The Rcs-regulated colanic acid capsule maintains membrane potential in *Salmonella enterica* serovar Typhimurium

Jasmine M Pando

A dissertation
submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

University of Washington

2017

Reading Committee

Ferric C. Fang, Chair

Caroline Harwood
Pradeep K. Singh

Program Authorized to Offer Degree:

Microbiology Department

University of Washington

Abstract
The Rcs-regulated colanic acid capsule maintains membrane potential in *Salmonella enterica* serovar Typhimurium
Jasmine M Pando

Chair of the Supervisory Committee:
Professor Ferric C. Fang, MD
Department of Microbiology

The Rcs phosphorelay and Psp (phage shock protein) systems are envelope stress responses that are highly conserved in γ-proteobacteria. The Rcs regulon was found to be strongly induced during metal deprivation of *Salmonella enterica* serovar Typhimurium lacking the Psp response. Nineteen genes activated by the RcsA-RcsB response regulator comprise an operon responsible for production of colanic acid capsular polysaccharide, which promotes biofilm development. Despite more than half a century of research, the physiological function of colanic acid has remained elusive. In this study we provide evidence that Rcs-dependent colanic acid production maintains the transmembrane electrical potential (Δψ) and proton motive force (PMF) in cooperation with the Psp response. Production of negatively-charged exopolysaccharide covalently bound to the outer membrane may enhance the surface potential by increasing the local proton concentration. This provides a unifying mechanism to account for diverse Rcs/colanic acid-related phenotypes, including susceptibility to membrane-damaging agents and biofilm formation. This work provides a new and fundamental insight into bacterial physiology.

Table of Contents

List of Figures and Tables

Figures for Chapter 2: *S.* Typhimurium Extracellular Polysaccharides

Figures for Chapter 3: The Rcs-regulated colanic acid capsule maintains membrane potential in *S.*Typhimurium

Figures for Chapter 4: Discussion

Figures for Chapter 5: Methods and Materials

Acknowledgements

I am grateful to my advisor Ferric. C. Fang for his patience and all his scientific mentorship. He has taught me so much about so many things. I will always remember his advice “a day in the library is worth two in the lab”.

I thank Joyce E. Karlinsey for making the initial observations that led to the development of my project. I am also grateful to Joyce for all her technical assistance (she knows so much about everything and will help with anything).

I am appreciative of all the technical assistance given by Stephen J. Libby during the isolation of RNA and for his overall kindness and cheerfulness that he provided everyday.

I sincerely appreciate all the help provided by Larissa A. Singletary. As a senior graduate student Larissa set the example for how obstacles in graduate school should be tackled and she has always been so gracious sharing her knowledge. Thanks Larissa.

I am fortunate to have been part of the Fang lab where all present and past members have been so supportive and friendly.

I thank my committee members Carrie Harwood, Pradeep K. Singh, Houra Merrikh and Lakshmi Rajagopal. Special thanks to Carrie for all her support, for writing me letters and for being on my reading committee. Special thanks to Pradeep as well for agreeing to be on my reading committee.

I could not have reached this point in my education without the endless support, encouragement and love provided by my Mom, Dad and Grandmother.

Finally, I am thankful to my boyfriend Diego Coca for his love and

friendship through all the years of graduate school and before (special thanks for driving me to the lab on all those weekends and late nights).

Dedications

To Mother, Dad and Gram, thanks for believing. To Diego, Olive and Papers, thanks for making me smile everyday no matter what.

CHAPTER 1:

Background

1.1 *Salmonella enterica* serovar Typhimurium as a pathogen

Salmonella enterica serovar Typhimurium (*S*. Typhimurium) is a prevalent cause of gastroenteritis in both developed and developing nations, while *S*. *enterica* ser. Typhi and Paratyphi cause enteric fever in less industrialized societies, in particular Asia, Africa and South America. According to the CDC, *S*. Typhimurium is the second most commonly laboratory-confirmed *Salmonella* serovar responsible for foodborne illness in the US (CDC Foodnet website). In addition, distinctive strains of *S.* Typhimurium have recently been associated with bacteremia in Africa (1).

Human gastroenteritis caused by *S.* Typhimurium is usually self-limiting and is characterized by neutrophils in the stool and fever. In mice, however, *S.* Typhimurium causes a systemic disease with some resemblance to typhoid fever, and diarrhea is absent unless the antibiotic streptomycin is administered to disrupt the intestinal microbiota (2, 3). Following ingestion of contaminated food or water, *S.* Typhimurium must survive multiple stresses present within the host environment. Within the gastrointestinal tract, *S.* Typhimurium encounters acidic pH, bile salts, low oxygen, and antimicrobial peptides. Following internalization by phagocytic cells, *S.* Typhimurium must withstand reactive oxygen and nitrogen species and nutrient deprivation. During diarrheal disease, *S*. Typhimurium is expelled from the host into the environment where it must adapt

to ambient temperatures and desiccation.

Outside of a host, *S*. Typhimurium may be found in association with various foods such as cantaloupe, peanut butter, eggs or egg products, meat and chili peppers. *S*. Typhimurium must be able to sense and induce responses necessary to cope with these multiple stresses as well as maintain essential physiological functions in different environmental conditions. The cell envelope, composed of the outer membrane, periplasm and inner membrane, is central to bacterial survival and thus requires stress responses to preserve its structure and function during adaptation to changing conditions. *S*. Typhimurium has five extracytoplasmic stress systems that sense and respond to damage of the cell envelope: the phage shock protein response (Psp) and regulons controlled by the alternative sigma factor σ^E (RpoE, σ^{24}) and the CpxAR, BaeSR, and RcsCDB phosphorelay systems.

1.2 Extracytoplasmic stress responses in *S.* Typhimurium

Bacteria use alternative sigma factors to direct RNA polymerase to specific promoters under stress conditions. The sigma factor σ^E was first identified in bacteria subjected to extreme heat and controls one of the most important extracytoplasmic stress response pathways in enteric bacteria. Heat stress activates σ^E by causing an increase in unfolded outer membrane proteins in the periplasm (4). The Cpx system also responds to the presence of misfolded proteins in the periplasm (5). The genes activated by Cpx and σ^E show some overlap in function (6). The inner membrane BaeS sensor histidine kinase and the response regulator BaeR control a regulon composed of three transcriptional

units: *mdtABCD-baeSR*, *acrD* and *spy* (6). Although the physiological function of the Bae system is unknown, the BaeSR activated *mdtD* gene encodes an efflux pump (designated IceT), which exports citrate (7). This helps to alleviate oxidative stress by arresting flux through the TCA cycle and reducing intracellular free iron concentrations (7). This dissertation primarily focuses on the Psp response and the RcsCDB regulon, which will be described in more detail.

1.3 The Psp Response

The Psp response was discovered by Brissette et al., who observed a 25 kDa protein specifically induced in *Escherichia coli* during infection with f1 phage (8). The protein was designated as PspA and subsequently shown to be a negative regulator and effector of the Psp response (9). The Psp response system is composed of genes in the *pspABCDE* operon, the divergently transcribed *pspF* gene and the unlinked *pspG* gene (10-12)**.** The cellular function of the Psp response became clearer through the observation by Weiner and Model that a *pspABC* mutation in *E. coli* dramatically decreases survival during prolonged stationary phase (13). The decreases survival in stationary phase could be exacerbated by alkaline pH (13). This suggested that the Psp response was necessary to maintain membrane potential ($\Delta\Psi$), which becomes the primary contributor to proton motive force (PMF) under these conditions. In this study, it was also demonstrated for the first time that the Psp response is induced by the ionophore CCCP, which causes collapse of both components of PMF (13). Additional Psp-inducing conditions are known to damage the cell membrane and impair PMF, for example: high heat, ethanol, osmotic shock and impaired protein

export (10, 14). Furthermore, the f1 pIV membrane protein decreases membrane potential in a *pspF* mutant but not in WT cells (15).

During non-inducing conditions, PspA binds to and sequesters the enhancer binding protein PspF that is necessary to promote σ^N-dependent *pspABCDE* transcription (9). Inducing stimuli cause PspA to interact with the inner membrane proteins PspB and PspC, thereby relieving the negative regulation of PspF (16). Following induction, PspA forms higher-order oligomers localized to the cell membrane, which have been observed to prevent proton leakage from damaged membrane vesicles *in vitro* (17, 18). Membrane stabilization by scaffold formation of PspA oligomers is considered to be the primary effector mechanism of the Psp response (19, 20). PspG is also a minor effector that has been shown to downregulate motility (11). PspD is an integral membrane protein, and PspE is found in the periplasm (16).

1.4 Rcs phosphorelay system

Expression of the Rcs system is observed in response to osmotic shock, growth on a solid surface, or exposure to β-lactam antibiotics (21-24). Effectors of the innate immune system including cationic antimicrobial peptides, complement and lysozyme, can also induce the Rcs system (25, 26). The Rcs response is initiated by autophosphorylation of the RcsC sensor kinase and proceeds via phosphotransfer by the RcsD protein to the RcsB response regulator (27-29). The Rcs system also includes RcsF, an outer membrane lipoprotein that acts upstream of RcsC (30). The phosphorylated RcsB response regulator can activate transcription of downstream genes either as a homodimer

or as a heterodimer with the auxiliary regulator RcsA (31) , which is unstable due to degradation by the Lon protease (32). RcsBA and RcsB homodimers regulate distinctive subsets of genes. RcsA-RcsB-regulated genes are primarily involved in exopolysaccharide production and include the 19-gene colanic acid capsular operon and the *yjbEFGH* operon, which encodes the biosynthesis of a distinct exopolysaccharide (33, 34). RcsB is required for expression of the Rcs response, and increased RcsB expression can compensate for the absence of RcsA with regard to capsular synthesis (29). Genes regulated by RcsB independently of RcsA include *ftsZ*, *osmC* and *rprA* (35-37) **.**

1.5 RpoE, Psp and Rcs are required for *S.* Typhimurium virulence

The σ^E controlled stress response is highly conserved between *E. coli* and *Salmonella* with regard to regulon members and regulation (38). However, σ^E is essential for viability in *E. coli* but not in *S*. Typhimurium, in which it is required for virulence and survival in macrophages (39, 40). *S*. Typhimurium carrying an *rpoE* mutation exhibits reduced survival after 30min exposure to 4 mM H_2O_2 *in vitro*, demonstrating that σ^E is needed for resistance to oxidative stress (39). The ability of *S*. Typhimurium to resist oxidative stress is relevant for infection, as an *rpoE* mutant of *S*. Typhimurium is virulent after i.p. inoculation of gp91phox-/- mice, which lack the superoxide-generating NADPH oxidase Nox2 (39) .

The Psp response contributes to *S.* Typhimurium survival within macrophages (41) . The importance of the Psp response has been demonstrated by Eriksson et al. (40), who found that *pspA* was highly upregulated during *S*. Typhimurium infection of murine macrophage-like J774-A.1 cells. A functional

role for the Psp response in macrophages was identified by observing that an *S*. Typhimurium *pspA* mutant is severely attenuated for virulence in C3H/HeN mice but exhibits virulence similar to wild type (WT) *S*. Typhimurium during infection of C57BL/6 mice (41). C57BL/6 mice lack the natural resistance-associated macrophage protein 1 (Nramp1), a proton-dependent divalent metal transporter expressed in the phagosomal membrane of phagocytic cells (42-44). *S*. Typhimurium contains a homolog of Nramp1 called MntH, which competes with Nramp1 for cations in the phagosome (45). Both *S*. Typhimurium MntH and Nramp1 require a proton gradient to facilitate transport of divalent cations (42, 43). Further analysis of *S*. Typhimurium MntH and the metal uptake systems ZupT and SitABCD demonstrated that they require PspA for transport of divalent cations (41). PspA is required for *S*. Typhimurium to maintain the proton gradient that allows metal transport systems to compete with the Nramp1 protein, which removes divalent metals from the phagosomal vacuole (41).

The Rcs stress response has been implicated in *S*. Typhimurium virulence during systemic infection. The *rcsC* gene was identified by microarray analysis to have expression patterns similar to that of *Salmonella* pathogenicity island-2 (SPI-2) in vitro (46). SPI-2 is required for *S*. Typhimurium intracellular growth. Further analysis demonstrated that an *S*. Typhimurium *rcsC* mutant is not defective in colonization but exhibits decreased virulence in mice and clearance from secondary sites of infection (47). Collectively, these observations suggest that the Rcs response contributes to the ability of *S*. Typhimurium to adapt to the intracellular environment of macrophages and cause systemic infection.

1.6 Integration of extracytoplasmic stress responses

Although the extracytoplasmic stress responses of enteric bacteria respond to different signals and control largely non-overlapping sets of genes, there is substantial evidence that these responses can act in an integrated fashion. Integration of the extracytoplasmic stress responses allows *Salmonella* to respond to a diverse array of environmental signals that threaten cell envelope integrity (48) .

Both the Psp response and σ^E regulon are involved in maintaining membrane potential during stationary phase and PMF following membrane damage by CCCP (49). During stationary phase in LB, the medium becomes alkaline, which abrogates the pH gradient across the inner membrane and makes membrane potential the major contributor to PMF. An *S*. Typhimurium *rpoE pspA* double mutant showed extremely reduced survival during stationary phase in association with a decreased membrane potential. An *rpoE pspA* double mutant also showed increased sensitivity to conditions other than stationary phase that are known to affect PMF. Killing by a peptide derived from bactericidal/permeability increasing protein, which leads to the dissipation of PMF, or by the protonophore CCCP, were both exacerbated in the double mutant (49). Additionally, beta-galactosidase expression by an *rpoE* mutant containing a *pspA*-lacZ fusion showed significantly increased *psp* expression relative to WT, which could be restored to baseline by introducing pBAD::*rpoE* and arabinose (49).

In addition to induction of the Psp response, *Salmonella rpoE* mutants

also show induction of the CpxAR two-component system (50). The presence of unfolded or misfolded proteins in the periplasm induces the Cpx system, which responds by activating protein folding and degrading factors (6). Similarly, production of σ^E is increased in response to the presence of misfolded outer membrane proteins in the periplasm and activates a regulon that maintains the homeostasis of the outer membrane (6), demonstrating functional overlap with the Cpx system.

The BaeSR envelope stress response prevents the accumulation of toxic compounds inside the cell by coordinating the activation of efflux pumps that expel drugs and heavy metals (7, 38). The CpxR regulator from the Cpx system can enhance BaeR binding to some target promoters in the BaeSR regulon (6), providing another example of separate stress responses coordinating with each other. Furthermore, the Cpx system maintains homeostasis following exposure to copper, demonstrating functional overlap with BaeSR in limiting the effects of heavy metal toxicity (6).

1.7 Summary

S. Typhimurium must be able to maintain the integrity of its cell envelope during adverse environmental conditions including exposure to effectors of the host immune system. The Psp response, RcsCDB phosphorelay, CpxAR and BaeSR two component systems and σ^E regulon are the five extracytoplasmic stress response systems that contribute to the ability of *S.* Typhimurium to survive as a pathogen and persist in the environment. Although the five extracytoplasmic stress response systems can act independently, integration and

functional overlap of these responses are evident. The contribution of the Psp response and σ^E regulon to maintenance of the PMF has been established (13, 49, 51). In this work, we evaluated whether biosynthesis of the CA capsule and the Yjb exopolysaccharide controlled by the RcsCDB extracytoplasmic stress response also play a role in the maintenance of PMF.

In chapter 2, some of the known functions of bacterial capsules are reviewed and the exopolysaccharides produced by *S*. Typhimurium are discussed. Chapter 3 details the results of our investigation of the maintenance of PMF by RcsCDB-regulated capsular polysaccharides.

CHAPTER 2:

S. Typhimurium Extracellular Polysaccharides

2.1 Introduction

Extracellular polysaccharides (EPS) are external to the bacterial cell wall and may be surface-associated or secreted into the surrounding milieu (52). EPS that remains firmly attached to the cell forms a discrete structure called a capsule (53). EPS released into the surrounding environment may be referred to as slime (52).

Capsular polysaccharide can be linked to the cell by covalent attachment to phospholipid or lipid A. Capsules are comprised of high-molecular-weight polysaccharides and can be homo- or heterosaccharides (55). Enormous structural diversity exists amongst bacterial capsules arising from variety of repeating monosaccharides, differences in glycosidic linkages and substitution with non-sugar moieties (53-55). The physical and chemical properties of secreted EPS also vary greatly (56) .

EPS can be neutral, polyanionic or cationic (56). As the production of EPS is a complex process that requires biosynthesis, assembly and translocation to the cell surface, EPS gene clusters are typically large and range from 6-19 genes (52, 56).

Bacterial EPS has diverse functions including biofilm formation, protection from desiccation, adhesion, resistance to phagocytosis and immune evasion (54, 57, 58). As capsular polysaccharides are comprised of over 95% water, they

protect from desiccation by providing a layer of hydration (59, 60). The production of EPS by bacteria can increase interaction with both abiotic and biotic surfaces. For instance, Group A *Streptococci* producing hyaluronic acid capsule interact with pharyngeal cells via CD44, a hyaluronic acid-binding protein (61). As biofilm formation can be a sequential process initiated by the attachment of cells to a surface, EPS can act as a molecular glue, increasing the persistence of surface-adhered bacteria while also providing architectural structure (56, 62). Bacteria within biofilms are extremely resistant to killing by antimicrobials and are protected from immune defenses (56, 62). Like biofilms, capsules can protect bacteria from immune cells. For example, *Klebsiella* producing the K21*b* type capsule is highly resistant to uptake by phagocytic cells (63).

S. Typhimurium can produce the following EPS: cellulose, O-antigen, polysaccharide associated with the *yjbEFGH* locus, and colanic acid. A summary of *Salmonella* EPS is provided in Table 2.

2.2 Cellulose

Cellulose is tough, fibrous, and one of the most abundant polysaccharides on earth. It is composed of repeating chains of β-1,4 linked D-glucose that form fibrils resembling cables. In *S.* Typhimurium, the *bcsABZC* and *bcsEFG* operons are required for cellulose biosynthesis (64, 65). The structural genes for cellulose biosynthesis are encoded by the *bcsABZC* operon and *bcsE* is required for maximal cellulose production. However, the exact role played by the *bcsEFG* genes is less well defined (64, 66, 67). Cellulose production in *S*. Typhimurium

occurs when c-di-GMP is present, which is produced by the GGDEF domain-containing protein AdrA (65, 68). CsgD is a member of the FixJ family of transcriptional response regulators and regulates transcription of *adrA* (68). Production of CsgD occurs during conditions of nutrient limitation such as when phosphorous, nitrogen and other trace elements are low, as well as during the stationary phase of growth (66).

S. Typhimurium grown on agar plates forms a colony biofilm, which binds the dye Congo red to produce what is referred to as a "red dry and rough" (rdar) morphotype (65). Colonies exhibiting the rdar morphotype are heterogeneous in structure and color and have large intensely staining red wrinkles in the center that diminish toward the colony edge, which is narrow with a smooth white rim (69). The formation of thin, aggregative fimbria (curli) and the production of cellulose create an extracellular matrix that forms the rdar colony biofilm (65). The ability to form rdar colonies is proposed to contribute to the environmental survival of *Salmonella* (70).

In addition to the rdar colony morphotype, *S*. Typhimurium can form biofilms on many surfaces including glass, plant surfaces, plastic and gallstones (64, 69, 71, 72). Cellulose is one of the main components of *S*. Typhimurium biofilms formed on glass surfaces (64). Colonization of plant surfaces by *S. enterica* is thought to involve biofilm formation, with cellulose as one of the EPS matrix constituents (73). In *S. enterica* serovar Enteritidis, a *bcsA* mutant unable to produce cellulose has a reduced ability to attach to and colonize alfalfa seeds and sprouts (74). Formation of a biofilm on gallstones by *S*. Typhimurium is also

disrupted when cellulose is not produced (71). Eliminating cellulose synthesis decreased biofilm formation in a gallstone model by 50% (71). Extreme resistance to antimicrobial agents is a characteristic of biofilms. *S.* Enteritidis cellulose-deficient biofilms demonstrate more sensitivity to the bactericidal action of chlorine in comparison to WT biofilms (64).

2.3 O-antigen capsule

Capsules comprised of O-antigen (O-Ag) are similar to the O-Ag of lipopolysaccharide. An O-Ag capsule was identified in *S.* Enteritidis and shown to be composed of a branched repeating tetrasaccharide containing rhamnose, mannose, galactose and tyvelose (58, 75). The repeating unit is partially substituted on the tyvelose and galactose sugars with a glucose-containing side chain (75). The repeating polysaccharide base unit is similar to the LPS O-Ag chain of *S*. Enteritidis (58) and *S*. Typhimurium, which contain galactose, rhamnose and mannose (76). In *S*. Enteritidis, the O-Ag capsule was found to be tightly cell-associated, and in S. Typhimurium, confocal and electron microscopy clearly show that it is present on the bacterial cell surface (58, 77).

The eight gene *yshAOPQRSTU* and divergent *yihVW* operons are required for O-Ag capsule assembly and translocation to the cell surface (58, 64, 75). The *yih* operons are conserved throughout *S. enterica* serovars and in *S. bongori* (58). In *S*. Enteritidis, *yihU* promoter::*lux* and *csgD* promoter::*lux* reporter fusions were found to have similar expression patterns (58). Additionally, luminescence activity expressed by a *yihU* promoter::*lux* reporter fusion was decreased 10-fold in a Δ*csgD* mutant background (58). These results suggest

that CsgD positively regulates the *yihU* operon in *S*. Enteritidis (58).

Capsule could not be detected on the cell surface of Δ*yihO* or Δ*yihQ* mutants when analyzed by ELISA and immunofluorescence using an O-Ag specific antiserum (58). The O-Ag capsule contributes to the ability of *S*. Enteritidis to survive desiccation; colonies formed by Δ*yihO* and Δ*yihQ* mutants were significantly more susceptible to desiccation stress than WT colonies (75, 77). In *S*. Typhimurium the O-Ag capsule has been found to impart resistance to serum-mediated killing and can decrease deposition of complement component C3 on the bacterial cell surface (77). As mentioned previously, *Salmonella* can form biofilms on gallstones (72) , and the O-antigen capsule contributes to this biofilm (71). In a competitive infection of 129X1/SvJ mice, a *S.* Typhimurium Δ*yihO* mutant was found to be less competitive than WT (71). Bacterial loads recovered from mouse gallbladder, gallstone and bile showed significantly more WT bacteria than the Δ*yihO* mutant (71).

2.4 *yjbEFGH* locus

Overexpression of the *yjbEFGH* locus in *E. coli* from a Ptrc promoter in the plasmid pTrc99a resulted in altered colony morphology and led to the production of an EPS that could bind Congo red (CR) and toluidine blue-O (TB) (33). Colonies of *E. coli* containing the *yjbEFGH* overexpression plasmid had a crater-like appearance and were translucent, whereas WT strains containing the vector control formed cone-shaped opaque colonies (33). To eliminate confounding results from the production of other surface components, colony analysis of *yjbEFGH*-overexpressing strains and relevant controls was performed in mutants

deficient in the production of curli, colanic acid and type I fimbriae (33). In all mutant backgrounds, overexpression of *yjbEFGH* resulted in crater-like colonies, indicating that the change in morphology was due to expression of this locus and not other surface structures or EPS (33) . The dye-binding observed in *E. coli yjbEFGH*-overexpressing strains led the authors (33) to conclude that this operon is involved in production of an EPS that may be helical (binds TB) and contain (1→ 4)-α-glucopyranoside units (binds CR). The dye-binding phenotype was only observed when the entire operon was overexpressed, indicating that CR and TB were not binding to an individual protein (33).

EPS was purified from *yjbEFGH*-overexpression strains and compared to strains containing the vector control (33). Carbohydrate analysis revealed an increase in reducing sugars, amino sugars and uronic acids in the overexpressing strain relative to the vector control (33). The carbohydrate analysis of EPS produced by the *yjbEFGH*-overproducing strain also showed a low level of glucose and an absence of galactose (33).

The *yjbEFGH* locus is regulated by the Rcs system (22). Microarray analysis of *E. coli* under Rcs-inducing conditions demonstrated that the *yjbEFG* locus is positively regulated by RcsC (22). The regulation of *yjbEFGH* is partially dependent on RcsA (33). A *yjbH-lacZ* fusion under Rcs-inducing conditions showed a 50-fold increase in expression in WT cells that was reduced to a 14-fold increase in an *rcsA* mutant (33). Additionally, *in silico* analysis of the region upstream of the *yjbE* start codon identified a potential RcsAB box (78) identical to the consensus sequence in 10 out of 14 nucleotides (33).

E. coli exposed to hyperosmotic stress imposed by growth in medium containing 0.7 M NaCl for 180 min showed induction of the *yjbEFGH* operon (79). The *yjbEFGH* operon was also found to be highly induced in an *rpoS* mutant exposed to 0.7M NaCl for 150 min (79). The induction of *yjbEFGH* in an *rpoS* mutant is adaptive, as an *rpoS yjbEFGH* mutant had impaired growth in LB with 0.7M NaCl when compared to mutant strains lacking either *rpoS* or *yjbEFGH* (79).

2.5 Colanic acid capsule

In 1963, a polysaccharide purified from “colicinogenic” *E. coli* isolates was designated as the colanic acid capsule (80). The colanic acid capsule is composed of a branched repeating sugar unit containing glucose, galactose, fucose and glucuronic acid (80). Production of the colanic acid capsule results in the formation of glistening mucoid bacterial colonies that are 5-6 mm in size (80). The colanic acid capsule is negatively-charged and covalently attached to LPS (63, 80, 81).

The operon encoding the proteins required for colanic acid capsule biosynthesis and export is composed of 19 genes and is 23kb in size (82). The Rcs system was first identified by its role in the transcriptional regulation of colanic acid biosynthesis in *E. coli* (83, 84). Production of colanic acid is activated by the RcsBA heterodimer response regulator of the Rcs system (31, 32). Maximal transcriptional activation of the colanic acid capsule operon requires the RcsBA heterodimer, but in a *rcsA* mutant the RcsBB homodimer can partially activate transcription (29) .

In contrast to many exopolysaccharide capsules, colanic acid does not protect against phagocytosis by polymorphonuclear leukocytes (PMNs) nor from killing following PMN uptake (63) , and confers only minimal resistance to the bactericidal actions of serum complement (25, 63). Adherence of uropathogenic *E. coli* to T84 colonic epithelial cells is impaired by the presence of colanic acid (63). Collectively, these observations do not suggest a primary role for colanic acid in *Salmonella* pathogenesis. Moreover, the production of colanic acid is increased at lower temperatures, consistent with an environmental function (21). Colanic acid capsule has been shown to contribute to biofilm formation in *E. coli* and *Salmonella* (57, 64). Colanic acid has been reported to confer resistance to environmental stresses including hyperosmolarity, acid pH, desiccation, oxidative stress and extreme temperatures (85-87).

Most *E. coli* and *S. enterica* strains are able to produce the colanic acid capsule (88, 89). However, *Salmonella* serovars that have a narrow host range contain pseudogenes in the colanic acid capsule operon (Table 1). Humans are the only known reservoir for the enteric fever *Salmonella* serovars *S*. Typhi, *S*. Paratyphi A, *S*. Paratyphi B, *S*. Paratyphi C and *S*. Sendai (90). All *S*. Typhi, *S*. Paratyphi A and *S*. Paratyphi B genomes that have been surveyed contain pseudogenes in the colanic acid capsule operon (Table 1). The genomes of the enteric fever serovars each contain more than one annotated pseudogene in the colanic acid capsule operon (Fig. 1). *S*. Gallinarum causes systemic disease in poultry but is avirulent in other animal species (90) , and also shows the presence of pseudogenes in 2 out of the 4 available annotated genomes (Table

1.). Enteric fever *Salmonella* serovars cause disseminated systemic infection in humans rather than localized gastroenteritis (90). The presence of numerous pseudogenes in the colanic acid capsule operons of enteric fever serovars further suggests that this capsule is not required for *Salmonella* virulence during systemic infection.

Table 1. List of *S*. enterica serovars containing annotated pseudogenes in the colanic acid capsule operon

***Salmonella enterica* serovar**	**# of sequenced genomes** *	**# of genomes with annotated pseudogenes in colanic acid capsule operon**#
Agona	1	0
Bareilly	1	1
Bovismorbificans	1	0
Choleraesuis	1	0
Cubana	1	0
Dublin	1	0
Enteritidis	184	14
Gallinarum	4	2
Heidelberg	5	1
Javiana	1	1
Newport	2	0
Paratyphi A	2	2
Paratyphi B	1	1
Paratyphi C	1	0
Schwarzengrund	1	0
Thompson	1	0
Typhi	4	4
Typhimurium	15	1

* *S*. enterica serovar genomes from the bacterial bioinformatics online database PATRIC http://www.patricbrc.org/ (91) .

Identified by analyzing the colanic acid capsule operon locus for annotated pseudogenes.

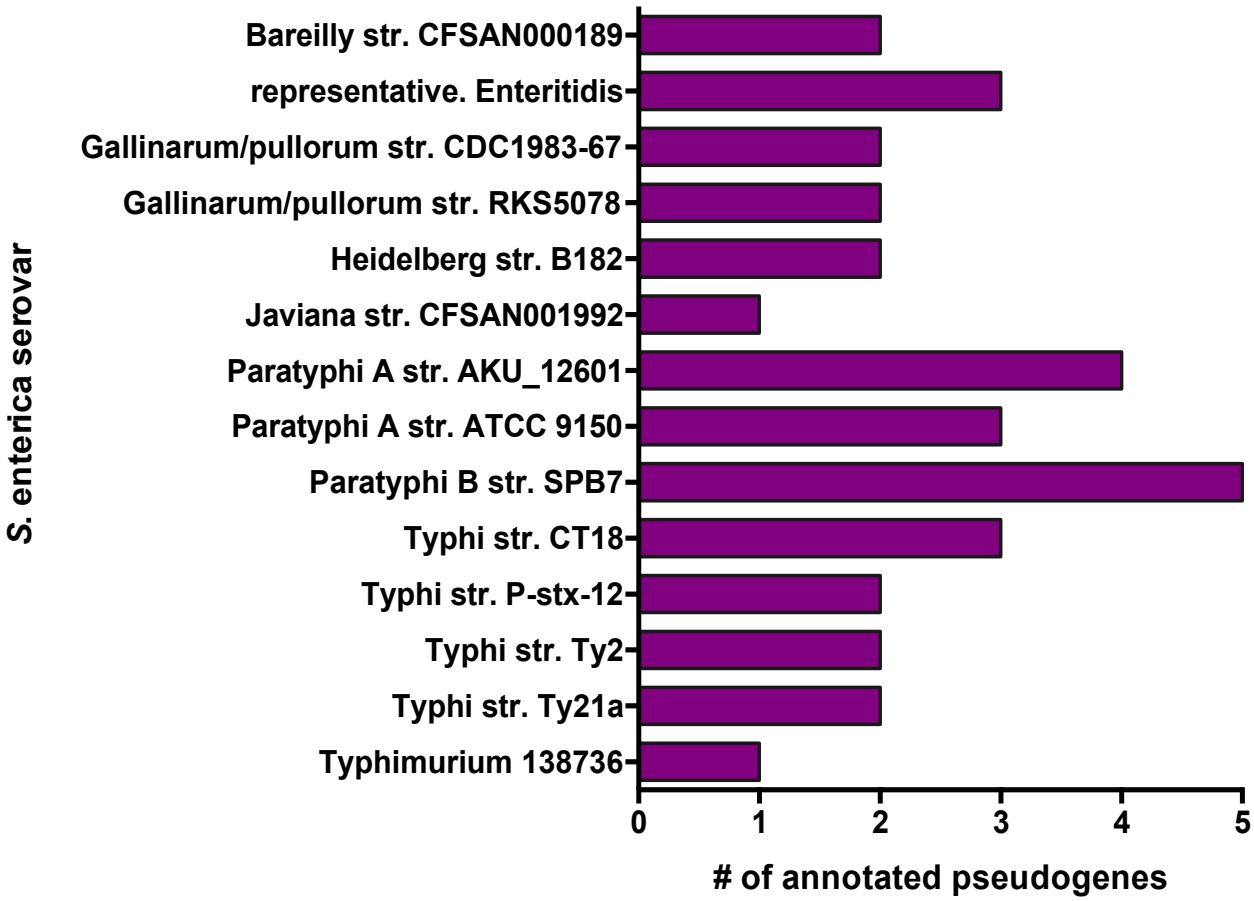

Figure 1. Total number of annotated colanic acid capsule pseudogenes in each *S*. enterica serovar. Strain designation from the bacterial bioinformatics online database PATRIC http://www.patricbrc.org/ (91) .

Table 2. Summary of *S*. enterica EPS information.

***S. enterica* EPS**	**Carbohydrate composition**	**Operons**	**Regulation**	**Functions**
Cellulose	Glucose	*bcsABZC* *bcsEFG*	CsgD	1.Contributes to RDAR morphotype. 2. Contributes to biofilm formation on gallstones, plant surfaces and glass.
O-Antigen capsule	Rhamnose Galactose subs. w/glucose. Tyvelose subs. w/glucose. Mannose	*yshAOPQRSTU* *yihVW*	CsgD	1. Improve tolerance to dessication. 2. Impart resistance to complement mediated killing. 3. Contributes to biofilm formation on gallstones.
***yjbEFGH* EPS**	Unknown	*yjbEFGH*	RcsCDB phosphorelay system	1. Increases adaptation of an *rpoS* mutant to hyperosmotic stress.
Colanic acid capsule	Glucose Galactose Fucose Glucuronic Acid	*wza wzb wzc* *wcA-F* *gmd* *wcaG-I*	RcsCDB phosphorelay system	1. Contributes to biofilm formation. 2. Confers resistance to hyperosmotic, acid pH,

		manC cpsG wcaJ wzxC wcaL-M		dessication, oxidative stress and extreme temperatures. 3. Slightly protective against complement mediated killing. 4. Maintains transmembrane electrical potential and PMF.

CHAPTER 3:

The Rcs-regulated colanic acid capsule maintains membrane potential in *S.*Typhimurium

3.1 Exopolysaccharide production is enhanced in metal-restricted *pspA* mutant *S.* Typhimurium.

The Psp (phage shock protein) system facilitates metal uptake by *S.* Typhimurium transport systems including SitABCD, MntH, and ZupT (41). Mutant strains deficient in metal transport exhibit impaired growth following treatment with the chelator 2,2'-dipyridyl (41). In previous studies, the introduction of a *pspA* (P) mutation to an *S.* Typhimurium strain lacking the ABC transporter SitABCD (S), the Fe2+ transporter FeoB (F) and the ZIP family permease ZupT (Z) resulted in cell death following treatment with dipyridyl, indicating that the Psp system is necessary for cell survival during extreme metal deprivation (41). In order to obtain mechanistic insights regarding the mechanism of cell death, a microarray analysis was performed to analyze the transcriptional response of SFZP (*sit feo zup psp*) mutant *S.* Typhimurium treated with dipyridyl for 2 hrs. The most strongly-induced genes were found to be those belonging to the colanic acid capsule operon (Table 3) regulated by the RcsA-RcsB heterodimer (31)**.** Other genes in the Rcs regulon, including *rcsA* and the *yjbEFGH* operon (33, 92) , were also strongly induced**.** The microarray results were confirmed by quantitative PCR (qPCR) analysis (Fig. 2A). As an SFZP mutant exhibited induction of the auxiliary regulator *rcsA,* we determined whether an *rcsA*-

expressing plasmid could improve growth in chelated medium. WT or mutant strains containing either a vector control or pRcsA were grown in chelated LB and growth monitored by OD_{600} (Fig. 2B). As previously observed (41) , the viability of an SFZP mutant declined after 12 hrs of growth. The *rcsA*-expressing plasmid allowed the SFZP mutant to reach a higher cell density and eliminated the decline in viability at 12 hrs, indicating that the induction of the Rcs response and exopolysaccharide production following metal deprivation of SFZP mutant *S.* Typhimurium is adaptive.

3.2 The Psp response maintains membrane integrity under metal-restricted conditions.

The cell morphology of SFZP mutant *S.* Typhimurium during metal-restriction was examined. SFZ and SFZP mutants grown in LB supplemented with 625 μM dipyridyl were sampled at 3 and 4 hrs and visualized by either differential interference contrast (DIC) or transmission electron (TE) microscopy. DIC images of cells from SFZ cultures (Fig. 3A and C) appeared smooth without surface defects, and TEM (Fig. 3B and D) revealed that the cell and outer membrane remained intact after 3 or 4 hrs of metal restriction. In contrast, DIC images of SFZP cells at 3 hrs (Fig. 3E) showed blebbing of the cell surface, and TEM (Fig. 3F) revealed cytoplasmic extrusion. Cell blebbing was frequently located near the septum of dividing cells. After 4 hrs of growth in dipyridyl, the surface blebs in SFZP cells had increased in size (Fig. 3G) with evident leakage of intracellular contents (Fig. 3H). These results show that metal restriction of SFZ mutant *Salmonella* does not compromise the integrity of the cell envelope

provided that the Psp response is intact. In the absence of the Psp response, membrane integrity is compromised when essential metal uptake is restricted.

3.3 The Rcs system maintains membrane potential (Δψ) in metal-restricted *pspA* mutant *S.* Typhimurium.

The membrane abnormalities observed in SFZP mutant *S*. Typhimurium adjacent to the septum of dividing cells are similar to what has been previously observed in mutant strains lacking the Pal lipoprotein (93). Pal comprises part of the Tol-Pal complex that bridges the inner and outer membranes via protein-protein and protein-peptidoglycan interactions (94)**.** The Tol-Pal complex is required for cell envelope integrity and dependent on PMF (94, 95). Thus, the morphology of SFZP *S.* Typhimurium cells during metal deprivation suggests that PMF is compromised under these conditions. As the Psp response is known to maintain PMF under stress conditions, and the Rcs system is strongly induced in metal-restricted SFZP mutant *S.* Typhimurium (Fig. 2A), we investigated whether the Rcs system helps to sustain the Δψ (membrane potential) component of PMF.

WT and mutant *Salmonella* cultures were grown for 2 hrs in LB with or without 625μM dipyridyl, and aliquots were removed and treated with $DiOC_2(3)$ dye for 15 min. $DiOC_2(3)$ bound to the cell surface emits green fluorescence, whereas internalized $DiOC_2(3)$ aggregates and emits red fluorescence. As $DiOC_2(3)$ internalization is Δψ-dependent, the ratio of red:green fluorescence provides a measure of Δψ. Fluorescence was measured by flow cytometry, with red:green fluorescence interpreted as proportional to Δψ (Fig. 4). No difference

in Δψ was observed between WT and mutant strains grown in LB under non-stress conditions, nor in SFZ (*sit feo zup*) or SFZR (*sit feo zup rcsA*) mutants under metal-restricted conditions. In contrast, the Δψ of a *pspA* mutant was significantly reduced in comparison to WT during metal restriction, demonstrating that PspA is required to maintain Δψ under stress conditions, in agreement with the earlier observation that the Psp response facilitates metal transport (41). An SFZPR (*sit feo zup psp rcsA*) mutant under metal-restricted conditions had the lowest Δψ, indicating that the Rcs system can sustain the membrane potential during stress when the Psp response is absent. Collectively these observations suggest the Psp response has a primary role in the conservation of Δψ, and that the Rcs system can partially compensate for the absence of the Psp response to maintain Δψ.

3.4 Construction of *S.* Typhimurium *yjb* and colanic acid capsule operon mutants.

The 19-gene colanic acid capsule operon and the *yjbEFGH* operon are regulated by the RcsA-RcsB heterodimer (34). To determine the contribution of colanic acid and the *yjbEFGH*-encoded exopolysaccharide to Rcs-related phenotypes, deletion mutations of each capsular operon were constructed. Recently, Ranjlt and Young reported that a mutation in the colanic acid capsule operon downstream of the initiating glycosylase WcaJ can result in the accumulation of toxic pathway intermediates (96). This is of concern because prior investigations have used the disruption of single pathway genes or genes downstream of WcaJ to infer the biological role of colanic acid (96). Complete operon deletions were constructed to avoid this problem.

Most *E. coli* and *S. enterica* strains are able to produce the colanic acid capsule (88, 89). Complete deletions of the colanic acid and *yjbEFGH* operons were constructed in *S. enterica* using λ-Red mediated recombination (97, 98). The mutations were verified by molecular (see methods) and functional assays involving the measurement of capsular carbohydrates (Fig. 5A). The colanic acid capsule is composed of glucose, galactose, fucose and glucuronic acid (63, 80). The structure of the Yjb exopolysaccharide has not been precisely determined but is known to contain an uronic acid component and lack fucose (33). WT cells overexpressing RcsA *in trans* showed a significant increase in both uronic acid and fucose relative to a vector control. The increase in uronic acid and fucose was eliminated by *wza* and *yjb* mutations. Overproduction of colanic acid capsule results in mucoid colonies (80). Colony morphology further confirmed the lack of colanic acid production by a *wza yjb* mutant (Fig. 5B). Additional confirmation that neither exopolysaccharide was being produced was provided by the failure to observe an increase in uronic acid (colanic acid and Yjb) or fucose (colanic acid), or the failure to generate mucoid colonies (colanic acid) in *wza yjb* mutants overexpressing RcsA.

3.5 Capsular deficiency enhances the sensitivity of *pspA* mutant *S.* Typhimurium to cationic antimicrobial peptides.

Cationic antimicrobial peptides (CAMPs) are amphipathic molecules that disrupt bacterial membranes and dissipate the PMF. The cationic P2 peptide derived from bactericidal/permeability-increasing protein (BPI-P2) permeabilizes the bacterial outer membrane and disrupts energy-dependent processes (99). An *rpoE pspA* mutant *S.* Typhimurium strain has been previously shown to

exhibit enhanced sensitivity to the BPI-derived P2 peptide (P2) (49). To determine whether the colanic acid capsule and Yjb exopolysaccharide protect cells from PMF-dissipating agents, we tested the susceptibility of WT and mutant strains to BPI-P2. The *pspA* and *wza yjb* mutants survived as well as WT following exposure to 8 μg/ml BPI-P2 for 45 min at 37˚C (Fig. 6A). However, *pspA yjb*, *pspA wza* and *pspA wza yjb* mutants were significantly more sensitive to BPI-P2 than an isogenic *pspA* mutant strain.

The Rcs system has been previously implicated in sensitivity to the CAMP polymyxin B (PMB), independent of the colanic acid capsule (47). Sensitivity to PMB was tested in WT and mutant strains to determine if the capsules are required for PMB resistance in a *pspA* mutant background. Exposure to 1 μg/ml PMB for 1 hr at 37˚C did not affect survival of *pspA, wza yjb* or *pspA yjb* mutant strains (Fig. 6B). However, *pspA wza* and *pspA wza yjb* mutants were significantly more sensitive to PMB than a *pspA* mutant, indicating that colanic acid but not the Yjb exopolysaccharide promotes cell survival following PMB-mediated membrane damage in a *pspA* mutant. As the antimicrobial activity of CAMPs is dependent in part on PMF disruption, these observations are consistent with a role of colanic acid in the maintenance of PMF.

3.6 The Psp and Rcs stress responses maintain membrane potential in stationary phase.

PspA is one of the most highly expressed proteins in stationary phase (13) , and the importance of the Psp response during stationary phase is well established. Membrane potential (Δψ) and survival are both decreased during

stationary phase in mutants lacking PspA (13, 49). The Δψ of WT and mutant bacteria was measured to determine whether the Rcs system, colanic acid capsule and Yjb exopolysaccharide contribute to maintaining this component of PMF in early stationary phase. Although our initial experiments focused on phenotypes dependent on RcsA, some residual capsular synthesis can be observed in *rcsA* mutant strains as the result of capsular operon activation by the RcsB homodimer (29). Therefore, measurement of membrane potential was performed in *rcsB* mutants that are completely incapable of capsule production.

Overnight cultures were diluted 1:1000 into fresh LB and grown at 37˚C with agitation to an OD_{600} of 1.5. Aliquots were taken and the Δψ measured using $DiOC_2(3)$ and flow cytometry. The Δψ of individual cells (Fig. 7A and C) are depicted as histograms representing the distribution of red:green ratio values for populations of 2×10^4 cells. The distribution of a WT population treated with the protonophore carbonyl cyanide m-chlorophenyl hydrazine (CCCP) is included as a control, showing a left-shifted histogram with a lower red:green ratio, indicating depolarization of the membrane potential. The Δψ was measured in four biological replicate experiments and the average mean fluorescent intensities (MFIs) calculated from histograms for statistical analysis (Fig. 7B and D).

The WT histogram (Fig. 7A) appears normally distributed with an average MFI of 570 ± 85 (Fig. 7B). All mutants showed left-shifted distributions relative to WT, with mean MFIs significantly different from that of WT, confirming the requirement of PspA for the maintenance of membrane potential during stationary phase and demonstrating a role for the Rcs response system in WT

cells. Although the histogram of an *rcsB* mutant appears slightly left-shifted in comparison to that of a *pspA* mutant, the mean MFIs were comparable (*pspA,* 413 ±110; *rcsB*, 411 ±118). The *pspA yjb* mutant histogram was only slightly left-shifted compared to that of a *pspA* mutant, and the mean MFIs were not significantly different, indicating the Yjb exopolysaccharide is not required for the maintenance of Δψ in stationary phase. The *pspA rcsB*, *pspA wza yjb* and *pspA wza* mutant histograms were all left-shifted relative to that of a single *pspA* mutant, and slightly left-shifted in comparison to that of a single *rcsB* mutant. Statistical analyses of the mean MFIs showed significantly reduced red:green ratios in *pspA rcsB*, *pspA wza yjb* and *pspA wza* mutant strains relative to single *pspA* or *rcsB* mutant strains.

Together, these observations demonstrate that the Psp and Rcs stress responses independently contribute to maintaining membrane potential in stationary phase, and that the production of colanic acid capsule is specifically required. Expression of *rcsB* on a plasmid fully complemented an *rcsB* mutation and also restored Δψ in a *pspA* mutant strain (Fig. 7C and D).

3.7 The Psp response and colanic acid capsule contribute to biofilm formation.

Salmonella can form biofilms on biotic and abiotic surfaces including glass, plastic, gallstones, HEp-2 cells and chicken intestinal epithelium (64, 69, 72, 100). The contribution of the colanic acid capsule to biofilm formation is well established (57, 64) , and induction of the Psp response has been observed in biofilms (101). Therefore, we tested whether the inability to mount the Psp

response and produce colanic acid capsule impacted biofilm formation in microtiter plates. For biofilm formation, overnight cultures were adjusted to an OD_{600} of 1.0, diluted 1:100 in LB, then added to microtiter plate wells and grown statically for 48 hrs at 25°C. Biofilms were quantified by the amount of crystal violet bound to exopolysaccharide as measured by absorbance at 595 nm. No defect in growth was observed for any of the strains under these assay conditions, as determined by optical density at 600nm (data not shown). The *pspA* mutant formed significantly less biofilm than WT cells (Fig. 8), demonstrating the importance of the Psp response for *Salmonella* biofilm formation. Biofilms formed by *pspA* and *pspA yjb* mutants were similar, whereas *pspA wza* and *pspA wza yjb* mutants formed significantly less biofilm than a *pspA* mutant. These observations indicate that the Psp response and colanic acid capsule contribute to *S*. Typhimurium biofilm formation, whereas the Yjb exopolysaccharide does not, as previously observed (22). Decreased biofilm formation by the *wza yjb* mutant is also likely to result from the absence of colanic acid capsule, further confirming that this exopolysaccharide is essential for *Salmonella* biofilm formation. The red, dry and rough (RDAR) colony morphotype is indicative of biofilm formation by *Salmonella* on agar plates containing dyes Congo red and Coomassie blue (69). Wild-type RDAR colonies are not formed by *pspA*, *wza yjb,* or *pspA wza yjb* mutants (Fig. 9), providing additional evidence that the Psp response and colanic acid capsule support biofilm development.

3.8 Colanic acid deficient mutants are more susceptible to ampicillin.

Beta-lactam antibiotics induce the colanic acid and Yjb exopolysaccharides as well as the Psp operon (23, 24, 102). Sensitivity to ampicillin was measured to determine if the Psp response and colanic acid or Yjb exopolysaccharides are protective against this clinically-relevant antibiotic. Overnight cultures were used to inoculate fresh LB and strains grown to logarithmic phase before the addition of 200µg/ml ampicillin and determination of survival by dilution, plating and enumeration of cfu. A *pspA* mutation did not affect the *Salmonella* sensitivity to ampicillin, nor did the introduction of a *yjb* mutation into a *pspA* mutant background (Fig. 10), at any time point measured. The *wza yjb*, *pspA wza,* and *pspA wza yjb* mutant strains, which lack the colanic acid capsular operon, all exhibited impaired survival following ampicillin treatment. Therefore, colanic acid supports *Salmonella* survival following ampicillin exposure.

Table 3. Genes induced four-fold or greater during growth of a *Salmonella* SFZP mutant in LB with 625 µM dipyridyl.

SFZP = *sit feo zup psp*.

STM#	Gene Symbol[a]	Function[a]	Fold Change[b]
Psp regulon			
STM1690	*pspA*	phage shock protein A	23
STM1689	*pspB*	phage shock protein B	47.1
STM1688	*pspC*	phage shock protein C	71.3
STM1687	*pspD*	phage shock protein D	44.6
STM1686	*pspE*	phage shock protein E	26.1
STM4244	*pspG*	phage shock protein G	4.3
RcsCDB regulon			
STM2262	*eco*	ecotin	5.6
STM1982	*rcsA*	transcriptional regulator	4.5
STM1705	*osmB*	osmotically inducible lipoprotein B	5.3
STM1212	*ycfJ*	outer membrane lipoprotein	6.3
STM0414	*yajI*	outer membrane lipoprotein	4.3
Colanic acid capsule operon			
STM2118	*wza*	polysaccharide export protein	31.5
STM2117	*wzb*	protein-tyrosine-phosphatase	25.1
STM2116	*wzc*	colanic acid tyrosine-protein kinase	36
STM2115	*wcaA*	glycosyl transferase	28.2
STM2114	*wcaB*	colanic acid biosynthesis acetyltransferase	20.5
STM2113	*wcaC*	glycosyl transferase	20.2
STM2112	*wcaD*	colanic acid polymerase	14.7
STM2111	*wcaE*	glycosyl transferase	30
STM2110	*wcaF*	colanic acid acetyltransferase	44
STM2109	*gmd*	GDP-D-mannose dehydratase	78.9
STM2108	*wcaG*	GDP fucose synthetase	64.1
STM2107	*wcaH*	GDP-mannose mannosyl hydrolase	34.9
STM2106	*wcaI*	glycosyl transferase	31.3
STM2105.S	*manC*	mannose-1-phosphate guanylyltransferase	35.2
STM2104	*cpsG*	phosphomannomutase	26.7
STM2103	*wcaJ*	UDP-glucose lipid carrier transferase	13.3
STM2102	*wzxC*	colanic acid exporter	8.8
STM2101	*wcaK*	pyruvyl transferase	8.6
STM2100	*wcaL*	colanic acid biosynthesis glycosyl transferase	14.3
STM2099	*wcaM*	colanic acid biosynthesis protein	9.1
yjb operon			
STM4223	*yjbF*	outer membrane lipoprotein	16.2
STM4224	*yjbG*	periplasmic protein	13.9
STM4225	*yjbH*	outer membrane lipoprotein	11.2
Others			
STM4274	*yjcH*	inner membrane protein	8.2
STM3645	*yiaD*	outer membrane lipoprotein	7
STM3157	*yghA*	Oxidoreductase	4.9
STM1697		diguanylate cyclase/phosphodiesterase domain containing protein	5.6
STM1685	*ycjX*	ATPase	31
STM1684	*ycjF*	hypothetical protein	18.4

[a] Gene symbol and predicted protein function was obtained from the *S. enterica* serovar LT2 database on the BioCyc Database Collection website.

[b] Fold change denotes an increase in the SFZP mutant's transcript levels relative to the SFZ mutant.

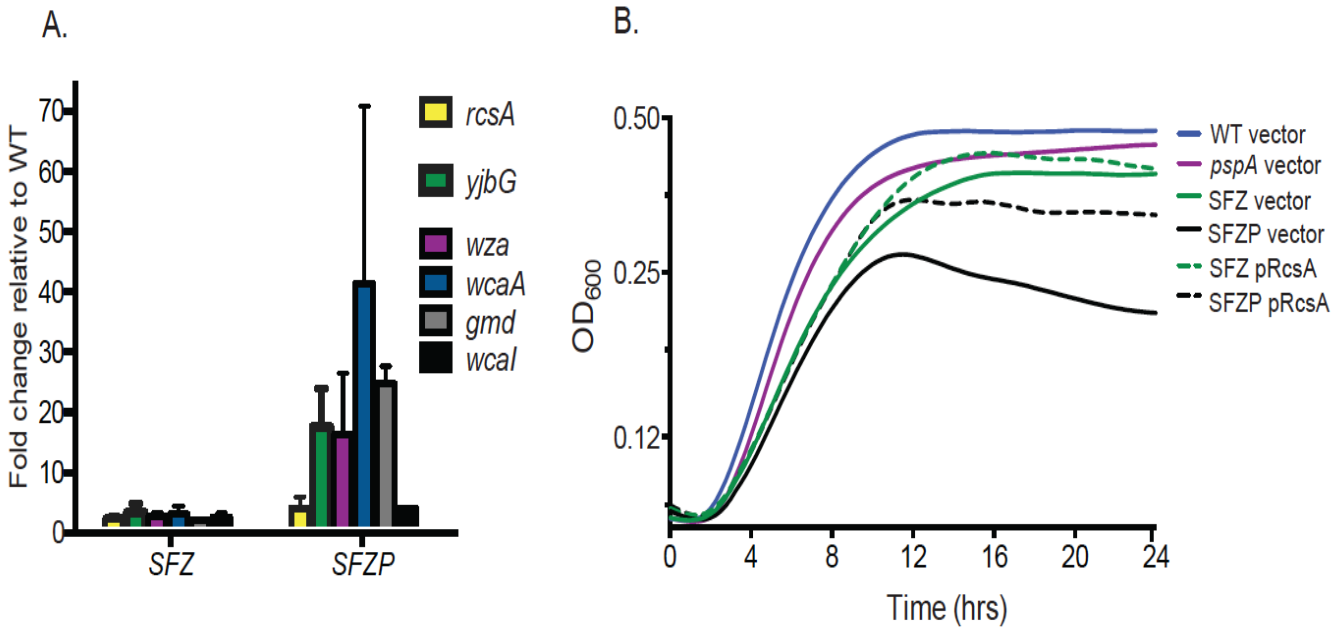

Figure 2. RcsA and exopolysaccharide genes are induced in *sitA feoB zupT pspA* (SFZP) mutant *Salmonella* during metal-restriction. (A) Quantitative PCR (qPCR) was performed with cDNA obtained from cultures grown in chelated LB for 2 hrs. Absolute qPCR values were normalized to the bacterial housekeeping gene *rpoD* and expressed as the fold-change over WT. Mean qPCR values from 3 biological replicates ± SD are shown. (B) Growth curves of strains in LB with 550 μM dipyridyl at 37°C.

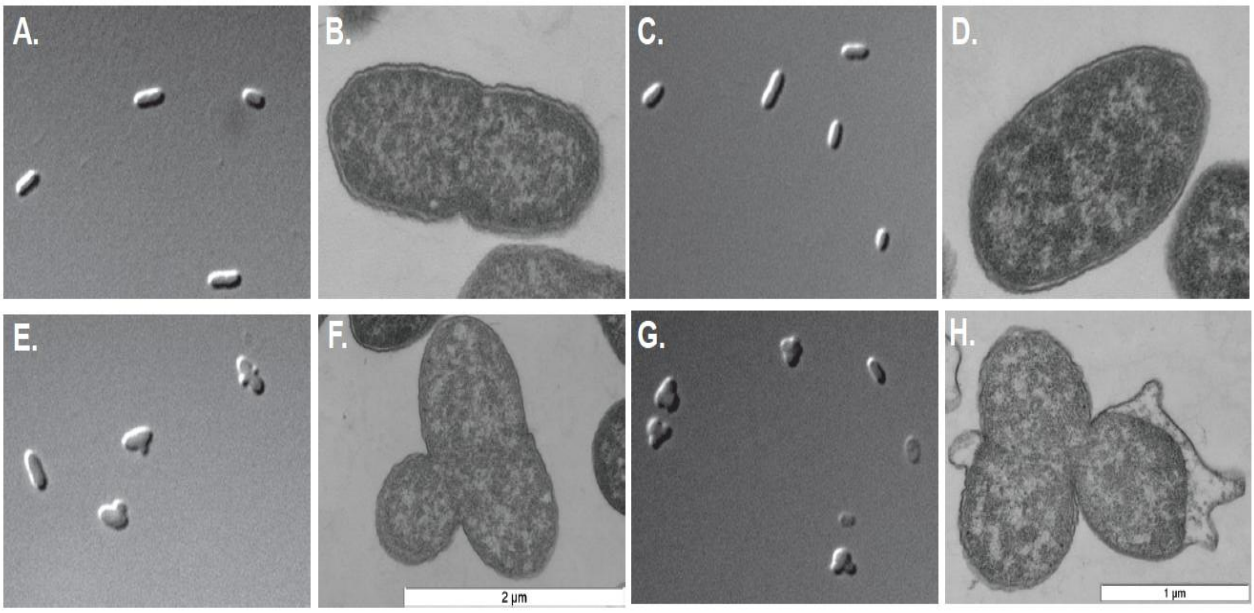

Figure 3. *S*. Typhimurium *sitA feoB zupT* metal transport mutants lacking the phage shock response lose cell membrane integrity. *Salmonella sitA feoB zupT* (SFZ) (A-D) or *sitA feoB zupT pspA* (SFZP) (E-H) mutants were diluted 1000-fold into LB with 625 µM dipyridyl and incubated for 3 (A-B and E-F) or 4 (C-D and G-H) hrs. Representative images from differential interference contrast (DIC) (A,C,E,G) or transmission electron microscopy (TEM) (B,F,D,H) are shown. DIC 100X, oil immersion; B,F, TEM 20,000X; D,H, TEM 30,000X.

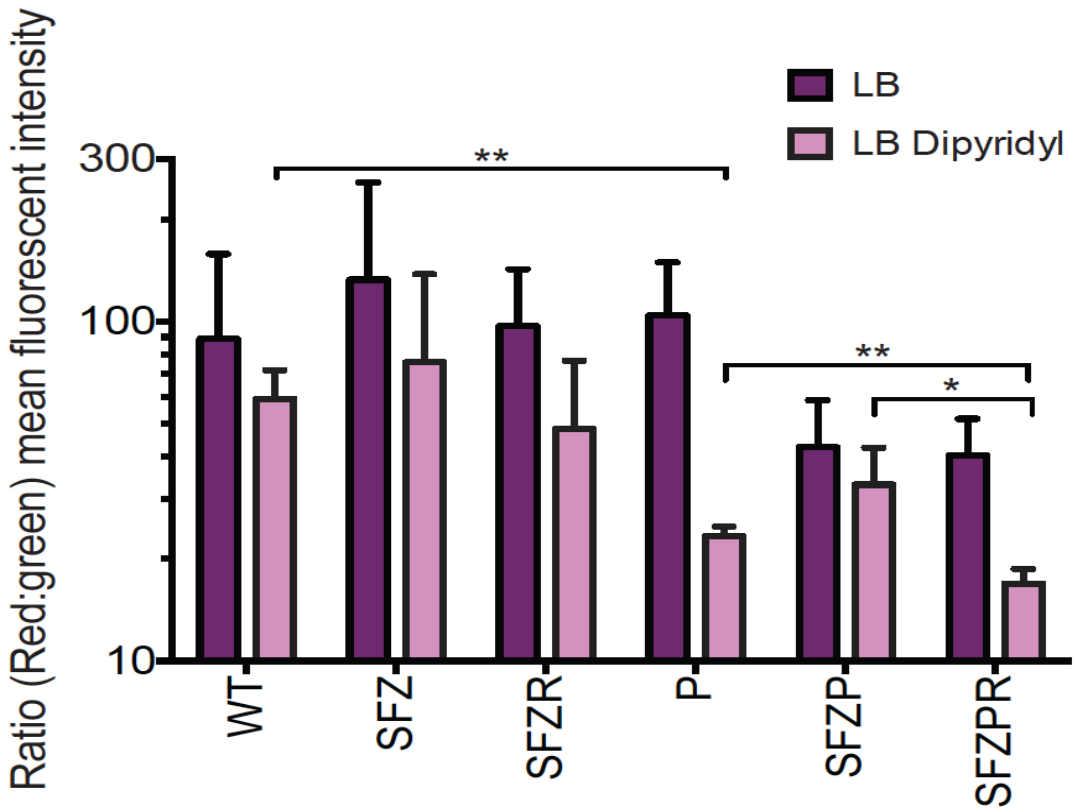

Figure 4. The Psp and Rcs responses maintain membrane potential in metal transport-deficient mutants during growth in metal limited media. Membrane potential (Δψ) was measured by flow cytometry of aliquots from cultures incubated for 2 hrs in LB with 625 μM dipyridyl. Flow cytometry was performed with live bacterial cells following 15 min incubation with the membrane potential-sensitive dye $DiOC_2(3)$, which exhibits green fluorescence that shifts towards red fluorescence following Δψ-dependent intracellular aggregation. Data were expressed as the mean ratio of red:green emission for a population of 2 x 10^4 cells; a representative plot of three biological replicates is shown. Statistical significance was determined using an unpaired t-test (*P < 0.05; **P < 0.01). Abbreviations: *sitA* (S), *feoB* (F), *zupT* (Z), *pspA* (P), *rcsA* (R).

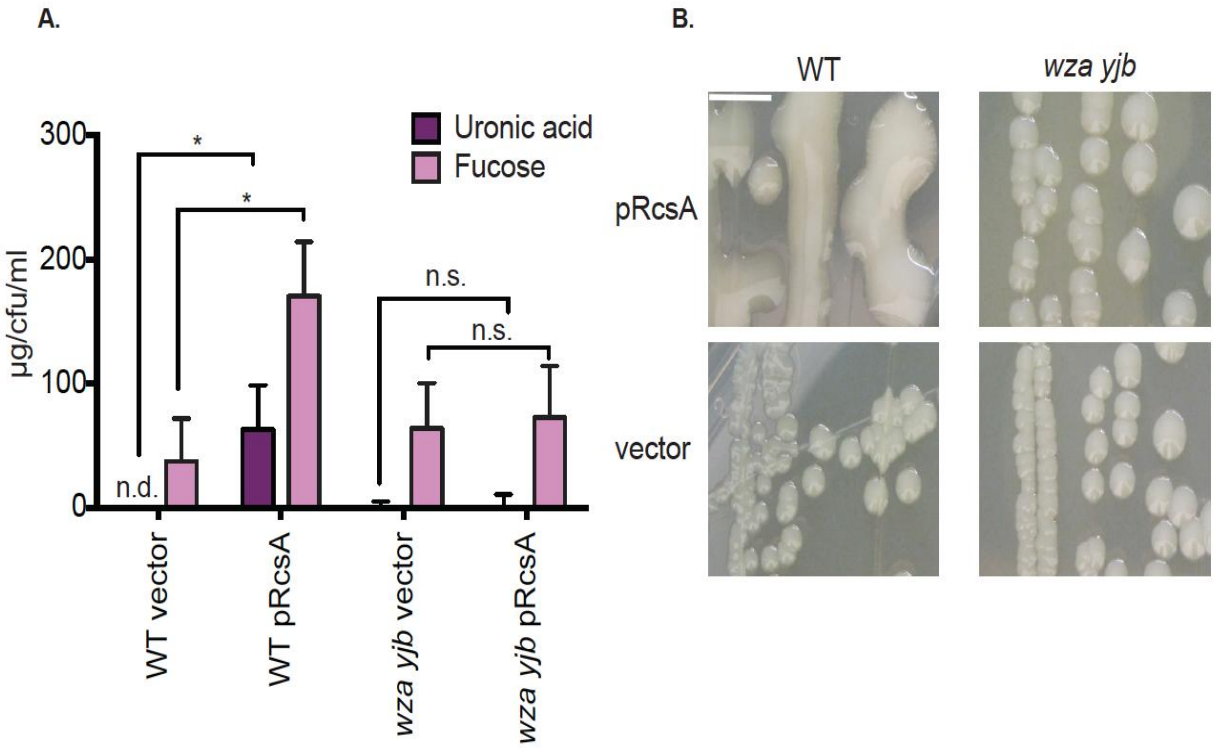

Figure 5. Mutations in the *wza* colanic acid and *yjbEFGH* operons eliminate the production of capsular exopolysaccharide. Expression of the colanic acid and *yjbEFGH* regulons is dependent on the RcsCDB phosphorelay. Capsule production was induced by RcsA expressed in trans on a pBR322 replicon. (A) Purified exopolysaccharide was subjected to a spectrophotometric assay for fucose and uronic acid. Values represent the mean of three biological replicates ± SD with significance determined using an unpaired t-test (*P < 0.05; n.s., not significant; n.d., not detected). (B) Representative images of colonies on LB agar plates formed by WT or the *wza yjb* capsular deficient mutant, each strain contains either the pRcsA expression plasmid or the vector control. Colonies were allowed to grow for 3 days at 25°C. Scale bar shows 5 mm.

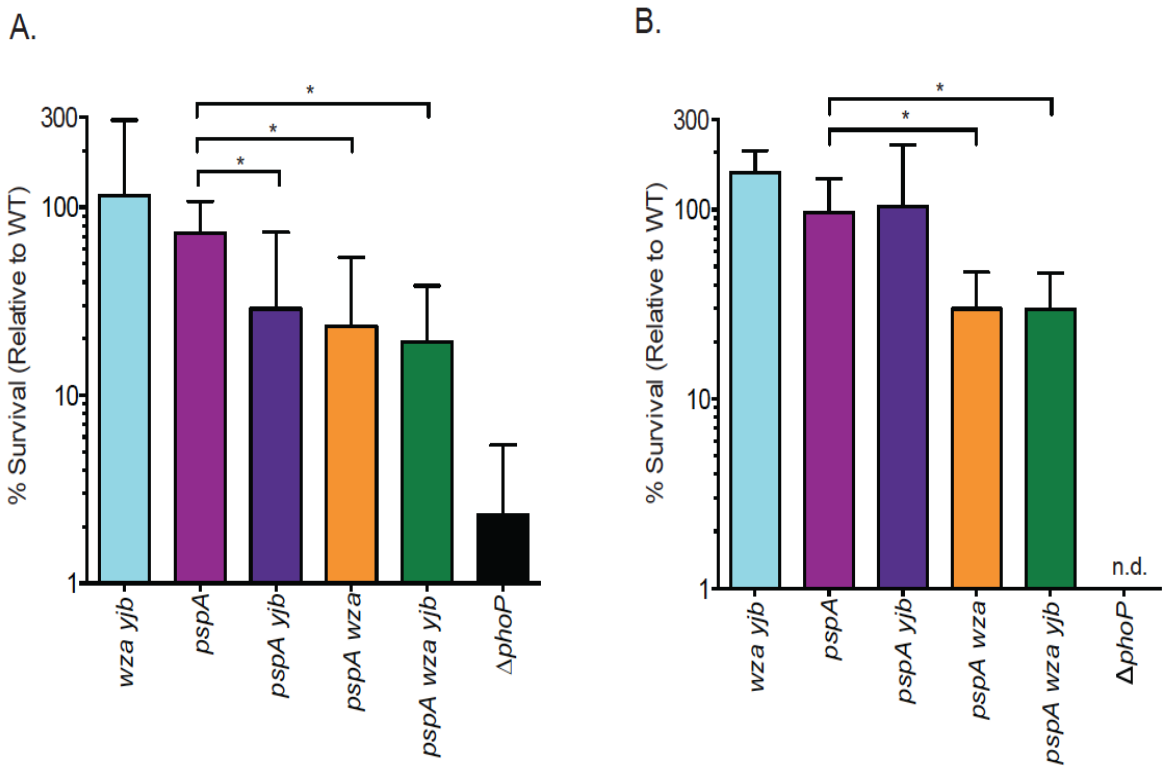

Figure 6. *S*. Typhimurium *pspA* mutants deficient in capsule production have increased sensitivity to antimicrobial peptides. (A) Sensitivity to the cationic Bactericidal Permeability Inducing peptide (BPI)-derived P2 peptide (8 µg ml-1) was measured by enumerating cfu after 45 min treatment at 37°C. Input cfu were calculated at time zero by plating untreated samples. Percent survival was calculated by dividing cfu at 1hr by input cfu and normalizing to wild-type. (B) Sensitivity to polymyxin B (1 µg ml^{-1}) was determined by enumerating cfu after 1 hr treatment at 37°C. Percent survival was calculated as described for P2. The mean percent survival ± SD from a minimum of 4 biological replicates is shown. Significance was determined by paired t-test (*P < 0.05; n.d., not detected)

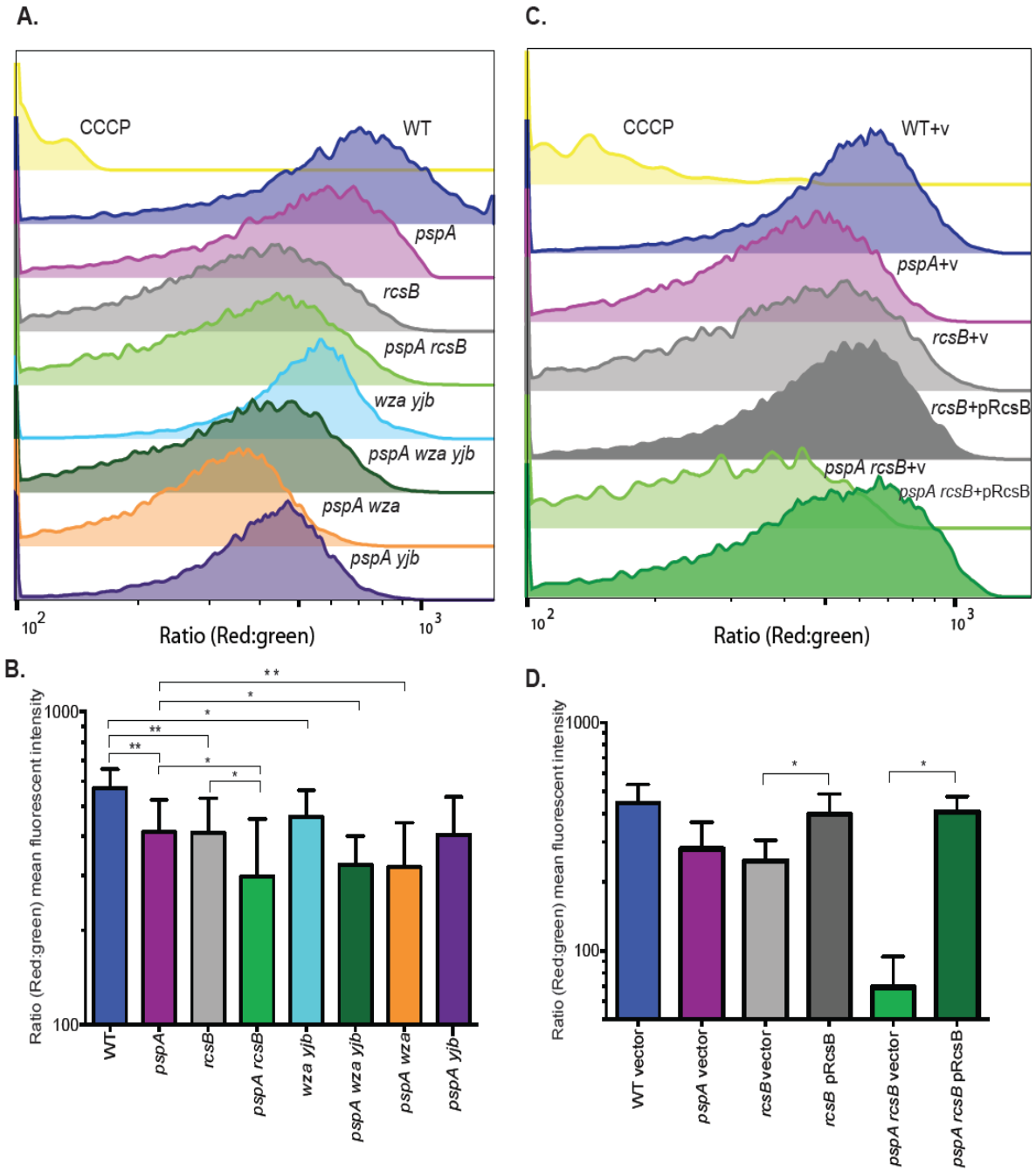
A.
CCCP
WT
pspA
rcsB
pspA rcsB
wza yjb
pspA wza yjb
pspA wza
pspA yjb
10^2
Ratio (Red:green)
10^3
C.
CCCP
WT+v
pspA+v
rcsB+v
rcsB+pRcsB
pspA rcsB+v
pspA rcsB+pRcsB
10^2
Ratio (Red:green)
10^3
B.
Ratio (Red:green) mean fluorescent intensity
1000
100
WT
pspA
rcsB
pspA rcsB
wza yjb
pspA wza yjb
pspA wza
pspA yjb
D.
Ratio (Red:green) mean fluorescent intensity
1000
100
WT vector
pspA vector
rcsB vector
rcsB pRcsB
pspA rcsB vector
pspA rcsB pRcsB

Figure 7. Rcs-regulated colanic acid capsule production maintains membrane potential in stationary phase. *Salmonella* cultures were grown to stationary phase and membrane potential measured by flow cytometry using $DiOC_2(3)$ as in Fig. 3. Histograms show the ratio of red:green emission as a measure of membrane potential (Δψ) distribution for a population of 2×10^4 cells. Mean fluorescent intensity (MFI) for each histogram was determined from 4 biological replicates. Wild-type cells depolarized by CCCP (carbonyl cyanide m-chlorophenyl hydrazone) is included as a control. (A) Representative histograms for wild-type and mutant cells. (B) Replicate MFIs for the strains represented in panel (A). (C) Representative histogram showing mutants complemented with pRcsB. (D) Replicate MFIs for strains represented in panel (C). Significance was determined by paired t-test (*$P < 0.05$; **$P < 0.01$).

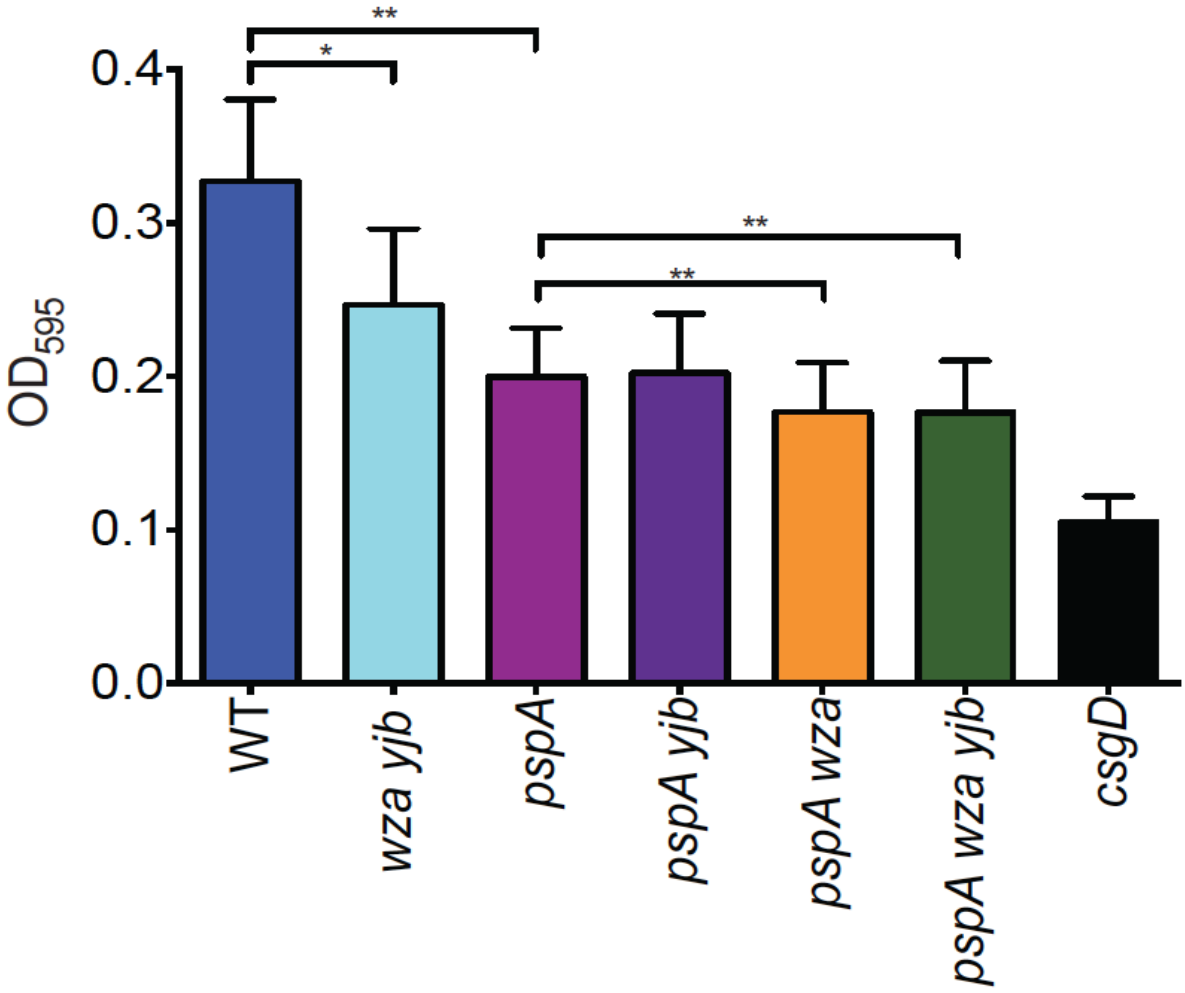

Figure. 8. Biofilms formed by wild-type and mutant *Salmonella* strains. Strains were added to 96-well PVC microtiter plates containing LB and incubated without agitation at 25°C for 48hrs. Crystal violet binding to biofilms was quantified by measuring absorbance at 595nm. A *csgD* mutant lacking curli and cellulose was included as a biofilm-negative control. Biofilm formation in 5 biological replicates was measured and the mean values ± SD are shown. Statistical significance was determined by paired t-test (**P < 0.01).

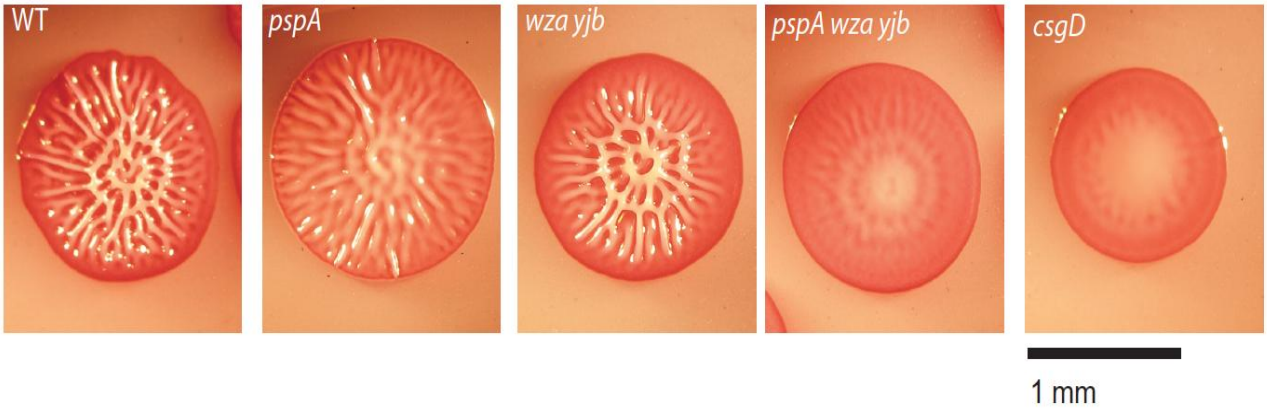

Figure 9. RDAR colony formation requires the Psp response and capsular polysaccharide production. *S.* Typhimurium strains were grown overnight in LB broth and plated onto LB agar containing the dyes Congo red and Coomassie blue without salt. Colonies were grown for 7 days at 25°C. The csgD mutant is unable to form RDAR colonies and is included as a negative control. Images shown are representative examples.

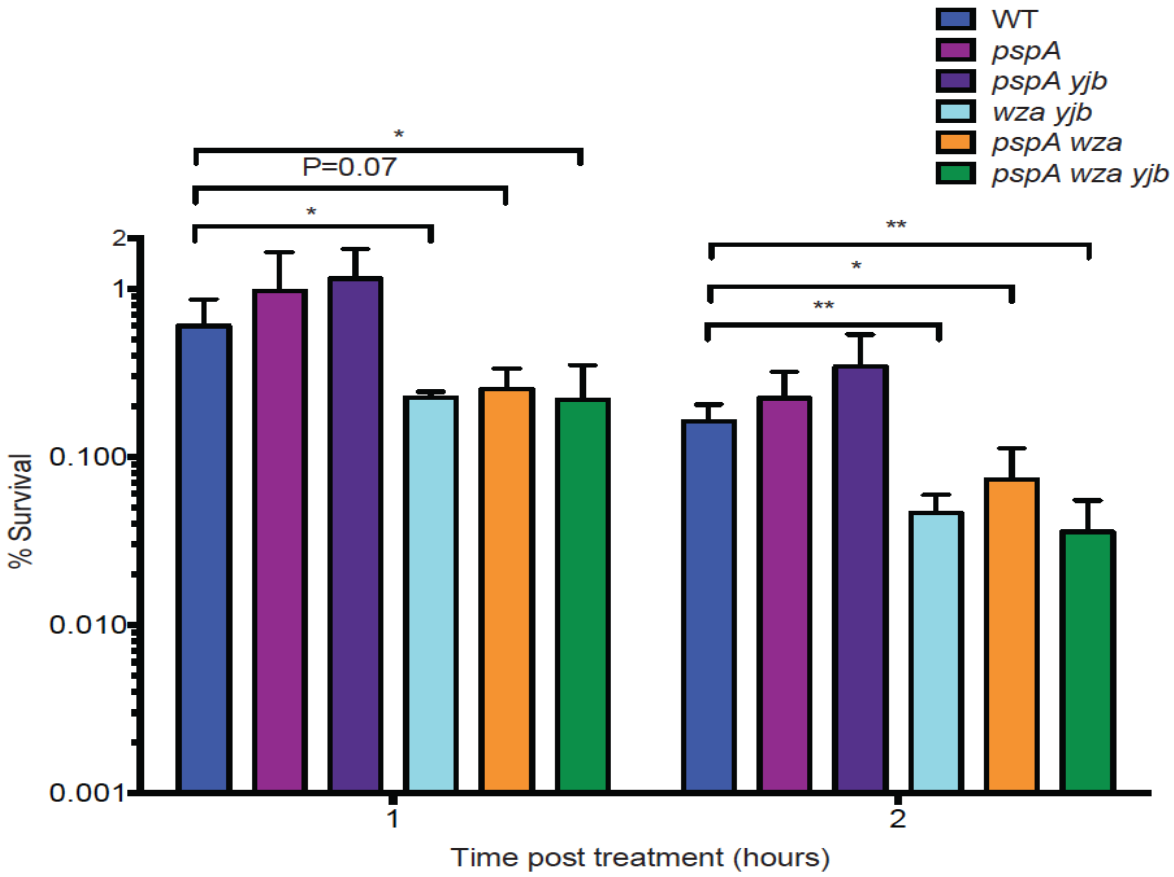

Figure 10. *S.* Typhimurium colanic acid capsule mutants show increased sensitivity to ampicillin. Bacteria were grown for three hours to reach logarithmic phase at which point 200 µg/ml ampicillin was added to culture tubes. Cultures were allowed to continue growth in the presence of antibiotic and samples were taken and plated at indicated timepoints and cfu were enumerated after 24 hrs. Susceptibility was determined by dividing the cfu at indicated times post treatment by the cfu enumerated from cultures immediately before antibiotic addition. The average survival ± SD from four biological replicates are shown. Significance was determined using a paired t-test (*, P<0.05; **, P<0.01).

CHAPTER 4:

Discussion

In this study, we describe the compensatory role of the *Salmonella* Rcs stress response system and colanic acid capsular production in the absence of the Psp response, and the role of colanic acid in preserving proton motive force (PMF).

The Psp response was originally described as a system that preserves PMF in response to cell envelope disruption by filamentous bacteriophages (1). Our laboratory subsequently demonstrated that the essential role of the Psp response in *Salmonella* virulence is to support energy-dependent metal importation in the host environment (40). Unexpectedly we observed that metal deprivation of a *Salmonella* strain lacking the Sit, Feo and ZupT (SFZ mutant) metal transport systems causes the cells to lose viability if the Psp system was also inactivated (SFZP mutant) (40 and Fig. 2B). In the present study, we demonstrate that the loss of viability of an SFZP mutant is accompanied by the loss of cell envelope integrity (Fig. 3). We hypothesize that metal depletion of an SFZP mutant impairs electron transport and results in energy depletion with a heightened dependency on the phage shock response to maintain PMF. The membrane instability observed in an SFZP mutant may result from disruption of the PMF-dependent formation of the cell envelope-stabilizing Tol-Pal complex (93).

A transcriptomic analysis of an SFZP mutant under metal-deprived conditions revealed expression of the Rcs system (Table 3), which was

confirmed by qPCR (Fig. 2A). Expression of the RcsA regulator from a plasmid is able to restore growth to the SFZP mutant in metal-deprived medium (Fig. 2B), indicating that the Rcs system is playing a compensatory role in the absence of PspA. *Salmonella* SFZP mutants continue to exhibit envelope structural defects (Fig. 3E-H) despite Rcs induction, indicating that the endogenous level of Rcs expression is insufficient to completely compensate for the loss of the Psp response under these environmental conditions.

In view of the established role of the Psp response in sustaining PMF during envelope stress (1), we investigated whether the Rcs system also affects the membrane potential (Δψ) component of PMF. We observed that metal deprivation of *Salmonella* results in depolarization of the Δψ, which is sustained by the Psp response (Fig. 4). Under these conditions, the Δψ of an SFZPR mutant lacking both the Psp and Rcs stress responses is significantly reduced in comparison to mutants lacking only the Psp response. This suggests that the Rcs system helps to preserve PMF in the absence of the Psp response. As an *S.* Typhimurium *pspA* mutant was previously found to be attenuated for virulence in mice expressing the metal transporter Nramp1 (41) , we determined whether a *pspA rcsB* mutant was less virulent than a *pspA* mutant during *S.* Typhimurium infection of Nramp1-expressing C3J/OuJ Nramp^{+} mice. However, a competitive infection experiment showed no effect of an *rcsB* mutation on virulence in this model (Fig. 11). Further attenuation in virulence of a *pspA rcsB* mutant was not observed, possibly because the effects of a *pspA* mutation on virulence are sufficiently marked that further attentuation could not be detected. Other

investigators have found that *rcsB* mutants can be outcompeted by WT *S.* Typhimurium after 3 weeks of competitive infection of 129SvC6 mice (47).

Other conditions that stimulate colanic acid production are also known to perturb membrane energetics. For example, the monoclonal IgA antibody Sal4 impairs membrane integrity, transiently reduces PMF (103) , and induces colanic acid synthesis (104). Low concentrations of the cationic anti-microbial peptide (CAMP) polymyxin B permeabilize the cell membrane and disrupt respiration, and higher polymyxin B concentrations result in Δψ depolarization (105) . Polymyxin B also induces colanic acid synthesis (106) , and we observed that absence of the colanic acid capsule renders *pspA* mutant *Salmonella* more susceptible to this antimicrobial agent (Fig. 6B).

Although elimination of the Psp response by itself did not affect survival of cells exposed to the CAMPs polymyxin B or BPI-derived P2, elimination of both the Psp response and colanic acid synthesis enhanced susceptibility to both peptides (Fig. 6). Deletion of the *yjbEFGH* operon did not increase the susceptibility of a *pspA* mutant to polymyxin B, but enhanced sensitivity to BPI-derived P2 antimicrobial peptide (67), suggesting that the Yjb exopolysaccharide subserves a similar function.

Both the Psp (13) and RcsB (107) responses are induced during stationary phase. Stationary phase cultures of *pspA* or *rcsB* mutant *Salmonella* exhibited significantly lower Δψ compared to WT (Fig. 7A and B), and the Δψ of a *pspA rcsB* double mutant was further reduced, demonstrating that both the Psp and Rcs stress responses maintain Δψ in stationary phase. Measurement of Δψ

in *pspA* mutants lacking either the *wza* or *yjb* operons indicated that colanic acid but not the Yjb exopolysaccharide is essential for the maintenance of Δψ during stationary phase. With the construction of an *rcsB* mutation, which completely abolishes expression of the Rcs regulon (14), we also found that the Rcs system is required for the preservation of the Δψ even in the presence of the Psp response (Fig. 7) and can be restored by the expression of RcsB *in trans* (Fig. 7B). We did not observe a decrease in membrane potential in a *wza yjb rcsB* mutant beyond what is observed in the *rcsB* mutant, suggesting that there are no additional RcsB-regulated factors required for the maintenance of PMF (Fig. 12).

Colanic acid is highly expressed in *Salmonella* biofilms, most likely to address membrane bioenergetic requirements during slow growth and nutrient limitation (71, 101, 108) . Decreased biofilm formation by a *pspA* mutant (Fig. 8) suggests that PMF is reduced in biofilms, and *Salmonella* mutants lacking both *pspA* and colanic acid capsule formed even less biofilm, suggesting that, in addition to its proposed structural role, colanic acid may function to maintain membrane energetics in biofilms as well. Bacteria in biofilms are notable for their resistance to killing by antibiotics (62) , and we observed that colanic acid capsular biosynthesis contributes to resistance to ampicillin (Fig. 10), an antibiotic used to treat *Salmonella* infections. Thus, the colanic acid capsule contributes to the antibiotic tolerance of *Salmonella* in biofilms.

The strongly negative charge of colanic acid (26) is likely to account for its ability to maintain the Δψ during stress conditions. Negative charge adjacent to the bacterial cell surface requires protons as counterions. A local increase in

proton concentration at the cell surface can enhance both the surface potential and ΔpH, which has been shown to increase ATP generation in *E. coli* (109) .

The *yjbEFHG* operon appears to be associated with production of a distinct type of EPS, but its structure and cell association have not been defined (33). We therefore cannot say why it is unable to preserve PMF in the absence of colanic acid. In the future, the analysis of other types of capsule whose structures and charge are characterized may provide further insight into the mechanism of PMF maintenance by colanic acid.

Our observations corroborate Model's original hypothesis that the Psp response conserves PMF under stress conditions and provides evidence that the Rcs system and specifically colanic acid also contribute to this function. This demonstrates a novel physiological role for colanic acid capsule that may provide a unifying mechanism to account for its diverse contributions to stress resistance in enteric bacteria.

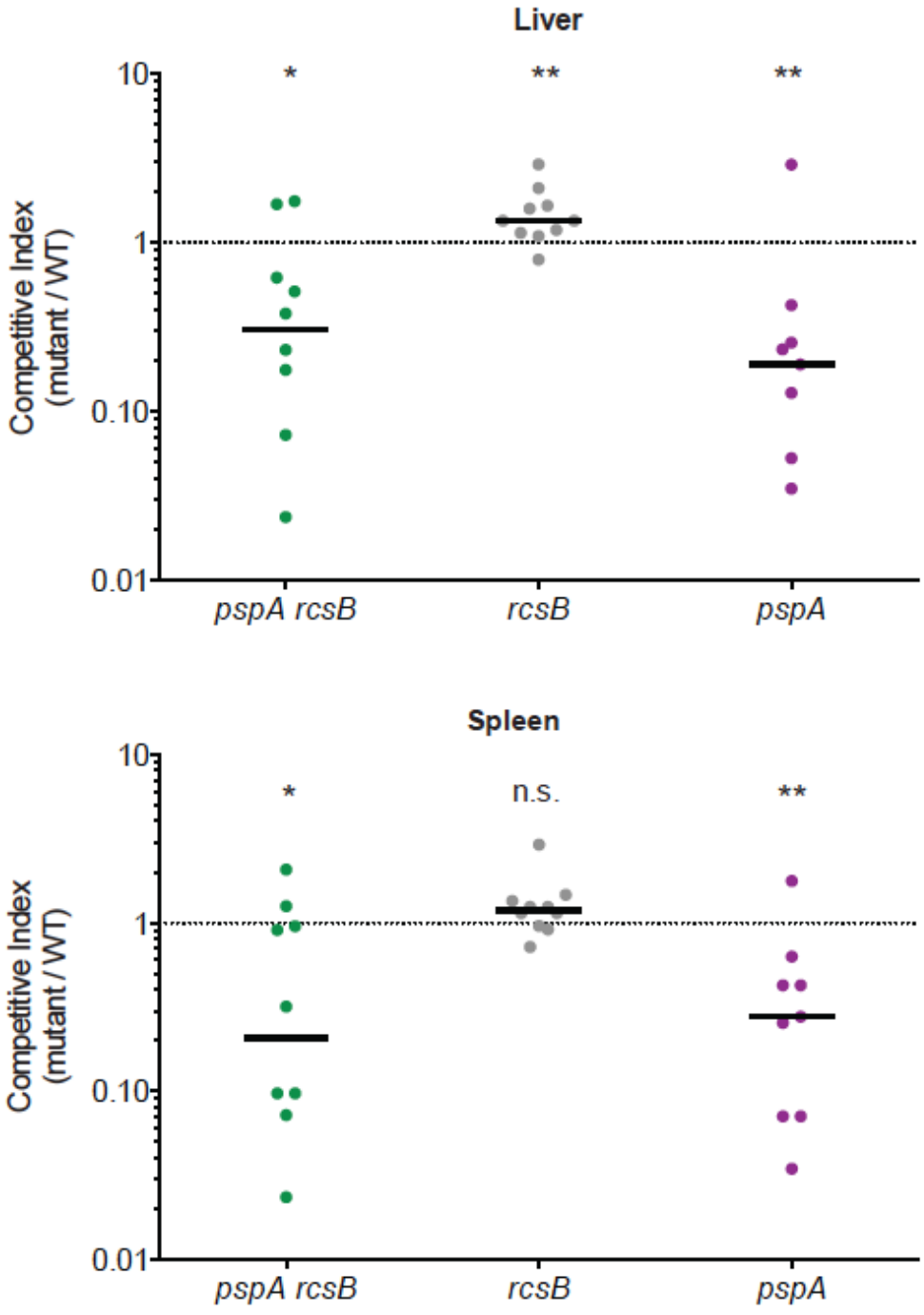
Liver
Competitive Index
(mutant / WT)
10
1
0.10
0.01
*
**
**
pspA rcsB
rcsB
pspA
Spleen
Competitive Index
(mutant / WT)
10
1
0.10
0.01
*
n.s.
**
pspA rcsB
rcsB
pspA

Figure 11. The Psp response is required for systemic virulence but the Rcs system is not. Six week old C3H/OuJ Nramp1+ mice were infected i.p with an ~1:1 mixture of mutant and wild-type *S.* Typhimurium. On day 5 post-infection, co-infected mice (n =10) were euthanized and CFU in liver and spleen were enumerated. Values less than 1 indicate a competitive advantage of WT over mutant. Bars represent the median values. Each symbol represents the result for 1 mouse. Statistical significance was determined using a Mann-Whitney test (*P < 0.05; **P < 0.01; n.s., not significant).

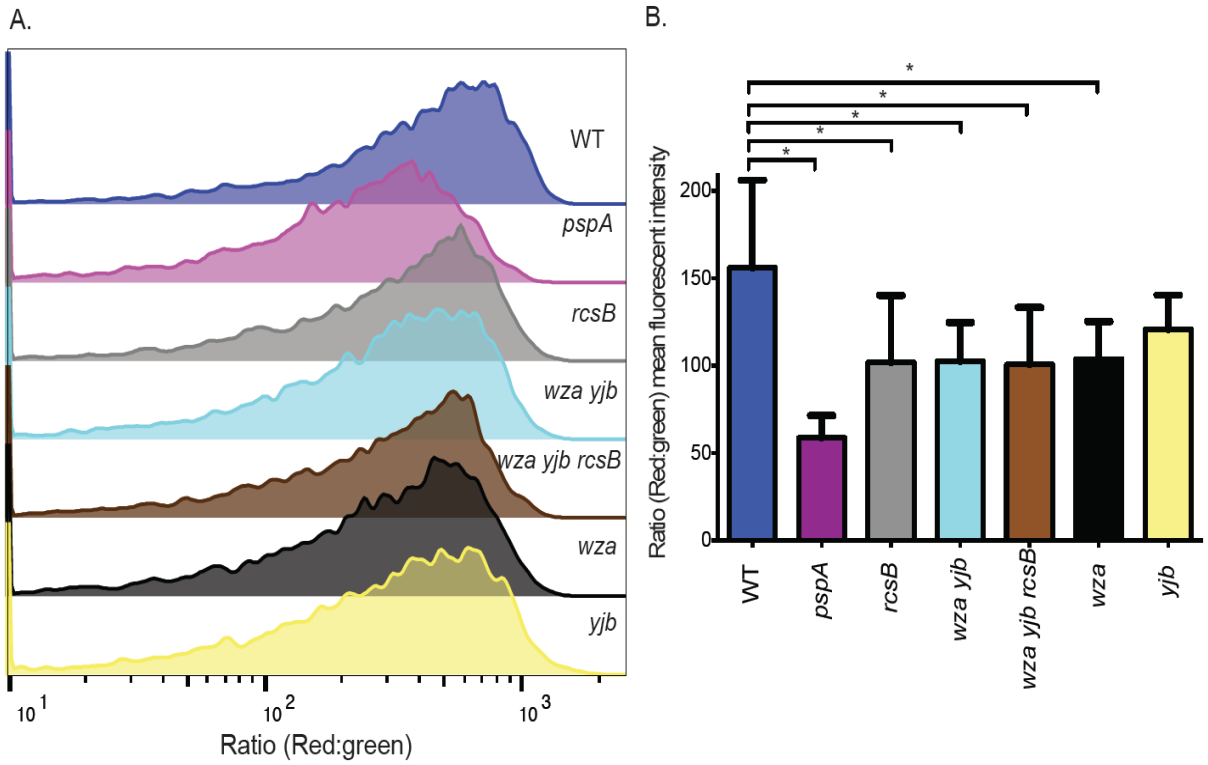

Figure 12. RcsB regulated colanic acid capsule maintains stationary phase membrane potential. *Salmonella* cultures were grown to early stationary phase and membrane potential was measured as in Fig. 7. (A) Representative histograms for wild-type and mutant cells. (B) Replicate MFIs from 4 biological replicates for the strains represented in panel A. Statistical significance was determined using a paired t-test (*P < 0.05).

CHAPTER 5:

Methods and Materials

Strains, plasmids and primers used are provided in Table 4.

5.1 Bacterial growth conditions.

All strains were routinely cultured in Luria-Bertani (LB) medium with shaking at 250 rpm at 37°C unless otherwise stated. Antibiotics were used at the following concentrations as indicated: ampicillin (100 μg ml^{-1}), kanamycin (50 μg ml^{-1}), chloramphenicol (20 μg ml^{-1}) and tetracycline (25 μg ml^{-1}).

5.2 Strain and plasmid construction.

Mutant strains were constructed using the λ- Red recombinase system (98). The *wza* colanic acid capsule mutant was constructed using the λ-Red *tetRA* replacement method (97). All mutations were verified by PCR using gene-specific primers and transduced into a clean 14028s background with bacteriophage P22. To generate plasmid JP102, plasmid pATC118 (32) was digested with *Eco*RI and *Hin*dIII to generate an 860-bp DNA fragment containing the Δ37 *rcsA* complementing fragment. The 860-bp fragment was then cloned into pJK392 using the *Eco*RI and *Hin*dIII sites and ligated with T4 DNA ligase (New England Biolabs, Ipswich, MA). To generate plasmid JP103, primers JPP249/250 were used to PCR-amplify the *rcsB* promoter and coding region (107) . Primers were designed to include the -35 and -10 elements of P_{rcsB}, which are located within the *rcsD* coding region (107) . The *rcsB* gene was cloned into

the stable low cloning vector pRB3-273C (110) using the *Sma*I site and verified by sequencing.

5.3 Flow cytometry.

Overnight cultures were diluted 1:1000 into fresh LB containing 625 μM of the metal chelator 2'2'-dipyridyl (Sigma-Aldrich) in a volume-to-flask ratio of 9:25. After 2 hrs growth, approximately 1 x 10^6 cfu were added to a 5 ml flow cytometry tube containing 1 ml of permeabilization buffer (10 mM Tris pH 7.5, 1 mM EDTA) and 30 μM of $DiOC_2(3)$ (Sigma-Aldrich), and incubated in the dark for 15 min at room temperature. A total of 2 x 10^4 cells were assayed by a LSRII flow cytometer using a 488nm excitation wavelength. Green emission was detected through a 505nm long pass filter with a 530nm, 30nm bandwidth band pass filter, and red emission was detected through a 600nm long pass filter with a 610nm, 20nm bandwidth filter. Gates for bacterial populations were based on the wild-type population using forward versus side scatter and red versus green emission. For measurements of stationary phase cultures, overnight cultures were diluted 1:1000 into fresh LB in a volume-to-flask ratio of 1:5, then grown to OD_{600} ~1.5 and assayed by flow cytometry as described. Flow cytometry data was processed using FlowJo v10.0.7 software (TreeStar, Inc.) and analyzed using a ratio of red to green fluorescence as described (111). Flow cytometry was performed at the University of Washington Pathology Flow Cytometry Core Facility.

5.4 Capsule purification and quantification.

An overnight culture was diluted 1:1000 into 50 ml of fresh LB with ampicillin and grown to OD_{600} ~2.0. One ml out of the 50 ml culture was used to enumerate cfu by dilution and plating onto LB agar, and 25 ml were pelleted and resuspended in an equal volume of PBS then boiled for 15 min to inactivate exopolysaccharide (EPS)-degrading enzymes and completely release EPS from the cell surface. The boiled sample was allowed to cool to room temperature then centrifuged at 25,400 x g for 30 min at 4°C, and the supernatant was combined with three volumes of 70% ethanol and incubated overnight at 4°C. Following overnight incubation, the sample was centrifuged at 25,400 x g for 30 min at 4°C and the resulting pellet resuspended in 1 ml sterile water and dialyzed against distilled water for 48 hrs. The final sample was stored at 4°C until quantification. Total fucose and uronic acid were quantified following established protocols (112, 113) . Total sugars were normalized to cfu and expressed as µg/cfu/ml.

5.5 Susceptibility Assays.

Measurement of polymyxin B sensitivity was performed in glass culture tubes by the method described in (114) . Polymyxin B stock was made in a glass tube, stored at 4°C and used for no longer than one week.

Synthesis of the (Bactericidal Permeability Inducing peptide) BPI-derived P2 peptide was described in (49) . P2 sensitivity was determined using a previously developed method (115) . Briefly, cultures were grown in trypticase soy broth (TSB) then diluted 1:100 into fresh TSB and grown to OD_{600} ~1.0. A

total of 10^6 bacteria/ml were treated with 8 μg/ml of BPI-derived P2 peptide and cells kept stationary at 37°C for 45 min. Input cfu were determined at time zero by plating unexposed samples onto LB agar and counting colonies after 24 hrs at 37°C. Percent survival was determined by dividing the cfu obtained after antimicrobial exposure by the input cfu and normalizing to wild-type percent survival.

Susceptibility to 200 μg ml^{-1} and 100 μg ml^{-1} ampicillin was performed as described (102) . Briefly, overnight cultures were diluted 1:1000 into fresh LB and grown for 3 hrs before plating to enumerate cfu before addition of ampicillin and at indicated time points following antibiotic treatment. Percent survival was calculated by dividing the cfu obtained after ampicillin exposure by the unexposed input cfu.

Growth kinetics in the presence of dipyridyl were performed as in (41) using a Bioscreen C Microbiology Microplate reader (Growth Curves USA).

5.6 Microscopy.

To prepare cells for microscopy, overnight cultures were diluted 1:1000 into 1 liter fresh LB with 625 μM of the metal chelator 2'2'-dipyridyl (Sigma-Aldrich) in a volume-to-flask ratio of 9:25. Cultures were grown with shaking at 37°C and aliquots taken at indicated time points, pelleted and kept on ice. For differential interference contrast microscopy, pelleted cells were re-suspended in 0.85% NaCl and 2 μl immobilized on an agarose pad and imaged with a Nikon Eclipse TE200 inverted microscope. For transmission electron microscopy, cells were pelleted and washed two times with PBS and resuspended in 1 ml of ½ x

Karnovsky's fixative. Transmission electron imaging was performed at the University of Washington Electron Microscopy Center.

5.7 RNA preparation, cDNA synthesis and qPCR.

Overnight cultures were diluted 1:1000 into 1 liter fresh LB with 625 μM of the metal chelator 2'2'-dipyridyl (Sigma-Aldrich) in a volume-to-flask ratio of 9:25, and 200 ml of cells were pelleted after 2 hrs of growth. The pellet was resuspended in 2.5 ml of Trizol reagent. Contaminating DNA was removed by a 1 hr DNAse (Fermentas) treatment. Following the DNAse step, the RNA was further purified using the acid/phenol method and stored at -80°C. RNA purity was determined on a 2% agarose gel and with a nanodrop spectrophotometer. The Qiagen QuantiTect reverse transcription kit was used to synthesize cDNA using 500 ng RNA as input. Quantitative PCR (qPCR) was performed using the SYBR Green Kit (Qiagen, Valencia, CA) and CFX96 real-time system (Bio-Rad, Hercules, CA) using *rpoD* as an internal control.

5.8 Crystal violet-based biofilm assays.

Overnight cultures were brought to an OD_{600} of ~1.0 with fresh LB. Adjusted cultures were then diluted 1:100 into fresh LB in a 96-well polystyrene microtiter plate. Plates were sealed with Parafilm and incubated at 25°C for 48 hrs. The OD_{600} was measured to determine growth, then culture supernatants were decanted and unbound bacteria removed by washing with PBS (pH 7.4). Remaining cells and cell-associated material were stained with 0.1% crystal violet (CV) for 10 min. After staining, wells were washed twice with PBS and the

dye solubilized with an 80:20 (v/v) ethanol/acetone mixture. CV absorbance was quantified at 595nm.

5.9 Mouse virulence assay

Mouse infections were performed similarly to those previously described, with modifications as noted (41) . C3H/OuJ mice were purchased from Jax Laboratories (Bar Harbor Maine) and housed at the Modified Specific Pathogen Free facilities at the University of Washington animal facilities under protocol 3373-01. For competitive infections, a 50/50 mixture of strains WT and mutant was used to infect mice intraperitoneally. Five days post infection, liver and spleen where removed and homogenized in PBS by using an Ultra Turrax T25 basic mixer (IKA). Homogenates were serially diluted and plated on LB agar plates. One hundred colonies from each tissue type were picked onto LB agar containing tetracycline (20 μg ml^{-1}). Competitive indices (CI) were determined as the CFU of mutant divided by the CFU of WT, normalized to the input inoculum.

5.10 Detection of the RDAR colonial morphotype.

For data regarding the RDAR colonial morphotype, bacteria were grown as in (116) with modifications. Overnight cultures grown in LB broth were diluted to a concentration of ~100 CFU/ml. Aliquots of 100 μl were plated onto LB agar without salt and supplemented with 40 μg ml^{-1} Congo red and 20 μg ml^{-1} Coomassie blue. Plates were inverted and incubated at 25°C for 7 days (27).

5.11 Microarrays

RNA was prepared from SFZ and SFZP cells as described in RNA preparation after 2 hours of growth in LB with 625 μM dipyridyl. Microarray was performed as described in (114) .

Table 4. Strains, Plasmids and Primers.

Strain, plasmid or primer	Genotype, relevant characteristics, or sequence	Source or reference
Strains		
JP1	*Salmonella* Typhimurium 14028s wild type	ATCC
JP54	*sitA*::MudCm *feoB*::Tn10 Δ*zupT*:: FRT	(41)
JP55	*sitA*::MudCm *feoB*::Tn10 Δ*zupT*:: FRT Δ*pspA*:: FRT	(41)
JP2	Δ*pspA*:: FRT	(41)
JP52	*sitA*::MudCm *feoB*::Tn10 Δ*zupT*:: FRT Δ*rcsA*:: FRT *kan*FRT	this paper
JP53	*sitA*::MudCm *feoB*::Tn10 Δ*zupT*:: FRT Δ*pspA*:: FRT Δ*rcsA*:: FRT*kan*FRT	this paper
JP57	14028s/ pJP102	this paper
JP65	14028s/pJK392	this paper
JP260	*wza*::tetRA	this paper

	Δ*yjbE*::FRT*kan*FRT /pJP102	
JP267	*wza*::tetRA Δ*yjbE*::FRT*kan*FRT /pJK392	this paper
JP312	Δ*pspA*:: FRT *wza*::tetRA	this paper
JP327	Δ*pspA*:: FRT Δ*yjbE*::FRT*kan*FRT	this paper
JP384	Δ*pspA*:: FRT *wza*::tetRA Δ*yjbE*::FRT*kan*FRT	this paper
JP391	Δ*rcsB*:: FRT*cat*FRT	this paper
JP400	Δ*pspA*:: FRT Δ*rcsB*:: FRT*cat*FRT	this paper
JP249	*wza*::tetRA Δ*yjbE*::FRT*kan*FRT	this paper
JP387	14028s/pRB3-273C	this paper
JP389	Δ*pspA*:: FRT /pRB3-273C	this paper
JP431	Δ*rcsB*:: FRT*cat*FRT /pRB3-273C	this paper
JP422	Δ*pspA*:: FRT Δ*rcsB*:: FRT*cat*FRT/ pRB3::14028sP_{rcsB}-*rcsB*	this paper
JP425	Δ*rcsB*:: FRT*cat*FRT/	this paper

	pRB3::14028sP_{rcsB}-*rcsB*	
JP412	Δ*pspA*:: FRT Δ*rcsB*:: FRT*cat*FRT/ pRB3-273C	this paper
CS051	14028s *phoP*102::Tn10dCm	(117)
SLN112	14028s *csgD::*FRT	this paper
JP245	*wza*::tetRA Δ*yjbE*::FRT*kan*FRT	this paper
JP447	Δ*yjbE*::FRT*kan*FRT	this paper
JP446	ΔrcsB::FRTcatFRT wza::tetRA Δ*yjbE*::FRT*kan*FRT	this paper
Plasmids		
pKD4	*bla* FRT *kan* FRT PS1 PS2 *ori6K*	(98)
pKD13	*bla* FRT *kan* FRT PS1 PS4 *ori6K*	(98)
pKD46	*bla araC*-P_{araB} γ β *exo* *oriR101* repA101(Ts)	(98)
pRB3-273C	*par* RK2 *bla* stable cloning vector	(110)
pJK392	Col E1 *bla*	
pRcsA	pJK392:: rcsA	this paper

JP103	pRB3::14028sP$_{rcsB}$-*rcsB*	this paper
Primers	**5' → 3'**	
JKP384-rscA-P1	GGGTATGCCATGTCAAC GATTATTATGGATTTGTG CAGTTGTGTAGGCTGGA GCTGCTTC	
JKP385-rscA-P2	ATGTCTCAGCGCATGTT AACAAAAATGCCGTTAG TGACGTCATATGAATATC CTCCTTAG	
JPP179-wza-tetR	CACAGGATAATGACTCT GCCAAAGTGATAAATAAT CAATGTTAAGACCCACTT TCACATT	
JPP180-wcaM-tetA	AACTTTTTCCCAGGAATT TTCGTAAAAATAGCGGT ACTAACTAAGCACTTGTC TCCTG	
JPP245-rcsB-P1	TACGTCAAAAGCTTGCT GTAGCAAGGTAGCCCAA TACATG GTG TAG GCT GGA GCT GCT TC	
JPP246-rcsB-P2	CCATCAGGCTGGGTAAC ATAAAAGCGATTTATTCT TTGTCCAT ATG AAT ATC CTC CTT AG	
JPP68-yjbE-P1	GGCACTGCGCTTATCCG GTC	
JPP69-yjbE-P2	CGATAAACTCAAGACGT GAG	
JPP42-*rpoD*-F	GTGAAATGGGCACTGTT GAACTG	

JPP43-*rpoD*-R	TTCCAGCAGATAGGTAATGGCTTC	
JPP140-*rcsA*-F	GATTTGTGCAGTTACACCCG	
JPP141-*rcsA*-R	GATAGCGAGTTCGTCAACGG	
JPP138-*yjbG*-F	GTGGTTTACCCTGATGGACG	
JPP139-*yjbG*-R	AGAATGTCGGCATTTATCGC	
JPP136-*wcaA*-F	ATCCGAAATCCCCTTATTCG	
JPP137-*wcaA*-R	TGCGCAGAAAAATGTCGTAG	
JPP8-*gmd*-F	CAACCCGAAATTTCATCTGC	
JPP9-*gmd-R*	CGCGTTTTCTTTTCAAGACC	
JPP12-*wza*-F	ACAGAGCACCCTCAAAATGG	
JPP13-*wza*-R	TGGCGTCAGACATATCAAGC	
JPP249-*rcsB* for plasmid-R	TAAGCGTAGCGCCATCAGGCTG	

JPP250-*rcsB* for plasmid-F	CGCCTGAAAGGGGTGTTTGCC	
delta*csgD*-F	CAGCTGTCAGATGTGCGATTAAAAAAAGTGGAGTTTCATCGTGTAGGCTGGAGCTGCTTC	
delta*csgD*-R	CTCTGCTGCTACAATCCAGGTCAGATAGCGTTTCATGGCCCATATGAATATCCTCCTTAG	

References

1. **Feasey, NA, Dougan, G, Kingsley, RA, Heyderman, RS, Gordon, MA.** 2012. Invasive non-typhoidal *Salmonellaa* disease: an emerging and neglected tropical disease in Africa. Lancet. **379:**2489-2499. doi: 10.1016/S0140-6736(11)61752-2; 10.1016/S0140-6736(11)61752-2.

2. **Santos, RL, Tsolis, RM, Zhang, S, Ficht, TA, Baumler, AJ, Adams, LG.** 2001. *Salmonella*-induced cell death is not required for enteritis in calves. Infect. Immun. **69:**4610-4617. doi: 10.1128/IAI.69.7.4610-4617.2001.

3. **Barthel, M, Hapfelmeier, S, Quintanilla-Martinez, L, Kremer, M, Rohde, M, Hogardt, M, Pfeffer, K, Russmann, H, Hardt, WD.** 2003. Pretreatment of mice with streptomycin provides a *Salmonella enterica* serovar Typhimurium colitis model that allows analysis of both pathogen and host. Infect. Immun. **71:**2839-2858.

4. **Alba, BM, Gross, CA.** 2004. Regulation of the *Escherichia coli* sigma-dependent envelope stress response. Mol. Microbiol. **52:**613-619. doi: 10.1111/j.1365-2958.2003.03982.x.

5. **Raffa, RG, Raivio, TL.** 2002. A third envelope stress signal transduction pathway in *Escherichia coli*. Mol. Microbiol. **45:**1599-1611. doi: 3112 [pii].

6. **Macritchie, DM, Raivio, TL.** 2009. Envelope Stress Responses. EcoSal Plus. **3:**10.1128/ecosalplus.5.4.7. doi: 10.1128/ecosalplus.5.4.7 [doi].

7. **Frawley, ER, Crouch, ML, Bingham-Ramos, LK, Robbins, HF, Wang, W, Wright, GD, Fang, FC.** 2013. Iron and citrate export by a major facilitator superfamily pump regulates metabolism and stress resistance in *Salmonella* Typhimurium. Proc. Natl. Acad. Sci. U. S. A. **110:**12054-12059. doi: 10.1073/pnas.1218274110 [doi].

8. **Brissette, JL, Russel, M, Weiner, L, Model, P.** 1990. Phage shock protein, a stress protein of *Escherichia coli.* Proc. Natl. Acad. Sci. U. S. A. **87:**862-866.

9. **Dworkin, J, Jovanovic, G, Model, P.** 2000. The PspA protein of *Escherichia coli i*s a negative regulator of sigma(54)-dependent transcription. J. Bacteriol. **182:**311-319.

10. **Brissette, JL, Weiner, L, Ripmaster, TL, Model, P.** 1991. Characterization and sequence of the *Escherichia coli* stress-induced psp operon. J. Mol. Biol. **220:**35-48.

11. **Lloyd, LJ, Jones, SE, Jovanovic, G, Gyaneshwar, P, Rolfe, MD, Thompson, A, Hinton, JC, Buck, M.** 2004. Identification of a new member of the phage shock protein response in *Escherichia coli*, the phage shock protein G (PspG). J. Biol. Chem. **279:**55707-55714. doi: 10.1074/jbc.M408994200.

12. **Jovanovic, G, Weiner, L, Model, P.** 1996. Identification, nucleotide sequence, and characterization of PspF, the transcriptional activator of the *Escherichia coli* stress-induced psp operon. J. Bacteriol. **178:**1936-1945.

13. **Weiner, L, Model, P.** 1994. Role of an *Escherichia coli* stress-response operon in stationary-phase survival. Proc. Natl. Acad. Sci. U. S. A. **91:**2191-2195.

14. **Kleerebezem, M, Tommassen, J.** 1993. Expression of the pspA gene stimulates efficient protein export in *Escherichia coli*. Mol. Microbiol. **7:**947-956.

15. **Jovanovic, G, Lloyd, LJ, Stumpf, MP, Mayhew, AJ, Buck, M.** 2006. Induction and function of the phage shock protein extracytoplasmic stress response in *Escherichia coli*. J. Biol. Chem. **281:**21147-21161. doi: 10.1074/jbc.M602323200.

16. **Adams, H, Teertstra, W, Demmers, J, Boesten, R, Tommassen, J.** 2003. Interactions between phage-shock proteins in *Escherichia coli*. J. Bacteriol. **185:**1174-1180.

17. **Engl, C, Jovanovic, G, Lloyd, LJ, Murray, H, Spitaler, M, Ying, L, Errington, J, Buck, M.** 2009. In vivo localizations of membrane stress controllers PspA and PspG in *Escherichia coli*. Mol. Microbiol. **73:**382-396. doi: 10.1111/j.1365-2958.2009.06776.x [doi].

18. **Kobayashi, R, Suzuki, T, Yoshida, M.** 2007. *Escherichia coli* phage-shock protein A (PspA) binds to membrane phospholipids and repairs proton leakage of the damaged membranes. Mol. Microbiol. **66:**100-109. doi: 10.1111/j.1365-2958.2007.05893.x.

19. **Standar, K, Mehner, D, Osadnik, H, Berthelmann, F, Hause, G, Lunsdorf, H, Bruser, T.** 2008. PspA can form large scaffolds in *Escherichia coli*. FEBS Lett. **582:**3585-3589. doi: 10.1016/j.febslet.2008.09.002 [doi].

20. **Jovanovic, G, Mehta, P, McDonald, C, Davidson, AC, Uzdavinys, P, Ying, L, Buck, M.** 2014. The N-terminal amphipathic helices determine regulatory and effector functions of phage shock protein A (PspA) in *Escherichia coli*. J. Mol. Biol. **426:**1498-1511. doi: 10.1016/j.jmb.2013.12.016 [doi].

21. **Sledjeski, DD, Gottesman, S.** 1996. Osmotic shock induction of capsule synthesis in *Escherichia coli* K-12. J. Bacteriol. **178:**1204-1206.

22. **Ferrieres, L, Clarke, DJ.** 2003. The RcsC sensor kinase is required for normal biofilm formation in *Escherichia coli* K-12 and controls the expression of a regulon in response to growth on a solid surface. Mol. Microbiol. **50:**1665-1682. doi: 3815 [pii].

23. **Sailer, FC, Meberg, BM, Young, KD.** 2003. beta-Lactam induction of colanic acid gene expression in *Escherichia coli*. FEMS Microbiol. Lett. **226:**245-249.

24. **Laubacher, ME, Ades, SE.** 2008. The Rcs phosphorelay is a cell envelope stress response activated by peptidoglycan stress and contributes to intrinsic antibiotic resistance. J. Bacteriol. **190:**2065-2074. doi: 10.1128/JB.01740-07; 10.1128/JB.01740-07.

25. **Miajlovic, H, Cooke, NM, Moran, GP, Rogers, TR, Smith, SG.** 2014. Response of extraintestinal pathogenic *Escherichia coli* to human serum reveals a protective role for Rcs-regulated exopolysaccharide colanic acid. Infect. Immun. **82:**298-305. doi: 10.1128/IAI.00800-13 [doi].

26. **Callewaert, L, Vanoirbeek, KG, Lurquin, I, Michiels, CW, Aertsen, A.** 2009. The Rcs two-component system regulates expression of lysozyme inhibitors and is induced by exposure to lysozyme. J. Bacteriol. **191:**1979-1981. doi: 10.1128/JB.01549-08 [doi].

27. **Clarke, DJ, Joyce, SA, Toutain, CM, Jacq, A, Holland, IB.** 2002. Genetic analysis of the RcsC sensor kinase from *Escherichia coli* K-12. J. Bacteriol. **184:**1204-1208.

28. **Takeda, S, Fujisawa, Y, Matsubara, M, Aiba, H, Mizuno, T.** 2001. A novel feature of the multistep phosphorelay in *Escherichia coli*: a revised model of the

RcsC --> YojN --> RcsB signalling pathway implicated in capsular synthesis and swarming behaviour. Mol. Microbiol. **40:**440-450. doi: mmi2393 [pii].

29. **Brill, JA, Quinlan-Walshe, C, Gottesman, S.** 1988. Fine-structure mapping and identification of two regulators of capsule synthesis in *Escherichia coli* K-12. J. Bacteriol. **170:**2599-2611.

30. **Majdalani, N, Heck, M, Stout, V, Gottesman, S.** 2005. Role of RcsF in signaling to the Rcs phosphorelay pathway in *Escherichia coli*. J. Bacteriol. **187:**6770-6778. doi: 10.1128/JB.187.19.6770-6778.2005.

31. **Gottesman, S, Trisler, P, Torres-Cabassa, A.** 1985. Regulation of capsular polysaccharide synthesis in *Escherichia coli* K-12: characterization of three regulatory genes. J. Bacteriol. **162:**1111-1119.

32. **Stout, V, Torres-Cabassa, A, Maurizi, MR, Gutnick, D, Gottesman, S.** 1991. RcsA, an unstable positive regulator of capsular polysaccharide synthesis. J. Bacteriol. **173:**1738-1747.

33. **Ferrieres, L, Aslam, SN, Cooper, RM, Clarke, DJ.** 2007. The *yjbEFGH* locus in *Escherichia coli* K-12 is an operon encoding proteins involved in exopolysaccharide production. Microbiology. **153:**1070-1080. doi: 153/4/1070 [pii].

34. **Wang, Q, Zhao, Y, McClelland, M, Harshey, RM.** 2007. The RcsCDB signaling system and swarming motility in *Salmonella enterica* serovar Typhimurium: dual regulation of flagellar and SPI-2 virulence genes. J. Bacteriol. **189:**8447-8457. doi: JB.01198-07 [pii].

35. **Carballes, F, Bertrand, C, Bouche, JP, Cam, K.** 1999. Regulation of *Escherichia coli* cell division genes *ftsA* and *ftsZ* by the two-component system *rcsC-rcsB*. Mol. Microbiol. **34:**442-450. doi: mmi1605 [pii].

36. **Majdalani, N, Hernandez, D, Gottesman, S.** 2002. Regulation and mode of action of the second small RNA activator of RpoS translation, RprA. Mol. Microbiol. **46:**813-826.

37. **Davalos-Garcia, M, Conter, A, Toesca, I, Gutierrez, C, Cam, K.** 2001. Regulation of *osmC* gene expression by the two-component system *rcsB-rcsC* in *Escherichia coli*. J. Bacteriol. **183:**5870-5876. doi: 10.1128/JB.183.20.5870-5876.2001 [doi].

38. **Raivio, TL.** 2005. Envelope stress responses and Gram-negative bacterial pathogenesis. Mol. Microbiol. **56:**1119-1128. doi: 10.1111/j.1365-2958.2005.04625.x.

39. **Testerman, TL, Vazquez-Torres, A, Xu, Y, Jones-Carson, J, Libby, SJ, Fang, FC.** 2002. The alternative sigma factor sigmaE controls antioxidant defences required for *Salmonella* virulence and stationary-phase survival. Mol. Microbiol. **43:**771-782.

40. **Eriksson, S, Lucchini, S, Thompson, A, Rhen, M, Hinton, JC.** 2003. Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. Mol. Microbiol. **47:**103-118.

41. **Karlinsey, JE, Maguire, ME, Becker, LA, Crouch, ML, Fang, FC.** 2010. The phage shock protein PspA facilitates divalent metal transport and is required for virulence of *Salmonella* enterica sv. Typhimurium. Mol. Microbiol. **78:**669-685. doi: 10.1111/j.1365-2958.2010.07357.x; 10.1111/j.1365-2958.2010.07357.x.

42. **Goswami, T, Bhattacharjee, A, Babal, P, Searle, S, Moore, E, Li, M, Blackwell, JM.** 2001. Natural-resistance-associated macrophage protein 1 is an H+/bivalent cation antiporter. Biochem. J. **354:**511-519.

43. **Forbes, JR, Gros, P.** 2001. Divalent-metal transport by NRAMP proteins at the interface of host-pathogen interactions. Trends Microbiol. **9:**397-403. doi: S0966-842X(01)02098-4 [pii].

44. **Cellier, MF, Courville, P, Campion, C.** 2007. Nramp1 phagocyte intracellular metal withdrawal defense. Microbes Infect. **9:**1662-1670. doi: S1286-4579(07)00286-9 [pii].

45. **Kehres, DG, Zaharik, ML, Finlay, BB, Maguire, ME.** 2000. The NRAMP proteins of *Salmonella* Typhimurium and *Escherichia coli* are selective manganese transporters involved in the response to reactive oxygen. Mol. Microbiol. **36:**1085-1100. doi: mmi1922 [pii].

46. **Detweiler, CS, Monack, DM, Brodsky, IE, Mathew, H, Falkow, S.** 2003. v*irK, somA and rcsC* are important for systemic *Salmonella enterica* serovar Typhimurium infection and cationic peptide resistance. Mol. Microbiol. **48:**385-400.

47. **Erickson, KD, Detweiler, CS.** 2006. The Rcs phosphorelay system is specific to enteric pathogens/commensals and activates *ydeI*, a gene important for persistent *Salmonella* infection of mice. Mol. Microbiol. **62:**883-894. doi: 10.1111/j.1365-2958.2006.05420.x.

48. **Shen, S, Fang, FC.** 2012. Integrated stress responses in *Salmonella*. Int. J. Food Microbiol. **152:**75-81. doi: 10.1016/j.ijfoodmicro.2011.04.017 [doi].

49. **Becker, LA, Bang, IS, Crouch, ML, Fang, FC.** 2005. Compensatory role of PspA, a member of the phage shock protein operon, in *rpoE* mutant *Salmonella*

enterica serovar Typhimurium. Mol. Microbiol. **56:**1004-1016. doi: 10.1111/j.1365-2958.2005.04604.x.

50. **Humphreys, S, Rowley, G, Stevenson, A, Anjum, MF, Woodward, MJ, Gilbert, S, Kormanec, J, Roberts, M.** 2004. Role of the two-component regulator CpxAR in the virulence of *Salmonella enterica* serotype Typhimurium. Infect. Immun. **72:**4654-4661. doi: 10.1128/IAI.72.8.4654-4661.2004 [doi].

51. **Kleerebezem, M, Crielaard, W, Tommassen, J.** 1996. Involvement of stress protein PspA (phage shock protein A) of *Escherichia coli* in maintenance of the protonmotive force under stress conditions. EMBO J. **15:**162-171.

52. **Reeves, PR, Hobbs, M, Valvano, MA, Skurnik, M, Whitfield, C, Coplin, D, Kido, N, Klena, J, Maskell, D, Raetz, CR, Rick, PD.** 1996. Bacterial polysaccharide synthesis and gene nomenclature. Trends Microbiol. **4:**495-503. doi: S0966842X97829125 [pii].

53. **Corbett, D, Roberts, IS.** 2008. Capsular polysaccharides in *Escherichia coli*. Adv. Appl. Microbiol. **65:**1-26. doi: 10.1016/S0065-2164(08)00601-1 [doi].

54. **Roberts, IS.** 1996. The biochemistry and genetics of capsular polysaccharide production in bacteria. Annu. Rev. Microbiol. **50:**285-315. doi: 10.1146/annurev.micro.50.1.285 [doi].

55. **Whitfield, C.** 2006. Biosynthesis and assembly of capsular polysaccharides in *Escherichia coli*. Annu. Rev. Biochem. **75:**39-68. doi: 10.1146/annurev.biochem.75.103004.142545 [doi].

56. **Limoli, DH, Jones, CJ, Wozniak, DJ.** 2015. Bacterial Extracellular Polysaccharides in Biofilm Formation and Function. Microbiol. Spectr. **3:**10.1128/microbiolspec.MB-0011-2014. doi: 10.1128/microbiolspec.MB-0011-2014 [doi].

57. **Danese, PN, Pratt, LA, Kolter, R.** 2000. Exopolysaccharide production is required for development of *Escherichia coli* K-12 biofilm architecture. J. Bacteriol. **182:**3593-3596.

58. **Gibson, DL, White, AP, Snyder, SD, Martin, S, Heiss, C, Azadi, P, Surette, M, Kay, WW.** 2006. *Salmonella* produces an O-antigen capsule regulated by AgfD and important for environmental persistence. J. Bacteriol. **188:**7722-7730. doi: 188/22/7722 [pii].

59. **Costerton, JW, Irvin, RT, Cheng, KJ.** 1981. The bacterial glycocalyx in nature and disease. Annu. Rev. Microbiol. **35:**299-324. doi: 10.1146/annurev.mi.35.100181.001503 [doi].

60. **Harshey, RM.** 2003. Bacterial motility on a surface: many ways to a common goal. Annu. Rev. Microbiol. **57:**249-273. doi: 10.1146/annurev.micro.57.030502.091014 [doi].

61. **Cywes, C, Wessels, MR.** 2001. Group A *Streptococcus* tissue invasion by CD44-mediated cell signalling. Nature. **414:**648-652. doi: 10.1038/414648a [doi].

62. **Mah, TF, O'Toole, GA.** 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. **9:**34-39. doi: S0966-842X(00)01913-2 [pii].

63. **Allen, PM, Fisher, D, Saunders, JR, Hart, CA.** 1987. The role of capsular polysaccharide K21b of *Klebsiella* and of the structurally related colanic-acid polysaccharide of *Escherichia coli* in resistance to phagocytosis and serum killing. J. Med. Microbiol. **24:**363-370. doi: 10.1099/00222615-24-4-363 [doi].

64. **Solano, C, Garcia, B, Valle, J, Berasain, C, Ghigo, JM, Gamazo, C, Lasa, I.** 2002. Genetic analysis of *Salmonella enteritidis* biofilm formation: critical role of cellulose. Mol. Microbiol. **43:**793-808. doi: 2802 [pii].

65. **Zogaj, X, Nimtz, M, Rohde, M, Bokranz, W, Romling, U.** 2001. The multicellular morphotypes of *Salmonella* Typhimurium and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. Mol. Microbiol. **39:**1452-1463. doi: mmi2337 [pii].

66. **Romling, U.** 2005. Characterization of the rdar morphotype, a multicellular behaviour in Enterobacteriaceae. Cell Mol. Life Sci. **62:**1234-1246. doi: 10.1007/s00018-005-4557-x [doi].

67. **Fang, X, Ahmad, I, Blanka, A, Schottkowski, M, Cimdins, A, Galperin, MY, Romling, U, Gomelsky, M.** 2014. GIL, a new c-di-GMP-binding protein domain involved in regulation of cellulose synthesis in enterobacteria. Mol. Microbiol. **93:**439-452. doi: 10.1111/mmi.12672 [doi].

68. **Romling, U, Rohde, M, Olsen, A, Normark, S, Reinkoster, J.** 2000. AgfD, the checkpoint of multicellular and aggregative behaviour in *Salmonella* Typhimurium regulates at least two independent pathways. Mol. Microbiol. **36:**10-23. doi: mmi1822 [pii].

69. **Simm, R, Ahmad, I, Rhen, M, Le Guyon, S, Romling, U.** 2014. Regulation of biofilm formation in *Salmonella enterica* serovar Typhimurium. Future Microbiol. **9:**1261-1282. doi: 10.2217/fmb.14.88 [doi].

70. **Gerstel, U, Romling, U.** 2001. Oxygen tension and nutrient starvation are major signals that regulate *agfD* promoter activity and expression of the

multicellular morphotype in *Salmonella* Typhimurium. Environ. Microbiol. **3:**638-648. doi: 235 [pii].

71. **Adcox, HE, Vasicek, EM, Dwivedi, V, Hoang, KV, Turner, J, Gunn, JS.** 2016. *Salmonella* extracellular matrix components influence biofilm formation and gallbladder colonization. Infect. Immun. . doi: IAI.00532-16 [pii].

72. **Prouty, AM, Gunn, JS.** 2003. Comparative analysis of *Salmonella enterica* serovar Typhimurium biofilm formation on gallstones and on glass. Infect. Immun. **71:**7154-7158.

73. **Cowles, KN, Willis, DK, Engel, TN, Jones, JB, Barak, JD.** 2015. Diguanylate Cyclases AdrA and STM1987 Regulate *Salmonella enterica* Exopolysaccharide Production during Plant Colonization in an Environment-Dependent Manner. Appl. Environ. Microbiol. **82:**1237-1248. doi: 10.1128/AEM.03475-15 [doi].

74. **Barak, JD, Jahn, CE, Gibson, DL, Charkowski, AO.** 2007. The role of cellulose and O-antigen capsule in the colonization of plants by *Salmonella enterica*. Mol. Plant Microbe Interact. **20:**1083-1091. doi: 10.1094/MPMI-20-9-1083 [doi].

75. **Snyder, DS, Gibson, D, Heiss, C, Kay, W, Azadi, P.** 2006. Structure of a capsular polysaccharide isolated from *Salmonella* Enteritidis. Carbohydr. Res. **341:**2388-2397. doi: S0008-6215(06)00311-9 [pii].

76. **Pescaretti Mde, L, Lopez, FE, Morero, RD, Delgado, MA.** 2011. The PmrA/PmrB regulatory system controls the expression of the *wzzfepE* gene involved in the O-antigen synthesis of *Salmonella enterica* serovar Typhimurium. Microbiology. **157:**2515-2521. doi: 10.1099/mic.0.050088-0 [doi].

77. **Marshall, JM, Gunn, JS.** 2015. The O-Antigen Capsule of *Salmonella enterica* serovar Typhimurium Facilitates Serum Resistance and Surface Expression of FliC. Infect. Immun. **83:**3946-3959. doi: 10.1128/IAI.00634-15 [doi].

78. **Wehland, M, Bernhard, F.** 2000. The RcsAB box. Characterization of a new operator essential for the regulation of exopolysaccharide biosynthesis in enteric bacteria. J. Biol. Chem. **275:**7013-7020.

79. **Ionescu, M, Belkin, S.** 2009. Overproduction of exopolysaccharides by an *Escherichia coli* K-12 *rpoS* mutant in response to osmotic stress. Appl. Environ. Microbiol. **75:**483-492. doi: 10.1128/AEM.01616-08 [doi].

80. **GOEBEL, WF.** 1963. Colanic acid. Proc. Natl. Acad. Sci. U. S. A. **49:**464-471.

81. **Meredith, TC, Mamat, U, Kaczynski, Z, Lindner, B, Holst, O, Woodard, RW.** 2007. Modification of lipopolysaccharide with colanic acid (M-antigen) repeats in *Escherichia coli*. J. Biol. Chem. **282:**7790-7798. doi: M611034200 [pii].

82. **Stevenson, G, Lan, R, Reeves, PR.** 2000. The colanic acid gene cluster of *Salmonella enterica* has a complex history. FEMS Microbiol. Lett. **191:**11-16.

83. **Stout, V, Gottesman, S.** 1990. RcsB and RcsC: a two-component regulator of capsule synthesis in *Escherichia coli*. J. Bacteriol. **172:**659-669.

84. **Trisler, P, Gottesman, S.** 1984. lon transcriptional regulation of genes necessary for capsular polysaccharide synthesis in *Escherichia coli* K-12. J. Bacteriol. **160:**184-191.

85. **Mao, Y, Doyle, MP, Chen, J.** 2001. Insertion mutagenesis of *wca* reduces acid and heat tolerance of enterohemorrhagic *Escherichia coli* O157:H7. J. Bacteriol. **183:**3811-3815. doi: 10.1128/JB.183.12.3811-3815.2001 [doi].

86. **Ophir, T, Gutnick, DL.** 1994. A role for exopolysaccharides in the protection of microorganisms from desiccation. Appl. Environ. Microbiol. **60:**740-745.

87. **Chen, J, Lee, SM, Mao, Y.** 2004. Protective effect of exopolysaccharide colanic acid of *Escherichia coli* O157:H7 to osmotic and oxidative stress. Int. J. Food Microbiol. **93:**281-286. doi: 10.1016/j.ijfoodmicro.2003.12.004 [doi].

88. **Stevenson, G, Andrianopoulos, K, Hobbs, M, Reeves, PR.** 1996. Organization of the *Escherichia coli* K-12 gene cluster responsible for production of the extracellular polysaccharide colanic acid. J. Bacteriol. **178:**4885-4893.

89. **Grant, WD, Sutherland, IW, Wilkinson, JF.** 1969. Exopolysaccharide colanic acid and its occurrence in the Enterobacteriaceae. J. Bacteriol. **100:**1187-1193.

90. **Baumler, A, Fang, FC.** 2013. Host specificity of bacterial pathogens. Cold Spring Harb Perspect. Med. **3:**a010041. doi: 10.1101/cshperspect.a010041 [doi].

91. **Wattam, AR, Abraham, D, Dalay, O, Disz, TL, Driscoll, T, Gabbard, JL, Gillespie, JJ, Gough, R, Hix, D, Kenyon, R, Machi, D, Mao, C, Nordberg, EK, Olson, R, Overbeek, R, Pusch, GD, Shukla, M, Schulman, J, Stevens, RL, Sullivan, DE, Vonstein, V, Warren, A, Will, R, Wilson, MJ, Yoo, HS, Zhang, C, Zhang, Y, Sobral, BW.** 2014. PATRIC, the bacterial bioinformatics database and analysis resource. Nucleic Acids Res. **42:**D581-91. doi: 10.1093/nar/gkt1099 [doi].

92. **Ebel, W, Trempy, JE.** 1999. *Escherichia coli* RcsA, a positive activator of colanic acid capsular polysaccharide synthesis, functions to activate its own expression. J. Bacteriol. **181:**577-584.

93. **Deatherage, BL, Lara, JC, Bergsbaken, T, Rassoulian Barrett, SL, Lara, S, Cookson, BT.** 2009. Biogenesis of bacterial membrane vesicles. Mol. Microbiol. **72:**1395-1407. doi: 10.1111/j.1365-2958.2009.06731.x [doi].

94. **Lloubes, R, Cascales, E, Walburger, A, Bouveret, E, Lazdunski, C, Bernadac, A, Journet, L.** 2001. The Tol-Pal proteins of the *Escherichia coli* cell envelope: an energized system required for outer membrane integrity? Res. Microbiol. **152:**523-529. doi: S0923-2508(01)01226-8 [pii].

95. **Cascales, E, Gavioli, M, Sturgis, JN, Lloubes, R.** 2000. Proton motive force drives the interaction of the inner membrane TolA and outer membrane pal proteins in *Escherichia coli*. Mol. Microbiol. **38:**904-915. doi: mmi2190 [pii].

96. **Ranjit, DK, Young, KD.** 2016. Colanic Acid Intermediates Prevent De Novo Shape Recovery of *Escherichia coli* Spheroplasts, Calling into Question Biological Roles Previously Attributed to Colanic Acid. J. Bacteriol. **198:**1230-1240. doi: 10.1128/JB.01034-15 [doi].

97. **Karlinsey, JE.** 2007. lambda-Red genetic engineering in *Salmonella enterica* serovar Typhimurium. Methods Enzymol. **421:**199-209. doi: S0076-6879(06)21016-4 [pii].

98. **Datsenko, KA, Wanner, BL.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U. S. A. **97:**6640-6645. doi: 10.1073/pnas.120163297 [doi].

99. **Crouch, ML, Becker, LA, Bang, IS, Tanabe, H, Ouellette, AJ, Fang, FC.** 2005. The alternative sigma factor sigma is required for resistance of *Salmonella enterica* serovar Typhimurium to anti-microbial peptides. Mol. Microbiol. **56:**789-799. doi: MMI4578 [pii].

100. **Ledeboer, NA, Jones, BD.** 2005. Exopolysaccharide sugars contribute to biofilm formation by *Salmonella enterica* serovar Typhimurium on HEp-2 cells and chicken intestinal epithelium. J. Bacteriol. **187:**3214-3226. doi: 187/9/3214 [pii].

101. **Beloin, C, Valle, J, Latour-Lambert, P, Faure, P, Kzreminski, M, Balestrino, D, Haagensen, JA, Molin, S, Prensier, G, Arbeille, B, Ghigo, JM.** 2004. Global impact of mature biofilm lifestyle on *Escherichia coli* K-12 gene expression. Mol. Microbiol. **51:**659-674.

102. **Kaldalu, N, Mei, R, Lewis, K.** 2004. Killing by ampicillin and ofloxacin induces overlapping changes in *Escherichia coli* transcription profile. Antimicrob. Agents Chemother. **48:**890-896.

103. **Forbes, SJ, Martinelli, D, Hsieh, C, Ault, JG, Marko, M, Mannella, CA, Mantis, NJ.** 2012. Association of a protective monoclonal IgA with the O antigen of *Salmonella enterica* serovar Typhimurium impacts type 3 secretion and outer membrane integrity. Infect. Immun. **80:**2454-2463. doi: 10.1128/IAI.00018-12 [doi].

104. **Amarasinghe, JJ, D'Hondt, RE, Waters, CM, Mantis, NJ.** 2012. Exposure of *Salmonella enterica* serovar Typhimurium to a Protective Monoclonal IgA Triggers Exopolysaccharide Production via a Diguanylate Cyclase-Dependent Pathway. Infect. Immun. . doi: 10.1128/IAI.00813-12.

105. **Daugelavicius, R, Bakiene, E, Bamford, DH.** 2000. Stages of polymyxin B interaction with the *Escherichia coli* cell envelope. Antimicrob. Agents Chemother. **44:**2969-2978.

106. **Farris, C, Sanowar, S, Bader, MW, Pfuetzner, R, Miller, SI.** 2010. Antimicrobial peptides activate the Rcs regulon through the outer membrane lipoprotein RcsF. J. Bacteriol. **192:**4894-4903. doi: 10.1128/JB.00505-10; 10.1128/JB.00505-10.

107. **Pescaretti Mde, L, Morero, R, Delgado, MA.** 2009. Identification of a new promoter for the response regulator *rcsB* expression in *Salmonella enterica* serovar Typhimurium. FEMS Microbiol. Lett. **300:**165-173. doi: 10.1111/j.1574-6968.2009.01771.x; 10.1111/j.1574-6968.2009.01771.x.

108. **Costerton, JW, Stewart, PS, Greenberg, EP.** 1999. Bacterial biofilms: a common cause of persistent infections. Science. **284:**1318-1322.

109. **Hong, Y, Brown, DG.** 2010. Alteration of bacterial surface electrostatic potential and pH upon adhesion to a solid surface and impacts to cellular bioenergetics. Biotechnol. Bioeng. **105:**965-972. doi: 10.1002/bit.22606 [doi].

110. **Berggren, RE, Wunderlich, A, Ziegler, E, Schleicher, M, Duke, RC, Looney, D, Fang, FC.** 1995. HIV gp120-specific cell-mediated immune responses in mice after oral immunization with recombinant *Salmonella*. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. **10:**489-495.

111. **Novo, D, Perlmutter, NG, Hunt, RH, Shapiro, HM.** 1999. Accurate flow cytometric membrane potential measurement in bacteria using diethyloxacarbocyanine and a ratiometric technique. Cytometry. **35:**55-63. doi: 10.1002/(SICI)1097-0320(19990101)35:1<55::AID-CYTO8>3.0.CO;2-2 [pii].

112. **Blumenkrantz, N, Asboe-Hansen, G.** 1973. New method for quantitative determination of uronic acids. Anal. Biochem. **54:**484-489.

113. **DISCHE, Z, SHETTLES, LB.** 1951. A new spectrophotometric test for the detection of methylpentose. J. Biol. Chem. **192:**579-582.

114. **Navarre, WW, Halsey, TA, Walthers, D, Frye, J, McClelland, M, Potter, JL, Kenney, LJ, Gunn, JS, Fang, FC, Libby, SJ.** 2005. Co-regulation of *Salmonella enterica* genes required for virulence and resistance to antimicrobial peptides by SlyA and PhoP/PhoQ. Mol. Microbiol. **56:**492-508. doi: MMI4553 [pii].

115. **Barker, HC, Kinsella, N, Jaspe, A, Friedrich, T, O'Connor, CD.** 2000. Formate protects stationary-phase *Escherichia coli* and *Salmonella* cells from killing by a cationic antimicrobial peptide. Mol. Microbiol. **35:**1518-1529. doi: mmi1820 [pii].

116. **Singletary, LA, Karlinsey, JE, Libby, SJ, Mooney, JP, Lokken, KL, Tsolis, RM, Byndloss, MX, Hirao, LA, Gaulke, CA, Crawford, RW, Dandekar, S, Kingsley, RA, Msefula, CL, Heyderman, RS, Fang, FC.** 2016. Loss of Multicellular Behavior in Epidemic African Nontyphoidal *Salmonella enterica* serovar Typhimurium ST313 Strain D23580. MBio. **7:**e02265-15. doi: 10.1128/mBio.02265-15 [doi].

117. **Gunn, JS, Miller, SI.** 1996. PhoP-PhoQ activates transcription of *pmrAB*, encoding a two-component regulatory system involved in *Salmonella* Typhimurium antimicrobial peptide resistance. J. Bacteriol. **178:**6857-6864.