PmrC and CptA-mediated regulation of outer membrane vesicle production in *Citrobacter rodentium*

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Abbreviations

A/E- attaching and effacing

AMP- antimicrobial peptide

DC- detergent compatible

Dsb- disulfide bonding protein

HK- histidine sensor kinase

HUS- hemolytic uremic syndrome

IM- inner membrane

KDO- 3-Deoxy-D-manno-oct-2-ulosonic acid

L-ara4N- aminoarabinose

LEE- locus of enterocyte effacement

Lpp- Braun's lipoprotein

LPS- lipopolysaccharide

LB- Luria-Bertani

OM- outer membrane

OMP- outer membrane protein

OMPLA- phosphoplipase A

OMV- outer membrane vesicle

PAMP- pathogen associated molecular pattern

PBS- phosphate buffered saline

pEtN- phosphoethanolamine

PG- peptidoglycan

PQS- 2-heptyl-3-hydroxy-4-quinolone

RR- response regulator

TCS- two-component system

TEM- transmission electron microscopy

Tir- translocated intimin receptor

TLR- toll-like receptor

TMCH- transmissible murine colonic hyperplasia

T3SS- type 3 secretion system

Contributions of Authors

Chapter 1

This chapter was written by AS and edited by SG and FV.

Chapter 2

This chapter was written by AS and HLM and edited by SG and FV. All experiments were performed by AS, except transmission electron microscopy imaging, which was performed by SN. Analysis of electron microscope images was performed by AS.

Chapter 3

This chapter was written by AS and edited by SG and FV.

Appendix

All experiments were performed by AS.

Abstract

Outer membrane vesicles (OMVs) are spherical structures produced by all Gram-negative bacteria. Since they are derived from the cell envelope, OMVs contain several periplasmic and OM proteins and lipids, which are important in host-pathogen and interbacterial interactions. While the functions of OMVs are fairly well defined, the mechanisms of OMV biogenesis remain poorly understood. In the murine pathogen, *Citrobacter rodentium,* PmrC and CptA, which are believed to add phosphoethanolamine (pEtN) to the lipid A and core regions of lipopolysaccharide (LPS) are regulated by the PmrAB two-component system (TCS). These enzymes are important in maintaining OM integrity and providing resistance against antimicrobial peptides and antibiotics. We hypothesize that PmrC and CptA regulate OMV production in *C. rodentium*. As measured by total protein and lipid content of OMV preparations, we show that iron, which induces the PmrAB TCS, increases OMV production. We also found that at various concentrations of iron, there was increased OMV production in Δ*pmrC* Δ*cptA* and Δ*pmrAB* strains in comparison to wild type, suggesting that PmrC and CptA are negatively regulating vesicle formation. Furthermore, we determined that *C. rodentium* OMVs contain the OM protease CroP and the periplasmic enzyme ß- lactamase. Our data suggests a novel mechanism by which enteric pathogens may negatively regulate OMV formation through modification of LPS by pEtN. By elucidating the processes which govern OMV biogenesis, we can pursue new avenues in the development of antibiotics and vaccines against Gram-negative pathogens.

Résumé

Les vésicules de membrane externe (OMV) sont des structures sphériques produites par toutes les bactéries à Gram négatif. Puisqu'elles dérivent de l'enveloppe des cellules, les OMV contiennent plusieurs protéines périplasmiques, de la membrane externe et lipides qui sont importants dans les interactions hôte-pathogène et inter-bactériennes. Tandis que les fonctions des OMV sont assez bien connues, les mécanismes de la biogénèse des OMV demeurent mal compris. Chez le pathogène murin, *Citrobacter rodentium*, les transférases de phosphoethanolamine (pEtN), PmrC et CptA, ajoutent le pEtN au lipide A et aux régions du noyau du lipopolysaccharide (LPS) et sont contrôlées par le système à deux composants (TCS) PmrAB. Ces modifications sont importantes pour le maintien de l'intégrité de la membrane externe et contribuent à la résistance contre les peptides antimicrobiens et les antibiotiques. Nous présumons que PmrC et CptA contrôlent la production d'OMV chez *C. rodentium*. Nous avons constaté que le fer, qui active le TCS PmrAB, augmente la production d'OMV, mesurée par la quantité de protéine totale et la teneur totale en lipide des préparations d'OMV. Nous avons également constaté qu'à chaque concentration de fer, il y avait production accrue d'OMV dans les souches Δ*pmrC* Δ*cptA* et de Δ*pmrAB* de par rapport au type sauvage, suggérant que les modifications de pEtN régulent négativement la formation de vésicule. En outre, nous avons déterminé que les OMV de *C. rodentium* contiennent la protéase de membrane externe CroP et l'enzyme périplasmique ß- lactamase. Nos données présentent un nouveau mécanisme par lequel la production d'OMV est négativement affectée, par des modifications de pEtN sur le LPS. En élucidant les processus qui contrôlent la biogénèse d'OMV, nous pouvons poursuivre de nouvelles avenues dans le développement des antibiotiques et de nouveaux vaccins contre les pathogènes à Gram négatif.

Preface to Chapter 1

Chapter 1 will present an overview of various concepts in the literature that form the rationale of this investigation. Gram-negative bacteria, including *C. rodentium* will be introduced, followed by a description of the structure and roles of the OM and LPS. This chapter will then highlight the importance of OMVs and the current theories which surround OMV biogenesis. Finally, the rationale and hypothesis of this thesis will be outlined.

Chapter 1: Literature Review

1.1 *Enterobacteriaceae*

Enterobacteriaceae are a family of Gram-negative bacteria, part of the phylum *Proteobacteria.* This family consists of commensal and pathogenic bacilli, which are non-sporulating, aerobic or facultative anaerobic and often inhabit the gastrointestinal tract, but can also be found in soil and water environments [1]. Several *Enterobacteriaceae* are prominent human pathogens including *Escherichia coli, Salmonella typhimurium*, and *Yersinia pestis.* In fact, several of these bacteria are multi-drug resistant, and the *Enterobacteriaceae* family represents the largest group responsible for hospital-acquired infections [2]. Among this family, *E. coli* is one of the most widely studied and impactful bacteria to human health.

1.1.1 *Escherichia coli*

E. coli is the most abundant facultative anaerobic species in the human gut [3]. In most cases, *E. coli* exists cooperatively with its human host, rarely causing disease. However, several *E. coli* pathotypes have acquired virulence factors necessary to cause a range of disease in their human hosts with different clinical outcomes. These *E. coli* pathotypes tend to have shared lipopolysaccharide O antigens and flagellar antigens (H antigens) and are often organized based on their ability to cause disease within the intestine (InPEC) or elsewhere in the body (ExPEC) [4]. Among the most common human extra-intestinal infections caused by *E.coli* are urinary tract infections caused by Uropathogenic *E. coli* (UPEC), and sepsis and meningitis, commonly caused by meningitis-associated *E. coli* (MNEC) [3]. Avian pathogenic *E. coli* (APEC), an extra-intestinal pathogen which infects poultry, causes respiratory disease and septicemia [5]. In humans, the most well characterized InPEC strains are: Enteropathogenic *E. coli* (EPEC), Enterohaemorrhagic *E.*

coli (EHEC), Enterotoxigenic *E. coli* (ETEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) [3]. While these pathotypes vary in how they cause infection, all are involved in causing some form of gastrointestinal disease. ETEC, EPEC, DAEC, and EAEC are the causative agents of infantile diarrhea, often in the developing world. EHEC is commonly associated with food poisoning, and EIEC causes an infection that is identical to shigellosis, which is characterized by fever and diarrehea[6].

1.1.2 Enteropathogenic and Enterohaemorrhagic *E. coli*

Of the diarrheal pathotypes of intestinal *E. coli*, EHEC and EPEC have great importance to human health. EPEC is a major pathogen in the developing world, causing highly persistent diarrhea with often fatal consequences [7]. EHEC occurs worldwide and commonly causes bloody diarrhea, which can progress to life-threatening hemolytic uremic syndrome (HUS) [8]. Much of the pathogenesis caused by EHEC is due to its production of the Shiga toxin, which inhibits protein synthesis and promotes apoptosis [9]. Despite certain differences in the pathogenesis and clinical outcomes of EHEC and EPEC, these pathogens share a common method of intestinal colonization. Both EHEC and EPEC form attaching and effacing (A/E) lesions, which are characterized by close bacterial attachment to an epithelial cell, formation of an actin-rich pedestal, and effacement of the brush border microvilli (Fig. 1). The genes responsible for the formation of these A/E lesions are carried on a 35 Kb pathogenicity island known as the locus of enterocyte effacement (LEE) [10]. The LEE genetic element encodes a type III secretion system (T3SS), which is a needle-like structure that carries several virulence factors from the cytoplasm of the pathogen directly into the host enterocyte [11]. Due to its ability to form A/E lesions, the LEE encoded T3SS is essential for virulence in EHEC and EPEC [12]. An important T3SS secreted protein is the translocated intimin

receptor (Tir), which is guided to the host cytoplasmic membrane and mediates intimate attachment to the bacterial cell [13]. While cell-line models have elucidated many of these pathogenic mechanisms of EHEC and EPEC, the ability to conduct *in vivo* studies has been inconvenienced by the fact that mice are inherently resistant to these pathogens [14].

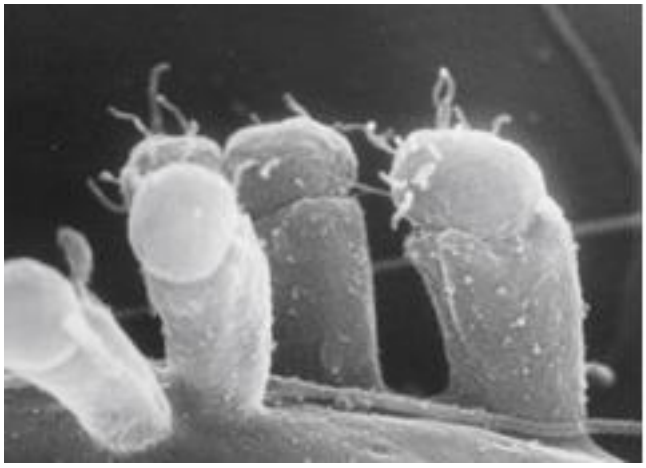

Figure 1. Attaching and effacing lesions. Scanning electron microscopy of A/E lesions formed by EPEC. A/E lesions are characterized by intimate attachment of the bacterial cell to the host enterocyte and the formation of an actin-rich pedestal. Reproduced from [15].

1.1.3 *Citrobacter rodentium*

C. rodentium is a natural Gram-negative bacterial mouse pathogen, which belongs to the *Enterobacteriaceae* family and has high genetic similarity to intestinal pathogens including EHEC, EPEC, and *Salmonella* (Fig. 2) [16]. As part of the family of A/E pathogens, *C. rodentium* contains the LEE pathogenicity island encoding a T3SS, allowing it to form A/E lesions, which are indistinguishable from those formed by EHEC and EPEC [17, 18]. In infected mice, *C. rodentium* displaces the normal colonic microflora and colonizes the descending colon 5-14 days post-infection [19]. In this time, infected mice develop transmissible murine colonic hyperplasia (TMCH),

which is characterized by thickening of the colonic mucosa and increased crypt length [20]. In cases of infection without mortality, *C. rodentium* is cleared from the colon 21-28 days post-infection [19].

Depending on the age and strain of mouse, the disease severity of *C. rodentium* infection can widely vary. In some cases, infection may manifest in self-limiting colitis, with little to no mortality [21, 22]. In more susceptible mouse strains, *C.* rodentium may cause retarded growth, dehydration, and fatal diarrhea [22]. Due to high similarities in genetic content and its process of colonization and infection, the use of *C. rodentium* as a model organism has emerged as an important tool in studying the various pathogenic mechanisms of the A/E pathogens, EHEC and EPEC [23].

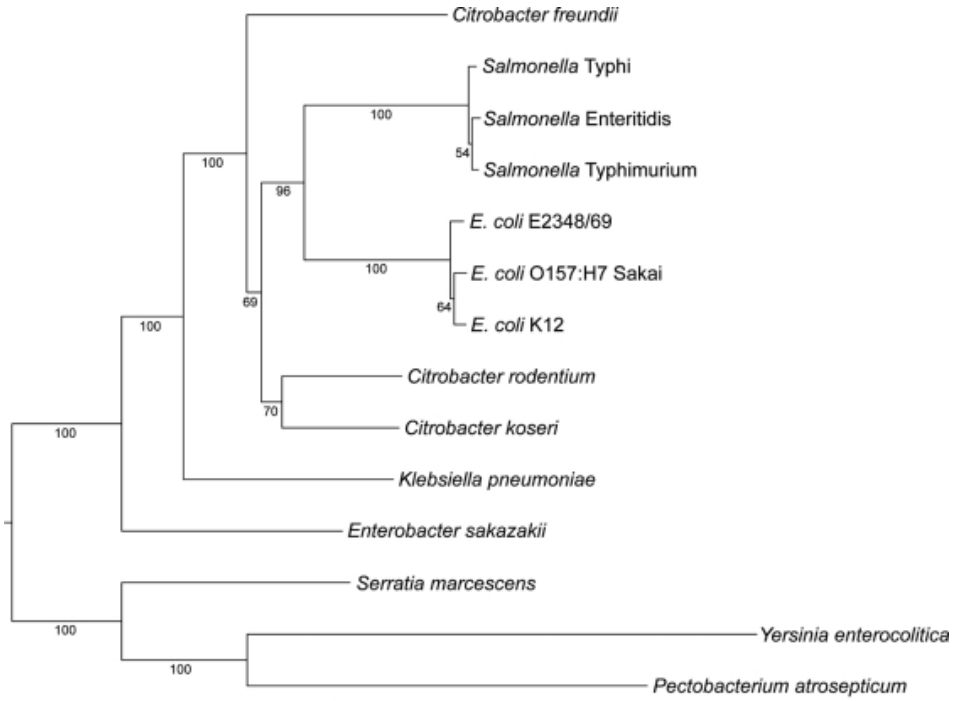

Figure 2. Phylogenetic relationship of *C. rodentium* to other enteric bacteria. Based on nucleotide similarity, *C. rodentium* is closely related to the intestinal pathogens, *Salmonella* and *E. coli*. Construction of phylogenetic tree was based on nucleotide similarities between seven housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA* and *recA*). Boostrap values as percentages are shown below the branches of the tree. Reproduced from [16].

1.2 Bacterial cell envelopes

The bacterial cell envelope is a complex structure, which provides an essential layer of protection and allows for the selective passage of nutrients and waste between the cell and the external environment. Bacterial cells face conditions that are often hostile and devoid of nutrients, highlighting the importance of a functional and organized cell envelope [24]. Bacterial cell envelopes can be generally separated into two major categories: Gram-positive and Gram-negative (Fig.3). The Gram-positive envelope consists of an inner cytoplasmic membrane (IM) surrounded by a thick layer of peptidoglycan (PG) (Fig. 3A). The Gram-negative envelope has a much thinner layer of PG, but is surrounded by an additional outer membrane (OM), which contains lipopolysaccharide (LPS) (Fig.3B). The OM of Gram-negative bacteria is important in excluding toxic molecules and providing an extra layer of physical protection, while the thick PG layer of Gram-positive bacteria is modified with anionic polymers, including teichoic acids, which provide stability to the cell envelope [25] .

1.2.1 Gram-negative cell envelope

The three main components of the Gram-negative bacterial cell envelope are: the OM, the IM, and an aqueous space in between the two membranes known as the periplasm, which contains the PG cell wall (Fig. 3B).

The IM is a phospholipid bilayer separating the periplasmic space from the cytoplasm. Most of the phospholipids in the IM are glycerophosphlipids such as phosphatidyl ethanolamine, phosphatidyl glycerol, and cardiolipin [26]. It is estimated that the IM contains up to 25% of the total proteins produced by *E. coli* [27]. Many of these proteins play important roles in energy production, lipid and protein transport and biogenesis, and signal transduction [28].

The periplasmic space, a viscous, oxidizing environment, provides Gram-negative bacteria an important compartment distinct from the cytoplasm where potentially harmful enzymes such as ribonucleases and phosphatases can elicit their enzymatic activity [29] . The oxidizing environment of the periplasm is essential to the formation of disulfide bonds, important in proper protein folding and activity. As such, several important disfulfide bonding protein (Dsb) exist in the periplasm to ensure correct protein folding prior to secretion [30] . The periplasm also contains chaperones involved in ensuring proper assembly of outer membrane proteins (OMPs) and lipoproteins in the OM. The LolA chaperone binds to the hydrophobic acyl chains of lipoproteins in the periplasm in order to protect them from the hydrophilic periplasm. LolA then transfers the lipoprotein to the LolB chaperone, which is responsible for insertion of the lipoprotein into the OM [31]. Several periplasmic chaperones including SurA, Skp, DegP, and FkpA are all involved in proper, transport, folding, and insertion of β – barrel proteins into the OM [30]. The periplasm also houses the PG cell wall, consisting of N-acetylglucosamine and N-acetylmuramic acid glycan chains, which are crosslinked by peptide bonds [32]. While much thinner than the cell wall of Gram-positive bacteria, the Gram-negative PG still provides important structural integrity to the cell envelope, dictates cell shape and helps resists turgor pressure [33, 34].

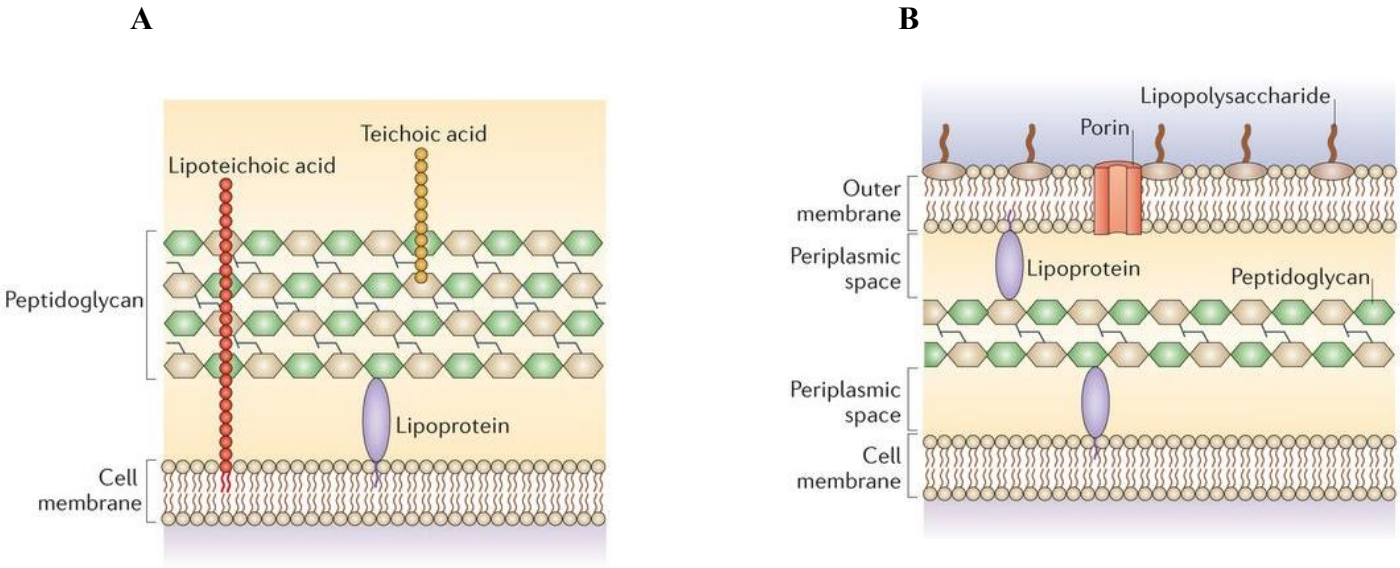

Figure 3. The Gram negative and Gram-positive cell envelopes. Cell envelopes in bacteria are generally separated into either A) Gram-positive or B) Gram-negative. A) The Gram-positive envelope contains a plasma membrane, which is surrounded by a thick layer of PG. The plasma membrane is decorated with lipoproteins and lipotechoic acids which connect to the PG. The PG contain techoic acids, which are important in regulating cell shape and stability. B) The cell envelope of Gram-negative bacteria contains the OM, the periplasm and the IM. The OM is an asymmetric bilayer consisting of phospholipids in the inner leaflet and LPS in the outer leaflet. Several integral membrane proteins and lipoproteins are found within the OM. The periplasmic space between the OM and IM contains the PG cell wall and houses several protein-folding chaperones involved in secretion and assembly. Reproduced from [35]

1.2.2 Gram-negative outer membrane

The OM is an important feature of Gram-negative bacteria, which allows for the selective passage of nutrients while preventing the entry of antimicrobial peptides (AMPs) and antibiotics. Unlike the IM, the OM is an asymmetric membrane, containing phospholipids in the inner leaflet and glycolipids such as LPS in the outer leaflet (Fig. 3B). LPS is particularly important in maintaining the barrier function of the OM by promoting envelope stability and preventing the diffusion of toxic hydrophobic molecules into the bacterial cell [36].

The OM is abundant with proteins, which are generally divided into two categories: β-barrel OMPs and lipoproteins. β-barrel proteins are mostly integral transmembrane proteins that contain approximately 8-24 β-sheets [37]. These OMPs have a diverse array of functions. General diffusion porins, such as OmpC and OmpF form hydrophilic channels that allow for the passage of small molecules, and hydrophilic antibiotics into the bacterial cell [38]. Some OM porins have higher substrate specificity, such as LamB, which has high affinity to maltose and maltodextrins [39]. The high molecular weight TonB-dependent receptors function as gated channels, which permit the entry of large substrates such as iron-siderophore complexes and vitamin B12 [40]. OmpX is a small β-barrel protein, which has been shown to mediate adhesion and entry into eukaryotic cells [41]. Some OMPs such as phospholipase A (OMPLA) and OmpT are enzymes, which hydrolyze the ester linkages of membrane phospholipids and cleave foreign and endogenous proteins and peptides, respectively [42, 43].

Bacterial lipoproteins contain a lipid-modified cysteine residue at the N-terminus, allowing them to be anchored into the OM. Lipoproteins are mostly localized in the inner leaflet facing the periplasm, but are sometimes found on the outer leaflet facing the external environment [44]. Lipoproteins tend to play diverse roles within bacterial physiology. Many lipoproteins, such as Braun's lipoprotein (Lpp) comprise an important structural component of the OM and are essential for growth and cell division [45]. Others are important for signaling, such as the RcsF and NlpE lipoproteins, which function in the RcsCBD and CpxRA envelope stress response pathways [46, 47]. Commensal Gram-negative bacteria, such as *Bacteroidetes* have a number of surface-exposed lipoproteins that have been found to be important in breaking down complex carbohydrates within the human gut [48].

Structurally important to the OM are protein and lipid crosslinks that exist between the OM and the underlying PG. Lpp, OmpA and the Tol-Pal system are the most abundant OM-PG crosslinks in Gram-negative bacteria [49]. Braun's lipoprotein (Lpp) is a highly abundant lipoprotein and the most abundant protein in *E. coli*, which covalently crosslinks to diaminopimelic acid residues of PG [50, 51]. The Tol-Pal system, an important component of cell-division, forms non-covalent interactions with the PG via its carboxy terminus region [52]. The porin, outer membrane protein A (OmpA) similarly provides membrane stability through non-covalent interactions with PG [53]. Strains which have defects in the ability to form these three crosslinks tend to exhibit increased envelope instability and susceptibility to various toxic agents [54, 53].

1.3 Lipopolysaccharide (LPS)

The barrier function provided by LPS is essential to the survival of Gram-negative bacteria in harsh environments. The protective advantage offered by LPS is in large part due to its negative charge, which allows the OM to interact with divalent cations, increasing the rigidity of the cell envelope [55]. The core region of LPS is particularly important in providing intrinsic resistance to hydrophobic antibiotics [56]. Several antibiotics and cationic AMPs that target Gram-negative bacteria function by disrupting the interactions between LPS and these divalent cations [57].

The LPS molecule is comprised of 3 moieties: lipid A, core oligosaccharide, and O-antigen (Fig. 4). Lipid A is the highly hydrophobic and endotoxic portion of the LPS molecule. Covalently attached to lipid A is the heterogeneous core oligosaccharide, which is made up of 6 to 10 sugar molecules comprising a highly conserved inner and more variable outer core [36] . At the distal end of the LPS is the O-antigen, consisting of several oligosaccharide repeats, which are the target of host antibody responses [58].

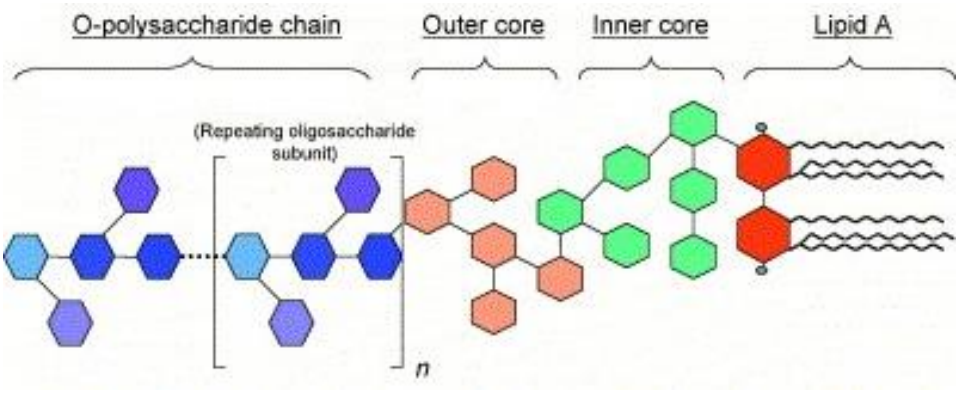

Figure 4. Structure of bacterial lipopolysaccharide. Bacterial LPS is composed of 3 main structures: A hydrophobic, endotoxic lipid A molecule, an inner and outer core oligosaccharide, and an antigenic repeating O-polysaccharide. Reproduced from [36].

1.3.1 Lipid A

Lipid A is the innermost portion of the LPS molecule, embedded within the outer leaflet of the OM. The general structure of lipid A is a glucosamine disaccharide with several attached hydrophobic acyl chains. The biosynthesis of lipid A is catalyzed by nine enzymatic reactions and begins with the UDP-N-acetylglucosamine and β-hydroxymyristoyl-ACP substrates [59]. LpxC, which deacetylates the lipid A precursor, is likely the control point of this pathway [60]. The LpxD acyltransferase, LpxH pyrophosphatase and LpxB disaccharide synthase then act on this precursor molecule to form the lipid A disaccharide [61, 62, 63]. Phosphate and 3-Deoxy-D-manno-oct-2-ulosonic acid (KDO) moieties are added to this molecule by LpxK and WaaA, respectively [64, 65]. Finally, the LpxL and LpxM acyltransferases complete the formation of the hexa-acylated lipid A disaccharide [66, 67].

Due to the conserved nature of lipid A in Gram-negative bacteria, these molecules are pathogen associated molecular patterns (PAMPs), which are powerful activators of the innate immune system. Lipid A is recognized by innate immune cells through the toll-like receptor (TLR4)/MD2 receptor complex [68]. Upon binding to TLR4/MD2, signal transduction is mediated via the cytoplasmic toll/interleukin-1 receptor homology domains, which recruit intracellular adaptor proteins that initiate downstream inflammatory cytokine production [69]. Picomolar amounts of lipid A are sufficient to initiate the production of pro-inflammatory cytokines [70]. In cases such as bacteremia where there are high amounts of lipid A in the bloodstream, septic shock can often develop [71]. Septic shock is caused by an uncontrolled immune response to PAMPs, leading to production of pro-inflammatory cytokines and organ dysfunction [72].

1.3.2 Core oligosaccharide

The core oligosaccharide tends to be well conserved within a given species. For instance, *E. coli* has 5 conserved core types: R1, R2, R3, R4, and K-12 [73]. The R1 core is generally associated with extra-intestinal *E. coli* infection, while the R3 core type has been associated with EHEC [74]. The structure of the core oligosaccharide is separated into a conserved inner core region and a more variable outer core region [36]. Biosynthesis of the inner and outer core occurs by sequential glycosyl transfer of nucleotide sugars to the lipid A molecule by genes found in the *waa* chromosomal region [73]. Proximal to the lipid A, the inner core region primarily contains heptose and KDO residues [73]. As discussed earlier, WaaA catalyzes the addition of KDO to the disaccharide head groups of lipid A, forming a glyosidic, acid-labile bond [64]. This primary KDO residue is highly conserved and is essential for bacterial cell viability, making it an obvious antibiotic target [75]. The outer core consists of hexose sugars such as glucose, galactose, and N-

acetyl-glucosamine. In *Salmonella* and *E. coli,* the first sugar in the outer core is commonly a glucose residue [73]. While the hexose backbone of the outer core remains conserved in *Enterobacteriacea,* the linkages, positions, and side chains of these sugars is highly variable [76] .

1.3.3 O-antigen

The O-antigen is the outermost, most highly variable region of LPS. It consists of repeating sugar subunits, usually between 3 and 5 sugars in length. It is suspected that 20 different sugars can make up the O-antigen, many of which are dideoxyhexoses such as paratose and tyvelose, which are rarely found in biological membranes [77]. Gene clusters involved in O-antigen biosynthesis are generally responsible for the synthesis of nucleotide sugar precursors, glycosyl transfer, translocation across the inner membrane, and polymerization of the oligosaccharide [78]. The WaaL ligase found on the inner membrane is responsible for joining the core oligosaccharide and the O-antigen [79]. The diverse nature of O-antigens provides different functions to different Gram-negative species. The Lewis blood group antigens found on the *Helicobacter pylori* O-antigen promote adhesion to the gastric epithelium of host cells [80]. Depending on the structure of the O-antigen in *Salmonella,* it can provide protection against complement-mediated cell lysis [81]. In *Shigella flexneri*, the O-antigen is thought to be important in correct polar localization of the IcsA protein, important in intracellular motility of the bacterium [82]. Along with flagellar H-antigens, the structure of the O-antigen is used for serotyping Gram-negative genera. In *Salmonella* alone over 2,600 serotypes have been identified [83]. Classifying bacteria by serotypes has been useful in tracking the sources of infectious outbreaks [83].

1.3.4 Two-component systems

The different structural components of LPS are often covalently modified in order to respond to environmental changes. A number of these covalent modifications are regulated by the PhoPQ and PmrAB two-component systems (TCS), which sense changes in the environment and respond by differentially regulating a number of genes. The general components of a TCS are an IM bound histidine sensor kinase (HK) and a cytoplasmic response regulator (RR). Upon ligand binding or environmental stimuli, there is auto-phosphorylation of the HK and phosphoryl transfer to the RR, changing its ability to bind to DNA and regulate target genes [84]. The PhoPQ and PmrAB TCSs, found in several *Enterobacteriaceae*, respond to different environmental conditions (Fig. 5). Both of these TCS' are required for virulence in *Salmonella* and have been implicated in resistance against antibiotics and AMPs [85, 86]. The PhoPQ TCS is activated in conditions of low Mg^{2+}, while PmrAB is activated in high concentrations of ferric (Fe^{3+}) iron [87, 88, 89].

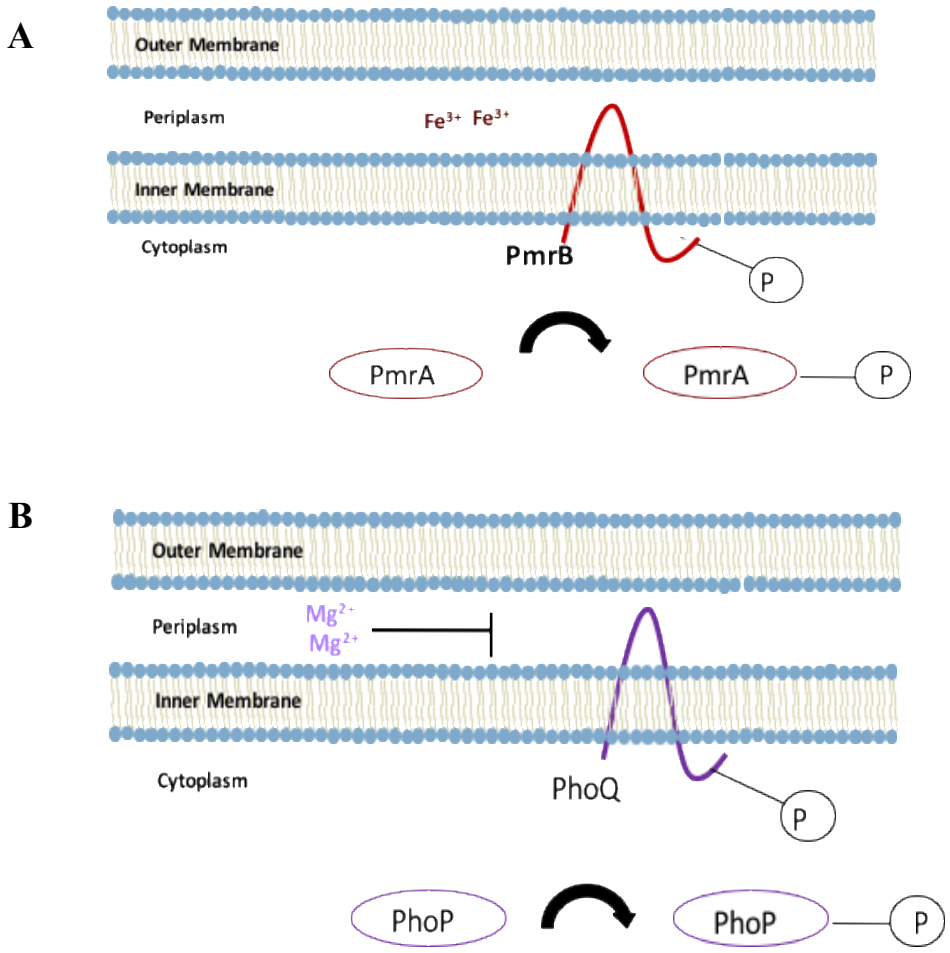

Figure 5. The PmrAB and PhoPQ two-component systems. Different environmental stimuli activate the PmrAB and PhoPQ TCS'. A) The PmrAB TCS is activated in high concentrations of Fe $^{3+}$. B) The PhoPQ TCS is activated in conditions of low magnesium. High concentrations of magnesium inhibit activation of this system, as noted by the inhibitory arrow. Upon activation of each TCS, auto-phosphorylation of the inner membrane bound histidine sensor kinase results in phosphorylation of the cytoplasmic response regulator and differential regulation of downstream gene targets.

In *C. rodentium,* PmrAB regulates genes involved in resistance against iron toxicity [90]. Iron is important for the growth of many bacteria. As such, bacteria use iron binding siderophores to acquire free iron from their environment. *E. coli* and other enteric bacteria possess siderophores known as enterobactins, which have high stability constants with Fe^{3+} [91]. While moderate concentrations of iron are generally beneficial to bacterial growth, high concentrations of iron can be toxic due to the participation of ferrous iron (Fe^{2+}) in the Fenton reaction [92]. In this reaction, hydrogen peroxide, which is a mildly reactive by-product of aerobic respiration, oxidizes Fe^{2+} to produce Fe^{3+} and hydroxyl radicals (Fig. 6). These hydroxyl radicals produced in the Fenton reaction are highly damaging to biological macromolecules [93]. It has been reported that the concentration of free iron in portions of the gastrointestinal tract are in μM range , which is PmrAB-activating [94, 89]. As such, PmrAB-mediated protection against iron is potentially relevant during *C. rodentium* infection.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + \bullet OOH + H^+$$

Figure 6. Fenton reaction. The Fenton reaction occurs when Fe^{2+} iron is oxidized by hydrogen peroxide to generate •OH. These radicals are highly damaging to biological macromolecules. Reproduced from [95]

1.3.5 Lipopolysaccharide modifications

A majority of covalent LPS modifications in Gram-negative bacteria occur on the lipid A and inner core oligosaccharide. While these modifications offer several distinct advantages, they are generally involved in increasing membrane stability and providing resistance against foreign

toxic compounds. A list of several LPS modifications in Gram-negative bacteria is shown in table 1.

The PmrAB TCS regulates the *arn* operon, and *pmrC* and *cptA*, which are genes responsible for the addition of 4- aminoarabinose (L-ara4N) and phosphoethanolamine (pEtN) to the lipid A head and core oligosaccharide in a number of *Enterobacteriaceae* [96]. The genes which comprise the *arn* operon are responsible for both the biosynthesis and incorporation of L-ara4N into the lipid A [97]. PmrC and CptA are both pEtN transferases, which add these groups to the lipid A and core regions of the LPS, respectively [98, 99]. Both L-ara4N and pEtN molecules possess amine groups, which neutralize the negative charge of the LPS. Anionic repulsion in the OM is known to cause increased membrane instability [100, 101]. Bridging divalent cations such as Mg^{2+} and Ca^{2+} play an important role in neutralizing these negative charges and maintaining OM integrity [101]. It is believed that L-ara4N and pEtN could play a similar role in neutralizing anionic repulsion and the associated membrane instability. Supporting this idea, PmrC and CptA have been shown to be important in maintaining OM integrity in *C. rodentium* [90]. Additionally, both L-ara4N and pEtN modifications have been shown to promote resistance to several AMPs and antibiotics in different enteric pathogens [102, 103, 90].

Other modifications of the lipid A alter the structure of the acyl chains. Regulated by PhoPQ, PagL deacylates the *R*-3-hydroxymyristoyl chain at position 3 of lipid A in *Salmonella* [104]. PagL has been shown to induce changes in membrane curvature and cause hypervesiculation [105]. The palmitoyltransferase PagP uses phospholipids as donors to add palmitate groups to the lipid A [106]. PagP has been shown to offer protection against cationic AMPs and prevent complement-mediated lysis [107, 108]. The biological function of the LpxO dioxygenase, which hydroxylates the 3' secondary acyl chain of lipid A has yet to be determined [109].

While the core oligosaccharide possesses less covalent modifications than lipid A, the *waaY* and *waaP* kinases and the *pmrG* phosphatase act on the inner core to alter the phosphorylation state of the molecule [110]. WaaP and WaaY add phosphate groups to the 1st and 2nd heptose molecules of the inner core, increasing the stability of the OM and contributing resistance to the AMP, LL-37 [111, 112]. PmrG removes phosphate groups on core heptose (II) molecule added by the WaaY kinase. The consequences of this phosphatase activity remain unknown, as a Δ*pmrG* strain was shown to exhibit wild-type levels of resistance to polymyxin and Fe^{3+} [110].

Gene name	Function	Regulation
pmrC	pEtN transferase to lipid A [103]	PmrAB TCS
pmrG	Core heptose (II) phosphatase [110]	PmrAB TCS
cptA	pEtN transferase to core heptose (I) [99]	PmrAB TCS
pagP	Palmitate biosynthesis and transferase to lipid A [113]	PhoPQ TCS
pagL	Lipid A deacylation [104]	PhoPQ TCS
waaP	Core heptose (I) kinase [111]	N/A
waaY	Core heptose (II) kinase [112]	N/A
arn operon	L-ara4N biosynthesis and transferase [114]	PmrAB TCS
lpxO	Hydroxylates lipid A acyl chains [115]	RamA TCS

Table 1. LPS modifications and functions in Gram-negative bacteria. The genes which modify LPS in bacteria, their target structure and their mode of regulation by TCSs. N/A indicates that the indicated gene has not been found to be regulated by a TCS.

1.3.6 Phosphoethanolamine modifications in *C. rodentium*

LPS produced by *C. rodentium* have not been well studied. However, the *C. rodentium* genome contains many of the genes found in other pathogenic enteric bacteria which modify the lipid A and core regions of the LPS. Particularly, pEtN LPS modifications are especially useful to study in *C. rodentium* since, unlike most enteric bacteria, these bacteria lack the *arn* operon, which catalyzes the similar addition of L-ara4N to LPS [116]. In several bacteria, PmrC and CptA are the pEtN transferases which add pEtN to LPS [103, 99]. Due to amino acid similarity, it is suspected that these enzymes similarly modify LPS in *C. rodentium*. However, structural and functional studies of *C. rodentium* LPS have yet to be performed to confirm that this is the case. In a recent study from our lab, *pmrC* and *cptA* were shown to play an important role in maintaining OM integrity in *C. rodentium*. Δ*pmrC* Δ*cptA* strains were shown to exhibit increased efflux of fluorescent dyes and increased leakage of the periplasmic protein ß- lactamase in the supernatant, suggesting that the barrier function of these strains is compromised [90]. Additionally, these strains showed decreased resistance to iron and several lipophilic antibiotics [90].

1.4 Outer membrane vesicles (OMVs)

The production of spherical vesicles is conserved by cells throughout all domains of life. Gram-negative bacteria ubiquitously produce outer membrane vesicles (OMVs), spherical structures 20-250 nm in size, which form when portions of the OM bud from the cell envelope [117]. OMVs were first discovered by Chatarjee and Das when they observed in electron microscope (EM) images that localized portions of the OM were bulging off from *Vibrio cholerae* cells [118] (Fig. 7). Initially, these findings were met with skepticism, with many suggesting that these bulges

were due to cell lysis or improper fixation of samples during electron microscopy [119] . In the following years, more vigorous EM protocols and careful monitoring of cell lysis confirmed that these spherical structures were indeed portions of the OM budding off of the cell [120]. After similar observations were performed for other bacteria, OMV formation became universally regarded as a novel form of secretion for Gram-negative bacteria [119]. Since their discovery, more recent studies on OMVs have primarily focused on their potential functions in bacterial physiology and pathogenesis, the mechanism of their formation, and their role in vaccine development.

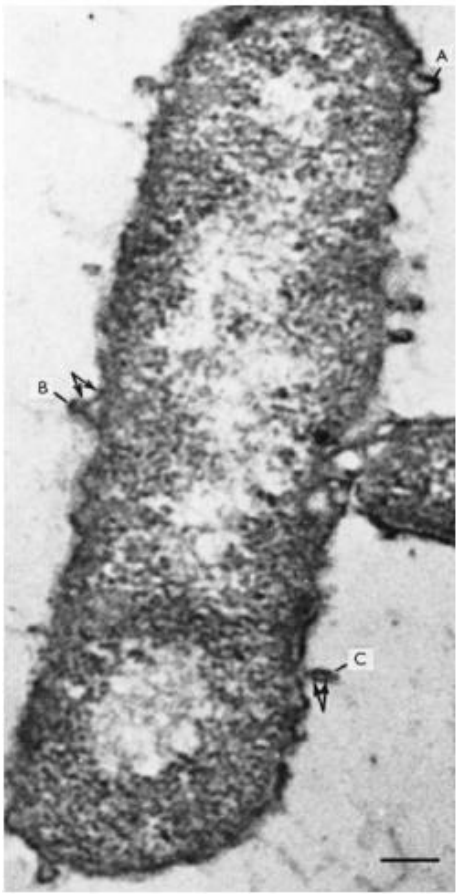

Figure 7. Discovery of outer membrane vesicles. Chatarjee and Das observed regions of the OM bulging from *V. cholerae* cells in the logarithmic phase of growth. Future studies have confirmed that these bulges are OMVs, secreted portions of the OM produced by all Gram-negative bacteria.

The sequence of bulging is indicated by A, B, and C. Scale bar represents 100 nm. Reproduced from [118].

Since they are products of the cell envelope, OMV cargo primarily includes contents from the periplasm and OM, including LPS, OM porins, toxins, proteases, PG, and signaling molecules (Fig. 8) [117]. Small amounts of DNA and RNA have also been observed within OMVs [121, 122]. Interestingly, several proteins and modified LPS species have been shown to be preferentially included or excluded from OMVs in certain environments, suggesting that the formation of these vesicles is a regulated, non-stochastic process [123, 124, 125].

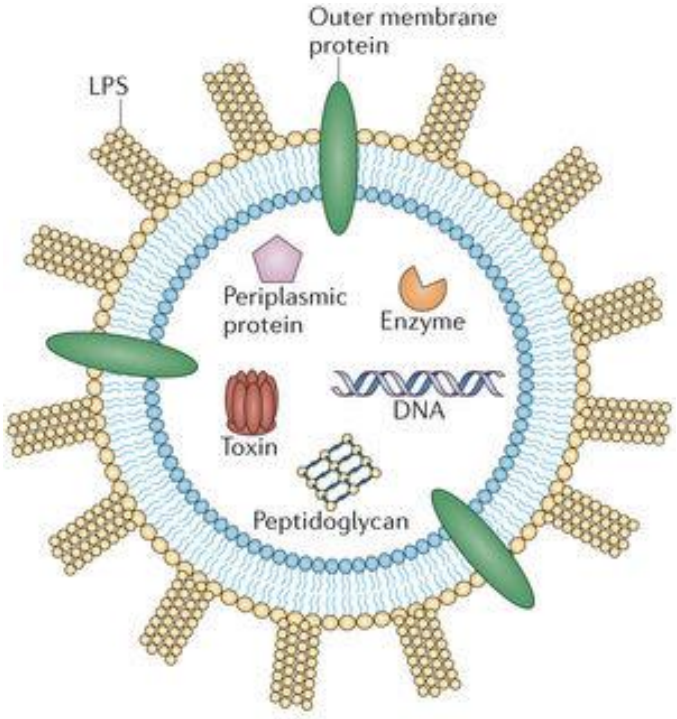

Figure 8. Outer membrane vesicles. OMVs are produced when sections of the cell envelope bud from the parent bacterial cell. The resultant vesicles contain mostly periplasmic and OM material, including LPS, OM proteins, PG, nucleic acids and different toxins and enzymes. Reproduced from [126].

1.4.1 Functions of OMVs

The constitutive secretion of OMVs has a number of consequences in terms of bacterial pathogenesis, physiology and interbacterial communication. Pathogenic Gram-negative bacteria, often secrete OMVs as a means to package and deliver toxins to host cells. For instance, EHEC OMVs, which carry Shiga toxin and hemolysin, are taken up by host endothelial cells via clathrin-mediated endocytosis and trigger apoptotic cell death [127]. Similarly, in a murine model, administration of OMVs from UPEC were sufficient to cause sepsis-induced cardiac dysfunction [128].

OMV proteomic studies have also revealed a potential role for OMVs in mediating interbacterial communication, as the coral pathogen *Vibro shilonii* was shown to secrete OMVs carrying quorum sensing autoinducers [129]. Furthermore, it is suspected that OMVs may also contribute to horizontal gene transfer, as the transfer of Carbapenem resistance genes has been demonstrated between different *Acinetobacter* isolates in an OMV-dependant manner [130].

The secretion of OMVs may be beneficial as a potential stress response or defense mechanism against membrane perturbing agents. In conditions where there is increased misfolded protein in the periplasm, OMVs are released at a higher rate in order to maintain homeostasis of the cell envelope [123]. Increased vesicle production in *E. coli* has also been associated with resistance to AMPs and T4 bacteriophage infection, as OMVs may act as targets for these molecules in order to provide an extra layer of protection to the bacterial cell [131].

Since OMVs carry many of the pathogen associated molecular patterns located on the surface of bacterial cells, they are similarly able to interact with the host immune system. Particularly, LPS found on *E. coli* and *Pseudomonas* OMVs has been shown to be a strong inducer of pro-inflammatory cytokines through its interactions with TLR 4 [132, 133]. OMVs from commensal

bacteria, on the other hand, are able to promote the production of anti-inflammatory cytokines via activation of regulatory T cells. OMVs containing a capsular polysaccharide carried on the surface of *Bacterioides fragilis* was shown to prevent mice from experimentally-induced colitis by inducing regulatory T cells through activation of TLR2 and IL-10 production [134].

1.4.2 OMV biogenesis

Due to the functional diversity of OMVs, much research has gone into the role of these vesicles in disease and their potential use as novel vaccine platforms. However, the mechanistic details of vesicle production remain largely unclear. In order for portions of the OM to bud from the cell envelope and form OMVs, there must be transient dissolution of the crosslinks between the OM and the underlying PG, highlighting a role of OM integrity in the formation of OMVs. Several studies have highlighted the importance of OM-PG crosslinks and OMV formation [135, 136, 137] . In *E. coli,* the absence of the Lpp causes a loss of structural integrity of the OM and an increase in OMV formation up to 150-fold [137]. Similar to Lpp, in *E. coli* and *Acinetobacter baumanii,* mutants in the Tol-Pal system and OmpA exhibit heightened OMV formation [138, 139]. Most mutations that have been described in OMV formation tend to increase vesicle production, further supporting the idea that OM integrity influences vesicle production [123]. This suggests that the formation of OMVs may be a product of decreased integrity in the OM caused by decreased amounts of integral membrane proteins and crosslinks, rather than a process that is reliant on the induction of the curvature in the membrane, as observed in eukaryotic vesicle formation [140]. Still, OMV production is not always associated with compromised OM integrity, indicating that several mechanisms may govern this complex process [141].

Many researchers have recently suggested that OMV formation is a response to maintain homeostasis in the cell envelope in response to stress. McBroom *et. al* demonstrated that overexpression of a misfolded protein analog in the periplasm of *E. coli* induced OMV formation as a means to remove this damaging material [123]. Accumulation of PG and LPS in the cell envelope was similarly found to increase vesiculation [137]. In *Salmonella enterica*, OMV secretion is used as a means to remodel the OM in stressful conditions. After transitioning to environments with low pH and low Mg^{2+}, *S. enterica* secrete OMVs that have less modified LPS species, leaving highly modified species, which are beneficial in these conditions, in the OM [124]. A number of different external stress factors have been implicated in OMV formation. In *P. aeruginosa,* oxidative stress in the form of hydrogen peroxide was shown to induce OMV formation [142]. Additionally, sub-lethal concentrations of several antibiotics and AMPs can also increase the production of OMVs in different species of Gram-negative bacteria [143, 144].

Other findings suggest that OMV formation relies on curvature-inducing compounds in the OM. In *P. aeruginonsa,* the negatively charged LPS-binding molecule, 2-heptyl-3-hydroxy-4-quinolone (PQS) is thought to induce OMV production through increased anionic repulsion in the OM [145]. Accumulation of phospholipids in the outer leaflet of the OM is also thought to facilitate vesicle formation by causing asymmetric expansion of the outer leaflet, initiating bulging of the OM [146]. Certain LPS modifications also alter the curvature of the OM, leading to bulging and formation of OMVs. Overexpression of *pagL*, a lipid A deacylase changes the hydrophobic cross-sectional area of lipid A, inducing membrane curvature and subsequent OMV formation [105]. Other LPS modifications similarly affect the topology and curvature of the OM. Thus, the regulation of these modifications may be a useful tool for bacteria to modulate the formation of OMVs.

However, the precise role of many covalent LPS modifications on OMV formation remains to be studied in detail.

Rationale

The current mechanistic details of OMV formation remain ill defined. Decreased OM integrity, changes in membrane topology, and envelope stress have been shown to be the main factors which manipulate OMV production in Gram-negative bacteria.

Covalent LPS modifications regulated by the PmrAB and PhoPQ TCS' have been suggested to play a role in vesicle formation since they are involved in remodeling the OM. However, the role of these modifications in OMV formation remains largely unknown. In the murine pathogen *C. rodentium*, PmrC and CptA are suspected to add pEtN groups to the lipid A and core regions of LPS. PmrC and CptA have been shown to reduce the negative charge of LPS, provide increased resistance against envelope stressing agents, and maintain OM integrity in *C. rodentium*.

Here, we hypothesize that PmrC and CptA negatively regulate OMV production in the *C. rodentium.*

Preface to Chapter 2

Chapter 2 will highlight the main findings of this thesis in the form of a manuscript. Here, we investigate the role of the putative pEtN transferases, PmrC and CptA on OMV biogenesis. We demonstrate that these enzymes negatively regulate vesicle formation in *C. rodentium*. This manuscript is currently under revision for resubmission to the Journal of Bacteriology.

Chapter 2:

PmrC and CptA negatively regulate outer membrane vesicle production in *Citrobacter rodentium*

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Keywords: Outer membrane vesicles, LPS modifications, two-component regulatory systems, *Citrobacter rodentium*, envelope stress response, oxidative stress

Running title: pEtN LPS modifications impact OMV biogenesis

Abstract

Outer membrane vesicles (OMVs) are naturally produced by Gram-negative bacteria by a bulging of the outer membrane (OM) and subsequent release in the environment. By serving as vehicles for various cargos, including proteins, nucleic acids and small metabolites, OMVs are central to interbacterial interactions and both symbiotic and pathogenic host bacterial interactions. However, despite their importance, the mechanism of OMV formation remains unclear. Recent evidence indicates that covalent modifications of lipopolysaccharide (LPS) influence OMV biogenesis. The murine intestinal pathogen *Citrobacter rodentium* possesses a limited number of LPS-modifying genes and relies primarily on phosphoethanolamine (pEtN) LPS modifications. In wild-type *C. rodentium*, the presence of increasing subtoxic concentrations of iron was found to stimulate OMV production 4- to 9-fold above baseline. *C. rodentium* uses the two-component system PmrAB to sense and adapt to environmental iron. When compared to wild-type, OMV production by the *C. rodentium* Δ*pmrAB* strain was further increased 1.5- to 2.5-fold at similar iron concentrations. PmrAB regulates transcription of the pEtN transferase genes *pmrC* and *cptA*. OMV production by the Δ*pmrC* Δ*cptA* double mutant was increased to similar extents in comparison to Δ*pmrAB*. Importantly, plasmid-complementation of the Δ*pmrC* Δ*cptA* strain with either *pmrC* or *cptA* resulted in a drastic inhibition of OMV production. Finally, we showed that OMVs contain β-lactamase and CroP protease activities, two enzymes found in the *C. rodentium* periplasm and OM, respectively. These data suggest a novel mechanism by which *C. rodentium* and possibly other Gram-negative bacteria can negatively regulate OMV production through pEtN LPS modifications.

Introduction

Outer membrane vesicles (OMVs) are spherical lipid structures of approximately 20-250 nm in size that are shed from the surface of all Gram-negative bacteria [1, 2] . OMVs contain various cargo including periplasmic and outer-membrane (OM) proteins, toxins, enzymes, signaling molecules, lipopolysaccharide (LPS), DNA and RNA [3, 4, 5, 6]. Due to their ability to carry these cargo over distances, OMVs were attributed multiple functions in both host-bacterial and interbacterial interactions. During infection, vesicles can be taken up by host cells and induce apoptosis through the release of toxins and enzymes [7]. The pathogen-associated molecular patterns, LPS and peptidoglycan, carried by OMVs are recognized by pattern-recognition receptors to initiate the host immune response [8, 9]. OMVs can also provide defense against environmental insults by acting as decoys that prevent antibiotics, antimicrobial peptides (AMPs) and bacteriophages to reach the bacterial cell [10]. They facilitate predatory activity by delivering active enzymes to adjacent bacteria to aid in the establishment of ecological niches [11]. Also, OMVs have been shown to carry quorum sensing autoinducers and genetic material to facilitate interbacterial communication and horizontal gene transfer, respectively [4, 12, 13, 14].

Although many biological functions have been described for OMVs, the exact mechanism of OMV biogenesis remains unclear. It was first proposed that OMVs are produced when crosslinks between specific lipoproteins of the OM and the underlying peptidoglycan dissociate, resulting in OM bulges that pinch off from the cell envelope [15]. Other studies have shown that OMV production may rely on the presence of certain molecules that induce membrane curvature in the OM. For example, the intercalation of the quorum sensing molecule 2-heptyl-3-hydroxy-4-quinolone (PQS) into the *Pseudomonas aeruginosa* OM is thought to induce membrane curvature

and subsequent vesicle budding [16]. Another study proposed that the presence of phospholipids in the outer leaflet of the OM facilitates OMV formation [17]. Other evidences suggest that OMV production is an adaptive response to environmental changes. Envelope and oxidative stresses, as well as the presence of AMPs or antibiotics, have been shown to result in increased vesiculation in several Gram-negative species [10, 18, 19]. It was also proposed that OMVs are secreted to rid the cell envelope of damaged material [20, 21].

LPS is an important component of the OM that acts as a permeability barrier [22]. LPS can be covalently modified in response to environmental cues to contribute additional protection to the OM against iron toxicity, antibiotics and AMPs [23]. These LPS modifications are regulated by the PmrAB and PhoPQ two-component systems (TCS). The PmrAB TCS is activated by the presence of ferric iron (Fe^{3+}) and mildly acidic pH, while PhoPQ is activated by low concentrations of Mg^{2+}, mildly acidic pH and AMPs [24, 25, 26]. In *Salmonella enterica*, the PhoPQ and PmrAB pathways are interconnected. PhoPQ controls PmrAB by regulating expression of the *pmrD* gene, which prevents dephosphorylation and inactivation of the PmrA response regulator [27]. In *S. enterica*, PmrAB regulates the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) to lipid A by the *arn* operon [28]. It also regulates the addition of phosphoethanolamine (pEtN) to the lipid A and core portions of LPS by PmrC and CptA, respectively [29, 30]. These modifications reduce the LPS negative charge and, in turn, provide increased resistance to AMPs and iron-induced oxidative stress [28, 29, 30, 31]. PhoPQ regulates expression of the PagL protein, which is responsible for deacylation of lipid A in *S. enterica*. PagL-mediated lipid A deacylation has been found to also induce OMV production by promoting positive curvature of the OM [32].

Citrobacter rodentium is a natural murine pathogen that causes transmissible murine colonic hyperplasia [33]. It is used as a surrogate model to study the human diarrheal pathogens enterohemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* (EPEC). The defining feature of these pathogens is the formation of attaching and effacing (A/E) lesions during colonization of the gut mucosa. These A/E lesions are characterized by adherence to intestinal epithelial cells, formation of a pedestal and effacement of brush-border microvilli [33, 34]. Unlike *S. enterica*, *C. rodentium* lacks the *pmrD* gene and, therefore, the PhoPQ TCS does not interfere with expression of PmrAB-regulated genes [31]. Furthermore, *C. rodentium* lacks the PmrAB-regulated *arn* operon that is responsible for L-Ara4N addition to lipid A [31]. Thus, *C. rodentium* largely relies on the PmrAB-regulated pEtN transferases PmrC and CptA for LPS modifications [31]. We previously showed that the absence of PmrAB-regulated pEtN LPS modifications compromises OM integrity in *C. rodentium*. Strains lacking the *pmrAB*, *pmrC* or *cptA* genes exhibited increased susceptibility to iron toxicity, increased OM permeability and loss of OM integrity [31].

Environmental iron is an important contributor to bacterial oxidative stress. In the Fenton reaction, hydrogen peroxide is converted to a more reactive hydroxyl radical through the oxidation of Fe^{2+} into Fe^{3+} [35]. These reactive hydroxyl radicals are highly damaging to lipid membranes, proteins and nucleic acids [35]. In this study, we explored the hypothesis that iron-induced and PmrAB-mediated pEtN LPS modifications influence OMV biogenesis in *C. rodentium.* We showed that production of OMVs is greatly increased in *C. rodentium* strains lacking the *pmrAB* or the *pmrC* and *cptA* genes. These results further relate LPS modifications to OMV biogenesis and reveal a new role for pEtN LPS modifications.

Results

Environmental iron influences OMV production. To examine the effect of environmental iron on OMV production, the *C. rodentium* wild-type strain was cultured statically for 48 hr at 28 °C in the presence of increasing concentrations of $FeCl_2$. OMVs were isolated as described in Material and Methods. OMV production was quantified by measuring the total protein and lipid contents of purified OMVs using the DC assay and the fluorescent probe FM1-43, respectively. Total protein concentrations increased by 5- and 9-fold in the presence of 25 and 50 μM $FeCl_2$, respectively (Fig. 1A). Increasing the concentration of $FeCl_2$ to 75 μM did not significantly increase OMV production (Fig. 1A). In addition, the presence of iron at concentrations of 100 μM or higher inhibited bacterial growth due to iron toxicity [31]. Similarly, 4- and 6-fold increases in the presence of 25 and 50 μM $FeCl_2$, respectively were observed by measuring the lipid content of OMV samples (Fig. 1B). Substitution of $FeCl_2$ by $FeSO_4$ or $FeCl_3$ resulted in very similar increases in OMV production by *C. rodentium* (Fig. S1). These results indicate that environmental iron increases OMV production in *C. rodentium*.

The PmrAB TCS regulates OMV production. *C. rodentium* and related *Enterobacteriaceae* use the PmrAB TCS to sense and respond to environmental iron [24]. Therefore, the role of PmrAB in OMV production was investigated by measuring the amounts of OMVs produced by the *C. rodentium* Δ*pmrAB* strain grown in the presence of increasing concentrations of $FeCl_2$. OMVs were quantified as described above for the wild-type strain. As noticed previously for the wild-type strain, increasing iron concentrations resulted in enhanced OMV production by the Δ*pmrAB* strain. Increasing the concentration of $FeCl_2$ from 0 to 50 μM resulted in a 7- and 4-fold increases in protein and lipid contents of OMV samples, respectively (Fig. 1A and 1B). When comparing OMV production between the wild-type and Δ*pmrAB* strains, we found that at each

concentration of $FeCl_2$, the Δ*pmrAB* strain systematically exhibited higher OMV production (Fig. 1A and 1B). Collectively, these results indicate that the PmrAB TCS regulates OMV production in the presence of environmental iron.

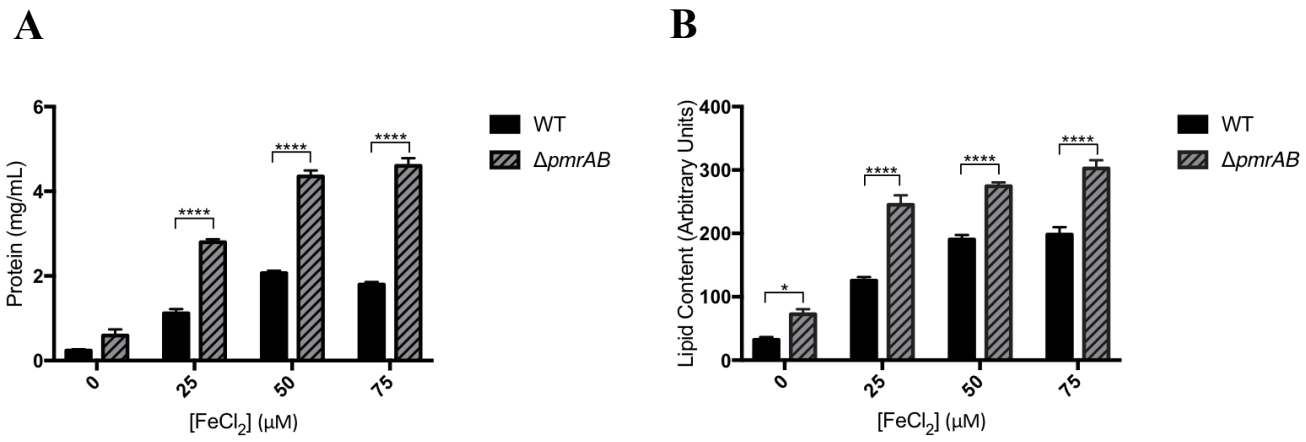

FIG 1 Deletion of the *pmrAB* TCS genes results in increased OMV biogenesis in *C. rodentium*. OMVs isolated from the *C. rodentium* wild-type and Δ*pmrAB* strains grown in the presence of increasing concentrations of iron were quantified by measuring total protein and lipid contents. (A) Total protein content of OMVs was measured using the DC protein assay. Protein concentration was calculated using BSA as a standard. (B) Total lipid content of OMV samples was determined by using the lipophilic dye, FM 1-43. Cultures were grown to mid-log phase and normalized to an OD_{595} of 0.5 prior to OMV isolation. Values shown are the means ± standard error from three independent experiments, each performed in triplicate. Significance was assessed using a two-way ANOVA, * $P \leq 0.05$, **** $P \leq 0.0001$.

The pEtN transferases, PmrC and CptA regulate OMV production. In *C. rodentium* and related species, the PmrAB TCS directly regulates transcription of the pEtN transferase genes *pmrC* and *cptA* [31] [36]. Therefore, OMV production by the *C. rodentium* Δ*pmrC* Δ*cptA* strain was measured in the presence of increasing concentrations of iron. As shown in Fig. 2, increasing the concentration of $FeCl_2$ from 0 to 50 μM resulted in 9- and 6-fold increases in protein and lipid contents, respectively, of OMV samples for the Δ*pmrC* Δ*cptA* strain. When comparing OMV production between the wild-type and Δ*pmrC* Δ*cptA* strains, we found that at each concentration

of $FeCl_2$, the Δ*pmrC* Δ*cptA* strain systematically exhibited a 2- to 3-fold increase in OMV production in comparison to the wild-type strain (Fig. 2). Complementation of the *C. rodentium* Δ*pmrC* Δ*cptA* strain with either pWSK*pmrC* or pWSK*cptA* resulted in drastic decreases of OMV production (Fig. 2). At all concentrations of iron, values of protein and lipid contents in OMV samples obtained for the two complemented strains were essentially similar to those observed for the Δ*pmrC* Δ*cptA* strain grown in the absence of $FeCl_2$ (Fig. 2). To confirm that complementation of the Δ*pmrC* Δ*cptA* strain with either plasmids results in the overexpression of the *pmrC* or *cptA* genes, the *C. rodentium* wild-type strain was transformed with the pWSK*pmrC* or pWSK*cptA* plasmids and grown in the presence of 50 μM $FeCl_2$. As expected, OMV production was found to be drastically decreased in both transformed wild-type strains, indicating that overexpression of either *pmrC* or *cptA* inhibits OMV production (Fig. S2). Altogether, these results show that the PmrAB-regulated pEtN transferases PmrC and CptA negatively affect OMV production in response to environmental iron in *C. rodentium*.

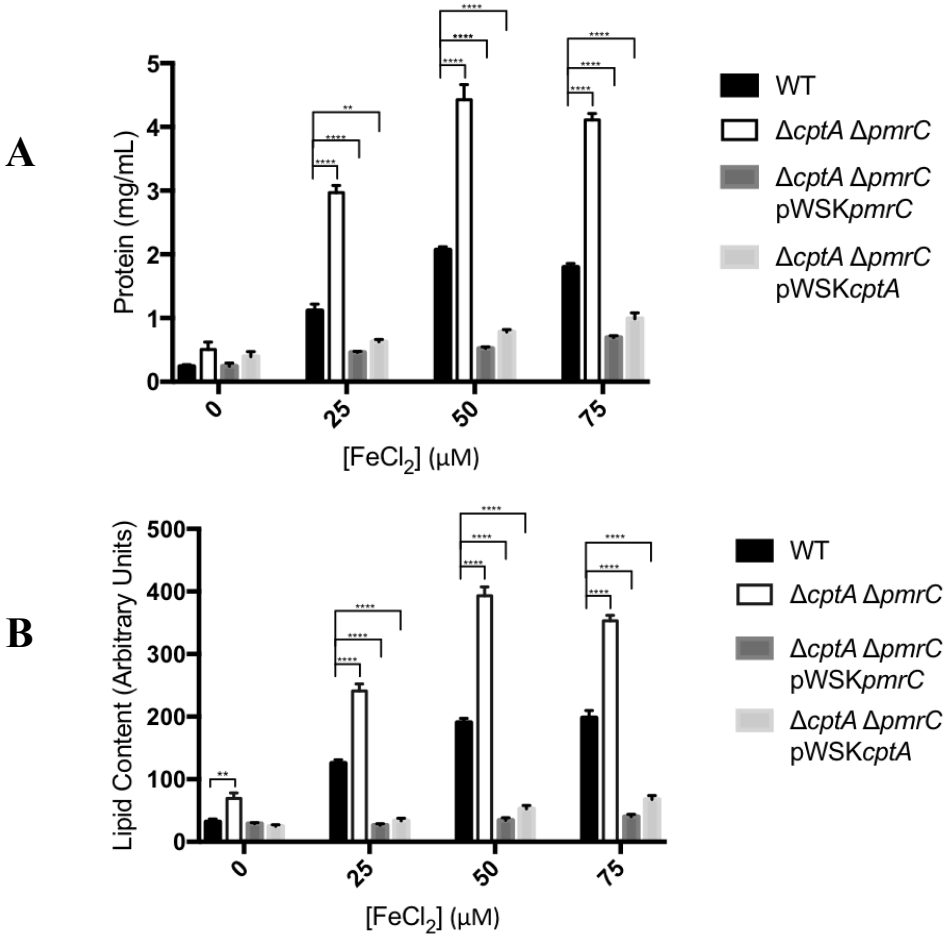

FIG 2 Deletion of the pEtN transferase genes *pmrC* and *cptA* results in increased OMV biogenesis in *C. rodentium*. The *C. rodentium* wild-type and Δ*pmrC* Δ*cptA* strains, and the Δ*pmrC* Δ*cptA* strain complemented with either pWSK*pmrC* or pWSK*cptA* were grown in the presence of increasing concentrations of iron to mid-log phase and normalized to an OD_{595} of 0.5. OMVs were isolated and quantified by measuring total protein and lipid content. (A) Total protein content of OMVs was measured using the DC assay. Protein concentration was calculated using BSA as a standard. (B) Total lipid content of OMV samples was determined by using the lipophilic dye, FM 1-43. Values shown are the means ± standard error from three independent experiments, each performed in triplicate. Significance was assessed using a two-way ANOVA, * $P \leq 0.05$, ** $P \leq 0.01$, **** $P \leq 0.0001$.

OMV quantification and size distribution by transmission electron microscopy (TEM). To determine whether increased OMV production by the *C. rodentium* Δ*pmrAB* and Δ*pmrC* Δ*cptA* strains is due to either increased number of OMVs or increased vesicle size, OMVs samples isolated from cells grown in the presence of 50 μM $FeCl_2$ were analyzed by TEM (Fig. S3). Consistent with the above data, TEM micrographs revealed a 2- to 3-fold increase in the absolute number of OMVs produced by the Δ*pmrAB* and Δ*pmrC* Δ*cptA* strains in comparison to

wild-type (Fig. 3A). Furthermore, micrographs of whole cells showed a greater number of vesicles budding from the membranes of the Δ*pmrAB* and Δ*pmrC* Δ*cptA* strains in comparison to wild-type (Fig. 4). The size distribution of OMVs isolated from the wild-type, Δ*pmrAB* and Δ*pmrC* Δ*cptA* strains showed a slightly higher proportion of OMVs between 20 and 40 nm in the Δ*pmrAB* and Δ*pmrC* Δ*cptA* strains in comparison to wild-type (Fig. 3B-D). This small difference in OMV size does not likely contribute to the observed differences in OMV production between the strains. Collectively, these data confirm that the amount of OMVs produced by *C. rodentium* is indirectly regulated by the PmrAB TCS through the action of PmrC and CptA.

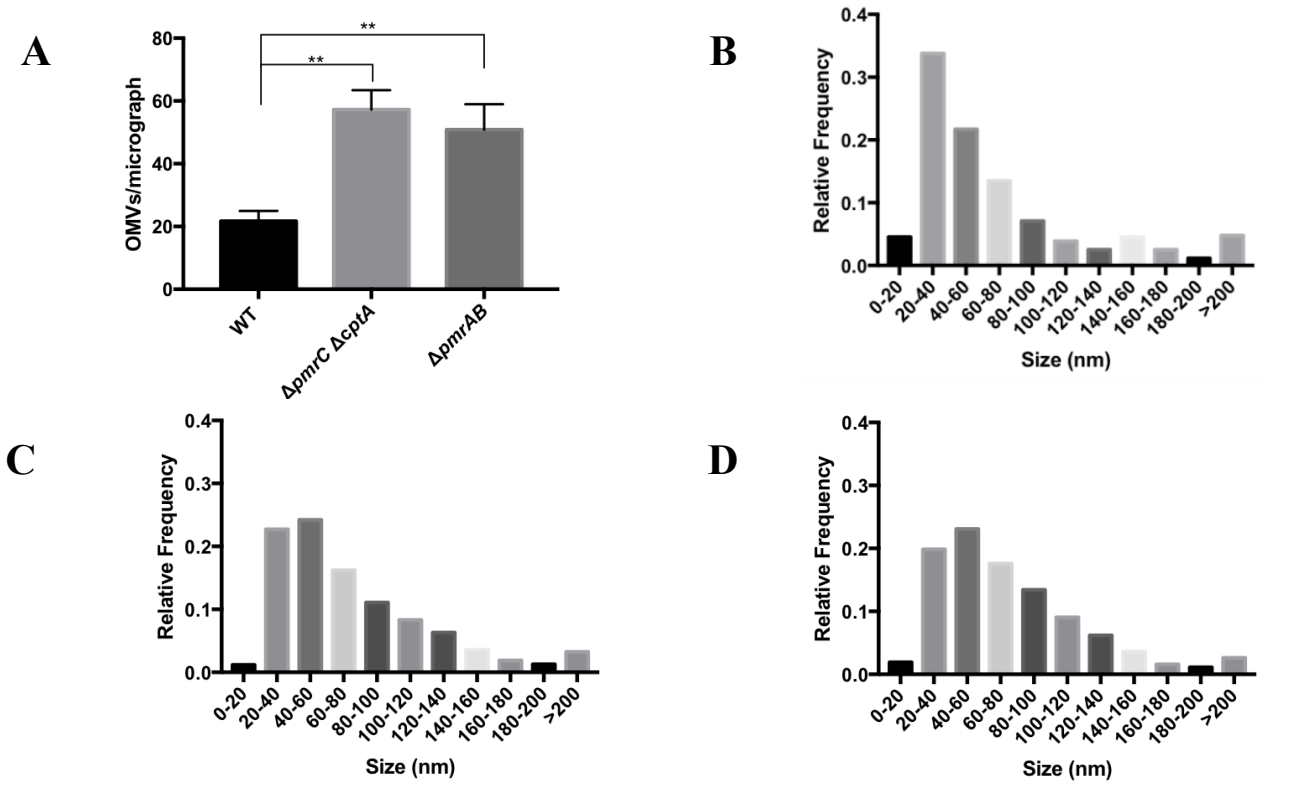

FIG 3 TEM of OMVs produced by *C. rodentium*. (A) Quantification of OMVs per micrograph. (B-D) Size distribution of OMVs. OMVs were isolated from *C. rodentium* wild-type (B), Δ*pmrAB* (C) and Δ*pmrC* Δ*cptA* (D) strains grown to mid-log phase with 50 μM $FeCl_2$. Twenty micrographs of OMVs isolated from each strain were imaged using TEM. Mean quantity and size of OMVs were determined using the Macnification software (n=1). Values shown are the means ± standard error. ** $P \leq 0.01$, two-way ANOVA.

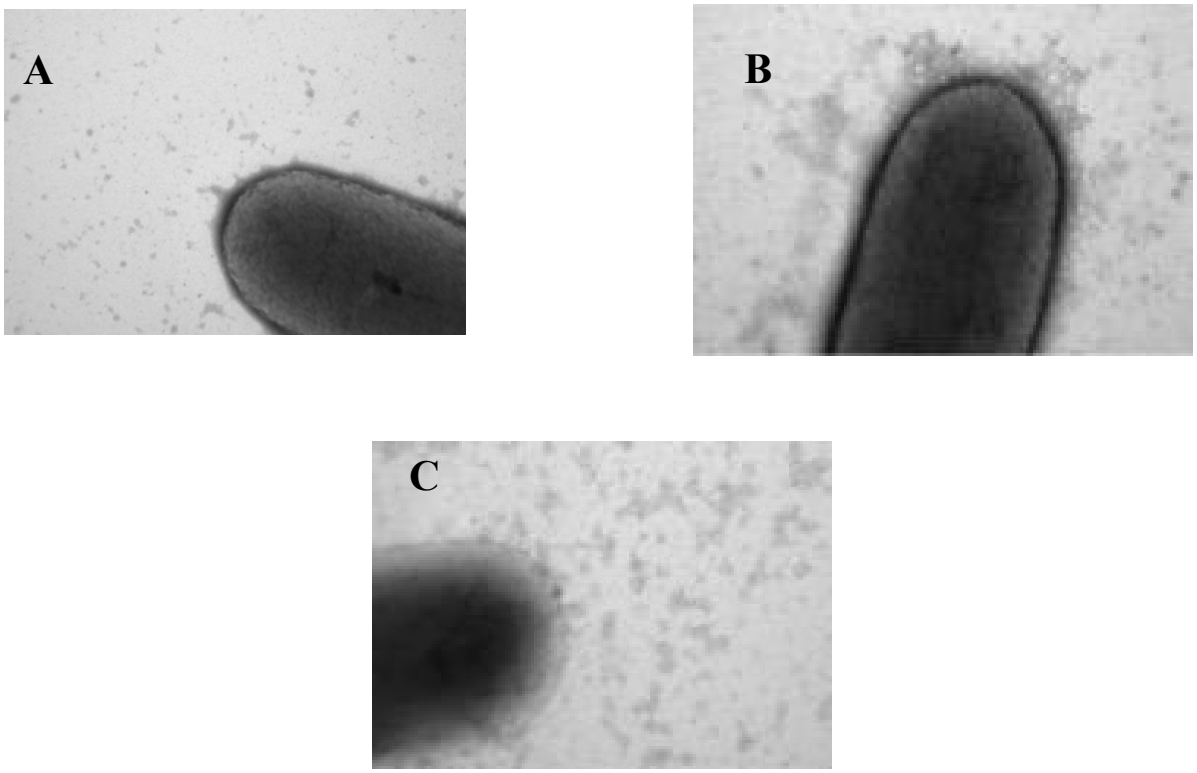

FIG 4 TEM of *C. rodentium* cells producing OMVs. *C. rodentium* wild-type (A), Δ*pmrAB* (B) and Δ*pmrC* Δ*cptA* (C) cells were grown to mid-log phase in N-minimal media supplemented with 50 μM $FeCl_2$. Culture dilutions in PBS were laid onto carbon-coated copper grids and stained as described in Materials and Methods. Samples were imaged using an accelerating voltage of 75 kV and at a magnification of 40,000 X. Images shown are representative of 20 micrographs per strain. Bars, 100 nm.

***C. rodentium* OMVs contain the periplasmic β-lactamase.** To assess the biological activity of *C. rodentium* OMVs, the presence of the chromosomally encoded periplasmic β-lactamase (ROD_12321) in OMVs was investigated. β-lactamase activity from OMVs isolated from the *C. rodentium* wild-type, Δ*pmrAB* and Δ*pmrC* Δ*cptA* strains grown in the presence of 50 μM $FeCl_2$ was measured using the chromogenic substrate nitrocefin [31]. Incubation of equal volumes of

solubilized OMVs with nitrocefin resulted in substrate cleavage, indicating the presence of β-lactamase within *C. rodentium* OMVs. In comparison to wild-type, solubilized OMVs from the Δ*pmrAB* and Δ*pmrC* Δ*cptA* strains resulted in increased β-lactamase activity over 25 min of incubation (Fig. 5). These results are consistent with increased OMV production by the Δ*pmrAB* and Δ*pmrC* Δ*cptA* strains.

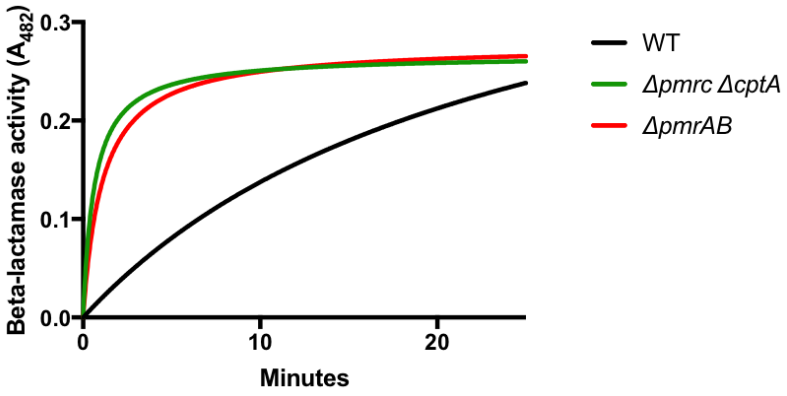

FIG 5 The periplasmic ß-lactamase is present in *C. rodentium* OMVs. OMVs were isolated from wild-type (black), Δ*pmrAB* (red) and Δ*pmrC* Δ*cptA* (green) *C. rodentium* strains grown to mid-log phase with 50 µM $FeCl_2$. Solubilized OMVs were incubated with the chromogenic substrate, nitrocefin. Cleavage of nitrocefin was monitored for 45 min at 22 °C by measuring the OD_{482}. Data shown are representative of three (n=3) independent experiments.

***C. rodentium* OMVs contain the outer-membrane protease CroP.** To further assess the biological activity of *C. rodentium* OMVs, the presence of CroP at the OMV surface was assessed. The relative amounts of CroP in equal volumes of OMVs from the *C. rodentium* wild-type, Δ*croP*, Δ*pmrAB* and Δ*pmrC* Δ*cptA* strains grown in the presence of 50 µM of $FeCl_2$ was determined by Western blotting. As estimated by measuring the intensity of the bands, the amount of CroP was approximately 2- to 3-fold higher in OMVs isolated from the Δ*pmrAB* and Δ*pmrC* Δ*cptA* strains in comparison to wild-type, reflecting the higher number of OMVs produced by these mutated strains (Fig. 6A). As expected, no band corresponding to CroP was observed in OMVs isolated

from the Δ*croP* strain (Fig. 6A). Activity of CroP on the surface of OMVs was measured by incubating equal volumes of OMVs isolated from the *C. rodentium* strains with the C2 FRET substrate [37]. In comparison to wild-type, equal volumes of OMVs from the Δ*pmrAB* and Δ*pmrC* Δ*cptA* strains resulted in a faster increase in fluorescence over 25 min of incubation, indicating increased CroP activity (Fig. 6B). OMVs isolated from the *C. rodentium* Δ*croP* strain produced minimal fluorescence, indicating that most of the substrate cleavage is mediated by the CroP protease (Fig. 6A). Together, these results show that OMVs secreted by *C. rodentium* contain biologically active CroP. Consistent with the TEM results, these data confirm that the Δ*pmrAB* and Δ*pmrC* Δ*cptA* strains produce more OMVs than the wild-type strain.

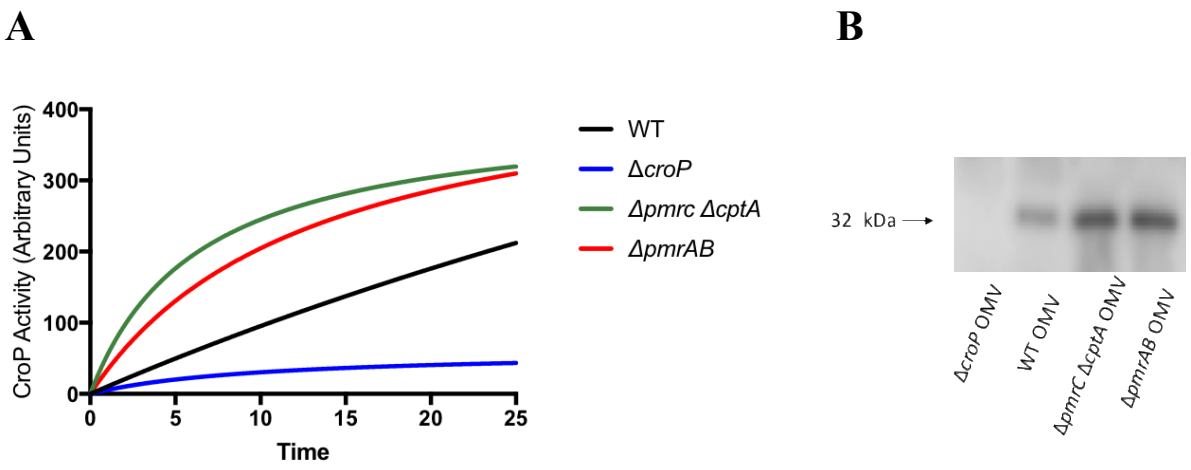

FIG 6 The outer membrane protease CroP is present in *C. rodentium* OMVs. OMVs were isolated from wild-type (black), Δ*croP* (blue), Δ*pmrAB* (red) and Δ*pmrC* Δ*cptA* (green) *C. rodentium* strains grown to mid-log phase in the presence of 50 μM $FeCl_2$. (A) The relative amount of CroP found within OMVs was determined by Western blot using an anti-CroP antibody. (B) CroP activity of OMVs were assessed by incubating OMVs with the C2 FRET substrate for 1 h. Cleavage of the FRET substrate was determined by measuring relative fluorescence at 430 nm after excitation at 325 nm. Data shown are representative of three independent experiments.

Discussion

The OM of Gram-negative bacteria is constantly remodelled in response to changing environments sensed by TCSs. Covalent modifications of LPS are viewed as an adaptive response to acidic pH, presence of AMPs, and changing concentrations of divalent cations [23]. The murine pathogen *C. rodentium* modifies its LPS relying primarily on the addition of pEtN to the lipid A and core. These modifications are catalyzed by the pEtN transferases PmrC and CptA, the expression of which are regulated by the PmrAB TCS [31]. Previously, we have shown that pEtN LPS modifications in *C. rodentium* confer protection against iron toxicity and contribute to the maintenance of OM integrity [31]. In the present study, we provide evidence that pEtN LPS modifications negatively regulate OMV production in *C. rodentium.* In the absence of *pmrAB* or *pmrC* and *cptA*, the production of OMVs is markedly enhanced during the adaptive response to environmental iron (Fig. 1-4). These data support the emerging hypothesis that LPS remodelling impacts OMV biogenesis by either enhancing or reducing OMV production in response to changing environmental cues [21, 32] .

Iron-induced oxidative stress is one form of envelope stress. Iron induces the formation of reactive oxygen species that damage the envelope of Gram-negative bacteria [35]. In *C. rodentium* wild-type, we found that OMV production is increased when cells are grown in the presence of up to 50 μM $FeCl_2$ (Fig. 1). Along the murine digestive tract, the concentration of free soluble iron was estimated to be approximately 50 μM [38]. Thus, during colonization of the murine gastrointestinal tract, *C. rodentium* is likely to encounter such concentrations of iron that may induce the formation of OMVs in vivo. Increasing the concentration of $FeCl_2$ to 75 μM did not yield a subsequent increase in OMV production for wild-type *C. rodentium* (Fig. 1). Further

increasing the concentration of $FeCl_2$ was toxic to *C. rodentium* (data not shown). To our knowledge, the present study is the first that shows the iron-mediated increase in OMV production.

Complementation of the Δ*pmrC* Δ*cptA* strain with either the pWSK*pmrC* or the pWSK*cptA* plasmid drastically reduced OMV production below wild-type levels (Fig. 2B). As shown by the transformation of these plasmids into *C. rodentium* wild-type (Fig. S1), the reduction of OMV production is likely due to the overexpression of these genes from the medium-copy-number plasmid pWSK129. We had previously showed that complementation of the Δ*pmrC* Δ*cptA* strain with either pWSK*pmrC* or pWSK*cptA* restores OM integrity and resistance to iron toxicity [31]. Collectively, these data indicate that pEtN LPS modifications mediated by *pmrC* and *cptA* are responsible for maintaining OM integrity and negatively regulating OMV production, suggesting that both processes may be important for *C. rodentium* resistance to iron toxicity. Given that the PmrAB TCS directly regulates *pmrC and cptA* expression [31], our data also support the notion that the increased OMV production observed in the *C. rodentium* Δ*pmrAB* strain is due to the downregulation of the *pmrC* and *cptA* genes. Although it remains possible that other PmrAB-regulated genes contribute to the increase in OMV production, most of the observed effects are likely mediated by *pmrC* and *cptA*, since no significant difference in OMV production was observed when comparing the Δ*pmrC* Δ*cptA* and Δ*pmrAB* strains (Fig. 1-4).

To assess putative biological functions of *C. rodentium* OMVs, we tested for the presence of the periplasmic enzyme β-lactamase and the OM protease CroP in isolated OMVs. Activity assays showed that both enzymes were present and active in OMVs isolated from the wild-type, Δ*pmrAB* and Δ*pmrC* Δ*cptA* strains (Fig. 5 and 6). Consistent with the higher numbers of OMVs produced by the deletion mutants (Fig. 3 and 4), equal volumes of OMVs isolated from the

Δ*pmrAB* and Δ*pmrC* Δ*cptA* strains exhibited higher β-lactamase and CroP enzymatic activities than an equal volume of OMVs isolated from the wild-type strain. These data indicate the presence of physiologically relevant enzymes on the surface and within *C. rodentium* OMVs.

In a recent study, Elhenawy *et al*. showed that the PhoPQ-regulated PagL lipid A deacylase enhances OMV formation in *S. enterica* [32]. Furthermore, overexpression of PagL resulted in a selective increase of deacylated lipid A species in OMVs in comparison to the OM, suggesting a mechanism whereby OMV production facilitates LPS turnover. The *C. rodentium* genome does not contain the *pagL* gene, however, it is possible that other PhoPQ-regulated LPS modifying enzymes fulfill a similar role in enhancing OMV production. In the current study, our data suggests that PmrC- and CptA-mediated pEtN LPS modifications are a means to control OMV formation. Together, these studies show that different LPS modifications have the ability to either enhance or repress OMV production. Since LPS modified with pEtN or L-Ara4N was shown to increase OM integrity, we propose that the decrease in OMV production mediated by CptA and PmrC is a response to maintain pEtN-modified LPS in the OM and, in turn, strengthen OM integrity during conditions of environmental stress. This idea is further supported by the study by Bonnington *et al*., that compared the distribution of LPS modifications between OMVs and the OM in *S. enterica* [21]. These authors found that lipid A species containing pEtN or L-Ara4N were more likely to be retained in the OM than secreted by OMVs under PhoPQ-activating conditions.

Our data indicate that OMV production is controlled by PmrC and CptA in order to retain pEtN-modified LPS species, which increase the robustness of the OM [39]. This *pmrC* and *cptA*-mediated repression of OMV production is consistent with the previously proposed polymorphic

model for regulation of membrane lipid, which suggests a relationship between lipid shape and membrane curvature [21, 32, 40, 41]. As pEtN is added to the LPS by PmrC, the effective size of the lipid A head groups is thought to increase, minimizing membrane curvature and the formation of OMVs. Although CptA does not modify lipid A, the CptA-mediated pEtN modification of the core first heptose is in close proximity to the lipid A head groups. Thus, PmrC and CptA likely play similar roles, both acting to subdue OMV formation and retain strengthening pEtN-modified LPS in the OM. This is the first study to report the negative regulation of OMV production in response to iron-induced environmental stress.

Materials and Methods

Bacterial strains, growth conditions and reagents. All bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown overnight in Luria-Bertani (LB) broth at 37°C with aeration. Bacteria were diluted 1:100 in N-minimal medium [50 mM Bis-Tris (pH 7.2), 7.5 mM $(NH_4)SO_4$, 5 mM KCl, 0.5 mM K_2SO_4, 0.5 mM KH_2PO_4, 38mM glycerol, 0.1% (w/v) casamino acids] supplemented with 0.2% glucose, 20 μM $MgCl_2$ and varying concentrations of $FeCl_2$, as indicated. Cultures were grown statically for 48 hr at 28 °C leading to an optical density at 595 nm (OD_{595}) of approximately 0.5. When appropriate for plasmid selection, kanamycin (50 μg/ml) was added to the medium.

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
C. rodentium strains		
DBS100	Wild-type *C. rodentium*	[42]
Δ*pmrAB*	DBS100 Δ*pmrAB*	[31]
Δ*pmrC* Δ*cptA*	DBS100 Δ*pmrC*Δ*cptA*	[31]
Δ*croP*	DBS100 Δ*croP*	[43]
Plasmids		
pWSK129	Cloning vector	[44]
pWSK*pmrC*	*pmrC* cloned into pWSK129	[31]
pWSK*cptA*	*cptA* cloned into pWSK129	[31]

OMV isolation. Bacterial cultures were normalized to an OD_{595} of 0.5 and centrifuged (12,000 x *g*, 4°C, 20 min). Supernatants were harvested and sequentially filtered through 0.8 μm and 0.45 μm MF membrane filters (Millex, Millipore). The resulting filtrates were concentrated using a 10 kDa molecular weight cut-off centrifugal filter units (Amicon Ultra-15, Millipore; 2360 x *g*, 4°C, 20 min). Concentrated filtrates were then centrifuged (200,000 x *g*, 4°C, 2.5 hr) in a tabletop Optima ultracentrifuge (Beckman Coulter). Pelleted OMVs were resuspended in 300 μl of phosphate buffered saline (PBS).

OMV protein quantification. Protein concentrations of OMV preparations were determined by using the detergent compatible (DC) protein assay (BioRad). OMVs were solubilized with 0.1 % Triton-X-100 (4°C, 15 min) and the DC protein assay was performed according to the manufacturer instructions. The OD_{750} was measured using a Hitachi U-2010 spectrophotometer. Protein concentrations were determined using bovine serum albumin (BSA) as a standard.

OMV lipid quantification. The lipid content of OMV samples was quantified using the lipophilic dye FM 1-43 (Molecular Probes, Life Technologies). OMV samples were diluted 1:55 in PBS and transferred to a quartz cuvette. The FM 1-43 dye was added at a final concentration of 4.5 μM. Following excitation at 479 nm, fluorescence emission at 600 nm was measured on a Varian Cary Eclipse fluorescence spectrophotometer, using 5 nm slit widths for both excitation and emission.

TEM. For bacterial cell imaging, culture aliquots were transferred onto carbon-coated copper grids and grown for an additional 24 hr. Grids were fixed with 2.5 % glutaraldehyde diluted in

50 mM sodium cacodylate (pH 7.2) and washed 3 times with 3 % saccharose diluted in the same buffer. For OMV imaging, purified OMV samples were diluted 1:20 with DMEM and 70 µl aliquots were laid onto carbon-coated copper grids by ultracentrifugation (Airfuge, 20.000 psi, 5 min). Excess liquid was discarded from the grids and samples were negatively stained with phosphotungstic acid 3% (w/v) for 5 min and dried with filter paper. Specimens were examined under the microscope (Hitachi H-7100) operated at an accelerating voltage of 75 kV and at a magnification of 40,000 x for cells and 30,000 x for OMVs. The size and number of OMVs per micrograph were determined using the Macnification software (Orbicular).

Nitrocefin assays. The activity of β-lactamase in OMVs was determined by measuring cleavage of the chromogenic β-lactam nitrocefin. OMVs were diluted 1:7 in PBS and solubilized with 0.1 % Triton-X-100 for 15 min at 4°C. Enzymatic assays were performed at 22°C in the presence of 43 µM nitrocefin and β-lactamase activity was monitored by measuring the OD_{482} for 45 min, using a Powerwave X340 microplate reader (Bio-Tek instruments). Data were fit to a Michaelis-Menten non-linear regression curve using the GraphPad Prism 7 software.

Western blotting. Purified OMVs (10 µl) were added to Laemmli sample buffer, boiled for 5 min and resolved on a 10% SDS-PAGE gel. Proteins were transferred to a polyvinylidene difluoride membrane and western blotting was performed using a rabbit polyclonal antibody raised against CroP and a peroxidase-conjugated anti-rabbit secondary antibody. Quantity One software (Bio-Rad) was used to image and quantify protein bands.

CroP activity assays. The activity of the outer membrane protease CroP in OMVs was determined by measuring the cleavage of the C2 FRET substrate (2Abz-SLGRKIQIK(Dnp)-NH_2, AnaSpec), as previously. Purified OMVs were diluted 1:37.5 in PBS, transferred into a quartz cuvette and incubated with the FRET substrate (6 μM) at 22°C. Cleavage of the FRET substrate was monitored over 60 min by measuring fluorescence emission at 430 nm at an excitation wavelength of 325 nm using a Varian Cary Eclipse fluorescence spectrophotometer. Excitation and emission slit widths were set at 5 nm. Data were fit to a Michaelis-Menten nonlinear regression curve using the GraphPad Prism 7 software.

Acknowledgements

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Supplementary Figures

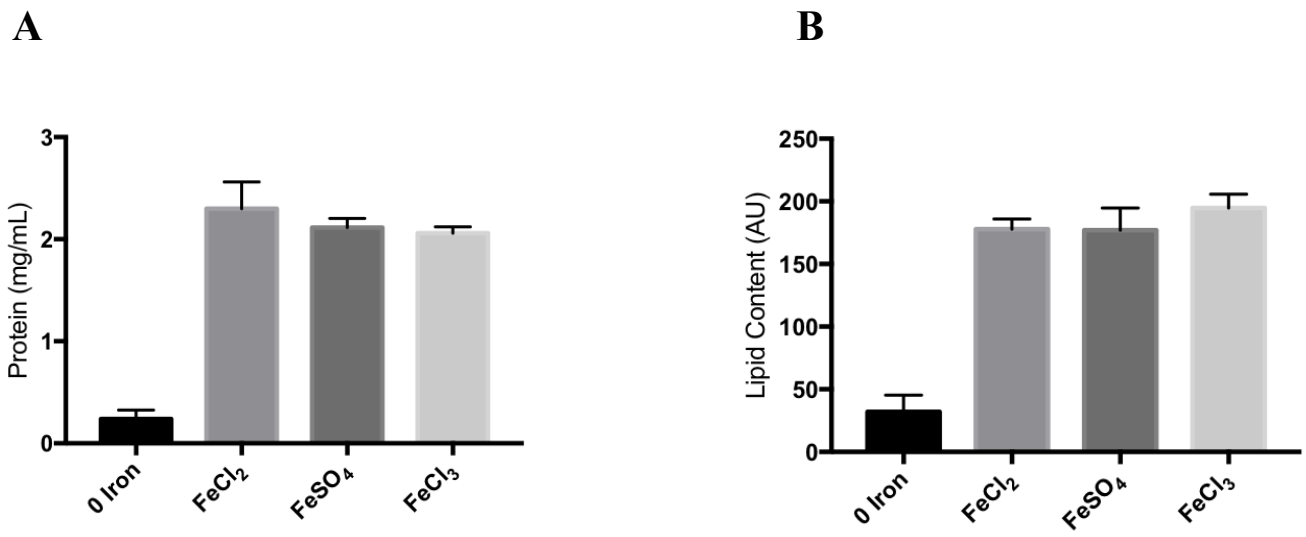

FIG S1 OMV production in wild-type *C. rodentium* grown in different iron sources. OMVs were isolated from wild-type *C. rodentium* grown with 50 µM of either $FeCl_2$, $FeCl_3$, or $FeSO_4$ or with no iron. (A) Total protein content of OMVs was measured using the DC assay (Bio-Rad). OMVs were solubilized using 0.1 % Triton-X-100 and Bio-Rad DC assay reagent A and B were added. After 15 mins, absorbance at 750 nm was measured. Protein concentration was calculated using bovine serum albumin (BSA) as a standard. (B) Total lipid content of OMV samples was determined by using the lipophilic dye, FM 1-43. OMV samples were diluted 1:55 and 50 µL of FM 1-43 molecular probe was added. After excitation at 479nm, relative fluorescence at 600 nm was measured. Values shown are the means ± standard error from a single independent experiment (n=1), repeated in triplicate, one-way ANOVA.

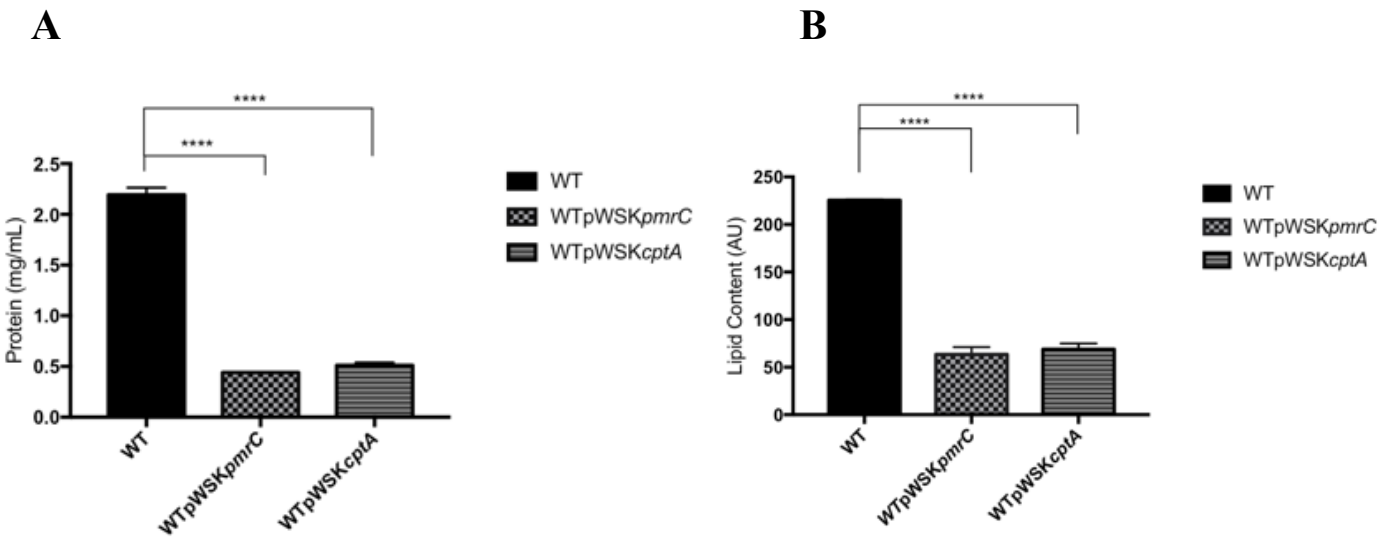

FIG S2 OMV production in wild-type *C. rodentium* transformed with plasmids containing *pmrC* or c*ptA*. OMVs were isolated from wild-type *C. rodentium* transformed with either the pWSK*pmrC* or the pWSK*cptA* plasmid and grown with 50 µM $FeCl_2$. (A) Total protein content of OMVs was measured using the DC assay (Bio-Rad). OMVs were solubilized using 0.1 % Triton-X-100 and Bio-Rad DC assay reagent A and B were added. After 15 mins, absorbance at 750 nm was measured. Protein concentration was calculated using bovine serum albumin (BSA) as a standard. (B) Total lipid content of OMV samples was determined by using the lipophilic dye, FM 1-43. OMV samples were diluted 1:55 and 50 µL of FM 1-43 molecular probe was added. After excitation at 479nm, relative fluorescence at 600 nm was measured. Values shown are the means ± standard error from a single independent experiment (n=1), repeated in triplicate. **** $p \leq 0.0001$, one-way ANOVA.

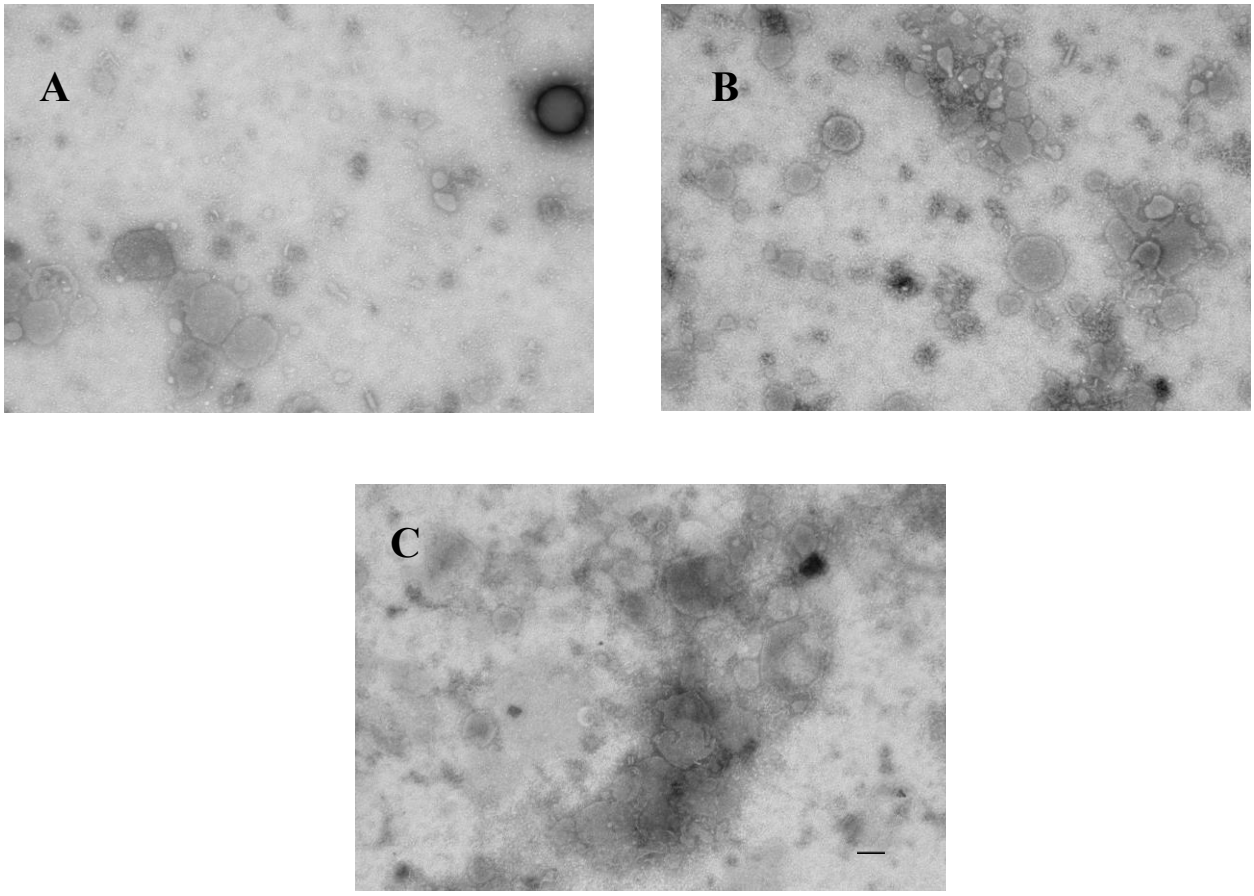

FIG S3 TEM of *C. rodentium* OMVs. *C. rodentium* wild-type (A), Δ*pmrAB* (B) and Δ*pmrC* Δ*cptA* (C) OMV samples were laid onto carbon-coated copper grids and stained as described in the Materials and Methods section. Transmission electron microscopy of specimens was conducted using an accelerating voltage of 75 kV and at a magnification of 30,000 x. Images shown are representative of 20 different fields for OMVs isolated from each strain, bars 100 nm.

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Preface to Chapter 3

This chapter discusses the main findings of this thesis. The limitations of our experimental approach and possible future directions of this work will first be mentioned. Next, the potential mechanisms regarding the PmrC and CptA-mediated repression and iron induction of OMV biogenesis will be outlined. Finally, the relevance of our work, followed by a summary of the main findings will be discussed.

Chapter 3: Discussion and Conclusion

3. 1 Discussion

The process of OMV production is complex and poorly understood. Among other mechanisms, OM integrity, envelope stress and membrane curvature have been implicated in regulating vesicle production [123, 136, 146, 105]. In several enteric Gram-negative pathogens, LPS modifications have been shown to provide resistance to AMPs and antibiotics, provide increased OM integrity and alter membrane topology [147, 90, 148]. The murine pathogen, *C. rodentium* is a useful model organism to study pEtN modifications since these bacteria are unable to modify their LPS with L-ara4N, which is similar in charge and size to pEtN [149]. The putative pEtN transferases, PmrC and CptA have been shown to provide increased OM integrity and increased resistance to iron toxicity, AMPs and antibiotics [90] . Here, we have shown that PmrC and CptA negatively regulate OMV production in *C. rodentium*. We have also shown that iron induces vesicle production, and that *C. rodentium* OMVs contain CroP and ß- lactamase, two enzymes normally found in the OM and periplasm, respectively.

3.1.1 Experimental limitations and future directions

Quantification of our isolated vesicle preparations clearly showed that the Δ*pmrC* Δ*cptA* and Δ*pmrAB* strains produce more OMVs in comparison to wild-type. However, purification steps need to be added to our protocol to ensure that our OMV samples are free of cellular debris or secreted large protein complexes. In our current protocol, these debris and proteins would pellet along with OMVs during ultracentrifugation. Since this material would be quantified in our protein and lipid quantification assays, it is possible that the perceived differences we see in OMV production may be due to differences in debris and protein produced by different strains. In order to address this issue we performed an additional centrifugation step (30,000 *x g*, 40 min, 4°C) prior

to our final ultracentrifugation step, which would pellet any large cellular debris [150]. We also performed density gradient centrifugation in order to remove large secreted protein complexes from our OMV preparations, which has become a standard for purification of vesicles prior to downstream application [151] . Due to differences in density between material, we would expect OMVs to float to higher fractions in the density gradient in comparison to secreted proteins [152]. We used Western Blot to measure differences in OMV production between wild type and the Δ*pmrC* Δ*cptA* strains after purification using the additional centrifugation step and using density gradient centrifugation. Measuring differences in pooled fractions from the density gradient centrifugation, we found that there were only marginal differences in OMV production between the strains in the upper fractions of the gradients (Fig. A1). However, it is possible that the 2-3 fold differences in OMV production we originally observed between wild type and the Δ*pmrC* Δ*cptA* strains became diluted over the course of density gradient centrifugation, as OMV material was found in several fractions (Fig. A1). Furthermore, TEM of both *C. rodentium* whole cells and isolated vesicle preparations still indicate that the Δ*pmrAB* and Δ*pmrC* Δ*cptA* strains produce more OMVs in comparison to wild type (Fig. 3 and 4).

In tandem with a previous paper from our lab, this current project focuses on the function of the *pmrC* and *cptA* genes in *C. rodentium* [90]. The role of these genes as pEtN transferases were first discovered in *Salmonella* [103, 99]. Since then, homologs of these genes with identical functions have been discovered in several other Gram-negative species [153, 154, 155, 156]. In *C. rodentium*, a BLAST search revealed that PmrC is 81% identical to *E. coli* K-12 MG1655, and 82% identical to *Salmonella enterica* serovar *typhimirium* LT2 (*S. typhimirium* LT2) based on amino acid similarity. Furthermore, CptA in *C. rodentium* was 87% identical to *E. coli* K-12 MG1655 and 89 % identical to *S. typhimiruum* LT2. The amino acid similarity of these proteins between these

bacteria suggests that PmrC and CptA are similarly responsible for modifying the lipid A and core regions of LPS with pEtN in *C. rodentium*. However, structural studies of the lipid A and core oligosaccharide in *C. rodentium* LPS still need to be performed in order to confirm that this is the case. Mass spectrometry and thin layer chromatography will need to be conducted to reveal the covalent modifications of LPS in *C. rodentium* and whether *pmrC* and *cptA* are indeed involved in adding pEtN to the lipid A and core.

While our study focuses on PmrC and CptA, which are believed to mediate pEtN modifications in *C. rodentium*, the role of other LPS modifications on OMV formation will need to be studied to gain a more profound understanding of vesicle formation. The *waaY* core heptose kinase and the *pmrG* phosphatase alter the phosphorylation state of LPS [110]. The impact that these changes have on the functionality of the OM remain to be fully determined. It is possible that increased negative charge favoured by *waaY* expression would induce OMV formation by promoting increased anionic repulsion. Furthermore, since pEtN has similarities in terms of size and charge to L-ara4N, it is suspected that both modifications would be involved in the negative regulation of OMV production. The *C. rodentium* genome does not possess the *arn* operon responsible for L-ara4N modifications. Consequently, we cloned the *S. typhimirium arn* operon on the pACYC plasmid, ectopically overexpressed it in *C. rodentium* and measured OMV production. We did not observe differences in OMV production between wild type and the wild type pACYC*arn* strain, suggesting that structural differences may exist between pEtN and L-ara4N that differentially impact OMV formation (Fig. A2). However, the structure of LPS produced by wild type pACYC*arn* will need to be elucidated to confirm that L-ara4N is being added to the lipid A before conclusions can be drawn.

Similar to the PmrAB TCS, the PhoPQ TCS regulates several genes involved in modifying LPS and OM remodeling [157]. As such, we suspected that PhoPQ might similarly negatively regulate OMV formation in *C. rodentium*. Based on quantification by total protein and total lipid content, we found that a Δ*phoPQ* strain produced more OMVs in comparison to wild type (Fig. A3). We then conducted microarray experiments to determine which genes might be regulated by PhoPQ and responsible for the negative regulation of OMV production. In total, we found that 706 genes were upregulated and 382 genes were downregulated by PhoPQ. Full analysis of these genes has yet to be performed. However, preliminary analysis should focus on genes which are highly regulated by PhoPQ and involved in OM structure and remodelling. In the future, it would be interesting to create knockout strains of candidate genes and assess OMV production.

3.1.2 Mechanism of PmrC and CptA-mediated regulation of OMV formation

Several studies have indicated that OMV production may be influenced by changes in the curvature of the OM. OMV production was found to be increased in *Haemophilus influenzae* and *Vibrio cholerae* strains which lacked the VacJ/YrB phospholipid transport system and displayed an accumulation of phospholipids in the OM [146]. The authors suggested that this increased phospholipid content in the OM caused asymmetric expansion of the outer leaflet of the OM initiating membrane curvature and subsequent formation of OMVs [146]. Another study found that overexpression of the PagL lipid A deacylase similarly induces OMV production by altering the shape of the OM [105]. It was proposed that the deacylation of lipid A causes a decrease in the hydrophobic cross-sectional area of the molecule, favouring the formation of "inverted-cone" structures, leading to increased membrane curvature and the formation of OMVs [105]. It has been suggested that lipid A pEtN modifications may also influence the shape of the OM [148]. Since we have shown that PmrC and CptA negatively regulate

OMV production in *C. rodentium*, it is possible that these results are due to changes in membrane shape. The model for polymorphic membrane lipid composition suggests that the intrinsic shape of an individual lipid molecule influences whether bilayer or non-bilayer structures, such as OMVs, are formed [158] . It is thought that pEtN modifications increase the size of the lipid A head groups, which is suspected to change the shape of the lipid A molecule from a conical to a cylindrical shape [159] . Lipids with a cylindrical shape are less likely to form non-bilayer structures such as OMVs. Therefore, pEtN modifications may be decreasing OMV production by promoting the formation of bilayer lipid structures. While CptA does not modify the lipid A head groups specifically, it adds pEtN to the core heptose (I), which is in close proximity to the PmrC-mediated modification. Thus, we expect that PmrC and CptA would have similar effects on influencing the membrane shape and subsequent vesicle formation.

Increased anionic repulsion between LPS molecules in the absence of PmrC and CptA may similarly induce OMV production by impacting the curvature of the OM. In *P. aeruginosa,* insertion of the PQS quorum sensing molecule into the OM facilitates OMV production [160]. It is thought that PQS disrupts interactions between LPS and the bridging divalent cations, Mg^{2+} and Ca^{2+} and causes increased anionic repulsion on the surface of the OM [161]. This electrostatic repulsion may cause strain on the OM, leading to increased membrane curvature and the formation of OMVs. In support of this idea, OMVs from *P. aeruginosa* were found to contain more of the B-band form of LPS rather than A-band, which are classified based on their O-antigen structure [162]. B-band is the more highly charged form of LPS and is found to be less abundant in the OM than A-band LPS [162]. In the case of *P. aeruginosa,* OMV formation may be driven by anionic repulsion in areas of the OM where B-band LPS is localized [161]. pEtN modifications decrease the negative charge of LPS as a result of the positively charged amine groups that they carry. As such,

our data suggests that PmrC and CptA may be negatively regulating OMV formation by decreasing the repulsion caused by proximal like charges in the OM. To further support the hypothesis that electrostatic repulsion is involved in vesicle formation, our data shows that increased concentrations of the bridging divalent cation, Mg^{2+} decreases OMV formation in *C. rodentium*, which is consistent with findings in other Gram-negative species (Fig. A4) [163].

In the absence of PmrC and CptA, the OM integrity of *C. rodentium* is compromised, which may lead to increased vesicle production. Numerous studies have studied the architecture of the cell envelope in the context of OMV formation [136, 164, 165]. While integral proteins, lipids, lipoproteins and PG provide a structural framework to the cell envelope, interactions between these components must be dynamic in order for cells to undergo processes such as cell division and OMV formation. The cell envelope is crosslinked by interactions between lipoproteins and proteins in the OM and the periplasmic PG. Lpp, OmpA, and the Tol-Pal system provide a large portion of the covalent and non-covalent interactions with PG that provide structural integrity to the OM [51 53 54] . Decreasing amounts of these crosslinks has been correlated with increased OMV production [136] . In the absence of these OM-PG crosslinks, the periplasmic dimension is thought to expand, resulting in bulging and formation of OMVs [90] . While pEtN modifications, do not crosslink the OM and PG, they are thought to be important for maintaining the barrier function of the OM in *C. rodentium* [90]. While full mechanistic details remain unclear, it is possible that structural integrity provided by PmrC and CptA may decrease the flexibility of the membrane, prevent expansion of the periplasmic space and reduce OMV formation. If this is the case, it would suggest that OMV production is a product, rather than the cause of decreased OM integrity in *C. rodentium* strains lacking PmrC and CptA. In support of this idea, there are numerous cases where strains that exhibit hypervesiculation do not show associated membrane instability [141]. However,

since the roles of PmrC and CptA are not well defined in *C. rodentium*, it is still possible that OMV formation in the absence these proteins may be increased in a manner that is independent to envelope instability. In such a case, increased vesicle formation might be disrupting the OM structure and driving envelope stability.

We have shown that ferrous chloride increases OMV formation in *C. rodentium.* Similar increases were observed when *C. rodentium* was grown in ferric chloride and ferrous sulphate, suggesting that OMV production is induced in an iron-dependent manner (Fig. S1). These findings are somewhat counterintuitive as PmrC and CptA are regulated by the PmrAB TCS, which is induced by Fe^{3+}. Since we have shown that PmrC and CptA negatively regulate OMV production, we would have expected that increased transcription of these genes with increasing concentration of iron would result in decreased amounts of vesicle production. It is then likely that iron and PmrC and CptA may independently regulate OMV production, explaining the differences in OMV production as iron concentration is increased. The iron-mediated increase in OMV production may be a result of reactive oxygen species produced during the Fenton reaction. Hydroxyl radicals may initiate an accumulation of misfolded protein in the periplasm, which has been established as a cause of stress-induced OMV formation [166]. In support of this idea, oxidative stress was shown to increase OMV formation in *P. aeruginosa* and *Neisseria meningitidis* [142, 167]. Furthermore, our preliminary data shows that when *C. rodentium* is grown with the antioxidant glutathione, the iron-mediated increase of OMV formation is dampened (Fig. A5). Glutathione has been shown to increase resistance of *E. coli* to oxidative stress [168]. Our data also shows that in the presence of glutathione, there is still increased OMV formation in Δ*pmrC* Δ*cptA* and Δ*pmrAB* strains in comparison to wild type, indicating that the iron mediated and PmrC and CptA-mediated responses to OMV formation are likely independent of one another (Fig. A5).

3.1.3 Relevance

Secretion of OMVs by Gram-negative bacteria can be beneficial towards pathogenesis in a number of ways. However, this process is energy-intensive, releasing a number of proteins and lipids from the cell envelope, which would need to be replaced by the parent cell. By repressing OMV formation in certain environmental conditions, bacteria would be able to conserve beneficial material while reducing metabolic costs. We propose that the negative regulation of OMV production by PmrC and CptA may be a mechanism to conserve pEtN residues in the OM. Based on previous work from our lab, it is believed that these residues are important in maintaining OM integrity and resistance to antibiotics[90]. In stressful conditions that *C. rodentium* would encounter during infection, reducing OMV production could maintain pEtN residues in the OM and provide increased integrity to the cell envelope. This idea of modulating OMV production in order to maintain LPS modifications is supported by a recent study, which compared the distribution of LPS modifications in the OM and in secreted OMVs from *S. typhimirium* [124]. The authors found that after transition into PhoPQ-activating conditions, there was a higher proportion of pEtN and L-ara4N residues maintained in the OM in comparisons to secreted OMVs [124].

Our findings that PmrC and CptA negatively regulate OMV formation add to the current understandings of OMV formation. It is becoming more clear that OMV biogenesis is governed by several different processes. By determining the individual genes and pathways involved in vesicle production, we can start to understand how these processes may be interconnected or differentially regulated based on environmental stimuli. Currently, OMVs are being used as a novel vaccine platform due to their stability at various temperatures and high amount of immuno-stimulatory molecule [169, 170, 171]. Manipulating the processes which govern OMV production will

allow for more effective vaccines to be produced at lower costs. It is also possible that once the mechanisms of OMV formation are elucidated, novel antibiotics could be developed to inhibit vesicle formation and limit the pathogenesis of Gram-negative bacteria. [172]

3.2 Conclusion

The mechanistic details of OMV production in Gram-negative bacteria remain poorly understood. OM integrity, envelope stress, and induction of membrane curvature have all been postulated to impact this process. In *C. rodentium,* PmrC and CptA, two enzymes regulated by the PmrAB TCS and believed to mediate pEtN modifications were shown to provide increased OM integrity and resistance against envelope perturbing agents. Here we have shown that OMV production in *C. rodentium* is induced by iron, which positively regulates the PmrAB TCS, and is negatively regulated by PmrC and CptA. We further show that *C. rodentium* OMVs contain the OM enzyme CroP and the periplasmic enzyme ß- lactamase. Our data presents a novel mechanism by which an enteric pathogen may negatively regulate OMV production using pEtN LPS modifications. Understanding the process of OMV formation will aid in the future development of vaccines and antibiotics against Gram-negative pathogens.

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Appendix

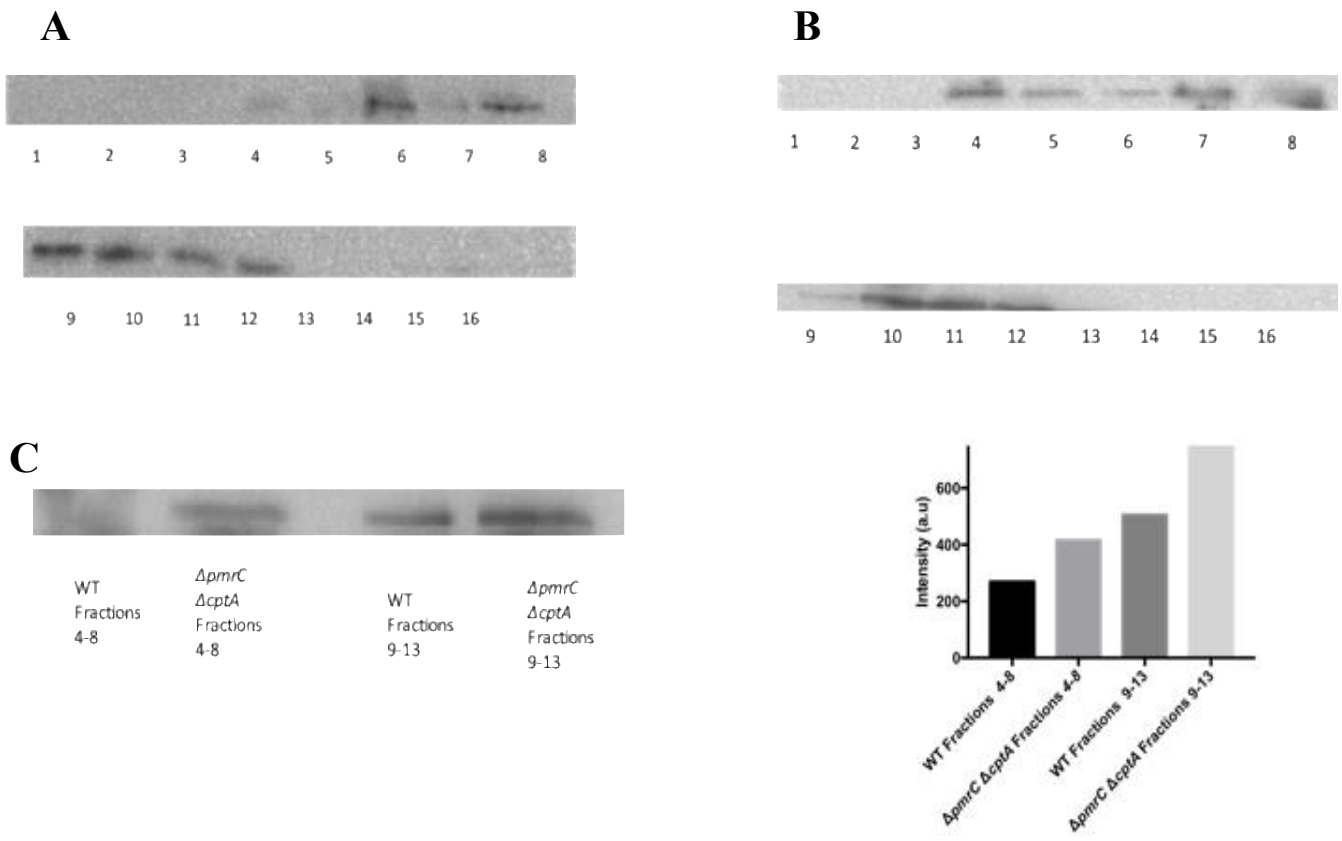

Figure A1. Density gradient centrifugation of *C. rodentium* OMVs. OMVs were isolated from A) wild-type and B) Δ*pmrC* Δ*cptA C. rodentium* strains and resuspended in 45 % iodixanol (Optiprep). Resuspended OMVs were layered at the bottom of an ultracentriufuge tube and a density gradient was made by sequentially layering 35%, 30%, 25%, 15%, and 10% iodixanol. Samples were ultracentrifuged for 3 hr (200,000 *x g*, 4°C). Equal volume fractions were taken sequentially from the top of the density gradient. In order to detect OMVs, Western Blot was conducted on equal volumes of each fraction using an anti-CroP antibody (32 kDa). C) Fractions 4-8 and 9-13 were pooled together in wild-type and Δ*pmrC* Δ*cptA C. rodentium* strains and Western Blot was conducted by taking equal volumes of pooled fractions from each strain using an anti-CroP antibody. Quantity One software was used to quantify protein bands from pooled fractions.

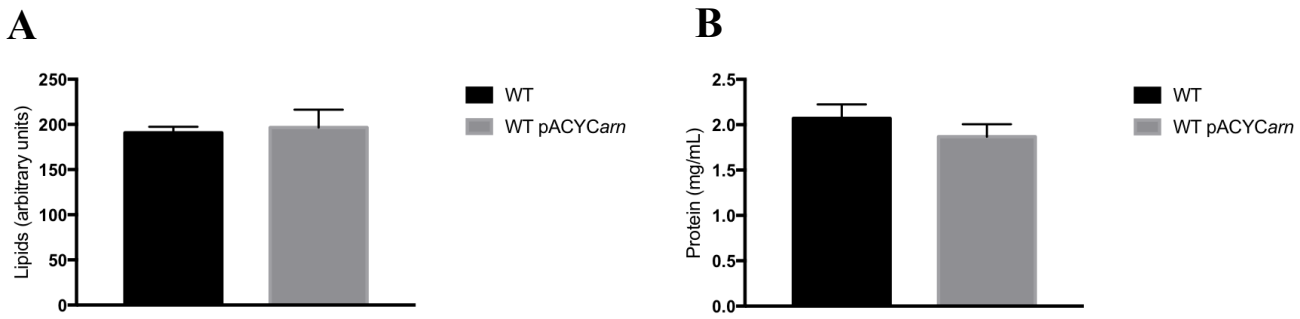

Figure A2. Ectopic expression of the *S. typhimirium arn* operon does not impact OMV production in *C. rodentium*. *C. rodentium* was transformed with pACYC*arn*, containing the *arn* operon from *S. typhimirium*. OMVs were isolated from *C. rodentium* grown in N-minimal media, supplemented with 50 µM $FeCl_2$. The wild type pACYC*arn* strain was grown with 35 µg/mL chloramphenicol (A) Total protein content of OMVs was measured using the DC assay (Bio-Rad). OMVs were solubilized using 0.1 % Triton-X-100 and Bio-Rad DC assay reagent A and B were added. After 15 mins, absorbance at 750 nm was measured. Protein concentration was calculated using bovine serum albumin (BSA) as a standard. (B) Total lipid content of OMV samples was determined by using the lipophilic dye, FM 1-43. OMV samples were diluted 1:55 and 50 µL of FM 1-43 molecular probe was added. After excitation at 479nm, relative fluorescence at 600 nm was measured. Values shown are the means ± standard error from a single independent experiment (n=1), repeated in triplicate, one-way ANOVA.

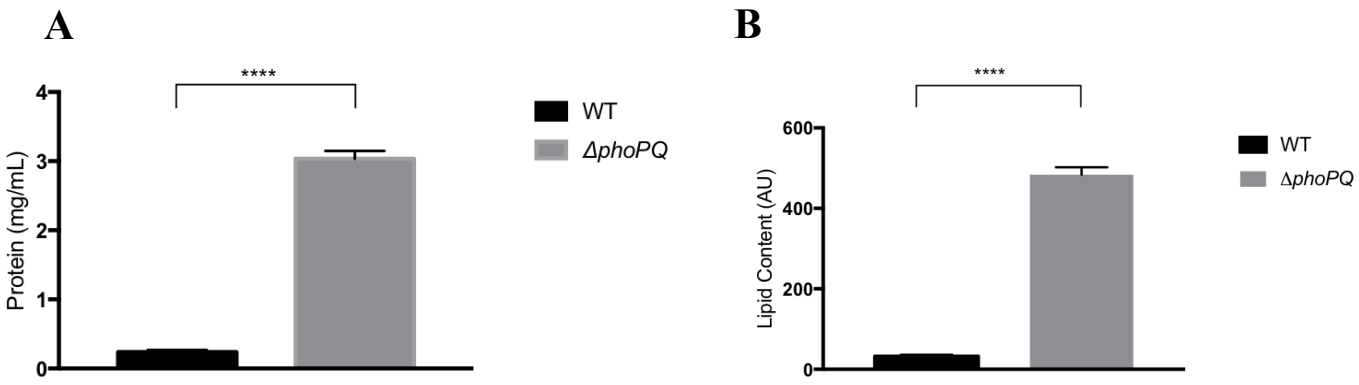

Figure A3. The PhoPQ TCS negatively regulates OMV production in *C. rodentium*. OMVs were isolated from *C. rodentium* grown in N-minimal media (A) Total protein content of OMVs was measured using the DC assay (Bio-Rad). OMVs were solubilized using 0.1 % Triton-X-100 and Bio-Rad DC assay reagent A and B were added. After 15 mins, absorbance at 750 nm was measured. Protein concentration was calculated using bovine serum albumin (BSA) as a standard. (B) Total lipid content of OMV samples was determined by using the lipophilic dye, FM 1-43. OMV samples were diluted 1:55 and 50 μL of FM 1-43 molecular probe was added. After excitation at 479nm, relative fluorescence at 600 nm was measured. Values shown are the means ± standard error from 3 independent experiments (n=3), repeated in triplicate, one-way ANOVA.

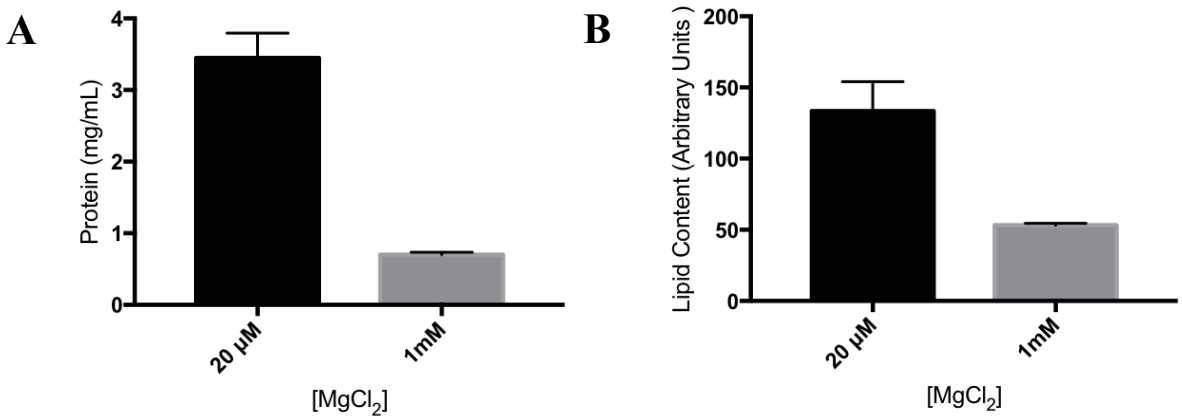

Figure A4. $MgCl_2$ reduces OMV production in *C. rodentium*.
OMVs were isolated from wild-type *C. rodentium* grown in N-minimal media with 50 µM $FeCl_2$ and either 20 µM or 1mM $MgCl_2$ (A) Total protein content of OMVs was measured using the DC assay (Bio-Rad). OMVs were solubilized using 0.1 % Triton-X-100 and Bio-Rad DC assay reagent A and B were added. After 15 mins, absorbance at 750 nm was measured. Protein concentration was calculated using bovine serum albumin (BSA) as a standard. (B) Total lipid content of OMV samples was determined by using the lipophilic dye, FM 1-43. OMV samples were diluted 1:55 and 50 µL of FM 1-43 molecular probe was added. After excitation at 479nm, relative fluorescence at 600 nm was measured. Values shown are the means ± standard error from a single independent experiment (n=1), repeated in triplicate, one-way ANOVA.

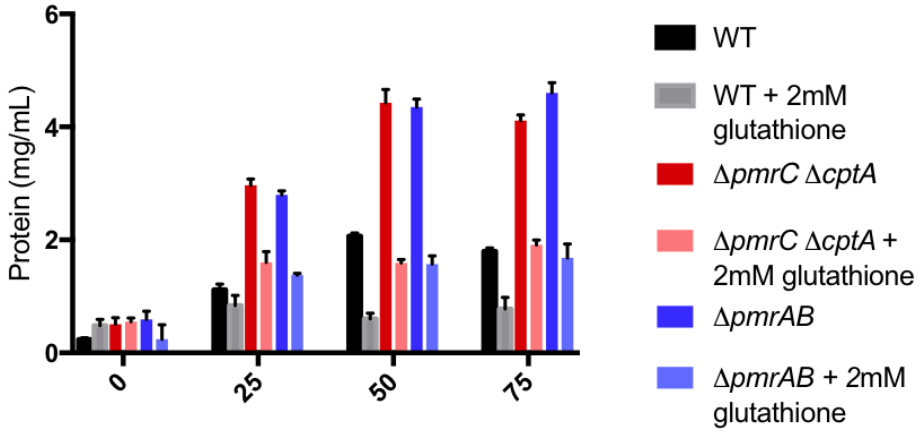

Figure A5. Glutathione reduces iron-mediated OMV production in *C. rodentium*. OMVs were isolated from *C. rodentium* grown in N-minimal media at various $FeCl_2$ concentrations with or without 1mM glutathione (A) Total protein content of OMVs was measured using the DC assay (Bio-Rad). OMVs were solubilized using 0.1 % Triton-X-100 and Bio-Rad DC assay reagent A and B were added. After 15 mins, absorbance at 750 nm was measured. Protein concentration was calculated using bovine serum albumin (BSA) as a standard. (B) Total lipid content of OMV samples was determined by using the lipophilic dye, FM 1-43. OMV samples were diluted 1:55 and 50 µL of FM 1-43 molecular probe was added. After excitation at 479nm, relative fluorescence at 600 nm was measured. Values shown are the means ± standard error from a single independent experiment (n=1), repeated in triplicate, one-way ANOVA.