, and Hill 2005).

The main mechanism of bile resistance in gram-negative bacteria is thought to be facilitated by the expression of multidrug resistance (MDR) efflux pumps that actively expel bile out of the cell (Prouty et al. 2004). Pumps that respond to other signals such as magnesium and iron have also been implicated in the removal of bile (Begley, Gahan, and Hill 2005). Another pump, AcrAB is found in both *Salmonella typhimurium* and *E. coli* is essential in pumping out intracellular bile above critical concentrations (Prouty et al. 2004).

A transcriptome analsysis of EHEC revealed that after treatment with bile salts, several genes were significantly upregulated: those involved in an efflux pump system (*acrAB*), a two-component signal transduction system (*basRS)*, and genes involved in lipid A modification (*arnBCADTEF* and *ugd*) (Kus et al. 2011). These changes suggest that EHEC is able to 'sense' bile and responds with both protective and virulence strategies. In addition, the gene encoding an outer membrane porin channel, OmpF, is down regulated in the presence of bile salts (Kus et al. 2011). A decrease in channel expression may lead to a decrease in the amount of bile that can enter the cell, thereby allowing the microorganism to be more resistant.

1.5.1.5 *Bile Exposure and Pathogenic Response*

Pumbwe *et al.* demonstrated that bile salts conferred increased resistance in *Bacteroides fragilis* to antimicrobial agents and also increased host cell adhesion and biofilm formation (Pumbwe et al. 2007). Previous studies of EHEC and bile have shown that various bile salt treatments of glycocholate, deoxycholate, chenodeoxycholate, ursodeoxycholate, and a mix of bile salts (BSM) do not significantly enhance the release of Shiga toxins Stx1 and Stx2 and those located in the LEE pathogenicity island (Kus et al. 2011).

1.5.2 **Cationic Antimicrobial Peptides**

Cationic antimicrobial peptides (CAMPs) are peptides of 12-40 amino acids with a net positive charge ranging from +2 to +7 (Jenssen, Hamill, and Hancock 2006) that play an important role in the natural defense of an organism by exhibiting broad-spectrum activity against both gram-positive and gram-negative bacteria (De Smet and Contreras 2005).

Human-derived CAMPs support the human innate immune response by distinguishing pathogenic microbes from host mammalian cells (De Smet and Contreras 2005; Sørensen, Borregaard, and Cole 2008; Vance, Isberg, and Portnoy 2009; Fellermann and Stange 2001).

1.5.2.1 *Human CAMP Structure and Function*

There are three main types of human CAMPs known as defensins, cathelicidins and histatins. This project will focus on members of the defensin and cathelicidin class. Defensins are non-glycosylated peptides characterized by a triple-stranded β-sheet structure formed through three disulphide bridges comprised of six cysteine residues (Cunliffe 2003; De Smet and Contreras 2005). There are two classes of defensins known as the α-defensins and β-defensins which vary in primary structure but retain β -sheet assembly due to analogous disulphide bridges (Cunliffe 2003). Figure 1 below shows how the classes of CAMPs are distributed. Defensins are mainly found at the mucosal epithelial surface at a concentration range of 1-5 μM, where their primary function is to protect against microbial invasion as well as in neutrophils (HNP 1-4) that kill engulfed bacteria. This project will focus on the α-defensin, human defensin-5 (HD-5) and on the cathelicidin LL-37.

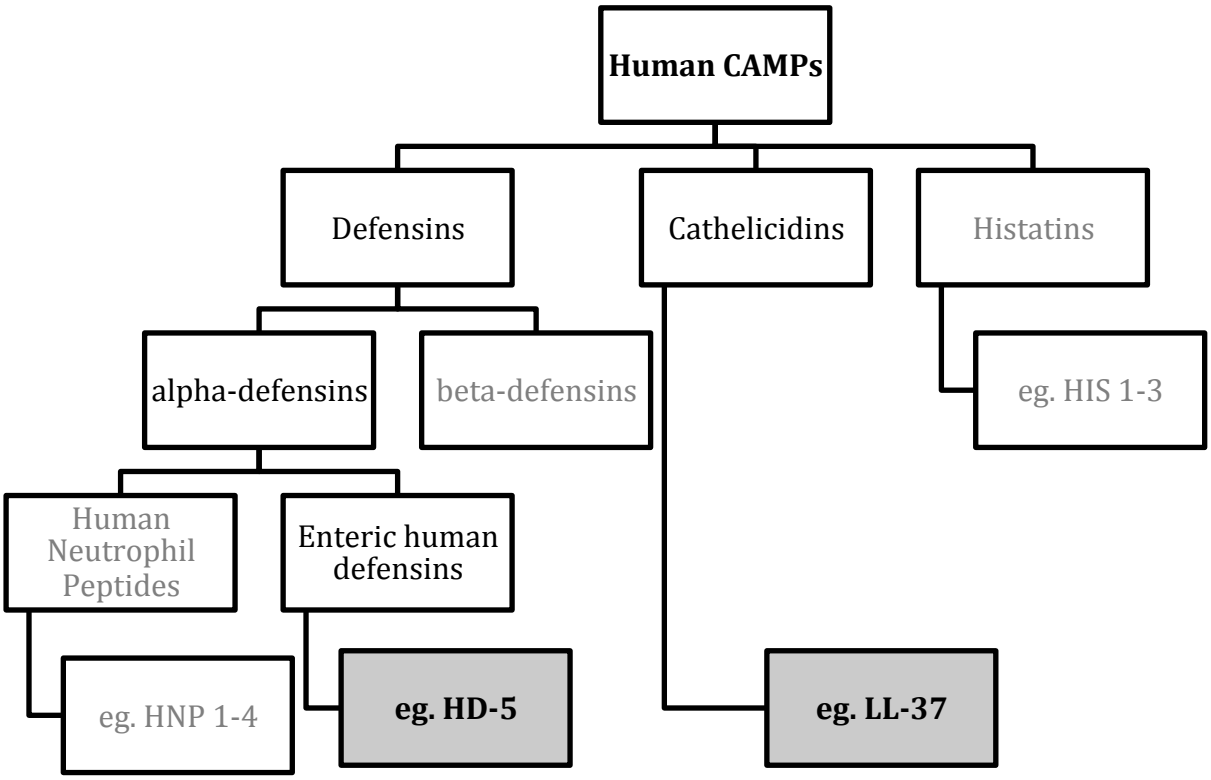


Figure 1 Classes of Human CAMPs

(Agerberth et al. 2000; Cunliffe 2003)

Cathelicidins are characterized by a tightly conserved cathelin domain located near the N-terminus. The cathelin domain is capped with a signal peptide on the N-terminus and an antimicrobial peptide on its C-terminus (Dürr, Sudheendra, and Ramamoorthy 2006; Bucki et al. 2010). The signal peptide ensures that the cathelicidin is transported to storage granules while the cathelin domain ensures that the cathelicidin remains in an inactive form until required (Dürr, Sudheendra, and Ramamoorthy 2006). The only human-derived cathelicidin is hCAP18, which generates LL-37 once the cathelin domain is cleaved (Gudmundsson et al.

1996). Figure 2 below shows a cartoon representation of hCAP-18 along with the sequence of LL-37 located on the C-terminus.

The secondary structure of LL-37 can be divided into three sections. The N-terminal region is relatively non-polar and hydrophobic while the C-terminal is hydrophilic with an LPS-binding domain (Burton and Steel 2009). This peptide is both constitutively expressed and induced in leukocytes such as neutrophils and monocytes, epithelial cells of the testis, skin, respiratory tract and lower gastrointestinal tract and has been shown to have potent antibacterial, antifungal, and antiviral activity (Cowland, Johnsen, and Borregaard 1995; Agerberth et al. 2000; Burton and Steel 2009).

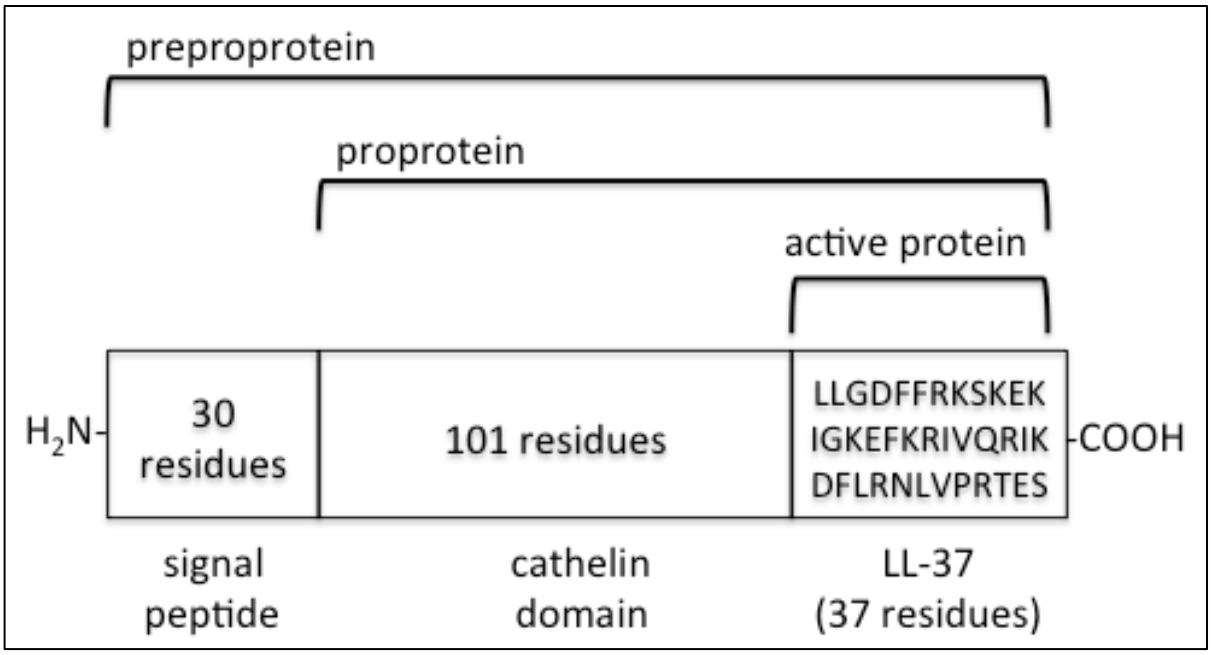


Figure 2 Cartoon Representation of Cathelicidin hCAP-18

(Bucki et al. 2010)

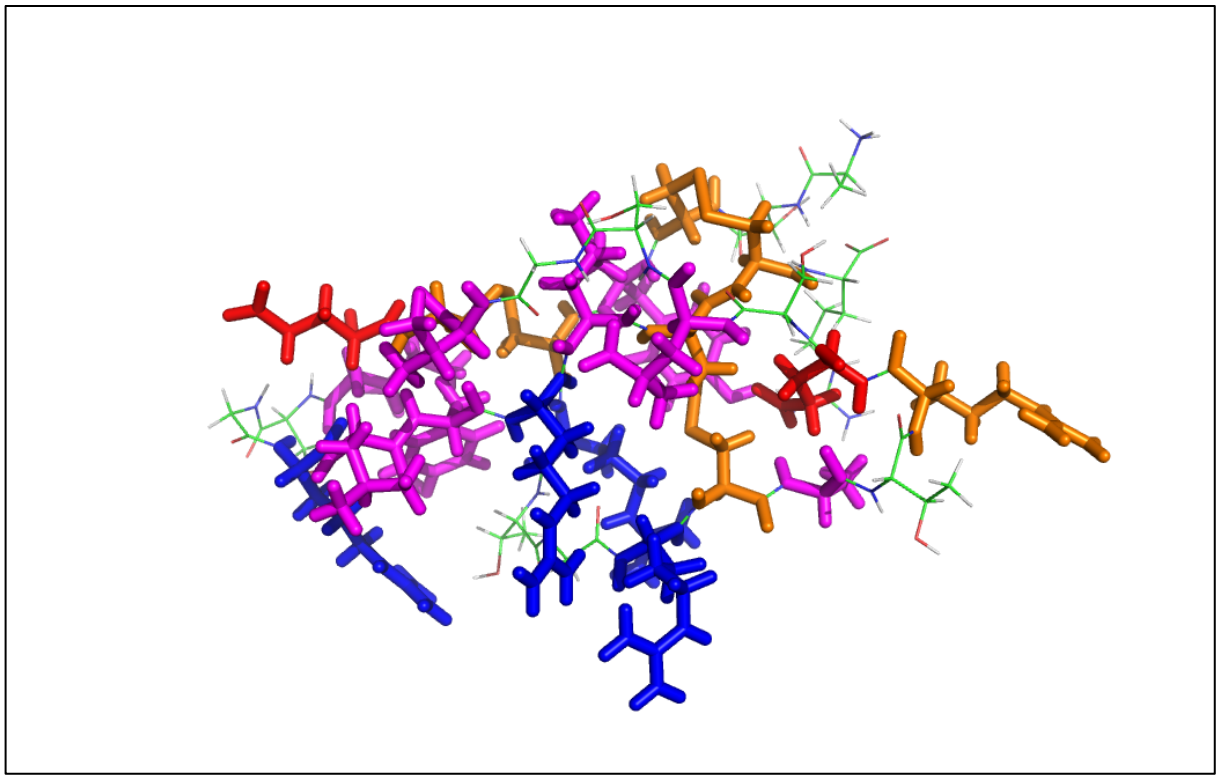


Figure 3 Structure of Human alpha-Defensin HD-5

Structure was generated from the pdb file 2LXZ in the Protein Database Bank (http://www.rcsb.org/pdb/home/home.do). Residues highlighted in blue are positively charged residues (lysine and arginine). Red residues highlighted are negatively charged residues (aspartic acid and glutamate). Residues highlighted in orange represent the six cysteine residues involved in the disulphide bridges. Image was kindly generated by Dr. Warren Wakarchuk (Ryerson).

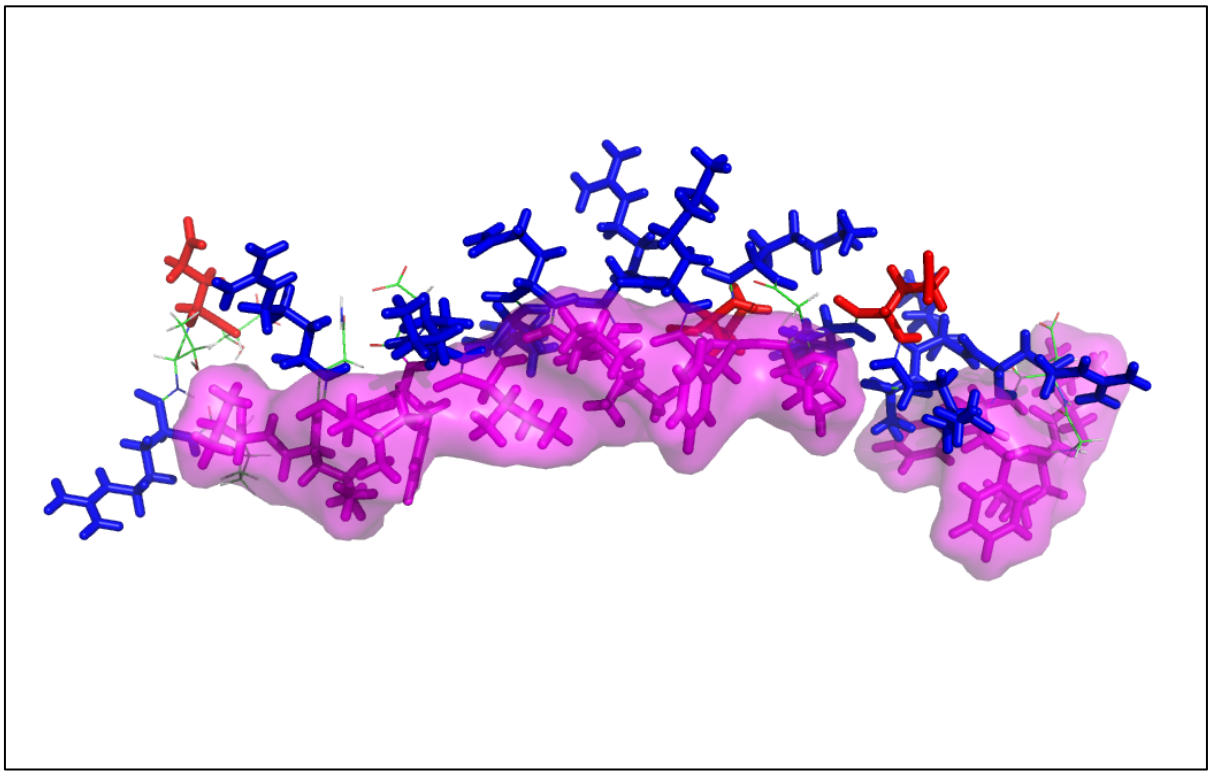


Figure 4 Structure of Human Cathelicidin, LL-37

Structure was generated from the pdb file 2K6O in the Protein Database Bank (http://www.rcsb.org/pdb/home/home.do). Residues highlighted in blue are positively charged residues (lysine and argine). Red residues highlighted are negatively charged residues (aspartic acid and glutamate). Purple residues within the space filling model are hydrophobic residues (phenylalanine, leucine, valine, and isoleucine). Image was kindly generated by Dr. Warren Wakarchuk (Ryerson).

1.5.2.2 *CAMP Molecular Mechanism of Action*

The outer surface of gram-negative bacteria is negatively charged due to the large number of LPS in contrast to neutrally charged mammalian cells. This means that cationic AMPs are primarily attracted to bacterial membranes instead of host cell membranes. The molecular mechanism of action against bacterial membranes for each class of CAMP has not been well studied. Several models that specifically address the mechanism of action of

defensins and cathelicidins agree that antibacterial activity is likely due to some type of pore formation through the membrane and/or membrane disruption (Pálffy et al. 2009).

In general, regardless of the specific mechanism, three main steps are outlined. First CAMPs must bind to the bacterial outer membrane, followed by some degree of non-covalent interaction of individual peptides, and potentially the formation of pores. Pore formation causes leakage of essential cell contents and eventually cell death (Burton and Steel 2009; Pálffy et al. 2009; De Smet and Contreras 2005; Dürr, Sudheendra, and Ramamoorthy 2006; Agerberth et al. 2000).

Three well-established models that support a pore-formation mechanism are: the barrel-stave, toroidal pore and carpet models (Figure 5). The barrel-stave pore model suggests that CAMPs first bind to the negatively-charged bacterial membrane and then form dimers or multimers. CAMP multimers then cross the cell membrane so that hydrophobic regions are in contact with the lipid bilayer while hydrophilic regions are oriented towards the lumen. Once assembled, the peptides form barrel-like channels Figure 5A. The toroidal pore model is similar to the barrel-stave pore model but also connects the outer and inner lipid leaflets in the center of the pore Figure 5B. The carpet model proposes that peptide monomers first coat the outer surface of the bacterial membrane. Once a threshold is achieved, the peptides exert detergent-like activity on the membrane and disrupt membrane integrity Figure 5C (Pálffy et al. 2009; Burton and Steel 2009).

It is generally accepted that the most likely CAMP mechanism of action involves some type of pore formation. However, some alternate mechanisms have been proposed that suggest that specific peptides can inhibit intracellular processes such as DNA and protein

synthesis by acting directly on the inside of the bacteria (Brogden 2005). In the case of *Streptococcus pyogenes*, non-lethal doses of LL-37 have been shown to induce extracellular capsule production producing bacteria that are more resistant to phagocytosis (Gryllos et al. 2008). Additional research to determine the precise mechanism of action of each human antimicrobial CAMP may provide new targets for antimicrobial therapy for infectious diseases.

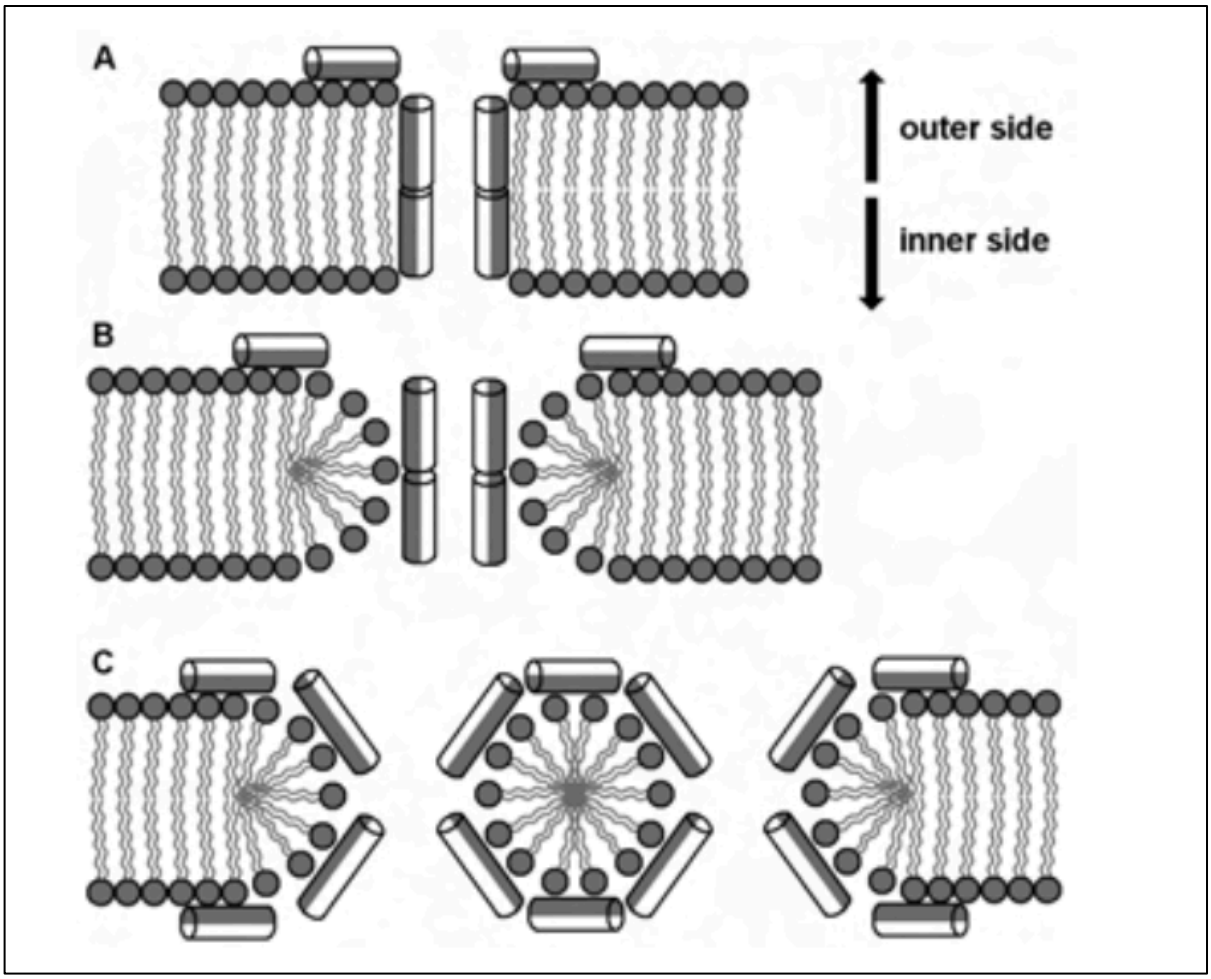


Figure 5 Proposed CAMP Molecular Mechanism of Action

A: Barrel-stave pore model; B: thoroidal pore model; C: carpet model. (Pálffy et al. 2009)

1.5.2.3 *Role of Human alpha-Defensin-5 in innate immune response*

The antimicrobial activity of α-defensins is well described in murine systems (Fellermann and Stange 2001; Cunliffe 2003; Ouellette 2006). The recombinant form of the human alpha defensin, HD-5 has been used for most research done on the peptide leaving the native peptide largely uncharacterized. Synthesized by Paneth cells of the ileum, HD-5 plays a significant role in defending the host against enteric pathogens such as *E. coli* O157:H7 (Cunliffe 2003). For example, studies with mice that were unable to produce mature defensins, reported that more time was required to clear *E. coli* KBC-236 infections and were more susceptible to *Salmonella* infections (Wilson 1999).

1.5.2.4 *Role of Human Cathelicidin LL-37 in Innate Immune Response*

LL-37 is also able to interact with mammalian cell membranes and stimulate a wide array of host cell receptors and transcription factors. In fact, LL-37 directly affects macrophage function as shown by the gene expression profile discussed below. Gene expression profile studies conducted by Scott et al. showed that LL-37 not only up-regulated the expression of chemokines in macrophages, human epithelial cells and whole human blood monocytes but also reduced production of proinflammatory cytokine TNF-α (Scott et al. 2002). Among others, chemokines IL-8 and MCP-1 were up-regulated after LL-37 stimulation which suggests that LL-37 directs leukocytes to the site of infection. LL-37 is known to bind bacterial products such as LPS and prevent their recognition by LPS-binding proteins. As a result, macrophages are not stimulated by bacterial products which prevents the development of sepsis in mild infections (Scott et al. 2002; Burton and Steel 2009; Bucki et al. 2010). LL-37 is thus aptly described as a multifunctional modulator of the host innate

immune response. Appendix D shows an info-graphic on the diverse activity profile of LL-37 and further cements its key role in the interplay of both immunomodulatory and microbial systems.

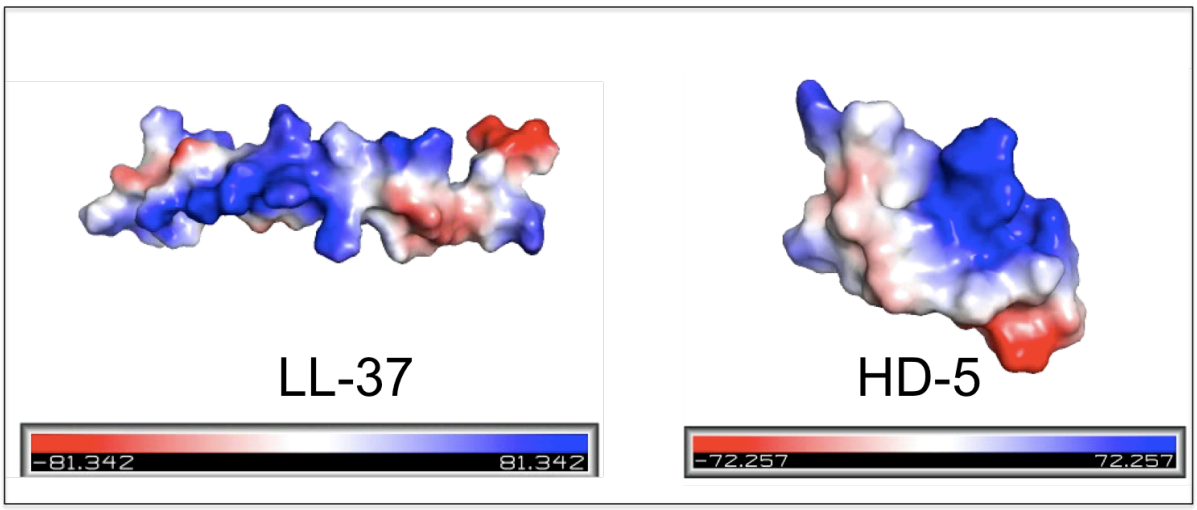


Figure 6 Electrostatic Potential Distribution of LL-37 and HD-5

Structure was generated from the Protein Database Bank (http://www.rcsb.org/pdb/home/home.do). Residues highlighted in blue are positively charged residues (lysine and argine). Red residues highlighted are negatively charged residues (aspartic acid and glutamate). Image was kindly generated by Dr. Warren Wakarchuk (Ryerson).

1.6 Pathogenic Response to CAMPs

Successful pathogens have evolved along with their host to develop a wide array of CAMP resistance mechanisms. Molecular microbiological studies have revealed many of these mechanisms and identified broad categories of resistance. Strategies involve the alteration of cell surfaces, activation of efflux pumps, proteolytic degradation of CAMPS, stimulation of bacterial regulatory networks, and even alteration of host processes (J. S. Gunn

et al. 2000; Moskowitz, Ernst, and Miller 2004; Li et al. 2008; Parkinson and Kofoid 2003). Appendix B summarizes common bacterial resistance mechanisms to human CAMPs.

Of interest, are a family of bacterial proteases known as omptins, located in the outer membrane of some Gram-negative bacteria (Stathopoulos 1998; Hritonenko and Stathopoulos 2007). This family of proteases includes, but is not limited to, OmpT and OmpP (*E. coli*), SopA (*Shigella flexneri*), Pla (*Yersinia pestis*), PgtE (*Salmonella enterica*) and have been reported to degrade a variety of different CAMPs found in their respective host niches (Hritonenko 2007; J.-L. Thomassin et al. 2012; Haiko et al. 2010; Hritonenko and Stathopoulos 2007). The OmpT protease in *E. coli* is the most characterized of the group and is known to be expressed in EHEC, and marginally in EPEC and UPEC (J.-L. Thomassin et al. 2012; Brannon et al. 2013). A recent study by Thomassin showed that *ompT* expression in EHEC was significantly higher than *ompT* expression in EPEC and is therefore able to efficiently degrade LL-37 much faster then OmpT in EPEC (J.-L. Thomassin et al. 2012). These researchers suggest that OmpT-mediated proteolysis of LL-37 is one of the main ways that EHEC develops resistance to LL-37 and that alternate mechanisms may be in play for other serotypes such as EPEC and UPEC (J.-L. Thomassin et al. 2012).

Another resistance mechanism explored in the present study is the production of extracellular capsules. This mechanism is used by EPEC to resist CAMPs such as HD-5 (J. L. Thomassin et al. 2013). These researchers suggest that the group four capsules may serve as a decoy for antimicrobial peptides based on an ionic attraction. The GFC operon is present in both EHEC and EPEC and is comprised of seven genes. The last gene in this operon, *etk,*

has been implicated in HD-5 resistance in EPEC (Lacour et al. 2006). The gene *etk* in EPEC is known as *yccC* in EHEC (Peleg et al. 2005).

1.6.1 **Two Component Regulatory Systems in EHEC**

One of the most basic bacterial signalling systems, the two-component system (TCS) consists of two protein modules: a sensor and a response regulator. The sensor is located in the cytoplasmic membrane of the cell while the response regulator facilitates changes in gene expression in response to signals received from the sensor (Nixon, Ronson, and Ausubel 1986). Figure 7 below shows the relationship between the sensor and the regulator. The two modules communicate via phosphorylation and dephosphorylation reactions. Transmitters attach phosphoryl groups from ATP to a histidine residue then they are transferred to an aspartate residue in the receiver (Parkinson and Kofoid 2003).

In EHEC, BasRS is a TCS whose genes were upregulated after BSM treatment (Kus et al. 2011). Kus established that the *basRS* promoter follows a concentration-dependent response to BSM treatment. Earlier work done on the regulation of *basR-basS* expression in *E. coli* or *Salmonella spp* showed that it was also linked to bile but has been associated with other stresses, including metal ion stress (Soncini and Groisman 1996; Chamnongpol et al. 2002; Hagiwara, Yamashino, and Mizuno 2004; L. J. Lee, Barrett, and Poole 2005), and mild acid stress (Soncini and Groisman 1996).

BasR (PmrA) and BasS (PmrB) control the expression of the *arnBCADTEF* (pmrHFIJKLM) operon (Raetz et al. 2007). Enzymes produced by these genes synthesize and transfer 4-amino-4-deoxy-L-arabinose (L-Ara4N), a cationic sugar, to lipid A (Trent et al. 2001). Figure 8 outlines the synthesis of L-Ara4N modified lipid A. Additional transcription units regulated by BasR is located in Appendix A.

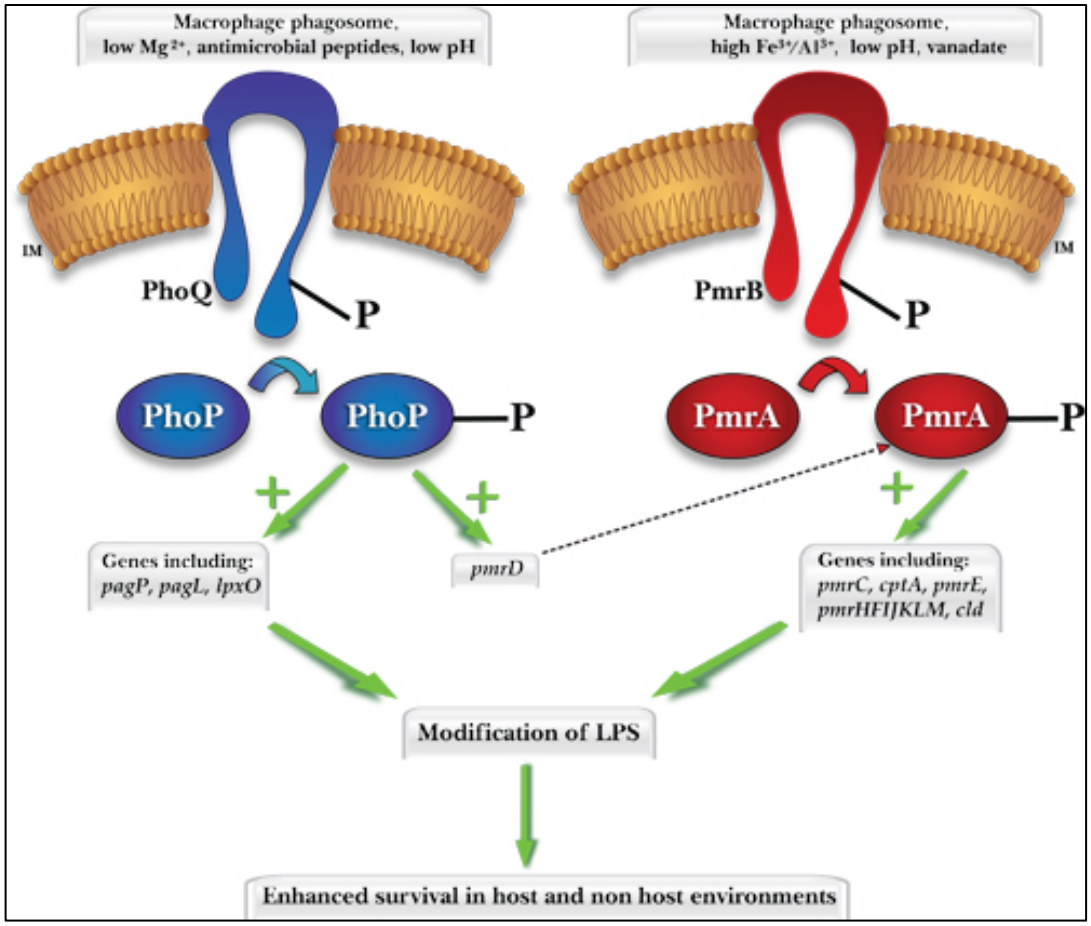


Figure 7 Two-component Signal Transduction Systems: PhoPQ and PmrAB (BasRS)

(John S. Gunn 2008)

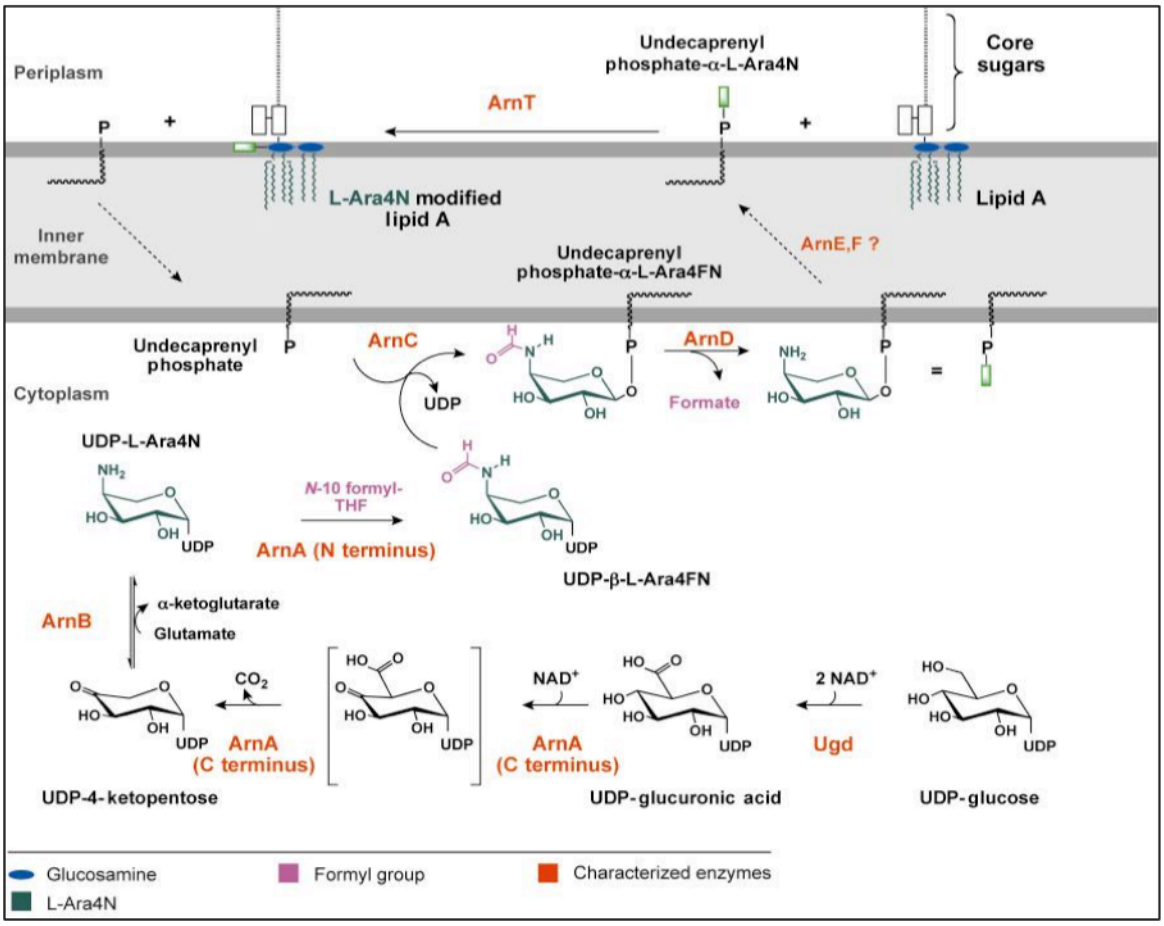


Figure 8 Biosynthesis of L-Ara4N Unit and Attachment to Lipid A

After transport to the outer surface of the inner membrane, the membrane protein, ArnT, transfers the L-Ara4N moiety (shown as a green rectangle) to lipid A. Reproduced from Raetz *et al.,* 2007 with permission from Russell Bishop.

1.6.2 **Consequences of Lipid A Modification**

Lipid A is the hydrophobic anchoring component of LPS, an endotoxin, found in the outer leaflet of the outer membrane of gram-negative bacteria (Raetz and Whitfield 2002; Nikaido 2003). Having a molecular weight of over one million Daltons (Da), LPS structures are fittingly named because they contain both a polysaccharide component and a lipid

component. LPS is unique in that it is soluble in water and insoluble in organic solvents. The structure of lipid A consists of a β,1′–6-linked disaccharide of glucosamine, modified with phosphate groups at the 1- and 4′-positions. Figure 9 below shows both the unmodified structure of lipid A in *E. coli* as well as the modified lipid A structure with aminoarabinose (L-Ara4N) shown in green (Pelletier et al. 2007; Pelletier et al. 2013).

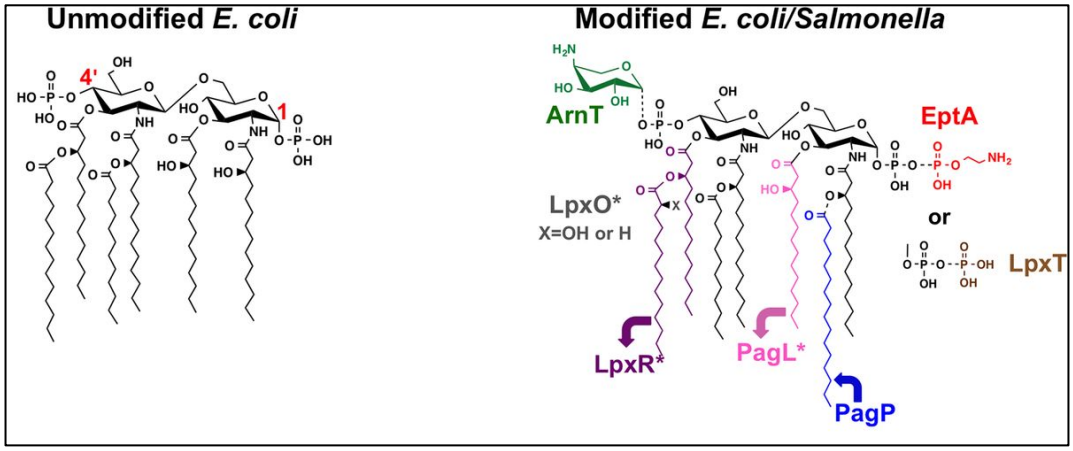


Figure 9 Structure of Unmodified and Modified *E. coli* Lipid A

(Rubin et al. 2015) Aminoarabinose shown in green is attached by enzyme ArnT as the last step in Figure 8 Biosynthesis of L-Ara4N Unit and Attachment to Lipid A. Alternate modifications can be made by numerous enzymes and under various growth conditions. Modifications include addition of phosphoethanolamine by EptA (red), addition of a phosphate group at the 1-phosphate by LpxT (brown), removal of the 3'-linked acyl chains by LpxR (purple), hydroxylation of the 3'-secondary acyl chain by LpxO (gray), removal of the 3-linked acyl chain by PagL (pink), and addition of a secondary palmitate chain at the 2-linked primary acyl chain by PagP (blue).

Highly conserved among gram-negative bacteria, lipid A plays an important role in the pathogenesis of gram-negative bacterial infections by activating toll-like receptors (TLRs) that recognize microbial pathogens and activate immune cell responses which include the

production of cationic antimicrobial peptides (CAMPs) among other molecules (Yan, Guan, and Raetz 2007; Round et al. 2011).

There have been studies that show that modification of lipid A with L-Ara4N in *Pseudomonas* and *Salmonella* increases resistance to common CAMPs and PMB (Raetz et al. 2007; Moskowitz, Ernst, and Miller 2004; John S. Gunn 2008) There have also been studies in *E. coli* illustrating a similar phenomenon (Kim et al. 2006; Yan, Guan, and Raetz 2007). However there has only been one study of EHEC developing CAMP resistance via lipid A modifications through bile salt stress (Kus et al. 2011). These authors examined resistance to the peptide PMB as it is the compound often used to test antimicrobial peptide resistance (J. S. Gunn et al. 2000; Kim et al. 2006; Herrera, Hankins, and Trent 2010; Nishino et al. 2006). However, since PMB is not a native human or bovine antibiotic, EHEC would not encounter this particular peptide on its journey through the gastrointestinal tract. Derived from *Bacillus polymyxa,* PMB is sometimes prescribed as an antibiotic for gram-negative infections including sepsis, meningitis and pneumonia (Cavaillon 2011).

The EHEC operon, *arnBCADTEF*, encodes a set of enzymes that are responsible for modifying lipid A with L-Ara4N (Kus et al. 2011; Raetz et al. 2007). Figure 8 shows the synthesis of L-Ara4N modified lipid A by the Arn enzymes. L-Ara4N is positively charged at physiologically relevant pH 7. Modification with L-Ara4N decreases the overall negative charge of the lipid A phosphate group and as a consequence, the overall charge of the bacterial membrane (Yan, Guan, and Raetz 2007).

1.7 **Role of Lipid A Modification in Innate Immune Response**

Lipid A, an essential core component of LPS that activates TLRs, is a predominant molecule on the bacterial surface that is difficult to conceal from the host (Finlay and McFadden 2006). Many gram-negative bacteria modify lipid A structures to make them less visible to host antimicrobials. As an example, *Salmonella*'s TCS (PhoP/PhoQ) regulates virulence genes that are involved in lipid A acylation (*pagP*) and lipid A palmitoylation (*pagL*). These modifications of lipid A result in a 30-100-fold decrease in activation of TLRs compared with unmodified lipid A (Kawasaki, Ernst, and Miller 2004).

The effect of L-Ara4N addition to the head group of LPS on TLR4 signalling has not yet been established. Hajjar et al. evaluated whether L-Ara4N affected TLR4 signalling by removing the sugar moiety from lipid A in *Pseudomonas aeruginosa* and observed no significant change in TLR4 activity (Hajjar et al. 2002). However, others have shown that L-Ara4N membrane modification inhibits PagL-mediated lipid A deacylation (Kawasaki, Ernst, and Miller 2005). To recap, L-Ara4N modification of lipid A have been shown to increase resistance to CAMPS whereas PagL-mediated lipid A deacylation reduces recognition by TLR4 thus lowering the possibility of a host immune response to the pathogen (Kawasaki, Ernst, and Miller 2004). Both mechanisms, which are under the control of TCSs, offer a competitive advantage to the pathogen to survive in host tissues. Authors suggest that there may be growth conditions in which PmrAB (regulates L-Ara4N) is repressed while PhoPQ (regulates PagL) is activated. Rapid and unique responses to various environmental conditions are key to bacterial survival in host tissues and highlight the importance of research into environmental cues that trigger bacterial pathogenesis. This study aims to

contribute to this area of research by exploring bile salts as an environmental cue that triggers L-Ara4N modification of lipid A.

2 Purpose of Investigation

To investigate the impact of bile salt treatment on EHEC resistance to human antimicrobial peptides.

2.1 **Research Rationale**

In order to colonize the large intestine, EHEC must successfully pass through the small intestine, where it is exposed to the detergent-like action of bile salts and membrane-damaging cationic antimicrobial peptides. Previously, we reported that bile salt treatment of EHEC upregulates genes encoding a two-component signal transduction system (*basRS*) and a lipid A modification pathway (*arnBCADTEF*). Bile salt treatment also enhances resistance to the cationic antimicrobial protein, polymyxin B, in an *arnT* and *basRS* dependent manner. The current study examines the effect of bile salt treatment on EHEC resistance to two *human* cationic antimicrobials, human defensin HD-5 and cathelicidin LL-37.

2.2 **Hypothesis**

Bile salt treatment enhances EHEC resistance to human cationic antimicrobial peptides.

2.3 **Main Objectives**

I. To investigate the effect of BS treatment on EHEC resistance to human CAMPs

   a. To evaluate the effect of BS treatment on EHEC resistance to human defensin, HD-5 and cathelicidin, LL-37

   b. To assess the roles of *basRS* and *arnT* in BS-induced resistance to HD-5 and LL-37

II. To evaluate lipid A modification after BS treatment by matrix-assisted laser desorption ionization- mass spectrometry

III. To evaluate the role of group four capsule in BS-induced resistance to CAMPs

3 Materials and Methods

3.1 **Bacterial Cultivation**

Briefly, the enterohemorrhagic *E. coli* strains analyzed in this study were: 86-24 (wt), 86-24 Δ*basS*, 86-24 Δ*basR*, 86-24 Δ*arnT*, 86-24 Δ*arnT*:pBADGr::*arnT,* EDL 933*,* EDL 933 Δ*ompT* and EDL 933 Δ*ompT* (pEH*ompT*). Bacteria were maintained as glycerol stocks (stored at -80 ˚C) and were routinely streaked onto Luria-Bertani (LB) agar plates (tryptone, 1% (w/v); yeast, 0.5% (w/v); sodium chloride, 0.5% (w/v); agar 1.5% (w/v)) with or without appropriate antibiotics and grown in a 37 ˚C static incubator for 16 to 18 hours. Isolated colonies were restreaked onto new LB plates with or without appropriate antibiotics and grown overnight. Mutants were checked to ensure they retained resistance to the appropriate antibiotics. Strains were stored at 4 ˚C and subcultured every three weeks (Riordan, *et al.*, 2000). Isolated colonies from the LB plates were grown overnight under standard conditions (shaking incubator at 37 ˚C for 16 to 18 hours at 200 rpm). Overnight cultures were diluted into fresh media. Subcultures were then grown statically at 37 ˚C with 5% CO_2 for three to four hours until mid-logarithmic phase was achieved. These growth conditions represent a microaerobic (decreased oxygen levels), which are physiologically relevant to infection sites of EHEC along the gastrointestinal tract (Gaines et al. 2005).

Strains 86-24 Δ*basS*, 86-24 Δ*basR*, and 86-24 Δ*arnT* were grown in the presence of 50 μg/mL kanamycin (Kan_{50}) (Sigma). Strain 86-24Δ*arnT*(pBADGr::*arnT*) was grown with both Gen_{20} and Kan_{50}. Strain EDL 933 Δ*ompT* (pEH*ompT*) was grown in the presence of 30 μg/mL chloramphenicol (Cm_{30}).

3.1.1 **Bile Salt and Peptide Suspensions and Concentrations**

Bile salt mixture (BSM) (Sigma-Aldrich B-3426) was used as a treatment in all assays at a final concentration of 1.5% (w/v). Human Defensin HD-5 was obtained from Dr. Charles Bevins, University of California, Davis and resuspended in 0.01% acetic acid. LL-37 was obtained from Anaspec Peptide and resuspended in water. Stock concentrations of peptides were prepared, aliquoted and stored at -20 °C.

3.1.2 **Modified Growth Conditions with N-minimal media**

Where identified, isolated colonies were inoculated into N-minimal media adjusted to pH 7.4 and supplemented with 0.2% glucose and 1mM $MgCl_2$. Strains were grown under standard growth conditions (shaking incubator at 37 ˚C) for 16 to 18 hours at 200 rpm. Overnight cultures were diluted into fresh media. Subcultures were then grown statically at 37 ˚C with or without 5% CO_2 for three to four hours until mid-logarithmic phase was achieved.

3.2 **Bacterial Strains Used in This Study**

The strains of bacteria used in this study are listed in Table 2.

Table 2 Strains used in this study

E. coli strains		
Strains	**Description**	**Source or reference**
86-24	Wild type EHEC O157:H7 strain 86-24	Dr. Jorge Giron, University of Florida
86-24 ΔbasS	86-24 with KanR disruption of basS gene; Kan^R_{50}	This study
86-24 ΔbasR	86-24 with KanR disruption of basR gene; Kan^R_{50}	This study
86-24 ΔarnT	86-24 with KanR disruption of arnT gene; Kan^R_{50}	This study
86-24 ΔarnT:pBADGr::arnT	86-24 transformed with vector pBADGr containing arnT gene; Kan^R_{50}, Gen^R_{20}	This study
EDL 933	Wild type EHEC O157:H7 strain EDL 933	Dr. Jorge Giron, University of Florida
EDL 933 ΔompT	EDL 933 ΔompT mutant created by sacB gene-based allelic exchange	Hervé Le Moual, McGill University
EDL 933 ΔompT (pEHompT)	EDL 933 ΔompT mutant expressing ompT from pEHompT; Cm^R_{30}	Hervé Le Moual, McGill University
Citrobacter rodentium	Negative capsule control	Ryerson Chemistry and Biology Dept.
Klebsiella pneumonia	Positive capsule control	Ryerson Chemistry and Biology Dept.

Amp^R_{100}: ampicillin resistant at 100 μg/mL; Kan^R_{50}: kanamycin resistant at 50 μg/mL; Gen^R_{20}: gentamycin resistant at 20 μg/mL

3.3 Lipid A Extraction and Preparation for MALDI-TOF

Phenol-prepped samples of laboratory strains under various growth conditions were made and sent to Dr. Robert Ernst at Department of Microbial Pathogenesis, University of Maryland School of Dentistry, Baltimore, Maryland, USA for matrix assisted laser desorption/ ionization- mass spectrometry (MALDI-TOF) analysis. Briefly, isolated colonies of each strain were inoculated into LB with or without 1.5% BSM and grown under standard growth conditions. Subcultures were grown in fresh media with CO_2. Where identified, media was spiked with 1mM $MgCl_2$. All other growth conditions remained the same. A treatment of 25mM metavanadate (NH_4VO_3) was used as a positive control to induce L-Ara4N moieties on Lipid A (Zhou et al. 1999).

Bacteria in mid-logarithmic phase was centrifuged, supernatant was removed then resuspended in 10 mM phosphate buffered saline (PBS) at pH 7.4. Phenol at a final concentration of 1% was added to each sample. Samples were then incubated at 37 °C for two hours while shaking at 250 rpm. Cells were harvested by centrifugation.

3.3.1 LPS Preparation and Lipid A Purification

LPS was extracted using a hot phenol-water method. Freeze-dried bacteria were resuspended in endotoxin-free water at a concentration of 10 mg/ml. A12.5-ml volume of 90% phenol was added, and the resultant mixture was vortexed and incubated in a hybridization oven at 65°C. The mixture was cooled on ice and centrifuged at 10,000 rpm at room temperature for 30 min. The aqueous phase was collected, and an equal volume of endotoxin-free water was added to the organic phase. The resultant pellet was resuspended at

a concentration of 10 mg/ml in endotoxin-free water, treated with DNase (Sigma, St. Louis,MO) at 100 μg/ml and RNase A (Sigma) at 25 μg/ml, and incubated at 37°C for one hour in a water bath. Proteinase K (Sigma) was added to a final concentration of 100 μg/ml and incubated for one hour in a 37°C water bath. The solution was then extracted with an equal volume of water-saturated phenol. The aqueous phase was collected and dialyzed against Milli-Q purified water and freeze-dried as above. The LPS was further purified by the addition of chloroform-methanol (2:1, vol/vol) to remove membrane phospholipids and further purified by an additional water-saturated phenol extraction and75% ethanol precipitation to remove contaminating lipoproteins. Lipid A was isolated using mild acid hydrolysis.

3.3.2 **MALDI-TOF Analysis**

Lipid A samples were purified as previously described (Kawasaki, Ernst, and Miller 2005). Samples were dissolved in 20 mg of 5-chloro-2-mercaptobenzothiazole matrices/ml in chloroform-methanol (1:1 [vol/vol]). The mixtures were allowed to dry at room temperature on the sample plate prior to mass spectrometry analysis. Spectra were obtained in the negative reflection mode by use of a matrix-assisted laser desorption ionization–time of flight (MALDI-TOF).

3.4 **Capsule Visualization**

Isolated colonies of each strain were inoculated into LB with or without 1.5% BSM and grown under standard growth conditions. A 20 μL bacterial sample was combined with 5 μL of 10% nigrosin. The suspension was then spread on a clean glass slide and allowed to air dry

at a 45 ° angle. Once dry 85% ethanol was added to the slide to fix the suspension for 2 minutes. Excess ethanol was removed from the slide and allowed to air dry. Crystal violet (2% crystal violet, 20% v/v ethanol, 0.2% ammonium oxalate) was added to the slide and allowed to stain for no more than two minutes. Slides were then washed with a gentle stream of sterile water. Slides were dried overnight and then visualized under oil immersion at a 1000x magnification.

A positive capsule formation is identified as the exclusion of the negative stain nigrosin and the incorporation of the positive stain crystal violet (J. L. Thomassin et al. 2013).

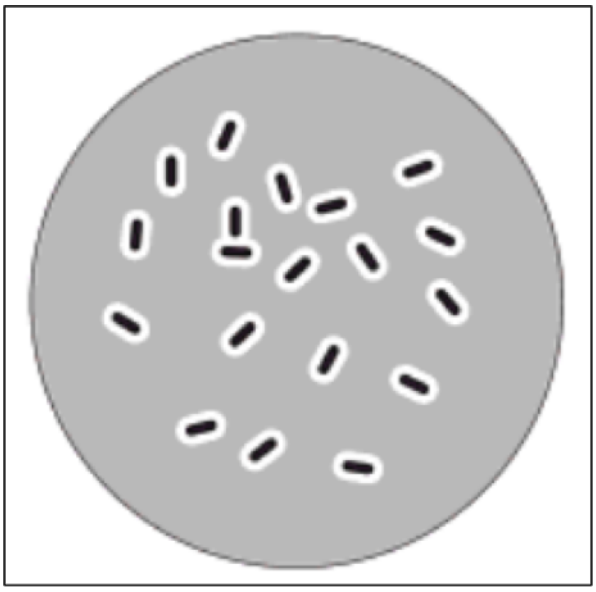


Figure 10 Schematic Diagram of Capsule Formation

A positive capsule formation is identified as the exclusion of the negative stain nigrosin and the incorporation of the positive stain crystal violet (J. L. Thomassin et al. 2013).

3.5 **Radial Diffusion Assay**

A modified version of a radial diffusion assay described by Lehrer was used to determine the effect of BSM on the survival of 86-24 when challenged with peptide (Lehrer et al. 1991). Isolated colonies of each strain were inoculated into LB with or without 1.5% BSM and grown under standard growth conditions. Overnight cultures were diluted into fresh media with or without BSM.

A 1/10 dilution of subcultures of overnight samples was prepared in LB in 150 mL Erlenmeyer Flasks with or without 1.5% BSM. Flasks were incubated at 37 ˚C, 5% CO2 under static conditions for 4 hours to allow cells to get to mid-exponential phase. Subcultures were transferred from Erlenmeyer flasks into precooled 50 mL Falcon tubes. After this step, bacterial cells, buffers and tubes were kept chilled on ice. Cells were spun at 900 xg for 10 minutes then washed with 50mL of 10mM sodium phosphate buffer (NAPB) pH 7.4. Cells were resuspended in 10mL NAPB. The OD600 of each sample was measured and approximately 4 x 10^6 CFU of each sample was inoculated into 10 mL of warm (47-50 ˚C) low nutrient agarose (components listed below in Table 3). After rapidly dispersing the bacteria with a vortex, seeded agarose was quickly poured into sterile round 100 x 25 mm petri dishes (VWR).

Table 3 Radial Diffusion Assay: Lower Agarose Components

10mM	NAPB pH 7.4
0.03% (w/v)	Tryptic Soy Broth (TSB) (Sigma)
1% (w/v)	Agarose Type I, low electroendosmosis (EEO) (Sigma-Aldrich A-6013)
0.02% (v/v)	Tween 20 (Sigma)

A 1000 µL pipette tip was used to make 2 mm wells in the hardened low nutrient agarose layer. A 3 µL volume of peptide was placed into individual wells and allowed to absorb into the agarose layer. Duplicate wells were made for each concentration of peptide. Plates were incubated at 37 ˚C for three hours. The lower agarose layer of each plate was then covered with 10 mL of (47-50 ˚C) sterile overlay agar that was kept liquid in a water bath. Table 4 lists the components of the overlay agar. The overlay was allowed to harden and then plates were inverted and incubated at 37 ˚C for no more than 16 hours. The zone of clearing around each well was measured.

Table 4 Radial Diffusion Assay Agarose: Overlay Components

6% (w/v)	Tryptic Soy Broth (TSB) (Sigma)
1% (w/v)	Agarose (Sigma Cat. No. A9539)

3.6 **Antimicrobial Peptide Susceptibility Assay**

A modified broth microdilution protocol as described by the REW Hancock Laboratory (http://cmdr.ubc.ca/bobh/methods/MODIFIEDMIC.html) was used to determine the effect of BSM on the survival of 86-24 when challenged with peptide LL-37. Briefly, isolated colonies of each strain were inoculated into LB or N-minimal media with or without 1.5% BSM and grown under standard growth conditions. Overnight cultures were diluted into fresh media with or without BSM.

A 1/10 dilution of subcultures of overnight samples was prepared in LB in 150 mL Erlenmeyer flasks with or without 1.5% BSM. Flasks were incubated at 37 ˚C, with or

without 5% CO_2 under static conditions for three to four hours to allow cells to get to mid-exponential phase.

During the bacterial subculture incubation step, 2x stocks of LL-37 were two-fold serially diluted (64 μg/mL to 2 μg/mL) in water. Fifty μL of each of these stocks was added to wells in 96 well polystyrene microtiter plates. Microtiter plates were kept on ice until bacteria was added.

Bacterial cells were spun at 900 xg for 10 minutes then washed with 50mL of PBS or 10 mM HEPES buffer at pH 7.4. Cells were resuspended in Mueller Hinton broth (Sigma) or HEPES. The OD 600 of each sample was measured and approximately 1 x 10^6 CFU (2X final concentration) was added to each well already containing LL-37. After 1 hour of growth at 37 °C, aliquots from each well were ten-fold serially diluted in fresh PBS. Ten μL of each dilution was plated on LB plates. Survival was assessed after 1 hour by plate count.

3.7 **Minimum Inhibitory Concentration Assay**

A modified broth microdilution protocol as described by the REW Hancock Laboratory (http://cmdr.ubc.ca/bobh/methods/MODIFIEDMIC.html) was used to determine the effect of BSM on the survival of 86-24 when challenged with peptide LL-37. The microtiter plates used in the antimicrobial peptide susceptibility (Section 3.6) were incubated for an additional 16 – 18 hours at 37 °C. Wells were then visually inspected for inhibition of growth. The lowest concentration of peptide which completely inhibited growth, (lack of visual turbidity) was determined to be the minimum inhibitory concentration (MIC) (Andrews 2001). MIC values of each sample/treatment were then compared.

3.8 **Statistical Analysis**

Results are presented as means ± standard error of mean. To test statistical significance among the groups, a one-way ANOVA was used followed by post-hoc comparisons with Tukey's method.

4 Results

4.1 BSM Treatment Limits but Does Not Terminate EHEC Growth

In the present study, EHEC O157:H7 cells in mid-logarithmic phase were quantified by measuring optical densities and direct enumeration on LB plates. Cells treated with BSM were found to grow to limited optical densities compared with untreated cells (Figure 11). The viability of 1.5% BSM treated cells decreased 6-fold relative to that of untreated cells and were observed to have 1.6-fold longer lag phase than cells that were treated with 0.15% BSM and cells that were grown in LB alone (Figure 12). These results are in concurrence with Pumbwe *et al.*, 2007.

Viability assays on all of the other strains tested (86-24 Δ*basS*, 86-24 Δ*arnT*, 86-24 Δ*arnT*:pBADGr::*arnT*, and 86-24 pBADGr) also showed a significant decrease in the 1.5% BSM treated cell densities however the wild type was the most dramatic and significant. From these data, it is evident that the same optical density for different strains does not equate to the same number of viable cells in solution. The most important information achieved from the viability assays are the mathematical equations used to quantify number of viable cells from OD measurements. These equations are used to standardize the number of cells inoculated for the radial diffusion assays.

These data suggest that BSM present in the small intestine, while it does decrease the total number of viable cells, still allows a significant number of BSM treated cells to grow into mid-logarithmic phase and continue their journey to the large intestines.

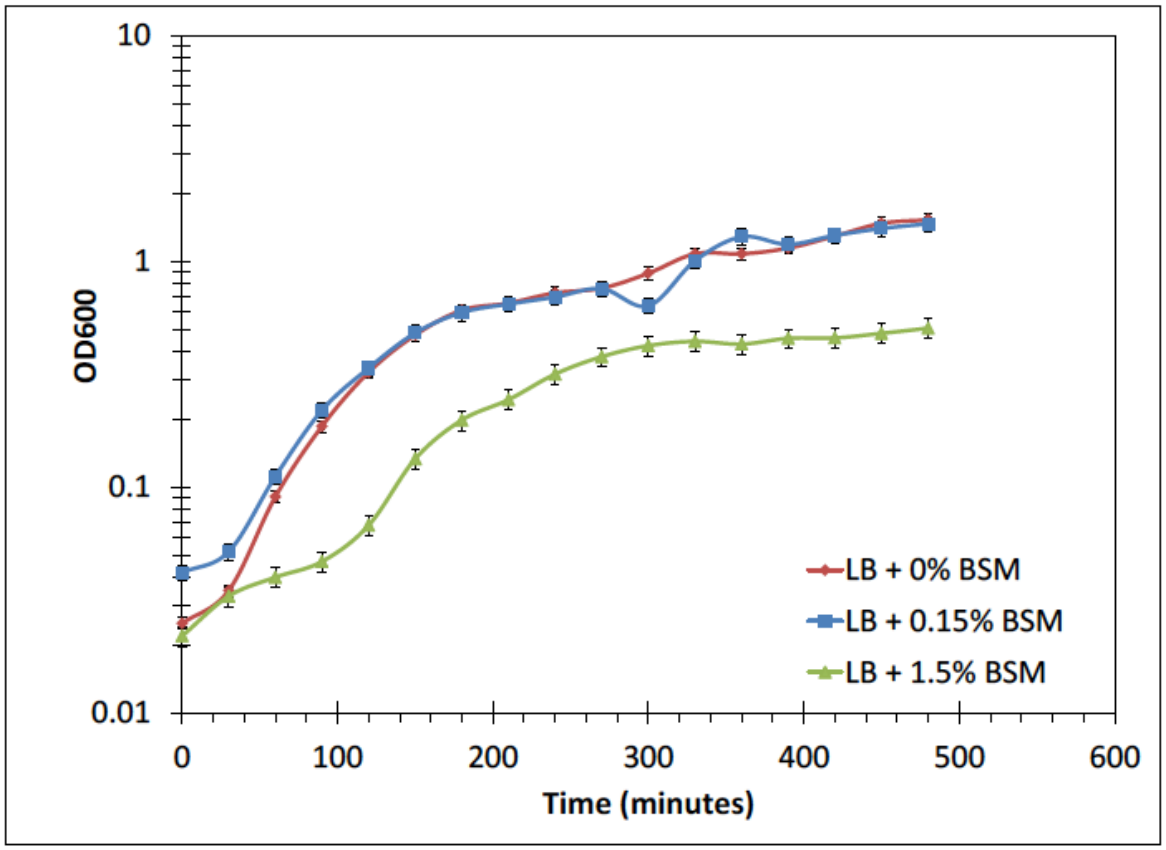


Figure 11 BSM Treatment Limits Wild Type *E. coli* Optical Density

Effect of BSM exposure on EHEC O157:H7 86-24 wt optical density was assessed by conducting a growth curve over an 8 hour period. All treatments were carried out at 37 ˚C, 5% CO_2, static conditions. OD was measured at a single wavelength of 600 nm. Each treatment is indicated by a single shaded line: (LB only (red diamonds), LB + 0.15% BSM (blue squares), LB + 1.5% BSM (green triangles). The data for each treatment was collected from a single biological sample with three replicates. Values represent mean ± _standard deviation. This data is representative of at least two other experiments.

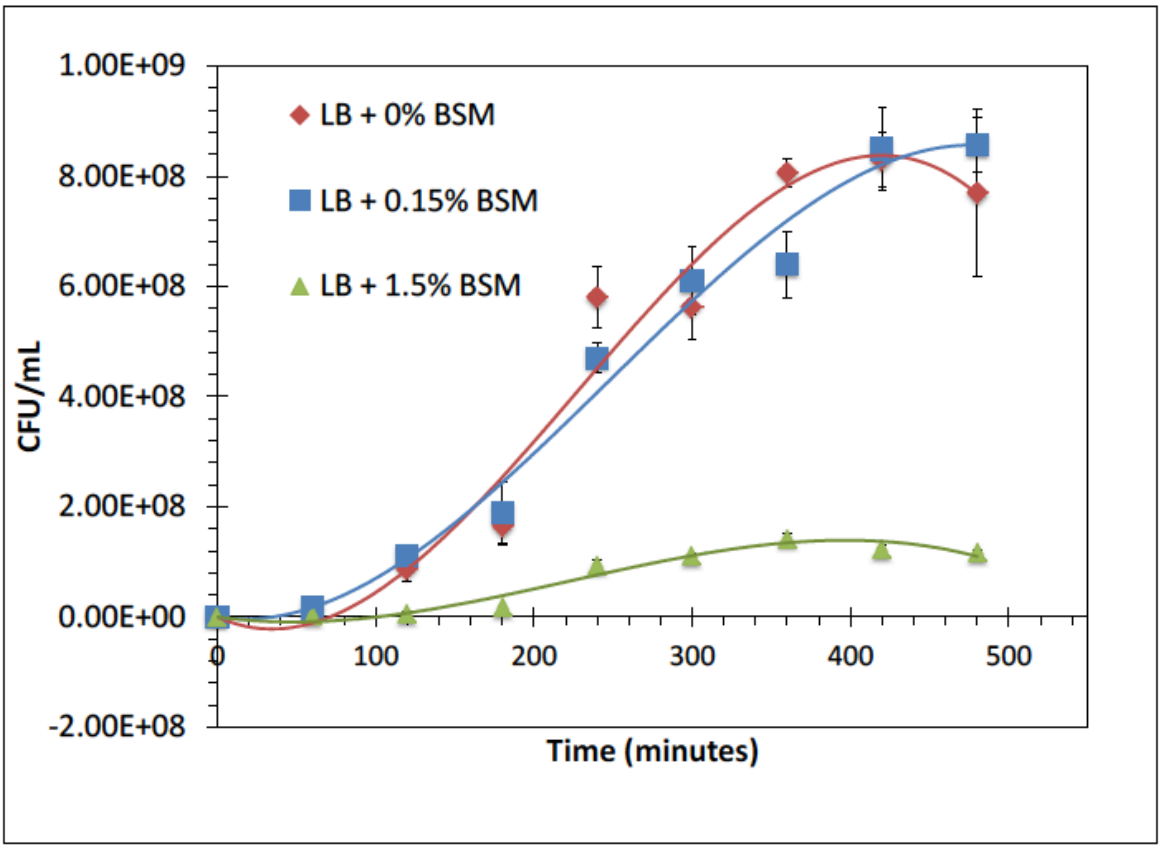


Figure 12 BSM Treatment Limits Overall Wild Type *E. coli* Cell Viability

EHEC 86-24 viability assay was conducted over a period of 8 hours in LB and treated with 0.15% BSM or 1.5% BSM. Serial dilutions at each 60 minute time intervals were made in PBS. Triplicates of 10 µl each (from one biological sample) were plated on agar plates then incubated at room temperature for a maximum of 14 hours. Values represent mean ± standard deviation.

4.2 BS-treatment Enhances Resistance of Wild Type EHEC to HD-5

BSM has been shown to induce LPS modifications in EHEC 86-24 that alter the charge of the bacterial membrane (Kus et al. 2011). This change in charge is hypothesized to be one of the reasons why EHEC 86-24 is able to gain resistance to HD-5 at physiological concentrations found in the small intestines. To assess the ability of BSM to induce this resistance, a radial diffusion assay was used with cells treated with 1.5% BSM and a control.

Figure 13 below the size of 86-24 wt clear zones around 2 mm wells of HD-5 when treated and untreated with BSM.

A smaller clear zone indicates an increased resistance to the peptide. In the presence of 0.056 μg/μL HD-5, the clear zones of BSM treated cells were approximately 16% smaller than those that were not treated with BSM but this difference was not statistically significant. When 0.11 μg/μL HD-5 was used, there was a 56 ± 20% decrease in the size of the clear zones of treated cells compared to untreated cells. A 0.33 μg/μL concentration showed a more modest yet significant decrease after BSM treatment. This data shows a dose dependent response to HD-5 concentration and suggests that BSM is able to induce resistance to a physiological range of concentrations of HD-5. These results suggest that *E. coli* O157:H7 uses bile in the small intestines as an environmental signal to prepare for evasion of local defensins also present in the environment.

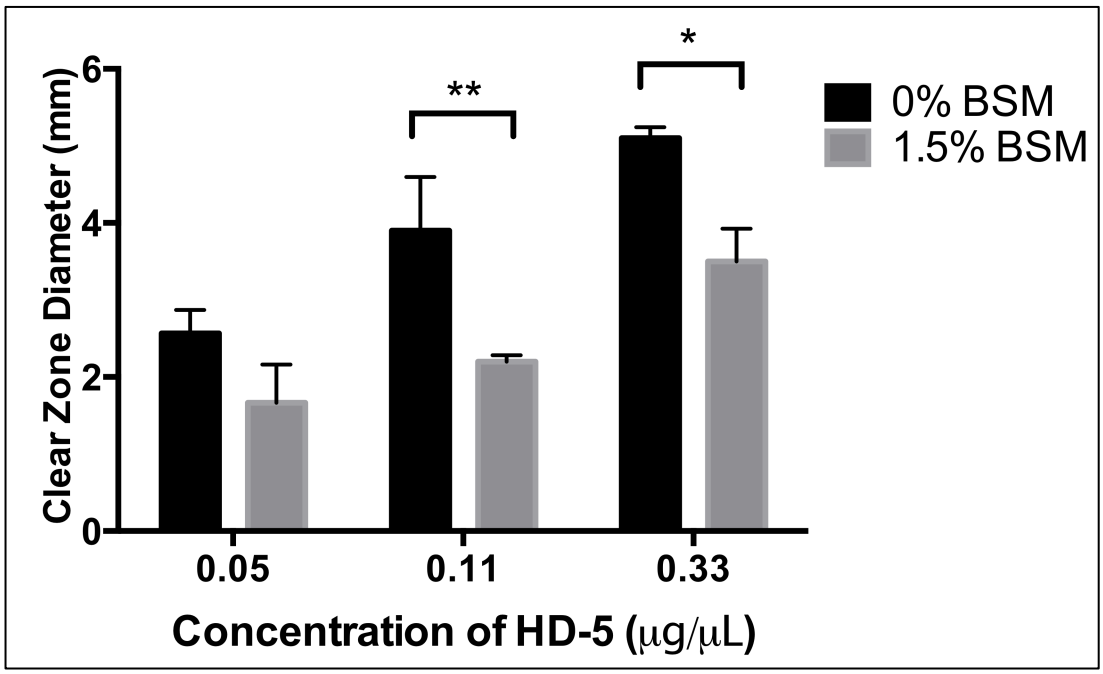


Figure 13 BS Treated Wild Type *E. coli* Shows Dose Dependent Increase in HD5 Resistance

Briefly, cells were grown in LB and subcultured in the same media and grown with 5% CO_2. Clear zones show extent of HD-5 killing of EHEC 86-24 at varying concentrations of HD-5 (0.05, 0.11, 0.33 μg/μL). Dark gray bars represent clear zones of cells that were not treated with BSM and light gray bars represent clear zones of cells that were treated with 1.5% BSM. Error bars represent mean ± standard error of mean. Statistical analysis conducted using Tukey's multiple comparisons test (n=4) ** $p < 0.005$. This graph is representative of at least three independent experiments.

4.2.1 **BasRS plays a role in BSM-induced EHEC resistance to HD-5**

Based on previous studies (Kus et al. 2011), BasS, the sensory component of the TCS BasRS, is expected to play a role in the ability of EHEC to sense bile. If BasS plays a role in bile-induced resistance to human defensins, then a *basS* deficient mutant should show no increase in resistance to HD-5 after bile treatment. Indeed, no significant increase in resistance of the Δ*basS* mutant strain to HD-5 after bile treatment as indicated by the radial diffusion assay shown in Figure 14. This evidence supports that BasS is acting as a sensor for bile salts and suggests that, in its absence, EHEC cannot continue with modifications to Lipid A that afford resistance to CAMPs such as HD-5.

Similarly, no significant increase in resistance of the Δ*basR* mutant strain to HD-5 after bile treatment is indicated by the radial diffusion assay shown in Figure 14. This data suggests that regardless of a functional sensor component (BasS), the Δ*basR* mutant strain remains unable to mount a BS-induced response. Therefore both components of the TCS, BasRS, are necessary to gain resistance to HD-5 through BSM-exposure.

Interestingly, the Δ*basR* mutant strain has significantly smaller clear zones than the Δ*basS* mutant strain when exposed to HD-5. In other words, the loss of BasS is more detrimental to *E. coli* O157:H7 than the loss of BasR. This data suggests that there may be a more complex interplay between the BasR-BasS and other TCSs such as PhoP-PhoQ under specific growth conditions.

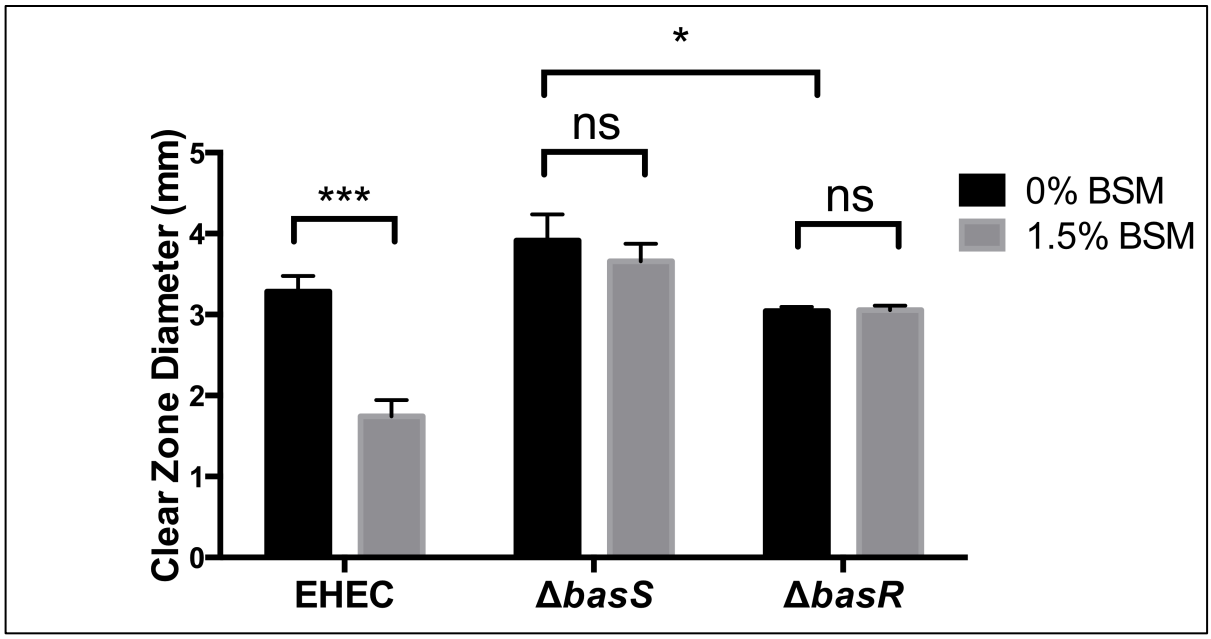


Figure 14 BS-induced Phenotype lost in Δ*basS* and Δ*basR E. coli* mutants

Briefly, cells were grown in LB and subcultured in the same media and grown with 5% CO_2. Clear zones show extent of HD-5 killing of EHEC 86-24 Δ*basS* at varying concentrations of HD-5 (0.056, 0.111, 0.333 μg/μL). Dark gray bars represent clear zones of cells that were not treated with BSM and light gray bars represent clear zones of cells that were treated with 1.5% BSM. Error bars represent means ± standard error of mean. Statistical analysis conducted using Tukey's multiple comparisons test (n=4) *** $p < 0.005$, * $p < 0.05$. This graph is representative of at least three independent experiments.

4.2.2 **ArnT plays a role in BSM-induced EHEC resistance to HD-5**

ArnT is an enzyme responsible for transferring the L-Ara4N moiety onto lipid A and as a result alters the charge of the bacterial membrane. Microarray analysis showed that *arnT* was significantly upregulated after BSM treatment. An *arnT* deficient mutant was constructed previously (86-24 Δ*arnT*) in the lab. It is expected that an *arnT* deficient strain, in the presence of BSM, will not show the BS-induced resistance phenotype to HD-5.

Figure 15 shows the size of 86-24 Δ*arnT* clear zones around 2 mm wells of HD-5 when treated and untreated with BSM. 86-24 Δ*arnT* shows no significant decrease in the size of the clear zones when treated with BSM compared to cells that were grown in just LB when 0.11 μg/μL HD-5 was placed in the wells.

To confirm the role of *arnT* in the BS-induced HD5 resistance phenotype, 86-24 Δ*arnT* was complemented with a plasmid pBADGr that contained *arnT* under an arabinose inducible promoter. A radial diffusion assay was used to test 86-24 Δ*arnT*:pBADGr:: *arnT*. It is expected that when *arnT* is expressed in this strain, it will restore the phenotype previously lost in the mutant. Figure 15 shows a significant decrease in the size of the clear zones for 86-24 Δ*arnT*::pBADGr:: *arnT* cells that were treated with BSM compared to cells that were untreated. A partial restoration of the wild type phenotype is observed in the complemented strain. This data further supports the integral role of ArnT in BSM-induced resistance to HD-5.

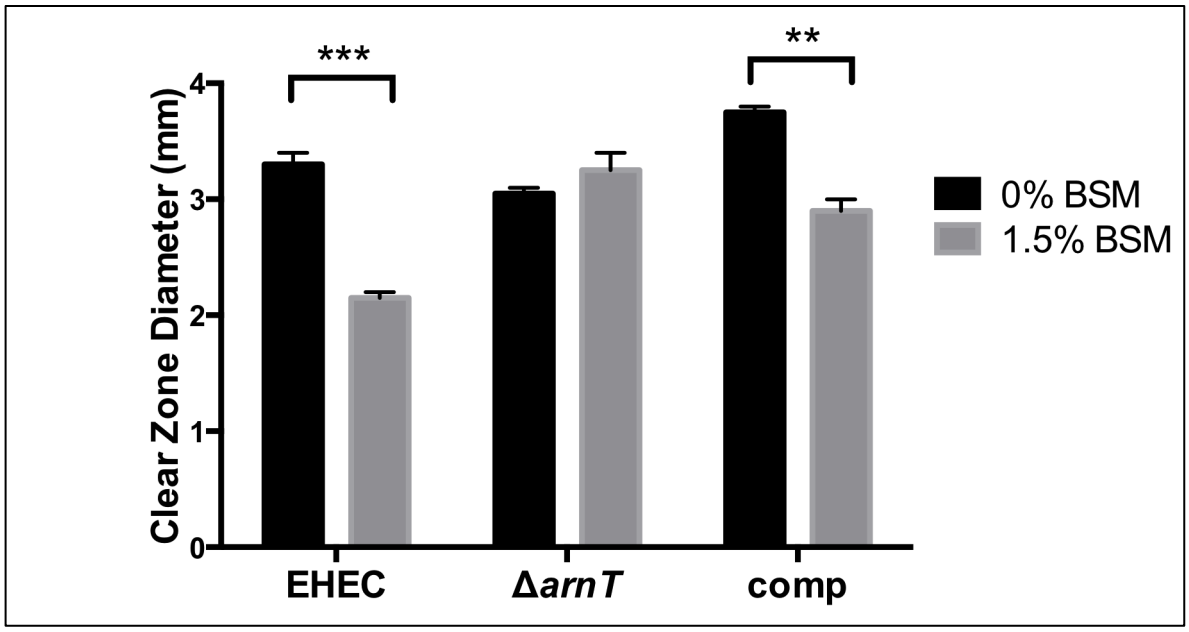


Figure 15 BS-induced Resistance to HD-5 Observed in Wild Type and *arnT* Complemented *E. coli*

Briefly, cells were grown in LB and subcultured in the same media and grown with 5% CO_2. Clear zones show extent of HD-5 killing of EHEC 86-24 Δ*arnT* with 0.11 μg/μL HD-5. Dark gray bars represent clear zones of cells that were not treated with BSM and light gray bars represent clear zones of cells that were treated with 1.5% BSM. Statistical analysis conducted using Tukey's multiple comparisons test (n=4) *** $p < 0.005$, ** $p < 0.05$. This graph is representative of at least three independent experiments.

4.3 **BSM treatment does NOT enhance EHEC resistance to LL-37**

Previous experiments (Figure 13 to Figure 15) support that BSM is able to enhance EHEC resistance to HD-5 which may be mediated by the TCS BasRS and the enzyme ArnT. As previously mentioned, ArnT catalyses the attachment of L-Ara4N to lipid A moieties in gram-negative bacteria. This modification reduces the overall negative charge of the bacterial membrane, which may be one of the primary ways *E. coli* O157:H7 mounts resistance to alpha-defensin, HD-5. It is expected that the same BSM-induced resistance is sufficient to confer EHEC resistance to a similar CAMP, cathelicidin LL-37, at physiological concentrations found in the large intestines.

Figure 16 and Figure 17 below show the size of EHEC wild type clear zones around 2 mm wells of LL-37 when treated and untreated with BSM. In the presence of 0.05 μg/mL LL-37, the clear zones of BSM treated cells were not significantly smaller than those that were not treated with BSM. Even when the concentration of LL-37 was increased to 0.1 μg/mL LL-37, no significant difference between the size of the clear zones of untreated and BSM-treated cells was observed. This data suggests that BSM-induced *E. coli* O157:H7 resistance previously observed to HD-5 is not sufficient to confer resistance to LL-37. However, when faced with 0.1 μg/mL LL-37, the *arnT* mutant showed a larger clear zone than wt *E. coli* O157:H7, suggesting that ArnT plays a role in LL-37 resistance.

The sheer number of EHEC infections annually is evidence that *E. coli* O157:H7 is able to bypass one of the body's important mechanisms of protection. There may be alternate environmental cues in the GI tract that *E. coli* O157:H7 senses in order to gain resistance to LL-37. For example, Vitamin D has been shown to increase production of LL-37 in

macrophages (W. J. Lee et al. 2012; Hertting et al. 2010). Interestingly, Group A *Streptococcus* responds to Vitamin D-induced expression of LL-37 by upregulating several virulence factors including hyaluronic acid capsule and streptolysin O mediated by the TCS CsrRS (Love, Tran-Winkler, and Wessels 2012).These virulence factors provide protection of the bacteria from LL-37 killing (Cole et al. 2010).

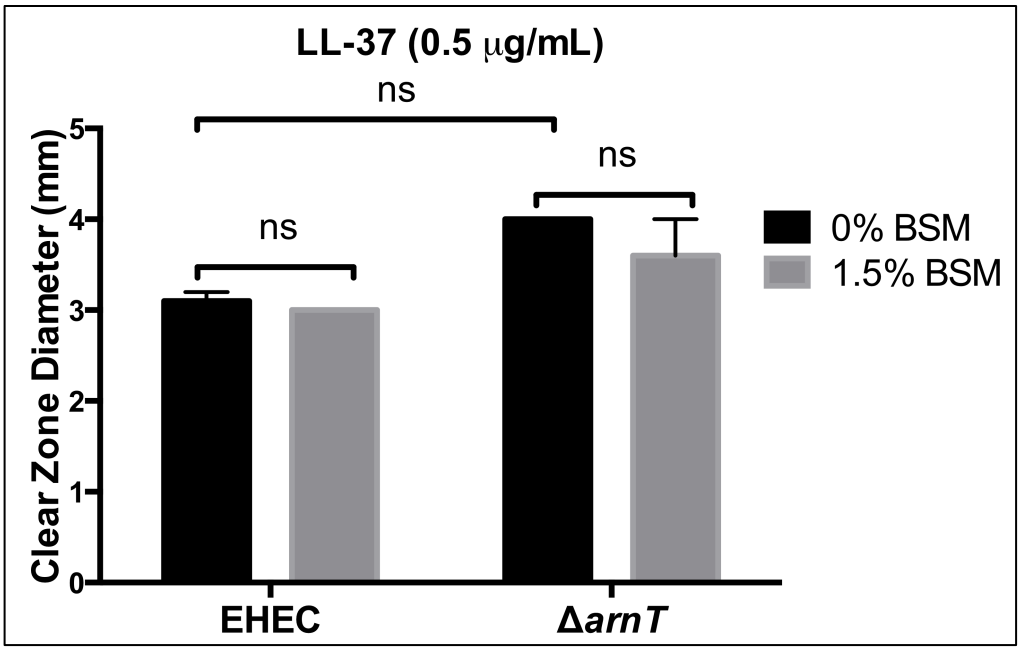


Figure 16 BS-treatment Does Not Induce Resistance to LL-37 in Wild Type *E. coli* or *arnT* mutant

Briefly, cells were grown in LB and subcultured in the same media. Clear zones show extent of LL-37 killing of EHEC 86-24 wt and 86-24 ΔarnT with 0.5 μg/mL LL-37. Dark gray bars represent clear zones of cells that were not treated with BSM and light gray bars represent clear zones of cells that were treated with 1.5% BSM. Error bars represent means ± standard error of mean. Statistical analysis conducted using Tukey's multiple comparisons test (n=4). This graph is representative of at least three independent experiments.

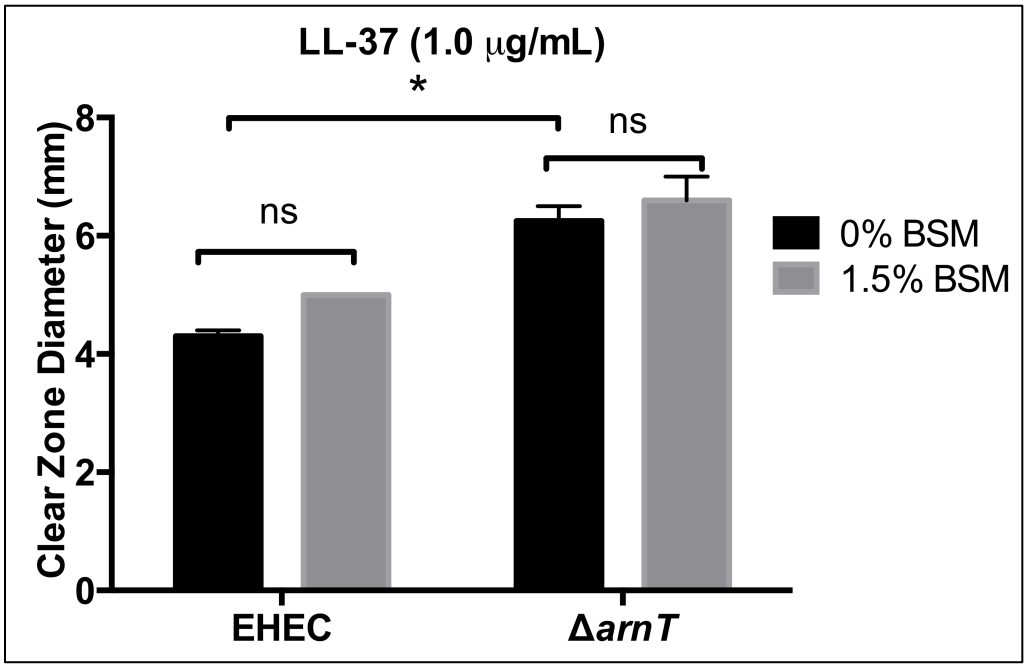


Figure 17 BS-treatment Does Not Induce Resistance to LL-37 in Wild Type *E. coli* or *arnT* mutant

Briefly, cells were grown in LB and subcultured in the same media. Clear zones show extent of LL-37 killing of EHEC 86-24 wt and 86-24 ΔarnT with 0.5 μg/mL LL-37. Dark gray bars represent clear zones of cells that were not treated with BSM and light gray bars represent clear zones of cells that were treated with 1.5% BSM. Error bars represent means ± standard error of mean. Statistical analysis conducted using Tukey's multiple comparisons test (n=4). This graph is representative of at least three independent experiments.

4.3.1 **OmpT plays a role in resistance of EHEC to LL-37**

As previously shown, EHEC is able to rapidly degrade LL-37 within 30 minutes of exposure and this response is mediated by OmpT (Thomassin et al. 2012). It is possible that BS-induced resistance via the *arn* operon is masked by rapid OmpT-mediated proteolysis of LL-37. It is hypothesized that in an OmpT-negative background, EHEC will need to rely on alternate mechanisms of resistance such as BS-induced lipid A modification. Therefore, a radial diffusion assay was conducted to compare relative sizes of clear zones of EHEC and an Δ*ompT* mutant (strain provided by Le Moual, McGill).

Figure 18 below shows a significant increase in the size of clear zones of the Δ*ompT* strain compared to the wild type strain. A larger clear zone indicates increased sensitivity to the peptide, LL-37. This data is in concurrence with previous studies which shows that OmpT plays a crucial role in EHEC resistance to LL-37 (J. L. Thomassin et al. 2012). Samples of both wild type EHEC strains and Δ*ompT* strains were treated with BSM to test whether BSM could also induce resistance to LL-37. In Figure 18 below, dark gray bars represent cells that were untreated and light gray bars represent cells that were treated with 1.5% BSM. There is no significant difference between clear zones of Δ*ompT* untreated cells compared to Δ*ompT* BSM-treated cells. This data implies that even in an OmpT-deficient background, BS-induced resistance mediated by the *arn* operon is not sufficient to confer resistance to LL-37.

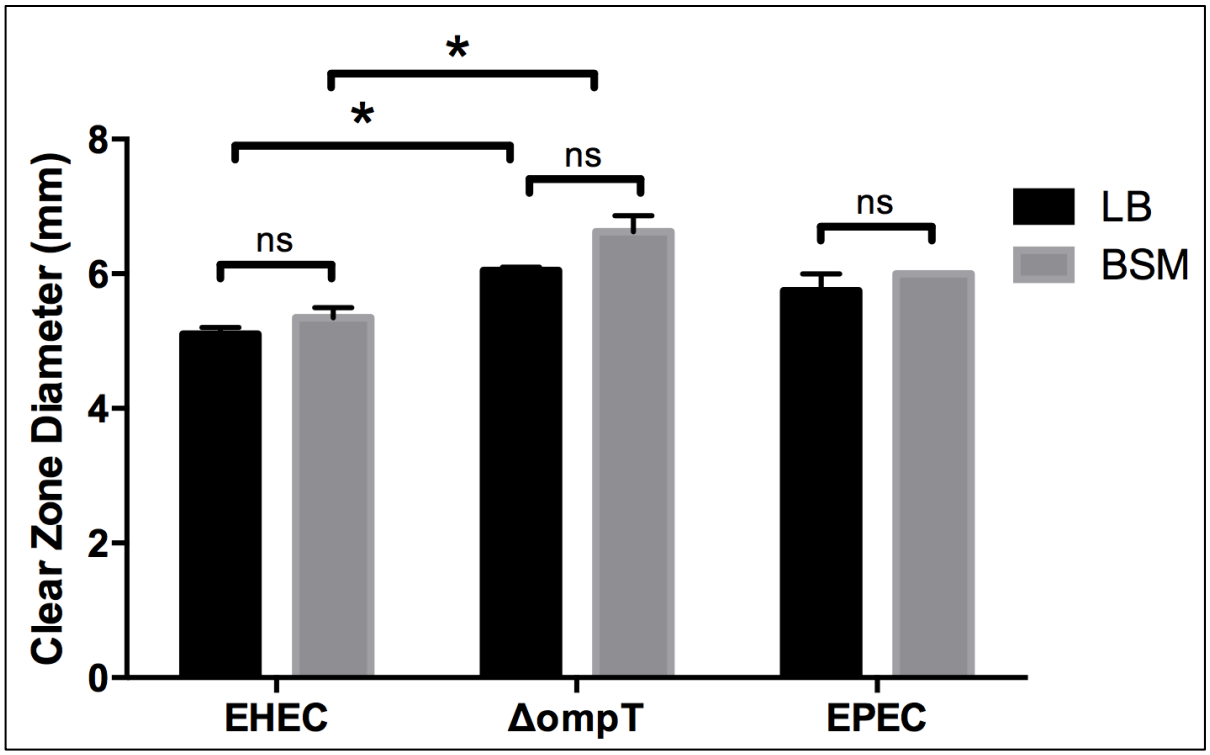


Figure 18 BS-treatment Does Not Induce Resistance to LL-37 in Wild Type *E. coli*, *ompT* Mutant or EPEC

Briefly, cells were grown in LB spiked with 1mM $MgCl_2$ (+/- 1.5% BSM). Subcultures were done in the same media and grown in 5% CO_2. Dark gray bars represent clear zones of cells that were not treated with BSM and light gray bars represent clear zones of cells that were treated with 1.5% BSM. Approximately 2E7 cells of each group were inoculated into agarose underlayer. Statistical analysis was conducted using Tukey's multiple comparisons test (n=4). This data is representative of at least three independent experiments * $p < 0.05$

4.4 **MALDI-TOF analysis of Lipid A Extracts Detects Protypical Lipid A Species**

The outer membrane of *E. coli* contains a heterogeneous mixture of lipid A variants. Approximately two-thirds of the lipid A in *E. coli* K-12 is a hexa-acylated glucosamine disaccharide (Figure 9) with a variety of different substituents depending on the growth conditions (Zhou et al. 1999; Rubin et al. 2015). To determine if BS induces L-Ara4N lipid A modification, cells were grown with and without BSM. Lipid A extracts were then analyzed by Dr. Robert Ernst and colleagues (University of Maryland).

Figure 19 below shows lipid A species identified after MALDI-TOF analysis. Lipid A from wild type *E. coli* grown in LB generated species at *m/z* 1797 and 2036 which correspond to hexa-acylated, bis phosphorylated lipid A and hepta-acylated lipid A respectively. Lipid A from wild type *E. coli* grown in LB with 1.5% BSM generated species at *m/z* 1797, 1825, 1956, and 2036. Novel species at 1825 and 1956 are not yet identified. Species at *m/z* 1797 and 2036 were previously identified in untreated lipid A species and correspond to hexa-acylated, bis phosphorylated lipid A and hepta-acylated lipid A respectively.

Treatment with metavanadate is known to stimulate lipid A species in *E. coli* (Zhou et al. 1999). In the present study, metavanadate is used as a positive control. Lipid A from wild type *E. coli* grown in LB with 25 mM NH_4VO_3 identified species at *m/z* 1797,1848, 1928, 1956, 2036, 2048, and 2087. Table 5 below shows the corresponding species associated with the *m/z* ratios identified.

To determine which lipid species arose from ArnT activity, lipid A species from Δ*arnT* and Δ*arnT:*pBADGr::*arnT* were analyzed under various growth conditions (+/- BSM). Figure 20 below shows lipid A species identified after MALDI-TOF analysis. Lipid A from Δ*arnT* grown in LB with or without 1.5% BSM generated the prototypical lipid A specie as well as species at *m/z*1956, and 2036. As previously stated, 1956 has not yet been identified and 2036 corresponds to hepta-acylated lipid A. Lipid A from Δ*arnT:*pBADGr::*arnT* grown in LB with or without 1.5% BSM generated the prototypical lipid A specie as well as species at *m/z*1956, 1928, 1956 and 2036. Additional MALDI-TOF data can be found in Appendix F.

To summarize, the prototypical hexa-acylated, bis phosphorylated lipid A, was identified under all conditions tested. This prototype is the predominant unmodified lipid A species expected in *E. coli*. It was expected that BSM-treatment of wild type *E. coli* would produce lipid A modified with L-Ara4N with *m/z* 1928. However, this lipid A species is only identified when wild type *E. coli* was treated with NH_4VO_3 or when the complement strain Δ*arnT:*pBADGr::*arnT* was treated with or without BSM. This data, in conjunction with the microarray data, sqRT-PCR (Kus et al. 2011), radial diffusion assays, along with analysis of the *arnT* mutant conducted (Kus et al. and current project), suggests that the methods used were not sensitive enough to evaluate or detect a modest increase in L-Ara4N addition to lipid A that has led to enhanced resistance to HD-5.

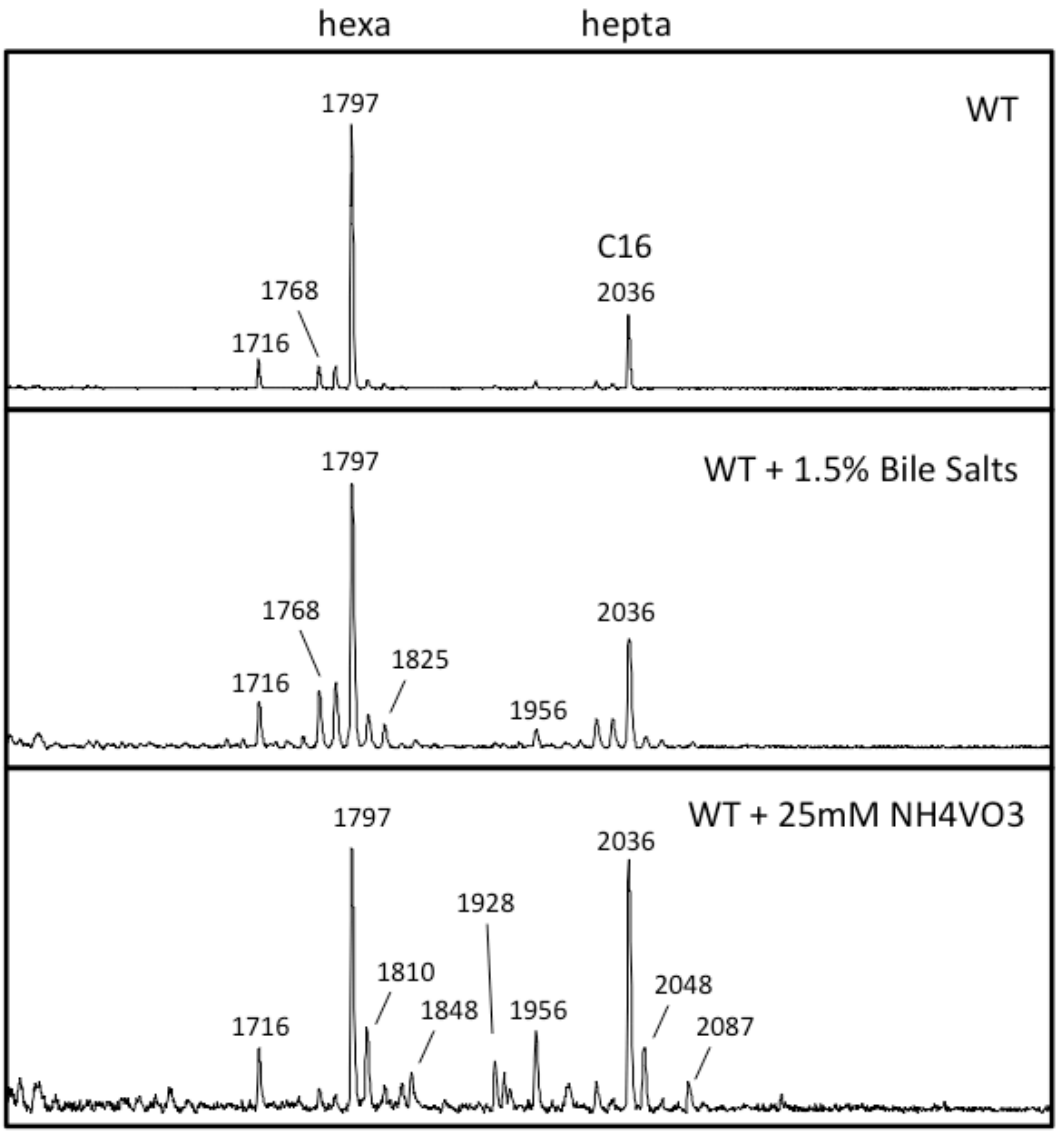


Figure 19 MALDI/TOF Mass Spectrometry of Lipid A species from *E. coli*

Cells were grown in LB spiked with 10mM $MgCl_2$ and either untreated, treated with 1.5% BSM or 25 mM metavanadate (NH_4VO_3)

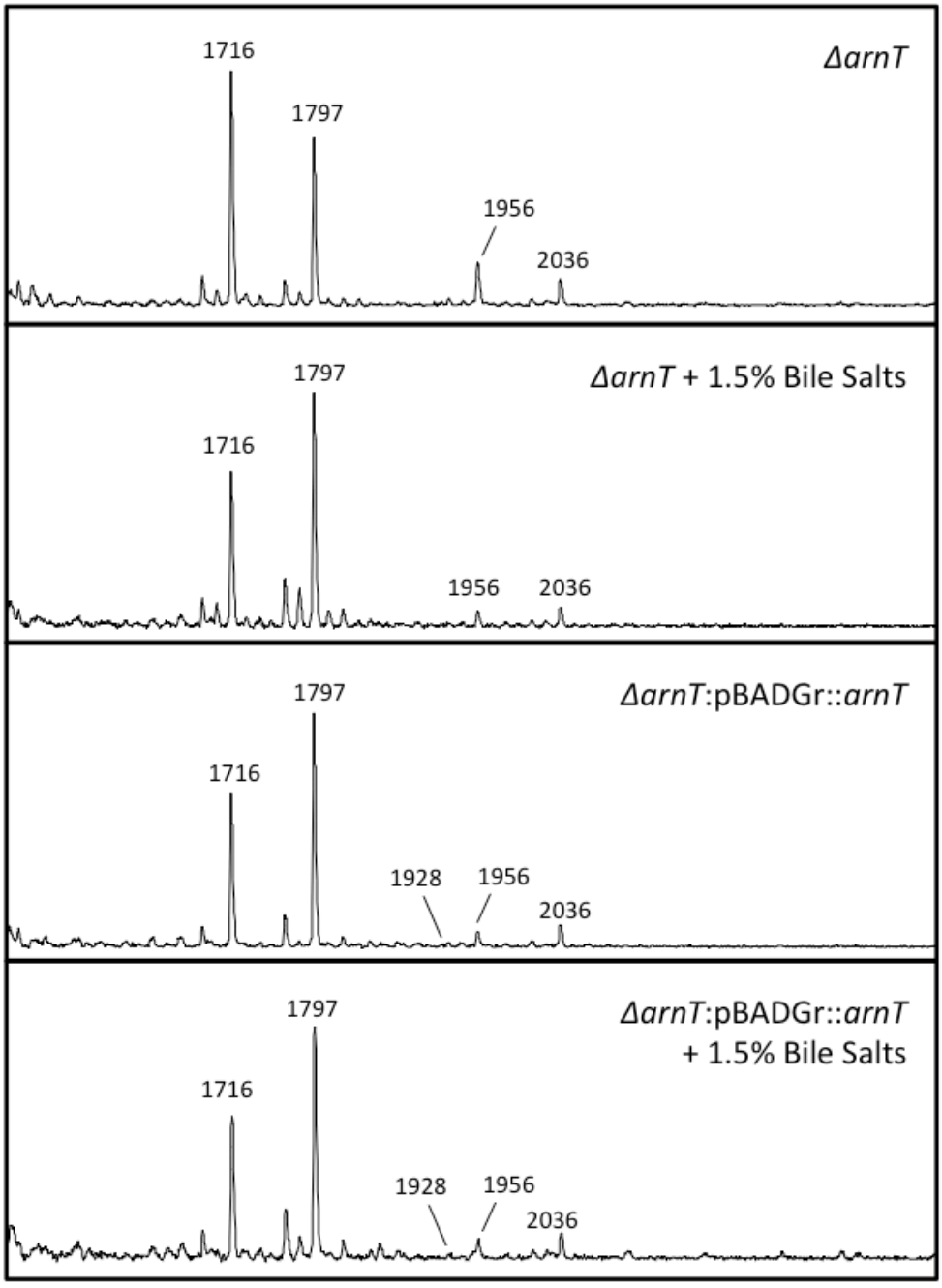


Figure 20 MALDI/TOF Mass Spectrometry of Lipid A species from *E. coli* Δ*arnT*

Cells were grown in LB spiked with 10mM $MgCl_2$ and either untreated or treated with 1.5% BSM.

Table 5 Lipid A Species Generated from *E. coli* Grown with NH_4VO_3

m/z	Lipid A Species	Δ*m/z*	Reference
1797	hexa-acylated, bis-phosphorylated lipid A (prototype)	N/A	(Rubin et al. 2015) (Kawasaki, Ernst, and Miller 2004)
1716	hexa-acylated, mono-phosphorylated lipid A	-80	(Rubin et al. 2015)
1810	hexa-acylated, bis-phosphorylated lipid A deacylated and palmitoylated	+12	This study*
1848	1-dephosphorylated hexa-acylated lipid A with one L-Ara4N	+51	(Rubin et al. 2015)
1928	hexa-acylated, bis-phosphorylated lipid A with one L-Ara4N	+131	(Rubin et al. 2015)
1956	not identified	+159	N/A
2036	hepta-acylated lipid A species	+238	(Kawasaki, Ernst, and Miller 2004)
2048	hexa-acylated, bis-phosphorylated lipid A with one L-Ara4N and one pEtN	+251	(Rubin et al. 2015)
2087	not identified	+290	N/A

4.5 **Capsule Production of Untreated EHEC and BS-treated EHEC**

Production of extracellular capsules is a mechanism used by EPEC to resist CAMPs such as HD-5 (Thomassin*, et al.*, 2013). Since BS-treatment has been shown to upregulate HD-5 resistance through ArnT-mediated lipid A modification, perhaps BS-treatment also affects capsular production. Group four capsule staining was carried out by negative staining with nigrosin and crystal violet. *Citrobacter rodentium* is used as a negative control because it does not form a capsule, while *Klebsiella pneumoniae* is commonly used as a positive control for capsule strains (Figure 21). A positive capsule is identified when an individual cell has a clear zone around it. Nigrosin only stains the background of the slide. Crystal violet stains the inside of the bacterial cell (Figure 10). Samples of untreated and BS-treated EHEC 86-24 were visualized to compare capsule formation.

Figure 21 below shows representative images of *C. rodentium* (negative), *K. pneumonia*, untreated EHEC and BS-treated EHEC. As shown in the figure below, most cells in both untreated and BS-treated EHEC show a positive capsule formation. However, day-to-day capsule staining results of wild type EHEC as well as EPEC were inconsistent with regards to positive identification of capsule. Staining techniques were adjusted by using varying durations of primary and secondary staining steps. Final concentrations of nigrosin and crystal violet were also adjusted in an attempt to visualize positive capsules. Images obtained with either a 60x objective or 100x objective were analyzed. If a positive capsule was identified, the length of this capsule was measured. However, due to poor resolution, this method of quantification proved to be challenging.

qRT-PCR was then employed to evaluate the differences between gene expression of a capsule related gene *yccC*.. YccC is the homolog for Etk in EPEC which facilitates group four capsule production. Preliminary qRT-PCR data shows that there is no significant difference between the levels of *yccC* transcription in BS-treated EHEC compared to untreated EHEC (Figure 22).

Microscopy and qRT-PCR capsule data suggest that BS do not further enhance group four capsule production which suggests that group four capsule production does not play a role in BS-induced resistance to HD-5. Therefore, lipid A modification with L-Ara4N remains the predominant mechanism *E. coli* O157:H7 utilizes in response to BS-induced resistance.

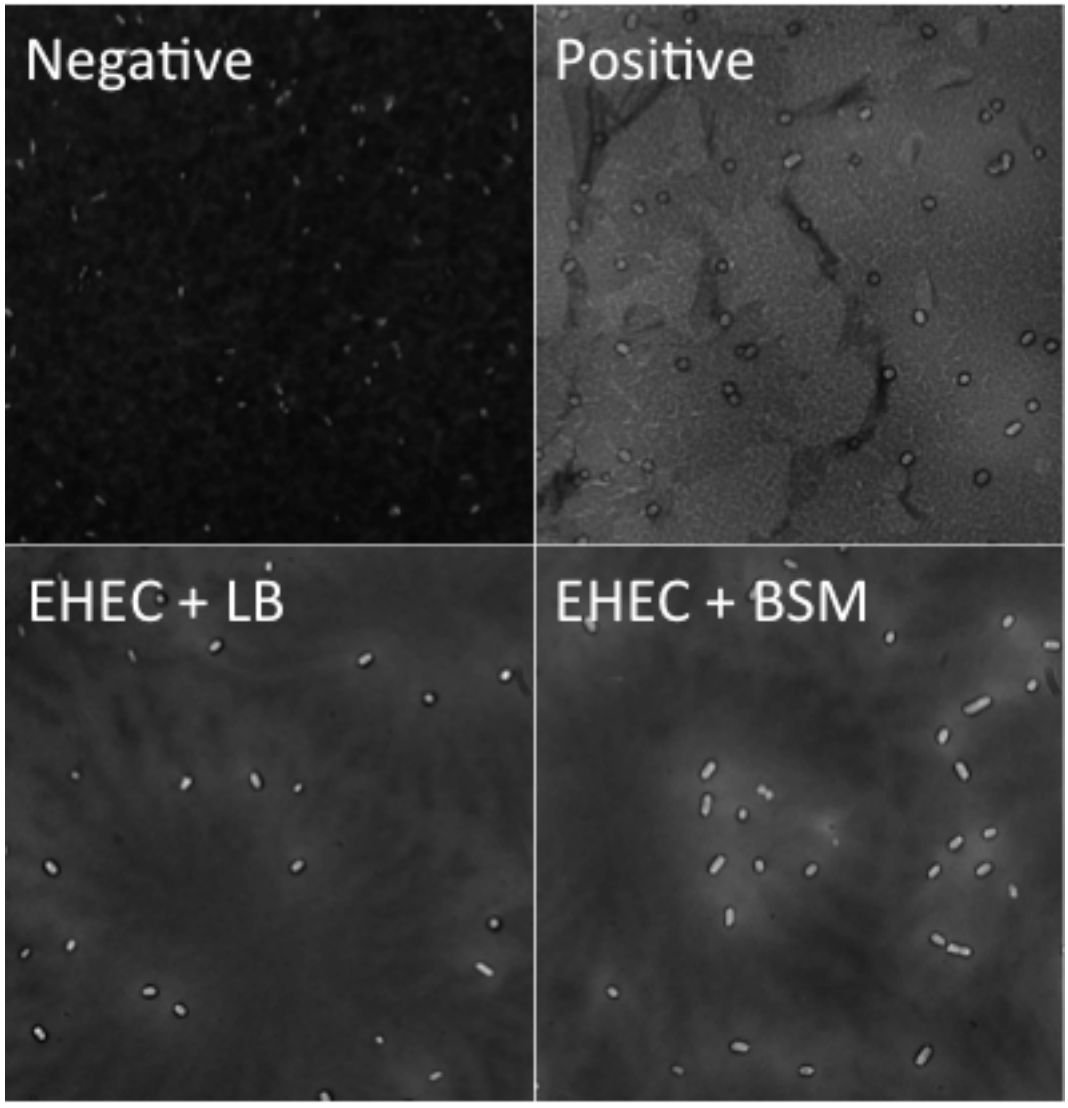


Figure 21 Capsule Stain with Nigrosin and Crystal Violet

A- *Citrobacter rodentium* (negative capsule control); B- *Klebsiella pneumonia* (positive capsule control); C- EHEC 86-24 wt (untreated); D- EHEC 86-24 wt (BSM treated)

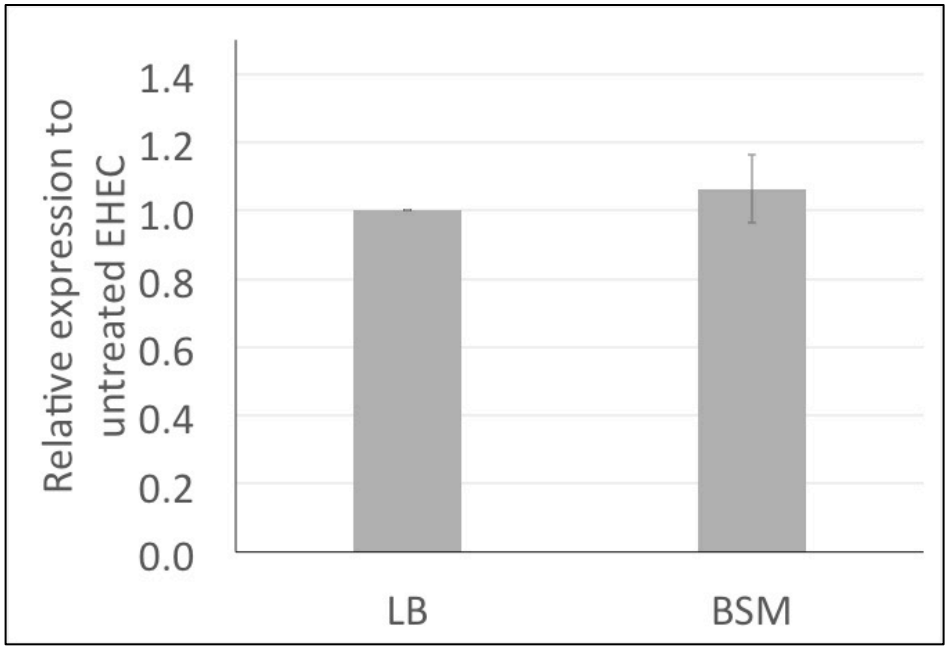


Figure 22 Relative qRT-PCR Expression of *yccC* in Wild Type *E. coli*

gapA was used as the housekeeping gene to normalize levels of DNA. Light cycler Fast Start DNA Master Sybr Green with a Roche Light Cycler was used to carry out qRT-PCR.

5 Discussion

The results of this study provide evidence that physiologically relevant mixes of bile salts, typically encountered during transit through the small intestine enhance EHEC O157:H7 resistance to the human defensin, HD5, also encountered in the lumen of the small intestine. This resistance is specific to HD-5 and is dependent on the modification of lipid A with aminoarabinose. These findings suggest that bile salts serve as an important environmental cue for EHEC by triggering protective modifications of the bacterial outer membrane, thereby increasing resistance to the deleterious human defensin encountered in the same locale.

Transcriptome expression profiling revealed that bile treatment of EHEC O157:H7 increases expression of genes encoding BasRS, a TCS homologous to PmrAB in *Salmonella typhimurium.* BasS is the sensory histidine kinase recently thought to function in self-regulation by bile salts (Kus et al. 2011). β-galactosidase assays report that in *basS* deficient mutants of 86-24 the *basRS* promoters were unable to respond to BSM. In addition, experiments with 86-24 Δ*basS* failed to show increased resistance to PMB when pretreated with 0.15% BSM (Kus et al. 2011).

Since BasS was required for bile salt induced resistance to PMB, it was reasonable to expect that BasS would also play a key role in bile salt induced resistance to human defensin. Results show that 86-24 Δ*basS* shows no significant increased resistance to HD-5 when treated with BSM. This data is consistent with work previously published by *Kus et al.*, (2011) which highlights the integral role of *basRS* in bile-induced resistance to PMB. The

current findings support BasS acting as a sensor for bile salts and suggests that, bile salts up regulates resistance to HD 5 in an *arnT* and *basS* dependent manner.

However, it is possible that there are other as yet uncharacterized sensors that can also sense bile and result in similar modifications of lipid A. Other studies on TCSs have reported that regulatory knockouts have been more effective at displaying a loss of phenotype (Derzelle et al. 2004; Li et al. 2008). To confirm the role of the response regulator of *basRS,* the *basR* deficient mutant constructed in this project was also tested and showed a similar loss of bile salt induced resistance phenotype.

ArnT is an enzyme that transfers the L-Ara4N moiety to lipid A in the last step of L-Ara4N modified lipid A shown in Figure 8. This modification alters the charge of the bacterial membrane to become less negatively charged. Lipid A modification with L-Ara4N is controlled through the *arnBCADTEF* system, which is regulated by *basRS*. Radial diffusion assays with 86-24 Δ*arnT* show a loss in BSM induced resistance to HD-5 when compared to wild type cells. This evidence supports that lipid A modification with L-Ara4N is able to confer resistance to *E. coli* O157:H7. Additionally when the *arnT* mutation was complemented, phenotype was restored 45% compared to the wild type. This is the first report of bile salt induced resistance of a pathogen to a human CAMP. Curiously, neither PmrAB nor its regulator PhoPQ in *Salmonella* have been linked to bile-induced regulation but yet they have shown to be important in bile sensing and antimicrobial resistance (J. S. Gunn et al. 2000; Begley, Gahan, and Hill 2005).

On the other hand, BS-treatment, even in an OmpT-negative background did not enhance resistance to LL-37 as previously shown to HD-5. The specific mechanism of action

for all human CAMPs, including LL-37, is not completely known (Pálffy et al. 2009). However, an ionic basis of interaction between CAMP and bacterial membrane is widely accepted (Pálffy et al. 2009; Nizet 2006; De Smet and Contreras 2005; Brogden 2005). The results of this study provide convincing evidence that there must be other factors that differentiate mechanisms of resistance to LL-37 and HD-5. Cationicity and hydrophobicity are known to play key roles in CAMP efficiency and may be one way to compare CAMPs (Bals et al. 1999; Bucki et al. 2010; Cunliffe 2003; Nagaoka et al. 2005) and therefore modification of these properties is expected to affect CAMP efficiency.

The net charge of LL-37 is +6 while the net charge of HD-5 is +4 (http://pepcalc.com/ppc.php). Figure 3 and Figure 4 show the structures of HD-5 and LL-37 respectively. In both figures, residues in highlighted blue represent cationic residues (L,K). Figure 6 Electrostatic Potential Distribution of LL-37 and HD-5 shows that LL-37 has a higher net charge and multi-ionic interaction sites. Residues highlighted in purple (Figure 3 and Figure 4) represent hydrophobic residues. Similarly, LL-37 shows a higher and more continuous hydrophobic plane than HD-5.

Specifically, an increase in overall charge of a CAMP may be sufficient to overcome some methods of bacterial resistance such as L-Ara4N lipid A modification. In fact, Nagaoka et al. aimed to increase bactericidal function of LL-37 by developing peptides with increased hydrophobicity and increased net positive charge (Nagaoka et al. 2005) to be used as potential therapeutic targets. Nagaoka et al. evaluated a shorter template of LL-37, K^{15}-V^{32}, which was shown to have the same antimicrobial activities as the full native peptide. This 17 amino acid structure was first modified by substituting E16 and K25 with L residues to

increase overall hydrophobicity and is referred to as 18-mer LL. The peptide 18-mer LL was additionally modified by substituting Q22, D26, and N30 with K residues, which increased the overall cationicity to an overall net charge of +9 and is referred to as 18-merLLKKK. Nagaoka et al. evaluated both antimicrobial activities and the ability to permeabilize membranes. These researchers found that while an increase in hydrophobicity increased percent killing of *E. coli* from ~1% to 20%, a further increase in cationicity (+3) increased percent killing to ~55% at a concentration of 0.1 μM. Higher concentrations of LL-37 (up to 1 μM) showed a similar enhancement. A 100% killing was observed for all peptide versions above 1 μM (Nagaoka et al. 2005). This research shows that a larger or more extended hydrophobic content and an increased net positive charge increases peptide activity. The bioactivity of polymixin B analogs has long been studied (HsuChen and Feingold 1973; Okhanov et al. 1987). Recent studies evaluated the role of hydrophobicity in polymyxin B efficiency, showed that a complete deletion of the hydrophobic tail cancelled all antimicrobial activity against *E. coli* and *P. aeruginosa* (Kanazawa et al. 2009).

Structural analysis shows that LL-37 has a higher net positive charge and a higher hydrophobic content than HD-5. These differences may explain why lipid A modifications with aminoarabinose induced by BS are sufficient to confer resistance to HD-5 and not to LL-37.

5.1 **Future Directions**

The results of this study provide compelling evidence that environmental conditions in gastrointestinal niches provide important information to bacterial pathogens that have the

ability to appropriately modulate their virulence properties in order to survive and cause infection. Future studies related to this work should explore the importance of positive charge and hydrophobic content on CAMP efficiency to determine the threshold at which lipid A modification is sufficient. These experiments could include antimicrobial assays with modified peptide 18-mer LLKKK and mutant *E. coli* strain *arnT*.

Additional evaluations may also involve the molecular response within two component systems identified, PhoPQ and BasRS. The interplay between these two TCSs will provide additional evidence as to how EHEC responds to different microenvironments such as high and low magnesium, low iron, low pH or presence of antimicrobials. This investigation may also highlight alternate environmental conditions that elicit a similar CAMP-resistance response.

This study also provides the foundation to be transitioned into an *in vivo* model. Murine models that do not produce bile salts are challenging, however the mutant strains Δ*basS* and Δ*basR* are unable to sense bile (Figure 14). It would be interesting to investigate whether these mutant strains are able to colonize as effectively when faced with native murine peptides.

In addition, therapeutic strategies for EHEC infection are always leading research pathways. The results of this study show that LL-37 is a dynamic and efficient peptide that is able to overcome some bacterial methods of resistance. Therefore, methods to either naturally increase concentrations of LL-37 such as Vitamin D (W. J. Lee et al. 2012) intake may be used to prevent or treat EHEC infection. This study supports research currently being

done to evaluate how host antimicrobial peptides could be used as anti-infective methods of treatment.

5.2 **Significance and Impact**

It is clear that all enteric microorganisms must overcome hostile conditions such as those encountered in the intestinal tract in order to effectively arrive at their colonization locations.

The survival and proliferation of EHEC O157:H7 strains within human hosts hinges critically on their ability to adapt to different microenvironments such as gastric acids, BS, and organic acids that present unique and diverse challenges (Alteri & Mobley, 2012). The effects of human intestinal stresses on EHEC O157:H7 are far from studied exhaustively.

The results of this study show that environmental conditions in the small intestine of the human host directly influence EHEC's ability to evade its host innate immune response of cationic antimicrobial peptides. This work suggests for the first time that EHEC O157:H7 uses BSM as a cue to signal Lipid A modifications that aid in evading human defensin HD-5.

Interestingly, overall net charge and charge distribution differences between HD-5 and LL-37 may play a larger role in the specific molecular mechanism of action than previously thought.

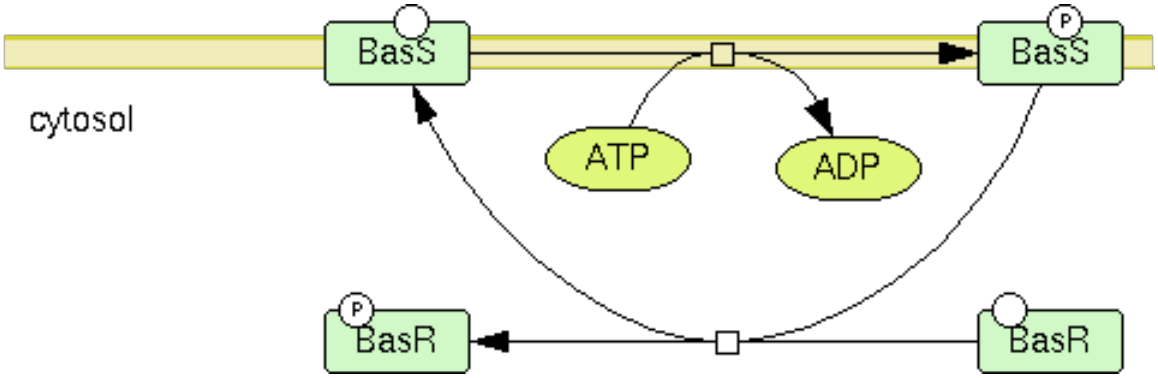
This work makes a valuable contribution to understanding how bile modulates the virulence potential of this pathogen and provides essential research necessary to decrease the number of infections, illnesses and fatalities in the future. Continued studies on how these

bacteria respond to different stresses will provide greater insights as to how exposure impacts subsequent survival and ability to proliferate.

Appendix

This section provides additional reference material to support the above project.

Appendix A: *Escherichia coli* K-12 substr. MG1655 Pathway: BasSR Two-Component Signal Transduction System

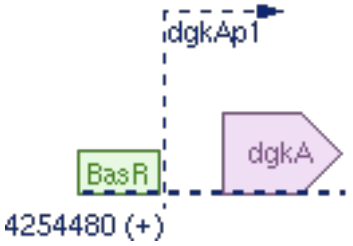


Transcription Units regulated by related protein BasR-Phosphorylated transcriptional regulator

1.

2.

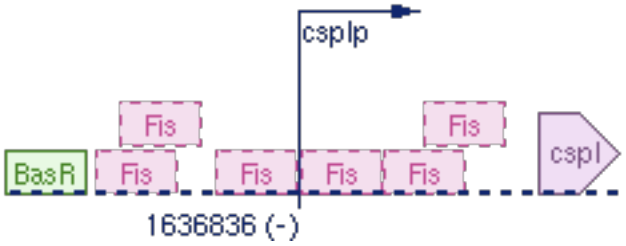
3.


dgkAp1
BasR
dgkA
4254480 (+)

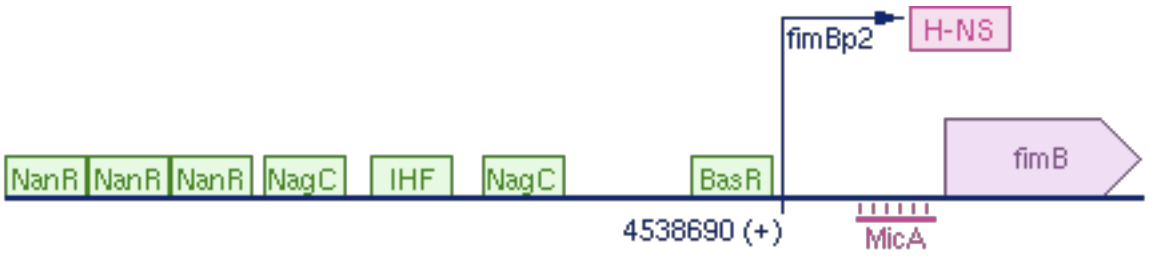
4.


BasR
waaH

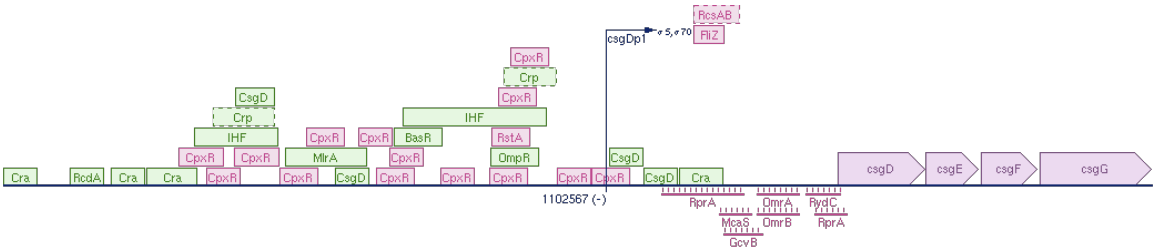
5.


cspIp
Fis
Fis
Fis
Fis
Fis
Fis
BasR
cspI
1636836 (-)

6.


fimBp2
H-NS
NanR
NanR
NanR
NagC
IHF
NagC
BasR
fimB
4538690 (+)
MicA

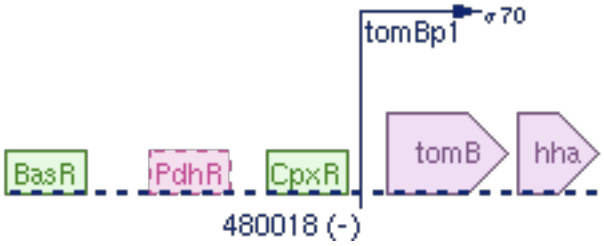
7.


RcsAB
FliZ
csgDp1
CpxR
Crp
CsgD
IHF
MlrA
BasR
RstA
OmpR
Cra
RcdA
csgD
csgE
csgF
csgG
1102567 (-)
RprA
OmrA
RydC
McaS
OmrB
GcvB

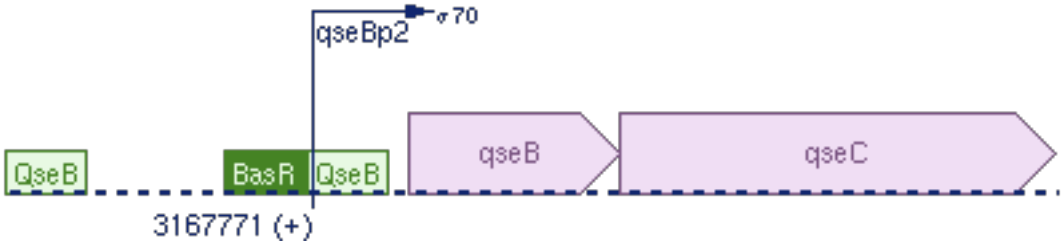
8.


putAp
MarA
PutA
PutA
PutA
BasR
putA
1078148 (-)

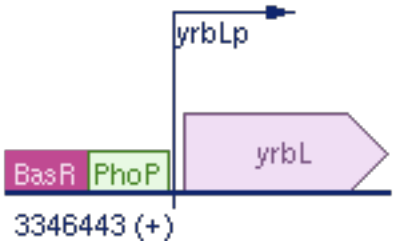
9.


tomBp1
σ70
BasR
PdhR
CpxR
tomB
hha
480018 (-)

10.


qseBp2
σ70
QseB
BasR
QseB
qseB
qseC
3167771 (+)

11.


yrbLp
BasR
PhoP
yrbL
3346443 (+)

Notes:

- Key to regulator colors: Green: activator; Magenta: inhibitor; Brown: dual; Gray: unspecified.
- A dashed baseline indicates that there is no high-quality evidence to confirm the extent of this transcription unit.
- A dashed outline for a transcription factor binding site or a dashed promoter indicates a lack of high quality evidence for the existence or location of that entity.
- Molecular Weight: 25.031 kD (from nucleotide sequence)
- Unification Links: ASAP:ABE-0013466 , CGSC:28168 , EchoBASE:EB1572 , EcoGene:EG11615 , OU-Microarray:b4113 , PortEco:basR , RefSeq:NP_418537 , RegulonDB:EG11615
- In Paralogous Gene Group: 121 (40 members)
- Reactions known to produce the compound:
- BasSR Two-Component Signal Transduction System : BasS sensory histidine kinase - phosphorylated + BasR → BasS + BasR-P^{asp51}

Page generated by SRI International Pathway Tools version 19.0 associated with the BioCyc database collection.

Appendix B: Summary of CAMP resistance mechanisms of human bacterial pathogens

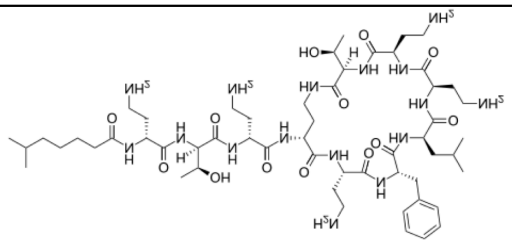
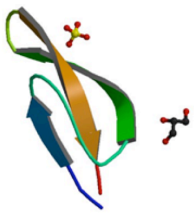
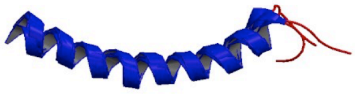
Resistance Phenotype	Gene (s)	CAMPs Affected	Organisms	Virulence Role	Reference
Cell Surface Alterations					
Addition of aminoarabinose to lipid A in LPS	*pmr* genes	Defensins, Cathelicidins	*Salmonella enterica, Proteus mirabilis, Pseudomonas aeruginosa*	Gastrointestinal infection (mice)	Gunn et al., 2000 McCoy et al., 2001 Moskowitz et al., 2004
Acylation of lipid A in LPS	*pagP rcp htrP*	Defensins, Cathelicidins	*Salmonella enterica, Legionella pneumophila, Haemophilus influenzae*	Lung colonization (mice)	Guo et al., 1998 Robey et al., 2001 Starner et al., 2002
Shedding of host proteoglycans that in turn neutralize CAMPs	*lasA*	Defensins, Cathelicidins	*Pseudomonas aeruginosa Enterococcus faecalis*	Pulmonary infection	Park et al., 2001 Schmidtchen et al., 2001
Active Efflux of CAMPS					
ATP-dependent efflux system	*mtr* genes	Cathelicidins	*Neisseria gonorrhoeae*	Genital tract infection	Jerse et al., 2003
K^+-linked efflux pump	*sap* operon *sapA*	Protamine Defensins	*Salmonella enterica, Haemophilus influenzae*	Gastrointestinal infection (mice)	Parra-Lopez et al., 1994 Mason, et al., 2005
Proteolytic Degradation of AMPS					
Elastase	*lasB*	Cathelicidins	*Pseudomonas aeruginosa*	Corneal infection (mice)	Schmidtchen et al., 20002
Surface protease	*pgtE*	Cathelicidins	*Salmonella enterica*	Unknown	Guina et al., 2000
Membrane peptidase	*ompT*	Cathelicidns	*Escherichia coli*	Gastrointestinal infection	(Stathopoulos 1998)
Regulatory Networks					
Two-component regulator	*phoP/phoQ*	Defensins	*Salmonella enterica, Pseudomonas aeruginosa*	Gastrointestinal infection (mice)	Ernst et al., 2001 Mcfarlane et al., 2000
Two-component regulator	*pmrA/pmrB*	Defensins, Polymyxin B	*Salmonella enterica Pseudomonas aeruginosa*	Gastrointestinal infection (mice)	Gunn et al., 2000 McPhee et al., 2003
Alteration of Host Processes					
Downregulation of CAMP transcription	Unknown	Cathelicidins	*Shigella dysenteriae*	Dysentery (humans)	Islam et al., 2001
Stimulation of host cathespins	Unknown	Defensins	*Pseudomonas aeruginosa*	Unknown	Taggart et al., 2003

Adapted from (Nizet 2006)

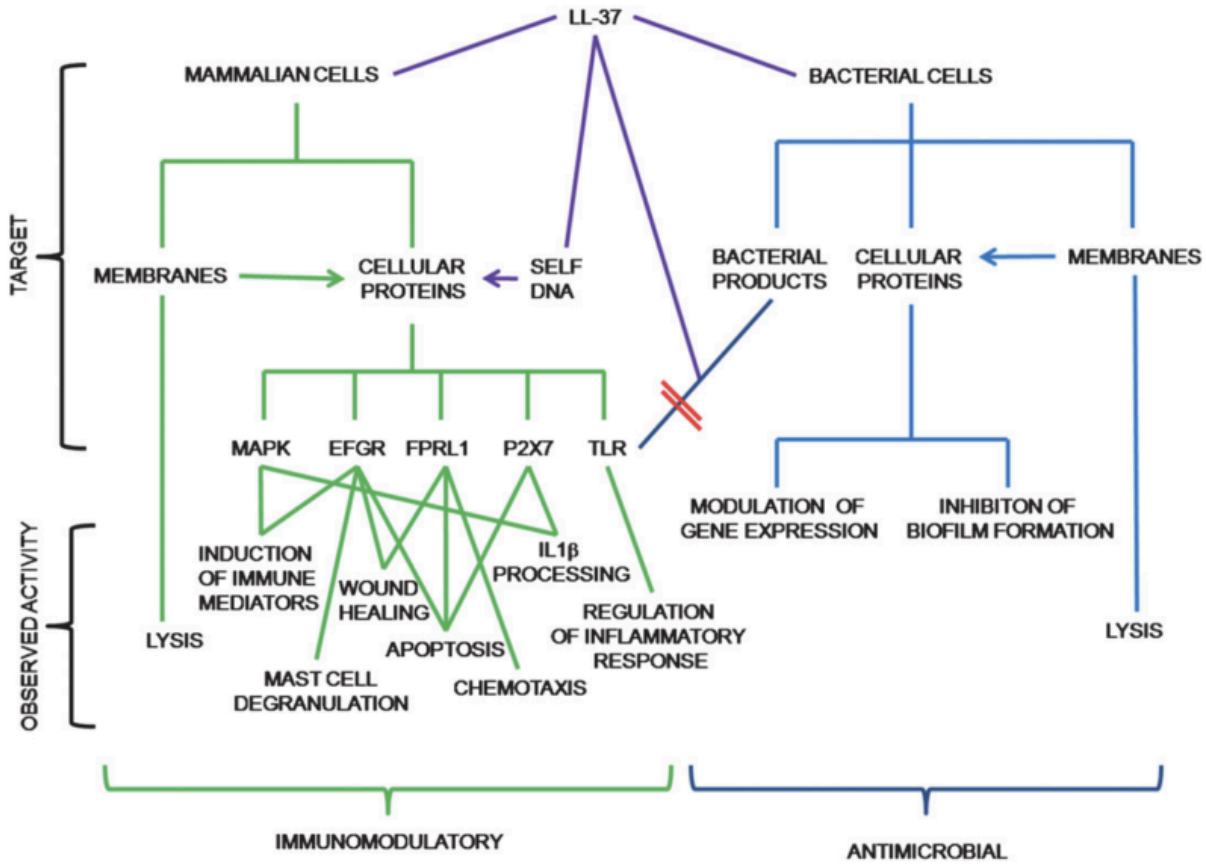
Appendix C: Summary Table for LL-37 Killing Assays

	EDL 933 (N-min + MgCl2)	**ΔompT (N-min + MgCl2)**	**EDL 933 (N-min + MgCl2 + 1.5% BSM)**	**ΔompT (N-min + MgCl2 + 1.5% BSM)**	**Notes**
Le Moual	16	8	-	-	(J.-L. Thomassin et al. 2012)
15 May 15	10 (16)	6 (8)	-	-	These concentrations were not tested by Le Moual. Brackets show next highest conc.
21 May 15	8*	8	16	8	**No enhanced resistance.** MIC for EDL 933 wt is lower than previously observed.
26 May 15	16	4	4	8	**Increased resistance** in ompT mutant
29 May 15	16	4	8	4 (8*)	Visual turbidity after 24 hours shows a MIC of 8 **Increased resistance** in ompT mutant
2 June 15	8	4	8	4	**No enhanced resistance.**

Appendix D: Comparison of CAMPs: PMB, LL-37 and HD-5

	Polymyxin B	Human Defensin 5	LL-37
Size g/mol	1385	3588	4504
Amino acid Sequence		ATCYCRTGRCATRES LSGVCEISGRLYRLCC R	NH2-LLGDFFRKSKEKIGKEFK RIVQRIKDFLRNLVPRTES-COOH (Bucki et al. 2010)
Notes on structure	diaminobutyric acid) residue at position 5 is the most important residue contributing to bactericidal activity (Kanazawa et al. 2009)	triple-stranded b-sheet structure with a b-hairpin loop containing cationic charged mole-cules	linear structure because it does not contain cysteine. The peptide adopts a lar- gely random coil conformation in a hydrophilic environment, and an a-helical structure in a hydrophobic environment (Turner et al. 1998)
Net Charge at pH 7.4	+5	+3.7 (Innovagen)	+6
Disulfide connectivity		Cys^{3}-Cys^{31}, Cys^{5}-Cys^{20}, Cys^{10}-Cys^{30}	No cysteines in structure
Hydrophobic ity	Strong hydrophobic tail		High content of basic and hydrophobic amino acids
Structure			

Appendix E: Info-web of LL-37 interactions *in vivo*



Interaction between mammalian and microbial systems shows a complex web of processes that support the multi-functional roles of LL-37 *in vivo*
(Burton and Steel 2009)

Appendix F Additional MALDI-TOF Samples prepared for analysis

Group #	Strain	Overnight Media	Subculture (Stress)	No. of Replicates
5	EHEC 86-24	LB	LB	3
6	EHEC 86-24 ΔarnT	LB	LB	3
7	EHEC 86-24	LB	LB + 25 mM Metavanadate	3
8	EHEC 86-24 ΔbasS	LB	LB	3
9	EHEC 86-24 ΔarnT: pBADGr::arnT	LB	LB	3
10	EHEC 86-24	LB + 1.5% BSM	LB + 1.5% BSM	6
11	EHEC 86-24 ΔarnT	LB + 1.5% BSM	LB + 1.5% BSM	3
12	EHEC 86-24 ΔbasS	LB + 1.5% BSM	LB + 1.5% BSM	3
13	EHEC 86-24 ΔarnT: pBADGr::arnT	LB + 1.5% BSM	LB + 1.5% BSM	3
14	EHEC 86-24 ΔarnT	LB	LB + 25mM Metavanadate	3
15	EHEC 86-24	LB + 1 mM $MgCl_2$	LB + 1 mM $MgCl_2$	3

References

Agerberth, B, J Charo, J Werr, B Olsson, F Idali, L Lindbom, R Kiessling, H Jörnvall, H Wigzell, and G H Gudmundsson. 2000. "The Human Antimicrobial and Chemotactic Peptides LL-37 and Alpha-Defensins Are Expressed by Specific Lymphocyte and Monocyte Populations." *Blood* 96 (9): 3086–93.

Andrews, J M. 2001. "Determination of Minimum Inhibitory Concentrations." *The Journal of Antimicrobial Chemotherapy* 48 Suppl 1: 5–16. doi:10.1093/jac/48.suppl_1.5.

Bals, R, D J Weiner, A D Moscioni, R L Meegalla, and J M Wilson. 1999. "Augmentation of Innate Host Defense by Expression of a Cathelicidin Antimicrobial Peptide." *Infection and Immunity* 67 (11): 6084–89.

Begley, Máire, Cormac G M Gahan, and Colin Hill. 2005. "The Interaction between Bacteria and Bile." *FEMS Microbiology Reviews* 29: 625–51. doi:10.1016/j.femsre.2004.09.003.

Bernstein, Carol, Harris Bernstein, Claire M. Payne, Shannon E. Beard, and John Schneider. 1999. "Bile Salt Activation of Stress Response Promoters in Escherichia Coli." *Current Microbiology* 39 (2): 68–72. doi:10.1007/s002849900420.

Besser, Richard E. 1993. "An Outbreak of Diarrhea and Hemolytic Uremic Syndrome From Escherichia Coli O157:H7 in Fresh-Pressed Apple Cider." *JAMA: The Journal of the American Medical Association* 269 (17). American Medical Association: 2217. doi:10.1001/jama.1993.03500170047032.

Brannon, John R., Jenny Lee Thomassin, Isabelle Desloges, Samantha Gruenheid, and Hervé Le Moual. 2013. "Role of Uropathogenic Escherichia Coli OmpT in the Resistance against Human Cathelicidin LL-37." *FEMS Microbiology Letters* 345: 64–71. doi:10.1111/1574-6968.12185.

Brogden, Kim A. 2005. "Antimicrobial Peptides: Pore Formers or Metabolic Inhibitors in Bacteria?" *Nature Reviews. Microbiology* 3 (3): 238–50. doi:10.1038/nrmicro1098.

Brown, Thomas, and Jiri Mestecky. 1985. “Immunoglobulin A Subclass Distribution of Naturally Occurring Salivary Antibodies to Microbial Antigens.” *Infection and Immunity* 49 (2): 459–62.

Bucki, Robert, Katarzyna Leszczyńska, Andrzej Namiot, and Wojciech Sokołowski. 2010. “Cathelicidin LL-37: A Multitask Antimicrobial Peptide.” *Archivum Immunologiae et Therapiae Experimentalis* 58: 15–25. doi:10.1007/s00005-009-0057-2.

Burton, Matthew F, and Patrick G Steel. 2009. “The Chemistry and Biology of LL-37.” *Natural Product Reports* 26: 1572–84. doi:10.1039/b912533g.

Cavaillon, Jean-Marc. 2011. “Polymyxin B for Endotoxin Removal in Vaccination against Meningitis Outside the.” *The Lancet Infectious Diseases* 11 (6): 426–27.

Chamnongpol, Sangpen, Walter Dodson, Michael J. Cromie, Z. Leah Harris, and Eduardo a. Groisman. 2002. “Fe(III)-Mediated Cellular Toxicity.” *Molecular Microbiology* 45 (3): 711–19. doi:10.1046/j.1365-2958.2002.03041.x.

Chowdhury, Rukhsana, Gautam K. Sahu, and Jyotirmoy Das. 1996. “Stress Response in Pathogenic Bacteria.” *Journal of Biosciences*. Indian Academy of Sciences.

Cole, Jason N, Morgan A Pence, Maren von Köckritz-Blickwede, Andrew Hollands, Richard L Gallo, Mark J Walker, and Victor Nizet. 2010. “M Protein and Hyaluronic Acid Capsule Are Essential for in Vivo Selection of covRS Mutations Characteristic of Invasive Serotype M1T1 Group A Streptococcus.” *mBio* 1 (4): e00191–10 – . doi:10.1128/mBio.00191-10.

Coleman, R, P J Lowe, and D Billington. 1980. “Membrane Lipid Composition and Susceptibility to Bile Salt Damage.” *Biochimica et Biophysica Acta* 599 (1): 294–300. doi:10.1016/0005-2736(80)90075-9.

Cooke, Edith Mary. 1974. *Escherichia Coli and Man*. Edinburgh: Churchill Livingstone.

Cowland, Jack B., Anders H. Johnsen, and Niels Borregaard. 1995. "hCAP-18, a Cathelin/pro-Bactenecin-like Protein of Human Neutrophil Specific Granules." *FEBS Letters* 368 (1): 173–76. doi:10.1016/0014-5793(95)00634-L.

Cunliffe, R N. 2003. "Alpha-Defensins in the Gastrointestinal Tract." *Molecular Immunology* 40 (7): 463–67.

Dare, R, J T Magee, and G E Mathison. 1972. "In-Vitro Studies on the Bactericidal Properties of Natural and Synthetic Gastric Juices." *Journal of Medical Microbiology* 5 (4). Microbiology Society: 395–406. doi:10.1099/00222615-5-4-395.

De Smet, Kris, and Roland Contreras. 2005. "Human Antimicrobial Peptides: Defensins, Cathelicidins and Histatins." *Biotechnology Letters* 27 (18): 1337–47. doi:10.1007/s10529-005-0936-5.

De Valdez, G F, G Martos, M P Taranto, G L Lorca, G Oliver, and a P de Ruiz Holgado. 1997. "Influence of Bile on Beta-Galactosidase Activity and Cell Viability of Lactobacillus Reuteri When Subjected to Freeze-Drying." *Journal of Dairy Science* 80 (9). Elsevier: 1955–58. doi:10.3168/jds.S0022-0302(97)76137-X.

Dean-Nystrom, Evelyn A., Brad T. Bosworth, Harley W. Moon, and Alison D. O'Brien. 1998. "Escherichia Coli O157:H7 Requires Intimin for Enteropathogenicity in Calves." *Infect. Immun.* 66 (9): 4560–63.

Derzelle, S., E. Turlin, E. Duchaud, S. Pages, F. Kunst, A. Givaudan, and A. Danchin. 2004. "The PhoP-PhoQ Two-Component Regulatory System of Photorhabdus Luminescens Is Essential for Virulence in Insects." *Journal of Bacteriology* 186 (5): 1270–79. doi:10.1128/JB.186.5.1270-1279.2004.

Drasar, B S, and M J Hill. 1975. "The Normal Colonic Bacterial Flora." *Gut* 16 (4): 318–23.

Dürr, Ulrich H N, U S Sudheendra, and Ayyalusamy Ramamoorthy. 2006. "LL-37, the Only Human Member of the Cathelicidin Family of Antimicrobial Peptides." *Biochimica et Biophysica Acta* 1758 (9): 1408–25. doi:10.1016/j.bbamem.2006.03.030.

Etienne-Mesmin, Lucie, Benoit Chassaing, Pierre Sauvanet, Jérémy Denizot, Stéphanie Blanquet-Diot, Arlette Darfeuille-Michaud, Nathalie Pradel, and Valérie Livrelli. 2011. "Interactions with M Cells and Macrophages as Key Steps in the Pathogenesis of Enterohemorrhagic Escherichia Coli Infections." *PloS One* 6 (8). Public Library of Science: e23594. doi:10.1371/journal.pone.0023594.

Fellermann, K, and E F Stange. 2001. "Defensins -- Innate Immunity at the Epithelial Frontier." *European Journal of Gastroenterology & Hepatology* 13 (7): 771–76.

Finlay, B Brett, and Grant McFadden. 2006. "Anti-Immunology: Evasion of the Host Immune System by Bacterial and Viral Pathogens." *Cell* 124 (4): 767–82. doi:10.1016/j.cell.2006.01.034.

Flores, Cristina, Ismael Maguilnik, Everton Haldich, and Luciano Goldani. 2003. "Microbiology of Choledochal Bile in Patients with Choledocholithiasis Admitted to a Tertiary Hospital." *Journal of Gastroenterology and Hepatology* 18 (3): 333–36. doi:10.1046/j.1440-1746.2003.02971.x.

Fox, JG, LL Yan, FE Dewhirst, BJ Paster, B Shames, JC Murphy, A Hayward, JC Belcher, and EN Mendes. 1995. "Helicobacter Bilis Sp. Nov., a Novel Helicobacter Species Isolated from Bile, Livers, and Intestines of Aged, Inbred Mice." *J. Clin. Microbiol.* 33 (2): 445–54.

Fujisawa, T., and M. Mori. 1997. "Influence of Various Bile Salts on Beta Beta-Glucuronidase Activity of Intestinal Bacteria." *Letters in Applied Microbiology* 25 (2): 95–97. doi:10.1046/j.1472-765X.1997.00180.x.

Gaines, Jennifer M, Nancy L Carty, Jane A Colmer-Hamood, and Abdul N Hamood. 2005. "Effect of Static Growth and Different Levels of Environmental Oxygen on toxA and ptxR Expression in the Pseudomonas Aeruginosa Strain PAO1." *Microbiology (Reading, England)* 151 (Pt 7): 2263–75. doi:10.1099/mic.0.27754-0.

Giannella, R. A., S. A. Broitman, and N. Zamcheck. 1972. "Gastric Acid Barrier to Ingested Microorganisms in Man: Studies in Vivo and in Vitro." *Gut* 13 (4): 251–56. doi:10.1136/gut.13.4.251.

Gryllos, Ioannis, Hien J Tran-Winkler, Ming-Fang Cheng, Hachung Chung, Robert Bolcome, Wuyuan Lu, Robert I Lehrer, and Michael R Wessels. 2008. "Induction of Group A Streptococcus Virulence by a Human Antimicrobial Peptide." *Proceedings of the National Academy of Sciences of the United States of America* 105 (43): 16755–60. doi:10.1073/pnas.0803815105.

Gudmundsson, G H, B Agerberth, J Odeberg, T Bergman, B Olsson, and R Salcedo. 1996. "The Human Gene FALL39 and Processing of the Cathelin Precursor to the Antibacterial Peptide LL-37 in Granulocytes." *European Journal of Biochemistry / FEBS* 238 (2): 325–32.

Gunn, J. S., S. S. Ryan, J. C. Van Velkinburgh, R. K. Ernst, and S. I. Miller. 2000. "Genetic and Functional Analysis of a PmrA-PmrB-Regulated Locus Necessary for Lipopolysaccharide Modification, Antimicrobial Peptide Resistance, and Oral Virulence of Salmonella Enterica Serovar Typhimurium." *Infection and Immunity* 68 (11): 6139–46. doi:10.1128/IAI.68.11.6139-6146.2000.

Gunn, John S. 2000. "Mechanisms of Bacterial Resistance and Response to Bile." *Microbes and Infection* 2 (8): 907–13. doi:10.1016/S1286-4579(00)00392-0.

———. 2008. "The Salmonella PmrAB Regulon: Lipopolysaccharide Modifications, Antimicrobial Peptide Resistance and More." *Trends in Microbiology* 16 (6): 284–90. doi:10.1016/j.tim.2008.03.007.

Hagiwara, Daisuke, Takafumi Yamashino, and Takeshi Mizuno. 2004. "A Genome-Wide View of the Escherichia Coli BasS-BasR Two-Component System Implicated in Iron-Responses." *Bioscience, Biotechnology, and Biochemistry* 68 (8): 1758–67. doi:10.1271/bbb.68.1758.

Haiko, Johanna, Liisa Laakkonen, Katri Juuti, Nisse Kalkkinen, and Timo K Korhonen. 2010. "The Omptins of Yersinia Pestis and Salmonella Enterica Cleave the Reactive Center Loop of Plasminogen Activator Inhibitor 1." *Journal of Bacteriology* 192 (18): 4553–61. doi:10.1128/JB.00458-10.

Hajjar, Adeline M, Robert K Ernst, Jeff H Tsai, Christopher B Wilson, and Samuel I Miller. 2002. "Human Toll-like Receptor 4 Recognizes Host-Specific LPS Modifications." *Nature Immunology* 3 (4): 354–59. doi:10.1038/ni777.

Herrera, Carmen M., Jessica V. Hankins, and M. Stephen Trent. 2010. "Activation of PmrA Inhibits LpxT-Dependent Phosphorylation of Lipid A Promoting Resistance to Antimicrobial Peptides." *Molecular Microbiology* 76 (6): 1444–60. doi:10.1111/j.1365-2958.2010.07150.x.

Hertting, Olof, Åsa Holm, Petra Lüthje, Hanna Brauner, Robert Dyrdak, Aino Fianu Jonasson, Peter Wiklund, Milan Chromek, and Annelie Brauner. 2010. "Vitamin D Induction of the Human Antimicrobial Peptide Cathelicidin in the Urinary Bladder." *PloS One* 5 (12): e15580. doi:10.1371/journal.pone.0015580.

Hofmann, AF, and KJ Mysels. 1992. "Bile Acid Solubility and Precipitation in Vitro and in Vivo: The Role of Conjugation, pH, and Ca2+ Ions." *J. Lipid Res.* 33 (5): 617–26.

Hofmann, Alan F. 1999. "Bile Acids: The Good, the Bad, and the Ugly." *News Physiol Sci* 14: 24–29.

House, B, J V Kus, N Prayitno, R Mair, L Que, F Chingcuanco, V Gannon, D G Cvitkovitch, and D Barnett Foster. 2009. "Acid-Stress-Induced Changes in Enterohaemorrhagic Escherichia Coli O157 : H7 Virulence." *Microbiology (Reading, England)* 155 (Pt 9). Microbiology Society: 2907–18. doi:10.1099/mic.0.025171-0.

Hritonenko, Victoria. 2007. "Omptin Proteins: An Expanding Family of Outer Membrane Proteases in Gram-Negative Enterobacteriaceae (Review)." *Molecular Membrane Biology* 24 (5-6): 395–406.

Hritonenko, Victoria, and Christos Stathopoulos. 2007. "Omptin Proteins: An Expanding Family of Outer Membrane Proteases in Gram-Negative Enterobacteriaceae." *Molecular Membrane Biology* 24 (5-6): 395–406. doi:10.1080/09687680701443822.

HsuChen, Chuen Chin, and David S. Feingold. 1973. "The Mechanism of Polymyxin B Action and Selectivity toward Biologic Membranes." *Biochemistry* 12 (11): 2105–11.

Jenssen, Håvard, Pamela Hamill, and R. E W Hancock. 2006. "Peptide Antimicrobial Agents." *Clinical Microbiology Reviews* 19 (3): 491–511. doi:10.1128/CMR.00056-05.

Kanazawa, Kazushi, Yuki Sato, Kazuhiro Ohki, Keiko Okimura, Yoshiki Uchida, Mitsuno Shindo, and Naoki Sakura. 2009. "Contribution of Each Amino Acid Residue in Polymyxin B(3) to Antimicrobial and Lipopolysaccharide Binding Activity." *Chemical & Pharmaceutical Bulletin* 57 (3): 240–44. doi:10.1248/cpb.57.240.

Kaper, James B, and Mohamed A Karmali. 2008. "The Continuing Evolution of a Bacterial Pathogen." *Proceedings of the National Academy of Sciences of the United States of America* 105 (12): 4535–36. doi:10.1073/pnas.0801435105.

Kawasaki, Kiyoshi, Robert K Ernst, and Samuel I Miller. 2005. "Inhibition of Salmonella Enterica Serovar Typhimurium Lipopolysaccharide Deacylation by Aminoarabinose Membrane Modification." *Journal of Bacteriology* 187 (7): 2448–57. doi:10.1128/JB.187.7.2448-2457.2005.

Kawasaki, Kiyoshi, Robert K. Ernst, and Samuel I. Miller. 2004. "3-O-Deacylation of Lipid A by PagL, a PhoP/PhoQ-Regulated Deacylase of Salmonella Typhimurium, Modulates Signaling through Toll-like Receptor 4." *Journal of Biological Chemistry* 279 (19): 20044–48. doi:10.1074/jbc.M401275200.

Kim, Sang Hyun, Wenyi Jia, Valeria R. Parreira, Russel E. Bishop, and Carlton L. Gyles. 2006. "Phosphoethanolamine Substitution in the Lipid A of Escherichia Coli O157 : H7 and Its Association with PmrC." *Microbiology* 152 (3): 657–66. doi:10.1099/mic.0.28692-0.

Kristiansen, T. Z. 2004. "A Proteomic Analysis of Human Bile." *Molecular & Cellular Proteomics* 3 (7): 715–28. doi:10.1074/mcp.M400015-MCP200.

Kus, Julianne V., Ahferom Gebremedhin, Vica Dang, Seav Ly Tran, Anca Serbanescu, and Debora Barnett Foster. 2011. "Bile Salts Induce Resistance to Polymyxin in Enterohemorrhagic Escherichia Coli O157:H7." *Journal of Bacteriology* 193: 4509–15. doi:10.1128/JB.00200-11.

Lacour, Soline, Patricia Doublet, Brice Obadia, Alain J. Cozzone, and Christophe Grangeasse. 2006. "A Novel Role for Protein-Tyrosine Kinase Etk from Escherichia Coli K-12 Related to Polymyxin Resistance." *Research in Microbiology* 157: 637–41. doi:10.1016/j.resmic.2006.01.003.

Lawhon, Sara D, Russell Maurer, Mitsu Suyemoto, and Craig Altier. 2002. "Intestinal Short-Chain Fatty Acids Alter Salmonella Typhimurium Invasion Gene Expression and Virulence through BarA / SirA" 46: 1451–64.

Lee, Lucy J, Jason A Barrett, and Robert K Poole. 2005. "Genome-Wide Transcriptional Response of Chemostat-Cultured Escherichia Coli to Zinc." *Journal of Bacteriology* 187 (3): 1124–34. doi:10.1128/JB.187.3.1124-1134.2005.

Lee, Weon Ju, Hyun Wuk Cha, Mi Yeung Sohn, Seok-Jong Lee, and Do Won Kim. 2012. "Vitamin D Increases Expression of Cathelicidin in Cultured Sebocytes." *Archives of Dermatological Research* 304 (8): 627–32. doi:10.1007/s00403-012-1255-z.

Lehrer, R I, M Rosenman, S S Harwig, R Jackson, and P Eisenhauer. 1991. "Ultrasensitive Assays for Endogenous Antimicrobial Polypeptides." *Journal of Immunological Methods* 137: 167–73. doi:10.1016/0022-1759(91)90021-7.

Li, Ming, Changjun Wang, Youjun Feng, Xiuzhen Pan, Gong Cheng, Jing Wang, Junchao Ge, et al. 2008. "SalK/SalR, a Two-Component Signal Transduction System, Is Essential for Full Virulence of Highly Invasive Streptococcus Suis Serotype 2." *PLoS ONE* 3 (5). doi:10.1371/journal.pone.0002080.

Liaqat, I. 2008. "Implication of Enterotoxigenic Escherichia Coli Pathogenicty from the Human Perspective." In *E. Coli Infections: Causes, Treatments and Preventions*, 139. New York: Nova Science Publishers.

Love, J. F., H. J. Tran-Winkler, and M. R. Wessels. 2012. "Vitamin D and the Human Antimicrobial Peptide LL-37 Enhance Group A Streptococcus Resistance to Killing by Human Cells." *mBio* 3 (5): e00394–12 – e00394–12. doi:10.1128/mBio.00394-12.

Marshall, John K., Marroon Thabane, Amit X. Garg, William F. Clark, Marina Salvadori, and Stephen M. Collins. 2006. "Incidence and Epidemiology of Irritable Bowel Syndrome After a Large Waterborne Outbreak of Bacterial Dysentery." *Gastroenterology* 131 (2): 445–50. doi:10.1053/j.gastro.2006.05.053.

Mead, P S, L Slutsker, V Dietz, L F McCaig, J S Bresee, C Shapiro, P M Griffin, and R V Tauxe. 1999. "Food-Related Illness and Death in the United States." *Emerging Infectious Diseases* 5 (5): 607–25. doi:10.3201/eid0505.990502.

Montenegro, M. a., M. Bulte, T. Trumpf, S. Aleksic, G. Reuter, E. Bulling, and R. Helmuth. 1990. "Detection and Characterization of Fecal Verotoxin-Producing Escherichia Coli from Healthy Cattle." *Journal of Clinical Microbiology* 28 (6): 1417–21.

Moskowitz, Samuel M., Robert K. Ernst, and Samuel I. Miller. 2004. "PmrAB, a Two-Component Regulatory System of Pseudomonas Aeruginosa That Modulates Resistance to Cationic Antimicrobial Peptides and Addition of Aminoarabinose to Lipid A." *Journal of Bacteriology* 186 (2): 575–79. doi:10.1128/JB.186.2.575-579.2004.

Nagaoka, I., K. Kuwahara-Arai, H. Tamura, K. Hiramatsu, and M. Hirata. 2005. "Augmentation of the Bactericidal Activities of Human Cathelicidin CAP18/LL-37-Derived Antimicrobial Peptides by Amino Acid Substitutions." *Inflammation Research* 54 (2): 66–73. doi:10.1007/s00011-004-1323-8.

Nikaido, H. 2003. "Molecular Basis of Bacterial Outer Membrane Permeability Revisited." *Microbiology and Molecular Biology Reviews* 67 (4): 593–656. doi:10.1128/MMBR.67.4.593-656.2003.

Nishino, Kunihiko, Fong F. Hsu, John Turk, Michael J. Cromie, M. M S M Wösten, and Eduardo a. Groisman. 2006. "Identification of the Lipopolysaccharide Modifications Controlled by the Salmonella PmrA/PmrB System Mediating Resistance to Fe(III) and Al(III)." *Molecular Microbiology* 61 (3): 645–54. doi:10.1111/j.1365-2958.2006.05273.x.

Nixon, B T, C W Ronson, and F M Ausubel. 1986. "Two-Component Regulatory Systems Responsive to Environmental Stimuli Share Strongly Conserved Domains with the Nitrogen Assimilation Regulatory Genes ntrB and ntrC." *Proceedings of the National Academy of Sciences of the United States of America* 83 (20): 7850–54. doi:10.1073/pnas.83.20.7850.

Nizet, V. 2006. “Antimicrobial Peptide Resistance Mechanisms of Human Bacterial Pathogens.” *Curr Issues Mol Biol* 8 (1): 11–26.

Noh, D O, and S E Gilliland. 1993. “Influence of Bile on Cellular Integrity and Beta-Galactosidase Activity of Lactobacillus Acidophilus.” *Journal of Dairy Science* 76 (5): 1253–59. doi:10.3168/jds.S0022-0302(93)77454-8.

Noris, Marina, and Giuseppe Remuzzi. 2005. “Hemolytic Uremic Syndrome.” *Journal of the American Society of Nephrology : JASN* 16 (4): 1035–50. doi:10.1681/ASN.2004100861.

Normanno, G. 2011. “Current Status on the Etiology, Food Safety Implications and Control Measures in Escherichia Coli O157:H7 Infections.” In *E. Coli Infections: Causes, Treatments and Prevention*, 177–200. New York: Nova Science Publishers.

Okhanov, V. V., D. I. Bairamashvili, M. N. Trakhanova, and A. I. Miroshnikov. 1987. “Structural and Functional Investigation of Polymyxins. 1H-NMR Spectra of Polymyxin B and Its Shortened Analog.” *Antibiotiki I Medicinskaya Biotechnologiya*.

Orr, Pamela, Bev Lorencz, Rosemary Brown, Robert Kielly, Ben Tan, Donna Holton, Helen Clugstone, et al. 2009. “An Outbreak of Diarrhea due to Verotoxin-Producing Escherichia Coli in the Canadian Northwest Territories.” *Scandinavian Journal of Infectious Diseases*, July. Taylor & Francis.

Ouellette, A.J. 2006. “Paneth Cell A-Defensin Synthesis and Function.” In *Antimicrobial Peptides and Human Disease*, edited by William Shafer, 1–25. doi:10.1007/3-540-29916-5_1.

Pálffy, Roland, Roman Gardlík, Michal Behuliak, Ludevit Kadasi, Jan Turna, and Peter Celec. 2009. “On the Physiology and Pathophysiology of Antimicrobial Peptides.” *Molecular Medicine (Cambridge, Mass.)* 15 (1-2): 51–59. doi:10.2119/molmed.2008.00087.

Parkinson, John S., and Eric C. Kofoid. 2003. “Communication Modules in Bacterial Signaling Proteins,” November. Annual Reviews 4139 El Camino Way, P.O. Box 10139, Palo Alto, CA 94303-0139, USA.

Paumgartner, G., and T. Sauerbruch. 1991. “Gallstones: Pathogenesis.” *The Lancet* 338 (8775): 1117–21. doi:10.1016/0140-6736(91)91972-W.

Peleg, Adi, Chen Nadler-yona, Shani Nov, Simi Koby, Kobi Baruch, Shoshy Altuvia, Maya Elgrably-weiss, et al. 2005. “Identification of an Escherichia Coli Operon Required for Formation of the O-Antigen Capsule.” *Journal of Bacteriology* 187 (15): 5259–66. doi:10.1128/JB.187.15.5259.

Pelletier, Mark R., Leila G. Casella, Jace W. Jones, Mark D. Adams, Daniel V. Zurawski, Karsten R O Hazlett, Yohei Doi, and Robert K. Ernst. 2007. “Determination of Acyl Chain Positioning of Lipid A Isolated from A. Baumannii LPS Using Tandem Mass Spectrometry” 1: 2–7. doi:10.1002/smll.

———. 2013. “Unique Structural Modifications Are Present in the Lipopolysaccharide from Colistin-Resistant Strains of Acinetobacter Baumannii.” *Antimicrobial Agents and Chemotherapy* 57 (10): 4831–40. doi:10.1128/AAC.00865-13.

Prouty, A M, I E Brodsky, S Falkow, and J S Gunn. 2004. “Bile-Salt-Mediated Induction of Antimicrobial and Bile Resistance in Salmonella Typhimurium.” *Microbiology (Reading, England)* 150 (Pt 4). Microbiology Society: 775–83. doi:10.1099/mic.0.26769-0.

Pumbwe, Lilian, Christopher A Skilbeck, Viviane Nakano, Mario J Avila-Campos, Roxane M F Piazza, and Hannah M Wexler. 2007. “Bile Salts Enhance Bacterial Co-Aggregation, Bacterial-Intestinal Epithelial Cell Adhesion, Biofilm Formation and Antimicrobial Resistance of Bacteroides Fragilis.” *Microbial Pathogenesis* 43 (2-3): 78–87. doi:10.1016/j.micpath.2007.04.002.

Raetz, Christian R H, C Michael Reynolds, M Stephen Trent, and Russell E Bishop. 2007. “Lipid A Modification Systems in Gram-Negative Bacteria.” *Annual Review of Biochemistry* 76 (January): 295–329. doi:10.1146/annurev.biochem.76.010307.145803.

Raetz, Christian R H, and Chris Whitfield. 2002. “Lipopolysaccharide Endotoxins.” *Annual Review of Biochemistry* 71 (January): 635–700. doi:10.1146/annurev.biochem.71.110601.135414.

Rampone, A. J. 1972. “Bile Salt and Non-Bile Salt Components in Bile Affecting Micellar Cholesterol Uptake by Rat Intestine in Vitro.” *Journal of Physiology* 227 (3): 889–98.

Round, June L, S Melanie Lee, Jennifer Li, Gloria Tran, Bana Jabri, Talal A Chatila, and Sarkis K Mazmanian. 2011. “The Toll-like Receptor 2 Pathway Establishes Colonization by a Commensal of the Human Microbiota.” *Science (New York, N.Y.)* 332 (6032): 974–77. doi:10.1126/science.1206095.

Rubin, Erica J, Carmen M Herrera, Alexander A Crofts, and M Stephen Trent. 2015. “PmrD Is Required for Modifications to Escherichia Coli Endotoxin That Promote Antimicrobial Resistance.” *Antimicrobial Agents and Chemotherapy* 59 (4). American Society for Microbiology: 2051–61. doi:10.1128/AAC.05052-14.

Safdar, Nasia, Adnan Said, Ronald E. Gangnon, and Dennis G. Maki. 2002. “Risk of Hemolytic Uremic Syndrome After Antibiotic Treatment of Escherichia Coli O157:H7 Enteritis.” *JAMA* 288 (8). American Medical Association: 996. doi:10.1001/jama.288.8.996.

Scott, M. G., D. J. Davidson, M. R. Gold, D. Bowdish, and R. E. W. Hancock. 2002. “The Human Antimicrobial Peptide LL-37 Is a Multifunctional Modulator of Innate Immune Responses.” *The Journal of Immunology* 169 (7). American Association of Immunologists: 3883–91. doi:10.4049/jimmunol.169.7.3883.

Soncini, F C, and E a Groisman. 1996. “Two-Component Regulatory Systems Can Interact to Process Multiple Environmental Signals.” *Journal of Bacteriology* 178 (23): 6796–6801.

Sørensen, Ole E, Niels Borregaard, and Alexander M Cole. 2008. “Antimicrobial Peptides in Innate Immune Responses.” *Contributions to Microbiology* 15 (January): 61–77. doi:10.1159/000136315.

Sperandio, Vanessa, James B Kaper, Mafalda Regina Bortolini, Bianca Cruz Neves, Rogeria Keller, and Luiz R Trabulsi. 1998. “Characterization of the Locus of Enterocyte Effacement (LEE) in Different Enteropathogenic Escherichia Coli (EPEC) and Shiga-Toxin Producing Escherichia Coli (STEC)

Serotypes." *FEMS Microbiology Letters* 164 (1). The Oxford University Press: 133–39. doi:10.1111/j.1574-6968.1998.tb13078.x.

Stathopoulos, C. 1998. "Structural Features, Physiological Roles, and Biotechnological Applications of the Membrane Proteases of the OmpT Bacterial Endopeptidase Family: A Micro-Review." *Membrane and Cell Biology* 12 (1): 1–8.

Thomassin, Jenny Lee, John R. Brannon, Julienne Kaiser, Samantha Gruenheid, and Hervé Le Moual. 2012. "Enterohemorrhagic and Enteropathogenic Escherichia Coli Evolved Different Strategies to Resist Antimicrobial Peptides." *Gut Microbes* 3 (6): 556–61.

Thomassin, Jenny Lee, Mark J. Lee, John R. Brannon, Donald C. Sheppard, Samantha Gruenheid, and Hervé Le Moual. 2013. "Both Group 4 Capsule and Lipopolysaccharide O-Antigen Contribute to Enteropathogenic Escherichia Coli Resistance to Human A-Defensin 5." *PLoS ONE* 8 (12): 1–13. doi:10.1371/journal.pone.0082475.

Thomassin, Jenny-Lee, John R Brannon, Bernard F Gibbs, Samantha Gruenheid, and Hervé Le Moual. 2012. "OmpT Outer Membrane Proteases of Enterohemorrhagic and Enteropathogenic Escherichia Coli Contribute Differently to the Degradation of Human LL-37." *Infection and Immunity* 80 (2): 483–92. doi:10.1128/IAI.05674-11.

Trent, M. Stephen, Anthony a. Ribeiro, Shanhua Lin, Robert J. Cotter, and Christian R H Raetz. 2001. "An Inner Membrane Enzyme in Salmonella and Escherichia Coli That Transfers 4-Amino-4-Deoxy-L-Arabinose to Lipid A: Induction in Polymyxin-Resistant Mutants and Role of a Novel Lipid-Linked Donor." *Journal of Biological Chemistry* 276 (46): 43122–31. doi:10.1074/jbc.M106961200.

Vance, Russell E., Ralph R. Isberg, and Daniel a. Portnoy. 2009. "Patterns of Pathogenesis: Discrimination of Pathogenic and Nonpathogenic Microbes by the Innate Immune System." *Cell Host and Microbe* 6 (1). Elsevier Inc.: 10–21. doi:10.1016/j.chom.2009.06.007.

Wilson, C. L. 1999. “Regulation of Intestinal -Defensin Activation by the Metalloproteinase Matrilysin in Innate Host Defense.” *Science* 286 (5437): 113–17. doi:10.1126/science.286.5437.113.

Wong, Craig S., Srdjan Jelacic, R Habeeb, S Watkins, and Phillip Tarr. 2000. “The Risk of Hemolytic-Uremic Syndrome after Antibiotic Treatment of Escherichia Coli O157:H7 Infections.” *N Engl J Med* 342 (26): 1930–36. doi:10.1056/NEJM200006293422601.

Yan, Aixin, Ziqiang Guan, and Christian R H Raetz. 2007. “An Undecaprenyl Phosphate-Aminoarabinose Flippase Required for Polymyxin Resistance in Escherichia Coli.” *The Journal of Biological Chemistry* 282 (49): 36077–89. doi:10.1074/jbc.M706172200.

Zhou, Zhimin, Shanhua Lin, Robert J Cotter, and Christian R H Raetz. 1999. “Lipid A Modifications Characteristic of Salmonella Typhimurium Are Induced by NH 4 VO 3 in Escherichia Coli K1.” *Biochemistry* 274 (26): 18503–14.