Eavesdropping and Mannitol Sensitivity in Bacteria

Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

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2024

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Abstract

Bacteria can communicate with each other through the production, release, and detection of small molecules called *N*-acyl homoserine lactones (AHLs). In a subset of the family Enterobacteriaceae, including the well-known genera *Salmonella* and *Escherichia*, AHLs are not produced but these bacteria retain the ability to detect them through the LuxR-type protein SdiA. This strategy is referred to as eavesdropping: where one species may listen in on the communication of another. The role of SdiA-mediated eavesdropping in the lifecycle of these bacteria is unknown.

To determine the function of eavesdropping, we first reviewed the available literature on SdiA. Since the initial discovery of SdiA, many studies have attempted to gain insight into its role by looking for mutant defects in various host systems, elucidating the SdiA regulon, or finding in vitro phenotypes. The literature on each topic is complex and interpretation must be measured and considerate of the methodology used.

We next examined the role of *Salmonella* SdiA in several host systems, including house flies, mice, and plants. We also determined the SdiA regulons of *Salmonella, E. coli*, and *Enterobacter cloacae*. The house fly is a known mechanical vector of *Salmonella* with some evidence of a more dynamic interaction between host and bacteria. Based on the abundance of AHL synthase homologs in insect metagenomes, we

hypothesized that SdiA played a role in the survival of *Salmonella* within house flies. After a series of experimental infections, the evidence suggests that *sdiA* mutants are highly advantaged over their wild-type competitor and that SdiA may have a negative effect on survival within house flies. Using a randomly barcoded transposon library (Barseq), we examined *Salmonella* fitness in mice that were co-infected with the AHL producing pathogen *Yersinia enterocolitica*. Consistent with previous reporting, *sdiA* and its regulon suffered no fitness defects during gastroenteritis. Finally, an experimental infection of plants indicated that SdiA is not active in either Angiosperms or soybeans.

The regulon of SdiA is poorly understood. We sought to elucidate the SdiA regulons of two clinically relevant *Salmonella* serovars, Typhimurium and Typhi, using RNA-seq. Although more than two-hundred genes were suggested to be *sdiA* regulated by expressing *sdiA* from a plasmid, only 13-20 genes across 5-6 loci are *sdiA* regulated when expressed from its native position on the chromosome. Most *sdiA* regulated genes are hypothetical or have no known function or phenotype. We also determined that *sdiA* regulons in other species, specifically *E. coli* and *E. cloacae*, have some overlap with each other. The partial overlap of regulons suggests a common response to foreign AHLs. It remains to be determined what phenotype or phenotypes when these *sdiA* regulated genes are activated.

Antibiotic resistance is a growing threat to the welfare of mankind. One of many approaches to tackling this great challenge is the identification of novel antimicrobial targets. One currently unexplored strategy is to attenuate bacteria by inducing sugar-phosphate toxicity. Bacterial metabolism uses many phosphorylated intermediates that

are quickly interconverted in the cell. Inhibiting enzymes in the cell essential for the processing of certain intermediates leads to their accumulation and subsequent growth defects: the phenomenon of sugar-phosphate toxicity. We evaluated the therapeutic potential of mannitol-1-phosphate (Mtl-1P) toxicity, which is induced by inactivation of Mannitol-1-phosphate 5-dehydrogenase (MtlD) and the exogeneous introduction of mannitol. We found that *mtlD* mutants in the genera *Cronobacter*, *Escherichia*, *Salmonella*, and *Pseudomonas* are all inhibited in vitro by mannitol at micromolar concentrations. In vivo, we observed that both gastrointestinal and systemic infections of *Salmonella mtlD* mutants could be attenuated by providing mannitol to mice in their drinking water, suggesting a hypothetical MtlD inhibitor would be effective in treating infections. While investigating the *mtlD* mutant in vitro, we discovered a previously unreported phenotype in *mtlD* mutants, termed recovery. Recovery is the resumption of growth following intoxication and the delay between intoxication and recovery is dependent on the initial quantity of mannitol in the solution.

Overall, the work in this thesis provides new insights into SdiA-mediated eavesdropping by identifying new regulon members in *Salmonella*, *E. coli*, and *E. cloacae* and investigating the role of SdiA in different host systems. In addition, we investigate the scope and therapeutic potential of mannitol sensitivity as a therapeutic target in bacteria, finding that all tested *mtlD* mutants are attenuated by the presence of mannitol and *mtlD* mutants are attenuated in multiple mouse models of infection.

Dedication

To my parents, Mike and Jenny, who have loved and supported me my entire life. And to my sister Katelyn, brother-in-law Ryan, and nephew, Oswald (Ozzie). Their love and support cannot be described in words alone.

Acknowledgments

I would first like to thank my mentor, Dr. Brian Ahmer. When I started my PhD, I knew nothing about science. Since then, he has taught me to be scientist. I would not be where I am today, nor would I be able to do what I plan to do in the future without his mentorship. I would like to thank my laboratory mentor, Dr. Anice Sabag-Daigle, who taught me how to work in the lab, do research, and provided invaluable advice on graduate school. I would also like to thank my fellow graduate students, Dr. Erin Boulanger and Emily Rego, who made working in the Ahmer lab more fun. Although they never joined the lab, the various rotation students who came and went throughout the years were fun to have around, if only for 6 weeks at a time.

Although most of my work was performed by myself, several other groups contributed in direct and indirect ways. This includes the labs of Kelly Wrighton, Vicki Wysocki, Mark Mitton-Fry, Adam Deutschbauer, Charles Bell, Venkat Gopalan, and John Gunn.

Finally, I would like to thank the Department of Microbiology and Department of Microbial Infection and Immunity. The PIs and students of these departments have provided scientific feedback, interesting conversation, and friendship during my time as a graduate student.

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Boulanger, E.F., A. Sabag-Daigle, M. Baniasad, K. Kokkinias, **A. Schwieters**, K.C. Wrighton, V.H. Wysocki, and B.M.M. Ahmer, Sugar-Phosphate Toxicities Attenuate *Salmonella* Fitness in the Gut. *Journal of Bacteriology*, 2022. 0(0): p. e00344-22.

Staats, A., P.W. Burback, **A. Schwieters**, D. Li, A. Sullivan, A.R. Horswill, and P. Stoodley, Rapid Aggregation of *Staphylococcus aureus* in Synovial Fluid Is Influenced by Synovial Fluid Concentration, Viscosity, and Fluid Dynamics, with Evidence of Polymer Bridging. *mBio*, 2022. 13(2): p. e00236-22.

Fields of Study

Major Field: Microbiology

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Chapter 1. Introduction

1.1 Quorum sensing is a phenomenon of bacteria mediated by the production and detection of small molecules

Quorum sensing (QS) is a process of cell-to-cell communication in bacteria enabling them to coordinate behaviors in response to changes in population density. This behavior is well documented and reviewed by excellent researchers in the field [1-5]. QS is mediated by autoinducers (often a small peptide or modified metabolite) that are produced by the cell using autoinducer synthases and released into the extracellular space. The accumulation of autoinducers occurs as a function of population density and time. External factors, including volume of the extracellular space, flow rate, pH, the presence of degrading enzymes (e.g. lactonases) or autoinducer analogs, etc, all factor into the overall autoinducer concentration [6-9]. At sufficient concentration, autoinducers are detected by response regulators. QS has been found to regulate a wide variety of biological processes, including bioluminescence [10], virulence [5,11], horizontal gene transfer [12,13], and phage biology [14-16]. It is thought these processes require sufficient bacterial population density (and coordination) to be effective, necessitating a mechanism like QS.

QS, originally proposed by Fuqua et al. [17], is an evolutionary explanation of the observed behavior of these bacteria. Diffusion Sensing (DS) was later proposed by

Redfield as an alternative interpretation [18]. In the DS paradigm, autoinducers are secreted to "figure out" if the more expensive extracellular molecules (e.g. proteases, virulence factors) would simply diffuse away and be ineffective to the cell. Another hypothesis, efficiency sensing, was proposed as a solution to the two competing ideas [19] though others consider QS and DS to not be mutually exclusive [20]. There have been several studies on the evolution and maintenance of cheaters within QS populations (i.e. mutants who do not produce autoinducers but still detect them) [21,22]. These observations suggest autoinducer production and detection is a social behavior (i.e. QS, not DS). Distinguishing QS from DS has also been discussed in terms of mathematical modeling (see [19,20,23]).

Regardless of the underlying evolutionary mechanism at play, the number of factors (mentioned above) that influence autoinducer concentration and whether bacteria reach their quorum allows them to take in a considerable amount of information about their environment and channel it into a regulated response. The sheer number of bacterial species across phyla and diversity of phenotypes regulated by autoinducer production and detection likely means any single evolutionary explanation is an over-simplification of the underlying dynamics at play. For simplicity, I will refer to these behaviors as QS in this document.

1.2 Eavesdropping bacteria use quorum sensing proteins but do not detect their own population density

Many bacterial QS circuits do not encode a cognate signal synthase (e.g. *luxI*) alongside their response regulator (*luxR*) [24]. Multiple new paradigms become possible from losing signal production but not signal detection [25]. One strategy is employed by the "third-wheel" LuxR solos that sense AHLs synthesized by another AHL synthase within their genome at a different locus than third-wheel LuxR. A known third-wheel is QscR in *P. aeruginosa*. This opportunistic pathogen encodes a complex QS network which includes two LuxRI pairs: LasRI (using oxoC12) and RhlRI (using C4). QscR is encoded elsewhere from either locus and binds oxoC12 and other long-chain length AHLs [26,27]. The QscR regulon is limited to a single locus, whose genes delay the activation of LasR and RhlR regulons by an unknown mechanism [28]. It is proposed that QscR may act as a "timing mechanism" in activating Las/Rhl during infection. Its ligand specificity also suggests it could alter expression in response to other nearby AHL producing species.

Eavesdropping, mediated by LuxR solo SdiA, is one of two major research topics presented in this document. SdiA is encoded in several genera within the Enterobacteriaceae, including notable genera *Escherichia*, *Salmonella*, *Enterobacter*, *Klebsiella*, *Citrobacter*, and *Cronobacter* [29]. SdiA allows bacteria to detect foreign AHL producing bacterial species and regulate specific genes (the SdiA regulon). Although this process is straightforward and easily demonstrable in the laboratory setting, its role in the lifecycles of $SdiA^+$ bacteria is essentially unknown. There are a few reasons for this issue. First, SdiA regulons mostly encode hypothetical or poorly understood genes. Second,

SdiA$^+$ genera occupy a diverse range of niches, yielding no immediately obvious relevant environment in which eavesdropping might occur. Third, clear *sdiA* mutant defects (both in vitro and in vivo) are either absent or their results are not easily interpreted. Finally, SdiA regulates genes in a complex manner: a mix of AHL-dependent and independent regulation that confuses which responses may be relevant to the detection of foreign bacteria. These issues are exemplified in the work presented in Chapter 3 on *Salmonella* SdiA regulons and expounded upon in Chapter 6.

1.3 *Salmonella* is a Gram-negative pathogen

The primary organism used in these studies is *Salmonella*, a genus of Gram-negative bacteria within the pathogen rich class Enterobacteriaceae. *Salmonella* is closely related to another model organism, *E. coli* [30]. *Salmonella* has two species, *enterica* and *bongori*, and species *enterica* is further divided into six sub-species [31]. *S. bongori* and the five subspecies other than *S. enterica* subspecies *enterica* are generally associated with cold-blooded hosts [32]. Subspecies *enterica* encodes over 1,500 serovars, a few of which have clinical and research significance, particularly Typhimurium and Typhi. The generalist *Salmonella* serovars (e.g. Typhimurium) are notable for their large host range. Other than infecting humans, hosts include livestock (including pigs, cows, chickens), plants, insects, reptiles, and wild birds [33-37]. Specialized serovars, such as *S.* Typhi, limit their scope to one or a few hosts and environmental niches (e.g. humans). Infection and transmission occur by the fecal-oral route.

1.4 Mouse models of *Salmonella* pathogenesis

The study of *Salmonella* pathogenesis relies primarily on mouse models. The origin of the name Typhimurium, the model organism for most *Salmonella* researchers, dates back to 1889 when a strain was isolated from infected mice by Loeffler (see reference [38] for some interesting history on *Salmonella*). *S.* Typhimurium infections can lead to gastroenteritis and/or systemic illnesses in humans.

To model human infection, the selection of mouse strain is important in the outcome of infection. The most significant host factor in whether *Salmonella* incurs a lethal infection is *NRAMP1* (aka *SLC11A1*). *NRAMP1* is a metal transporter which restricts the growth of *Salmonella* within macrophages [39]. There is a considerable volume of research on the mechanism(s) by which this occurs. Recently, it was proposed that magnesium deprivation is the primary factor [40] though others have argued that iron is the relevant restricted element [41]. Mouse strains without a functional copy (*NRAMP1*$^{-}$) are highly susceptible to lethal systemic infections, with more than 50% of mice dying to inoculums as low as 10 CFU [42-44]. *NRAMP1*$^{-/--}$ mouse strains include BALB/c and C57BL/6. The lethal dose (LD_{50}) of *Salmonella* in mice that are wild-type at the *NRAMP1* locus is much higher, estimated to be somewhere between 10^3 and 10^4 CFU per mouse [45-47]. *NRAMP1*$^{+}$ mouse strains include Swiss Websters, Cba/J, and 129/SvJ. The selection of vendor can also influence outcomes: mice from certain facilitates harbor small populations of Enterobacteriaceae that can protect against *Salmonella* expansion in the gut [48].

Mice are generally resistant to *Salmonella* mediated inflammation of the gut. This has been attributed to the gut microbiota, whose protective mechanisms are collectively referred to as colonization resistance (see [49-51]). In order to study gastroenteritis, researchers often pre-treat with antibiotics (e.g. streptomycin), colloquially referred to as "strep-treated". Broad spectrum antibiotics deplete the host microbiota, allowing *Salmonella* to expand [52,53]. Treatment can increase colonization efficiency by a factor of 100,000 [54,55]. The strep-treatment approach arguably models pathogen expansion and not necessarily the interactions that allow *Salmonella* to overcome initial colonization resistance. In *NRAMP1*$^{-}$ mice, gastrointestinal infections are lethal because of the invasive subpopulation of *Salmonella* that reach and replicate within the liver and spleen. In *NRAMP1*$^{+}$ mice, gastroenteritis is typically nonlethal.

Newer models for studying *Salmonella* gastroenteritis in the absence of antibiotic perturbation have been developed. The Cba/J mouse allows for long term colonization of *Salmonella* by an unknown mechanism. While most mice maintain a small *Salmonella* population in the gut, a subset (10-30%) will eventually develop inflammation and a high *Salmonella* burden. In these mice, inflammation can be studied in the absence of a significant perturbation to the microbiota (e.g. antibiotics). Our lab, in collaboration with other groups, performed a multi-omics study on these inflamed mice [56]. Another approach is to alter the mouse diet. Laboratory mice are typically maintained on a plant-based diet. When maintained on a high-fat diet, they become permissive to *Salmonella*-mediated inflammation and expansion without the need for antibiotics (unpublished data and [57]). Finally, the use of gnotobiotic mice colonized with small, defined communities

allows for both natural inflammation and controlled experiments to study interactions between *Salmonella* and the microbiota [58].

The study of human typhoid illness historically relied on infections of mice using *S.* Typhimurium as *S.* Typhi cannot colonize mice. Recently, a collaborative cross study identified CC003/Unc mice as permissive to colonization [59]. This mouse model was used in Chapter 5 to study mannitol sensitivity in *S.* Typhi. Other groups have used 129/SvJ mice, which are defective in clearing *S.* Typhimurium bacteria, allowing researchers to model a persistent infection [60,61]. Finally, *NRAMP1*$^{+}$ mice can be fed a lithogenic diet that induces the formation of gallstones, then infected with *S.* Typhimurium. This is used to model chronic typhoid infections, which is mediated by the colonization of gallstones in humans [62,63].

1.5 Pathogenesis of *Salmonella* gastroenteritis

Three primary disease states of *Salmonella* infections have been described: gastroenteritis, typhoid (or enteric) fever, and bacteremia. The primary virulence factors of *Salmonella* are two type 3 secretion systems encoded on *Salmonella* pathogenicity islands 1 (SPI1, T3SS1) and 2 (SPI2, T3SS2). SPI1 is a defining genetic trait of *Salmonella* compared to *Escherichia* [64] and SPI2 distinguishes *S. enterica* from *S. bongori* [65]. Additional pathogenicity islands have been identified and characterized to varying degrees and other virulence factors include a virulence plasmid and adhesins (e.g. fimbriae) (reviewed in [66]) as well as the lipocalin resistant siderophore salmochelin [67].

Salmonella mediated gastroenteritis, for which serovar Typhimurium is the model organism, is a self-limiting infection of the gut marked by several days of intestinal inflammation and fecal shedding that can last several weeks [68,69]. The pathogenesis of gastroenteritis is a well-reviewed topic as many excellent researchers occupy the field [51,70-75]. Infections are initiated by the consumption of *Salmonella* in contaminated food or drink. Modeling studies suggest the infectious dose of humans in virulent strains like Typhimurium is low in humans (<1,000 CFU) which is on par with the ID_{50} of inflammation susceptible mouse models [54,55,76]. It was reported that the acidity of the stomach kills a significant percentage of invading bacteria [77], though more recent work shows that population bottlenecking occurs after the onset of gut inflammation [78]. Within this location, *Salmonella* faces considerable competition for attachment and nutrients by the resident microbiota. The induction of inflammation in the gut invokes the host immune system to contain the infection, but at the expense of depleting resident flora in the process (and colonization resistance with it). Thus, inducing inflammation in the gut can also be considered a strategy by pathogens to facilitate colonization [74]. Other bacterial pathogens have been observed to take advantage of inflammation-mediated depletion of host microbiota other than *Salmonella* [49].

The primary virulence factor responsible for intestinal inflammation is SPI1, with SPI2 contributing to the overall level of inflammation [79,80]. T3SS1 injects a collection of effectors that performs two main functions: mediating entry into host cells and inducing inflammation [66]. SPI1-mediated entry into host cells occurs by a trigger mechanism, where effectors modulate host proteins in such a way as to induce cytoskeletal

rearrangement and bacterial engulfment [66,81]. Three effectors, SopB, SopE, and SopE2, are essential for invasion [82]. SopB is a lipid phosphatase whose activity leads to the recruitment of multiple phosphatidylinositols [83]. The exact mechanism by which this leads to entry is complex and not fully understood (reviewed in [81]). Both SopE and SopE2 are guanine nucleotide exchange factors (GEFs) [84]. The host targets of SopE/SopE2 are Cdc42 and Rac1, two Rho family GTPases whose downstream activity is regulated by host (and pathogen) GEFs [85,86]. The mechanisms by which effectors trigger inflammation is also complex, but the pathways appear to converge on central host regulators NF-kB and AP-1 (see reference [74] for a detailed review on this subject). Interestingly, *Salmonella* activates both pro- and anti-inflammatory pathways through various effectors, suggesting a fine-tuned manipulation of host signaling.

1.6 *Salmonella* causes diarrhea by several possible mechanisms

Diarrhea is a defining trait of many gastrointestinal pathogens. The underling physiological process underling diarrhea the secretion of water into the lumen of the gastrointestinal tract. The movement of water is secondary to the movement of solutes, primarily sodium (moving inward) and chloride (moving outward) [87]. Diarrhea can occur in non-inflammatory and inflammatory contexts, either mediated by an infectious agent or through non-infectious disease (e.g. inflammatory bowel disease). *Salmonella* induces diarrhea in an inflammatory environment in the gut and three possible contributors to diarrhea have been investigated: chloride secretion, loss of vascular permeability, and neutrophil-mediated damage [88].

Several SPI1 effectors are essential for fluid secretion [88] but it is difficult to distinguish the relative contributions of individual effectors as a direct process (i.e. modulating host targets) or indirectly through induction of inflammation and recruitment of neutrophils. SopB is an inositol phosphatase [89], mutants of which are defective in the induction of both fluid secretion and neutrophil influx into the lumen of calf ileal loops [89]. SopB activity leads to the accumulation of inositol-1,4,5,6-tetrakisphosphate (IP_4), which counteracts epidermal growth factor inhibition of potassium efflux indirectly by increasing Cl^- secretion [90]. Mutants of *sopB* are in fact defective in chloride secretion through this mechanism [90]. In further support of the chloride secretion explanation of diarrhea, work from Barrett lab found that epithelial cell proliferation is increased during infection and immature epithelial cells are less effective in water regulation due to defects in the expression and function of transporters compared to mature cells [91]. Changes in cell maturity as a result of infection may therefore be a contributor to fluid secretion as well.

It has been established that vascular permeability is compromised through the observation of plasma protein loss during infection and this loss was thought to occur through neutrophil-mediated damage to the epithelia [88]. An early study on the role of neutrophils in diarrhea used nitrogen mustard to induce neutropenia in rabbit ileal loops, which reduced fluid secretion resulting from *Salmonella* infection [92]. However, nitrogen treatment also reduces fluid secretion from cholera toxin, which triggers diarrhea through chloride secretion, not neutrophil recruitment [92,93]. Another study used mice unable to produce IL-8, which are defective in neutrophil migration into the lumen. *Salmonella*

proliferates faster in these mice, but there is no change in inflammation, transporter expression, or epithelial cell proliferation [94]. While these results suggest neutrophils are irrelevant to diarrhea, it was recently shown that neutrophils are protective against epithelial cell damage, evidenced by increased epithelial cell efflux in mice treated with α-Ly6G (an anti-neutrophil antibody) [78]. Barrier integrity was also compromised by α-Ly6G treatment during infection [78].

Chloride secretion, or more generally, defects in solute transport, as a mechanism in *Salmonella* mediated diarrhea has several supporting studies. Loss of vascular permeability during infection has been observed across several groups, and neutrophils appear to be a protective, not damaging, mechanism against infection and diarrhea. The intertwined nature of *Salmonella* pathogenesis makes it difficult to quantify individual contributors to overall net fluid secretion. Additionally, some of these studies are performed in mice. Although mice do have increased water content in their feces during infection [95], the differences in feces morphology and consistency between infected and un-infected mice is indistinguishable to the naked eye. It is fair to say that mice experience *Salmonella*-mediated diarrhea much differently than their natural hosts (humans and livestock) and results derived from mice should take this caveat into account.

1.7 The efficacy of antibiotics is threatened by antibiotic resistance

The invention of antibiotics represents one of the great advancements in the control of infectious disease. The concept of antibiotics was developed by Paul Ehrlich,

who also developed the first synthetic antibiotic, Salvarsan, in the early 20th century [96]. Antibiotics are antimicrobial compounds that either kill or inhibit bacterial growth, which can be classified by structure and mechanism of action. The golden age of antibiotic discovery (the 1950s and 1960s), in which numerous new classes of antibiotics were discovered, has since ended [97]. The central issue challenging the gains humanity has made against infectious disease with antibiotics is antibiotic resistance. It is estimated that antibiotic resistance will lead to 2 million deaths per year by 2050 [98]. Another issue with the use of antibiotics is their unintended effects on gut microbiota. Most antibiotics are broad-spectrum, meaning their target or mechanism of action works against most or all bacteria. This is useful clinically: antibiotics can be administered empirically rather than needing to identify the causative agent and fewer antibiotics need to be developed overall. The depletion of the microbiota can have negative consequences, such as *Clostridium difficile* infections that arise after antibiotic treatment [99]. Ideally then, antibiotics would be specific against pathogenic bacteria but not host microbiota. However, mechanisms specific to pathogenic bacteria but broad enough to target all or many different pathogens are rare.

Treating *Salmonella* with antibiotics is challenging for multiple reasons. As discussed above, susceptibility to gastroenteritis in mice is mediated by the depletion of host microbiota through a broad-spectrum antibiotic. Correspondingly, disease longevity and severity in humans can increase with antibiotic treatment [52,100-106]. Thus, infections cannot be easily treated by conventional means. Systemic infections (bacteremia and typhoid fever) can be treated by broad-spectrum antibiotics but nearly half of isolates

causing invasive nontyphoidal salmonellosis (iNTS) are multi-drug resistant [107,108] and many isolates of multi-drug-resistant *S.* Typhi are spreading rapidly [109]. Novel antibiotics may help combat the rising threat of *Salmonella* and, as discussed below, could be used in the treatment of gastroenteritis.

1.8 Sugar-phosphate toxicity could be a new antimicrobial strategy

In bacteria, many sugars are imported into the cytoplasm concurrently with their phosphorylation, donated by their transporter (who itself receives a phosphate from phosphoenolpyruvate, also known as the phosphoenolpyruvate transport system or PTS). This provides an energy source for transport and limits diffusion of sugars back out of the cell. Phosphorylated sugars and transporters are also important regulators in metabolism [110]. Although intermediates quickly interconvert to meet the needs of the cell (further oxidation to generate energy, synthesis of precursor molecules, etc.), they can accumulate within the cytoplasm when enzymes essential for their processing are disrupted by mutation or an inhibitor. Sugar-phosphate toxicity is a phenomenon that occurs when accumulation of those intermediates leads to defects in the cell [111]. Cells that experience defects upon exposure to the sugar, even in the presence of another carbon source, are referred to as sugar sensitive.

We have explored these defects as a possible anti-microbial strategy, where an antibiotic targeting the enzyme that processes the intermediate is co-administered with the sugar. Recently, our lab reviewed the available literature on sugar-phosphate toxicity, summarizing 11 identified toxicities and their essential processing genes [111]. Of the

fifteen enzymes whose mutants lead to some form of toxicity, 8 are encoded in humans and therefore unsuitable as an antibiotic target [111]. We previously screened 9 mutants in *S.* Typhimurium. looking for fitness defects in the strep-treated Swiss webster model of gastroenteritis [112,113]. Using competitive infections against a wild-type counterpart, significant defects were observed in mutants sensitive to galactose (*galE*), mannose (*manA*), rhamnose (*rhaD*), arabinose (*araD*), fructose-asparagine (*fraB*), and mannitol (*mtlD*). The most significant attenuation phenotypes are in mutants conferring sensitivity to arabinose, rhamnose, and mannitol. The scope and efficacy of MtlD as a drug target against bacteria is evaluated in Chapter 5.

1.9 Mannitol and Mannitol sensitivity

Mannitol is a highly soluble sugar alcohol synthesized by plants and fungi for use in osmoregulation [114]. Humans do not metabolize mannitol, enabling its use in the food industry and medicine [115]. In the food industry, mannitol (and sorbitol) can be used as a low-calorie sweetener [116]. In medicine, mannitol has many uses based on its osmotic properties. It can be used to treat increased intracranial pressure and promote diuresis in the oliguric phase of acute renal failure [117,118]. It has also been used in the treatment of cystic fibrosis to reduce mucus viscosity [119]. This may have implications for the treatment of the pathogens *S. aureus* and *P. aeruginosa*, both of which encode *mtlD* and mutation leads to mannitol sensitive (Chapter 5 and [120-122]). Humans excrete circulating mannitol unaltered in urine within a few hours [115]. Orally and intraperitoneally provided mannitol appears to be accessible to *Salmonella* residing within the liver and spleen, as indicated

by our work in Chapter 5. The excretion of mannitol through the bladder also suggests it may be a suitable strategy to treat uropathogenic *E. coli*, which are responsible for most urinary tract infections and whose *mtlD* mutants are mannitol sensitive (Chapter 5 and [123]).

In *Salmonella* and *E. coli*, mannitol is imported across the inner membrane by MtlA, the EIICBA component of a phosphoenolpyruvate-dependent sugar phosphotransferase system [124,125]. Its cytosolic product, mannitol-1-phosphate (Mtl-1P) is the substrate for mannitol-1-phosphate 5-dehydrogenase, whose reaction generates fructose-6-phosphate and two reducing equivalents ($NAD^+ \rightarrow NADH$). To my knowledge, no other enzmye has been published to use Mtl-1P as a substrate in *E. coli* and *Salmonella*. Other bacteria have slightly different metabolic processes. For example, *S. aureus* two mannitol transporters [121] and *P. aeruginosa* encodes an ABC family transporter complex with two nearby kinases. The *mtlADR* operon is regulated by MtlR, which does not bind the locus directly and regulates by an unknown mechanism [126-130].

Mutants of *mtlD* exposed to mannitol have been shown to accumulate mannitol-1-phosphate (Mtl-1P) and this correlates to growth defects in vitro [131-133]. An early mutant of *Salmonella mtlD* (isolated by a genetic screen) accumulated an intracellular concentration of ~20 mM Mtl-1P after one hour mannitol exposure in nutrient broth (leading to lysis shortly after) [134]. Most in vitro assays on mannitol sensitivity in the work presented here uses defined, minimal medium (M9). We find that mannitol concentrations up to 300 mM are bacteriostatic, not bactericidal (unpublished data) in M9. In rich complex media (e.g. LB), decreases in OD_{600} during growth, suggestive of

lysis, can be observed in stationary phase after exposure to mannitol at a concentration of 1 mM (Chapter 5). Bactericidal activity at these timepoints has not been confirmed. I speculate that this early reported mannitol sensitive mutant could have a nonsense mutation in *mtlD*, leading to polarity effects on *mtlR*. Our lab confirmed that polar *mtlD* mutations increase mannitol sensitivity phenotypes in vitro [131]. The mechanism by which Mtl-1P intoxicates cells is unknown [131]. In Chapter 5, the kinetics of toxicity in-vitro are elucidated. In Chapter 6, I discuss the current knowledge and hypothetical mechanisms of Mtl-1P toxicity.

1.10 Organization of the work presented in this thesis

In this thesis, I lay out a collection of works performed under mentorship of Brian Ahmer at The Ohio State University. Chapters 2-4 include work on the LuxR solo SdiA and our efforts to understand its role in the Enterobacteriaceae. Chapter 5 includes work on mannitol sensitivity, in which disruption of mannitol dehydrogenase leads to sensitivity. In this chapter, we show that many pathogens mutated for *mtlD* become mannitol sensitive, and their infections are attenuated when the host is provided mannitol. Mannitol sensitivity, we propose, could be used as an anti-microbial strategy, with MtlD being the target of a small molecule inhibitor introduced alongside a mannitol solution. In both studies, the primary organism used is *Salmonella*, a well-studied, genetically tractable, model pathogen. In Chapter 6, these works are summarized and future directions for both projects are discussed.

Chapter 2: SdiA-mediated eavesdropping in the Enterobacteriaceae

2.1 Contributions

This work is a review article with the first draft written by myself, and then multiple rounds of editing by myself and Dr. Brian Ahmer.

2.2 Abstract

Bacteria can cooperate to engage in complex behaviors by coordinating their gene expression through the production, release and detection of small molecules, a phenomenon known as quorum sensing. Many bacteria encode what are known as LuxR solos: response regulators with no cognate signal synthase. In a subset of Enterobacteriaceae, including *E. coli* and *Salmonella*, the LuxR solo SdiA is used to detect the quorum sensing molecules of foreign bacteria, a behavior known as eavesdropping. Despite significant research on the topic, the role of SdiA-mediated eavesdropping in these bacteria remains unknown. In this review we discuss the phenotypes and regulons of SdiA in the Enterobacteriaceae.

2.3 Introduction

Quorum sensing (QS) is a behavior that allows bacteria to measure their population density by producing and releasing small molecules into the surrounding environment. In suitable environments (e.g. sufficient density, areas of low diffusion), these small molecules reach a detectable threshold concentration, leading to coordinated behaviors in the population through a ligand bound response regulator. QS itself is a highly reviewed topic (see references [2-4,18,135]). In this review, we refer only to QS in Gram-negative bacteria utilizing small molecules of the *N*-acyl-homoserine lactone (AHL) class. AHLs contain a homoserine lactone ring, amide group, and variable length acyl side group that can be modified by carbonyl or hydroxyl substitution on the third carbon [136]. A QS circuit encodes an AHL synthase (LuxI or LuxM), response regulator (LuxR), and regulon whose expression is controlled by the regulator bound to the AHL. LuxR-type proteins are more abundant in genomes than AHL synthases [24], suggesting that some bacteria synthesize AHLs with proteins other than LuxI and LuxM. This has been found in at least one protein, HdtS [137]. Ratios of regulator to synthase greater than one also indicate cells may utilize a LuxR-type protein for functions outside the standard QS paradigm. Those LuxR proteins that lack a known cognate synthase have been broadly termed "LuxR solos" [25]. The LuxR solos have a variety of demonstrated functions, including regulation of intra-species QS circuits (third-wheels) and inter-kingdom communication through detection of eukaryote produced AHL analogs [25].

The subject of this review is one LuxR solo, SdiA, which engages in eavesdropping. Eavesdropping species do not encode a cognate AHL synthase adjoining

their LuxR family protein, nor do they participate in other QS circuits within the genome like third-wheels. Instead, they rely on foreign AHL-producing bacteria for activation (one-way inter-species communication) [25,29,138-141]. A large subset of the Enterobacteriaceae family encode *sdiA*, including the prominent genera *Escherichia* and *Salmonella* (Fig. 1) [29]. At the same locus, related genera like *Erwinia* and *Pantoea* also encode LuxR homologs with a cognate AHL synthase, indicating SdiA was once part of an ancestral LuxR/LuxI pair [29]. The speciation of *Salmonella* and *Escherichia* has been estimated to be between 60 and 100 million years ago, making the adoption of SdiA-mediated eavesdropping a relatively old event [64]. Despite such a long time frame, SdiA (and its ability to detect foreign AHLs) appears largely or completely conserved within these genera, even in recently emerged lineages like *Salmonella enterica* serovar Typhi [142] (Chapter 3 paper).

The ability of SdiA to detect the AHLs of other bacteria has been clearly demonstrated [143-146]. In simple terms, we still do not understand why eavesdroppers engage in this behavior. This is due in part to the absence of clear-cut phenotypes of *sdiA* mutants but also the complex, sometimes contradictory collection of previously published studies that require more nuanced consideration. In this review, we discuss the current body of literature on SdiA, focusing on its reported phenotypes and regulons. Each section is labeled by an important and unanswered question in the field.

2.4 How is SdiA activity regulated?

SdiA is a LuxR-type protein, a family named for the response regulator which controls bioluminescence in *Vibrio fischeri*. This is not to be confused with the LuxR protein of *Vibrio harveyi,* which contains a TetR-type helix-turn-helix domain [147]. LuxR-type proteins are two-domain proteins encoding a N-terminal ligand binding domain and C-terminal helix-turn-helix domain responsible for binding to DNA by recognizing a specific motif, usually as a homodimer. Depending on the specific protein, transcriptional activation can occur by both class I and class II mechanisms while repression has been shown to occur through steric hindrance [148-150]. The regulatory mechanisms for SdiA activation and repression have not been experimentally determined except for one study suggesting a class II mechanism of *E. coli* SdiA on the *ftsQ* promoter [151]. The relevance of *E. coli* SdiA's reported interaction with the *ftsQ* promoter is questionable (see below).

Schuster and Greenberg proposed a classification scheme for LuxR-type proteins based on folding and ligand binding characteristics [152]. Class I proteins, such as TraR *of Agrobacterium tumefaciens*, require AHL for folding and bind them irreversibly [153]. Class II proteins, such as *V. fischeri* LuxR, also require AHL for folding but bind them reversibly [154]. Class III proteins, such as *ExpR* of Erwinia, do not require AHLs for folding and bind them reversibly [149]. *E. coli* SdiA can be purified in the absence of AHLs (possibly requiring an endogenous ligand, 1-octanoyl-*rac*-glycerol) suggesting it fits into class III, alongside its close evolutionary homolog ExpR [155,156]. Consistent with the ability to fold in the absence of AHL, orthologs of SdiA in *Salmonella*, *E. coli*, and *E. cloacae* have both AHL dependent and independent regulatory phenotypes (Chapter 3)

[157,158]. Apo-SdiA forms an open ligand-binding pocket that limits the size of the acyl chain through two residues, F59 and L77 [155]. In *Salmonella*, these residues are flipped (L59 and F77). Both *Salmonella* and *E. coli* SdiA preferably interact with mid chain length AHLs, specifically N-Hexanoyl-DL-homoserine lactone (oxoC6) and N-Octanoyl-DL-homoserine lactone (oxoC8). At least for *Salmonella*, AHL dependent SdiA activity can be induced by both shorter and longer chain lengths, suggesting the detectable range of foreign AHL producing bacteria is relatively large. [138].

The role of AHLs in regulating SdiA activity remains unclear. It was initially proposed that AHLs induce a large conformational change in protein structure (a folding switch mechanism) [159] but it was later shown that AHLs only have a minor impact on conformation [155]. It is possible this small change is sufficient for altering the binding motif: the *ler* promoter of *E. coli* O157::H7 contains two SdiA binding sites: one AHL-independent and one AHL-dependent [155]. A SdiA box has been proposed based on DNase I footprinting of *E. coli* SdiA at the *ftsQAZ* promoter, but this site cannot be found in the *ler* promoter or upstream of *Salmonella* SdiA regulated genes [151,160]. AHLs also increase the stability of SdiA, which could impact regulatory phenotypes [155]. The hypothesis that AHLs regulate SdiA activation solely through stability is not consistent with the presence of both AHL-independent and AHL-dependent regulated loci within a single strain's regulon [157]. Adding to the confusion is the fact that AHL-dependent regulation at promoters becomes semi-AHL independent at 30°C (instead of 37°C) in *E. coli* and *Salmonella* [146,158]. The underlying mechanism of temperature dependent effects on

regulation has not been investigated and no model explains the reported range of *sdiA*-dependent regulation.

2.5 When and where is SdiA relevant?

Of considerable significance to understanding SdiA-mediating eavesdropping is identifying the relevant environment in which it occurs. Eavesdropping bacteria are found in diverse environmental niches, but experimental data primarily come from infections of animal models using two intestinal pathogens: Enterohemorrhagic *E. coli* (EHEC) and *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*S.* Typhimurium). Below we describe the techniques used and the relevant studies for each environment evaluated.

2.5.1 Methodology

The preferred method of evaluating an environment for relevance is the competition assay, where a 1:1 ratio of wild-type and *sdiA* mutant are inoculated into the system. Later, the bacteria can be sampled from the environment and their ratio measured again. Changes from the initial ratio then indicate fitness phenotypes and relevance may be inferred. In addition, our lab has utilized a reporter strain of *S.* Typhimurium that measures if SdiA becomes active during transit through an environment. This reporter heritably deletes an antibiotic resistance marker from its chromosome in the presence of AHLs [145,161,162]. By inoculating environments or hosts with wild-type and *sdiA* mutant reporter strains of *S.* Typhimurium, fitness and activity can be evaluated simultaneously. The relevance of each site can also be considered indirectly by the presence of AHL synthase genes within the metagenome of the environment.

2.5.2 Mice and humans

Humans are a hypothesized site of relevance to SdiA-mediated eavesdropping due to their role as a host of many $SdiA^+$ pathogens. As a proxy, mice have been used as a model system to determine whether eavesdropping occurs during gastroenteritis. The only pathogen evaluated in mice is *S.* Typhimurium, whose pathogenesis includes the induction of inflammation [74]. Using the aforementioned methodology, the *S.* Typhimurium SdiA reporter strain is not active in the gastrointestinal tract of mice nor do *sdiA* mutants exhibit significant fitness defects [144,145]. One explanation for the absence of colonization defects of *sdiA* is the lack of AHLs in the mouse gut. This can be solved by introducing an AHL-producer into the system. By co-infecting *S.* Typhimurium with *Yersinia enterocolitica* (another gastrointestinal pathogen), SdiA becomes active in the gut [144]. Even in this environment, the *sdiA* mutant has no fitness defect. The first and most obvious interpretation of these results is that *sdiA* is not relevant to *Salmonella* in the gastrointestinal tract of mice. The co-localization of *Y. enterocolitica* and *S.* Typhimurium may also be insufficient for phenotypes to emerge. *Y. enterocolitica* preferably resides in the small intestine while *S.* Typhimurium colonizes the cecum and large intestine [163,164]. Activity of the genetic reporter strain does not increase significantly after passaging from the small intestine to the cecum, suggesting activation may occur prior to *Salmonella's* arrival at its preferred colonization site. Activation is highest in the Peyer's patches and *Y. enterocolitica* facilitates the survival of *Salmonella* within this immune organ. This may be due to the effect of *Y. enterocolitica* virulence factors that have anti-phagocytic activity, preventing uptake of *Salmonella* into host cells [165].

Interestingly, there is still no *sdiA* mutant fitness defect within Peyer's patches. Given that at least two *Salmonella* SdiA regulated genes are virulence factors (*rck* and *srgE*, discussed below), anti-phagocytic activity could prevent the emergence of fitness defects [166,167].

Using a second pathogen as a strategy for introducing AHLs into the system is effective at inducing activity but risks pathogen-specific confounding results. Determining mutant fitness with competitive infections also carries the risk of false negatives if the mutant's defect is rescued by the wild-type. The latter can be controlled by single infection studies (an experimental approach absent in animal studies of *Salmonella* SdiA). The former issue was addressed by infecting mice with wild-type and *sdiA* mutant *Salmonella* in a genetic background encoding *yenI* from *Y. enterocolitica*, enabling *Salmonella* to produce AHLs without the need for another bacteria. In this model, the *sdiA* mutant is attenuated in the gut during the infection and it is the largest observed mutant defect to our knowledge (>100-fold) [144]. The fitness advantage conferred by *sdiA* requires its regulated virulence factors, *pefI-srgC* and *srgE* [144]. These results suggest that the absence of *Salmonella sdiA* mutant defects in the *Yersinia* study was due to *Yersinia* itself (co-localization defects and/or anti-phagocytic activity) and that wild-type does not rescue *sdiA* mutants. It is still unclear if this model is biologically relevant. During infection, *Salmonella* reaches very high population densities, and an AHL-producing genetic background would have a dramatic effect on luminal AHL concentrations. This could lead to over-activation of SdiA or unintended effects on the host's immune response, which can also detect AHLs [168-170].

Humans cannot be tested directly, but the possibility of QS in the human gut was recently reviewed [171]. Bioinformatic searches find almost no LuxI homologs in the human gut microbiome [172,173], yet AHLs have been detected in both the gut and feces [173-175]. AHL concentrations are in the low nanomolar range, near the detection limit of SdiA [138,176], but could be effectively increased in microenvironments [3]. QS potential is further complicated by antagonistic compounds in the gut (e.g. indole), quorum quenching activity (e.g. lactonases), and compositional shifts during infection [6,51,177-180]. The relevance of QS in the human/mouse gut to eavesdropping bacteria like *Salmonella* remains an open question.

2.5.3 Cattle

Cattle have been explored as a site of SdiA activity using *S.* Typhimurium and *E. coli* O157::H7. The *Salmonella* reporter system has only been tested in a single calf, but there was no activation of SdiA or *sdiA* mutant defect [145]. *E. coli* has been tested in larger cohorts using competition assays and single infections that indicate fecal shedding and colonization defects to varying degrees (up to ~4-fold) [181-184]. AHLs have also been extracted from the rumen with seasonal and dietary effects on concentration [181-183]. The intensity of *sdiA* mutant defects positively correlates with diets that increase AHL concentration in the rumen. From these studies, a model has been proposed whereby pathogenic *E. coli* sense AHLs in the rumen to activate their acid response system and suppress virulence [182,183,185]. Suppression of virulence is alleviated upon leaving the rumen, allowing for colonization of the gastrointestinal tract. Both acid tolerance and virulence have a degree of AHL-independent regulation by SdiA and the rumen

microbiota member(s) producing AHLs have yet to be identified. The cattle infection model is unfortunately difficult to manipulate to clearly establish a causal and quantifiable contribution of the ruminal AHLs to SdiA fitness.

2.5.4 Reptiles

Turtles are an asymptomatic carrier of non-typhoidal *Salmonella* and a source of outbreaks in the United States [35,36]. A study in our lab found that SdiA activation occurs within the turtle intestine at levels comparable to those observed after growing in the presence of AHLs in-vitro [145]. The source of AHLs was most likely the co-colonizing aquatic pathogen, *Aeromonas hydrophila*, an AHL producer that *Salmonella* can detect [145]. Despite strong activation, *sdiA* mutants have no fitness defect in this system. Like the mouse studies, it is possible that mutant defects were occluded by the presence of the wild-type. The microbiota of turtles appears to be more abundant in proteobacteria than that of humans though their composition is impacted by many factors including location, age, and captivity status (reviewed in [186]). Other *Salmonella* subspecies (that generally do not infect humans) are often isolated from turtles and encode *sdiA*, but in vivo studies are limited to *S.* Typhimurium [36].

2.5.5 Insects

Insects are known reservoirs and transmission vectors of $SdiA^+$ genera and pathogens like *Salmonella* [37,187] . Insect microbiota are commonly colonized with Proteobacteria, the only known phyla that produces AHLs and this includes known AHL

producers like *Pseudomonas* and *Pantoea* [188]. Direct examination of insects as a site of SdiA activity is limited. AHL producing *Rahnella* species were isolated from the gut of wax moth larvae (*Galleria mellonella*) [189]. Subsequent infections of *G. mellonella* with *Salmonella* Enteritidis (a serovar similar to Typhimurium) pre-incubated with AHLs (C12) increased their persistence in the hemolymph with minor to no significant effects on host survival or health [190]. It was not determined if this phenotype was *sdiA* dependent, and *S.* Typhimurium SdiA was previously shown to be unresponsive to C12 [138]. Our lab has investigated the potential of *Salmonella* SdiA activation and fitness in house flies (*Musca domestica*). In adult flies raised from vendor acquired pupae, we observed some activation in experimental infections (the microbiota responsible could not be identified), though not as much as in turtles or *Yersinia* infected mice. Unfortunately, subsequent studies revealed a complex interaction between SdiA, antibiotic markers, and infectious dose that could not be sufficiently controlled to draw conclusions (unpublished data).

2.5.6 Plants

A bioinformatic search of metagenomes we performed showed that the rhizosphere encodes LuxI homologs most frequently, followed by insects (unpublished data) and plant microbiomes have previously described AHL producing pathogens and commensals [191-194]. Plants have been probed for *Salmonella* SdiA activation in tomato soft rot caused by plant pathogen *Pectobacterium carotorvorum*. Although *P. carotovorum* produces AHLs detectable by *Salmonella* in vitro, detection is lost during co-infection within the plant (and *sdiA* mutants have no fitness defects) [143]. The lack of

detection was attributed to lack of transcription of *sdiA* [143]. Transcription of *Salmonella sdiA* is primarily regulated by FliA, Crp, and LeuO. It is unknown if transcription inhibition in tomato soft rot is inhibited through these regulators [195,196]. A second study on a possible plant-*sdiA* relationship found that rice root extracts have detectable concentrations of AHLs (detected via biosensor strains) and a *sdiA* mutant of *Enterobacter cloacae* can better colonize their roots [197]. The directionality of this mutant phenotype suggests plants are not the sought after relevant environment. In a small pilot study, our lab inoculated the soil and leaves of a variety of commercially available angiosperms (leeks, parsley, tomato, and soybeans) with our *Salmonella* reporter strain but found no activation phenotypes or fitness defects in the *sdiA* mutant (unpublished data). It is interesting to note that the closest homologs of SdiA, ExpR and PhzR, are encoded in *Erwinia* and *Pantoea*, respectively [29]. Both genera contain known plant pathogens and have also been isolated from insects [193,198,199].

2.5.7 Other

Along with those listed, experimental infections of guinea pigs, rabbits, pigs, and chickens (chicks) have been evaluated as a site of *Salmonella* SdiA activity [145]. No activation occurred in any tested host. Mutant phenotypes of *sdiA* were only found in chicks, but the mutation was advantageous, and magnitude of the phenotype was small (<3-fold).

2.6 What are phenotypes of SdiA?

Other than a role in virulence or colonization of hosts (described above), SdiA has a small number of reported phenotypes with a significant amount of literature (cell division, drug resistance, and biofilm formation). Others are either discussed in other sections or not included in this review. A major source of confusion regarding SdiA phenotypes comes from issues of reproducibility. Phenotypes have been described using plasmid-based expression of SdiA or *sdiA* mutants. The former method often produces phenotypes and regulatory changes that are not observed when *sdiA* is expressed on the chromosome under its own promoter. This disparity can be interpreted as artifacts arising from increasing the copy number of the gene in question, or the observed behaviors require environmental conditions that are currently unknown. Studies relying on *sdiA* mutants sometimes produce phenotypes and regulatory changes not observed in independent constructs, other strains/species, or occur only in AHL independent manners. Thus, discerning behaviors relevant to the eavesdropping paradigm requires a more nuanced consideration of the data underlying each purported phenotype.

2.6.1 Cell division

SdiA was initially discovered in an early study on nearby gene *uvrC* in *E. coli* [200]. Shanna et al. described it as a "28kd protein" with a LexA binding site in its terminator region and a higher rate of rare codons suggesting a regulatory protein as proposed by Konigsberg and Godson [201]. After its initial description, SdiA was identified in a screen for genes involved in cell division performed by Wang, de Boer, and Rothfield [202].

Specifically, they selected for genes in a plasmid-based *E. coli* DNA library that could rescue growth in an inducible *minCD* genetic background. When over-expressed, MinCD inhibits assembly of the Z-ring and thus septation and cell division. They observed that two genes, *ftsZ* and the 28kd protein, could suppress division inhibition (hence SdiA). Based on their findings that a) over-expressing *sdiA* produced mini-cells, b) *sdiA* mutants had no cell division phenotypes, and c) *sdiA* could not complement *ftsZ* mutants, they surmised that *sdiA* was a positive regulator of the *ftsQAZ* locus [202].

A follow-up study described two promoters of *ftsQAZ*: one regulated by RpoS and one by SdiA [203]. The SdiA regulated promoter (P2) could indeed be activated by over-expression and activity increased by introduction of exogenous AHLs. The P2 promoter has also been shown to be bound by SdiA directly using gel-shift assays [151,156,204]. The mini-cell phenotype resulting from SdiA interaction with this *ftsQ* promoter has been observed by multiple researchers [139,202,204]. The major caveat to this finding is that the cell division phenotype as well as the regulation of the P2 promoter has only ever been observed by plasmid-based over-expression of SdiA in *E. coli*. When examining a native expression system (i.e. wild-type *E. coli*), the introduction of AHLs has no effect on cell division nor *ftsQAZ* expression and a *sdiA* mutation has no effect on cell division or *ftsQ* promoter regulation [158,202]. Recently, a *sdiA* mutant of *Klebsiella* was reported to have a filamentation phenotype that could be rescued by plasmid complementation [205]. It is not clear why the presence of foreign AHL producers should be linked to a basic and essential function like cell division. Other than the obvious interpretation (an artifact of

plasmid expression of *sdiA*), it is possible that SdiA manipulates transcription of *ftsQAZ* in a specific condition yet to be discovered.

2.6.2 Multiple-drug resistance

The multi-drug resistance phenotype of SdiA was implicated in a microarray study by Wei et al. comparing *E. coli* over-expressing SdiA on a plasmid to a vector control [206]. The AcrAB system, a TolC-dependent efflux pump which confers resistance to multiple compounds, was upregulated [206,207]. Over-expression of SdiA in *E. coli* and *Cronobacter* has also been shown to increase resistance to several antibiotics [158,208-210]. Mutation of *sdiA* alone has little effect on drug resistance in *E. coli* [158,208], *Salmonella* [158], or *Cronobacter* [211] and AHLs have no effect on resistance in *E. coli* or *Salmonella* [158] (Chapter 3).

2.6.3 Biofilms

The relationship between SdiA and biofilms was suggested in two studies from Jintae Lee [212,213]. Using microarrays and mutant studies in *E. coli*, there were four observations: 1) *sdiA* mutants have increased biofilm formation, 2) mutants differentially express curli and flagella genes (regulation of flagella but not curli has been independently observed [158]), 3) biofilm formation can be suppressed by indole in a *sdiA*-dependent manner, and 4) this occurs primarily at lower temperatures (30°C) [212,213]. A later study evaluated the role of SdiA, AHLs, and indole in biofilms of both *E. coli* and *Salmonella* [177]. For *E. coli*, mutation of *sdiA* had no effect on biofilm formation at any temperature (25, 30, and 37°C) and indole suppressed biofilm formation in *E. coli*, but in

a *sdiA*-independent manner. For *Salmonella*, neither *sdiA* nor indole has any effect on biofilms [177]. Interestingly, indole can suppress AHL-dependent activation of SdiA regulated genes, which may have implications for eavesdropping in the human gut where indole is at relevant concentrations [177,214,215]. Biofilm phenotypes have also been reported in *sdiA* mutants of *Cronobacter* [211,216], *Enterobacter* [197], and *Klebsiella* [205] but no study has reported significant AHL-dependent changes in biofilm formation. Interestingly, motility is implicated in both *E. coli* biofilms and SdiA (SdiA regulates motility) [158,217]. In *Salmonella* motility is also implicated in both biofilms and SdiA (motility, via FliA, regulates SdiA) [195,218,219]. Thus, SdiA is situated in a position where it may regulate biofilm formation and/or dispersal. These early and late aspects of biofilm phenotypes may not have been observable in experiments described above.

2.7 What genes does SdiA regulate?

As SdiA is a transcription factor, perhaps the most obvious question is "what does it regulate?". Possible regulon members have been identified with microarrays [206,213], genetic screens [139,157,158,220], and RNA-seq [211](Chapter 3). These studies compare wild-type to mutant [211,213] (Chapter 3) or use plasmid over-expression to induce activity [139,206] (Chapter 3). Some but not all have used AHLs as part of their initial screen, either with (Chapter 3) or without [157,158,220] a *sdiA* mutant control. Screens often identified dozens or hundreds of putative *sdiA* regulated genes, but viewed stringently, the size of the regulons may be much smaller (<20 genes). The regulon of each genus is described below.

2.7.1 *Salmonella*

We performed have attempted to identify the SdiA regulon of Salmonella, one with a genetic screen and one with RNAseq [139] (Chapter 3). A major limitation in studying *Salmonella* SdiA is its direct regulation by FliA [195]. Activity is strongest in motility agar [146] and we have been unable to extract viable RNA from semi-solid media. As an alternative approach, we identified putative hits by expressing *sdiA* on a plasmid. In our first study, a genetic screen of MudJ fusions that did not use AHLs, two loci were found and validated (i.e. *sdiA* expressed on the chromosome is sufficient to activate their promoters in the presence of AHLs). When comparing wild-type and *sdiA* mutant *S.* Typhimurium transcriptomes in the presence of AHLs using RNA-seq, three putative *sdiA* regulated genes were found (and only 1 was validated) (Chapter 3). Plasmid expression increased the putative number of hits to 209, but after extensive validation only six loci (representing 18 genes) were validated (Chapter 3). The first genetic screen had a low frequency of false negatives, but it missed a significant portion of the regulon. The RNA-seq study re-identified the hits from the genetic screen and found four new loci, at the expense of a significant frequency of false positives that required individual validation experiments to distinguish from true positives. Neither approach appears to be optimal for the identification of regulon members.

As a broad-host range serovar, serovar Typhimurium can be found in numerous human food related environments and can colonize a wide range of hosts including humans, livestock, plants, reptiles, and insects [33,37,221]. The possible sites where SdiA could be used to eavesdrop on foreign bacteria is vast. At the same time, the recently

emerged (~50,000 years ago) serovar of *Salmonella*, Typhi, is believed to use humans as its sole host and reservoir [222]. *S.* Typhi also encodes *sdiA*. The limited niche overlap, we hypothesized, represented a selective pressure on their *sdiA* regulons. Interestingly, Typhimurium and Typhi have semi-conserved regulons: four loci regulated in both serovars (SrgF, SrgKJ, SrgGH, and MenFDHBCE), two specific to Typhimurium (PefI-SrgC and SrgE), and one specific to Typhi (SrgIL) (Chapter 3).

Ideally, identifying the regulon of *sdiA* would provide insights into its in vivo role or at least possible phenotypes. The *Salmonella sdiA* regulon proves to be as nebulous as the gene itself. SrgF is a putative ATP-dependent RNA helicase like protein, though bioinformatic tools find no similarity to known protein domains [223,224]. SrgF has a high degree of basal expression in *S.* Typhimurium, especially compared to *sdiA* and other regulon members [225]. It appears sparsely in the literature with putative mutant phenotypes in chicken colonization motility, and phage defense [226-230]. We found no *srgF* fitness defects in mice, and *sdiA* mutants of serovar Typhimurium have no motility defects ([146] and Chapter 3 paper). SrgKJ encode a band 7/mec-2 family protein and NfeD family protein, respectively [231]. Previous characterization of *E. coli* found that SrgK ortholog QmcA could rescue lethal mutations in proteases, indicating a role in protein turnover with YbbJ acting as a helper protein [231]. Orthologs of SrgKJ are conserved in Gram-negative bacteria and *sdiA* regulation of *srgKJ* orthologs occurs in *E. cloacae* and *Salmonella* but not *E. coli*. The protein target(s) of SrgKJ remain unidentified and its connection to eavesdropping is unclear. SrgGH are both truncated fragments of hypothetical proteins. SrgG encodes the N-terminus of a full length, putative citrate

transporter in *S. bongori* and *E. cloacae*, and *E. cloacae* SrgG is *sdiA* regulated (Chapter 3). SrgH, like SrgG, is a fragment of a nearby protein, UshB (Cdh in *E. coli*). It is unknown if SrgG or SrgH are made and if these truncated proteins perform any relevant functions in *Salmonella*. The last *sdiA* regulated locus common to both Typhimurium and Typhi is the *menFDHBCAE* operon, which produces menaquinones (aka vitamin K2) that are involved in electron transport [232,233].

PefI-SrgC is a six gene operon encoding *pefI*, *srgD*, *srgA*, *srgB*, *rck*, and *srgC* encoded on the virulence plasmid pSLT (reviewed in [234]). This regulon member is not found in Typhi (Typhi does not harbor the virulence plasmid) and, like all members of the *Salmonella* SdiA regulon, our understanding of these genes is still limited. SrgB, a putative lipoprotein, and SrgC, a transcriptional regulator, have yet to be characterized. PefI and SrgA are involved in expression of Pef fimbriae through their roles as a transcriptional regulator and in post-translational maturation of PefA, respectively [235]. Two studies have suggested a role for PefI and/or SrgD in regulation of flagellar motility [236,237]. Although motility, through FliA, is an essential regulator of SdiA [195], neither mutation of *sdiA* nor AHLs have any effect on either transcription of motility genes, or motility phenotypes (Chapter 3 and [146]). The best characterized among these six genes is Rck, an outer membrane protein which confers resistance to complement killing and mediates invasion of host cells [167,238]. Rck binds epidermal growth factor receptor (EGFR) [239] and is only weakly expressed during infection of mice [240].

The second *S.* Typhimurium specific SdiA regulated locus is *srgE*, which encodes a secreted effector [166]. *Salmonella* encodes two type three secretion systems involved in

invasion (SPI1) and intracellular survival and replication (SPI2) [66]. SrgE is secreted in a SPI2 dependent manner, indicating a role in intracellular pathogenesis [166]. *S.* Typhi encodes *srgE*, but it is not regulated by *sdiA* in serovar Typhi (Chapter 3). Preliminary studies in our lab suggest SrgE may target retrograde trafficking proteins, a well-known target of other effector proteins (unpublished data) [241-243]. Within a host cell, *S.* Typhimurium secretes numerous effector proteins that remodel the phagosome in which it resides [244,245]. Oddly, *srgE* is the only known *sdiA* regulated effector and no others were implicated in our RNA-seq study (Chapter 3). While SrgE activity alone could alter intracellular growth or survival, we suspect that expression of other effectors would be altered as well. RNA was extracted from cells growing in LB, which mimics the intestinal lumen more than the endosome of a host cell. In media mimicking the intracellular environment, oxidative stress appears to be a strong inducer of *sdiA* transcription [246]. A future study focused on intracellular conditions may yield new virulence related regulon members.

The sole regulon member specific to *S.* Typhi is the *srgIL* operon, which encodes two small lipoproteins orthologous to *yfgHI* in *E. coli* (Chapter 3). Based on the reported sensitivity of *E. coli yfgI* mutants to nalidixic acid [247], we examined a possibility relationship between eavesdropping and resistance to DNA damage. Neither serovar Typhimurium nor Typhi exhibit any *sdiA* or AHL-dependent changes in resistance to nalidixic acid or UV damage (Chapter 3).

2.7.2 *Escherichia*

The glutamate dependent acid fitness island (*gad*, reviewed in [248]) and Locus of Enterocyte Effacement (LEE) are the two best described regulon members of *E. coli*. Regulation of *gad* occurs in both nonpathogenic (K12) and pathogenic (O157::H7) strains and has been described in multiple labs [158,183,249]. A significant amount of *sdiA*-dependent regulation of *gad* is AHL-independent [158,183] and acid resistance phenotypes are stronger at lower temperatures, at least in K12 [158,220]. Regulation of LEE by SdiA occurs directly at the promoter of virulence regulator *ler*, with stronger AHL-dependent phenotypes than *gad* despite the presence of both AHL-dependent and independent binding sites on the promoter [155,183,204]. SdiA also represses flagellar genes with in *E. coli*, with sdiA mutants reported to have motility defects [158,206,250]. A few other regulon members have been reported including the transcription factor, *uvrY* [220,251] and the O-antigen chain length determinant *fepE* (Chapter 3). The *uvrY* gene is activated by *sdiA*, while *fepE* is repressed. Although the regulatory mechanism is unknown, it was found that AHLs can induce temperate phages in *E. coli* in an *sdiA*-dependent manner [252].

2.7.3 *Enterobacter*

The SdiA regulon of *Enterobacter cloacae* includes a handful of *Enterobacter* specific hypothetical genes, the copper transporter, CopA, the O-antigen chain length determinant, FepE, signal transduction proteins, components of a putative type 6 secretion system, a phage integrase, SrgKJ, the menaquinone biosynthesis operon, and a full length version of citrate transporter SrgG (Chapter 3 and [157]). The regulatory action

of SdiA is more complex: a mix of activation and repression occurring in both AHL-dependent and independent manners. The strain used in these studies was isolated from a laboratory mouse (ref). Adding to the complexity is the fact that the genes identified do not appear related in function and no phenotypes for these genes have been identified (other than the aforementioned plant study of a different isolate [197]).

2.7.4 Other eavesdropping genera

Of the other genera encoding *sdiA*, *Klebsiella* has one study on the subject [205] and *Cronobacter* two [211,216]. Although no regulon screening experiments were performed, it was reported that that a *sdiA* mutation in *Klebsiella pneumoniae* alters the expression of *rpoS* and *ftsQ* (<2-fold). Additionally, the mutant had increased expression of Type 1 fimbriae, which is also regulated by phase variation [253-255]. It is unclear if *sdiA* has any effect on phase variation directly. Gel-shift assays support SdiA binding to both *ftsQ* and *fimA* in *K. pneumoniae*, but it was not determined if regulation was AHL-dependent [205]. In our most recent study, we tested whether *S.* Typhimurium regulated type 1 fimbriae (which is controlled by a different phase variation mechanism [253](Chapter 3)). In *S.* Typhimurium, plasmid-based expression could repress expression of the operon encoding structural genes and its three regulators (*fimW*, *fimY*, and *fimZ*), but there was no regulation under endogenous expression conditions or evidence that *sdiA* controls phase variation (Chapter 3 and unpublished data). *Cronobacter sakazakii* has been examined with a RNA-seq experiment comparing wild-type to *sdiA* mutant [211,216]. The transcriptome of the *sdiA* mutant suggested *sdiA* represses flagellar genes and activation of biofilm component genes (cellulose and extracellular polysaccharide). Mutant

phenotypes were consistent with those changes, but it was not determined if motility or biofilm formation phenotypes were AHL-dependent.[216].

2.8 Conclusions and future directions in the field of SdiA-mediated eavesdropping

Several genera within the Enterobacteriaceae encode SdiA, a LuxR-type protein. By loss of the corresponding AHL synthase, these bacteria no longer use AHLs to facilitate population-density dependent behaviors (quorum sensing) but instead detect other AHL-producing bacterial species in their environment (eavesdropping). Here we have discussed three questions fundamental to the nature of SdiA. If one thing is clear, it is that there are no clear answers to these questions in the data currently available. Many studies have probed various hosts as a relevant site of SdiA activation, but significant mutant defects (a strong indicator of relevance) are lacking. Most in vivo research relies on model systems, given the amount of control they offer compared to the real world. If SdiA is detecting foreign bacteria and/or its role in survival comes from an interaction with something in the environment, the selective pressure placed on the mutant could be lost in a model system. After evaluating the available literature, we note that two hosts, insects and plants, are largely unexplored in the literature and may be a suitable venue for future investigation.

A significant number of studies on the SdiA regulon used plasmid over-expression for identification of regulon members and in-vitro phenotypes. As discussed above, current evidence suggests that expressing SdiA on a plasmid is an artifact-prone approach given their conflicting results with chromosomal expression. Interpretation of the regulons are further complicated by ligand-independent activity, which demands

further consideration in interpreting results that are *sdiA*-dependent versus AHL-dependent. In some species (e.g. *E. coli*), SdiA regulated genes are relatively well characterized. In others (e.g. *Salmonella* and *E. cloacae*), the regulons include poorly or completely uncharacterized genes with no known relationship. Elucidating their function is complicated by the lack of relevant in vivo and in vitro phenotypes of *sdiA* that would normally be used as a starting point for further characterization. Identifying in vivo and in vitro phenotypes for *sdiA* rather than its regulon may be a more productive avenue for future research.

SdiA is conserved in both *Salmonella* and *E. coli*, indicating eavesdropping behavior was likely acquired and subsequently maintained for millions of years [64]. This time frame provided ample opportunity for changes in niches where one might expect some loss of an eavesdropping environment long enough to allow for loss of SdiA, yet this did not occur. We interpret this to mean that evolving lineages have always been in environments where eavesdropping is advantageous. Has this always been the same environment, did they spread to unique environments, or both? An apparent paradox then is the conservation of foreign AHL detection and the divergent transcriptional responses among species. If eavesdropping occurs in a common environment, why are the regulons so different? SdiA regulons were known to be completely unique between species until recently, when we found a degree of overlap within *E. cloacae*, *E. coli*, and *Salmonella* (no regulon member is common to all three species) (Chapter 3). Alternatively, a phenotype may be common to all bacteria undergoing SdiA-mediating eavesdropping but each bacteria uses different genes. We hypothesize that responses to phage may be that

unifying behavior. In *E. coli*, AHLs can induce temperate phage lysis [252]. In *Salmonella*, plasmid expression of SdiA represses a significant number of prophage genes and many regulon members are implicated in phage defense (Chapter 3). A phage integrase is regulated by SdiA in *E. cloacae* [157]. O-antigen chain length determinant *fepE* is *sdiA* regulated in both *E. coli* and *E. cloacae*, which could influence phage adhesion.

2.9 Figures

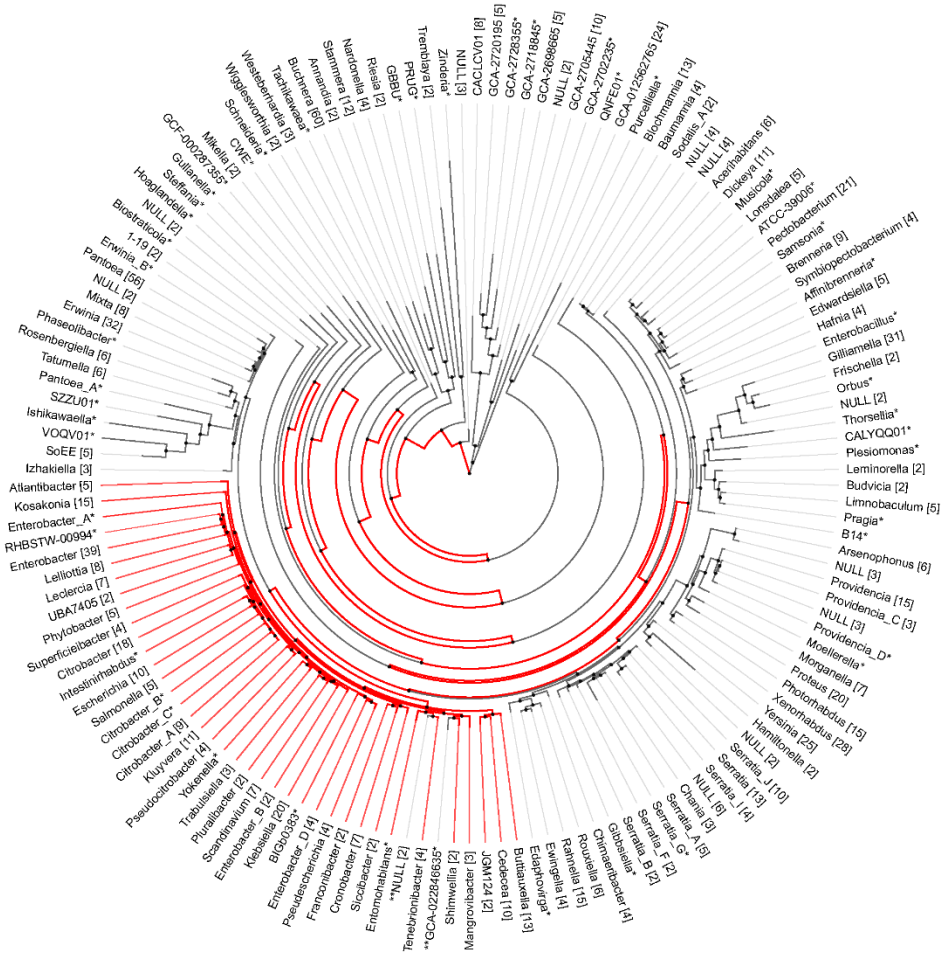


Figure 1. Bacterial genera predicted to undergo SdiA-mediated eavesdropping in the Enterobacteriaceae

Genomes within the family Enterobacteriaceae were searched with BLAST using Salmonella enterica subspecies enterica serovar Typhimurium 14028 SdiA as an input. The results were overlayed using AnnoTree version 214.

For hits outside of the identified subset spanning Atlantibacter to Buttiauxella (e.g. Erwinia ExpR), genomes were accessed and individually examined to determine whether sdiA was encoded and if it is adjcantent to an AHL synthase. Those encoding AHL synthases were omitted from the results.

Numbers in brackets indicate number of numbers per taxonomic group

*only one genome from that Taxonomic group

** Genomes unavailable

Chapter 3: Identification of new SdiA regulon members of *Escherichia coli*, *Enterobacter cloacae*, and *Salmonella enterica* serovars Typhimurium and Typhi

3.1 Contributions

This chapter is a first author research article that was published in the journal Microbiology Spectrum in 2024 [256]. I performed all of the experiments and wrote the paper in collaboration with Dr. Brian Ahmer.

3.2 Abstract

Bacteria can coordinate behavior in response to population density through the production, release, and detection of small molecules, a phenomenon known as quorum sensing. *Salmonella enterica* is among a group of Enterobacteriaceae that can detect signaling molecules of the *N*-acyl homoserine lactone (AHL) type but lack the ability to produce them. The AHLs are detected by the LuxR-type transcription factor, SdiA. This enables a behavior known as eavesdropping, where organisms can sense the signaling molecules of other species of bacteria. The role of SdiA remains largely unknown. Here we use RNA-seq to more completely identify the *sdiA* regulons of two clinically significant serovars of *Salmonella enterica*: Typhimurium and Typhi. We find that their *sdiA* regulons are largely conserved despite the significant differences in pathogenic

strategy and host range of these two serovars. Previous studies identified *sdiA*-regulated genes in *E. coli* and *Enterobacter cloacae* but there is surprisingly little overlap in regulon membership between the different species. This led us to individually test orthologs of each regulon member in the other species and determine that there is indeed some overlap. Unfortunately, the functions of most *sdiA*-regulated genes are unknown, with the overall function of eavesdropping in these organisms remaining unclear.

3.3 Introduction

Quorum Sensing (QS) is a bacterial strategy of coordinating behavior based on population density through the production, release, and detection of small molecules [17]. In this study, we refer specifically to QS that utilizes the detection of *N*-acyl-homoserine lactones (AHLs) by transcription factors of the LuxR type [4]. A complete QS circuit of this type includes an AHL synthase of the LuxI or LuxM type and a corresponding AHL receptor of the LuxR type. AHLs can differ based on acyl chain length (4 to 18 carbons) and acyl chain differences including the degree of saturation, and the presence of hydroxyl or ketone groups [136]. AHL nomenclature is based on these characteristics (e.g. N-(3-Oxooctanoyl)-DL-homoserine lactone or oxoC8, shown in Fig. 1A). Each LuxR/LuxI pair synthesizes and responds to a single (or a few closely related) type of AHLs, providing a degree of species-specificity. Within a confined space, or in a space with low diffusion, the AHLs accumulate to a threshold concentration and are detected by a LuxR-type transcription factor [3,18,153,154]. LuxR family members often regulate genes that affect fitness when a species is at a high population density [257]. Numerous QS-

regulated phenotypes have been described in bacteria, including bioluminescence in *Vibrio fischeri* [258] and virulence in *Pseudomonas aeruginosa* [259].

A subset of Enterobacteriaceae encode a LuxR homolog named SdiA [29]. The evolutionary history of SdiA appears to have begun as a LuxR/LuxI pair. The *Erwinia* and *Pantoea* still encode this pair where it is called ExpR/ExpI and PhzR/PhzI, respectively [29]. The LuxI homolog is absent in the *Escherichia*, *Shigella*, *Salmonella*, *Klebsiella*, *Enterobacter*, *Citrobacter*, and *Cronobacter*, leaving SdiA as a LuxR solo [25,29]. Without the cognate signal synthase, SdiA detects the AHLs produced by other bacterial species [138,139], a phenomenon referred to as eavesdropping [140,141]. Interestingly, *sdiA* has not been lost in any lineage suggesting a function important to all these organisms despite their differing environmental niches.

The role of SdiA-mediated eavesdropping remains unknown. One key piece of information to understanding this behavior is the environment in which SdiA is relevant. The genera encoding *sdiA* include many notable gastrointestinal residents and consequently the gut has been the environment most tested [144,145,183]. The possibility of quorum sensing in the gut was recently reviewed [171]. To briefly summarize, AHLs have been detected in both the gut and feces at low concentrations [173-175] but bioinformatic searches find few to no AHL synthases in the gut microbiome [172,173]. Although the reported concentrations of AHLs are near the detection limit of SdiA (low nanomolar [138,176]), microenvironments in the gut could have higher concentrations [3]. The implication of these findings for eavesdropping is complicated by other factors including antagonistic compounds in the gut (e.g. indole), quorum quenching activity (e.g. lactonases), and

compositional shifts during infection [6,51,177-180]. To determine if SdiA becomes active during bacterial transit through the gut, a reporter of SdiA activity was constructed in which *Salmonella* heritably deletes an antibiotic resistance marker from its chromosome in the presence of AHLs [145,161]. This reporter was inactive when *Salmonella* transited the gastrointestinal tract of an individual guinea pig, rabbit, and cow as well as several mice and chickens, indicating an absence of AHLs or a concentration below its detection threshold in these animals [143-145]. However, the reporter strain does indicate SdiA activity when mice are concurrently infected with *Yersinia enterocolitica*, an organism known to produce AHLs [144]. The reporter strain also indicates activity during transit through the gastrointestinal tract of turtles (likely due to the presence of *Aeromonas hydrophila*, a known AHL producer) [145]. However, the *sdiA* mutant of *Salmonella enterica* serovar Typhimurium has no fitness defect during transit through any of these scenarios even when SdiA is active [144,145]. Thus, it is unclear if these are scenarios in which SdiA is relevant.

Another way to determine the function of SdiA is to identify the genes it regulates. Genetic screens for *sdiA*-regulated fusions have been performed in three genera: *Escherichia*, *Enterobacter*, and *Salmonella* [139,157,158,220]. Each screen tested ~10,000 transposon-based fusions which is roughly 68% coverage of the genome, so currently unknown regulon members may reside within the remaining 32%, or among essential genes. Microarrays and RNA-seq have also been used to identify *sdiA*-regulated genes in *Escherichia* and *Cronobacter* [183,211]. Very few genes have been tested for direct binding by SdiA so their regulons likely include direct and indirect effects (we use *sdiA*

regulon throughout, rather than SdiA regulon, to emphasize this). In *Salmonella*, *sdiA* regulates two loci: the *pefI-srgD-srgA-srgB-rck-srgC* operon (hereafter referred to as the *pefI-srgC* operon) and *srgE* [139,260-262]. The *pefI-srgC* operon is known to be directly regulated by SdiA while *srgE* has not yet been tested [160]. PefI and SrgA are involved in expression of Pef fimbriae through their roles as a transcriptional regulator and in the post-translational maturation of PefA, respectively [235,263,264]. SrgB, a putative lipoprotein, and SrgC, a transcriptional regulator, have yet to be characterized. PefI and/or SrgD are involved in the regulation of flagellar motility, although mutation of *sdiA* has no effect on motility in *Salmonella*, regardless of the presence of AHLs [146,236,237]. Rck mediates invasion of host cells by binding to epidermal growth factor receptor (EGFR) [167,234,239,265-267]. SrgE is an effector protein of unknown function that is injected into host cells using the type three secretion system (T3SS) encoded within *Salmonella* Pathogenicity Island 2 (SPI2) [166]. In *E. coli*, *sdiA* regulates the acid fitness island, flagellar motility, prophage induction, and the virulence regulator, *ler* [155,158,183,204,206,249,250,252]. Ler is reported to be directly regulated by SdiA [155,183,204]. In *Enterobacter cloacae*, mutation of *sdiA* affects a collection of genes encoding hypothetical proteins along with a putative type 6 secretion system, copper transporter (CopA), O-antigen chain length determinant (FepE), and phage integrase [157]. Interestingly, there is no overlap between the SdiA regulons of these three genera. The conservation of SdiA and eavesdropping represents an interesting aspect of evolution. The ligand for SdiA is externally sourced, thus limiting its activity to environments containing AHL synthesizing microbiota at sufficient population density. Despite this, *sdiA* has survived multiple speciation events spanning millions of years

while maintaining completely unique regulons with no clearly related functions [139,157,158,268]. This paradox of simultaneous conservation and diversification across a large time frame remains one of many unsolved mysteries on the nature of SdiA and eavesdropping.

In the last 50,000 years, a serovar of *Salmonella enterica*, Typhi, has emerged and is currently undergoing reductive evolution as its host range becomes restricted to humans [222]. Serovars Typhi and Typhimurium have significant differences in their pathogenic strategy. Serovar Typhimurium invades intestinal epithelial cells, inducing inflammation to eliminate competitors in the lumen [51,52,70]. Serovar Typhi limits intestinal inflammation and replication in the lumen in favor of colonization at systemic sites [269-271]. Additionally, Typhi infections can develop into a chronic carrier state through the formation of biofilms on gallstones and gallbladder epithelium [63,272]. This change in host range and pathogenesis could impart selective pressure on the response of serovar Typhi to foreign AHLs, yet the *sdiA* regulon of Typhi has not been investigated.

We sought to identify the regulons of *sdiA* more thoroughly and determine what effect, if any, the reduction in host range has had on serovar Typhi's transcriptional responses to foreign AHLs. Using RNA-seq, we measured the *sdiA*-dependent response of serovars Typhimurium and Typhi to AHLs. Differentially regulated genes were validated via the construction and testing of transcriptional fusions, revealing regulons comprising six loci in Typhimurium and five in Typhi with four common to both. Other genes were identified that respond to plasmid-based expression of *sdiA*, but these could not be validated using *sdiA* expressed from its native position in the chromosome. These

may be artifactual or require additional unknown stimuli for expression. Additionally, we constructed fusions to orthologs of genes in *Salmonella, E. coli,* and *E. cloacae* that were known to be regulated by *sdiA* in one genus but not the others. No new regulon members in *Salmonella* were discovered by this approach, but one new regulon member was found in *E. coli* and three new regulon members were found in *E. cloacae*.

3.4 Results

3.4.1 Identification of SdiA-regulated loci in Typhimurium and Typhi

A genetic screen previously revealed seven members of the *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) SdiA regulon encoded in two loci: the *srgE* gene located in the chromosome and the *pefI-srgD-srgA-srgB-rck-srgC* (*pefI-srgC*) operon located on the virulence plasmid pSLT [138,139]. The *sdiA* regulon of *Salmonella enterica* serovar Typhi (*S.* Typhi) has never been investigated. To identify additional regulon members, we performed RNA-seq. Unfortunately, *sdiA*-dependent regulation is weak in broth culture and higher in motility agar due to its direct regulation by alternative sigma factor FliA [146,195]. Other regulators of the *sdiA* promoter have been described, including Crp and LeuO [273]. RNA for downstream analysis of *sdiA*-dependent changes would preferably be sourced from bacteria grown in motility agar but we have not been able to isolate quality RNA from bacteria grown in this manner. Therefore, we isolated RNA from wild-type and *sdiA* mutant bacteria grown in liquid culture containing AHL (oxoC8 is the optimal AHL for SdiA activation [138] and was the sole AHL used in this study). In a second experiment, RNA was isolated from *sdiA* mutant strains containing *sdiA* under the

control of an arabinose-inducible promoter on plasmid pBAD18, compared to a vector control, also in liquid culture containing AHL (Fig. 2). After processing, sequencing, and analysis, we observed a small number of differentially expressed genes in both serovars when expressing *sdiA* from its native position in the chromosome (Fig. 2C and E). Plasmid-based expression of *sdiA* increased the number of differentially regulated genes (Fig. 2D and F). Results from all four RNA-seq experiments can be found in Tables 4-7.

To validate the RNA-seq results, we constructed transcriptional fusions to each differentially regulated gene (defined as fold change greater than 4 and p-value ≤ 0.05). Each putative *sdiA* regulated promoter was cloned upstream of the *luxCDABE* operon of plasmid pSB401 or had already been constructed in previous studies (see table 2) [274]. In some cases, multiple regions were cloned for a single locus as it was not always clear where the promoter might be. Additionally, if a gene was differentially regulated in one serovar but an ortholog is present in the other serovar, reporters were constructed for both serovars. This turned out to be a wise choice as some regulon members were identified in this manner.

For *S*. Typhimurium, we tested approximately 60 reporters representing 50 loci. For *S*. Typhi, we tested 15 reporters representing 15 loci. Each reporter was placed into wild-type and *sdiA* mutant strains of the relevant serovar. Some were also placed into strains with plasmid-encoded *sdiA* (*sdiA* mutant strains containing *sdiA* under the control of an arabinose-inducible promoter on plasmid pBAD18). Luciferase activity of these strains was measured over time in the presence or absence of 1 µM AHL. Some fusions were regulated by plasmid-encoded but not chromosome-encoded *sdiA* (Fig. 8) while

others were regulated under neither condition (Fig. 9). Here, genes were only 'confirmed' as *sdiA*-regulated if they respond to *sdiA* expressed from its native position in the chromosome. In total, four loci are regulated by *sdiA* in both serovars, two are exclusively regulated by *sdiA* in *S.* Typhimurium (totaling six), and one is exclusively regulated by *sdiA* in *S.* Typhi (totaling five). Genes of limited characterization were renamed to *srg* (*sdiA*-regulated gene) and are described below. For each reporter, we calculated the maximum fold activation (wild-type vs *sdiA* mutant) in both motility agar and broth with or without AHL. In motility agar, raw luciferase values are shown while broth culture luciferase readings were normalized to growth (OD600) at that time point (neither serovar's *sdiA* mutant exhibits growth or motility defects). These values are shown in Table 1.

3.4.2 SrgF, SrgGH, SrgKJ, and MenFDHBCE are regulated by SdiA in both Typhimurium and Typhi

The first newly identified gene, *srgF* (*STM14_3820* in Typhimurium and *T06040* in Typhi), was identified in the RNA-seq results as upregulated by plasmid-based expression of *sdiA* in both serovars, but not in either RNA-seq experiment using endogenous expression of *sdiA*. Reporter constructs of the *srgF* promoter were generated for each serovar and placed into wild-type and *sdiA* mutant strains. These strains were grown in the presence or absence of AHL in LB broth or motility agar and luciferase activity was recorded over time. AHL increased the activity of the reporter in a *sdiA*-dependent manner in both media, with higher activation in motility agar (Fig. 3A). The *srgF* reporter of *S.* Typhi behaved similarly (Fig. 3B). As observed with other regulon

members, there is a small amount of *sdiA*-dependent but AHL-independent regulation. SrgF is annotated as a putative ATP-dependent RNA helicase-like protein in serovar Typhimurium. We examined SrgF using bioinformatic tools HHPred and FoldSeek, which found no similarity to previously identified protein domains [223,224]. Phobius identified a possible transmembrane domain in the first 30 residues and cytoplasmic orientation of the remaining protein [275]. Literature searches for SrgF revealed occasional hits in genetic screens involving colonization of chickens [226], motility [227,228], aquatic survival [229] and phage infection [230]. We have previously observed no effect of AHL or *sdiA* on motility [146]. We tested a *srgF* mutant of *S.* Typhimurium for fitness in mice rendered susceptible to gastroenteritis by a high fat diet [57] and found the mutant to have little or no phenotype (Fig. 10A). Interestingly, transcriptomic studies indicate a significant amount of basal expression of *srgF* during in vitro growth, especially compared to other regulon members [225,246]. The role of this gene in *Salmonella* requires additional study.

The second locus, which we refer to as *srgGH (STM14_4893-4894)*, was identified by RNA-seq of *Typhimurium* expressing *sdiA* from a plasmid. The P_{srgGH} reporter (pAMS145) exhibits both *sdiA* and AHL-dependent activation in motility agar and LB (Fig. 3C). Although not identified in either *S.* Typhi RNA-seq experiment, a promoter fusion of the *S.* Typhi *srgGH* orthologs (pAMS265) is also regulated (Fig. 3D). The *srgG* and *srgH* genes appear to be remnants of functional genes present in other bacterial species. SrgG encodes a 55 amino acid fragment of the N-terminus of a putative citrate transporter in *Salmonella bongori* (SBG_RS18665) and *Enterobacter cloacae* (this

gene is *sdiA*-regulated, see below). SrgH is a 44 amino acid fragment homologous to the C-terminal domain of *ushB (cdh* in *E. coli*). UshB is non-functional in *Salmonella* [276] and no published literature on either SrgG or SrgH was found. A mutant of *S.* Typhimurium lacking *srgH* has no fitness defect during gastrointestinal infection of mice (Fig. 10A).

SrgKJ (*ybbKJ* in Typhimurium and *T2359-2360* in Typhi) was identified in three RNA-seq experiments and both reporters (pJLD202 and pAMS050) exhibit *sdiA* and AHL-dependent regulation (Fig. 3E and F). Previous characterization of *E. coli* orthologs *qmcA-ybbJ* indicates that QmcA is likely involved in protein turnover and YbbJ acts as a helper protein [231]. The protein target(s) of QmcA and its orthologs are unknown. Like *srgF, srgK* and *sdiA* were implicated in phage resistance in a recent Tn-seq study [230].

Finally, we identified the *menFDHBCE* operon. This locus was only identified by RNA-seq using plasmid-based expression of *sdiA* in *S.* Typhi but is encoded in both serovars. Reporters pAMS291 and pAMS202 show weak regulation by *sdiA* in both serovars (Fig. 3G and H). *E. coli* and *Salmonella* encode two isochorismate synthases, which make isochorismate for synthesis of both menaquinone (*menF*) and enterobactin (*entC*) [232,233,277]. Menaquinones have a role in respiration induced by anaerobic conditions while enterobactin is a siderophore used to acquire iron from the environment [233]. It is unclear what role menaquinones may play in SdiA-mediated eavesdropping. We observed no *sdiA* or AHL-dependent regulation of *entC* or any effect of iron availability on SdiA activity (data not shown).

3.4.3 Serovar specific regulon members

A past genetic screen for *sdiA* regulated genes in Typhimurium yielded two loci: *srgE* and *pefI-srgC* [138,139]. As previously published, the reporters for these loci (pJNS25 and pBA428) are regulated by *sdiA* and AHL (Fig. 3I and K) [138,146]. *S.* Typhi does not harbor the virulence plasmid that encodes *pefI-srgC* but does encode *srgDAB* orthologs (*T4538-4540*) in the chromosome as well as a *srgE* ortholog [166,278]. Neither constructed fusion exhibited regulation by *sdiA* (Fig. 3J and L). These two loci have been previously examined in a third serovar, Enteritidis [160,166]. The virulence plasmid of *S.* Enteritidis has lost *sdiA*-dependent regulation of its *pefI-srgC* operon and does not encode *srgE* [160,166]. In the context of these three serovars, *sdiA*-dependent regulation of the *pefI-srgC* operon and *srgE* is an exclusive trait of *S.* Typhimurium despite the significant host range overlap of *S.* Typhimurium and *S.* Enteritidis. A competitive infection between wild-type *S.* Typhimurium and a *srgE* mutant revealed no fitness defects in a mouse model of gastrointestinal infection (Fig. 10A).

A locus encoding T0351-0350 (*srgIL*), orthologous to *yfgHI* in *E. coli*, respectively, was found by RNA-seq to be upregulated by plasmid-based expression of *sdiA* in *S.* Typhi. Orthologs are not found in Typhimurium (Fig. 3M). The reporter for *srgIL*, pAMS201, is strongly regulated by *sdiA* and AHL (Fig. 3N). The first gene of the operon, *T0351*, is annotated as a pseudogene. However, an alternative reading frame can be found within this pseudogene that produces a SlyB-like lipoprotein, the same protein family as YfgH. The original annotation may be incorrect but the expression of the SrgI protein was not confirmed here. In *E. coli*, *yfgH* is predicted to be involved in outer

membrane integrity [279] while *yfgI* mutants have been shown to be susceptible to DNA damage [247]. The hypothesis that SdiA could mediate resistance to DNA damage was assessed for both serovars using two stressors: nalidixic acid and ultraviolet light (UV). Inhibitory concentrations of nalidixic acid were quantified for wild-type, *sdiA* mutant, and plasmid complementation strains grown in AHL or solvent control (Fig. 10B). A difference (<2-fold) was only observed using plasmid-based expression of *sdiA*. Given that *sdiA* had much stronger effects on P_{srgIL} in motility agar, we assessed this putative phenotype using a disk diffusion assay in motility agar, using a two-fold dilution series of nalidixic acid. No differences in zones of inhibition were apparent (Fig. 10D). The results of the nalidixic acid challenge are also consistent with our previous report that *sdiA* has no effect on antibiotic resistance in *S.* Typhimurium [158]. For UV mediated DNA damage, we generated survival curves against increasing doses of UV. Differences between wild-type and *sdiA* mutant strains were never observed in either endogenous or plasmid-based *sdiA* backgrounds (Fig. 10C). Thus, we find no evidence for protection from DNA damage by *sdiA* in either serovar.

3.4.4 Unconfirmed regulon members

It is worth noting that several virulence associated loci were found to be regulated by plasmid-encoded *sdiA* but not under endogenous expression conditions, including promoters of *Salmonella* pathogenicity island 1 (SPI1), flagellar genes, and type 1 fimbriae (Fig. 8). The absence of regulation at the endogenous level could be artifacts from plasmid-based expression of *sdiA*, or true regulon members for which the proper

environmental conditions for *sdiA*-dependent expression have not yet been found. We identified several fusions that were also differentially regulated by plasmid-encoded *sdiA* that are known to be regulated by other extrinsic elements: P_{leuA} (pAMS173) and leucine, P_{proVWX} (pAMS172) and osmotic stress, and P_{dpiBA} (pAMS143) and citrate [280-282]. We manipulated leucine and citrate levels as well as osmolarity of the medium. This did alter activity of the corresponding reporter, but it did not cause *sdiA*-dependent regulation at the endogenous level (data not shown). Based on RNA-seq results, a significant number of prophage genes were repressed by plasmid-based expression of *sdiA* in serovar Typhimurium. These have not yet been tested using fusions and the potential relationship between *sdiA* and prophage elements requires further investigation.

3.4.5 Cross screening SdiA regulon members reveals semi-conservation between species

The published *sdiA* regulon members are different in *E. coli*, *S. enterica*, and *Enterobacter cloacae* [139,157,158]. We hypothesized that at least a portion of the regulons are evolutionarily conserved. To test this, we identified orthologs of each regulon member in species where that ortholog is not known to be regulated by *sdiA*. We then constructed transcriptional fusions to each and tested them for *sdiA*-dependent regulation. For clarity, reporters were tested only in the species from which the promoter was amplified.

In *E. cloacae*, orthologs of four *sdiA*-regulated loci of *Salmonella enterica* were identified: *srgKJ*, *menFDHBCE*, *srgF*, and *srgG*. The *srgG* ortholog, *ENC_00800*, is full

length in *E. cloacae*. One ortholog of the *E. coli sdiA* regulon was identified in *E. cloacae*: *fliE*.

Using constructed luciferase fusions, we find three to be regulated by *sdiA* in *E. cloacae*: *srgKJ*, *srgG*, and *menFDHBCE* (Fig. 4A). Regulation of $P_{menFDHBCE}$ is strongly *sdiA* regulated but fully AHL-independent, a trait observed in some other *E. cloacae* regulon members [157]. This adds three members to the *sdiA* regulon of *E. cloacae*, all of which are the first regulon members conserved between *Salmonella* and *E. cloacae*.

In *E. coli*, orthologs of three *sdiA* regulated loci from *Salmonella enterica* were identified: *qmcA(ybbK)-ybbJ*, *menFDHBCE*, and *yfgHI* (Fig. 5B). One ortholog of the *E. cloacae sdiA* regulon was identified in *E. coli*: *fepE*. We were unable to construct a transcriptional reporter for *copA* of *E. coli*. One of the four constructed fusions is regulated by *sdiA*: *fepE*, and this occurs in an AHL-independent manner (Fig. 4B). The *fepE* gene is the only *sdiA* regulon member conserved between *E. coli* and *E. cloacae*.

In *S.* Typhimurium, two orthologs of *sdiA*-regulated loci from *E. cloacae* were identified: *fepE* and *copA*. Neither were regulated by *sdiA* or AHL in either broth or motility agar (Fig. 4C, motility agar not shown). A *fliE* reporter was not tested based on the negative results of other flagella reporters (Fig. 8). The *copA* gene is regulated by CueR, whose gene is adjacent and inversely oriented [283]. We tested both orientations of the reporter (i.e. measuring *copA* or *cueR* transcription) in broth and motility agar, with and without copper at stress inducing concentrations, none of which led to AHL- or *sdiA*-dependent regulation (data not shown). The regulons of these three species as currently understood are summarized in Figure 6.

3.4.5 SdiA affects pSLT conjugation efficiency independent of AHL

A subset of *Salmonella* serovars, including Typhimurium but not Typhi, harbor IncF plasmids that range in size from 50-90 kb [284]. The 90 kb plasmid of serovar Typhimurium, pSLT, is self-transmissible [285-287]. The RNA-seq dataset from plasmid-based expression of *sdiA* showed an upregulation of pSLT conjugation genes. To determine whether or not there was an effect on transmission frequency we used a conjugation assay [285]. Wild-type or *sdiA* mutant donor strains with a *spv*::Mu*dJ* mutation (kan^r) on their pSLT plasmid were mated with a recipient strain lacking pSLT (BA770, nal^r) in the presence or absence of AHL. The frequency of kan^r nal^r transconjugants obtained per donor was 3- to 6-fold lower in the wild-type compared to *sdiA* mutant, suggesting that *sdiA* represses conjugation (Fig. 5B). Expression of *sdiA* from a plasmid increases conjugation frequency, though this only occurred in the absence of the inducer (arabinose) (Fig. 6A). When arabinose was provided to induce *sdiA* expression, *sdiA* no longer had any effect on conjugation efficiency (Fig. 5A). AHL had no significant effect on conjugation efficiency in either strain background.

Since plasmid-based expression of *sdiA* gave different effects on conjugation frequency than expression of *sdiA* from its native position in the chromosome, we complemented the chromosomal *sdiA* mutation with a functional copy of *sdiA* inserted at a neutral location in the chromosome located downstream of *pagC* (strain AMS203, Fig. 5D) [288]. To confirm that this strain restored *sdiA* activity, we measured luciferase activity from the P_{srgE} reporter plasmid pJNS25 and observed complementation of *sdiA* function

(Fig. 5C). Conjugation efficiency is restored to wild-type levels by this method of complementation (Fig. 5B). We conclude from these findings that *sdiA* has a small negative effect on the frequency of pSLT transmission and AHL do not alter this phenotype. The mechanism(s) by which *sdiA* regulates plasmid transmission and reasons for the confounding effects of plasmid-based expression and arabinose are unclear at this point. It should be noted that the recipient, BA770, encodes *sdiA*. We did not determine if *sdiA* can affect conjugation efficiency as a recipient but a previous study suggests that *sdiA* can repress plasmid transmission between a donor *Pseudomonas aeruginosa* and recipient *E. coli* [289].

3.5 Discussion

Quorum sensing is a strategy used by bacteria to coordinate behavior within a species upon reaching a population density threshold. Bacteria have evolved to link a diverse array of behaviors to population density, including competence, virulence, biofilm formation, bioluminescence, and phage defense [7,11,16,290-292]. A subset of Enterobacteriaceae, including model organisms like *Salmonella* and *E. coli*, have lost their signal synthase to facilitate an alternative behavior: eavesdropping [29]. The LuxR solo SdiA detects foreign AHL, preferably with acyl chain lengths of 6 or 8 and a ketone modification on the third carbon (oxoC6, oxoC8) [146]. Experimentally, SdiA has been shown to detect the AHL produced by a wide range of genera, including *Agrobacterium*, *Aeromonas*, *Hafnia*, *Pantoeae*, *Pectobacterium*, and *Yersinia* [143-146]. However, *sdiA* mutants have almost no reported in vivo defects (even when AHL are present), leaving it

unclear in which scenarios *sdiA*-mediated eavesdropping is relevant. Additionally, the body of literature on phenotypes is complex and ultimately inconclusive on what exactly these bacteria do differently when they detect foreign AHL.

The *sdiA* regulon of *Salmonella enterica* has only been studied in one serovar, Typhimurium, and only using a genetic screen that was 68% saturated [139]. That study identified two *sdiA*-regulated loci: the *pefI-srgC* operon and *srgE* [138,139]. Here we investigated the *sdiA*-dependent transcriptional responses of this same organism using RNA-seq, allowing for full coverage of the genome. An identical experiment was performed using serovar Typhi, representing an interesting contrast as a host-adapted serovar [270]. *Salmonella* SdiA is most active in motility agar (because FliA directly regulates the *sdiA* promoter) [146,195]. As we have been unable to isolate quality RNA in semi-solid media, we instead collected RNA from wild-type and *sdiA* mutants grown in LB. Additionally, RNA was collected from strains expressing *sdiA* on a plasmid, substantially increasing its activity and the number of differentially expressed genes. Over 200 potential members of the *Salmonella sdiA* regulon in serovars Typhimurium and Typhi were found, mostly from plasmid-based expression of *sdiA* in serovar Typhimurium. Increasing the copy number of *sdiA* on a plasmid is a commonly used approach as it bypasses the need for AHL entirely [138]. This is also quite risky given the propensity for phenotypes that occur in plasmid-based expression backgrounds to disappear under endogenous expression conditions (e.g., multiple drug resistance and mini-cell formation) [158,208,293]. Using transcriptional fusions, we tested almost all of the genes identified using plasmid-based expression of *sdiA*. Most were confirmed to be

regulated by *sdiA* expressed from a plasmid but not by *sdiA* expressed from its native position in the chromosome (Fig. 8). However, in this study, we considered a gene to be a verified member of the *sdiA* regulon only if the gene has been confirmed to be regulated by *sdiA* expressed from its native position in the chromosome. This greatly limits the size of the regulon and is likely excluding real members. It is probable that our in vitro growth conditions are not permissive for expression of some of the regulon members. Thus, the list of genes that respond to plasmid-based *sdiA* should not be dismissed entirely. It should also be noted that we have not yet determined which genes are directly regulated by SdiA and which are indirect (which is why we refer to the *sdiA* regulon rather than the SdiA regulon). Thus, our current understanding of the regulons of these different species includes the direct and indirect effects.

In serovar Typhimurium, the regulon includes four new loci (six total). In serovar Typhi, five loci are *sdiA* regulated (all newly discovered here), four of which are shared with serovar Typhimurium (Fig. 4). The first two regulon members identified in Typhimurium, *pefI-srgC* and *srgE*, were not regulated in serovar Typhi [139]. Another broad host range non-typhoidal serovar, Enteritidis, has lost the SdiA-specific promoter of its *pefI-srgC* operon and does not encode *srgE* at all [160,166]. Host-range reduction alone therefore may not be sufficient to explain this change in regulon membership. In addition, *sdiA* regulates transmission of the virulence plasmid, pSLT, independently of AHL. Transmission is known to be regulated by multiple factors, including nutrient availability, osmolarity, and microaerophilic conditions and occurs both in vitro and in vivo [285,287,294]. SrgIL is the single Typhi-specific regulon member found in this study. The four

conserved regulon members include an ATP-dependent RNA helicase-like protein (SrgF), two proteins likely involved in protein turnover (SrgKJ), truncated versions of a CDP-diacylglycerol pyrophosphatase and citrate transporter (SrgGH), and the menaquinone biosynthesis operon (*menFDHBCE*). The evolutionary maintenance of *sdiA*-dependent regulation at four loci suggests a common response to an AHL-laden environment.

Some *sdiA* regulon members have orthologs in other *sdiA*$^+$ genera, but these have not been tested specifically for *sdiA*-dependent regulation in those genera. Therefore, we constructed transcriptional fusions to genes hypothesized to be regulated by *sdiA*, based on *sdiA*-dependent regulation in other genera. This led to three newly identified *sdiA* regulated loci in *E. cloacae* (citrate transporter *ENC_00800*, *srgKJ*, *menFDHBCE*), one in *E. coli* (*fepE*), and none in *Salmonella* (Fig. 5). This is the first reported instance of inter-genus conservation of the *sdiA* regulon. We have speculated on the existence of a "core regulon" common to all SdiA-mediated eavesdroppers that could link these apparently disparate responses to AHL together. While we were successful in identifying loci conserved between two genera, no locus was identified that was conserved among all three.

Understanding the purpose of SdiA-mediated eavesdropping is hampered by the absence of in vivo and in vitro phenotypes. We hypothesized that one or both may be deduced from the *sdiA* regulon: applying known roles or functions of regulated genes to SdiA and elucidating environments from there. Although we were able to find new regulon members, there is very little known about them. SrgKJ, likely involved in protein

turnover based on the activity of *E. coli* orthologs *qmcA-ybbJ*, has no reported defects or targets [231]. The menaquinone biosynthesis operon (*menFDHBCE*) is known to be activated in anaerobic conditions, but we have no hypothesis as to its relationship to *sdiA*-mediated eavesdropping [233,277]. SrgF, a putative ATP-dependent RNA helicase-like protein, has not been characterized, but has been hit in several genetic screens [226-230]. Those genetic screens suggested roles in colonization, motility, aquatic survival, and phage defense [226-230]. We tested mutants lacking *srgF* or two other *sdiA* regulon members in serovar Typhimurium (*srgE, srgH*) for colonization defects in a mouse gastroenteritis model and observed no fitness defects (Fig. 9). Transcriptional fusions of *flhDC*, *fliA*, and *fliC* promoters had no *sdiA* or AHL-dependent differential expression in serovar Typhimurium (Fig. 8), and *sdiA* mutants of Typhimurium [146] and Typhi (data not shown) have no motility defects. Therefore, while *sdiA* is regulated by FliA, *sdiA* does not regulate motility in *Salmonella*. In *E. coli*, mutants of *yfgI* (orthologous to *srgL*) are reported to have a DNA repair defect. We tested both Typhi and Typhimurium for *sdiA*-dependent changes in sensitivity to DNA damage caused by either nalidixic acid or UV (Fig. 10) [247]. No significant differences were found.

One explanation for the absence of colonization defects of *sdiA* and regulon mutants could be the lack of AHL in the mouse gut. Our lab has previously found that SdiA is not active in the mouse gut and *sdiA* mutants have no fitness defect [144,145]. AHLs can be introduced into the gastrointestinal tract by co-infection with an AHL-producing pathogen (*Yersinia enterocolitica*) [144]. Although *Salmonella* can detect those AHLs, *sdiA* mutants still have no fitness defects. An interesting effect is observed when wild-type and

sdiA mutant *Salmonella* are co-infected in a genetic background encoding *yenI* from *Y. enterocolitica*, enabling *Salmonella* to produce AHL without the need for another bacteria. The *sdiA* mutant is attenuated in the gut during the infection and it is the largest observed phenotype of *sdiA* to our knowledge (>100-fold) [144]. The differences in fitness phenotypes in an infection from foreign AHLs versus those endogenously produced may be due to *Yersinia* specific factors (e.g. limited co-localization with *Salmonella*, anti-phagocytic activity) [165]. Additional studies are needed to determine if AHLs are relevant to *Salmonella* in the gastrointestinal tract.

The study of SdiA-mediated eavesdropping has proven to be challenging. Numerous studies relating to in vivo and in vitro phenotypes have been performed with no clear answer as to the role of SdiA in the lifecycle of these bacteria. In terms of relevant environments, mammals and livestock are the most studied [144,145,181-183]. Very few studies have been performed in insects [190] and plants [143,197], which are colonized by both *sdiA*$^+$ genera and AHL-producers (including *Erwinia* and *Pantoea*) [33,191-194,198,199,295]. Experiments in our lab indicate that serovar Typhimurium SdiA is active within house flies but elucidating the fitness of the *sdiA* mutant requires additional study (unpublished data). We have also tested for *sdiA*-mediated gene regulation or fitness phenotypes in plants and rhizomes with no activity observed so far (unpublished).

Most *sdiA*-regulated genes are uncharacterized. We interpret this to mean that SdiA-mediated eavesdropping is part of a relatively unexplored aspect of these organisms' lifestyle. There may be an interesting connection in *Enterobacter*, *E. coli*, and *Salmonella*: phage infection. Previous studies found *sdiA*-dependent regulation of a

phage integrase in *E. cloacae* as well as prophage induction in *E. coli* [157,252]. In this study, we found several pieces of circumstantial evidence linking *Salmonella sdiA* to phage biology. First, expressing *sdiA* from a plasmid in serovar Typhimurium induces the downregulation of dozens of prophage genes (Supplemental Table 4). Second, we found that *sdiA* represses transmission of the virulence plasmid pSLT, whose pilus is a likely phage target [296]. We also found *sdiA*-dependent regulation of O-antigen chain length determinant *fepE* in both *E. coli* and *E. cloacae* (but not *Salmonella*), which could potentially impact phage attachment. Finally, a transposon screen identified differential fitness of mutants during infection against certain phages: including *srgB*, *srgF*, *srgG*, and *sdiA* [230]. Quorum sensing phage interactions have been previously reported in both directions (host regulation of phage defense and phage regulation of lysis-lysogeny decision making using quorum sensing receptors [15,16,292]). Further study is needed to determine if *sdiA* plays a role in phage biology.

3.6 Methods

3.6.1 Bacterial strains and media

Bacteria were grown in Lysogeny Broth (LB) or on LB agar (1.5% w/v) unless otherwise stated. For motility experiments, agar was used at a final concentration of 0.25% w/v. Antibiotics were used at the following final concentrations: tetracycline (tet) at 10 µg/mL, kanamycin (kan) at 50 µg/mL, chloramphenicol (cam) at 30 µg/mL, ampicillin (amp) at 100 µg/mL, nalidixic acid (nal) at 50 µg/mL. Arabinose (ara) was

used at a final concentration of 0.2%. N-(3-Oxooctanoyl)-DL-homoserine lactone (oxoC8) was obtained from Sigma Aldrich (Cat# O1639) and dissolved in ethyl acetate (EA) acidified with glacial acetic acid at a concentration of 0.1 mL per Liter [297]. OxoC8 was used at a final concentration of 1 μM and acidified EA at 0.1% v/v. Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetra-acetic acid (EGTA) was used at a final concentration of 10 mM. Anhydrotetracycline (AHT) was used at a final concentration of 5 μg/mL. Evan's Blue Uranine (EBU) plates were made by adding tryptone (10 g/L), yeast extract (5 g/L), NaCl (5 g/L), glucose (2.5 g/L), and agar (15 g/L) to water, autoclaving, cooling to roughly 50°C, and then adding K_2HPO_4 (40 mL/liter of 12.5% w/v), Evans Blue (1.25 mL/L of 1% w/v), Uranine (also known as sodium fluorescein, 2.5 mL/L of 1% w/v) [298].

3.6.2 Strain and plasmid construction

Strains and plasmids used in this study are listed in Supplemental Table 1. Primers used in this study are listed in Supplemental Table 2. New mutations were constructed as described below. Other strains were created by moving existing mutations into new strain backgrounds via P22 phage transduction. For P22 transductions, phage lysates were first grown on strains encoding the desired mutation. Recipient strains were then infected with the phage lysate for 25 minutes. The infection was halted by addition of LB + EGTA and outgrown for 1-3 hours before plating on selective media. Isolates were sub-cultured twice on selective media with EGTA, then cross-struck on EBU to confirm a lack of P22 pseudolysogeny and no P22 resistance mutations. The specific

donor and recipients for each strain are described in Table S1. Plasmids were constructed as described below and moved into strains via electroporation [299].

3.6.3 Chromosomal complementation strain AMS203

For chromosomal complementation, we inserted *sdiA* and its surrounding intergenic sequences between *pagC* and *STM14_1503*, which has been previously identified as a neutral insertion site [288]. This position is depicted in Figure 4D. Insertion of *sdiA* into the *pagC-STM14_1503* intergenic region was engineered by allelic exchange with suicide vector pFOK [300]. The construct was assembled using Gibson assembly of four fragments: vector, the upstream region of *pagC* homology, the *sdiA* gene, and the downstream region of *STM14_1503* homology [301]. The vector was linearized by PCR with primers BA3875 and BA3876. The upstream homology fragment was constructed by PCR with primers BA3883 and BA3884, which bind upstream of *STM14_1499* and downstream of *pagC*, respectively. The *sdiA* fragment was construct by PCR with primers BA3885 and BA3886, which bind immediately downstream of *yecC* and immediately downstream of *yecF*, respectively. The downstream homology fragment was constructed by PCR with primers BA3887 and BA3888, which bind downstream of *pagC* and within *pliC*, respectively. Primers include overhangs with homology to their adjacent fragments. PCR was performed with Q5 Polymerase; fragments were purified by gel extraction and quantified by Nanodrop. Gibson assembly was performed per manufacturer's instruction.

Gibson product was transformed into TransforMax EC100D *pir*$^{+}$ *E. coli* by electroporation (Lucigen ECP09500). The resulting plasmid, pAMS150, was moved into mating strain Jke201 by electroporation. Allelic exchange was performed by mating Jke201 + pAMS150 with BA612 on LB agar containing DAP then resuspending colonies and selecting on LB Kan to obtain single crossovers. Isolates were grown without selection and dilution plated on LB + AHT + 10% sucrose to select for a second crossover that eliminates the vector. Individual colonies were screened for loss of kan resistance and the insertion of *sdiA* was confirmed by PCR. The final strain is named AMS203.

3.6.4 Construction of strains AMS001, AMS002, and JLD1221

Mutants of Typhi *sdiA* and Typhimurium *srgE* were created using Wanner mutagenesis [302]. Chloramphenicol and kanamycin cassettes were amplified from pKD3 and pKD4, respectively. Primers BA3454 and BA3455 were used to generate insertions for mutants AMS001 and AMS002. Primers BA1563 and BA1564 were used to generate the insertion for JLD1221. Strains Ty2 and 14028 carrying helper plasmid pKD46 were transformed with gel purified DNA and isolated on selective media as previously described [302]. The helper plasmid was eliminated from the strains by growth at 42°C. Mutations were confirmed by PCR. Strain AMS001 encodes the cam^{r} cassette oriented opposite *sdiA* while AMS002 encodes the kan^{r} cassette oriented with *sdiA*. Strain JLD1221 encodes the cam^{r} cassette oriented opposite *srgE*.

3.6.5 Reporter plasmid construction

Transcriptional reporters of genes of interest were made by subcloning into luciferase reporter plasmid pSB401 [274]. Promoters were amplified with Q5 Polymerase using primers listed in Table S2. Genomic DNA from strains 14028, Ty2, K12, and JLD401 served as the templates. DNA fragments were cloned into TOPO vector pCR2.1 (Invitrogen), and then removed by digestion with EcoRI and gel purified. The vector pSB401 was digested with EcoRI (NEB) and gel purified to remove the fragment encoding *luxR*. The vector and insert were ligated using T4 DNA ligase (NEB) and then transformed into chemically competent *E. coli*. Transformants were screened for insertion and orientation by PCR using forward primers binding the desired promoter and a universal reverse primer binding *luxC* downstream of the EcoRI site (BA1090). For transformation of plasmids into *Salmonella,* plasmids were first passaged through the restriction$^-$ modification$^+$ strain JS198.

The conditional expression plasmid pAMS130, encoding the *sdiA* gene from strain Ty2, was made by restriction cloning. The *sdiA* gene was amplified from the genome with primers BA3601 and BA3602. Vector pBAD33 was digested with SmaI. The digested vector and PCR product was blunt-end ligated using T4 DNA ligase, then transformed into competent cells (Stellar) and grown on selective media. Isolates were screened for insertion and orientation using two primer pairs: BA3601-BA2475 and BA3602-BA2474. Purified plasmid was transformed into strains by electroporation.

3.6.6 RNA-seq and analysis

Overnight cultures of 14028, BA612, Ty2, and AMS001 were grown in LB Broth at 37°C shaking. At a 1:100 dilution, they were sub-cultured in LB oxoC8 then incubated at 37°C shaking until late exponential phase. For plasmid over-expression, cultures of BA612 + pJVR2 and BA612 + pBAD33, AMS002 + pAMS130, and AMS002 + pBAD33 were grown overnight, supplemented with cam then sub-cultured in LB cam ara oxoC8. Three biological replicates were collected per strain. RNA was extracted from cell pellets by affinity purification using the PureLink RNA Mini Kit (Invitrogen #C12183018A) followed by DNase I treatment using TURBO DNA-free Kit (Invitrogen #AM2238). RNA quantity and quality was confirmed by Bioanalyzer. RNA was sent to the OSU Genomics Shared Resources center for cDNA library synthesis and sequencing. Reads were assessed for quality and trimmed with FastQC and Trimmomatic, respectively [303,304]. Reads were mapped to *Salmonella* reference genomes (14028 – accession number CP001363; Ty2 – accession number AE014613) with Bowtie2 [305]. Mapped reads were assembled, quantitated, and assigned to annotations using Stringtie [306]. Differential expression analysis was performed using DESeq2 in R Studio [307]. Results from the differential gene expression analysis provided $\log_2$ fold-changes, p-values, and adjusted p-values for all genes. The adjusted p-value was calculated using the Benjamin-Hochberg method.

3.6.7 Liquid and motility agar assays for lux reporter activity

Cultures of wild-type and *sdiA* mutants harboring reporter plasmids were grown shaking in LB with appropriate antibiotics at 37°C overnight. They were then sub-cultured 1:100 in LB broth or motility agar containing appropriate antibiotics and supplements (e.g. arabinose). For liquid assays, the bacteria were grown in a white plate with clear bottom, reading both OD_{600} and luminescence (Fisher Scientific, Catalog # 265302). Measurements were taken every hour for 20 hours in the SpectraMax i3x at 37°C. Each sample was tested in technical triplicate per run, with three independent runs per strain or condition. For motility agar assays, only luminescence was measured. In both serovar Typhimurium and Typhi, no growth or motility defects were observed in *sdiA* mutants in either oxoC8 or a solvent control (data not shown).

3.6.8 Conjugation assays

Conjugation assays for transmission of virulence plasmid pSLT were performed as previously described [285]. Briefly, donor strains encode a plasmid marker, *spv::*Mu*d*J (kan^r) and the recipient strain BA770 is a spontaneous nalidixic acid resistant mutant (nal^r). Overnight cultures of donor and recipient were washed once in PBS and mated on LB agar (+ supplements) on a 0.45 µm filter disk at a MOI of 0.1. Disks were incubated overnight at 37°C. Filter disks were removed from the plate, resuspended in 3 mL PBS, and dilution plated on LB Kan and LB Kan Nal to enumerate donors and transconjugants,

respectively. Conjugation efficiency was calculated as the ratio of transconjugants to donors.

3.6.9 DNA damage assays

To evaluate resistance to nalidixic acid, strains were grown overnight in LB at 37°C shaking. Cells were washed and diluted 1:100 into growth media (2 µL into 198 µL) in a 96-well plate. Endogenous expression strains were grown in LB + oxoC8 or LB + EA. Plasmid expression strains were grown in LB cam +/- ara + oxoC8 or EA. Nalidixic acid was added into the media in a two-fold dilution series from 50 to 0.15 µg/mL and a no nal control. IC_{50} was calculated using GraphPad software (Prism Version 10) as the relative growth at 20 hours (OD_{600}) compared to a no-antibiotic control and maximum concentration of antibiotic (no growth). Each strain and condition were tested on three separate occasions. For the disk diffusion assay, each strain was grown overnight in LB, washed, and inoculated into motility agar containing 1 µM oxoC8. Disks were inoculated with nalidixic acid in a two-fold dilution series, starting at 250 µg (7 dilutions, 1 control) in a volume of 5 µL per disk. Plates were incubated overnight at 37°C and images were taken in the morning. This was performed on three separate occasions.

To evaluate UV Damage, strains were grown overnight in LB at 37°C shaking. Each strain was drip plated onto LB agar in a ten-fold dilution series. Once dry, plates were placed in a UV Crosslinker (HybriLinker HL-2000 UVP Laboratory Products). Plates were challenged with zero to 150 x 100 µJ/CM^2 in intervals of 25 µJ/CM^2 (6

conditions and 1 control). Plates were then grown overnight at 37°C and quantified. Survival was calculated as the ratio of CFU at dose to CFU at no UV.

3.6.10 Mouse experiments

All mice used in this study were six-to-eight-week-old female CBA/J mice purchased from Jackson Labs. This study used mice maintained on a high-fat diet, which confers susceptibility to inflammation and pathogen expansion in C57BL/6 [57] and CBA/J mice (unpublished data). The high-fat diet was purchased from vendor Research Diets Inc. (Cat#1705i) and provided three days prior to infection. Mice were maintained on the diet throughout the duration of the study. Wild-type and mutant strains were grown overnight in LB, washed in water, and mixed in a 1:1 ratio. Mice were orally gavaged to deliver $1x10^9$ CFU (day 0). Fecal pellets were collected and plated on LB containing selective media to quantify wild-type and mutant on days 1, 3, and 5. On day 7, mice were humanely euthanized, ceca were harvested and plated for CFU on selective media. The fitness of *Salmonella* mutants was compared to that of the wild-type by calculating the ratio of the mutant to the wild-type divided by initial ratio of mutant to wild-type (~1:1). Values below one (or negative $\log_{10}$(CI)) indicate fitness defects in the mutant.

3.6.11 Animal assurance

All animal work was performed using protocols approved by our Institutional Animal Care and Use Committee (IACUC; OSU 2009A0035) and in accordance with the relevant guidelines set forth in the Guide for the Care and Use of Laboratory Animals [308].

3.6.12 Data availability

The RNA-seq data have been deposited in the NCBI Gene Expression Omnibus repository under accession number GSE275322.

3.7 Figures

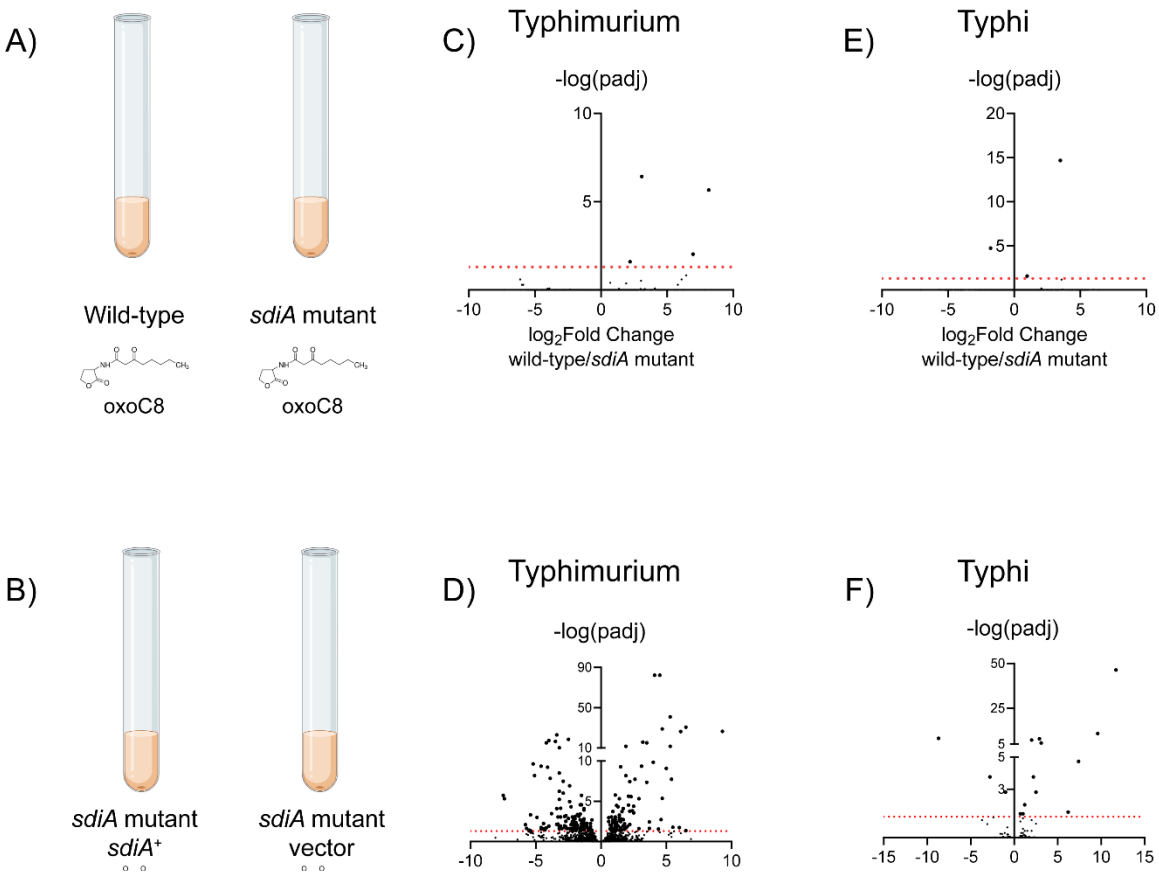


Figure 2. RNA-seq of *Salmonella enterica* serovars Typhimurium and Typhi to identify putative *sdiA*-regulated genes.

A) RNA was isolated from serovar Typhimurium wild-type strain 14028 or *sdiA* mutant BA612, or serovar Typhi strain Ty2 or *sdiA* mutant AMS001. All strains were grown in LB with 1 mM AHL (oxoC8) to late exponential phase.

B) RNA was isolated from serovar Typhimurium strain BA612 + pJVR2 (*sdiA*+),, which is a *sdiA* mutant expressing $sdiA^{Typhimurium}$ from the P*araBAD* promoter, or the vector control strain, BA612 + pBAD33, or from serovar Typhi strain AMS002 + pAMS130 (*sdiA*+), which is a *sdiA* mutant expressing $sdiA^{Typhi}$ from the P*araBAD* promoter, or the vector control strain AMS002 + pBAD33. All strains were grown in LB with 1 mM AHL (oxoC8) and arabinose (0.2%) to late exponential phase.

(C-F) Volcano plots of gene expression differences between wild-type and *sdiA* mutant strains described in panel A (C, E), or between strains described in panel B (D, F). Each dot represents one gene. X-axes are Log_2 fold-change in gene expression (wild-type/*sdiA* mutant or $sdiA^+$/vector control) and Y-axes are $-log_{10}$ of *p* values (padj). Red line indicates p = 0.05. See supplemental tables 3-6 for values of specific genes. Figures 1A and 1B were designed in Biorender.

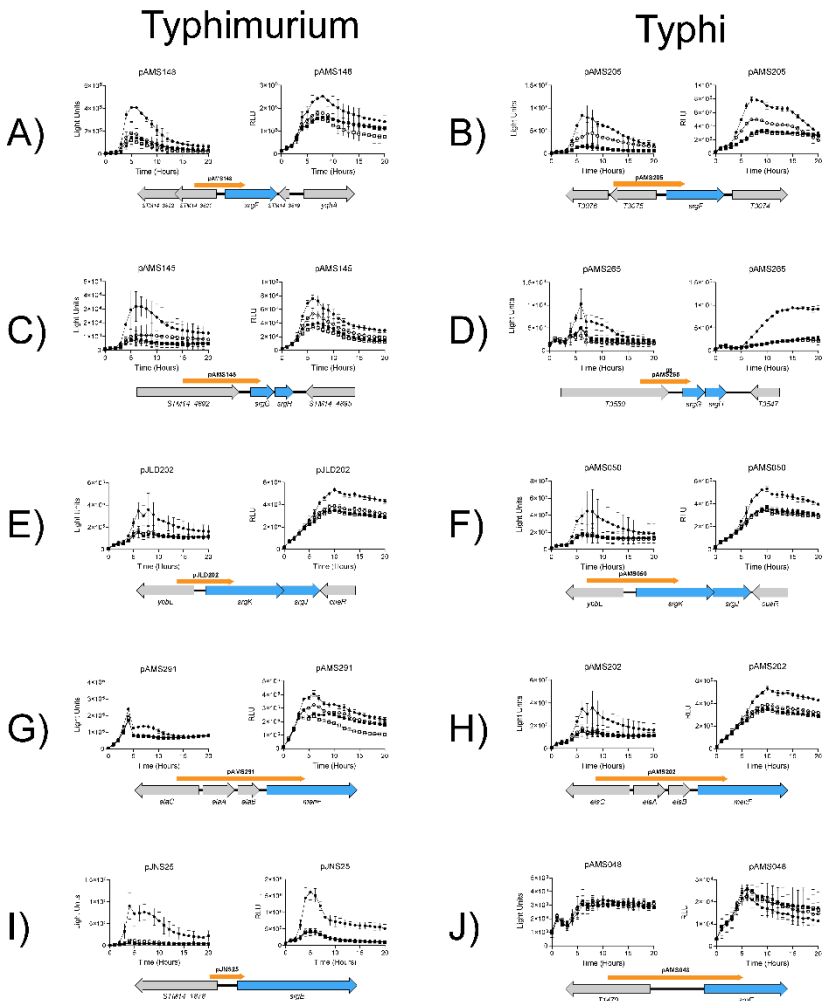


Figure 3. Validation of *sdiA*-regulated genes in serovars Typhimurium and Typhi.

Dependence of luciferase reporters on *sdiA* and AHL. Each reporter was tested in motility agar (left graph) and LB (right graph) for luciferase activity in wild-type (circles) and *sdiA* mutant (squares) backgrounds. Each media was supplemented with either AHL (oxoc8) at 1 μM (closed symbols) or solvent (EA) at 0.1% v/v (open symbols). Diagrams of genes identified using RNA-seq (in blue) and their genomic context (not to scale) are shown under their corresponding luciferase data. The cloned promoter is displayed as an orange arrow. Graphs 2A, C, E, G, I, K, and M show data in *S.* Typhimurium whose wild-type is 14028, *sdiA* mutant is BA612. Graphs 2B, D, F, H, J, L, N show data in *S.* Typhi, whose wild-type is Ty2 and *sdiA* mutant is AMS002. In each graph, X-axes is time (in hours). Y-axes is either raw luciferase activity (motility agar) or luciferase activity normalized to growth (OD_{600}) at the corresponding time point. Each time point represents the mean +/- standard deviation of 9 replicates (3 technical x 3 biological replicates).

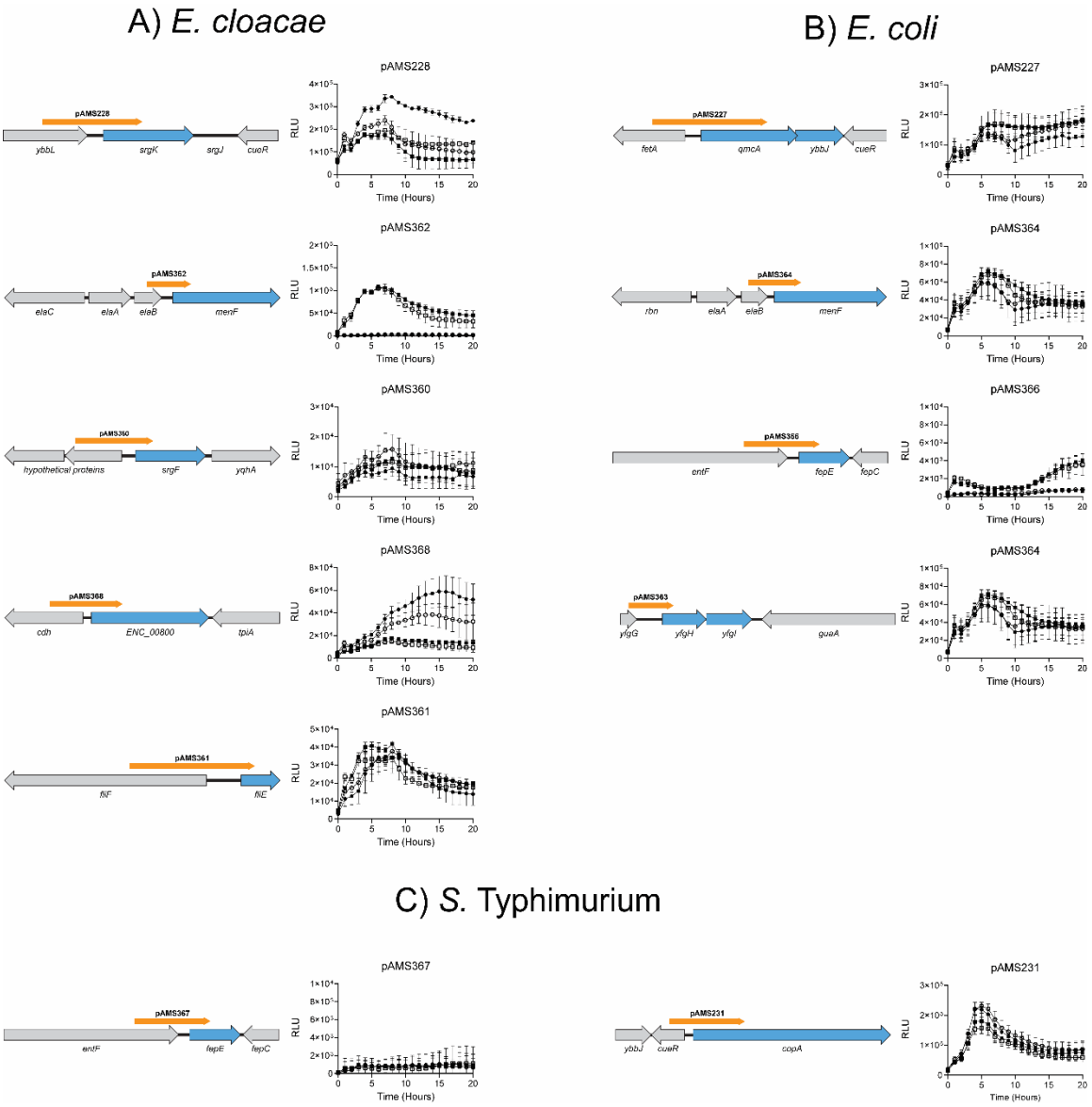


Figure 4. Cross-species validation of *sdiA*-regulated genes in *Salmonella*, *E. coli* and *E. cloacae.*

Dependence of luciferase reporters on *sdiA* and AHL. Each reporter was tested LB (right graph) for luciferase activity in wild-type (circles) and *sdiA* mutant (squares) backgrounds. Each media was supplemented with either AHL (oxoc8) at 1 µM (closed symbols) or solvent (EA) at 0.1% v/v (open symbols). In each graph, X-axes is time (in hours). Y-axes is luciferase activity normalized to growth (OD_{600}) at the corresponding time point. Diagrams of genes of interest (in blue) and their genomic context (not to scale) are shown under their corresponding luciferase data. The cloned promoter is displayed as an orange arrow. Figure 2A indicates promoters and activity in *E. cloacae*, whose wild-type is JLD401 and *sdiA* mutant is ASD401. Figure 2B is *E. coli*, whose wild-type is MG1655 and *sdiA* mutant is JNS21. Figure 2C is *S.* Typhimurium, whose wild-type is 14028 and *sdiA* mutant is BA612. Each time point represents the mean +/- SD of 9 replicates (3 technical x 3 biological replicates).

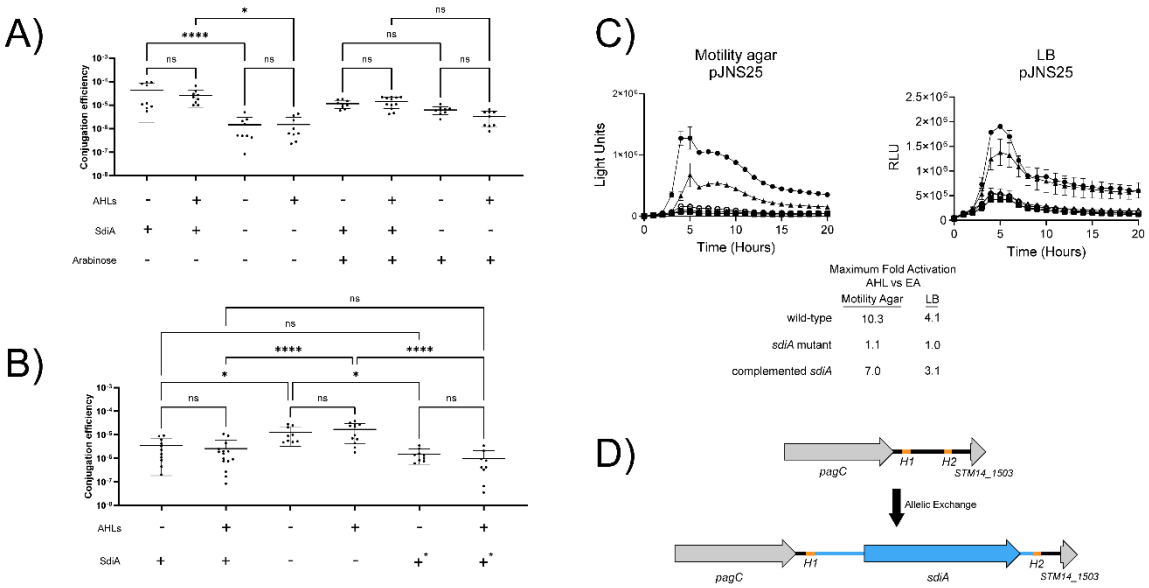


Figure 5. Repression of pSLT self-transmission by *sdiA.*

A, B) Conjugation efficiency (transconjugants per donor) was determined for matings between donor strains carrying pSLT$^{spv::MudJ}$ and recipient strain BA770. Matings were performed overnight on LB agar with indicated supplements: AHL +/- (oxoC8 at 1 mM or EA at 0.1% v/v) and +/- arabinose (0.2% or none) A) SdiA+ uses donor BA612 + pJVR2; SdiA- uses donor BA612 + pBAD33. B) SdiA+ uses donor BA1541; SdiA- uses donor AMS171; SdiA+* donor is AMS246 (complemented *sdiA*).

C) Activity of P*srgE* reporter plasmid in wild-type 14028 (circle), *sdiA* mutant BA612 (square) and complemented *sdiA* mutant AMS203 (triangle) in motility agar or LB, + AHL (closed) or EA (open). Fold-activation of each strain was calculated as the expression in AHL vs solvent. The highest value is listed in the table.

D) Graphical representation of placement of *sdiA* into the intergenic region between *pagC* and *STM14_1502* (see methods for details of construction)

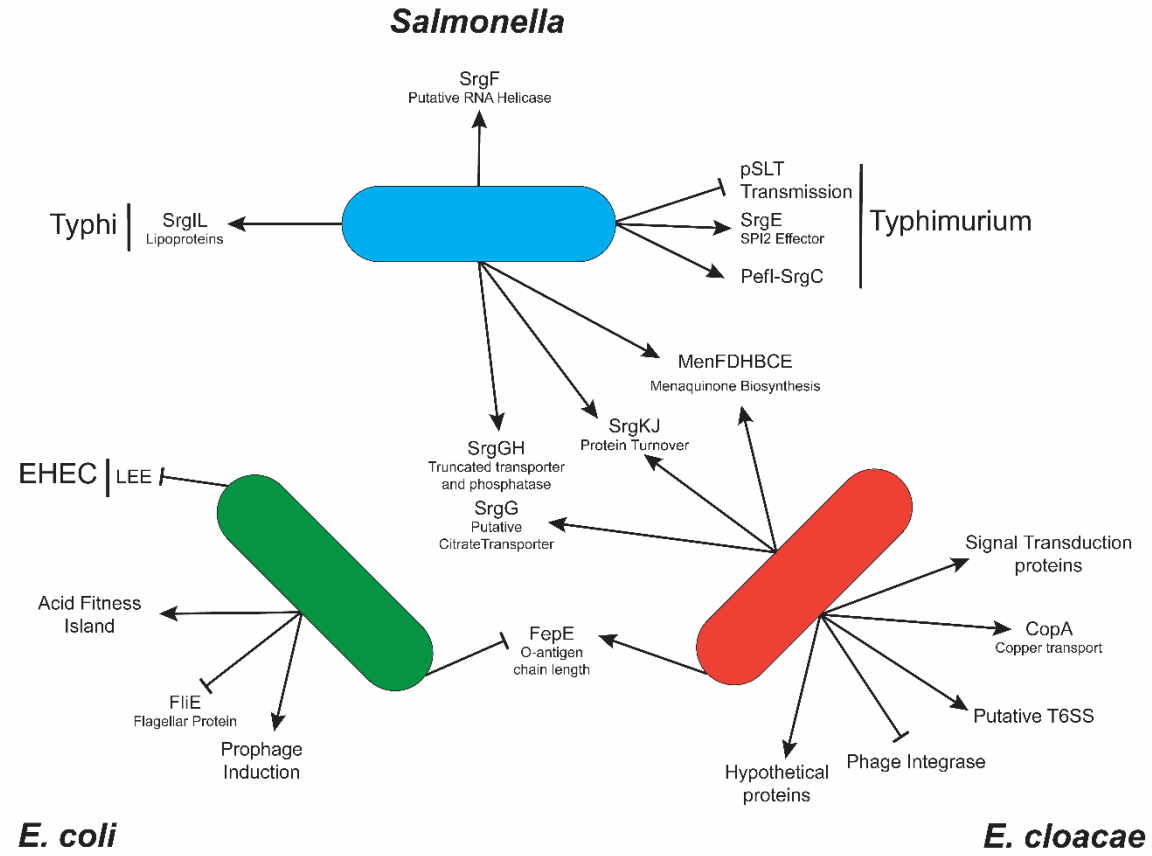


Figure 6. Summary of the *sdiA* regulons

Diagram of known *sdiA* regulons of *Salmonella*, *E. coli*, and *E. cloacae* based on this study and previous literature. Arrows indicate transcriptional activation or increased phenotype. Blunt arrows indicate transcriptional repression or decreased phenotype. Abbreviations: EHEC (Enterohemorrhagic *E. coli*), LEE (Locus of Enterocyte Effacement).

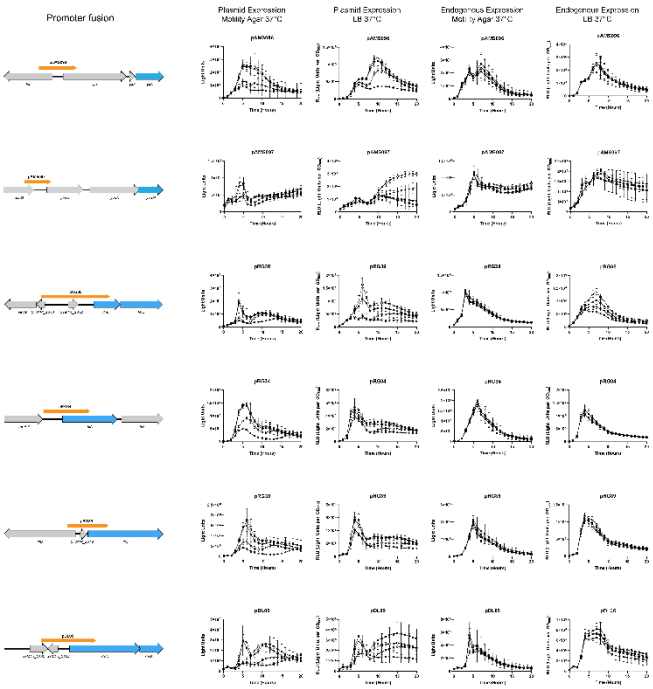


Figure 7. Reporters that respond to plasmid-based expression of *sdiA*, but not *sdiA* expressed from its natural position in the chromosome.

Diagrams of reporter fusion constructs and their expression data for putative *sdiA*-regulated genes in serovar Typhimurium. This figure includes reporter constructs that are regulated by *sdiA* expressed from a plasmid but not from the chromosome. Diagrams show the genomic context of each gene of interest identified by RNA-seq (blue) and the putative promoter region cloned into pSB401 to measure transcriptional activity (orange arrow). Size of each figure is not to scale with length of region represented. Each reporter was tested for *sdiA*-dependent regulation in strain backgrounds expressing sdiA from a plasmid or from the chromosome in motility agar or LB. In motility agar graphs, Y-axes represent raw light units. In LB graphs, Y-axes represent raw light units normalized to growth (OD_{600}) at the corresponding time point. Each time point is mean +/- SD of 9 replicates (3 technical x 3 biological).
For *sdiA* expressed from a plasmid, the strains are: *sdiA*$^+$ – BA612 + pBA321, *sdiA* mutant – BA612 + pBAD18. For sdiA expressed from the chromosome, the strains are: *sdiA*$^+$ – 14028, *sdiA* mutant - BA612 Open circles: *sdiA*$^+$ + solvent (EA). Closed circles: *sdiA*$^+$ + AHL. Open squares: *sdiA* mutant + solvent (EA). Closed squared: *sdiA* mutant + AHL. When using *sdiA* expressed from a plasmid, all media were supplemented with arabinose (0.2%).

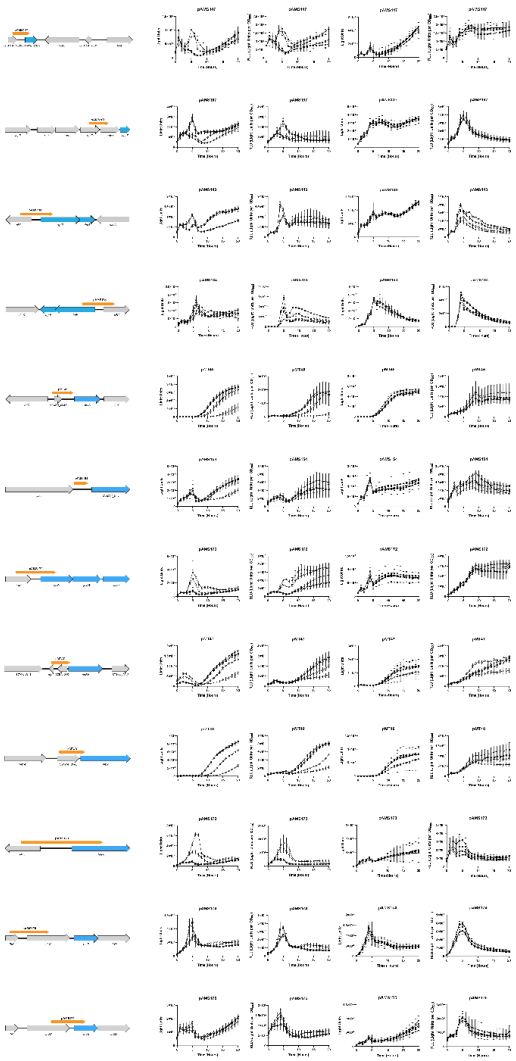


Figure 7 continued

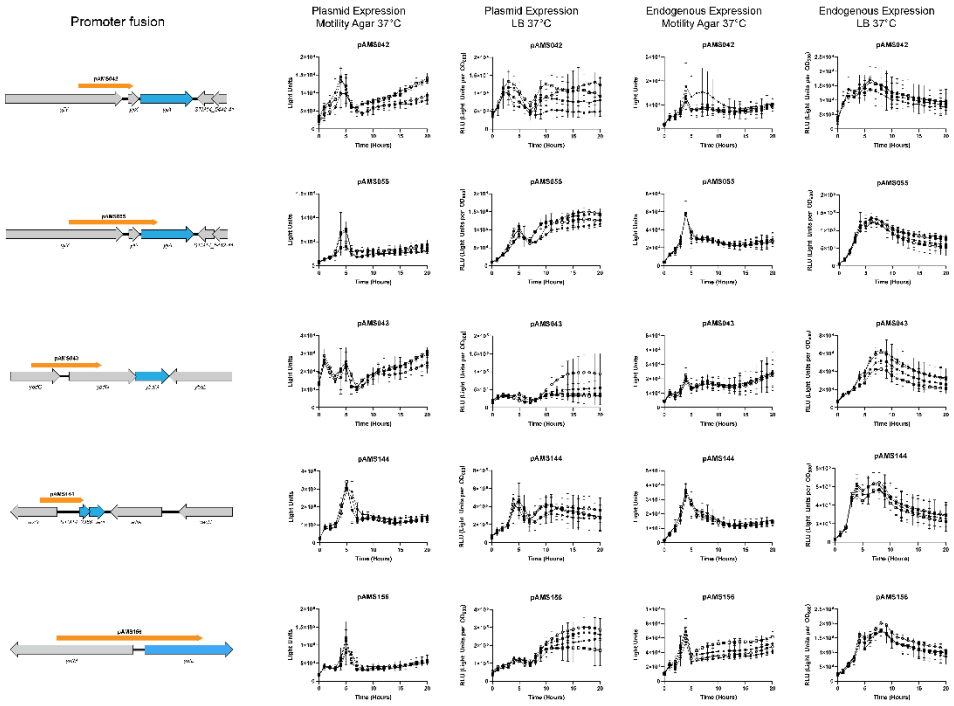


Figure 8. Reporters that do not respond to plasmid-based expression of *sdiA*, or *sdiA* expressed from its natural position in the chromosome.

Diagrams of reporter fusion constructs and their expression data for putative *sdiA*-regulated genes in serovar Typhimurium. This figure includes reporter constructs that are not regulated by *sdiA* under any condition tested to date. Diagrams show the genomic context of each gene of interest identified by RNA-seq (blue) and the putative promoter region cloned into pSB401 to measure transcriptional activity (orange arrow). Size of each figure is not to scale with length of region represented. Each reporter was tested for *sdiA*-dependent regulation in strain backgrounds expressing sdiA from a plasmid or from the chromosome in motility agar or LB. In motility agar graphs, Y-axes represent raw light units. In LB graphs, Y-axes represent raw light units normalized to growth (OD_{600}) at the corresponding time point. Each time point is mean +/- SD of 9 replicates (3 technical x 3 biological).
For *sdiA* expressed from a plasmid, the strains are: *sdiA*$^+$ – BA612 + pBA321, *sdiA* mutant – BA612 + pBAD18. For sdiA expressed from the chromosome, the strains are: *sdiA*$^+$ – 14028, *sdiA* mutant - BA612
Open circles: *sdiA*$^+$ + solvent (EA). Closed circles: *sdiA*$^+$ + AHL. Open squares: *sdiA* mutant + solvent (EA). Closed squared: *sdiA* mutant + AHL. When using *sdiA* expressed from a plasmid, all media were supplemented with arabinose (0.2%).

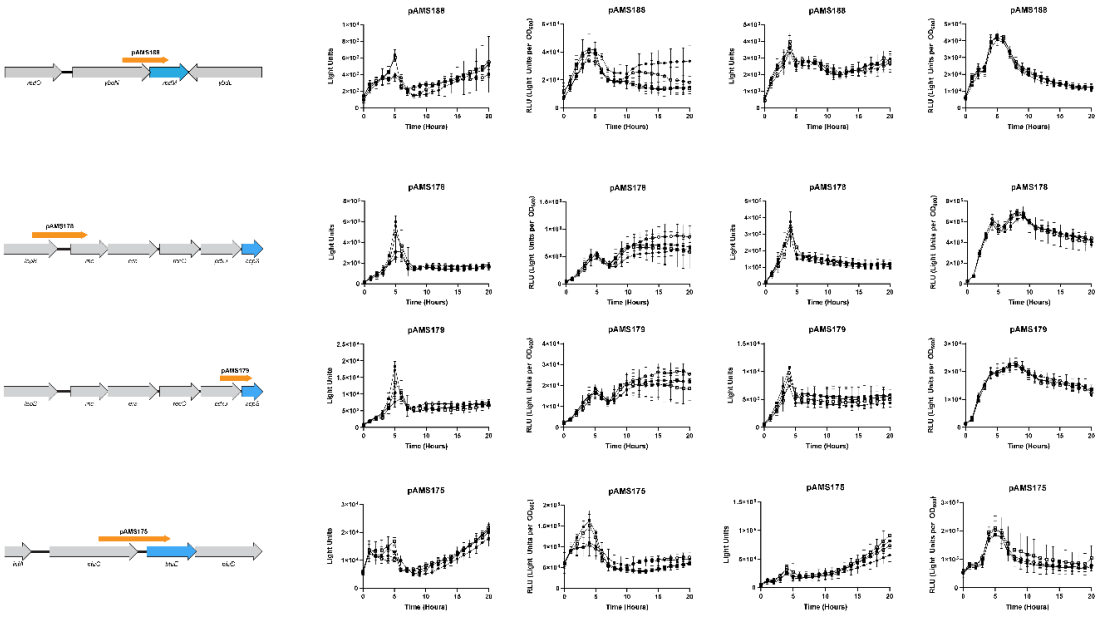


Figure 8 continued

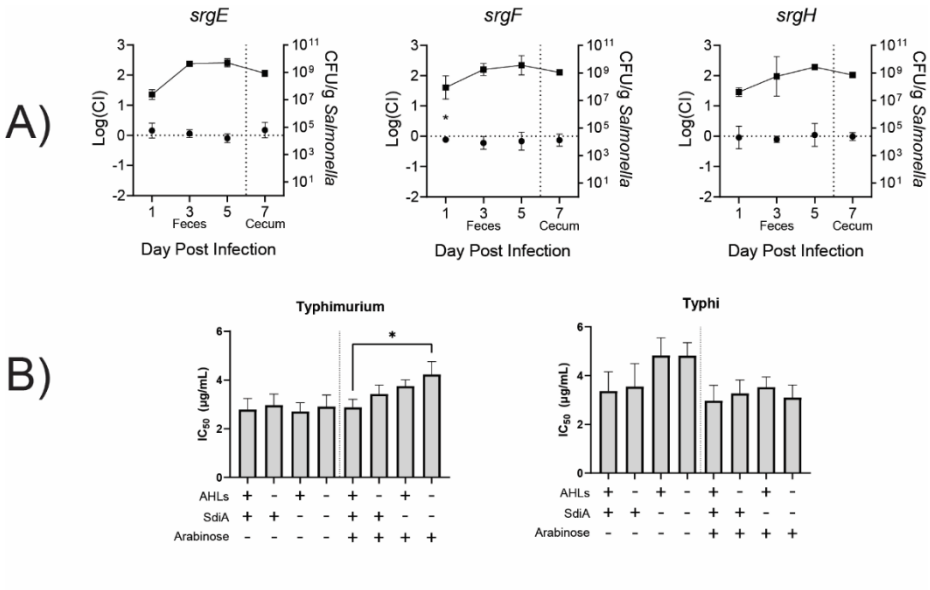
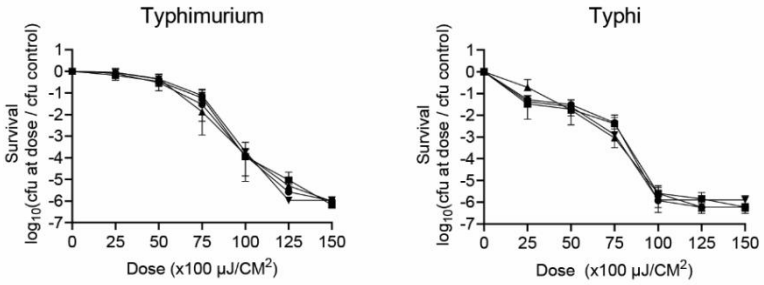


Figure 9. Tested phenotypes of SdiA and regulated genes.

A) Competition assays between wild-type (14028) and mutants of *sdiA*-regulated genes. SrgE competition (EFB051 vs JLD1221), SrgF competition (JLD1214 vs AMS254), SrgH (EFB051 vs AMS264). Mice were inoculated orally with both strains in a 1:1 ratio. Fecal pellets were collected and CFU quantified (squares, right axis) and competitive index (circles, left axis). Cecum was collected and CFU quantified on Day 7. Competitive index at each time point was calculated as the ratio of mutant to wild-type divided by the ratio of mutant to wild-type in the inoculum. Each competition was performed with five female CBA/J mice. Statistical significance was evaluated using a one-sample student's t-test. * $P < 0.05$
B) Minimum inhibitory concentration of nalidixic acid for serovars Typhimurium and Typhi. See methods for details on growth conditions and IC_{50} calculations. Strains used in assay: Typhimurium – 14028, BA612, BA612 + pJVR2, BA612 + pBAD18; Typhi – Ty2, AMS002, AMS002 +pAMS130, AMS002 + pBAD33. The left four strains utilize endogenous expression of *sdiA*. The right four strains utilize sdiA expressed from a plasmid. Mean and standard deviation was calculated from three independent experiments. Significance was evaluated using a student's t-test. * $P < 0.05$
C) Resistance of serovars Typhimurium and Typhi to UV-mediated killing. See methods for details on assay. Circles – 14028 and Ty2, squares – BA612 and AMS002, upward triangle BA612 + pJVR2 and AMS002 + pAMS130, downward triangle BA612 + pBAD33 and AMS002 + pBAD33. No timepoints were significant as determined by student's t tests. Mean and standard deviation was calculated from three independent experiments.
D) Resistance of serovars Typhimurium and Typhi to nalidixic acid in motility agar. Strains were inoculated into motility agar containing 1 mM AHL. Disks were implanted with indicated quantities of Nalidixic Acid in 5 mL of water. Plates were incubated overnight at 37 °C. Images are representative of three independent experiments.

C)



D)

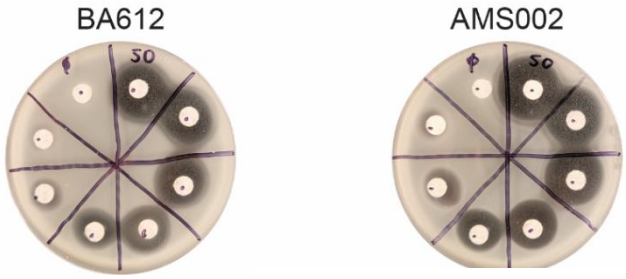


Figure 9 continued

Table 1. *sdiA*-dependent regulation of transcriptional fusions in *S. enterica*, *E. coli*, and *E. cloacae*.

a. Value is the largest fold change in *sdiA*-dependent expression of each fusion throughout the time course in the media of each column (kinetics shown in Figures 2 and 3). Positive values indicate a *sdiA*-dependent increase in expression while negative values indicate *sdiA*-dependent decrease in expression.

b. The AHL is 1 μM oxoC8

c. The solvent control is 0.1% EA

d. ND = not determined

			Motility agar[a]		**LB[a]**	
Species/Serovar	**Gene(s)**	**Plasmid**	**AHL[b]**	**Solvent[c]**	**AHL[b]**	**Solvent[c]**
Salmonella enterica serovar Typhimurium	*srgE*	pJNS25	19	2. 3	5.2	1.2
	srgF	pAMS148	5.0	1.6	1.6	1.5
	srgGH	pAMS145	4.5	2.2	2.1	1.6
	srgKJ	pJLD202	3.0	1.3	1.6	1.5
	menFDHBCE	pAMS291	1.8	1.1	1.6	1.8
	pefI-srgC	pBA428	8.2	1.3	4.9	2.5
Salmonella enterica serovar Typhi	*srgIL*	pAMS201	14	2.3	1.7	1.4
	srgF	pAMS205	6. 3	3.7	3.2	2.0
	srgGH	pAMS265	2.5	1.0	4.4	1.1
	srgKJ	pAMS050	2.8	1.1	1.5	1.1
	menFDHBCE	pAMS202	2.7	1.2	1.5	1.4
	$srgE^{Typhimurium}$	pJNS25	ND[d]	ND[d]	2.8	2.1
E. coli	*fepE*	pAMS366	ND[d]	ND[d]	-7.3	-8.0
E. cloacae	*srgKJ*	pAMS228	ND[d]	ND[d]	1.9	1.7
	menFDHBCE	pAMS362	ND[d]	ND[d]	-105	-70
	srgG	pAMS368	ND[d]	ND[d]	4.2	3.6

Table 2. Strains and plasmids used in this study

Strain	**Genotype or Description**	**Source, Construction, or Reference**
ATCC 14028 (14028)	Wild-type *Salmonella enterica* subspecies *enterica* serovar Typhimurium strain 14028	American Type Culture Collection (ATCC)
BA612	14028 *sdiA*::mTn3	[139]
JSG624 (Ty2)	Wild-type *Salmonella enterica* subspecies *enterica* serovar Typhi strain Ty2	John Gunn
AMS001	Ty2 *sdiA1*::cam. Made by Wanner mutagenesis with primers BA3454 and BA3455	This Study
AMS002	Ty2 *sdiA2*::kan. Made by Wanner mutagenesis with primers BA3454 and BA3455	This Study
AMS203	BA612 *pagC* IG::*sdiA1*	This Study
AMS246	AMS203 *spv154::*MudJ. P22 transduction $P22_{BA1541}$ X AMS203	This Study
AMS171	BA612 *spv1541*::MudJ. P22 transduction $P22_{BA1541}$ X BA612	This Study
BA1541	14028 *spv1541*::MudJ	[285]
BA770	14028 – pSLT nal^R	[285]
Jke201	Mating strain of *E. coli*, see reference [309] for full genotype and description	Gift from Dirk Bumann, [309]
JLD401	*Enterobacter cloacae* Nal^R	[157]
ASD401	JLD401 *sdiA32*::mTn5-FC	[157]
MG1655	Wild-type Escherichia coli K-12 strain MG1655	*E. coli* Genetic Stock Center
JNS21	MG1655 *sdiA25*::EZ-Tn5,kan-2.	[158]
JS198	LT2 *metE551 metA22 ilv452 trpB2 hisC527(am) galE496 xyl-404 rpsL120 flaA66 hsdL6 hsdSA29 zjg8103 : :* pir+ *recA1*	[310]

AMS254	14028 *srgF1::*kan. P22 transduction $P22_{srgF1::kan}$ X 14028	This Study
AMS264	14028 *srgH1::*kan. P22 transduction $P22_{srgH1::kan}$ X 14028	This Study
JLD1214	14028 *IG(pagC-STM14_1502)*::Cam	[113]
EFB051	14028 *IG(pagC-STM14_1502)6*::Kan	[131]
JLD1221	14028 *srgE42::cam*. Constructed by wanner mutagenesis. Insertion amplified with primers BA1563 and BA1564. Transduced into a clean 14028 background by P22	This Study
*srgF1::*kan	Mutation from McClellend Collection	[311]
*srgH1::*cam	Mutation from McClellend Collection	[311]
Plasmid	**Genotype or Description**	**Source, Construction, or Reference**
pKD46	PBAD *gam bet exo* pSC101 oriTS	[302]
pKD3	FRT-cam-FRT *oriR6K*	[302]
pKD4	FRT-kan-FRT *oriR6K*	[302]
pBAD33	pACYC vector for arabinose-conditional expression	[312]
pAMS130	pBAD33 $sdiA^+$ (Typhi). Insert amplified with primers BA3601 and BA3602	This Study
pJVR2	pBAD33 $sdiA^+$ (Typhimurium)	[139]
pBAD18	ColE1 origin vector for arabinose-conditional expression	[312]
pSB401	$luxR^+$ *luxI::luxCDABE*	[274]
pBA321	pBAD18 $sdiA^+$ (Typhimurium)	[138]

pFOK	Suicide vector for allelic exchange.	Dirk Bumann [300]
pAMS150	pFOK-SdiA. See methods	This Study
pAMS148	Reporter Plasmid of Typhimurium *srgF* (*STM14_3820*). Insert amplified with primers BA3869 and BA3870.	This Study
pAMS145	Reporter Plasmid of Typhimurium *srgGH* (*STM14_4893-4894*). Insert amplified with primers BA3863 and BA3864	This Study
pJLD202	Reporter Plasmid of Typhimurium *srgKJ* (*STM14_0589-0588*). Insert amplified with primers BA1218 and BA1219.	[313] disseration
pAMS291	Reporter Plasmid of Typhimurium *menFDHBCE* (*STM14_2848-2843*). Insert amplified with primers BA4031 and BA4032	This Study
pJNS25	Reporter Plasmid of Typhimurium *srgE* (*STM14_1877*).	[146]
pBA428	Reporter Plasmid of Typhimurium *pefI-srgC*.	[138]
pAMS205	Reporter Plasmid of Typhi *srgF* (*T06040*). Insert amplified with primers BA3953 and BA3964	This Study
pAMS265	Reporter Plasmid of Typhi *srgGH* (*T3549-3548*). Insert amplified with primers BA4025 and BA4026	This Study
pAMS050	Reporter Plasmid of Typhi *srgKJ* (*T2359-2360*). Insert amplified with primers BA3710 and BA3711	This Study
pAMS202	Reporter Plasmid of Typhi *menFDHBCE* (*T0553-0558*). Insert amplified with	This Study

	primers BA3950 and BA3961	
pAMS048	Reporter Plasmid of Typhi *srgE* (*T1468*). Insert amplified with primers BA3706 and BA3707	This Study
pAMS347	Reporter Plasmid of Typhi *srgDAB* (*T4538-4540*). Insert amplified with primers BA4075 and BA4076	This Study
pAMS201	Reporter Plasmid of Typhi (*T0351-0350*). Insert amplified with primers BA3949 and BA3960	This Study
pAMS042	Reporter Plasmid of Typhimurium *yjiXA* (*STM14_5444-5443*). Insert amplified with primers BA3722 and BA3723	This Study
pAMS055	Reporter Plasmid of Typhimurium *yjiA* (*STM14_5443*). Insert amplified with primers BA3720 and BA3721	This Study
pAMS096	Reporter Plasmid of Typhimurium *yjiYXA* (*STM14_5445-5443*). Insert amplified with primers BA3828 and BA3829	This Study
pAMS043	Reporter Plasmid of Typhimurium *ybdNM* (*STM14_0704-0703*). Insert amplified with primers BA3724 and BA3725	This Study
pAMS097	Reporter Plasmid of Typhimurium *ybdO* (*STM14_0705*). Insert amplified with primers BA3830 and BA3831	This Study
pRG38	Reporter Plasmid of Typhimurium *flhD* (*STM14_2341*).	[262]

pRG34	Reporter Plasmid of Typhimurium *fliA* (*STM14_2374*).	[262]
pRG39	Reporter Plasmid of Typhimurium *fliC* (*STM14_2378*).	[262]
pDL05	Reporter Plasmid of Typhimurium *rtsA* (*STM14_5188*). Insert amplified with primers BA1631 and BA1632	[314]
pDL83	Reporter Plasmid of Typhimurium *invF* (*STM14_3498*). Insert amplified with primers BA1978 and BA1979	[314]
pBA409	Reporter Plasmid of Typhimurium *sopB* (*STM14_1237*).	[262]
pAMS144	Reporter Plasmid of Typhimurium *yecF* (*STM14_2367*). Insert amplified with primers BA3861 and BA3862	This Study
pAMS146	Reporter Plasmid of Typhimurium *yciG* (*STM14_2091*). Insert amplified with primers BA3865 and BA3866	This Study
pAMS147	Reporter Plasmid of Typhimurium *STM14_1829*. Insert amplified with primers BA3867 and BA3868	This Study
pAMS188	Reporter Plasmid of Typhimurium *ybdM* (*STM14_0703*). Insert amplified with primers BA3946 and BA3948	This Study
pAMS187	Reporter Plasmid of Typhimurium *pdxJ-acpS* (*STM14_3158-3157*). Insert amplified with primers BA3945 and BA3947	This Study

pAMS143	Reporter Plasmid of Typhimurium *dpiA* (*STM14_0728*). Insert amplified with primers BA3859 and BA3860	This Study
pAMS184	Reporter Plasmid of Typhimurium *citA* (*STM14_0804*). Insert amplified with primers BA3860 and BA3859	This Study
pMT45	Reporter Plasmid of Typhimurium *fimA* (*STM14_0635*).	[315]
pAMS154	Reporter Plasmid of Typhimurium *STM14_0979*. Insert amplified with primers BA3889 and BA3890	This Study
pAMS156	Reporter Plasmid of Typhimurium *ynfL* (*STM14_1798*). Insert amplified with primers BA3893 and BA3894	This Study
pAMS172	Reporter Plasmid of Typhimurium *proVWX* (*STM14_3391-3393*). Insert amplified with primers BA3902 and BA3914	This Study
pAMS178	Reporter Plasmid of Typhimurium *rnc-acpS* (*STM14_3161-3157*). Insert amplified with primers BA3908 and BA3920	This Study
pAMS179	Reporter Plasmid of Typhimurium *acpS* (*STM14_3157*). Insert amplified with primers BA3909 and BA3921	This Study
pMT47	Reporter Plasmid of Typhimurium *fimY* (*STM14_0642*).	[315]
pMT48	Reporter Plasmid of Typhimurium *fimW* (*STM14_0644*).	[315]

pAMS173	Reporter Plasmid of Typhimurium *leuABCD* (*STM14_0134-0131*). Insert amplified with primers BA3903 and BA3915	This Study
pAMS174	Reporter Plasmid of Typhimurium *btuCED* (*STM14_1627-1629*). Insert amplified with primers BA3906 and BA3918	This Study
pAMS175	Reporter Plasmid of Typhimurium *btuED* (*STM14_1628-1629*). Insert amplified with primers BA3905 and BA3917	This Study
pAMS362	Reporter Plasmid of *E. cloacae menFDHBCE*. Insert amplified with primers BA4081 and BA4082	This Study
pAMS228	Reporter Plasmid of *E. cloacae ybbKJ*. Insert amplified with primers BA4003 and BA4004	This Study
pAMS360	Reporter Plasmid of *E. cloacae srgF* (*ENC_32410*). Insert amplified with primers BA4077 and BA4078	This Study
pAMS368	Reporter Plasmid of *E. cloacae ENC_00800*. Insert amplified with primers BA4093 and BA4094	This Study
pAMS367	Reporter Plasmid of Typhimurium *fepE* (*STM14_0687*). Insert amplified with primers BA4091 and BA4092	This Study
pAMS231	Reporter Plasmid of Typhimurium *copA* (*STM14_0586*). Insert amplified with primers BA4001 and BA4002	This Study

pAMS364	Reporter Plasmid of E. coli *menFDHBCE*. Insert amplified with primers BA4085 and BA4086	This Study
pAMS227	Reporter Plasmid of *E. coli ybbKJ*. Insert amplified with primers BA3999 and BA4000	This Study
pAMS363	Reporter Plasmid of *E. coli yfgHI*. Insert amplified with primers BA4083 and BA4084	This Study
pAMS361	Reporter Plasmid of *E. cloacae fliE*. Insert amplified with primers BA4079 and BA4080	This Study
pAMS366	Reporter Plasmid of *E. coli fepE*. Insert amplified with primers BA4089 and BA4090	This Study

Table 3. Primers used in this study.

Primer	**Sequence**	**Description**
BA1090	GAATGTATGTCCTGCGTCTTGAGTA	Universal reverse verification primer for pSB401 reporter constructs
BA1218	AGGGCTTATTAACGAGGCCACCATT	Primer for amplification of insert in reporter plasmid pJLD202
BA1219	TTGGTCATGGTCAGGTTAATGATCG	Primer for amplification of insert in reporter plasmid pJLD202
BA1563	AGTGAAGCTATACCTAACGTGGCTGTTCCTGCAAAATGTGTAGGCTGGAGCTGCTTCG	Primer for generating mutant JLD1221
BA1564	TAGATTCATCCTGAAAGAGCTAATTAGCTCTCCCGACATATGAATATCCTCCTTAG	Primer for generating mutant JLD1221
BA1631	TACCTCATGCTAACTACCTCC	Primer for amplification of insert in reporter plasmid pDL05
BA1632	TGGGGCCGAAAAGTCTGCATGTT	Primer for amplification of insert in reporter plasmid pDL05

BA1978	GAAGAAGGTGAGCGCCTGTTCTTTG	Primer for amplification of insert in reporter plasmid pDL83
BA1979	CGATCTTGCCAAATAGCGCGAAACTC	Primer for amplification of insert in reporter plasmid pDL83
BA2474	ACCACCCCCTGACCGCGAATGGTGA	Verification primer for insertions into pBAD18 and pBAD33 vectors
BA2475	AAGCATTTATCAGGGTTATTGTCTC	Verification primer for insertions into pBAD18 and pBAD33 vectors
BA3454	GACCATAAAATATGCAGGAAAATGATTTCTTCACCTGGCGGTGTAGGCTGGAGCTGCTTC	Primer for generating mutants AMS001 and AMS002
BA3455	CGTCAGCACGTCATATCAGACCTGTCGCCGCAGCGTAGCACATATGAATATCCTCCTTAG	Primer for generating mutants AMS001 and AMS002
BA3601	ATGCAGGAAAATGATTTCTT	Primer for amplifying Typhi *sdiA* in construction of pAMS130
BA3602	TCATATCAGACCTGTCGCCG	Primer for amplifying

		Typhi *sdiA* in construction of pAMS130
BA3706	GACATCATAAGCTTCACATAATAAAA	Primer for amplification of insert in reporter plasmid pAMS048
BA3707	ATAAGTAGCGTAATCCATTTTTCTAT	Primer for amplification of insert in reporter plasmid pAMS048
BA3710	CTGTAGATTACGAATTAGAGCAATAC	Primer for amplification of insert in reporter plasmid pAMS050
BA3711	AATTTCGATACGGGTAACTTTAATTC	Primer for amplification of insert in reporter plasmid pAMS050
BA3720	ATCATTGAACCGGGTCTCTACTTC	Primer for amplification of insert in reporter plasmid pAMS055
BA3721	CATAATACGTCATGGGAGAAAAAG	Primer for amplification of insert in reporter plasmid pAMS055
BA3722	CTTTGTGATTTCCCCGGAACAAATC	Primer for amplification of insert in reporter

		plasmid pAMS042
BA3723	TAGTCCGGAATACCAATCAACATTTT	Primer for amplification of insert in reporter plasmid pAMS042
BA3724	AATAATCGTTCTATTGTTTGTACTCA	Primer for amplification of insert in reporter plasmid pAMS043
BA3725	TGATACATCAAATTATACAAAGGGTT	Primer for amplification of insert in reporter plasmid pAMS043
BA3828	TGGTAGATATCATAGGTTCGTTTGAT	Primer for amplification of insert in reporter plasmid pAMS096
BA3829	CGAGGATGATGATCATGATTAAGAAG	Primer for amplification of insert in reporter plasmid pAMS096
BA3830	TCATTAATCAGAATCAGCAGCTAATG	Primer for amplification of insert in reporter plasmid pAMS097
BA3831	AATTCCTTTGCCTGAACGAATAAATA	Primer for amplification of insert in reporter plasmid pAMS097

BA3859	CATCACAATACAGCCAATTTTCTTTC	Primer for amplification of insert in reporter plasmid pAMS143/pAMS184
BA3860	ATTTTACTGACCAGATAGCCAATTGA	Primer for amplification of insert in reporter plasmid pAMS143/pAMS184
BA3861	CATGATCACTTTGATATCCGCTGTC	Primer for amplification of insert in reporter plasmid pAMS144
BA3862	TACCATAAGCTACGCTAAAAATAGCA	Primer for amplification of insert in reporter plasmid pAMS144
BA3863	ACTATCTCTATATTTCGCGTATTCGT	Primer for amplification of insert in reporter plasmid pAMS145
BA3864	AAAAATAGCAGTGCGGTCATAAACTC	Primer for amplification of insert in reporter plasmid pAMS145
BA3865	TTGTGAACAGGTTGGCGTAGATTC	Primer for amplification of insert in reporter plasmid pAMS146

BA3866	GTTGCGGATCGTTTTTGAAATTC	Primer for amplification of insert in reporter plasmid pAMS146
BA3867	GGTGGCTTGATTGCCAAAGATTTATT	Primer for amplification of insert in reporter plasmid pAMS147
BA3868	CTTCTGAGGCTTTCTCTTTATCTTCT	Primer for amplification of insert in reporter plasmid pAMS147
BA3869	GTTTCAATTTTAGCCACACAATACAG	Primer for amplification of insert in reporter plasmid pAMS148
BA3870	ATTAATACTGAGAAATGATCTTCGCC	Primer for amplification of insert in reporter plasmid pAMS148
BA3875	ATCGAATTCCTGCAGCCCGGGGGATCCACT	Primer for construction of suicide vector pAMS150. See methods
BA3876	ATCAAGCTTATCGATACCGTCGACCTCGAG	Primer for construction of suicide vector pAMS150. See methods
BA3883	CTCGAGGTCGACGGTATCGATAAGCTTGATATCGGAGCGGGAATAAAGCG	Primer for construction

		of suicide vector pAMS150. See methods
BA3884	AGCGTTGAAAAGGCAGAGAGAAAAGACAGGCAGGT	Primer for construction of suicide vector pAMS150. See methods
BA3885	CCTGTCTTTTCTCTCTGCCTTTTCAACGCTCGC	Primer for construction of suicide vector pAMS150. See methods
BA3886	ACATTGTGATTAATTTAAAAAACCGGCTGTTAGCATCG	Primer for construction of suicide vector pAMS150. See methods
BA3887	ACAGCCGGTTTTTTAAATTAATCACAATGTCATCAAGA	Primer for construction of suicide vector pAMS150. See methods
BA3888	AGTGGATCCCCCGGGCTGCAGGAATTCGATAAATTACGAAGCCATAGACA	Primer for construction of suicide vector pAMS150. See methods
BA3889	TTTTTATTTTTTCCGAATGCAATGTG	Primer for amplification of insert in reporter plasmid pAMS154
BA3890	ACAATATGTTTACCACAAAATATATTCG	Primer for amplification of insert in reporter

		plasmid pAMS154
BA3893	AATAGCTGAAAAGATAAAGTGACGAG	Primer for amplification of insert in reporter plasmid pAMS156
BA3894	GTATTCATTTCACGCGTTTGCATAT	Primer for amplification of insert in reporter plasmid pAMS156
BA3902	AGATTATTTTAGCTCATTACGTCAGC	Primer for amplification of insert in reporter plasmid pAMS172
BA3903	TCTACACGATTATAAATCTGTGACGT	Primer for amplification of insert in reporter plasmid pAMS173
BA3905	TAATGTACTGGATGATGGGAGGATTT	Primer for amplification of insert in reporter plasmid pAMS175
BA3906	CGTCTTTATTGAGCATAACGATAACT	Primer for amplification of insert in reporter plasmid pAMS174
BA3908	CGAGCGATTTTGTACAGACTTTT	Primer for amplification of insert in reporter plasmid pAMS178

BA3909	ATCCAGGTTTCGCTCTTTATCGAT	Primer for amplification of insert in reporter plasmid pAMS179
BA3914	GGAAAACGCTTCATCCATTAATAAGA	Primer for amplification of insert in reporter plasmid pAMS172
BA3915	CTGGCGTCATGATTTCATAGTTTT	Primer for amplification of insert in reporter plasmid pAMS173
BA3917	CACAATAGGTTTTAATCTCCTCTTCG	Primer for amplification of insert in reporter plasmid pAMS175
BA3918	GTTAGAGGTACACAGCACGTTAC	Primer for amplification of insert in reporter plasmid pAMS174
BA3920	GGAATCCGCCGCTTTTTAATTC	Primer for amplification of insert in reporter plasmid pAMS178
BA3921	ATCGTTAAACACTTCGAACTGATTG	Primer for amplification of insert in reporter plasmid pAMS179
BA3945	TCATCTGGGGTATGGCGTCAAT	Primer for amplification

		of insert in reporter plasmid pAMS187
BA3946	TTATGGCTGTATCATGTTATCGAACC	Primer for amplification of insert in reporter plasmid pAMS188
BA3947	CGATAAAGAGCGAAACCTGGAT	Primer for amplification of insert in reporter plasmid pAMS187
BA3948	AAAACCGTCTACAATCTCGTACTC	Primer for amplification of insert in reporter plasmid pAMS188
BA3949	TTAAGGAACCCTTTGTAAGTCAGG	Primer for amplification of insert in reporter plasmid pAMS201
BA3950	CTTGTCATGTTCAACAACGCGATATC	Primer for amplification of insert in reporter plasmid pAMS202
BA3953	TATTGGTTTCGCGAGTGATTAAATTA	Primer for amplification of insert in reporter plasmid pAMS205
BA3960	GAGCCCACGTTATGACCAGTAAC	Primer for amplification of insert in reporter

		plasmid pAMS201
BA3961	GGCTTAATACCGACCAGAGAAG	Primer for amplification of insert in reporter plasmid pAMS202
BA3964	ATTAATACTGAGAAATGATCTTCGCC	Primer for amplification of insert in reporter plasmid pAMS205
BA3999	CCAGGGAAAGATCAGATTATCGTATA	Primer for amplification of insert in reporter plasmid pAMS227
BA4000	CTTTCAGGATTTGCGACTGTTTTTC	Primer for amplification of insert in reporter plasmid pAMS227
BA4001	CATCATGTTATCGCCGATCATC	Primer for amplification of insert in reporter plasmid pAMS231
BA4002	GGATCGTTAAACAGATTGACCAGTTC	Primer for amplification of insert in reporter plasmid pAMS231
BA4003	CAATGCTAATGAATTCCCTACCCTA	Primer for amplification of insert in reporter plasmid pAMS228

BA4004	CTTCAAGAATATAGGCACGCTTGGTA	Primer for amplification of insert in reporter plasmid pAMS228
BA4025	CGATAGAAAAAGTTGAGGCGATTTTA	Primer for amplification of insert in reporter plasmid pAMS265
BA4026	GATAGTAATGCCAACGATGATGGAAG	Primer for amplification of insert in reporter plasmid pAMS265
BA4031	CTGATAAAAATGCGCTCAAGCTTA	Primer for amplification of insert in reporter plasmid pAMS291
BA4032	AAAAGTGGTAGCAGTTGAGATTTAAA	Primer for amplification of insert in reporter plasmid pAMS291
BA4075	TTCCACTGTCGGTTATAATAAAAACC	Primer for amplification of insert in reporter plasmid pAMS347
BA4076	GTTTGTAATGATGGATTCACCCATAA	Primer for amplification of insert in reporter plasmid pAMS347
BA4077	GCGTTTTCTCGGTCATTATTTGA	Primer for amplification

		of insert in reporter plasmid pAMS360
BA4078	CCTCCTCTTTCGACATCAATTCAG	Primer for amplification of insert in reporter plasmid pAMS360
BA4079	GTATTTCAGCTGGGCGTCATTG	Primer for amplification of insert in reporter plasmid pAMS361
BA4080	CTCTGATTACGTGCGGTCATCG	Primer for amplification of insert in reporter plasmid pAMS361
BA4081	GCCTCCGACAATTACTACTACC	Primer for amplification of insert in reporter plasmid pAMS362
BA4082	AGATACGGGTGTCATTGGCTAC	Primer for amplification of insert in reporter plasmid pAMS362
BA4082	AAAAAGAAGCTCAGCAATCCAC	Primer for amplification of insert in reporter plasmid pAMS363
BA4084	TTTGCGGGAAGAATGGAAATAATATT	Primer for amplification of insert in reporter

		plasmid pAMS363
BA4085	CAGTTTATCGTGCTGATGACTAC	Primer for amplification of insert in reporter plasmid pAMS364
BA4086	ATAAATTCTTTTGCCTGAATCGCATC	Primer for amplification of insert in reporter plasmid pAMS364
BA4089	AACTTCCACAGTTACCACTTAGC	Primer for amplification of insert in reporter plasmid pAMS366
BA4090	GACTGAAACTTCTTGATAAACAGGTT	Primer for amplification of insert in reporter plasmid pAMS366
BA4091	CAGGAAAAATTACGCCAAAAACTTC	Primer for amplification of insert in reporter plasmid pAMS367
BA4092	TAATAAACAGATTAAATACGCTGCCC	Primer for amplification of insert in reporter plasmid pAMS367
BA4093	GTTTCGCTGACCATCAACTCCC	Primer for amplification of insert in reporter plasmid pAMS368

BA4094	ATGTTCGACGGCATCACGAATG	Primer for amplification of insert in reporter plasmid pAMS368

Table 4. Differentially expressed genes in S. Typhimurium 14028 vs sdiA mutant BA612

Gene name	**symbol**	**log2FoldChange**	**padj**	**product_accession**	**Description**
STM14_1877	srgE	3.08	3.70 E-07	WP_000987828.1	hypothetical protein
STM14_5443	yjiA	8.15	2.19 E-06	WP_000187839.1	GTPase
STM14_0703	ybdM	6.96	9.39 E-03	WP_001164756.1	ParB-like nuclease domain-containing protein
STM14_2368	sdiA	2.19	2.54 E-02	WP_001157166.1	transcriptional regulator SdiA

Table 5. Differentially expressed genes in S. Typhimurium expressing plasmid sdiA (pJVR2) vs vector (pBAD33)

Gene name	symbol	log2FoldChange	padj	product_accession	Description
STM14_0588	ybbj	4.5	4.40E-83	WP_000561177.1	NfeD family protein
STM14_0589	ybbk	4.1	5.90E-83	WP_000906146.1	SPFH/Band 7/PHB domain protein
STM14_2367	yecF	5.3	1.20E-41	WP_000106483.1	DUF2594 family protein
STM14_5537	srgD	6.5	1.90E-31	WP_001526811.1	helix-turn-helix transcriptional regulator
STM14_3820	srgF	4.7	1.10E-29	WP_000433046.1	RNA helicase
STM14_1877	srgE	9.3	3.00E-27	WP_000987828.1	hypothetical protein
STM14_5538	pefI	6.1	3.80E-27	WP_000004313.1	transcriptional regulator PefI
STM14_3483	sipC	-3.4	7.90E-24	WP_000909019.1	SPI-1 type III secretion system needle tip complex protein SipC
STM14_2378	fliC	-2.5	2.70E-19	WP_000079805.1	FliC/FljB family flagellin
STM14_3894	aer	-4	3.60E-18	WP_000094651.1	PAS domain-containing methyl-accepting chemotaxis protein
STM14_2380	fliD	-3.5	3.00E-17	WP_000146802.1	flagellar filament capping protein FliD
STM14_3392	proW	3.2	1.50E-16	WP_000775022.1	glycine betaine/L-proline ABC transporter permease ProW
STM14_2368	sdiA	3.5	6.90E-16	WP_001157166.1	transcriptional regulator SdiA
STM14_2334	tar	-4.2	7.20E-16	WP_000483274.1	methyl-accepting chemotaxis protein II
STM14_0534	hupB	1.9	1.90E-12	WP_001043544.1	DNA-binding protein HU-beta
STM14_5534	rck	5.3	1.90E-12	WP_000725062.1	complement resistance protein Rck
STM14_5446	tsr	-3.2	5.20E-11	WP_000919519.1	methyl-accepting chemotaxis protein
STM14_2335	cheW	-3.2	6.60E-11	WP_000147295.1	chemotaxis protein CheW

STM14_5596	traY	4	1.40E-10	WP_001676655.1	conjugal transfer relaxosome protein TraY
STM14_1236	sigE	-5.2	2.20E-10	WP_000444724.1	type III secretion system chaperone SigE
STM14_2338	motA	-4.6	4.30E-10	WP_000906312.1	flagellar motor stator protein MotA
STM14_3391	proV	3.1	4.30E-10	WP_000985529.1	glycine betaine/L-proline ABC transporter ATP-binding protein ProV
STM14_1891	adhP	1.5	5.20E-10	WP_000642447.1	alcohol dehydrogenase AdhP
STM14_1237	sopB	-4.1	5.90E-10	WP_001166946.1	SPI-1 type III secretion system effector inositol phosphate phosphatase SopB
STM14_2366	yecF buddy	5	8.20E-10	WP_000546186.1	hypothetical protein
STM14_4305	tcp	-3.2	3.10E-09	WP_000789683.1	methyl-accepting chemotaxis citrate transducer
STM14_2110	oppA	1.9	6.50E-09	WP_001521098.1	oligopeptide ABC transporter substrate-binding protein OppA
STM14_2374	fliA	-5.1	6.50E-09	WP_001087453.1	RNA polymerase sigma factor FliA
STM14_1966		-3.9	1.40E-08	WP_000528484.1	Tar ligand binding domain-containing protein
STM14_3393	proX	2.6	1.80E-08	WP_001216622.1	glycine betaine/L-proline ABC transporter substrate-binding protein ProX
STM14_979		5.4	1.80E-08	WP_010988985.1	DUF4261 domain-containing protein
STM14_1355	flgL	-2.9	3.20E-08	WP_001223033.1	flagellar hook-associated protein FlgL
STM14_2108	oppC	2.2	3.60E-08	WP_000979653.1	oligopeptide ABC transporter permease OppC
STM14_5536	srgA	3.5	4.30E-08	WP_000178592.1	DsbA family protein
STM14_2340	flhC	-2.4	1.20E-07	WP_000605987.1	flagellar transcriptional regulator FlhC

STM14_2332	cheB	-3.2	5.60E-07	WP_000036392.1	protein-glutamate methylesterase/protein glutamine deamidase
STM14_3893		-2.9	9.90E-07	WP_000478472.1	MCP four helix bundle domain-containing protein
STM14_1898	ompD	1.1	1.70E-06	WP_000769035.1	porin OmpD
STM14_3823		-1.5	1.80E-06	WP_000019032.1	SDR family oxidoreductase
STM14_5188	rtsA	-7.5	1.80E-06	WP_000921674.1	AraC family transcriptional regulator
STM14_2109	oppB	2.1	2.30E-06	WP_000911097.1	oligopeptide ABC transporter permease OppB
STM14_4672	mioC	2.3	2.60E-06	WP_000763722.1	FMN-binding protein MioC
STM14_5597	traA	4.7	4.40E-06	WP_001274201.1	type IV conjugative transfer system pilin TraA
STM14_5535	srgB	2.9	4.70E-06	WP_010999938.1	YjiK family protein
STM14_2333	cheR	-7.4	4.80E-06	WP_000204362.1	protein-glutamate O-methyltransferase CheR
STM14_3377	stpA	1.4	4.80E-06	WP_001051100.1	DNA-binding protein StpA
STM14_2337	motB	-3.2	5.80E-06	WP_000795653.1	flagellar motor protein MotB
STM14_2443		-2.4	1.10E-05	WP_000779218.1	hypothetical protein
STM14_2341	flhD	-1.6	2.70E-05	WP_001518146.1	flagellar transcriptional regulator FlhD
STM14_4398	yiaG	-1.5	2.70E-05	WP_000455790.1	HTH-type transcriptional regulator
STM14_2839	arnT	2.1	2.90E-05	WP_000978038.1	lipid IV(A) 4-amino-4-deoxy-L-arabinosyltransferase
STM14_1416	icd	1.2	3.30E-05	WP_000444507.1	NADP-dependent isocitrate dehydrogenase
STM14_1878		2.5	4.10E-05	WP_000201080.1	LacI family DNA-binding transcriptional regulator
STM14_2106	oppF	1.3	4.90E-05	WP_000994696.1	murein tripeptide/oligopeptide ABC transporter ATP binding protein OppF

STM14_2440		-2.3	4.90E-05	WP_000515952.1	phage tail tube protein
STM14_3485	sicA	-3.1	7.40E-05	WP_000386309.1	SycD/LcrH family type III secretion system chaperone SicA
STM14_0379	proA	1.7	7.80E-05	WP_000893231.1	glutamate-5-semialdehyde dehydrogenase
STM14_3481	sipA	-3.4	7.80E-05	WP_000258812.1	SPI-1 type III secretion system effector SipA
STM14_2473		-1.3	8.00E-05	WP_001191666.1	helix-turn-helix transcriptional regulator
STM14_4258	glgB	-1.3	1.20E-04	WP_000098543.1	1,4-alpha-glucan branching enzyme
STM14_2574	ugd	2.4	1.50E-04	WP_000704831.1	UDP-glucose 6-dehydrogenase
STM14_5079		2.2	1.70E-04	WP_000750804.1	YjbH domain-containing protein. Outer membrane?
STM14_0146	rsmH	0.9	1.80E-04	WP_000970444.1	16S rRNA (cytosine(1402)-N(4))-methyltransferase RsmH
STM14_1509		1.6	1.90E-04	WP_001218118.1	Hsp20 family protein
STM14_0235	stfC	2.5	2.80E-04	WP_000951687.1	fimbrial biogenesis outer membrane usher protein
STM14_2093	yciE	-3.8	3.40E-04	WP_001109977.1	ferritin-like domain-containing protein
STM14_0201		-1.6	3.90E-04	WP_000829730.1	pyrroloquinoline quinone-dependent dehydrogenase
STM14_2475		-1.8	4.30E-04	WP_000997190.1	hypothetical protein
STM14_4346	yhjH	-5.4	4.70E-04	WP_000595626.1	cyclic-guanylate-specific phosphodiesterase
STM14_2107		1.4	5.10E-04	WP_000058857.1	ABC transporter ATP-binding protein
STM14_2091	yciG	-2.7	6.00E-04	WP_000807657.1	general stress protein
STM14_1882	treY	-1.7	7.20E-04	WP_000613145.1	malto-oligosyltrehalose synthase
STM14_3493	invC	-2.8	7.60E-04	WP_000856766.1	SctN family type III secretion system ATPase InvC
STM14_3338	fljB	-2	8.10E-04	WP_000079794.1	FliC/FljB family flagellin

STM14_0147	ftsL	1.1	8.80E-04	WP_000625651.1	cell division protein FtsL
STM14_1354	flgK	-3.3	8.80E-04	WP_000096425.1	flagellar hook-associated protein FlgK
STM14_2857	nuoN	0.8	8.80E-04	WP_000156671.1	NADH-quinone oxidoreductase subunit NuoN
STM14_3473	prgH	-2.2	8.80E-04	WP_000450192.1	type III secretion system inner membrane ring protein PrgH
STM14_5563	spvA	-3.1	8.80E-04	WP_001526990.1	virulence protein SpvA
STM14_5562	spvB	-4.9	1.00E-03	WP_001676648.1	SPI-2 type III secretion system effector NAD(+)--protein-arginine ADP-ribosyltransferase SpvB
STM14_1945		1.4	1.10E-03	WP_001259025.1	DUF3313 domain-containing protein
STM14_4813		0.6	1.10E-03	WP_001541209.1	hypothetical protein
STM14_3498	invF	-2.5	1.40E-03	WP_001674874.1	type III secretion system transcriptional activator InvF
STM14_0378	proB	1.5	1.50E-03	WP_001285275.1	glutamate 5-kinase
STM14_2070	acnA	-1.3	1.50E-03	WP_000099475.1	aconitate hydratase AcnA
STM14_2441		-2	1.50E-03	WP_001007991.1	phage tail sheath subtilisin-like domain-containing protein
STM14_3495	invA	-2.1	1.70E-03	WP_000927219.1	type III secretion system export apparatus protein InvA
STM14_5430		-1.3	1.70E-03	WP_000331411.1	NAD-dependent succinate-semialdehyde dehydrogenase
STM14_2069	ribA	4.6	1.90E-03	WP_001192557.1	hypothetical protein
STM14_3305		1.7	1.90E-03	WP_000248794.1	SEC-C domain-containing protein
STM14_4208	igaA	1.1	1.90E-03	WP_000104094.1	intracellular growth attenuator protein IgaA

STM14_1214	ompA	-0.7	2.00E-03	WP_001674965.1	porin OmpA
STM14_2094		-1.6	2.00E-03	WP_000488349.1	manganese catalase family protein
STM14_4027	nanK	-2.1	2.10E-03	WP_000208976.1	N-acetylmannosamine kinase
STM14_0305	mltD	1.4	2.30E-03	WP_000644706.1	murein transglycosylase D
STM14_0482	secD	0.9	2.30E-03	WP_000934811.1	protein translocase subunit SecD
STM14_3033	tal	-1.4	2.30E-03	WP_001072448.1	transaldolase
STM14_1881	glgX	-1.5	2.50E-03	WP_000210601.1	glycogen debranching protein GlgX
STM14_2244	sopE2	-2.7	3.10E-03	WP_000182072.1	SPI-1 type III secretion system guanine nucleotide exchange factor SopE2
STM14_0732	cspE	0.6	3.20E-03	WP_000034826.1	transcription antiterminator/RNA stability regulator CspE
STM14_3352		1.5	3.30E-03	WP_000178733.1	VirK family antimicrobial peptide resistance protein
STM14_2438		-2.2	3.50E-03	WP_000785385.1	phage tail tape measure protein
STM14_0134	leuA	3.7	3.60E-03	WP_000082819.1	2-isopropylmalate synthase
STM14_4418		1.1	4.40E-03	WP_001575119.1	protein bax
STM14_4752	uvrD	1.2	4.40E-03	WP_000383441.1	DNA helicase II
STM14_2795	ada	-1.3	4.60E-03	WP_000975956.1	bifunctional DNA-binding transcriptional regulator/O6-methylguanine-DNA methyltransferase Ada
STM14_3482	sipD	-1.9	4.60E-03	WP_000932246.1	SPI-1 type III secretion system needle tip complex protein SipD
STM14_2737	mepS	0.9	4.90E-03	WP_000241015.1	bifunctional murein DD-endopeptidase/murein LD-carboxypeptidase
STM14_2452		-2	5.10E-03	WP_000088182.1	terminase large subunit

STM14_1600	katE	-1.1	5.40E-03	WP_000019119.1	catalase HPII
STM14_5489	deoB	-0.8	5.60E-03	WP_000816454.1	phosphopentomutase
STM14_2336	cheA	-4.4	5.70E-03	WP_000061302.1	chemotaxis protein CheA
STM14_2852	CheV	-1.5	5.90E-03	WP_000368558.1	chemotaxis protein CheV
STM14_3818		1.8	6.20E-03	WP_000439335.1	TIGR00645 family protein. Unknown function, predicted transmembrane
STM14_2476		-2.1	6.60E-03	WP_000080415.1	DUF2303 family protein
STM14_2393	fliI	-3.3	6.70E-03	WP_000213257.1	flagellum-specific ATP synthase FliI
STM14_3471	prgJ	-3.4	7.20E-03	WP_000020431.1	type III secretion system inner rod protein PrgJ
STM14_4331	FraB	2.2	7.50E-03	WP_010989080.1	SIS domain-containing protein
STM14_2266		-5.8	8.10E-03	WP_000275418.1	tail fiber assembly protein
STM14_2432		-2.3	8.10E-03	WP_001207832.1	DUF2313 domain-containing protein
STM14_0383		-1.1	8.70E-03	WP_001539227.1	DUF1889 family protein
STM14_0533	lon	0.7	8.80E-03	WP_001067723.1	endopeptidase La
STM14_2448		-1.8	9.50E-03	WP_000257528.1	phage major capsid protein
STM14_2019		1.3	1.00E-02	WP_000275493.1	hypothetical protein
STM14_0014	dnaJ	1.1	1.10E-02	WP_001119009.1	molecular chaperone DnaJ
STM14_0418		1.3	1.10E-02	WP_001651666.1	type III restriction-modification system endonuclease
STM14_0818	speF	-2.8	1.10E-02	WP_001292400.1	ornithine decarboxylase SpeF
STM14_2445		-2.1	1.10E-02	WP_000702408.1	phage head closure protein
STM14_2454		-2	1.10E-02	WP_001135225.1	HNH endonuclease

STM14_3034	tkt	-1.3	1.10E-02	WP_000087323.1	transketolase
STM14_3627	mutH	1.7	1.10E-02	WP_001274930.1	DNA mismatch repair endonuclease MutH
STM14_0075	dapB	-1.5	1.20E-02	WP_000544031.1	4-hydroxy-tetrahydrodipicolinate reductase
STM14_0154	murG	0.8	1.20E-02	WP_000016613.1	undecaprenyldiphospho-muramoylpentapeptide beta-N-acetylglucosaminyltransferase
STM14_1348	flgE	-4	1.20E-02	WP_000010567.1	flagellar hook protein FlgE
STM14_1628	btuE	-3.1	1.20E-02	WP_001181565.1	glutathione peroxidase
STM14_4013	yrbL	-1.8	1.20E-02	WP_000602196.1	PhoP regulatory network protein YrbL
		1.1	1.20E-02	WP_001804776.1	type I toxin-antitoxin system Ibs family toxin
STM14_0587	cueR	1.2	1.30E-02	WP_001026760.1	Cu(I)-responsive transcriptional regulator
STM14_2430		-1.8	1.30E-02	WP_015701331.1	tail fiber assembly protein
STM14_4206	nudE	1.8	1.30E-02	WP_000045725.1	ADP compounds hydrolase NudE
STM14_4256	glgC	-1.1	1.30E-02	WP_000253995.1	glucose-1-phosphate adenylyltransferase
STM14_1639	ppsA	-0.8	1.40E-02	WP_000069340.1	phosphoenolpyruvate synthase
STM14_5533	srgC	2	1.40E-02	WP_000417898.1	AraC family transcriptional regulator
STM14_0158	ftsA	0.8	1.50E-02	WP_000588463.1	cell division protein FtsA
STM14_1342	flgM	-2.8	1.50E-02	WP_000020893.1	anti-sigma-28 factor FlgM
STM14_3491	spaN	-2.7	1.50E-02	WP_000503098.1	SPI-1 type III secretion system protein SpaN
STM14_3497	invG	-3.2	1.50E-02	WP_000848113.1	type III secretion system outer membrane ring protein InvG
STM14_0549		-1.1	1.60E-02	WP_000779803.1	YbaY family lipoprotein

STM14_2453		-1.9	1.70E-02	WP_000929191.1	phage terminase small subunit P27 family
STM14_5598	traL	5.5	1.70E-02	WP_000012129.1	type IV conjugative transfer system protein TraL
STM14_0483	secF	0.8	1.80E-02	WP_000046629.1	protein translocase subunit SecF
STM14_0796	nagB	-1.4	1.80E-02	WP_001237059.1	glucosamine-6-phosphate deaminase
STM14_1341	flgN	-1.9	1.80E-02	WP_000197547.1	flagella biosynthesis chaperone FlgN
STM14_3496	invE	-4.6	1.80E-02	WP_000612171.1	type III secretion system gatekeeper InvE
STM14_4871	rhaT	6	1.80E-02	WP_000063541.1	L-rhamnose/proton symporter RhaT.
STM14_1662		1.5	1.90E-02	WP_000261866.1	L-cystine transporter
STM14_4255	glgA	-1.1	1.90E-02	WP_001197669.1	glycogen synthase GlgA
STM14_2437		-1.8	2.00E-02	WP_000863818.1	DNA circularization N-terminal domain-containing protein
STM14_3484	sipB	-4.2	2.00E-02	WP_000245788.1	SPI-1 type III secretion system needle tip complex protein SipB
STM14_5162	pmrR	1.9	2.00E-02	WP_000844399.1	LpxT activity modulator PmrR
STM14_0149	murE	0.8	2.10E-02	WP_000775077.1	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate--2,6-diaminopimelate ligase
STM14_0249	degP	0.8	2.10E-02	WP_000753958.1	serine endoprotease DegP
STM14_1829		-2.7	2.10E-02	WP_000807642.1	general stress protein
STM14_2450		-1.8	2.20E-02	WP_000466254.1	phage portal protein
STM14_3552	cysI	3.7	2.30E-02	WP_001290660.1	assimilatory sulfite reductase (NADPH) hemoprotein subunit
STM14_3849	ygiX	-5.7	2.30E-02	WP_001221574.1	two-component system response regulator QseB

STM14_0716	ybdR	-2.6	2.40E-02	WP_000646113.1	glutathione-dependent formaldehyde dehydrogenase
STM14_1798	ynfL	2.9	2.40E-02	WP_001019577.1	LysR family transcriptional regulator
STM14_1921	mcbR	-2.4	2.40E-02	WP_001119592.1	colanic acid/biofilm transcriptional regulator McbR
STM14_2330	cheZ	-2.1	2.40E-02	WP_000983586.1	protein phosphatase CheZ
STM14_2446		-1.8	2.40E-02	WP_000927378.1	phage gp6-like head-tail connector protein
STM14_2460		-2.3	2.40E-02	WP_001527046.1	phage holin, lambda family
STM14_3157		-3	2.40E-02	WP_000986043.1	holo-ACP synthase
STM14_1272	wrbA	-0.8	2.50E-02	WP_001062899.1	NAD(P)H:quinone oxidoreductase
STM14_2092	yciF	-3.2	2.50E-02	WP_001022822.1	ferritin-like domain-containing protein
STM14_0153	ftsW	1	2.60E-02	WP_001239803.1	cell division protein FtsW
STM14_0156		0.7	2.60E-02	WP_000763905.1	D-alanine--D-alanine ligase
STM14_1347	flgD	-4.4	2.70E-02	WP_000020450.1	flagellar hook assembly protein FlgD
STM14_2642	fbaB	-1	2.70E-02	WP_000129590.1	class I fructose-bisphosphate aldolase
		-1.6	2.70E-02	WP_014343856.1	baseplate J/gp47 family protein
STM14_2439		-2	2.80E-02		phage tail assembly protein
STM14_3064	purM	4.4	2.80E-02	WP_000130477.1	phosphoribosylformylglycinamidine cyclo-ligase
STM14_4671	mnmG	1.2	2.80E-02	WP_000499872.1	tRNA uridine-5-carboxymethylaminomethyl(34) synthesis enzyme MnmG
STM14_2381	fliS	-4.6	2.90E-02	WP_000287764.1	flagellar export chaperone FliS
STM14_0048	nhaA	0.8	3.00E-02	WP_000681340.1	Na+/H+ antiporter NhaA

STM14_4883	cpxP	1.8	3.00E-02	WP_001233463.1	cell-envelope stress modulator CpxP
STM14_0481	yajC	1.1	3.10E-02	WP_000007628.1	preprotein translocase subunit YajC
STM14_2442		-1.9	3.10E-02	WP_000497739.1	DUF2635 domain-containing protein
STM14_966	dps	-1	3.20E-02	WP_000100805.1	DNA starvation/stationary phase protection protein Dps
STM14_0454	psiF	-1.3	3.30E-02	WP_000705165.1	phosphate starvation-inducible protein PsiF
STM14_1015	ybjG	1.6	3.50E-02	WP_001738110.1	undecaprenyl-diphosphate phosphatase
STM14_2455		-1.6	3.50E-02	WP_001292890.1	hypothetical protein
STM14_3492	spaM	-4.3	3.50E-02	WP_001520714.1	SPI-1 type III secretion system protein SpaM
STM14_0422		-1.5	3.60E-02	WP_000393711.1	cytochrome ubiquinol oxidase subunit I
STM14_5574		-0.8	3.60E-02	WP_000728917.1	Rpn family recombination-promoting nuclease/putative transposase
STM14_2858	nuoM	0.9	3.80E-02	WP_000926431.1	NADH-quinone oxidoreductase subunit M
STM14_5119	siiC	-5.5	3.80E-02	WP_001541306.1	SPI-4 type I secretion system protein SiiC
STM14_1512		1.4	3.90E-02	WP_000929982.1	cytochrome b
STM14_2431		-1.9	3.90E-02	WP_000554737.1	hypothetical protein
STM14_2231	htpX	1	4.00E-02	WP_000984498.1	protease HtpX
STM14_2458		-2.1	4.00E-02	WP_001050825.1	lysis protein
STM14_2849	elaB	-0.8	4.00E-02	WP_001522308.1	stress response protein ElaB
STM14_4254	glgP	-0.7	4.00E-02	WP_000993428.1	glycogen phosphorylase
STM14_0727	dpiB	6.5	4.40E-02	WP_001277836.1	sensor histidine kinase DpiB

STM14_0150	murF	0.8	4.60E-02	WP_000626630.1	UDP-N-acetylmuramoyl-tripeptide--D-alanyl-D-alanine ligase
STM14_3489	spaP	-5.4	4.60E-02	WP_000526016.1	SPI-1 type III secretion system export apparatus protein SpaP
STM14_5404		0.9	4.60E-02	WP_001240360.1	membrane protein
STM14_0635	fimA	-2.2	4.70E-02	WP_000681030.1	type 1 fimbrial protein subunit FimA
STM14_1329	solA	1.1	4.70E-02	WP_000872773.1	N-methyl-L-tryptophan oxidase
STM14_3724		-5.5	4.70E-02	WP_000104231.1	Ldh family oxidoreductase. putative malate/L-lactate dehydrogenase

Table 6. Differentially expressed genes in S. Typhi Ty2 vs sdiA mutant AMS001

Gene name	**symbol**	**log2FoldChange**	**padj**	**product_accession**	**name**
T0926	sdiA	3.49	2.10E-15	WP_001157173.1	transcriptional regulator SdiA
T0927	yecC	-1.78	1.80E-05	WP_001273033.1	L-cystine ABC transporter ATP-binding protein YecC
T2359	ybbK	0.98	2.70E-02	WP_000906145.1	SPFH/Band 7/PHB domain protein

Table 7. Differentially expressed genes in S. Typhi Ty2 expressing plasmid sdiA (pAMS130) vs vector (pBAD33)

Gene Name	symbol	log2FoldChange	padj	product_accession	name
T0926	sdiA	11.7	4.3E-47	WP_001157173.1	transcriptional regulator SdiA
T0350	srgJ	9.6	1.3E-11	WP_000755800.1	DUF5384 family protein
T4028	cysG	-8.7	6.0E-09	WP_000349908.1	uroporphyrinogen-III C-methyltransferase
T0553	menF	2.9	1.0E-08	WP_000555672.1	isochorismate synthase MenF
T0554	menD	2	6.5E-08	WP_000116387.1	2-succinyl-5-enolpyruvyl-6-hydroxy-3-cyclohexene-1-carboxylic-acid synthase
T2359	YbbK	3.1	2.5E-06	WP_000906145.1	SPFH/Band 7/PHB domain protein
T2835	cysN	7.4	1.9E-05	WP_001092273.1	sulfate adenylyltransferase subunit CysN
T0167	ygbK	-2.8	1.7E-04	WP_000783298.1	D-threonate kinase
T2360	YbbJ	2.2	1.7E-04	WP_000561177.1	NfeD family protein
T0199	fhuA	-1	1.5E-03		ferrichrome porin FhuA
T06040	srgF	2.5	1.5E-03	WP_000433048.1	hypothetical protein
T4011	igaA	1.2	9.1E-03	WP_000104086.1	intracellular growth attenuator protein IgaA
T0351	srgI	6.2	2.6E-02	WP_001131774.1	glycine zipper 2TM domain-containing protein

T2456	secD	0.7	3.3E-02	WP_000934811.1	protein translocase subunit SecD
T2528	proA	1	3.3E-02	WP_000893213.1	glutamate-5-semialdehyde dehydrogenase

Chapter 4: The in vivo relevance of SdiA in house flies, mice, and plants

4.1 Contributions

The work presented in this chapter was carried out by me. I thank Dr. Sarah Short for her assistance in developing and carrying out the work on house flies and for the use of her insectary. Without her, none of the house fly experiments would have been possible. I thank Dr. Brian Ahmer for his guidance in the development of hypotheses and experimental design while performing these studies and for purchasing the plants used in this study. I thank Dr. Adam Deutschbauer for aiding in the development of the barcoded transposon library used in this study and for performing and analyzing the Bar-seq results. I thank the Department of Microbiology for their feedback and ideas given at seminars throughout the duration of this study.

4.2 Abstract

SdiA is a LuxR family protein found in a subset of Enterobacteriaceae, including model pathogen *Salmonella*. Unlike other LuxR encoding bacteria, those encoding SdiA have no cognate AHL synthase. Thus, these bacteria rely on foreign bacterial species for the activation of SdiA and eavesdrop on the communication of those bacteria. Despite significant effort, no relevant environment in which SdiA detects foreign AHLs has been identified. In this study, we examine three host sites as potentially relevant sites to *Salmonella* SdiA-mediated eavesdropping: house flies, mice co-infected with *Yersinia enterocolitica*, and plants. Using a combination of a genetic reporter strain and

competitive infections between wild-type and *sdiA* mutant, we find that SdiA is active within house flies. The effect of *sdiA* mutation on survival within house flies is unclear due to complex results in different genetic backgrounds. To study a possible role for SdiA in the gut of mice co-infected with *Y. enterocolitica*, we screened a barcoded transposon library of *Salmonella* in mice both with and without a co-infecting *Y. enterocolitica*. No mutant phenotypes were observed for *sdiA* or its known regulon members. Finally, we looked for SdiA activity in Angiosperms and soybeans using the genetic reporter strain and found no evidence of SdiA activity.

4.3 Introduction

As discussed in Chapter 2, the LuxR solo SdiA is conserved in a group of Enterobacteriaceae including the genus *Salmonella*. SdiA detects the AHLs produced by foreign species, leading to activation of its regulon [260]. A major limitation in our understanding of SdiA-mediated eavesdropping is lack of a clearly relevant in vivo site of activity. *Salmonella* is a highly versatile organism: colonization occurs in humans, livestock (including chickens, pigs, and cows), reptiles, wild birds, plants, and wastewater [33,34,221,316-322]. Some potentially relevant sites have been previously investigated; the literature is discussed in detail in Chapter 2. In this study, we evaluate a sparsely examined host, the insect, as a site of SdiA activity as well as the previously examined *Yersinia-Salmonella* co-infection model and a pilot study on plants.

Insects (class Insecta) are a group within the phylum Arthropoda with the following traits: six-legged, three-part bodies (head, thorax, and abdomen), compound

eyes, and antennae. Insects were initially divided by number of wings (wingless Aptera, 2-winged Diptera, and 4-winged) though modern classification is more complex [323]. For *Salmonella*, Arthropods can act as both a reservoir and transmission vector [37]. The most important hosts to *Salmonella* include flies (Diptera) and beetles (Coleoptera). In this study, we used the house fly (*Musca domestica*) based on its ease of use, availability, and previously described interactions with *Salmonella* [324-330]. We did not use the more commonly used fruit fly model as SdiA appears inactive in this host (Ahmer lab, unpublished data).

The house fly, *Musca domestica*, is a relevant pest in livestock and agriculture implicated in the transmission of *Salmonella* and other enteric pathogens [37]. *Salmonella* and other enteric pathogens are frequently isolated from house flies associated with livestock [318,324,331]. Associations between house flies and *E. coli* O157:H7 (another *sdiA*$^+$ species) have been described as well [331,332]. In 1964, Greenberg demonstrated the vector potential of house flies experimentally [333]. *Salmonella* exposed house flies were allowed to contaminate a beverage subsequently consumed by volunteers. Later, *Salmonella* was recovered from their fecal samples. In this scenario, the house fly acts as a mechanical vector: a vessel for the movement of a pathogen to a host with no underlying biological relationship in the intermediary. This stands in contrast with biological vectors, which develop more intertwined biological relationships like *Yersinia pestis* and the rat flea *Xenopsylla cheopis* [334].

Although the *Salmonella*-fly relationship is considered mechanical, some studies suggest otherwise. *S.* Typhimurium can proliferate within house flies [330,335] and horn flies

[326]. An interesting yet abandoned topic is the putative relationship between typhoidal *Salmonella* (*S.* Typhi) and insects, which is believed to have no environmental reservoir other than humans [336-338]. In 1900, a military camp undergoing an outbreak of typhoid fever was investigated, revealing an abundance of flies with internalized *S.* Typhi [336]. Greenberg later demonstrated that flies reared on contaminated media can maintain both Typhi and Paratyphi B into the larval phase and Paratyphi B survives in flies all the way into adulthood [338]. A preliminary experiment in our lab also found that *S.* Typhi CT18 could be introduced into house flies by free feeding (this method is discussed in depth below) and recovered from those flies 3 days later (N=5 flies, range 10-7,500 bacteria per fly). Additionally, another SdiA-mediated eavesdropping organism, *E. coli* O157::H7, has also been shown to be capable of multiplication in the mouthparts of house flies [339,340]. A critical missing element in establishing a biological relationship between *Salmonella* and flies is one or more genes relevant to bacterial survival on or within the fly itself. To my knowledge, no such gene has been reported and the insect as a site of SdiA relevance is almost entirely unexplored in the literature [190].

We hypothesized that SdiA promotes fitness (or transmission) of *Salmonella* in house flies through its detection of AHL-producing host microbiota and subsequent regulation of relevant proteins. If true, this would demonstrate a new dynamic in *Salmonella*'s (and possibly other eavesdroppers') lifecycle. Here we evaluate the relevance of SdiA-mediated eavesdropping in insects using the house fly as a model. In addition, we re-evaluated animals as a relevant host in SdiA-mediated eavesdropping using high throughput genetic screening (Barseq). Finally, the largely unexplored host

system of plants was evaluated by challenging commercially available plants with genetic and luciferase reporters of SdiA activity. In this chapter, we exclusively use *S.* Typhimurium in experiments, referred to as *Salmonella*.

4.4 Results

4.4.1 The microbiome of hosts

Literature evidence on the relevance of SdiA and AHL-mediated quorum sensing in different niches is discussed in Chapter 2. In examination of the potential of environments for relevance, a collaborator at Joint Genome Institute (JGI), Simon Roux, ran a bioinformatic search of metagenomes for homologs of the AHL synthase *luxI* (Table 8). Results were considered positive if at least one member of the metagenome encoded a *luxI* ortholog. These results therefore do not serve as predictors of AHL abundance but merely as suggested sites where AHLs may be. The most frequent *luxI*$^{+}$ sites are in plants, followed by fish, arthropods, and segmented worms. In humans, a mere 0.8% of the 2,364 gut metagenomes encodes at least one *luxI*. These findings support our lab's earlier assessment of AHL synthases being absent in the human gut microbiome [172], but stands in contrast with direct measurements of very low concentrations of AHLs in the human gut, suggesting synthesis by an enzyme other than LuxI [173-175].

4.4.2 Approach to infecting flies with *Salmonella*

To determine the relevance of *sdiA* in the house fly, we used a previously developed genetic tool named RIVET (recombination-based in vivo expression technology). Determining the expression of specific genes during infection is inherently difficult and several techniques have been developed [162,341]. The RIVET tool was originally developed by James Slauch and adapted for specific use in our lab [145,162,342]. To study *sdiA*, a resolvase was transcriptionally fused to the *sdiA* regulated gene *srgE*. Once transcribed, the resolvase targets and removes a tetracycline resistance gene flanked by two resolution sites, leaving one behind. The loss of this gene (and its resistance phenotype) is heritable. Thus, any activation within AHL containing environments is quantifiable as a percentage of isolates sensitive to tetracycline, aka percent resolution. A limitation of this approach is the loss of precision. Transcription that occurs either for a short period of time or continuously would register as positive. Numerous in vivo and in vitro experiments show that transcription of *srgE* and its fused resolvase is extremely dependent on both *sdiA* and AHLs, and maximal resolution hovers around 25% [143-145]. It is unknown why only ~25% of isolates resolve in activating conditions. This system is considered a reliable indicator of whether *Salmonella* detects AHLs by SdiA.

The infection protocol is summarized in Figure 10 and described in detail in the methods. In brief, a 1:1 mix of wild-type and *sdiA* mutant RIVET strains were introduced into flies by allowing them to free feed on a solution of sucrose for 2-4 hours. Infected flies were sampled back and enumerated for CFUs and competitive fitness calculations. A limitation of our methodology was the delivery of bacteria by free-feeding, leading to

no defined infectious dose. There are methods for delivering a specific volume of liquid to flies, which involves anesthetizing, immobilizing, and delivery via a pipette. As these studies only used competitive infections, which deliver identical ratios of bacteria per unit volume, we elected to use the less precise but more time efficient approach of free-feeding.

4.4.3 Infectious dose determines *Salmonella* fitness in house flies

We initially infected flies with our wild-type and *sdiA*::mTn3 (amp^r) mutant RIVET strains via infected sucrose water containing 10^8 CFU/mL *Salmonella*. For simplicity, I will refer to these concentrations as doses, though they are not strictly a dose in the traditional sense of the word. On days 1 and 3, a subset (5 flies) were collected, homogenized, and plated for enumeration of CFU and strain fitness. The per fly recovery of *Salmonella* was between 10^2 and 10^6 CFU (Fig. 11A). A significant defect in the *sdiA* mutant was observed on both day 1 and 3 (1.7-fold, Fig. 11B). The wild-type resolved in 30% of flies (9 of 30) on day 3, indicating the presence of AHLs in some flies (Fig. 11C). As expected, no *sdiA* mutants resolved. *Salmonella* burden was plotted against resolution, revealing a negative relationship between SdiA activity and burden (i.e. activation only occurs in flies with low overall burden) (Fig. 11D). There was no correlation between *Salmonella* burden and competitive index (data not shown). I hypothesized that the high feeding concentration of 10^8 CFU/mL, and presumably high inoculum, was leading to an over-representation of *Salmonella* in the fly gut compared to the host microbiota. By reducing the feeding dose, I hypothesized that fewer initial *Salmonella* may be able to

interact more dynamically with the fly gut and microbiota, perhaps increasing resolution. Additionally, if the fly gut indeed imposed a negative selective pressure on *sdiA* mutants, fitness phenotypes may be higher in a smaller initial population that could expand over the course of the infection.

The infection was repeated, reducing the feeding concentration to 10^6, 10^5, and 10^4 CFU/mL. This had little effect on *Salmonella* burden on day 1 but appears to trend the *Salmonella* burden downward by day 3 (Fig. 11A). The magnitude of the fitness phenotype was indeed altered by this change, and dramatically so (Fig. 2B). Unexpectedly however, it was the wild-type experiencing defects, not the *sdiA* mutant. In the lower doses (10^4 and 10^5 CFU/mL), there was almost no detectable wild-type in any flies. Due to the lack of wild-type isolates, resolution could not be determined to any degree of significance in these low-dose infected flies. In the few wild-type isolates I was able to recover, many were resolved (data not shown). Around two-hundred (non-*Salmonella*) isolates were screened for AHL production by cross streaking against a biosensor (wild-type *Salmonella* harboring pJNS25). No isolates were confirmed to be AHL producers (data not shown).

Two hypotheses were developed to explain the *sdiA* mutant advantages over the wild-type. One, SdiA is a toxic gene in flies (i.e. its expression leads to survival defects or death of the bacteria itself). As a transcription factor, this would likely be mediated by one or more of its regulon members. The second hypothesis is that the beta-lactamase encoded in the mutant (*sdiA*::mTn3) confers a significant advantage within the fly during carriage. This may also explain the effect of inoculum on wild-type fitness: *sdiA*::mTn3

Salmonella produce and secrete beta-lactamases to protect the wild-type which might be achieved only at high population densities within the fly itself, but this could be incorrect as fitness was unrelated to over *Salmonella* burden on days 1 and 3. This hypothesis requires that beta-lactams or beta-lactam like antibacterial compounds are in the house fly gut. The beta-lactamase hypothesis was selected for further investigation, as it seemed unlikely that SdiA would confer such toxic effects within the fly.

4.4.4 Beta-lactamase not responsible for *Salmonella* survival or *sdiA* mutant fitness in house flies

To investigate the role of beta-lactamase in *Salmonella* survival, the *sdiA*::mTn3 RIVET strain was replaced with a *sdiA*::cam RIVET strain, removing beta-lactamase from the system entirely. Flies were infected by feeding at 10^5 CFU/mL. In this competition between wild-type and *sdiA*::cam RIVET, almost no *Salmonella* could be recovered on day 1 (Fig. 12). The lack of *Salmonella* isolates prevented quantification of fitness and resolution, but these results are consistent with the beta-lactamase hypothesis.

The putative relevance of beta-lactamase to survival prompted us to engineer additional strains more conducive to the fly gut. The beta-lactamase gene and promoter from *sdiA*::mTn3 was cloned and moved in the intergenic region between *pagC* and *STM14_1502*, a commonly used site in our lab that confers no fitness defects in mouse models of infection [288]. This mutation was moved into the wild-type and *sdiA*::cam RIVET strains, generating wild-type (AMS039) and *sdiA*::cam RIVET (AMS040) strains

resistant to ampicillin and carbenicillin (Fig. 13A-D). Strain construction had no effect on RIVET function (Fig. 4E).

Using this new background, we infected flies, sampling at 2 hours as well as 1- and 3-days post infection. No *Salmonella* was recovered at 3 days. After 2 hours, *Salmonella* was only noticeably recovered in the 10^6 and 10^7 CFU/mL feeding concentrations (Fig. 5A). In flies with recoverable quantities of *Salmonella*, the *sdiA*::cam RIVET strain was defective compared to the wild-type (Fig. 14B). At 1 day, flies free-feeding on a concentration of 10^8 CFU/mL of the *bla*$^+$ background strains led to identical *Salmonella* recovery as the original strain pair (Fig. 14C). *Salmonella* was sparsely recovered from those feeding on lower concentrations, despite the presence of *bla*. Thus, beta-lactamase is unlikely to explain the fitness of *sdiA*::mTn3 RIVET *Salmonella* at low feeding doses.

The slight increase in overall *Salmonella* recovery at the 10^5 cfu/mL dose allowed us to measure strain fitness at 24 HPI. Interestingly, the *sdiA*::cam RIVET strain in the *bla*$^+$ background phenocopies the *sdiA*::mTn3 RIVET strain from the original experiment (Fig. 14D). At high feeding concentrations, the wild-type is slightly advantaged against either *sdiA* mutant. At the lower concentration (10^5 CFU/mL), either mutant will win against the wild-type, regardless of the antibiotic marker used to inactivate *sdiA*. We conclude from this that the antibiotic markers used in this study are unlikely to be the source of *sdiA* mutant fitness phenotypes in house flies, though *bla* may slightly contribute to survival.

In addition to these assays, fungal isolates from *Salmonella* infected flies were recovered by plating homogenized flies on acidified potato dextrose agar. Five unique morphologies were abundant and sub-cultured for further analysis. Their identities were not determined. To determine if any fungi made antimicrobial compounds, each isolate was grown in LB and YPD for several days at 30°C. Filtered supernatant was supplemented into fresh media at 10% v/v and inoculated with *Salmonella*. No growth defects were observed during regrowth, indicating no antimicrobial compounds present (data not shown) Fungal isolates were also cross-struck against wild-type and *sdiA*::mTn3 *Salmonella*. No zones of inhibition were observed at the intersection of fungus and wild-type *Salmonella*, indicating no antimicrobial compounds (data not shown).

These experiments evaluated my hypothesis that beta-lactamase was the source of *sdiA* mutant phenotypes in house flies. Based on the results of these experiments, this appears to be incorrect. By disproving the beta-lactamase hypothesis, my "SdiA is a toxic gene" hypothesis remains as the sole explanation for the observed results. Unfortunately, the only strain in these experiments capable of survival in house flies is the *sdiA*::mTn3 RIVET strain. Further considerations and future approaches are in the discussion section.

4.4.5 The relevance of SdiA in mammals

Currently, no strong evidence supports the notion that SdiA is relevant in mammalian gastrointestinal infection. The possibility of quorum sensing in the gut has been recently reviewed and is also discussed in Chapter 2 [171]. However, there are aspects to SdiA biology that suggest the opposite. In *E. coli* O157::H7, SdiA regulates both an

acid resistance island and virulence regulator *ler*, suggesting a role in mammalian pathogenesis [260,343]. The *S.* Typhimurium SdiA regulon includes an effector protein secreted within host cells, SrgE, that is strongly regulated by SdiA and preferably at mammalian body temperature (37°C) [138,146,166]. An outer membrane protein, Rck, is also SdiA regulated, preferably at 37°C [138,146,160]. Rck protects against complement mediated killing and mediates entry into host cells by binding epidermal growth factor receptor [234]. Neither *srgE* nor *rck* are SdiA-regulated in *S.* Typhi or *S.* Enteritidis and found only selectively within the *Salmonella* (Chapter 3 and [160,166,278,344]), suggesting this regulon module may be specific to *S.* Typhimurium or just a few serovars.

4.4.6 The genetic fitness of *Salmonella* in mice co-infected with *Yersinia enterocolitica*

It was previously found that AHL-producing gastrointestinal pathogen *Yersinia enterocolitica* activates *Salmonella* SdiA during co-infections but a *sdiA* mutant has no fitness defect in this infection model [144]. The standard approach of studying *Salmonella* gastroenteritis is an antibiotic pre-treated mouse [52]. Mice are generally regarded as resistant to *Salmonella*-mediated inflammation of the gut due to the protective effect of their microbiota (the mechanisms by which this occurs is collectively referred to as colonization resistance) [345]. A single dose of a broad-spectrum antibiotic (e.g. streptomycin) administered one-day prior to infection clears away the microbiota, allowing *Salmonella* to expand and outcompete residual microbiota [52]. The previous

study in our lab did not use any method of inducing susceptibility (i.e. antibiotic pre-treatment) and thus *sdiA* fitness was determined in a non-inflamed environment.

To re-examine this interaction, we first induced susceptibility to infection by switching the mice to a high-fat diet. This approach bypasses the need for antibiotics, which may negatively impact *Yersinia* that colonize prior to *Salmonella* [57]. On this diet, mice were infected with *Y. enterocolitica* by oral gavage. The next day, mice were infected with *Salmonella*. Rather than test mutants individually, we opted to infect with a barcoded transposon library containing ~100,000 independent insertions in the wild-type strain 14028, referred to as AMS100K (manuscript in preparation). This allowed us to examine all non-essential genes for fitness in a single assay. Both *Salmonella* and *Yersinia* burden were tracked in the feces for four days. On day 5, organs were harvested for bacterial quantification and downstream assessment of genetic fitness by sequencing (Barseq).

Based on the work of our lab and others, the high-fat diet produces an expected *Salmonella* fecal burden of ~10^7 CFU/g on day 1, rising to 10^9 CFU/g on day 3 (unpublished data and [57]). However, the burden in this experiment remained between 10^5 and 10^6 CFU/g in feces (Fig. 15A). The age of the mouse chow may have contributed to this outcome. Interestingly, the *Yersinia* co-infection drastically increased *Salmonella* burden in the feces, with a *Salmonella* burden of ~10^9 CFU/g. The mice displayed signs of severe infection toward the end of the experiment, which is unusual for Cba/J mice infected with *Salmonella* via the oral route. *Y. enterocolitica* can cause lethal infections in mice, but the median time to death is later than seen here [164].

The *Salmonella* to *Yersinia* ratio in the feces ranged between 10:1 and 100:1 throughout the study (Fig. 15A). In the gastrointestinal organs, including the cecum, large intestine, and ileum, the co-infection potentiated *Salmonella* colonization and reduced variability between mice. *Yersinia* preferentially resided in the ileum as has been previously reported [346]. In systemic organs, the co-infection aided *Salmonella* recovery from Peyer's patches, which we previously observed [144]. This is likely due to the anti-phagocytic effects of *Yersinia* virulence factors [165].

There was no apparent effect of *Yersinia* in the mutant fitness of *sdiA* or any of its regulon members, consistent with our previous study (Fig. 16) [144]. Mutants of *srgF* and *srgH* appear to have fitness defects in the gastrointestinal tract independent of *Yersinia*, but a follow-up experiment found neither gene has fitness defects in the high-fat diet mouse model (Chapter 3). Although SdiA is strongly activated by *Yersinia* in the mouse gut during co-infection [144], the interaction appears to confer no selective pressure on *sdiA* or its regulon.

Other than the SdiA regulon, we also looked for any putative *Salmonella* genes with differential fitness in the cecum after co-infection (Fig. 17). The most prominent were involved in maltose utilization. Specifically, inactivation of transport genes was advantageous in single infection but not during co-infection. We have previously observed this mutant phenotype in the cecum of mice in two other genetic screens ([113] and unpublished data). Three other genes of interest were identified: *ynfL*, *rob*, and *yaiZ*. Both *ynfL* and *rob* are transcriptional factors while *yaiZ* is a protein of unknown function.

4.4.7 No evidence of SdiA activity in commercially available angiosperms

Based on the findings of our metagenomic search (Table 8) and the paucity of studies on SdiA-plant interactions [143,197], we screened commercially available Angiosperms for SdiA activity using RIVET strains. A collection of Angiosperms was purchased from Lowe's. Plants used in this experiment included Leeks, Parsley, Oregano, Sage, Tomato, and Cauliflower plants as well as Soybeans sourced both commercially and from a farm. Plants were inoculated with two *Salmonella* strains. One was the wild-type RIVET strain. The second was a wild-type *Salmonella* harboring luciferase reporter plasmid pJNS25, which measures the transcriptional activity of the *srgE* promoter. Plants were inoculated in the soil near the root and on leaves (both intact and wounded by a pipette tip). Activity was measured over 10 days. To measure RIVET activity, soil and leaves were sampled on days 3 and 10 and plated for *Salmonella*. No isolates resolved in any plants. To measure luciferase activity, Plants were photographed in the Kino (Spectral Instruments Imaging). There was no indication of significant luciferase activity in any plant at 1, 3 or 10 days. In summary, we find no evidence of SdiA activity in any tested plant.

4.5 Discussion

Salmonella can be isolated from a diverse set of environmental niches. SdiA-mediated eavesdropping could hypothetically occur in any of these; the relevant site of activity is not easily elucidated by our current understanding of the *Salmonella* lifecycle

alone. In the body of literature on SdiA, no host or environment has been conclusively shown to be relevant experimentally (Chapter 2). Insects, a known vector and reservoir of *Salmonella* and other Enterobacteriaceae, have not been examined in this regard. SdiA in not active in laboratory mice, but the presence of a secondary, AHL-producing pathogen such as *Yersinia enterocolitica* can induce activation. Plants, like insects, are largely understudied. A search of metagenomes revealed that insects and plant microbiomes frequently encode homologs of AHL synthase LuxI while mammals do not (Table 8). Thus, we investigated these three systems in hopes of identifying activation of SdiA and a co-occurring fitness defect.

4.5.1 Insects

To evaluate the potential of insects as a relevant site of SdiA activity in *Salmonella*, we infected house flies with *Salmonella* encoding an in vivo reporter of SdiA activity (RIVET). The resulting experiments revealed two important factors in both *Salmonella* burden and *sdiA* mutant fitness that were not initially anticipated: Infectious dose and antibiotic markers. *Salmonella* was introduced into house flies by allowing them to free feed on contaminated sucrose water for a set period. By doing so, we uncovered an odd effect of feeding concentration on *sdiA* mutant fitness: the mutant was significantly advantaged over the wild-type at lower feeding doses but neutral or slightly disadvantaged at high doses. Two hypotheses were developed to explain this effect. First, *sdiA* is a toxic gene in house flies (i.e. the genes it regulates mediate the elimination of *Salmonella* from the host). This would explain why the *sdiA* mutant is advantaged over the wild-type. The loss of this defect at high feeding doses may be due to changes that a

larger initial *Salmonella* burden may induce in the fly environment, but how this occurs is unknown. The second hypothesis was that the beta-lactamase gene that inactivates *sdiA* confers a significant fitness advantage. In support of this second hypothesis, we found that *Salmonella* recovery from flies was lost in competitions between wild-type and *sdiA*::cam RIVET strains. However, a competition between wild-type and *sdiA*::cam RIVET *Salmonella* where both strains encode *bla* on another locus led to only minor increases in *Salmonella* recovery at lower feeding doses. The *sdiA* mutant phenotypes were also identical at high (10^8 CFU/mL) and low (10^5 CFU/mL) feeding doses regardless of the antibiotic marker used. The *bla* gene alone is insufficient to explain the ability of *Salmonella* to colonize at low feeding doses and the fitness advantage conferred by the *sdiA::mTn3* mutation.

From these results, I suspect that the *sdiA*::mTn3 RIVET strain is uniquely suited for survival in the house fly. The toxic gene hypothesis is not disproven by the experiments presented in this study, and both the inactivation of *sdiA* and the presence of a beta-lactamase may be positive contributing factors. Beta-lactamase activity and inactive *sdiA* cannot explain survival, as a *bla* encoding *sdiA*::cam RIVET strain (which has both phenotypes) fails to colonize at low feeding doses. Therefore, an unknown phenotype of *sdiA*::mTn3 RIVET *Salmonella* is likely to be a fly colonization factor. The genomes of wild-type and *sdiA*::mTn3 (BA612) have been sequenced in our lab, but no mutations unique to BA612 were identified by variant analysis (unpublished data). If there is a mutation, it may have been acquired during the addition of the RIVET components to the BA612 background.

he mutation may have been acquired during the construction of *sdiA*::mTn3 RIVET from the BA612 background.

Going forward, a series of experiments may unwind these complex observations. First, the suitability of each strain in colonizing house flies can be determined by single infections of each strain and lineages (e.g. *sdiA*::mTn3, RIVET, and *sdiA*::mTn3 RIVET). By comparing the capacity of each strain to colonize, the underlying genetic factor and possible point of secondary mutation may be deduced and further examined by whole genome sequencing and SNP analysis.

Second, determining whether *sdiA* is a toxic gene in house flies through new genetic constructs. Based on the premise that the *sdiA*::mTn3 RIVET strain is a uniquely suitable colonizer, intact *sdiA* can be moved into the *pagC IG* locus by allelic exchange (this mutation was constructed in Chapter 3) into this background. As a control, a chloramphenicol resistance gene (or the *sdiA*::cam allele) can be moved into the same location. Competitive infections between these two strains would allow for quantification of *sdiA* mutant fitness independent of the genetic background necessary for colonization of house flies. If this works, further experiments can be performed by mutating *sdiA* regulon members to determine which are necessary or sufficient for the observed fitness phenotypes.

Finally, A causal relationship between the detection of AHLs from underlying microbiota and mutant defects can be established using gnotobiotic house flies. These flies would be colonized with AHL-producing isolate and a mutant unable to produce AHLs. We were unsuccessful in identifying the bacterial species responsible for SdiA

activity in these studies. Alternatively, collecting the metagenome of these flies would allow for identification of putative AHL producing microbiota. Another isolate of the same species or strain could be acquired commercially or through another researcher and used as an alternative to the uncultured native isolate, which may have issues with genetic tractability. Mutant phenotypes should be dependent on the production of AHLs by that microbiota member.

Regardless of whether *sdiA* itself is a survival factor in house flies, these studies suggest *Salmonella* does have intrinsic factors involved in colonization of house flies. Identifying these factors will require additional studies.

4.5.2 Mice

Studies from our lab have probed various mammalian hosts for SdiA activity and fitness by competitively infecting them with the wild-type and *sdiA*::mTn3 RIVET strains [144,145]. Activity has only been found in mice when they are co-infected with the AHL-producing pathogen *Yersinia enterocolitica* [144]. Mice are resistant to *Salmonella*-mediated inflammation of the gut and establish an infection without a reduction in microbiota-mediated colonization resistance. Traditionally, this is achieved by pre-treating mice with a broad-spectrum antibiotic like streptomycin, allowing *Salmonella* to induce inflammation and expand to high concentrations by day one post infection (~10^8-10^9 CFU/g in the cecum/feces) [52]. More recently, two other strategies have been developed that make mice permissive to inflammation: gnotobiotic mice colonized in low-complexity microbiomes [347] and high-fat diets [57]. In antibiotic-pretreatment models,

Salmonella inflames and reaches maximal burden within 24 hours of inoculation, a rate more like a typical human infection. The infection kinetics in the other two models are slower, establishing a fecal burden of ~10^7 CFU/g 1 day after inoculation and expanding to ~10^9 CFU/g on day 3, with inflammation occurring between 3-4 days post infection. Despite their differences in approach, both models have similar infection kinetics.

Here we used a high-fat diet as a means of inducing susceptibility as it has been used in other studies in our lab and is easier to implement than gnotobiotic mice. Unfortunately, our model failed to establish a strong *Salmonella* burden (Fig. 15A). The reason for this is unknown, but I suspect there may have been an issue with mouse chow quality. Although this weakened our *Salmonella* infection, it revealed that *Y. enterocolitica* could rescue whatever defect occurred and increase day 1 fecal burden closer to 10^9 CFU/g, exceeding our estimated day one burden (Fig. 15A). The intestinal organ burden was consistent with our observations in the feces (Fig. 15B). The co-infection also seems to facilitate colonization of the intestinal immune sites (Peyer's Patches and Mesenteric Lymph Nodes) but not in deeper systemic organs (Fig. 15C). Although not quantified here, co-infected mice were noticeably sicker by the end of the study and would likely have reached early removal criteria within 1-3 more days of infection. There are very few studies on co-infections between *Salmonella* and *Y. enterocolitica* and none involving a high-fat diet. It is possible that severity was mediated by *Salmonella*, though this is often attributed to sepsis after *Salmonella* reaches higher burdens in systemic organs than observed here. Alternatively, severity may have been caused by *Yersinia* bacteria. More study is needed to determine how these two factors,

Yersinia and high-fat diet, might work independently or together to facilitate *Salmonella* infections in mice.

The goal of this study was to identify genetic factors in *Salmonella* that contribute to survival in mice during a co-infection with *Y. entercolitica*, with special consideration to *sdiA* and its regulon. After analysis, we saw no fitness defect in *sdiA* (Fig. 16). Two members of the regulon, *srgF* and *srgH*, were defective in mice independent of the presence of *Y. enterocolitica*. When these two mutants were tested individually, this defect did not reproduce (Chapter 3). Our results concur with the findings of our lab's previous study: although *Salmonella* can detect *Y. enterocolitica* AHLs during a co-infection with SdiA, there is no appreciable advantage or disadvantage in doing so [144].

By doing a genome-wide genetic selection, we were able to identify *Salmonella* genes that may interact with *Y. enterocolitica* during co-infection other than *sdiA* and its regulon. The primary finding was genes involved in maltose utilization. Maltose is a disaccharide of two glucose molecules with an α 1-4 linkage. The utilization of maltose and maltodextrins (α 1-4 linked glucose chains larger than 4 monomers) has been previously reviewed [348]. Transport genes are encoded on two adjacent and divergent operons (Fig. 8A). Within the cytoplasm, amylomaltase (*malQ*) and maltodextrin phosphorylase (*malP*) metabolize maltose. These two genes form an operon, next to regulator *malT* (Fig. 17B). Maltose acts as a substrate for MalQ, which transfers one glucose onto existing maltose chains (e.g. maltotriose, maltosetetraose, etc), forming a larger chain and one glucose monomer. MalP, alternatively, liberates glucose monomers

from the chain as glucose-1-phosphate (which then enters glycolysis as glucose-6-phosphate via phosphoglycerate mutase).

All transport gene mutations (except *malM*) were advantageous within the cecum of *Salmonella* infected mice but neutral in *Salmonella – Yersinia* co-infected mice (Fig. 17A). MalM is a periplasmic protein of unknown function [348]. The cytoplasmic genes were neutral in both conditions (Fig. 17B). The regulator MalT (essential for expression of the transport genes) had an identical pattern to the transport genes (Fig. 17B). A previous genetic screen in our lab also suggested that maltose transport mutants were advantageous to *Salmonella* during gastroenteritis [113]. More recently, another study in our lab using the AMS100K library in mice suggested the same result, but also indicated that *malQ* and *malP* mutants may be attenuated in the same environment (unpublished data). We hypothesize *malQ* mutant attenuation in vivo could be caused by its inability to maintain maltose chain lengths, a defect reported to occur in vitro during growth on maltose [349-351].

It is unclear why mutations in the transport of maltose and maltodextrins would be advantageous in an infection. Maltodextrin is a significant component of the high-fat diet and thus is the likely source of the nutrient itself. *Y. enterocolitica* encodes maltose utilization genes. If they consumed enough maltodextrin, this may have been sufficient to alleviate the negative selective pressure imposed on *Salmonella*. Alternatively, *Y. enterocolitica* may have altered the environment in such a way to eliminate the negative selective pressure mediated by maltose transport in *Salmonella*. *Y. enterocolitica* could potentially also benefit from maltose transport mutations, though this does not appear to

be universal to all gastrointestinal pathogens. Maltose transport mutations in *E. coli* O157::H7 and *Vibrio cholerae* leads to fitness defects, not advantages, in vivo [351,352]. One hypothesis on the mechanism of maltose transport mutant fitness is that the maltose transport proteins act as a receptor of cryptic prophages encoded within the *Salmonella* genome (LamB is a known receptor of phage lambda in *E. coli*). This hypothesis has not been confirmed.

4.6 Methods

4.6.1 Bacteria strains and media

Strains and plasmids used in this study are listed in Table 2. Primers used in this study are listed in Table 3. Bacteria were grown in Lysogeny Broth (LB) or on LB agar (1.5% w/v) unless otherwise stated. For motility experiments, agar was used at a final concentration of 0.25% w/v. Antibiotics were used at the following final concentrations: kanamycin (kan) at 50 µg/mL, chloramphenicol (cam) at 30 µg/mL, ampicillin (amp) at 100 µg/mL, carbenicillin (carb) at 50 µg/mL. Diaminopimelic acid (DAP) was used at a final concentration of 100 µM. Sucrose was used at a final concentration of 10%. Anhydrotetracycline (AHT) was used at a final concentration of 0.5 µg/mL. Minimal media (M9) contained 1X M9 Salts, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.01 mM Thiamine, and trace elements [353]. N-(3-Oxooctanoyl)-DL-homoserine lactone (oxoC8) was obtained from Sigma Aldrich (Cat# O1639) and dissolved in ethyl acetate (EA) acidified with glacial acetic acid at a concentration of 0.1 mL per Liter [297]. OxoC8 was used at a final

concentration of 1 μM and acidified EA at 0.1% v/v. Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetra-acetic acid (EGTA) was used at a final concentration of 10 mM. Evan's Blue Uranine (EBU) plates were made by adding tryptone (10 g/L), yeast extract (5 g/L), NaCl (5 g/L), glucose (2.5 g/L), and agar (15 g/L) to water, autoclaving, cooling to roughly 50°C, and then adding K_2HPO_4 (40 mL/liter of 12.5% w/v), Evans Blue (1.25 mL/L of 1% w/v), Uranine (also known as sodium fluorescein, 2.5 mL/L of 1% w/v) [298].

4.6.2 House fly infections

House flies were purchased from a vendor as pupae, the penultimate stage of their life cycle. Within 2-4 days, adults emerge from the pupae. Adult flies had no *Salmonella* prior to infection (data not shown). The experimental protocol is visualized in Figure 10. Cages containing ~50 fly pupae and cotton fiber soaked in sterile 5% sucrose were assembled. To infect, the two strains used in each experiment were resuspended in 5% sucrose and applied to cotton fiber. Bacterial viability was not lost in any strain over a period of 4 hours in 5% sucrose (data not shown). The sterile sucrose cotton fiber was replaced with the infected one for a period of 2-4 hours, allowing the flies to free feed upon it. Flies were observed to interact with the fiber almost immediately upon placement. After infection, the fiber was removed and replaced with a new sterile sucrose-soaked fiber. Cages were then moved into a secondary containment system and incubated. The incubator was maintained at 27°C, 80% relative humidity in a 12-hour day/night cycle. Although this temperature is lower than what is traditionally used in SdiA experiments (normally 37°C), activity is observable in vitro at 30°C under motile

conditions [146]. At indicated timepoints, a random sampling of flies was collected from each cage for analysis (5 flies per time point).

To quantify *Salmonella*, flies were first removed from the cage and anesthetized by a short freezing period. They were then washed in a bleach solution (1%) followed by PBS to remove external bacteria. This method does not kill internal bacteria (data not shown). Flies were then individually homogenized in PBS and dilution plated on XLD kan (selecting for RIVET *Salmonella*). No fly microbiota that grow on XLD produce black colonies, a phenotype found only in *Salmonella* and a few other bacterial species (data not shown). Isolates were then patched on either LB amp (selecting for *sdiA*::mTn3) or LB cam (selecting for *sdiA*::cam) as well as LB tet (identifying resolved isolates). Fitness was calculated as the ratio of mutant to wild-type divided by in the initial mutant to wild-type ratio. Resolution (%) was calculated as the percent of each strain that did not grow on LB tet.

4.6.3 Screening insects for AHL producing bacterial isolates

House flies were purchased from a vendor as pupae. After reaching adulthood. Flies were knocked out and surface sterilized as described above, then moved into sterile phosphate buffered saline (PBS). Flies were then homogenized using sterile plastic pestles. Homogenate was dilution plated on LB Agar and MacConkey Agar. Isolates were then screened in sets of 8 using cross streak assays with 14028 + pJNS25 on LB agar. Presumptive positive plates, indicating by increased light production at the intersection of *Salmonella* and an isolate, were individually streaked against 14028 +

pJNS25 and BA612 + pJNS25 for confirmation. No isolates were confirmed to produce AHLs.

4.6.4 Strain and plasmid construction

A strain of *Salmonella* encoding beta-lactamase at the *pagC* IG locus was constructed by allelic exchange. A suicide plasmid, pAMS015, was constructed in the pTOX6 vector backbone [354]. This plasmid was constructed using Gibson assembly of four components. The first component is the backbone (vector pTOX6), amplified by primers BA3666 and BA3667 (template was purified pTOX6). The second fragment, approximately 1kb of upstream homology of *pagC*, was amplified by primers BA3668 and BA3669 (template was 14028 gDNA). The third fragment, encoding *bla* and its promoter, was amplified with BA3670 and BA3671 (template was BA612 gDNA). The fourth fragment, approximately 1kb of downstream homology from the *pagC* IG insertion site, was amplified with primers BA3672 and BA3673 (template was 14028 gDNA). The fragments were amplified by PCR with polymerase Q5, gel purified, and assembled according to manufacturer's instructions (NEB, catalog # E2611). The plasmid was transformed into TransforMax EC100D *pir*$^{+}$ *E. coli* by electroporation (Lucigen ECP09500), selecting on LB + 2% glucose + cam. The resulting plasmid, pAMS015, was moved into mating strain BW20767 by electroporation, selecting on LB + 2% glucose + cam.

Allelic exchange was performed by first mating BW20767 + pAMS015 with BA612 on LB agar containing 2% glucose. Single crossovers were selected for on M9 +

2% glucose + cam. Individual colonies were outgrown in LB + 2% rhamnose (which induces toxicity) overnight then plated on LB. Isolates were patched on LB (master plate), LB cam (confirming loss of integrated plasmid), M9 + glucose (confirming isolate is not mating strain), and LB carb (indicating acquisition of *bla*). Isolates growing on LB, M9 + glucose, and LB carb, but not LB cam, were screened for the desired mutation by PCR. the final isolate, AMS037, was selected for use.

A P22 lysate was made of *sdiA*::cam. Strain AMS3206 was made by transduction of $P22_{sdiA::cam}$ X JNS3206. To transduce, the recipient strain was mixed with the lysate at MOIs of 0.1, 1, and 10 for 25 minutes. The reaction was halted by addition of LB + 10 mM EGTA and incubated at 37°C for 1 hour. Outgrowths were dilution plated on LB EGTA cam. Individual isolates were sub-cultured twice on LB cam + 10 mM EGTA, then cross-struck against P22 on EBU plates. An isolate still sensitive to P22 and containing no residual phage were kept as AMS3206.

A P22 lysate was made of AMS037. Strain AMS039 was made by transduction of $P22_{AMS037}$ X JNS3206. Strain AMS040 was made by transduction of $P22_{AMS037}$ X AMS3206. To transduce, the recipient strain was mixed with the lysate at MOIs of 0.1, 1, and 10 for 25 minutes. The reaction was halted by addition of LB + 10 mM EGTA and incubated at 37°C for 3 hours. Outgrowths were washed 3 times in fresh LB, then dilution plated on LB Carb. Individual isolates were sub-cultured twice on LB agar + 10 mM EGTA, then cross-struck against P22 on EBU plates. Isolates still sensitive to P22 and containing no residual phage were kept as AMS039 and AMS040.

4.6.5 Growth assays

Growth was measured over time in the Spectramax i3x (Molecular Devices) in flat, clear-bottom plates (Corning, catalog # 3370). Readings of the optical density at 600nm (OD_{600}) were taken at the times indicated in each figure. Overnight cultures of strains were washed and resuspended in water, then inoculated into designated media at a dilution of 1:100 (2 µL of culture and 198 µL of media). A Breathe-easy membrane film (Sigma, catalog # Z380059) was placed over the top of each plate to allow for gas exchange. All experiments were incubated at 37°C. All growth assays were performed on at least three separate occasions.

4.6.6 Mouse experiments

All mice used in this study were six-to-eight-week-old female CBA/J mice purchased from Jackson Labs. This study used mice maintained on a high-fat diet, which confers susceptibility to inflammation and pathogen expansion in C57BL/6 [57] and CBA/J mice (unpublished data). The high-fat diet was purchased from vendor Envigo and provided three days prior to infection. Mice were maintained on the diet throughout the duration of the study. An overnight culture of *Yersinia enterocolitica* was washed and resuspended in water. A total of 10^7 CFU of bacteria in 200 µL of water was delivered to each mouse by oral gavage. The next day, an overnight culture of the AMS100K library was washed and resuspended in water. A total of 10^9 CFU was delivered in a total volume of 200 µL to each mouse by oral gavage. On each indicated day, a sample of

feces was collected and plated for bacterial burden on XLD. *Salmonella* was differentiated from *Yersinia* by color (black and yellow, respectively). On day 5, mice were euthanized by CO_2 and cervical dislocation. Organs were harvested and plated for CFU on XLD. Remaining organ homogenate was outgrown in LB Kan. gDNA was harvested from outgrowths using the Quick-DNA Fecal/Soil Microbe Microprep Kit (Zymo Research). DNA was sent for sequencing and analysis to the Deutschbauer lab.

4.6.7 Animal assurance

All animal work was performed using protocols approved by our Institutional Animal Care and Use Committee (IACUC; OSU 2009A0035) and in accordance with the relevant guidelines set forth in the Guide for the Care and Use of Laboratory Animals [308].

4.7 Figures

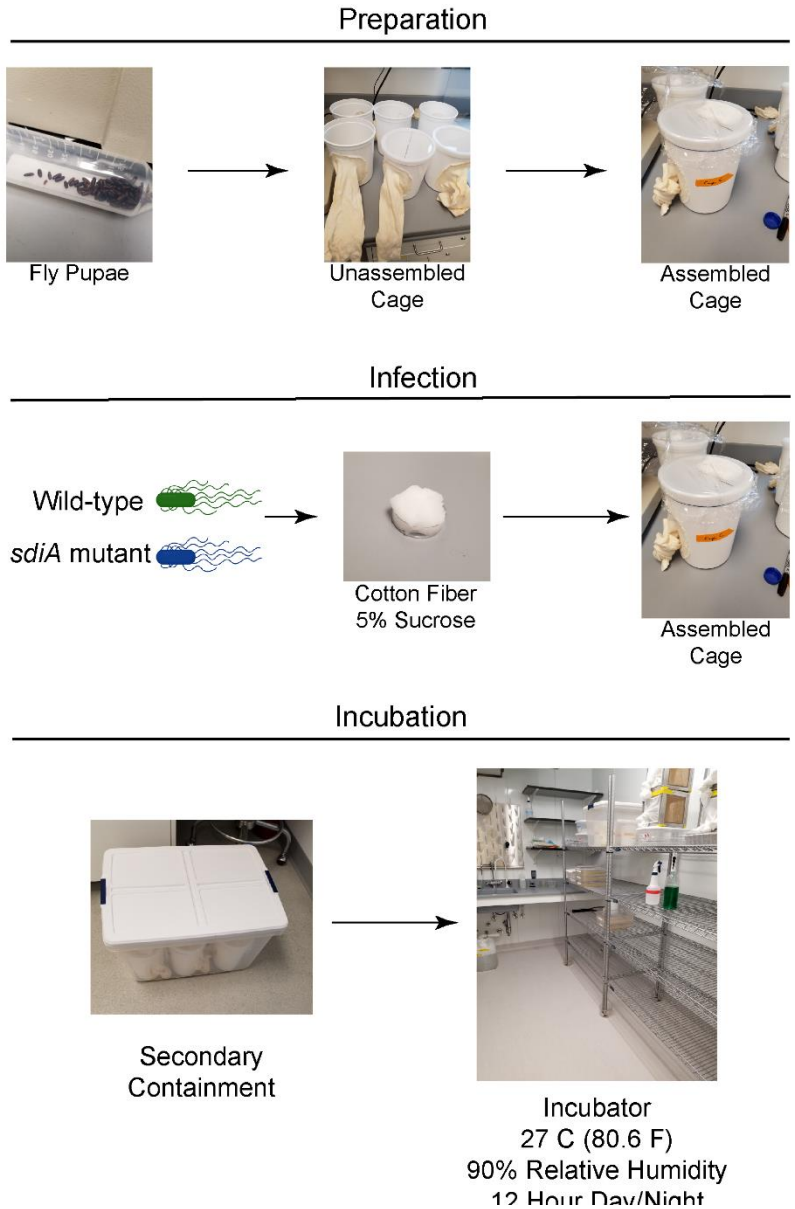


Figure 10. Overview of the fly infection protocol
Preparation. Vendor-acquired pupae were added to an unassembled cage, along with sterile 5% sucrose on cotton fiber. The nylon sock was tied to prevent escape. Cotton fiber soaked with sterile water was applied to the top of the cage and kept in place with sarin wrap and a rubber band. This forms the assembled cage. Infection. A 1:1 mix of strains was resuspended in 5% sucrose and applied to cotton fiber. The cage's sterile sucrose is removed and replaced with the contaminated sucrose. After 2 hours, the contaminated sucrose was removed and replaced with new sterile sucrose. Incubation. Cages were kept in the incubator in a secondary containment vessel to reduce risk of escape.

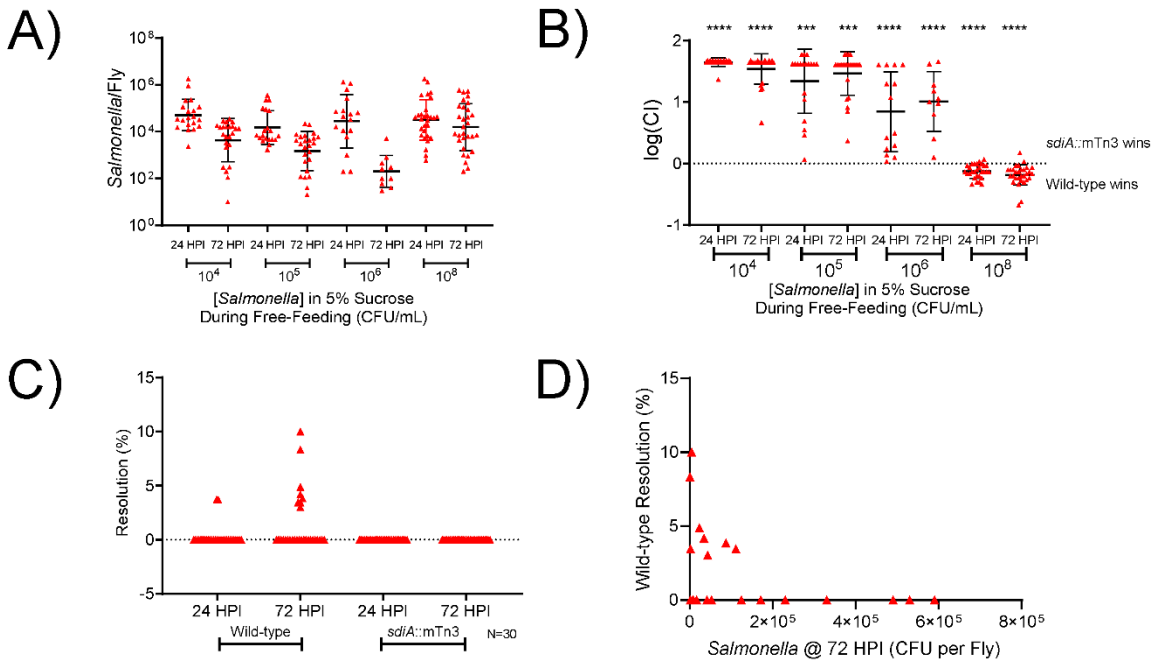


Figure 11. Free-feeding inoculum concentrations alter *Salmonella* fitness in house flies.

Cages of adult house flies were infected by allowing them to free feed on sucrose containing RIVET *Salmonella* (wild-type and *sdiA*::mTn3 in a 1:1 ratio) in concentrations ranging from 10^4 to 10^8 CFU/mL. Flies were sampled back out at 24 and 72 hours post infection (HPI). A) Total recovery of *Salmonella* (wild-type and mutant) from each fly from each feeding concentration. B) Competitive indices (mutant to wild-type ratio) in each fly at the indicated time point and feeding concentration. Competitive fitness was first normalized to the initial mutant to wild-type ratio. Significance was determined using a one-sample t-test. **** $p<0.0001$, *** $p<0.001$. C) Resolution of wild-type and *sdiA*::mTn3 *Salmonella* in each fly. Resolution data comes from 10^8 CFU/mL group. D) Total burden of *Salmonella* in 10^8 CFU/mL group in each fly (X-axis) and their corresponding resolution (Y-axis). Each symbol represents one fly. Data comes from two independent experiments, with two or three cages per experiment, and five flies sampled per cage per time point.

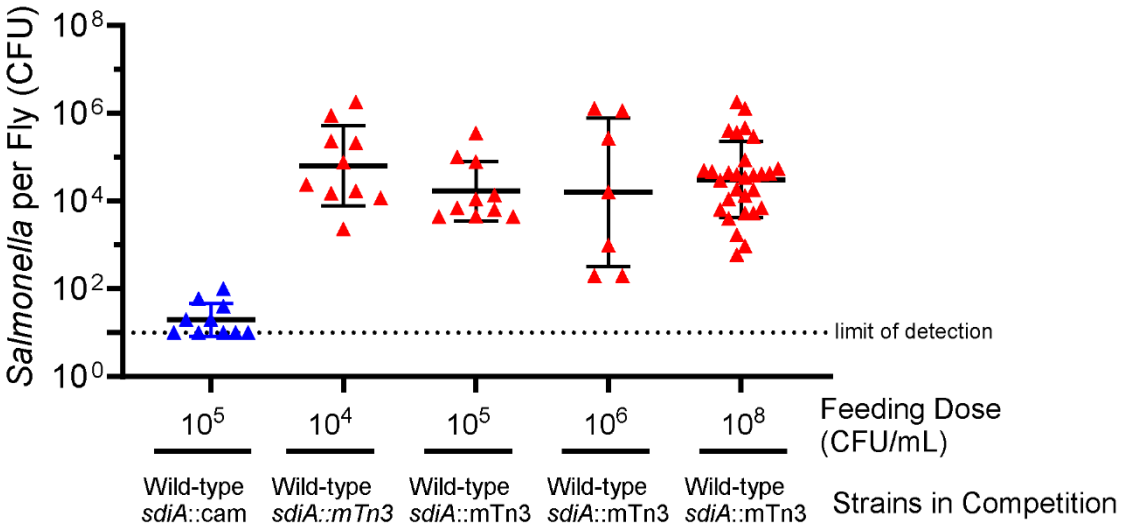
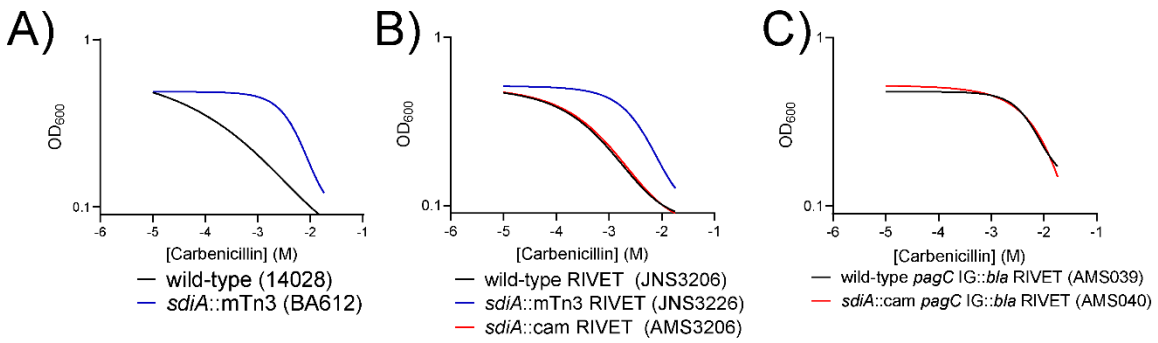


Figure 12. Loss of *sdiA*::mTn3 allele prevents recovery of *Salmonella* from house fly.

Cages of adult house flies were infected by allowing them to free feed on sucrose containing RIVET *Salmonella* (wild-type and *sdiA* mutant) in concentrations ranging from 10^4 to 10^8 CFU/mL. In red, wild-type and *sdiA*::mTn3 RIVET strains were used. In blue, the *sdiA*::mTn3 RIVET strain was replaced with the *sdiA*::cam RIVET strain. Data in red is the same as shown in Figure 2.



D)

Strain name	*sdiA* genotype	*pagC* genotype	Ampicillin IC_{50} (mM)
14028	wild-type	wild-type	0.1
BA612	*sdiA*::mTn3	wild-type	4.9
JNS3206	wild-type	wild-type	0.4
JNS3226	*sdiA*::mTn3	wild-type	3.4
AMS3206	*sdiA*::cam	wild-type	0.4
AMS039	wild-type	*pagC* IG::*bla*	5.3
AMS040	*sdiA*::mTn3	*pagC* IG::*bla*	7.5

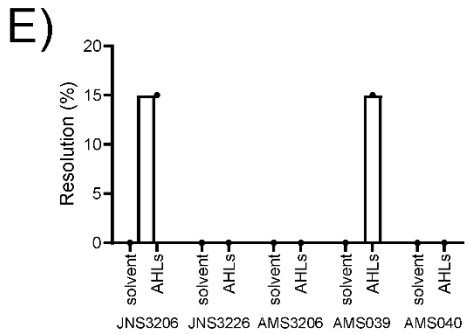


Figure 13. Engineered RIVET strains are resistant to beta lactam antibiotics.

A-C) The final OD_{600} of different strains of *Salmonella* grown in the presence of carbenicillin for 20 hours. Strains include A) ancestral wild-type (14028) and *sdiA*::mTn3 (BA612) backgrounds, B) RIVET strains used in house fly infections shown in Figures 2 and 3, C) engineered RIVET strains encoding a beta-lactamase at a neutral location on the chromosome. D) Calculated mean IC_{50} of ampicillin for strains used in house fly studies. E) Resolution of RIVET strains after growth in the presence of AHL (oxoC8)

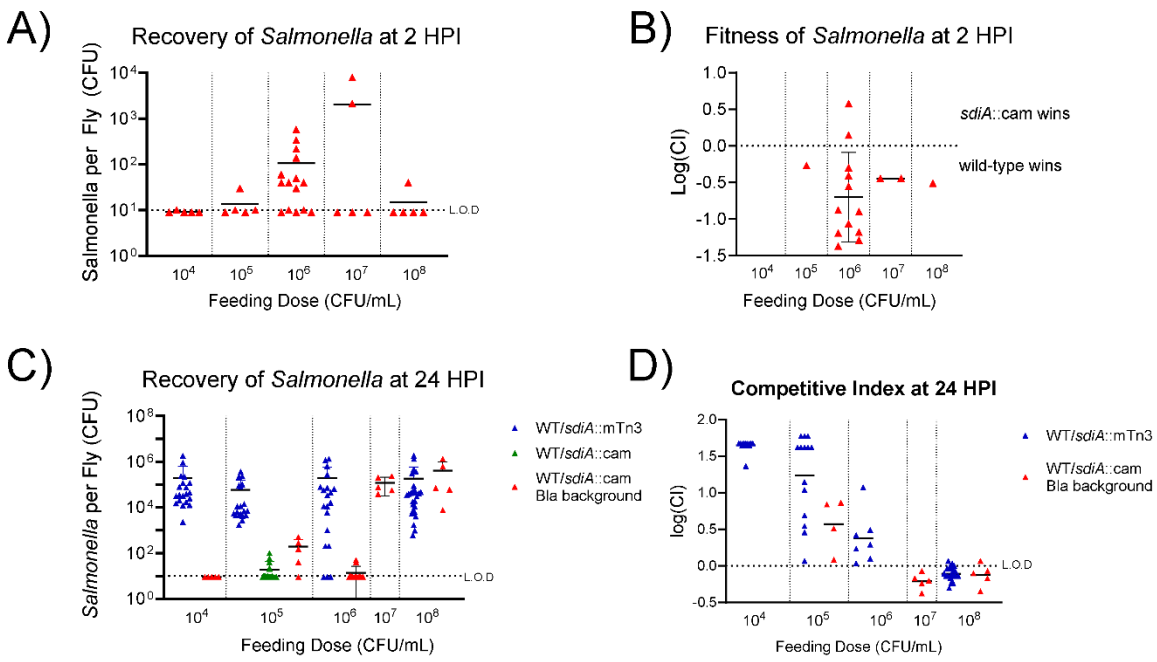


Figure 14. Beta-lactamase is not sufficient for colonization of house flies.

The engineered strains of RIVET *Salmonella* encoding *bla* at the *pagC* IG locus were inoculated into house flies by free-feeding at the indicated concentration on the X-axes in a 1:1 ratio (AMS039 and AMS040). A) Total recovery of *Salmonella* at 2 hours. B) Competitive fitness at 2 hours. C) Recovery and D) competitive fitness at 24 hours of indicated strains. Data includes previous findings shown in Figures 2 and 3. Competitive indices (mutant to wild-type ratio) in each fly at the indicated time point and feeding concentration. Competitive fitness was first normalized to the initial mutant to wild-type ratio.

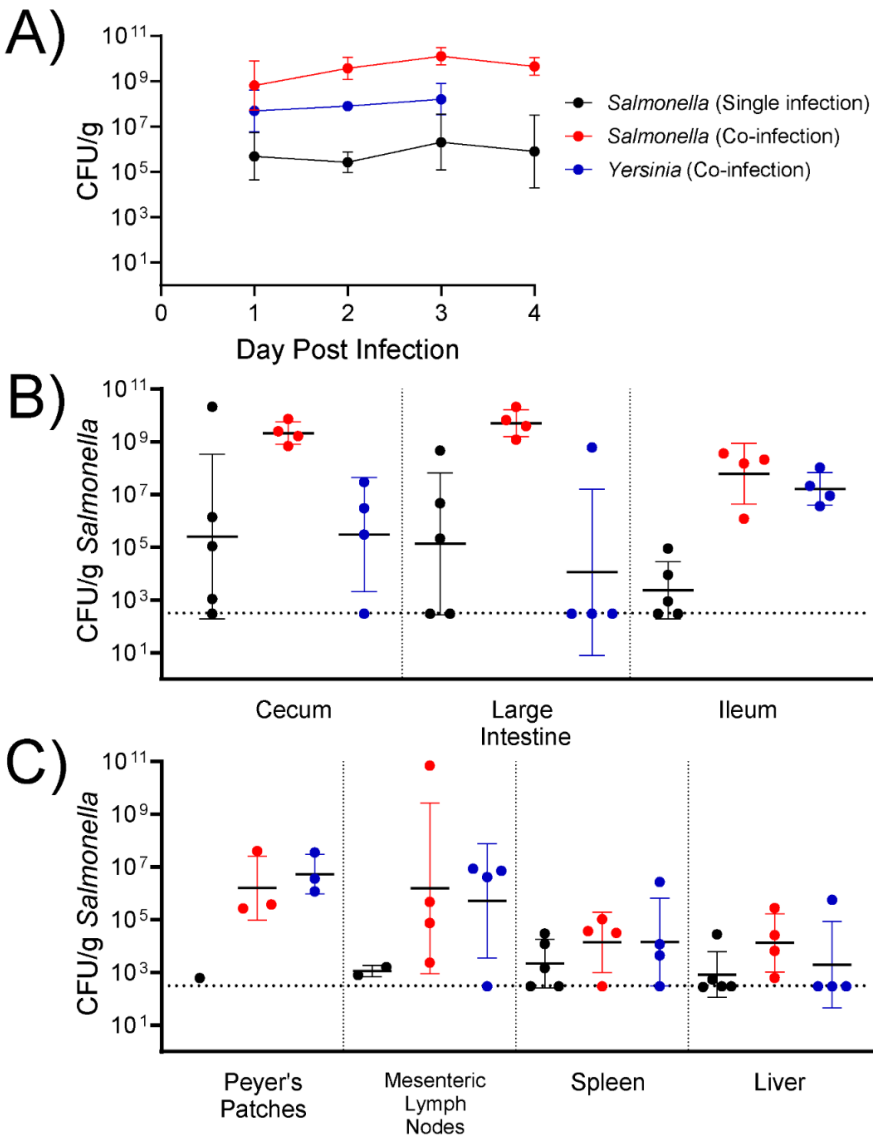


Figure 15. *Yersinia enterocolitica* co-infection facilitates *Salmonella* colonization in mice.

Cba/J mice maintained on a high-fat diet were infected with 10^7 CFU of *Y. enterocolitica* by oral gavage or not at all (day -1). One day later, 10^9 CFU of a *Salmonella* transposon library (AMS100K) was administered to each mouse by oral gavage (day 0). Burden of *Salmonella* was tracked in mice infected with *Salmonella* alone (single infection, black) or with both bacteria (co-infection, red). The burden of *Y. enterocolitica* in the co-infection was also determined (blue). A) Burdens in feces on days 1-4. B) burden of each bacteria in gastrointestinal organs on day five. C) Burden of each bacteria in systemic organs on day five.

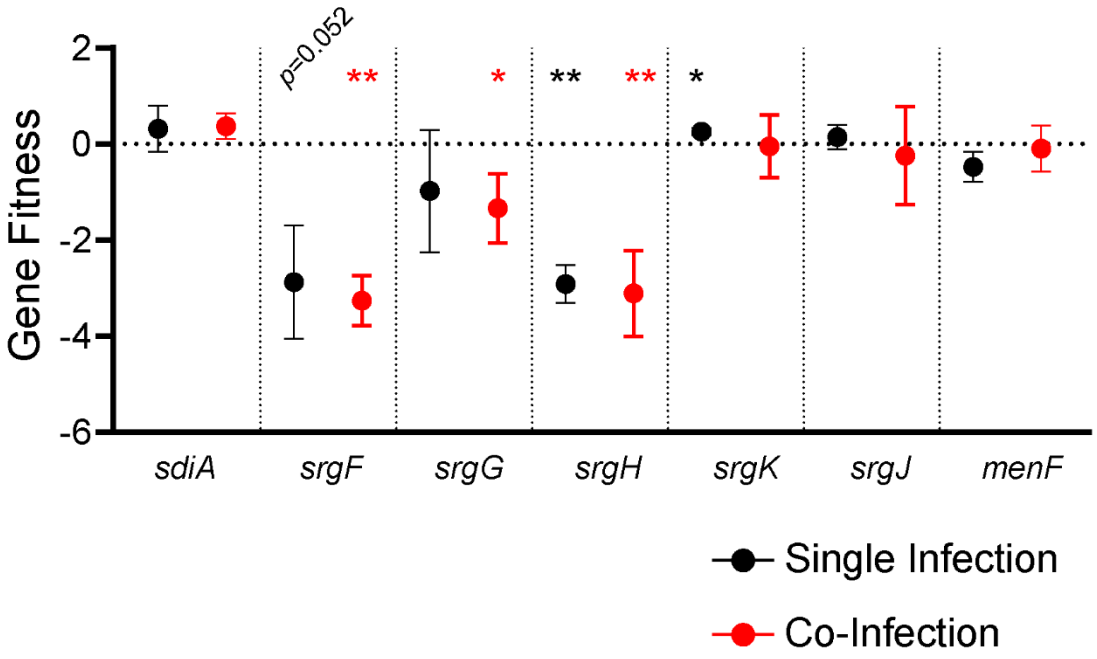


Figure 16. Fitness of *sdiA* and its regulated genes in mice.

Gene fitness, a measurement of fitness based on barcode abundance mapping to the indicated genes, of each indicated mutant in the ceca of mice infected with either *Salmonella* alone (black) or *Salmonella* and *Y. enterocolitica* (red). Negative values indicate fitness defects * $p<0.05$, ** $p<0.01$. Symbols and bars indicate mean +/- standard deviation.

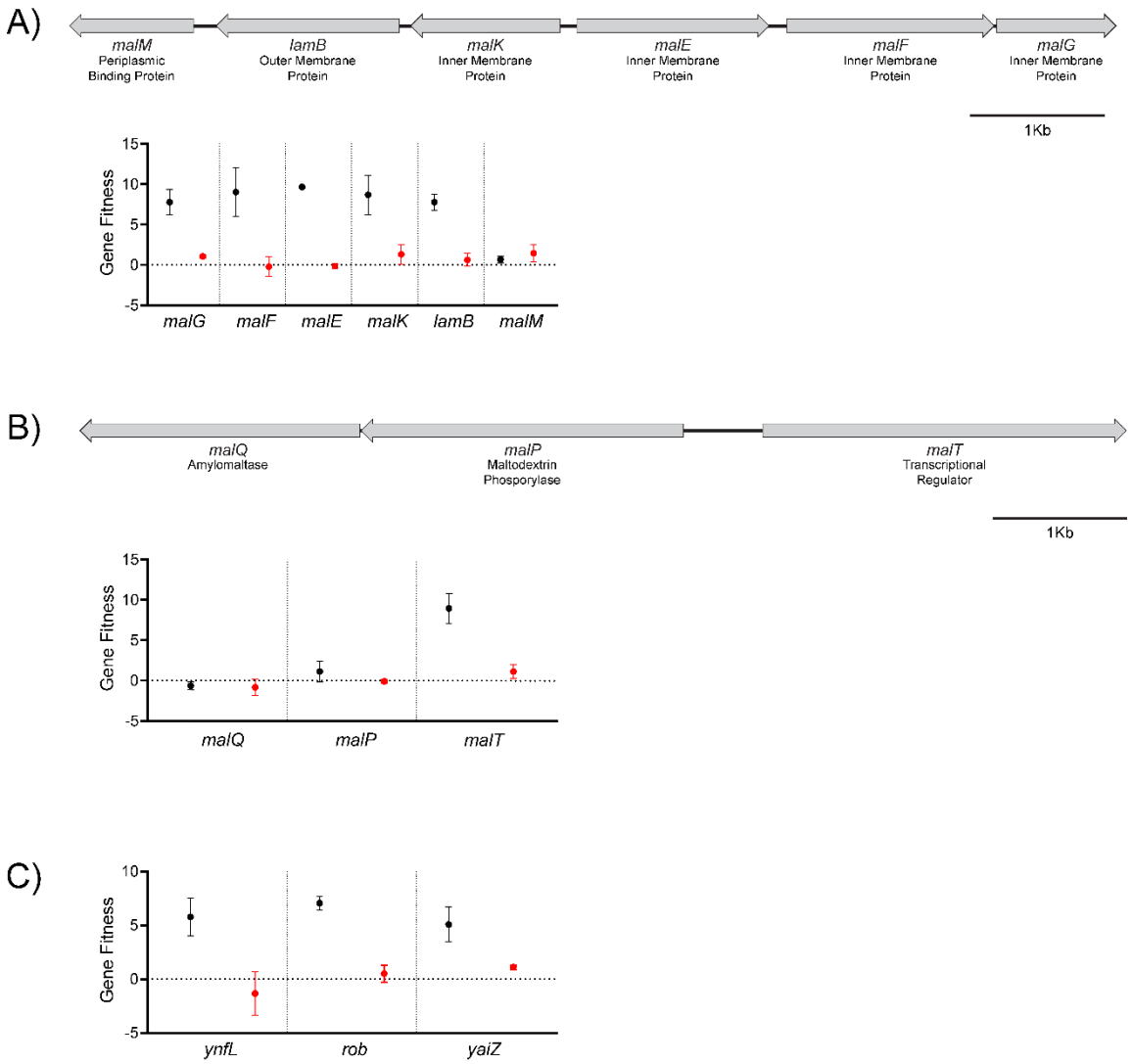


Figure 17. Maltose utilization genes exhibit *Y. enterocolitica* dependent changes in mutant fitness.

Gene fitness, a measurement of fitness based on barcode abundance mapping to the indicated genes, of each indicated mutant in the ceca of mice infected with either *Salmonella* alone (black) or *Salmonella* and *Y. enterocolitica* (red). A) a schematic of maltose transport genes in *Salmonella*, and their corresponding gene fitness. B) A schematic of the cytoplasmic components of maltose utilization and their corresponding gene fitness. C) Three genes in *Salmonella* (unrelated to maltose) which also exhibit *Y. enterocolitica* dependent changes in mutant fitness Significance was not determined for graphs in A-C). Symbols and bars indicate mean +/- standard deviation.

Table 8. The frequency of *luxI* orthologs in metagenomes.

Metagenomes of the indicated hosts were searched for homologs of AHL synthase *luxI* (*V. fisheri*). Any metagenome with at least one hit was considered positive. Searches were performed by Simon Roux of the Joint Genome institute.

Host	**Number of Metagenomes (total)**	**Number of metagenomes with 1 or more LuxI homologs**	**Percent of metagenomes with 1 or more LuxI homologs**
Annelida (segmented worms)	149	33	22.1%
Arthropoda (Digestive)	124	21	17.0%
Arthropoda (Other)	231	67	29.0%
Birds	38	6	15.8%
Fish	11	6	54.4%
Human (Digestive)	2,364	20	0.8%
Human (Skin)	674	121	18.0%
Human (other)	356	4	1.1%
Non-human mammal (Digestive)	734	24	3. 3%
Non-human mammal (other)	21	8	38.0%
Plants (Phyllosphere)	572	138	24.1%
Plants (Rhizome)	180	138	76.7%
Plants (roots)	630	392	62.2%
Plants (other)	62	20	32.2%

Table 9. Strains and plasmids used in this study

Strain	**Genotype or Description**	**Source, Construction, or Reference**
ATCC 14028 (14028)	Wild-type *Salmonella enterica* subspecies *enterica* serovar Typhimurium strain 14028	American Type Culture Collection (ATCC)
JNS3206	*14028 zjg8103::res1-tetRA-res1srgE10-tnpR-lacZY (kanr)*	[145]
JNS3226	*BA612 zjg8103::res1-tetRA-res1srgE10-tnpR-lacZY (kanr ampr)*	[145]
sdiA::cam	*14028 sdiA::cam*	[311]
AMS3206	JNS3206 *sdiA::cam*. Made by transduction. P22*sdiA*::cam X JNS3206	This Study
AMS037	*14028 pagC IG::bla*	This Study
AMS039	JNS3206 pagC IG::bla. Made by transduction. P22AMS037 X JNS3206	This Study
AMS040	AMS3206 pagC IG::bla. Made by transduction. P22AMS037 X AMS3206	This Study
BA612	14028 *sdiA*::mTn3	[139]
JB580v	*Wild type Yersinia enterocolitica Serogroup O:8; Nalr yenR*	[355]
BW20767	*E. coli RP4-2tet::Mu-1kan::Tn7-integrant uidA(deltaMlu1)::pir+ recA1 creB510 leu-63 hsdR17 endA1 zbf-5 thi*	ATCC
Plasmids		
pJNS25	Luciferase reporter plasmid of P_{srgE} from 14028	[146]
pAMS015	Suicide vector for construction of strain AMS037	This study
pTOX6	Suicide vector backbone	[354]

Table 10. Primers used in this study

Primer	**Sequence**	**Description**
BA3666	ACAGGACACTTGGTATACGT	pTOX6 linearization primer
BA3667	TTTCTTGCCGCCAAGGATCT	pTOX6 linearization primer
BA3668	ATCGGACCGCGGCCGCTAGCACGTATACCAAGTGTCCTGTTAATGACATGTTTTTAGCCG	pAMS015 construction primer
BA3669	GAGAGAAAAGACAGGCAGGTACGTGTCACTGGTAAAGAAGCCCTGTTTTATTGACTGGCG	pAMS015 construction primer
BA3670	CTTCTTTACCAGTGACACGTACCTGCCTGTCTTTTCTCTCAATTCTTGAAGACGAAAGGG	pAMS015 construction primer
BA3671	CGAAGGCGGTCACAAAATCTTGATGACATTGTGATTAATTGGATTTTGGTCATGAGATTA	pAMS015 construction primer
BA3672	AATTAATCACAATGTCATCAAGATTTTGTGACCGCCTTCGCATATTGTACCTGCCGCTGA	pAMS015 construction primer
BA3673	TCTTGATCCCCTGCGCCATCAGATCCTTGGCGGCAAGAAATTAAGAAATGCCTGAAGACT	pAMS015 construction primer

Chapter 5: Mannitol can be used to inhibit a *mtlD* mutant of *Salmonella enterica* serovars Typhimurium and Typhi in mouse models of gastroenteritis and systemic infection

5.1 Contributions

The work presented in chapter 5 is a manuscript accepted for publication in the *Journal of Bacteriology*. I am the first author of this work, in collaboration with the co-authors listed below. I would like to thank Drs. Anice Sabag-Daigle and Erin Boulanger for their work on the topic of sugar-phosphate toxicity and mannitol sensitivity that is not included in this work. I also would like to thank Dr. Sheryl Justice for her assistance in experiments on uropathogenic *E. coli* that were not included in this work. I would also like to thank Sabrina Lamont and Daniel Wozniak for providing *Pseudomonas aeruginosa* and guidance in its growth and manipulation.

Andrew Schwieters[a,b], Allysa L. Cole[b,c,d], Emily Rego[a,b], Chengyu Gao[e], Razieh Kebriaei[j], Vicki H. Wysocki[b,f,g], John S. Gunn[b,c,h], and Brian M. M. Ahmer[a,b,i,#]

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[#]Corresponding author.

5.2 Abstract

The ability to treat infections is threatened by the rapid emergence of antibiotic resistance among pathogenic microbes. Therefore, new antimicrobials are needed. Here we evaluate mannitol-1-phosphate 5-dehydrogenase (MtlD) as a potential new drug target. In many bacteria, mannitol is transported into the cell and phosphorylated by MtlA, the EIICBA component of a phosphoenolpyruvate-dependent sugar phosphotransferase system. MtlD catalyzes the conversion of mannitol-1-phosphate (Mtl-1P) to fructose-6-phosphate, which enters the glycolytic pathway. Mutants lacking *mtlD* are sensitive to mannitol due to accumulation of Mtl-1P. Here, we constructed *mtlD*

mutants in four different bacterial species (*Cronobacter sakazakii*, *Pseudomonas aeruginosa*, five serovars of *Salmonella enterica*, and three strains of *Escherichia coli*), confirming and quantifying their mannitol sensitivity. The quantification of mannitol sensitivity in vitro was complicated by an inoculum effect and a resumption of growth following mannitol intoxication. The rate of resumption at different mannitol concentrations and cell population densities is fairly constant and reveals what is likely a mannitol processing rate. Provision of mannitol in drinking water, or by intraperitoneal injection, attenuates infection of a *Salmonella enterica* serovar Typhimurium *mtlD* mutant in mouse models of both gastroenteritis and systemic infection. Using CC003/Unc mice, we find that a *mtlD* mutant of *Salmonella enterica* serovar Typhi is also attenuated by provision of mannitol in drinking water. Therefore, we postulate that MtlD could be a valuable new therapeutic target.

5.3 Introduction

Bacterial infections are becoming harder to treat due to the growing prevalence of antibiotic resistance, motivating the need to identify new drug targets. We highlight here the difficulties in treating *Salmonella enterica*, a Gram-negative bacterial species comprising over 2500 serovars that cause diseases ranging from gastroenteritis to typhoid fever. Non-typhoidal serovars like Typhimurium typically cause self-limiting gastroenteritis with severe inflammation lasting 4-10 days [68] and shedding of bacteria continuing for up to five weeks [69]. Serovar Typhimurium induces inflammation in the gut using two type 3 secretion systems, T3SS1 and T3SS2, encoded on separate

pathogenicity islands (SPI1 and SPI2, respectively) [74,79,356]. Inflammation is advantageous for the pathogen as it eliminates competing microbial species and generates respiratory electron acceptors such as nitrate [357] and tetrathionate [358] (reviewed in [51,71,74]). Paradoxically, antibiotic treatment can prolong shedding and worsen outcomes, presumably due to the depletion of the protective host microbiota [52,100-106]. Therefore, antibiotic use is typically reserved for those with severe illness or at risk for invasive disease, with treatment focusing instead on hydration therapy to replace lost water and salts [359].

Strains of non-typhoidal serovars are evolving rapidly to cause invasive systemic disease in Africa, where invasive disease is coincident with malaria, sickle cell disease, and AIDS [360,361]. Two sequence types (ST) currently predominate in infections leading to salmonellosis: serovar Typhimurium ST313 and serovar Enteritidis ST11 [362]. These invasive non-typhoidal *Salmonella* (iNTS) are now the most common cause of bacteremia in Africa, and they have a high prevalence of multiple drug resistance (>47% of isolates) [107,108]. The typhoidal serovars (Typhi, Paratyphi A, and a few others) are adapted to humans and cause typhoid (or enteric) fever. The case fatality rate was 10-20% prior to the discovery of antibiotics, which subsequently reduced mortality to 1% primarily through the use of chloramphenicol [363]. Between 2% and 5% of people infected with serovar Typhi become chronic carriers that shed the bacterium in their stool for years, a condition recalcitrant to chloramphenicol treatment [62,363-365]. Multi-drug resistant (and recently, extensively-drug resistant) strains of serovar Typhi have emerged and are spreading rapidly [109]. These strains are particularly concerning as they are resistant to the

once successful fluoroquinolones (e.g. ciprofloxacin) and third generation cephalosporins [366]. Every year there are more than 10 million cases of typhoid fever that result in 100,000 deaths [367,368]. The CDC and the WHO have listed both the typhoidal and non-typhoidal *Salmonella* serovars as a threat because multiple-drug resistance is prevalent and increasing among these organisms [369,370].

Sugar-phosphate toxicity is a phenomenon in which the blockade of a sugar utilization pathway, either with a mutation or an inhibitor, leads to the accumulation of a toxic phosphorylated intermediate that attenuates growth [111]. These toxicities were first observed in the late 1950s during the initial discoveries of sugar utilization pathways in *Escherichia coli* (*E. coli*) and *Salmonella* [371-376]. The phenotypic defects suffered by mutants that accumulate a toxic intermediate can vary and include both bacteriostatic and bactericidal outcomes [111]. Sugar sensitive mutants are inhibited by the presence of the sugar (e.g., *mtlD* in Fig. 18C) as opposed to those that simply cannot utilize the sugar, which are referred to as sugar negative (e.g., *mtlA* in Fig. 18C). The mechanisms underlying sugar-phosphate toxicity remain largely unknown (reviewed in [111]) and their induction as a therapeutic strategy has not been widely explored [121].

Mannitol is a sugar alcohol widely present in nature and synthesized by plants and fungi for use in osmotic regulation and redox protection. Mannitol is metabolically inert in humans [377]. In *Salmonella* and *E. coli*, mannitol is catabolized by two gene products: MtlA and MtlD (Fig. 18A). MtlA is the EIICBA component of a phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) that imports and phosphorylates D-mannitol (hereafter, mannitol), forming mannitol-1-phosphate (Mtl-1P) [124,125]. Mtl-1P is

converted to fructose-6-phosphate by mannitol-1-phosphate 5-dehydrogenase (MtlD, M1PDH) [378,379]. Some differences in metabolism among species exist, such as *Staphylococcus aureus* (which encodes a second mannitol dehydrogenase) and *Pseudomonas aeruginosa*, which appears to use an ABC transporter complex and may phosphorylate intracellular mannitol with a separate kinase or kinases [121,133,380] (Fig. 18B). The sensitivity of *mtlD* mutants to mannitol has been previously demonstrated in *Salmonella enterica* serovar Typhimurium, *E. coli*, and *S. aureus* [121,122,134,380,381]. In serovar Typhimurium and *E. coli*, toxicity appears to be bacteriostatic in vitro, though lysis has been reported in some mutants [132].

MtlD is encoded by many pathogenic species, suggesting it may be a suitable drug target against a variety of infectious bacteria [131]. However, there is limited information on the conservation of mannitol sensitivity and the degree of attenuation of *mtlD* mutants in vivo. Here, we report on mutants of *mtlA* and *mtlD* in serovars of *Salmonella* representing typhoidal, nontyphoidal, and invasive nontyphoidal (iNTS) lineages. Mutations of *mtlA* and *mtlD* were also constructed in enterohemorrhagic and uropathogenic *E. coli* (EHEC and UPEC, respectively), *Cronobacter sakazakii*, and *P. aeruginosa*. We find that all *mtlD* mutants are sensitive to mannitol and all *mtlA* mutants are unable to catabolize mannitol. We discovered that quantifying mannitol sensitivity is complicated by both an inoculum effect and the ability of *mtlD* mutants to recover from intoxication.

We previously found that serovar Typhimurium *mtlD* mutants are highly attenuated in the gastrointestinal tract of streptomycin-treated mice while in competition

against their wild-type counterpart [131]. However, the presence of the wild-type precluded the measurement of inflammation caused by the mutant. By infecting mice with only the *mtlD* mutant, we find that the provision of mannitol in the drinking water can drastically reduce both burden and inflammation. Next, we examined the potential of mannitol to treat systemic infections and find that mannitol provided either intraperitoneal (IP) or in drinking water attenuates a *mtlD* mutant in the spleen and liver. Providing mannitol in drinking water also enhanced the survival of mice infected with the *mtlD* mutant. Finally, a pilot experiment using a recently described typhoidal mouse model [59] suggests that mannitol can attenuate systemic infections by a *mtlD* mutant of serovar Typhi. In conclusion, we find that mannitol sensitivity is conserved among *mtlD* mutants, and providing mannitol to infected hosts attenuates infections in the gastrointestinal tract, spleen, and liver.

5.4 Results

5.4.1 *mtlD* mutants of four species are mannitol sensitive while *mtlA* mutants are mannitol negative.

In the 1970s, it was reported that *mtlD* mutants of *Salmonella enterica* serovar Typhimurium are sensitive to mannitol [132,134] and we recently confirmed this with serovar Typhimurium strain 14028 [131]. Mutation of *mtlD* by insertion of antibiotic resistance genes can influence mannitol toxicity phenotypes, likely by altering the expression of the downstream regulatory gene, *mtlR* (Fig. 18B) [129-131]. To avoid this issue, we constructed

non-polar, in-frame deletions of *mtlD* and *mtlA* using allelic exchange. Mutations of this type were made in several strains. For *Salmonella*, these included serovar Typhimurium strains 14028 and ST4/74, the invasive nontyphoidal (iNTS) strain D23580 (ST313 lineage 2), serovar Typhi strain Ty2, and three paratyphoid serovars (A, B, and C). We constructed *mtlA* and *mtlD* mutations in *E. coli* K12 strain MG1655 and two pathogenic *E. coli* strains (enterohemorrhagic strain 700927 (EHEC) and uropathogenic strain UTI89 (UPEC)). We also constructed mutants of *C. sakazakii* MZ0686 lacking *mtlA* or *mtlD*, as well as a mutant of *P. aeruginosa* PA01 lacking *mtlD*. Along with the phenotyping and quantification of mannitol sensitivity below, we complemented the 14028 D*mtlD2* mutation (Fig. 18D). This restored growth of the mutant on mannitol as a sole carbon source and eliminated the sensitivity phenotype, confirming that mannitol sensitivity is due to the loss of *mtlD*.

Each wild-type strain and its isogenic *mtlA* and *mtlD* mutant were assayed for in vitro growth phenotypes (Fig. 24). When grown in rich media (LB) either with or without mannitol, *mtlA* mutants grew comparably to wild-type. In the presence of mannitol, all *mtlD* mutants have growth defects by late exponential phase. In some cases (e.g. 14028, Paratyphi B), *mtlD* mutants have subtle growth defects in LB that occur during mid-exponential phase of growth and resolve by stationary phase. This growth defect may be due to the presence of mannitol in LB broth, which we measured at 67 ± 2.3 µM. In some cases (e.g. 14028, ST4/74), *mtlD* mutants grown in LB with 5 mM mannitol have decreases in OD_{600} during stationary phase. This decrease may be from lysis of

intoxicated cells, as was previously observed in nutrient broth supplemented with mannitol [132].

Each strain was also assayed in a defined minimal medium (M9) containing either glucose, fructose, or mannitol as a sole carbon source (Fig. 24). For auxotrophic strains, the media were supplemented with casamino acids and tryptophan (referred to as M9 Supp). The presence of mannitol in cultures growing on fructose inhibited the growth of all *mtlD* mutants (but not *mtlA* mutants), indicating that they are mannitol sensitive. Growth inhibition of the *mtlD* mutants is not as severe when glucose is used as the primary carbon source, possibly due to catabolite repression [126,127]. When the strains are grown with mannitol as the sole carbon source, neither *mtlA* nor *mtlD* strains can grow, indicating they are mannitol negative and that both genes are essential for mannitol utilization (Fig. 18C, 24). For strains grown in M9 Supp, mannitol increases growth of wild-type but not *mtlA* mutants. In defined media, mannitol is bacteriostatic to *mtlD* mutants even at mannitol concentrations up to 300 mM (data not shown).

While each strain background behaved similarly, there are four exceptions worth noting. First, the EHEC *mtlD* mutant has a partial growth defect in M9 fructose (but not M9 glucose). Second, the *mtlA* mutant of serovar Paratyphi A grows in M9 Supp with no primary carbon source added but fails to grow in M9 Supp containing 5 mM mannitol. Third, wild-type UPEC strain UTI89, but not its *mtlA* mutant, exhibits apparent sensitivity to mannitol at high concentrations (5 mM) and lyses beginning 5 hours into growth. This sensitivity in the wild-type strain is unlike that seen in *mtlD* mutants which are unable to grow at all. Interestingly, the UTI89 *mtlD* mutant exhibits a similar drop in

OD_{600} after recovering from intoxication (Fig. 25). Finally, *P. aeruginosa mtlD* mutants (both our constructed strain and a transposon mutant from the Manoil collection [382]) lack significant mannitol sensitivity when grown in LB or M9 glucose but display typical mannitol sensitivity in M9 fructose. These four observations were not investigated further in this study. In conclusion, *mtlA* is essential for mannitol catabolism and mutation of *mtlD* confers sensitivity to mannitol in all strains tested.

5.4.2 The serovar Typhimurium *mtlD* mutant is attenuated in C57BL/6 mice at systemic infection sites when mannitol is provided

The extraintestinal sites of infection are of major importance in *Salmonella* pathogenesis, particularly for the typhoidal and iNTS lineages. It has been previously noted that a *mtlD* mutant of *Staphylococcus aureus* is attenuated in the liver of C57BL/6 mice [121]. To determine if *Salmonella mtlD* mutants are attenuated during systemic infection, a competition experiment was performed. C57BL/6 mice were infected IP with 14028 *mtlA* and *mtlD* mutants together in a 1:1 ratio. The *mtlA* mutant was used instead of wild-type to avoid the wild-type gaining an advantage from utilizing mannitol as a carbon source. Mice were then treated with mannitol by two routes, IP and through drinking water. In human medicine, IV bags contain between 5% and 25% mannitol w/v (274 mM to 1.37 M), with 20% mannitol being the most common. A human dose ranges between 0.5 to 2.0 g/kg; 1.0 g/kg was used here (Fig. 19A). After four days, mice were euthanized, and the number of each mutant present in the spleen and liver was determined by plating homogenized organs (Fig. 19B). The *mtlA* and *mtlD* mutants had

equal fitness in the absence of mannitol treatment. Providing mannitol in either drinking water or by IP injection led to the *mtlA* mutant outnumbering the *mtlD* mutant in both the liver and spleen by >100-fold. These results indicate that mannitol is accessible to *Salmonella* in both the liver and spleen and that inactivation of *mtlD* confers significant, mannitol-dependent defects during systemic infections.

To determine if Mtl-1P intoxication could promote survival of infected mice, we performed infections in which each mouse was infected with only one strain. C57BL/6 mice were infected IP to initiate systemic infection and then treated with mannitol in drinking water at three different concentrations (10 mM, 100 mM, or 320 mM), or not treated (Fig. 19C). We arrived at the 320 mM concentration by using as reference the typical concentration of sugar in a can of soda. On day 4, half of the mice were euthanized for enumeration of bacterial burden in spleen and liver. The remaining mice were tracked for survival. The highest dose was required to significantly reduce bacterial burden in the liver and spleen (Fig. 19E and F). Both 100 mM and 320 mM mannitol treatments delayed mortality compared to the other groups (Fig. 19D).

The survival experiment was repeated using Swiss Webster mice (Fig. 20A). The untreated groups infected with either wild-type or *mtlD* were unable to survive past day 5. In the treatment groups, survival rates increased in a dose-dependent manner: 20% at 10 mM, 40% at 100 mM, and 60% at 320 mM mannitol (Fig. 3B). In the surviving mice, there were some residual bacteria in the spleen and liver at 21 days post-infection (Fig. 20C). In a repeat of this experiment at a 10-fold higher infectious dose, treatment significantly prolonged survival, but only one treated mouse reached the end of the study

(Fig. 20D and E). Treatment with mannitol had no apparent adverse effects on the surviving animals, even after 21 days. Swiss webster mice have an intact *Nramp1* (*SLC11A1*) gene and are more resistant to systemic infection than C57BL/6J mice, which may explain their differences in survival rates [383-385]. In conclusion, mannitol in drinking water can reduce bacterial burden, prolong survival, and reduce mortality in mice infected IP with a serovar Typhimurium *mtlD* mutant.

5.4.3 Mannitol in drinking water can prevent serovar Typhimurium *mtlD* mutant expansion and inflammation in the gastrointestinal tract

In a previous study, we determined that serovar Typhimurium *mtlD* mutants are highly attenuated in the gastrointestinal tract of streptomycin-treated Swiss Webster mice during competitive infection against the wild-type [131]. This attenuation was largely independent of the presence of mannitol in the drinking water [131]. The lack of mannitol-dependence could be due to the *mtlD* mutant having additional defects beyond mannitol sensitivity or due to the presence of mannitol in mouse chow, which we have measured at 1.6 ± 0.057 mM. An unresolved question in this competition experiment was whether the *mtlD* mutant can cause inflammation of the gastrointestinal tract [131].

To answer this question, we inoculated mice with either the wild-type or *mtlD* mutant alone. The Swiss Webster mice were pre-treated with streptomycin, which disrupts the microbiota and renders them susceptible to *Salmonella*-mediated inflammation [52]. One day later they were infected with serovar Typhimurium by oral gavage. Mannitol was provided in the drinking water. Feces were collected daily and mice were euthanized on day five for enumeration of bacteria in the cecum and for

histopathology of the proximal colon. As expected, the mice infected with wild-type *Salmonella* had high bacterial counts and severe inflammation (Fig. 41B and C). The *mtlD* mutant burden in mice treated with 320 mM mannitol dropped below the detection limit (1 CFU/mg) by day 2 and was not detected in the cecum on day 5. All treatment groups (and the untreated *mtlD* mutant) had significantly lower inflammation than the wild-type, dramatically so in the 320 mM mannitol group (Fig. 41C). In conclusion, mannitol can inhibit a *Salmonella mtlD* mutant in the gut and prevent inflammation.

5.4.4 Pilot study on the in vivo efficacy of mannitol treatment against a *mtlD* mutant of S. Typhi.

Based on our in vitro and in vivo findings above, we hypothesized that *mtlD* mutants of other pathogens could also be attenuated by mannitol during infection. We performed a small pilot study using a recently developed typhoidal mouse model [59]. CC003/Unc mice were infected with serovar Typhi by the IP route to initiate a systemic infection and then provided mannitol in drinking water at 100 mM. This treatment led to an 18-fold reduction in burden of the *mtlD* mutant in the spleen but no change in the liver or gallbladder (Fig. 22B).

5.4.5 Bacterial inoculum density and recovery from mannitol intoxication both affect IC_{50}

To determine the minimal inhibitory concentration (MIC) of mannitol, we grew each *mtlD* mutant in M9 fructose (or M9 Supp fructose) supplemented with mannitol at various concentrations. These assays revealed that intoxicated *mtlD* mutants eventually resume growth, a phenotype we refer to as recovery. The recovery phenotype occurs in a mannitol concentration-dependent manner: growth resumes faster when the mannitol concentration is lower (Fig. 23A). Additionally, beginning the growth assays with fewer cells appears to delay the onset of recovery (Fig. 23B and C). Changes in MIC from changes in initial population size, referred to as inoculum effects, have been observed in the study of antibiotics (particularly beta-lactams) [386-390]. Both phenotypes complicate the calculation of a mannitol MIC because the inhibitory concentration (IC_{50}) changes as a function of both the initial population size and the time point selection.

We first determined the effect of inoculum size on MIC. The IC_{50} of a 1:100 diluted *ΔmtlD2* mutant (AMS302, ~10^7 CFU/mL) is 27.4 µM after 20 hours of growth (Fig. 23D). When the inoculum is diluted further, the IC_{50} is reduced 6-fold at a 1:1,000 dilution (~10^6 CFU/mL), and 24-fold at a 1:10,000 dilution (~10^5 CFU/mL), confirming an inoculum effect. It should be noted that the time point used for the calculation was 4 hours later for each 10-fold dilution to compensate for the delay in reaching an equivalent OD_{600}. The time point selected for IC_{50} readings does not abolish the inoculum effect, as we calculated the IC_{50} at hourly intervals for all cultures (Fig. 23E). No matter the time point chosen, the starting inoculum size affects the IC_{50}. The IC_{50} changes over time are largely linear. This

prompted us to calculate the IC_{50} as the number of mannitol molecules per cell. These plots were also linear over time, but the slopes of the lines are quite similar, ranging between 1.2 x 10^8 molecules of mannitol per cell per hour for the highest concentration inoculum (1:100 dilution) to 4.5 x 10^8 molecules of mannitol per cell per hour for the lowest concentration inoculum (1:10,000 dilution). We propose that this represents a rate for resolving the effects of, or processing, mannitol intoxication.

In Table 13 we present the inhibitory concentration (IC_{50}) of *mtlD* mutants using either a 1:100 or 1:10,000 dilution from a washed overnight culture at the 20- or 28-hour time point, respectively, in M9 fructose supplemented with mannitol. The time point used for M9 Supp was 10 or 14 hours (for the 1:100 or 1:10,000 dilutions, respectively) to compensate for the faster growth rate and reduced lag phase of the strains. Using these criteria, the IC_{50} of mannitol for *mtlD* mutants of all species and strains tested is <50 mM. The inoculum effect and recovery phenotypes are observed in all *mtlD* mutants (Table 13 and Fig. 25). In Fig. 27, we present the data using Suppression Index [391]. Suppression Index offers quantified values for evaluating the efficacy of mannitol-dependent inhibition of growth, in which the area under the OD-time curve of treated and untreated cells is compared. Because the *Salmonella mtlD* mutant recovers from mannitol over time, the suppression index is dependent on how long the growth measurements are performed. Therefore, we calculated the suppression index as if the growth measurements had been performed for 18 different time periods and plotted the results (Fig. 27B). As expected, the suppression index increases with increasing mannitol concentrations (Fig. 27C) referring to better efficacy in a dose dependent manner. The mannitol concentration that provides

Suppression Index of 0.5 (IC_{50}) is about 12.5 mM, comparable to the value obtained in Table S3. Therefore, this may be a useful method of presenting inhibition data in situations where there are inoculum effects and recovery of growth.

5.5 Discussion

We became interested in sugar-phosphate toxicities (loosely defined) after characterizing a toxic metabolic intermediate within the fructose-asparagine utilization pathway [392]. We then reviewed the literature surrounding other sugar-phosphate toxicities [111] and tested the induction of seven of these toxicities for attenuation of *Salmonella* in the murine gastrointestinal tract [131]. Of the seven, the provision of rhamnose to a *rhaD* mutant, arabinose to an *araD* mutant, or mannitol to a *mtlD* mutant, caused severe attenuation of *Salmonella* in the gastrointestinal tract [131]. We hypothesize that any of these three enzymes could be used as a therapeutic target for the treatment of *Salmonella*-mediated gastroenteritis. However, of these three we suspect that MtlD is the most promising therapeutic target, primarily because as we show here, mannitol can reach the *mtlD* mutant of *Salmonella* at systemic sites. In contrast, injection of fructose-asparagine or rhamnose does not inhibit *fraB* or *rhaD* mutants at systemic sites, respectively (unpublished data and Fig. 26). We have not yet tested the effect of arabinose on an *araD* mutant at systemic sites. Because *Salmonella* is an intracellular pathogen, we were surprised that mannitol administered orally or IP to mice could reach *Salmonella* in the

spleen and liver. However, there are two previous publications demonstrating that, at least in tissue culture cells, mannitol in the growth medium is metabolized by intracellular *Salmonella* [393,394]. IV injection of mannitol has also been shown to attenuate a *mtlD* mutant of *S. aureus* in the kidneys and liver [121]. How mannitol gains entry to eukaryotic cells is not known.

Another advantage of MtlD as a therapeutic target is that the safety profile and pharmacokinetics of mannitol are well known. Mannitol is a natural product, synthesized by plants and fungi as a compatible solute to regulate osmolarity, and also as a storage molecule and a redox protectant [377]. In humans, mannitol is metabolically inert with 80% of the mannitol injected intravenously being secreted into the urine within three hours [115]. The osmotic properties of mannitol enable its use in medicine as an osmotic diuretic to reduce intracranial pressure/cerebral edema, to reduce intraocular pressure, or to promote diuresis in the oliguric phase of acute renal failure (OSMITROL, NDC0338-0357-03) [118]. Mannitol has also found application in the respiratory tract, both as a diagnostic for asthma and as a therapeutic to enhance mucociliary clearance in cystic fibrosis patients (reviewed in [119]). The host microbiota appears to metabolize a significant percentage of orally consumed mannitol, reducing the efficiency of uptake via the oral route [115]. While mannitol is clearly safe for humans, there are some caveats. Some polyols can cause osmotic diarrhea, intestinal bloating, or flatulence when consumed in high quantities (especially glucitol, also known as sorbitol) [395-397]. The FDA requires that any human food that may result in more than 20 g of mannitol ingestion per day, be labeled as potentially having a laxative effect. Thus, identifying the lowest concentration of

mannitol, and most effective route of delivery, will be important if this strategy is to be used to treat infections.

Overall, our data indicate that a MtlD inhibitor coupled with mannitol may be an effective therapeutic strategy in combating gastroenteritis or systemic infection caused by the non-typhoidal *Salmonella* serovars including the invasive non-typhoidal serovars that have recently emerged in Africa. It is likely that the typhoidal serovars could also be treated with this strategy. MtlD mutants of serovars Typhi, Paratyphi A, B, and C are all similar to serovar Typhimurium with regard to mannitol sensitivity. Additionally, we used a new mouse model that is permissive to serovar Typhi infection to demonstrate that mannitol can reduce the quantity of a serovar Typhi *mtlD* mutant in the spleen. Unfortunately, these mice are expensive and slow to reproduce so we have only tried one dose of mannitol by one route. The route and concentration chosen, 100 mM in drinking water, is likely not optimal. When more mice become available, we would like to test the hypothesis that a higher concentration of mannitol in the drinking water, or the use of the IV route, could more thoroughly eliminate serovar Typhi from the mice.

We constructed a *mtlD* mutant of *Cronobacter sakazakii* and confirmed that it is sensitive to mannitol. This organism can contaminate powdered infant formula and then cause lethal infections in the neonates fed the formula. The administration of mannitol and a MtlD inhibitor may be able to treat these infections, as well.

MtlD is highly conserved among the *Escherichia, Salmonella*, *Cronobacter, Streptococcus, Vibrio,* the CRE pathogens (carbapenem-resistant Enterobacteriaceae), and most of the ESKAPE pathogens including *Enterococcus*, *Staphylococcus, Klebsiella*,

Pseudomonas, and *Enterobacter* [131]. Gene presence is likely to predict drug effectiveness as MtlD is highly conserved among the genera listed above (>50% identity) and X-ray crystal structures of MtlD from a Gram-positive organism, *S. aureus*, and a Gram-negative organism, *Shigella flexneri*, reveal highly conserved NAD^+ and mannitol-binding residues as well as the catalytic triad (Lys, Asn, Asn) [121,398]. MtlD is found in only 2% of the Bacteroidota and 40% of the Firmicutes, thus inhibitors of MtlD would likely spare much of the normal microbiota in the gastrointestinal tract [131]. MtlD is a narrow-spectrum target, but not too narrow to limit utility.

Our efforts to quantify the MIC of mannitol for *mtlD* mutants were complicated by both an inoculum effect and a recovery phenotype. Inoculum effects are changes in inhibitory concentrations of compounds (e.g. antibiotics) due to changes in the initial bacterial population [386-390,399]. The IC_{50} of mannitol is reduced significantly by diluting the initial population. These inoculum effects are greatly reduced by presenting the IC_{50} as molecules of mannitol per cell rather than simply mannitol concentration (Fig. 6D). The second complication is that the bacteria recover from intoxication over time. Thus, choosing a time point for the IC_{50} calculation has large effects on the result. When we calculated the IC_{50} at every time point (hourly), we noted a linear relationship between IC_{50} and time. The slope of the line in molecules of mannitol per cell per hour provides what we propose to be a processing rate for the effects of mannitol toxicity (Fig. 23). "Processing" could represent the elimination of Mtl-1P either by cleavage, conversion to another molecule, or efflux from the cell [400]. Repairing damage caused by Mtl-1P accumulation may also be necessary. To our knowledge, antibiotic challenged cells do

not exhibit recovery phenotypes. The unique nature of this phenotype may prompt the need for alternative approaches in quantifying toxicity (i.e. processing rate rather than MICs or suppression indices). The underlying mechanisms of both intoxication and recovery are under active investigation in our lab and could inform the application of a future therapeutic.

5.6 Methods

5.6.1 Bacterial strains and media

Strains used in this study are listed in Table 11. Bacteria were routinely grown in Lysogeny Broth (LB) or on LB agar (1.5% w/v). Minimal media (M9) contained 1X M9 Salts, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.01 mM thiamine, and trace elements [353]. M9 Supp is M9 with casamino acids (final concentration 0.2%) and tryptophan (final concentration 1 mM). Sugars were supplemented into the media at the designated concentration in the text. Antibiotics were used at the following final concentrations: kanamycin (kan) at 50 µg/mL, chloramphenicol (cam) at 30 µg/mL, ampicillin (amp) at 100 µg/mL, and gentamicin at 10 or 50 µg/mL. Diaminopimelic acid was used at a final concentration of 100 µM. Sucrose was used at a final concentration of 10%. Anhydrotetracycline (AHT) was used at a final concentration of 0.5 µg/mL.

5.6.2 Construction of mutants

Primers used in this study are listed in Table 12. Deletions of *mtlA* and *mtlD* in *Salmonella enterica*, *Escherichia coli*, *Cronobacter sakazakii*, and *Pseudomonas aeruginosa* were constructed using allelic exchange. Each mutation was made by a strain-specific suicide vector made with Gibson assembly in the vector backbone pFOK (*Salmonella*, *Escherichia*, *Cronobacter*) or pEX18 (*Pseudomonas*). Each plasmid had two inserted fragments (the upstream and downstream regions of the target gene, to create an in-frame deletion) with overhangs homologous to the first or last 30 nucleotides of the deleted gene. The final product encodes the first and last 10 amino acids in each gene. We identified regions upstream, downstream, and within the *mtl* locus to act as sites of conserved overlap homology (sequences were conserved in all strains within the species at those specific sites). This reduced the number of primers needed for construction of mutations in different strains. However, for each strain, a unique suicide plasmid was made. The *Salmonella* upstream *mtlA* fragment was amplified with primers BA4111 and BA4113 and downstream fragment with primers BA4114 and BA4112. The upstream *mtlD* fragment was amplified with primers BA4111 and BA4115 and downstream fragment with primers BA4116 and BA4112. The *E. coli* upstream *mtlA* fragment was amplified with primers BA4127 and BA4128 and downstream fragment with primers BA4129 and BA4120. The upstream *mtlD* fragment was amplified with primers BA4117 and BA4118 and downstream fragment with primers BA4119 and BA4120. The *Cronobacter* upstream *mtlA* fragment was amplified with primers BA4143 and BA4144 and downstream fragment with primers BA4145 and BA4148. The

upstream *mtlD* fragment was amplified with primers BA4143 and BA4146 and downstream fragment with primers BA4147 and BA4148. The *P. aeruginosa* upstream *mtlD* fragment was amplified with primers BA4138 and BA4136 and downstream fragment with primers BA4137 and BA4135.

The pFOK vector was linearized by PCR with primers BA3875 and BA3876. The pEX18 vector was linearized with primers BA4130 and BA4131. Vector and fragments were purified by gel extraction, quantified by nanodrop, and assembled according to the manufacturer's instruction (NEB cat # E5510). Product was transformed into TransforMax EC100D pir+ *E. coli* by electroporation (Lucigen ECP09500), selecting on LB kan at 50 mg/mL (pFOK) or LB gent at 10 mg/mL (pEX18). Plasmids were confirmed by PCR, purified from EC100D pir+ cells and moved into mating strain Jke201 by electroporation (LB DAP kan or LB DAP gent at 10 mg/mL). Allelic exchange was performed by mating Jke201 + plasmid with a recipient strain on LB DAP. Exconjugants were isolated on LB kan or LB gent at 50 mg/mL. Isolates were outgrown without selection in LB and dilution plated on LB AHT sucrose (pFOK) or LB sucrose (pEX18). Individual colonies were screened for loss of vector resistance to identify isolates in which the vector has recombined out of the chromosome. Mutants were distinguished from wild-type by screening on M9 Mtl which identifies both *mtlA* and *mtlD* mutants. Auxotrophic strains were screened on M9 Supp Mtl. Mutations were confirmed by PCR.

5.6.3 MtlD complementation plasmid

The complementation plasmid of MtlD was constructed into the low copy number vector pWSK29 (amp^r) [401]. The *mtlD* gene of *Salmonella* strain 14028 was amplified by PCR with primers BA4123 and BA4124. The PCR product was cloned into pCR2.1 (TOPO, kan^r). This plasmid was digested with EcoRI to remove the *mtlD* insert and ligated into the EcoRI site of pWSK29 with T4 ligase, transformed into competent *E. coli*, selecting on LB amp. Isolates were screened for insertion and orientation using BA2473 and BA4124. The confirmed plasmid (pAMS394) and vector (pWSK29) were transformed into 14028 *ΔmtlD2* (AMS302) by electroporation. Isolates were selected and maintained on LB amp.

5.6.4 Growth assays

Growth was measured over time in the Spectramax i3x (Molecular Devices) in flat, clear-bottom plates (Corning, catalog # 3370). Readings of the optical density at 600nm (OD_{600}) were taken at the times indicated in each figure. Overnight cultures of strains were washed and resuspended in water, then inoculated into designated media at a dilution of 1:100 (2 µL of culture and 198 µL of media). For further dilutions, washed cultures were serially diluted in water 10-fold before inoculating. A breathe-easy membrane film (Sigma, catalog # Z380059) was placed over the top of each plate to allow for gas exchange. All experiments were incubated at 37°C. All growth assays were performed on at least three separate occasions.

5.6.5 Minimum inhibitory concentrations

Inhibitory concentration (IC_{50}) was determined for each strain by growth assays in M9 or M9 Supp (M9 + 0.2% casamino acids + 1mM tryptophan) + 5 mM fructose. Mannitol was added in a series of concentrations varying by two-fold. Each strain was grown overnight in LB, washed and diluted in water, then inoculated into the media. Growth was measured every hour (measuring OD_{600}) at each time designated in each experiment. IC_{50} was calculated by nonlinear regression analysis, using normalized growth, bracketed by a no mannitol control representing maximum growth (100%) and the no growth control (0%) at the specified time. In each assay, the highest concentration used was sufficient to prevent growth of the culture (recovery) for the time point used in the calculation. All MIC assays were done on at least 3 independent occasions.

5.6.6 Systemic infections with *S. enterica* serovar Typhimurium

Six-to-eight week old C57BL/6 and Swiss Webster mice were acquired from Jackson Labs and Taconic Farms, respectively. Overnight cultures of strains designated for each experiment were washed and resuspended in water. After diluting to the desired concentration, mice were infected by intraperitoneal injection (IP) in a total volume of 200 µL. Mice were monitored daily for weight loss and early removal criteria, including weight loss >20%.

In the competition experiment using C57BL/6J mice, where specified, mice were treated, or not, with mannitol by either IP injection or drinking water. For IP treatment, one dose per day for three (days 1-3) of 1 g/kg were delivered to each mouse. For drinking water treatment, mannitol was supplemented into their drinking water to specified final concentrations, beginning after infection (day 0). Mice were euthanized on day 4. Homogenized liver and spleen were dilution plated on LB kan and LB cam for determining wild-type and mutant burdens. Competitive index (CI) was calculated as the ratio of mutant to wild-type divided by the initial mutant-to-wild-type ratio.

In the competition experiment using C57BL/6J mice testing rhamnose-dependent fitness, the experiment was performed identically as the mannitol experiment above except different strains were used and rhamnose was used instead of mannitol. Rhamnose was delivered IP at 1 g/kg per day for three days and drinking water treatment containing rhamnose at 100 mM was provided beginning after infection.

In the survival study using C57BL/6J mice, where specified, mice were treated or not with mannitol in drinking water. For drinking water treatment, mannitol was supplemented into their drinking water to the specified final concentrations beginning after infection (day 0). On day 4, half of each group was euthanized for determining bacterial burden. Homogenized liver and spleen were plated on LB. The remaining mice were monitored and euthanized upon reaching removal criteria.

In the survival studies using Swiss Webster mice, where specified, mannitol was provided in drinking water. For drinking water treatment, mannitol was supplemented into their drinking water to the specified final concentrations beginning after infection

(day 0). After infection, mice were monitored using the same criteria. Mice reaching the end of the study (day 21) were euthanized for determining *Salmonella* burden in the liver and spleen. Homogenized organs were plated on LB.

5.6.7 Gastroenteritis infections with *S. enterica* serovar Typhimurium

Six-to-eight week old Swiss Webster mice were acquired from Taconic Farms. Mice were pre-treated with 20 mg in 200 µL of streptomycin, delivered by oral gavage (day -1). One day later, mice were infected with *Salmonella* by oral gavage in 200 µL water (day 0). Where specified, mice not treated or treated with mannitol by drinking water, supplemented into their drinking water to specified final concentrations beginning after infection (day 0). Feces were collected daily for 4 days, homogenized, and plated on XLD for quantification. On day 5, mice were euthanized, and ceca were collected for determining *Salmonella* burden. Proximal colon was collected, stored in formalin, and sent to HistoWiz (Brooklyn NY, USA) for histopathology, which was analyzed without knowledge of group conditions (e.g. control vs treatment).

5.6.8 Systemic infections with *S. enterica* serovar Typhi

CC003/Unc mice were bred in-house and used at 7 weeks of age. 2×10^4 CFU of *Salmonella enterica serovar* Typhi were delivered IP in a total volume of 200 µL of PBS. Where indicated, mice were provided 100 mM mannitol in their drinking water beginning

immediately after infection (day 0). On day 6, mice were euthanized, and organs (liver, spleen, and gallbladder) were harvested for enumeration of CFU by plating on LB agar.

5.6.9 Animal assurance

All animal work was performed using protocols approved by our Institutional Animal Care and Use Committee (IACUC; OSU 2009A0035) and in accordance with the relevant guidelines set forth in the Guide for the Care and Use of Laboratory Animals [308].

5.6.10 Quantification of mannitol in LB and mouse chow

D-Mannitol was purchased from Sigma Aldrich (MO, USA). D-Mannitol($^{13}C_6$) was purchased from Cambridge Isotope Laboratories (MA, USA). Optima LC/MS grade formic acid, water, and acetonitrile were purchased from Fisher Scientific (MA, USA). Precellys lysing kit was purchased from Bertin Technologies (France).

For quantification of mannitol in mouse chow, 20 mg of sample and 1 mL DCM/Methanol/water (3:2:1; v/v/v) extract solution were added to 2 mL Precellys lysing kit (Bertin, France). Each sample was homogenized at 6800 RPM for 4 cycles (30 s per cycle with 45 s pause) using Bertin Precellys Homogenizer (Bertin, France). The homogenized sample was sonicated in a water bath for 10 minutes at room temperature and followed by centrifugation at 10,000 rcf for 5 minutes. To minimize matrix effect, 2 µL of aqueous phase extract was diluted 50 times with water and spiked with 1 ppm

internal standard. External calibration was prepared in water with spiked internal standard at 1 ppm. Five microliters of calibration and samples were analyzed by LC-MS/MS.

For quantification of mannitol in LB, 20 µL broth and 1 mL DCM/Methanol/water (3:2:1; v/v/v) extract solution was added to a 2 mL Eppendorf tube. The sample was vortexed for 20 seconds, sonicated in water bath for 10 minutes, and followed by centrifugation at 10,000 rcf for 5 minutes at room temperature. The aqueous phase was transferred out and diluted 20 times before preparing the standard addition. The individual calibration was prepared by spiking 0.5, 1, 2, 3, 4 ppm of D-Mannitol in the diluted aqueous extract. The internal standard of D-Mannitol($^{13}C_6$) was spiked in each calibration levels at 1 ppm. Finally, five microliters of the standard addition levels were analyzed by LC-MS/MS. Both calibration curves and samples were analyzed in triplicates.

The quantification was carried out on a Vanquish UHPLC coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher, MA, USA). The analytes were separated on a Accucore C18 2.6 µm 2.1 × 100 mm column using the binary solvents of water with 0.1% formic acid (v/v) (solvent A) and acetonitrile with 0.1% formic acid (v/v) (solvent B). The gradient was: 0–1 min, 2% B; 1–3 min, 2 %–5 % B; 3–5 min, 5%–50 % B; 5–6 min, 50-95% B; 6-8 min, holding at 95 % B; 8-8.01 min, 95 %–2% B; 8.01-10 min, holding at 2% B. The flow rate of 0.3 mL/min. The following mass spectrometer instrument settings were used: ion source = H-ESI; positive ion = 3500 V; sheath gas = 35; aux gas = 7; ion transfer tube temperature = 320°C; vaporizer temperature = 275°C; HCD collision energy = 60%; RF lens = 60%. The mannitol (205.0683 m/z) and

mannitol($^{13}C_6$) (211.0884 m/z) were detected by tMS^2 mode between 0-9 minutes. Both external calibration and standard addition curves demonstrated great linearity with R^2 > 0.99.

5.7 Figures

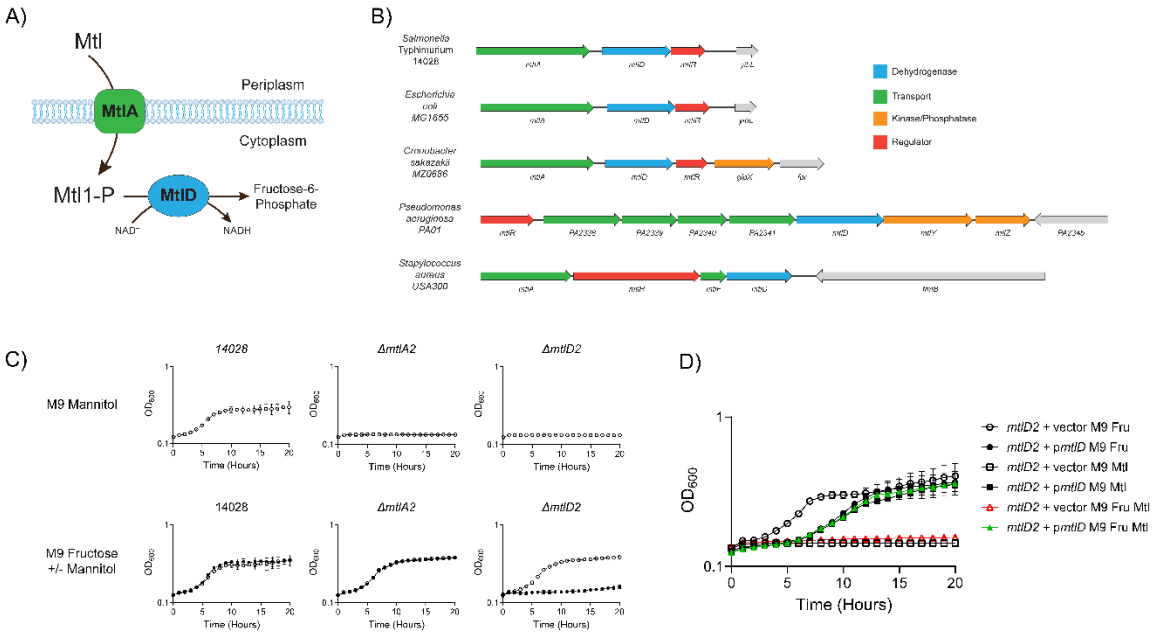


Figure 18. Mannitol catabolism and sensitivity in bacteria

A) Schematic of mannitol catabolism in *E. coli* and *Salmonella*. Periplasmic mannitol is imported into the cytoplasm by MtlA (in green, the EIICBA component of a phosphoenolpyruvate-dependent sugar phosphotransferase system), producing Mannitol-1-Phosphate (Mtl-1P). Mtl-1P is oxidized to fructose-6-phosphate by D-Mannitol-1-phosphate 5-Dehydrogenase (MtlD, Blue), generating NADH from NAD^+.

B) Mannitol utilization loci in *Salmonella enterica* serovar Typhimurium (14028), *Pseudomonas aeruginosa* (PA01), *Staphylococcus aureus* (USA300), *Escherichia coli* (MG1655), and *Cronobacter sakazakii* (MZ0686).

C) Growth of *Salmonella* wild-type (14028), D*mtlA2* (AMS300), and D*mtlD2* (AMS302) in M9 + 5 mM mannitol (top row), or M9 + 5 mM fructose + /- 1 mM mannitol (bottom row). In the bottom row, open symbols are M9 fructose and closed symbols are M9 fructose mannitol.

D) Plasmid complementation of D*mtlD2* mutation restores function. Growth of D*mtlD2* mutant carrying plasmid-encoded *mtlD* (pAMS394) or vector (pWSK29) in M9 + 5 mM fructose, M9 + 5 mM mannitol, or M9 + 5 mM fructose and 1 mM mannitol

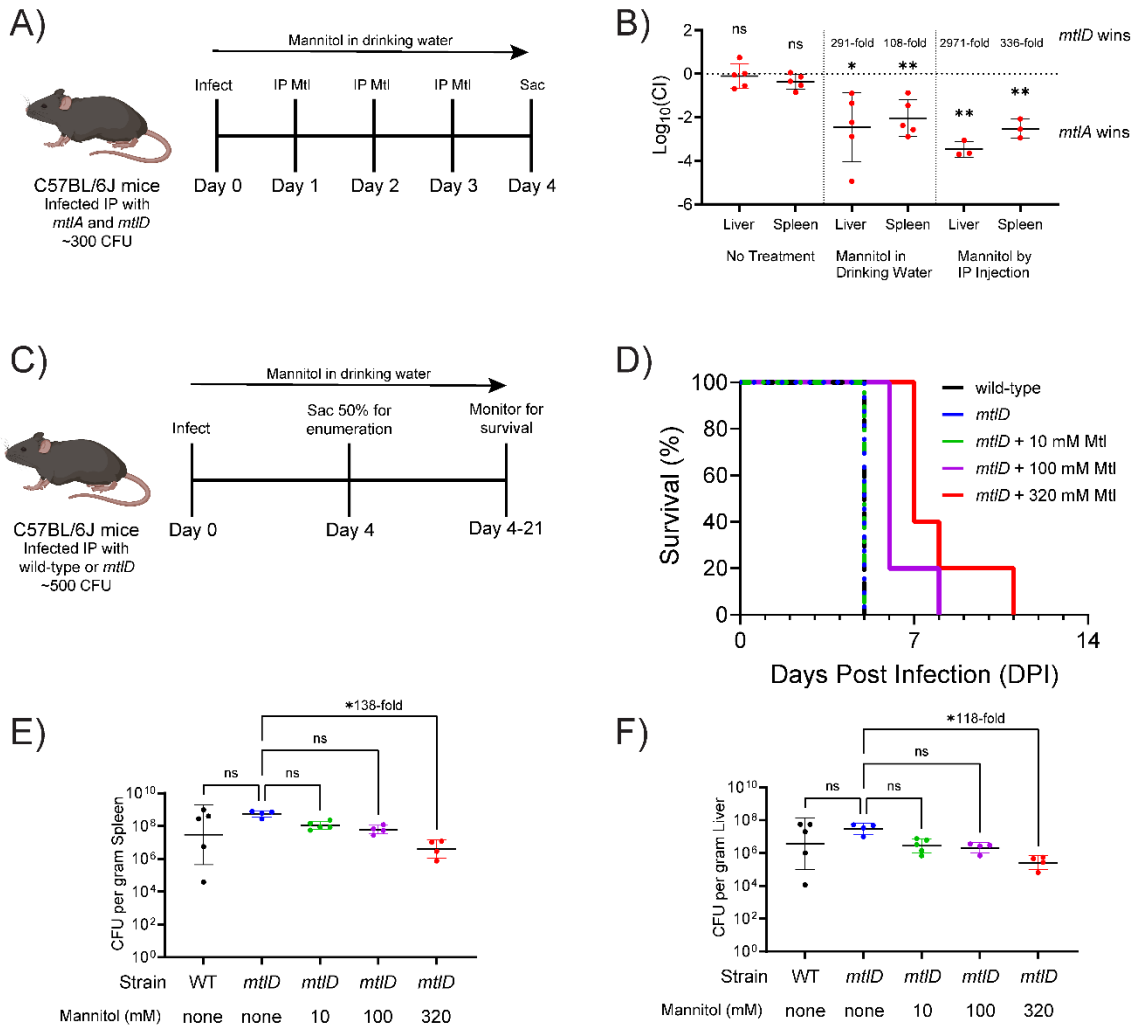


Figure 19. A *Salmonella enterica* serovar Typhimurium *mtlD* mutant is attenuated during systemic infection of C57BL/6 mice when mannitol is provided.

A, B) Groups of five C57BL/6 mice (female) were inoculated with a 1:1 ratio of *mtlA1*::*cam* (EFB036) and D*mtlD1* mutant (AMS276) by the IP route, totaling 300 CFU. One group was provided mannitol (100 mM) in their drinking water immediately after infection for the duration of the experiment. Another group was provided mannitol (100 µL of 1 M, equivalent to ~1 g/kg) by the IP route on days 1, 2, and 3 post-infection. A third group received no mannitol. B) On day 4, the burden of D*mtlA1*::*cam* and D*mtlD1* mutant serovar Typhimurium in the spleen and liver was determined by dilution plating on LB cam (*mtlA*) and LB kan (*mtlD*) to distinguish the two strains. The competitive index is plotted, calculated as $\log_{10}$ of the *mtlA* to *mtlD* ratio, normalized to the initial ratio (0.8:1). Statistical significance was evaluated using a one sample, two-tailed t test. * $P < 0.05$, ** $P < 0.01$. C-F) Groups of ten C57BL/6 mice (female) were inoculated with 500 CFU of wild-type (14028) or D*mtlD2* mutant (AMS302) serovar Typhimurium by the IP route. Mannitol was provided in drinking water immediately after infection at either 0, 10, 100, or 320 mM for the duration of the experiment. D) Kaplan-Meier plot of survival. The log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test both indicate that the 100 mM and 320 mM groups are different than the other three groups ($P < 0.01$). The Gehan-Breslow-Wilcoxon test, but not the Mantel-Cox test, indicates that the 320 mM group is different than the 100 mM group ($P < 0.05$). On day 4, five mice from each group of ten were sacrificed for enumeration of bacterial burden in the spleen (E) and liver (F). Fold-differences in burden are indicated and statistical significance was evaluated using Tukey's multiple comparison test. * $P < 0.05$.

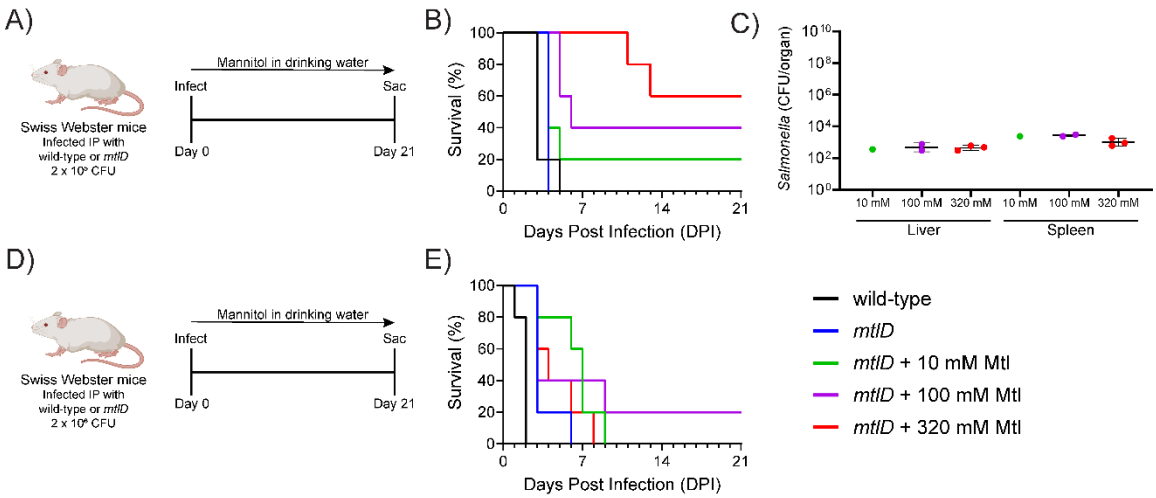


Figure 20. A *Salmonella enterica* serovar Typhimurium *mtlD* mutant is attenuated during systemic infection of Swiss Webster mice.

Groups of five Swiss Webster mice (female) were inoculated IP with 2 x 10^5 CFU (A-C) or 2 x 10^6 CFU (D-E) of wild-type serovar Typhimurium (14028) or D*mtlD2* mutant (AMS302). Mannitol was provided in drinking water immediately after infection at either 0, 10, 100, or 320 mM for the duration of the experiment. Survival was monitored over 21 days (B, E). On day 21, surviving mice from panel B were sacrificed for enumeration of bacterial burden in the spleen and liver (C). B) The Gehan-Breslow-Wilcoxon test indicates that the 320 mM group is different than the wild-type and *mtlD* groups ($P < 0.01$) and the 10 mM group ($P < 0.05$) but not the 100 mM group. The log-rank (Mantel-Cox) test indicates that the 320 mM group is different than the wild-type and *mtlD* groups ($P < 0.01$) but not the 10 mM or 100 mM groups. E) The log-rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test both indicate that the wild-type group is different than the other four groups ($P < 0.01$).

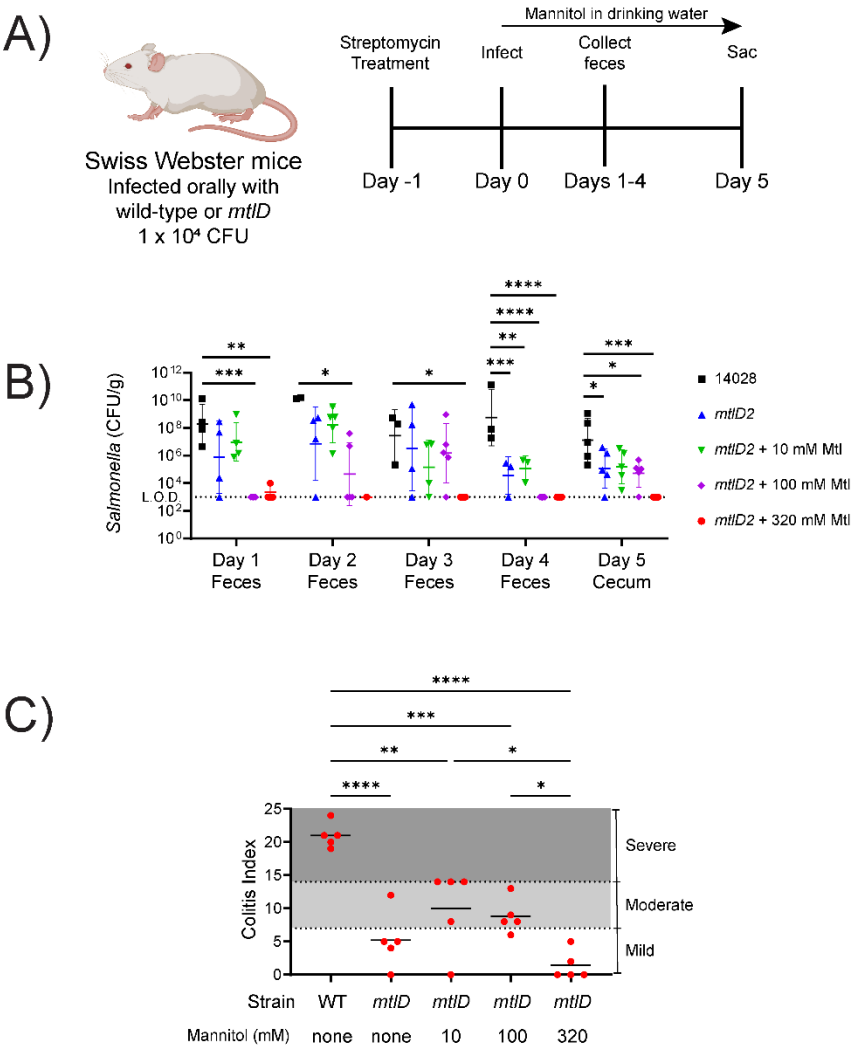


Figure 21. A *Salmonella enterica* serovar Typhimurium *mtlD* mutant is attenuated during gastrointestinal infection of streptomycin-treated Swiss Webster mice.

One day after streptomycin treatment, groups of five mice (female) were inoculated orally with 1 x 10^4 CFU of wild-type serovar Typhimurium (14028) or D*mtlD2* mutant (AMS302). Mannitol was provided in drinking water immediately after infection at either 0, 10, 100, or 320 mM for the duration of the experiment. B) Fecal samples were collected daily for enumeration of CFU. On day 5 post-infection, mice were sacrificed for enumeration of CFU in the cecum. C) Histopathological analysis was performed on the proximal colon. Statistical significance was evaluated using Dunnett's multiple comparisons test (B) or Tukey's multiple comparison test (C). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

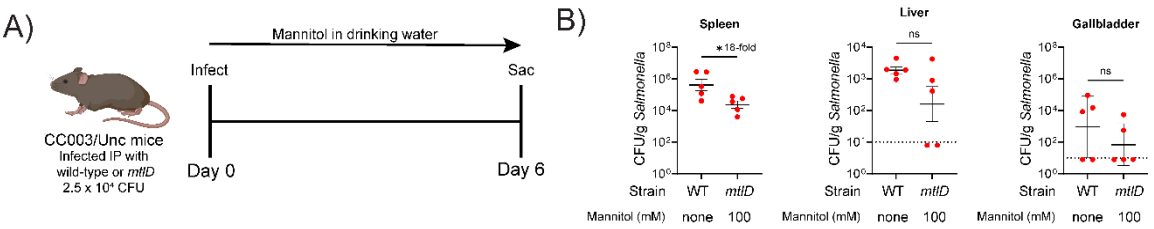
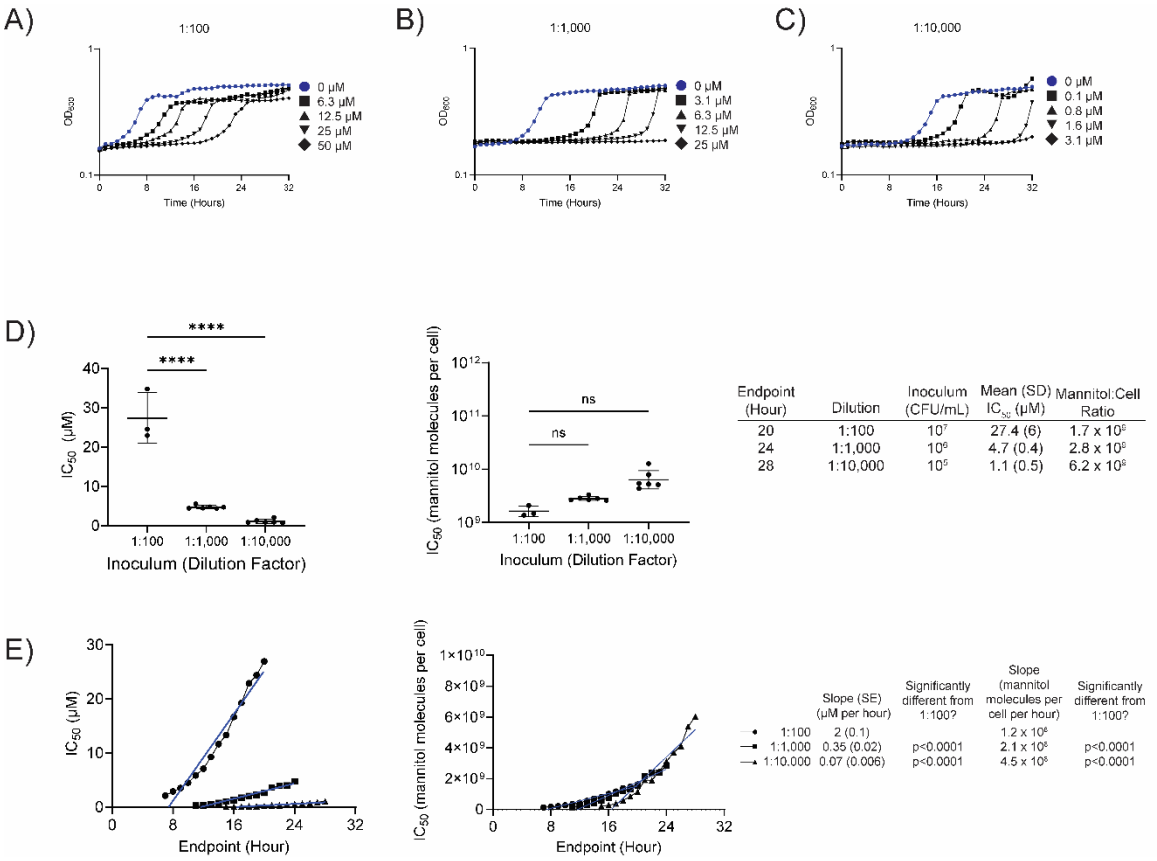


Figure 22. A *Salmonella enterica* serovar Typhi *mtlD* mutant is sensitive to mannitol in CC003/Unc mice.

A, B) Groups of five CC003/Unc mice were infected with 2 x 10^4 CFU of either wild-type *Salmonella enterica* serovar Typhi (Ty2) or D*mtlD6* mutant (AMS310) by the IP route. Mannitol was provided in drinking water immediately after infection at either 0 or 100 mM for the duration of the experiment. On day 6, mice were sacrificed for enumeration of bacterial burden in the spleen, liver, and gallbladder. Statistical significance was determined using Tukey's multiple comparison test. * $P < 0.05$



Endpoint (Hour)	Dilution	Inoculum (CFU/mL)	Mean (SD) IC_{50} (µM)	Mannitol:Cell Ratio
20	1:100	10^7	27.4 (6)	1.7 x 10^8
24	1:1,000	10^6	4.7 (0.4)	2.8 x 10^8
28	1:10,000	10^5	1.1 (0.5)	6.2 x 10^8

	Slope (SE) (µM per hour)	Significantly different from 1:100?	Slope (mannitol molecules per cell per hour)	Significantly different from 1:100?
1:100	2 (0.1)		1.2 x 10^8	
1:1,000	0.35 (0.02)	p<0.0001	2.1 x 10^8	p<0.0001
1:10,000	0.07 (0.006)	p<0.0001	4.5 x 10^8	p<0.0001

Figure 23. There is an inoculum effect and recovery from mannitol intoxication.

A-C) Growth of the *Salmonella enterica* serovar Typhimurium D*mtlD2* mutant (AMS302) in M9 + 5 mM fructose supplemented with mannitol at various concentrations. Concentrations are indicated to the right of each graph. The control (blue) contains no mannitol. The initial population of cells comes from an overnight culture washed and diluted 1:100 (1 x 10^7 CFU/mL) (A), 1:1,000 (1 x 10^6 CFU/mL) (B), or 1:10,000 (1 x 10^5 CFU/mL) (C). Growth (OD_{600}) was monitored for 32 hours. D) The IC_{50} of mannitol for the D*mtlD2* mutant (AMS302) grown in M9 fructose with units of µM (left) or number of mannitol molecules per cell (middle). The table (right) shows the time point that was chosen, the dilution, the inoculum (in CFU/mL), the mean IC_{50}, and the mannitol to cell ratio for each dilution. Statistical significance in D) was determined using two-tailed student's t-test. E) IC_{50} of mannitol for the *mtlD2* mutant (AMS302) grown in M9 fructose using different time points for three inoculum dilutions (1:100, 1:1,000, 1:10,000), with units of µM (left) or number of mannitol molecules per cell (middle). Slopes were determined by linear regression analysis. Statistical significance was evaluated using Dunnett's multiple comparisons test. **** $P < 0.0001$. The table (right) summarizes data and statistical analysis for the two graphs. SD - Standard deviation, SE - Standard error.

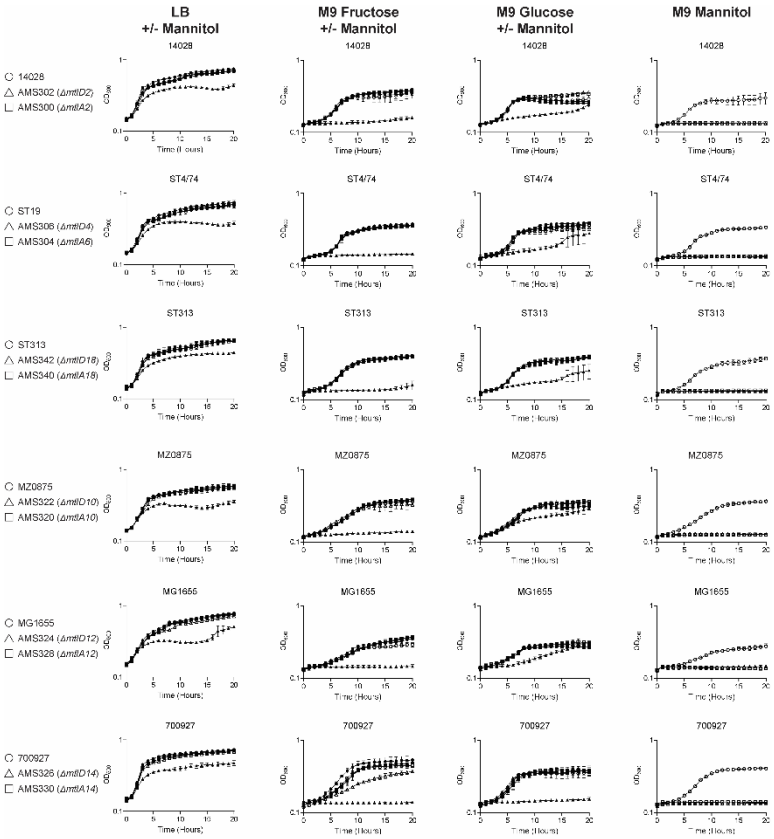


Figure 24. Growth kinetics of wild-type, *mtlA*, and *mtlD* mutants of different bacterial species and strains.

Overnight cultures of strains were washed and diluted 1:100 into different media, listed as columns in the figure. Growth (OD_{600}) was measured every hour for 20 hours. Each row is a wild-type strain (circle), *mtlA* mutant (square), and *mtlD* mutant (triangle). In growth curves performed with LB, M9 Fructose, M9 Glucose, M9 Supp Fructose, and M9 Supp Glucose media, mannitol was added at 1 mM and mannitol containing cultures are indicated as closed symbols. Fructose was added at 5 mM and Glucose was added at 5 mM. For M9 Mannitol and M9 Supp Mannitol, mannitol was added at 5 mM. For M9 Supp Mannitol graphs, open symbols are M9 Supp with no mannitol. M9 Supp contains M9 + 0.2% casamino acids + 1 mM Tryptophan.

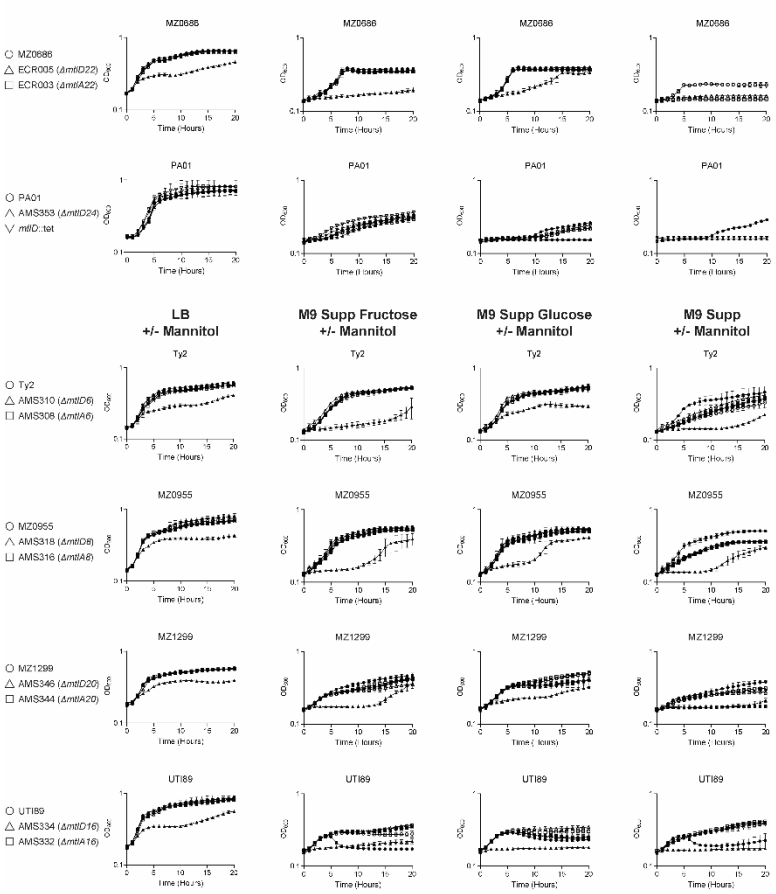


Figure 24 (cont.)

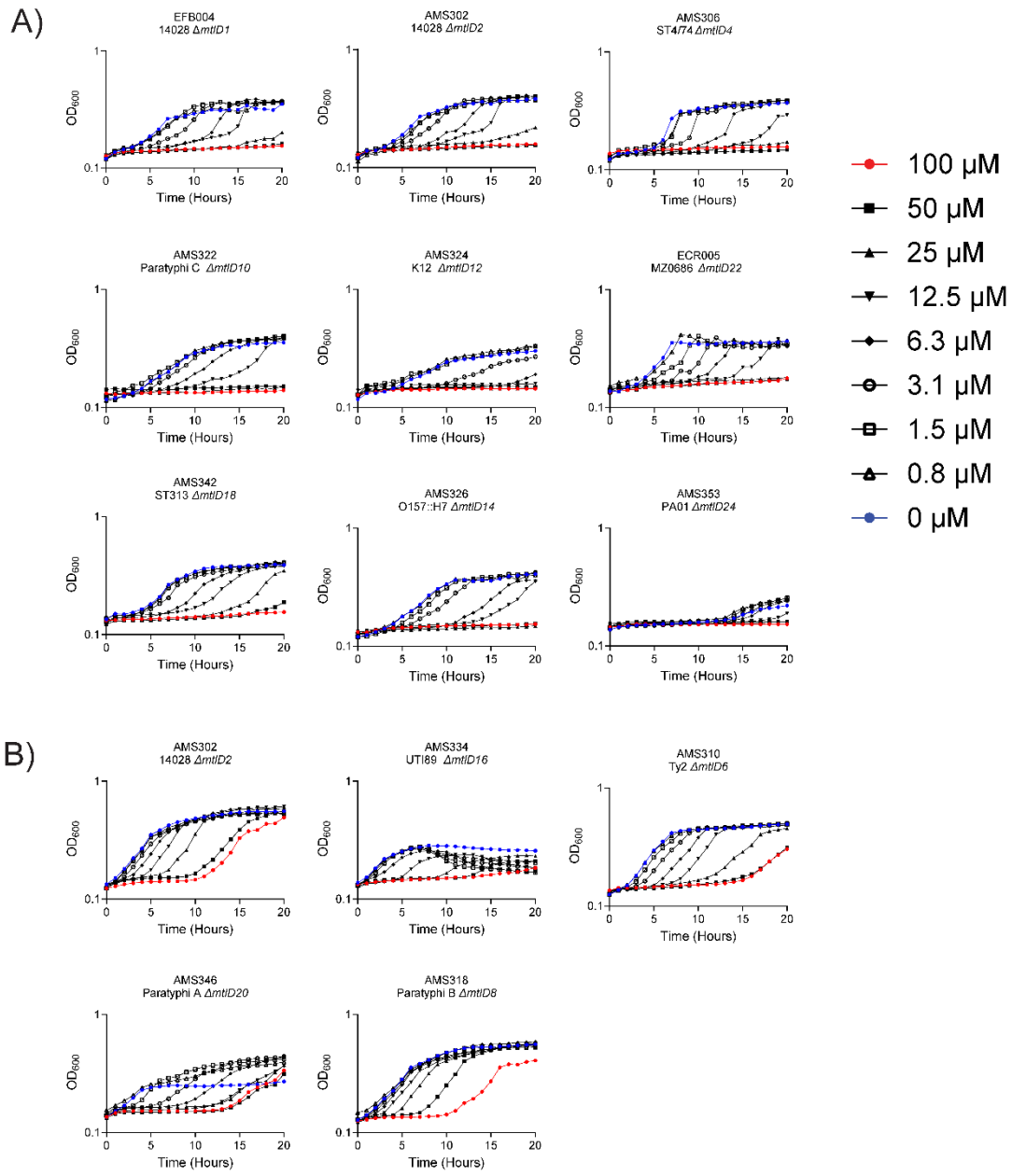


Figure 25. Recovery from mannitol intoxication is conserved among *mtlD* mutants of different bacterial species and strains.

Overnight cultures of each *mtlD* mutant were washed and diluted 1:100 into M9 + 5mM fructose (A) or M9 Supp + 5 mM fructose (B) in varying concentrations of mannitol indicated by the legend in the figure. Growth (OD_{600}) was measured every hour for 20 hours. Each strain is indicated in the title of each figure.

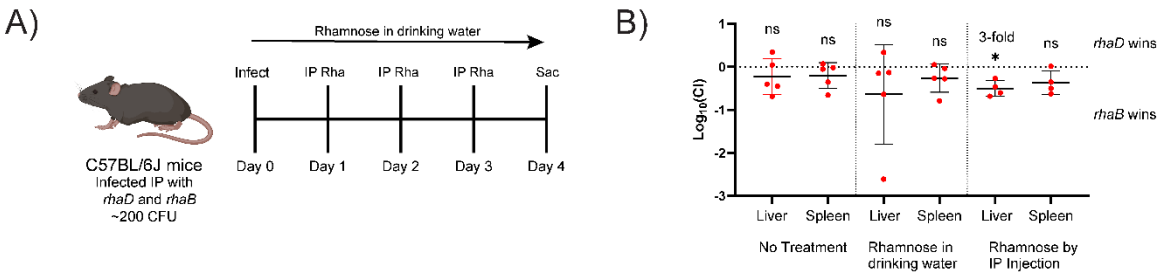


Figure 26. Treatment of mice infected with *Salmonella rhaD* mutant using IP rhamnose.

A) Groups of five C57BL/6 mice (female) were inoculated with a 1:1 ratio of *rhaB*::kan (EFB063) and *rhaD*::cam mutant (EFC015) by the IP route, totaling 200 CFU. The *rhaB*::kan mutant acts as a 'wild-type' control that cannot benefit from rhamnose as a nutrient. One group of mice was provided rhamnose in their drinking water (100 mM) immediately after infection for the duration of the experiment. Another group was provided rhamnose (100 µL of 1 M, equivalent to ~1 g/kg) by the IP route on days 1, 2, and 3 post-infection. A third group received no mannitol. B) On day 4, the burden of *rhaB*::kan and *rhaD*::cam mutant serovar Typhimurium in the spleen and liver was determined by dilution plating on LB cam (*rhaD*) and LB kan (*rhaB*) to distinguish the two strains. The competitive index is plotted, calculated as $\log_{10}$ of the *rhaD* to *rhaB* ratio, normalized to the initial ratio (1:1).

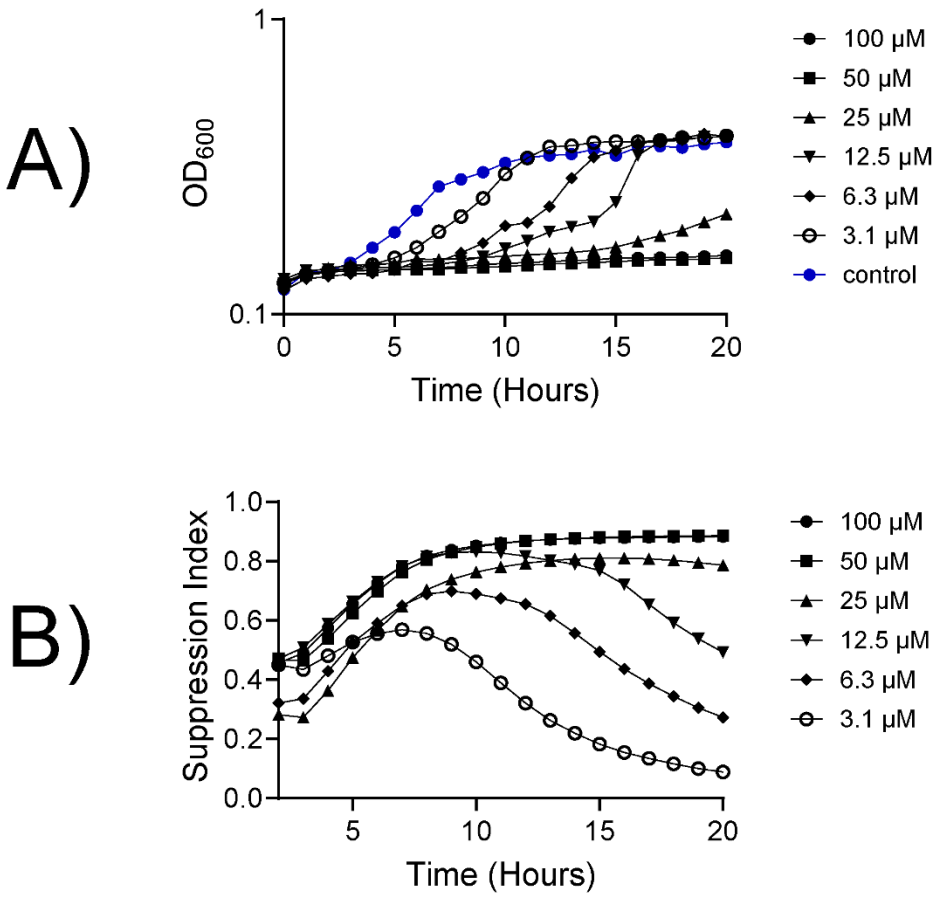


Figure 27. Suppression index for mannitol intoxicated *Salmonella mtlD* mutant (AMS302).

The suppression index calculates the ratio between the area under a growth curve (AUC) for untreated cells (control) compared to treated cells (Suppression index = ($AUC_{untreated}$ - $AUC_{treated}$)/$AUC_{untreated}$). Since *Salmonella mtlD* mutant cells recover from low concentrations of mannitol intoxication, the suppression index can be quite different depending on how long the growth curves are performed. Therefore, we took the growth curves in panel A and calculated the suppression index as if the growth curves had been performed for 1 hour, 2 hours, 3 hours, etc, and plotted each of these values (panel B). A bar graph of the suppression index for 20 hours of growth is shown in panel C. Statistical significance was evaluated using Dunnett's multiple comparisons test. * $P < 0.05$, **** $P < 0.0001$.

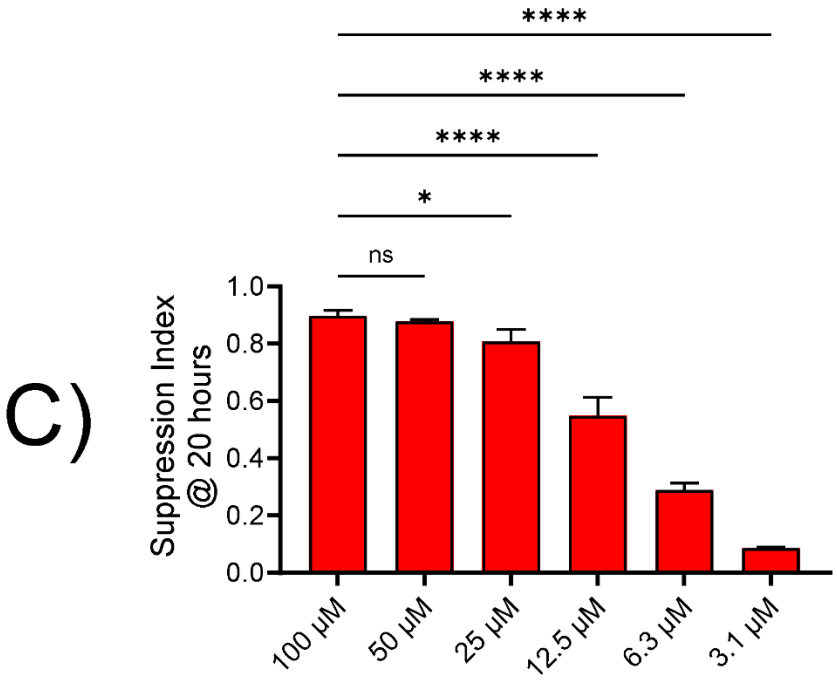


Figure 27 (cont.)

Table 11. Strains and plasmids used in this study.

Strain	**Genotype or Description**	**Source, Construction, or Reference**
ATCC 14028 (14028)	*Salmonella enterica* serovar Typhimurium	American Type Culture Collection (ATCC)
ST4/74	*Salmonella enterica* serovar Typhimurium	[402]
Ty2	*Salmonella enterica* serovar Typhi	John Gunn
D23580	*Salmonella enterica* serovar Typhimurium ST313 lineage 2	Jay Hinton [403]
MZ1299 (SGSC4902)	*Salmonella enterica* serovar Paratyphi A	Michael McClelland
MZ0955 (SGSC4150)	*Salmonella enterica* serovar Paratyphi B	Michael McClelland [404]
MZ0875 (SGSC2290)	*Salmonella enterica* serovar Paratyphi C	Michael McClelland
MG1655	*Escherichia coli* K-12	*E. coli* Genetic Stock Center
700927	*Enterohemorrhagic Escherichia coli* O157:H7 (EHEC)	ATCC
UTI89	Uropathogenic *Escherichia coli* (UPEC)	Sheryl Justice
Jke201	Mating strain of *E. coli*, see reference [309] for full genotype and description	Gift from Dirk Bumann, [309]
PA01	*Pseudomonas aeruginosa* strain PA01	Daniel Wozniak
MZ0686	*Cronobacter sakazakii*	Michael McClelland
JLD1214	14028 *IG(pagC-STM14_1502)*::cam	[113]
EFB004	14028 *ΔmtlD1*	[131]
EFB036	14028 *mtlA1::cam*	[131]
EFB063	*14028 rhaB1*::kan	[131]
EFC015	14028 *rhaD1*::cam	[131]
AMS276	14028 *ΔmtlD1 IG(pagC-STM14_1502)*::cam. P22 transduction of *IG(pagC-STM14_1502)*::cam from JLD1214 to EFB004	This Study
AMS300	14028 *ΔmtlA2*	This Study

AMS302	14028 *ΔmtlD2*	This Study
AMS304	ST4/74 *ΔmtlA4*	This Study
AMS306	ST4/74 *ΔmtlD4*	This Study
AMS308	Ty2 *ΔmtlA6*	This Study
AMS310	Ty2 *ΔmtlD6*	This Study
AMS316	MZ0955 *ΔmtlA8*	This Study
AMS318	MZ0955 Δ*mtlD8*	This Study
AMS320	MZ0875 *ΔmtlA10*	This Study
AMS322	MZ0875 *ΔmtlD10*	This Study
AMS324	MG1655 *ΔmtlD12*	This Study
AMS326	700927 *ΔmtlD14*	This Study
AMS328	MG1655 *ΔmtlA12*	This Study
AMS330	700927 *ΔmtlA14*	This Study
AMS332	UTI89 *ΔmtlA16*	This Study
AMS334	UTI89 *ΔmtlD16*	This Study
AMS340	D23580 *ΔmtlA18*	This Study
AMS342	D23580 *ΔmtlD18*	This Study
AMS344	MZ1299 *ΔmtlA20*	This Study
AMS346	MZ1299 *ΔmtlD20*	This Study
AMS353	PA01 *ΔmtlD24*	This Study
mtlD::tet	PA01 *mtlD*::tet	[382]
ECR003	MZ0686 Δ*mtlA22*	This Study
ECR005	MZ0686 Δ*mtlD22*	This Study
Plasmid	**Genotype or Description**	**Source, Construction, or Reference**
pFOK	Suicide vector backbone for allelic exchange. kan^r	Dirk Bumann [300]
pWSK29	pSC101 cloning vector. amp^r	[405]
pAMS394	pWSK29-$MtlD_{14028}$	This Study
pAMS370	Suicide vector for construction of 14028 *mtlA*	This Study
pAMS371	Suicide vector for construction of 14028 *mtlD*	This Study
pAMS373	Suicide vector for construction of ST4/74 *mtlA*	This Study
pAMS374	Suicide vector for construction of ST4/74 *mtlD*	This Study
pAMS375	Suicide vector for construction of Ty2 *mtlA*	This Study
pAMS376	Suicide vector for construction of Ty2 *mtlD*	This Study

pAMS377	Suicide vector for construction of MZ1299 *mtlA*	This Study
pAMS378	Suicide vector for construction of MZ1299 *mtlD*	This Study
pAMS379	Suicide vector for construction of MZ0955 *mtlA*	This Study
pAMS380	Suicide vector for construction of MZ0955 *mtlD*	This Study
pAMS381	Suicide vector for construction of MZ0875 *mtlA*	This Study
pAMS382	Suicide vector for construction of MZ0875 *mtlD*	This Study
pAMS383	Suicide vector for construction of MG1655 *mtlD*	This Study
pAMS384	Suicide vector for construction of 700927 *mtlD*	This Study
pAMS385	Suicide vector for construction of MG1655 *mtlA*	This Study
pAMS386	Suicide vector for construction of 700927 *mtlA*	This Study
pAMS387	Suicide vector for construction of UTI89 *mtlA*	This Study
pAMS388	Suicide vector for construction of UTI89 *mtlD*	This Study
pAMS403	Suicide vector for construction of PA01 *mtlD*	This Study
pECR001	Suicide vector for construction of MZ0686 *mtlA*	This Study
pECR003	Suicide vector for construction of MZ0686 *mtlD*	This Study

Table 12. Primers used in this study

Primer	**Sequence**	**Description**
BA2473	CGGCATTCGCCATTCAGGCTGCCTC	Verification of pWSK29 insertions
BA3875	ATCGAATTCCTGCAGCCCGGGGGATCCACT	pFOK linearization
BA3876	ATCAAGCTTATCGATACCGTCGACCTCGAG	pFOK linearization
BA4111	CTCGAGGTCGACGGTATCGATAAGCTTGATGGGATATCGACATAAGGGGGATTGTAACGT	For constructing *Salmonella* specific suicide plasmids
BA4112	AGTGGATCCCCCGGGCTGCAGGAATTCGATGATCGCTCAGGCGTTTAATTTCGTTTTTTT	For constructing *Salmonella* specific suicide plasmids
BA4113	TTAAGCTTTTTTACCTGCCAGCAGTTCCAGTTGCACTTTGATCTTAATATCGGATGACAT	For constructing *Salmonella* specific suicide plasmids
BA4114	ATGTCATCCGATATTAAGATCAAAGTGCAACTGGAACTGCTGGCAGGTAAAAAAGCTTAA	For constructing *Salmone*

		lla specific suicide plasmids
BA4115	TCATTTGGTCGCGTTATATGCGTTAACCGCATTACCTGCGCCAAAATGTAATGCTTTCAT	For constructing *Salmonella* specific suicide plasmids
BA4116	ATGAAAGCATTACATTTTGGCGCAGGTAATGCGGTTAACGCATATAACGCGACCAAATGA	For constructing *Salmonella* specific suicide plasmids
BA4117	AGTGGATCCCCCGGGCTGCAGGAATTCGATCCGGTATGGGTTCCAGTGCG	For constructing *E. coli* specific suicide plasmids
BA4118	TTATTGCATTGCTTTATAAGCGGTTACCGCATTACCTGCGCCAAAATGTAATGCTTTCAT	For constructing *E. coli* specific suicide plasmids
BA4119	ATGAAAGCATTACATTTTGGCGCAGGTAATGCGGTAACCGCTTATAAAGCAATGCAATAA	For constructing *E. coli* specific suicide plasmids
BA4120	CTCGAGGTCGACGGTATCGATAAGCTTGATGCGGGGTAATACGGAGATACATCATGG	For constructing *E.*

		coli specific suicide plasmids
BA4123	ATGAAGGTTAATACTATGAAAGCATT	For amplifying *mtlD* from *S.* Typhimurium 14028
BA4124	CGTCATTTGGTCGCGTTATA	For amplifying *mtlD* from *S.* Typhimurium 14028
BA4127	AGTGGATCCCCCGGGCTGCAGGAATTCGATCGTAAGTTAAAACAATCAATAGATCCATAA	For constructing *E. coli* specific suicide plasmids
BA4128	TTACTTACGACCTGCCAGCAGTTCCAGCACTTGCACTTTGATCTTAATATCGGATGACAT	For constructing *E. coli* specific suicide plasmids
BA4129	ATGTCATCCGATATTAAGATCAAAGTGCAAGTGCTGGAACTGCTGGCAGG	For constructing *E. coli* specific suicide plasmids
BA4130	GGGTACCGAGCTCGAATTC	pEX18-GM linearization

BA4131	GGGGATCCTCTAGAGTCGAC	pEX18-GM linearization
BA4135	ACCATGATTACGAATTCGAGCTCGGTACCCACGCCAGAAGAGGAAGGAAAGCGACCATTA	For constructing *P. aeruginosa* specific suicide plasmids
BA4136	TCATTCGCCGAGTACCTGGCGCAGGGTTTCCAGGGGGAGGTGCTGCCGGTTGAGTTTCAT	For constructing *P. aeruginosa* specific suicide plasmids
BA4137	ATGAAACTCAACCGGCAGCACCTCCCCCTGGAAACCCTGCGCCAGGTACTCGGCGAATGA	For constructing *P. aeruginosa* specific suicide plasmids
BA4138	ATGCCTGCAGGTCGACTCTAGAGGATCCCCTATTCCGCCATGGCTGATCTCAAGATCCGC	For constructing *P. aeruginosa* specific suicide plasmids
BA4143	CTCGAGGTCGACGGTATCGATAAGCTTGATGCGACGGAAATTGAGATAGCCGATG	For constructing *C. sakazakii* specific suicide plasmids

BA4144	TTACGCCACGGTTTTACCGGAAAGCAGCGCTTGCACTTTGATCTTGATATCGGATGACAT	For constructing *C. sakazakii* specific suicide plasmids
BA4145	ATGTCATCCGATATCAAGATCAAAGTGCAAGCGCTGCTTTCCGGTAAAACCGTGGCGTAA	For constructing *C. sakazakii* specific suicide plasmids
BA4146	TTACGCTGTTGCGTTATACGCGTTTACTGCATTACCTGCGCCAAAATGTAATGCTTTCAT	For constructing *C. sakazakii* specific suicide plasmids
BA4147	ATGAAAGCATTACATTTTGGCGCAGGTAATGCAGTAAACGCGTATAACGCAACAGCGTAA	For constructing *C. sakazakii* specific suicide plasmids
BA4148	AGTGGATCCCCCGGGCTGCAGGAATTCGATTCAGCTTTTCCATATACATATCGGGCGCGT	For constructing *C. sakazakii* specific suicide plasmids

Table 13. The IC_{50} of mannitol for *mtlD* mutants.

The IC_{50} calculations were performed on cultures grown in M9 minimal medium containing 5 mM fructose and a variable concentration of mannitol (M9) or in the same medium supplemented with 0.2% casamino acids and 1 mM tryptophan (M9 Supp) on three separate occasions (representative graphs are shown in Supplementary Figure S2). 95% confidence intervals are shown. The cultures were initiated with cells that had been previously grown overnight in LB, then washed, and diluted either 1:100 or 1:10,000. The IC_{50} was calculated for cultures grown from a 1:100 dilution in M9 using the 20 hour time point; from a 1:10,000 dilution in M9 using the 28 hour time point; from a 1:100 dilution in M9 Supp using the 10 hour time point; and from a 1:10,000 dilution in M9 Supp using the 14 hour time point. ND – not determined.

					1:100 dilution		**1:10,000 dilution**	
Media	**Species**	**Strain Background**	**Mutant**	**Strain**	**Mean IC_{50} (μM)**	**95% CI (μM)**	**Mean IC_{50} (μM)**	**95% CI (μM)**
M9	*Salmonella enterica*	14028	*ΔmtlD1*	EFB004	28.3	16.0-51.7	ND	ND
M9	*Salmonella enterica*	14028	*ΔmtlD2*	AMS302	26.9	16.7-44.3	0.70	0.51-0.95
M9	*Salmonella enterica*	ST19	*ΔmtlD4*	AMS306	14.7	9.8-22.1	0.73	0.53-1.0
M9	*Salmonella enterica*	D23580	*ΔmtlD18*	AMS342	35.0	23.4-53.0	1.3	0.78-2.2
M9	*Salmonella enterica*	Paratyphi C	*ΔmtlD10*	AMS322	22.0	13.0-38.3	0.47	0.33-0.65
M9	*Escherichia coli*	K12	*ΔmtlD12*	AMS324	5.2	3.6-7.6	1.2	0.76-2.1
M9	*Escherichia coli*	700927	*ΔmtlD14*	AMS326	9.5	5.9-15.3	2.3	1.2-4.2
M9	*Cronobacter sakazakii*	MZ0686	*ΔmtlD22*	ECR005	17.7	13.2-23.6	3.6	2.6-4.9
M9	*Pseudomonas aeruginosa*	PA01	*ΔmtlD22*	AMS353	14.1	7.5-27.2	5.3	3.0-9.4
M9	*Pseudomonas aeruginosa*	PA01	*mtlD*::tet	unnamed	4.3	2.9-6.1	ND	ND
M9 Supp	*Salmonella enterica*	14028	*ΔmtlD2*	AMS302	27.2	18.5-40.5	1.6	1.1-2.2
M9 Supp	*Salmonella enterica*	Ty2	*ΔmtlD6*	AMS310	9.4	6.9-13.0	0.7	0.5-1.1
M9 Supp	*Salmonella enterica*	Paratyphi A	*ΔmtlD20*	AMS346	11.5	2.4-72.2	0.29	0.21-0.39
M9 Supp	*Escherichia coli*	UTI89	*ΔmtlD16*	AMS334	24.9	13.7-45.9	0.48	0.27-0.84
M9 Supp	*Salmonella enterica*	Paratyphi B	*ΔmtlD8*	AMS318	47.7	30.5-76.6	2.3	1.6-3.3

Chapter 6: Discussion

6.1 SdiA mediates eavesdropping on foreign AHL producers

SdiA is a LuxR family protein. SdiA is orthologous to the LuxR proteins ExpR and PhzR, of *Erwinia* and *Pantoeae* species, respectively [29]. Unlike true quorum sensing bacteria, the subset of Enterobacteriaceae encoding *sdiA* have no cognate *luxI* homolog. Instead, they rely on AHLs produced by nearby foreign bacteria. This change leads to the eavesdropping phenotype, where one species "listens in" on the AHL-based communication of another without reciprocating information back to the AHL producer.

In Chapter 2, we lay out the current body of literature on SdiA-mediated eavesdropping, focusing on three questions that may yield insight into "why" bacteria adopted this behavior. This included how SdiA regulates genes, where SdiA is active (and relevant), and what SdiA regulates.

The mechanism by which SdiA regulates genes is unknown as there are only a few studies on the matter [151,155,156,406]. It has been found in multiple species that *sdiA* mutation alone can change transcriptional activity of loci, indicating that SdiA has both ligand dependent and independent activity (Chapter 3 and [157,158]). In support of this, the *ler* promoter of *E. coli* O157::H7 has direct binding sites for both apo-SdiA and SdiA bound to AHL [155]. SdiA activity independent of AHLs complicates how we define what transcriptional and phenotypic changes occur during eavesdropping.

The second question, “where is SdiA active and relevant”, has historically yielded a significant number of negative results. SdiA is active in *Aeromonas hydrophila* infected turtles and *Yersinia entercolitica* infected mice [144,145]. SdiA is not active in tomato soft rot, mice, cattle, pigs, or chickens [143,145]. In turtles and *Y. enterocolitica* infected mice, where SdiA is active, *sdiA* mutants have no fitness defect. In environments with no SdiA activity, no fitness defect is observed either. One system has been found to cause significant fitness phenotypes: a competitive infection between wild-type and *sdiA* mutant *Salmonella* in a mouse gastroenteritis model where both strains encode an AHL synthase (*yenI*). In this model, the wild-type outnumbers the mutant at least ~100-fold. The relevance of this system is unknown and has not been investigated further.

The final question of our review of SdiA literature, “what does SdiA regulate?” has been studied using genetic screens and RNA-seq. In this section we also considered the reported in vitro phenotypes (cell division, multiple-drug resistance, and biofilm formation). The regulons of SdiA, primarily investigated in *E. coli*, *Salmonella*, and *Enterobacter cloacae*, are quite diverse, with no published overlapping genes [138,139,146,157,158]. As we show in Chapter 3, a few genes are SdiA regulated in at least two species from other genera (but no regulon member is common to all three). A clear SdiA phenotype, I would argue, should require a change occurring in both AHL and *sdiA*-dependent manners. None of the three reported phenotypes have such results.

6.2 The biologically relevant environment of SdiA

As discussed in this thesis, we sought to determine "why" bacteria engage in SdiA-mediated eavesdropping. On a basic level, the gene must aid in survival or reproduction to be maintained evolutionarily. Considering the apparent conservation within this subset of Enterobacteriaceae, I assume that *sdiA* is important to the survival of bacteria encoding it. In simple terms, the question of "why" remains unanswered. Like most genes, the significance of *sdiA* to the bacteria encoding it is evaluated through probing for mutant defects in different environments and conditions. Using a genetic reporter strain, we have been able to simultaneously check for SdiA activation and mutant defects in various environments with some activating SdiA but with no *sdiA* mutant defects [143-145]. Based on a search of metagenomes for AHL synthase homologs, we found that insect and plant microbiomes are the strongest candidates for AHL producer containing environments. Thus, we set out to examine these using house flies as a model for insects and Angiosperms and soybeans as models for plants. Our study on house flies was complicated by an apparent dose dependent change in *sdiA* mutant fitness within house flies where lower doses led to the *sdiA* mutant drastically outnumbering the wild-type by day one post infection. It was hypothesized that the antibiotic marker used to inactivate *sdiA*, *bla*, was the determining factor of this phenotype. This was controlled for by mutating *sdiA* with another marker (cam^r) and encoding *bla* in both wild-type and *sdiA* mutant. Surprisingly, this had almost no effect on *Salmonella* survival within house flies and no effect on mutant phenotypes.

Since two different *sdiA* mutants, each disrupted with a different antibiotic resistance gene, are advantaged over wild-type, I hypothesize that inactivation of *sdiA* is advantageous in flies (i.e. *sdiA* is a toxic gene in house flies). In Chapter 4, I lay out future experiments that may unravel these complex results. Another interesting aspect to these results was the finding that *sdiA* mutants are disadvantaged over wild-type early on. Samples of flies taken at the time of removing the contaminated sucrose used to infect the flies by free-feeding (~2 hours) indicate that wild-type outnumber *sdiA* mutant bacteria in early stages of colonization by an unknown mechanism. This phenotype is flipped by 24 hours. Determining the dynamics of SdiA-mediated interactions with the house fly may also require sampling at early time points.

Although our experiments in house flies suggest SdiA toxicity, they are still valuable sites of investigation into the relevant site of SdiA activity. House flies represent the sole environment in which SdiA activation and mutant phenotypes have co-occurred. The magnitude of mutant advantage over wild-type certainly suggests biological activity that has not been previously demonstrated. Determining the mechanism by which SdiA mediates loss of colonization in the house fly would provide new insights into the phenotypes it mediates. There is little research on how *Salmonella* interacts with the house fly or its microbiota. One study has shown that *Salmonella* localizes to the midgut and becomes highly motile within hours of ingestion [335]. Motility likely leads to increased transcription of *sdiA* and possibly more activity [195]. Insects recognize pathogens by their pathogen associated molecule patterns (PAMPs) like humans [407]. SdiA activity (e.g. expression of Rck) could lead to more efficient targeting by the host's immune

components (e.g. phagocytes). It would be interesting to see if certain *Salmonella* SdiA regulon members interacted with specific fly immune components. Alternatively, SdiA activity could lead to elimination by interaction with the fly microbiome. At least one fly microbiota species, *Proteus mirabils*, protects against *Salmonella* colonization and the house flies used in these experiments were colonized by this species (data not shown) [330]. Determining if specific microbiota facilitate SdiA-mediated elimination from the house fly would be difficult without first identifying the bacteria responsible for SdiA activity, which we were unable to do.

6.3 The regulons and phenotypes of SdiA

In Chapter 3, we used RNA-seq to identify members of the SdiA regulon in *Salmonella*, focusing on serovars Typhimurium and Typhi. *S.* Typhimurium is a broad host range serovar known to colonize a wide range of hosts. In contrast, *S.* Typhi is a host restricted serovar in which humans are its only known host and reservoir [271]. Their environmental overlap, therefore, is quite small. This presented an interesting intersection with SdiA, which we currently understand to be an environmental sensor whose overlapping environment (humans) does not appear to be conducive for SdiA-mediated eavesdropping.

After constructing and screening a large array of transcriptional fusions, we found that the *sdiA* regulons of *S.* Typhimurium and *S.* Typhi are quite similar, sharing four members. These four members include a putative ATP-dependent RNA helicase-like protein (SrgF), two small putative proteins of unknown function (SrgGH), a prohibitin

family protein and helper protein (SrgKJ), and the menaquinone biosynthesis operon (MenFDHBCE). Based on existing literature, new functions or phenotypes of these genes were hypothesized and tested. No *sdiA* or regulon member mutant phenotypes were found. *S.* Typhimurium SdiA regulates an additional two loci (six total), and *S.* Typhi SdiA one (five total). The *S.* Typhimurium specific regulon members include *srgE* and *pefI-srgC*, whose functions as virulence factors of unknown significance have been previously reported [166,234]. The sole *S.* Typhi specific regulon members are SrgIL, lipoproteins of unknown function. Further insight into how these specific regulon members contribute to SdiA-mediated eavesdropping will likely first require identifying a relevant environment.

As part of our regulon study in Chapter 3, we also searched the genomes of *E. coli* and *E. cloacae*, two species whose regulons had been previously investigated in our lab [157,158]. Some of each species' regulon members are conserved (genetically) in the other two species. I constructed additional transcriptional fusions and looked for *sdiA* and AHL dependent regulation. This revealed that *sdiA* regulons are also semi-conserved between genera. SdiA may have a common regulon member among all species that encode it, but this does not appear to be the case (at least among currently known regulated genes).

We hypothesize the existence of a common SdiA regulated gene or genes that could explain the conservation of SdiA-mediated eavesdropping within this group of Enterobacteriaceae. In this scenario, eavesdroppers have a conserved 'core' SdiA regulon and variable 'auxiliary" regulons meet the specific needs of the strain's lifestyle. Alternatively, these bacteria may have a 'core' phenotype that is mediated by strain

specific regulon members. In Chapter 2, we speculate this phenotype may be related to phage biology. Phages are ubiquitous in nature, representing a constant selective pressure, and new mechanisms of phage defense are frequently identified [408-410]. A future study could collect and isolate phages specific to *E. coli*, *Salmonella*, and *E. cloacae*. These phages could be screened for plaque formation against a SdiA-expressing strain versus mutant. From there, any hits could be further screened against known regulon members. Alternatively, transposon libraries could be constructed in SdiA-active backgrounds (e.g. encoding *yenI*) and challenged with phages like a recent study from Adam Arkin [230]. This may reveal both a phenotype of SdiA and novel functions for uncharacterized genes.

In conclusion, the field of SdiA research is complex due to the absence of clear phenotypes and difficult to interpret literature. The absence of phenotypes does not make this gene unimportant; its conservation within this subset of Enterobacteriaceae suggests it is quite important. Identifying its role in bacteria will reveal new aspects to their lifecycle that are currently unknown and unappreciated. The study of this gene requires careful consideration of its history and behavior to perform experiments that might gain meaningful insight into its role in bacteria.

6.4 Mannitol sensitivity as an anti-microbial strategy

Antibiotics interfere with essential biological processes in cells, inhibiting their growth or causing loss of viability. The crisis of antibiotic resistance must be addressed, or once treatable illnesses will again become a leading source of morbidity and mortality.

Developing new classes of antibiotics is one of many approaches that must be implemented. Since 2014, our lab has investigated sugar-phosphate toxicities as a possible novel antimicrobial strategy [113]. Sugar-phosphate toxicity is a phenomenon in bacteria where inactivation of enzymes that are essential for processing phosphorylated metabolic intermediates leads to their accumulation in the cell and this accumulation confers growth defects.

In Chapter 5, we investigate mannitol sensitivity as a possible anti-microbial strategy. Mannitol is transported into the cell by MtlA, producing mannitol-1-phosphate (Mtl-1P). Mtl-1P is oxidized by mannitol-1-phosphate 5-dehydrogenase (MtlD) or else it will accumulate, as seen in *mtlD* mutants exposed to mannitol [122,131,132]. It is believed that accumulated Mtl-1P is toxic to the cell, leading to growth defects by an unknown mechanism.

We first constructed a collection of mutants in various species, lacking either *mtlA* or *mtlD*, using allelic exchange. While investigating other sugar phosphate toxicities, our group has found that construction of mutations can influence their phenotypes [131]. This is most likely caused by polarity effects on downstream genes in their utilization operons. In the case of mannitol (*mtlADR*), polar mutations in *mtlD* would decrease expression of the repressor *mtlR*, leading to increased expression of transporter *mtlA* and subsequent accumulation of Mlt-1P. Thus, we constructed scarless deletions of *mtlA* and *mtlD* to more accurately assess how inactivation of MtlD by a small molecule (which would occur post-transcriptionally) might impact virulence. Mutation of *mtlD* in seven strains of *Salmonella*, three strains of *E. coli*, one strain of *Pseudomonas aeruginosa*, and one strain

of *Cronobacter sakazakii* all led to mannitol sensitivity. Additionally, a *Staphylococcus aureus mtlD* mutant is also mannitol sensitive ([121,122] and unpublished data). We hypothesize that mannitol sensitivity occurs in all bacterial *mtlD* mutants.

We next set out to identify the therapeutic potential of mannitol sensitivity in bacterial infections. Prior to the studies shown in Chapter 5, our lab had previously established that *mtlD* mutants of *S.* Typhimurium have significant defects in the strep-treated model of gastroenteritis in competition with the wild-type [131]. Another group has shown that *S. aureus mtlD* mutants are attenuated during system infections [121]. Finally, *S.* Typhimurium *mtlD* mutants can access mannitol within host cells and *mtlD* mutants are unable to replicate in this environment (when mannitol is present) [393,394]. In our previous study, we used competition assays to identify fitness defects in the gastrointestinal tract [131]. The presence of the wild-type prevented us from determining if the attenuation suffered by the mutant would actually attenuate the infection overall. We repeated the infection using single infections and found the *mtlD* mutant unable to significantly induce inflammation in the strep-treated mouse model of gastroenteritis compared to the wild-type. By 'treating' mice with a high dose of mannitol in their drinking water (320 mM), we were able to prevent inflammation and fecal shedding entirely. Thus, we propose that mannitol sensitivity can interfere with gastrointestinal infections caused by nontyphoidal *Salmonella*.

An important aspect of *Salmonella* pathogenesis is its systemic element, especially for typhoidal and invasive nontyphoidal strains [270,361]. We first established that mannitol sensitivity using a competitive infection between *S.* Typhimurium *mtlA* and

mtlD mutants and found mannitol-dependent defects in *mtlD* mutants (i.e. defects required providing mannitol to the mouse). Like with our gastroenteritis experiments, we were curious if an infection could be attenuated by treating a *mtlD* mutant infected mouse with mannitol. Perhaps surprisingly, mortality could be delayed and even prevented by treatment depending on the dose and strain of mouse used (i.e. *NRAMP1* had to be functional to prevent death). One of the weaknesses of some sugar-phosphate toxicities as therapeutic modalities is that they cannot attenuate *Salmonella* at systemic sites (e.g. rhamnose and fructose-asparagine, Chapter 5). As we have reported here, mannitol is accessible to pathogens in systemic sites like the liver and spleen. The attenuation conferred by treatment could be caused at least partially by virulence defects in phagocytes, but the host factors mediating attenuation during infection remain to be determined.

In conclusion, mannitol could be used to treat mannitol sensitive *Salmonella* (*mtlD* mutants or wild-type bacteria targeted by a small molecule inhibitor of MltD) in both gastrointestinal and systemic sites. We hypothesize that the same strategy could be used to treat other infectious bacteria, such as *E. coli* and *P. aeruginosa*. In future studies, the therapeutic potential of treatment could be established by a similar approach used here (providing mannitol to *mtlD* infected hosts). Different doses and treatment regimens will need to be explored to determine the most effective approach and this will likely vary with each infection.

6.5 The recovery phenotype of mannitol sensitive bacteria

As part of our study, we sought to quantify mannitol sensitivity in *mtlD* mutants by finding minimum inhibitory concentrations of each strain. However, this revealed a previously unreported behavior that we termed “recovery”. Mutants of *mtlD* exposed to mannitol eventually resume growth, and the time that growth resumes is a function of the initial concentration of mannitol introduced into the culture. All *mtlD* mutants appear to have the ability to recover from toxicity in this manner, suggesting its mechanism is conserved as well.

I initially suspected that recovery was the outgrowth of suppressor mutations (e.g. *mtlA* mutations). However, bacteria from recovered cultured are still mannitol sensitive (unpublished data). Another hypothesis was that mannitol sensitivity was causing a dose-dependent amount of lysis in the population and a small group of survivors was regrowing. However, no loss of cell viability has been found in *mtlD* mutants intoxicated in minimal media (unpublished data). Another possibility was that the bacteria were engaging in a population-dependent behavior, secreting a recovery accelerating or depleting a toxicity potentiating compound into the cultural media. Consist with this idea, the spent supernatant of *Salmonella* accelerates the rate of recovery (unpublished data).

I tested these ideas by decreasing the size of the initial population. I hypothesized that smaller initial populations would lead to variability in recovery if either suppressors or lysis mechanisms were at play (e.g. some cultures would never recover). If population-dependent behaviors were at play, smaller initial populations would be much slower at recovery or unable to do so. It was determined that smaller populations still recover from

toxicity (and do so consistently) but are much more sensitive to mannitol. This inoculum effect further complicated our attempts to quantify mannitol sensitivity. Based on the time-dependent aspect of recovery, we calculated the inhibitory concentration of mannitol (IC_{50}) on an hourly basis, revealing a linear increase in IC_{50} over time. Recovery occurs linearly regardless of initial population size, but the rate at which cultures recover (in μM per hour) still has an inoculum effect. We then recalculated IC_{50} values as a ratio of mannitol molecules per cell. Surprisingly this eliminated the inoculum effect almost entirely and revealed that cultures of *mtlD* mutants recover from toxicity at a rate of 1-4 x 10^8 mannitol molecules per cell per hour, with smaller initial populations recovering faster than larger populations. We propose that this 'recovery rate' represents the speed at which intoxicated cells process toxicity and this may be largely or entirely an intrinsic process. Using the estimate that *Salmonella* cells have a volume of one femtoliter (10^{-15} L) and cells process 10^8 molecules per hour, this equates to an internal concentration of 166 mM processed over one hour or 2.8 mM processed every minute.

This phenotype raises questions about how intoxication occurs. If cells can process toxicity, why do they get attenuated? If intoxication occurs from excess intake compared to processing (output), then a sufficiently large quantity of mannitol in the media could eventually lead to death. At least after 24 hours, a concentration of 300 mM mannitol in media has no significant bactericidal effects. If input is equal to or lower than output, then toxicity wouldn't occur at all. One possibility is that bacteria have an early phase where input > output, then changes in expression allow for processing, where input ≤ output. This could allow for eventual recovery. If "recovery genes" are identified, it

may be interesting to track their expression over the course of intoxication and recovery. We also hypothesized that intoxicated bacteria may simply stop importing mannitol to reduce or halt intoxication. The media of recovered cells are depleted for mannitol, suggesting all mannitol in a culture is processed (unpublished data). This does not rule out intoxication-dependent changes in transport as a mechanism of managing accumulation of Mtl-1P.

6.6 The mechanism of mannitol toxicity and recovery

Recently, our group reviewed the available literature on sugar-phosphate toxicities [111]. Only a handful of sugar-phosphate toxicity mechanisms have a significant amount of characterization, and the mechanism of mannitol toxicity is not among them. The handful of older studies on the subject may provide insight into this mechanism.

In 1972, Jensen et al. reported on mannitol sensitive isolates of *Salmonella* [132]. A sensitive isolate, DB82, lyses in nutrient broth after exposure to mannitol. This strain reached internal Mtl-1P concentrations of ~ 20 mM. They also fed intoxicated bacteria several radiolabeled precursors to look for defects in synthesizing cell envelope (via diaminopimelate), proteins (via phenylalanine), DNA (via uracil), and lipids (via acetate). The earliest defect was in the incorporation of acetate into lipids, suggesting the sensitive mutant is limited in this capacity. The transport and efflux of acetate is regulated by the metabolic state of the cell and so this result could be due to issues in transport or in internal defects in lipid synthesis [411]. While most radiolabeled mannitol formed Mtl-1P, a small amount became a nucleoside diphosphate mannitol [132]. Another study of an *E. coli*

mtlD mutant found that radiolabeled mannitol ends up as ribose sugar [412]. Interestingly, the formation of adenosine 5-diphosphate mannitol in *Salmonella* was also reported but a connection between these three observations has yet to be established [413]. In a preliminary experiment, the Wysocki lab at OSU performed metabolomics on cell pellets of a *mtlD* mutant exposed to mannitol for 10 minutes. Compared to a wild-type control, the *mtlD* mutant had alterations in concentrations of purines, glutamate, and glycolytic intermediates 2- and 3- phosphoglycerate (unpublished data). The available data suggests a relationship between mannitol and nucleic acids.

I hypothesize that mannitol toxicity is a result of three potential factors. One, that Mtl-1P directly interacts with a host target or targets (e.g. proteins) that modulates its activity leading to physiological dysbiosis. Two, that excess intracellular Mtl-1P could lead to osmotic stress in the cell (specific hypo-osmotic stress). Three, accumulation of Mtl-1P toxicity causes damage from the first two mechanisms that require repair to overcome toxicity. I hypothesize that recovery occurs because the cell processes (i.e. enzymatically) Mtl-1P, forming a different, non-toxic molecule.

The existence of a direct target of Mtl-1P is still hypothetical. The sole target I have hypothesized is glyceraldehyde-3-phosphate dehydrogenase (GpsA), which is conserved in most bacteria. GpsA catalyzes the formation of sn-glycerol-3-phopshate (sn-G3P or G3P) from glycolytic intermediate dihydroxyacetone phosphate (DHAP). This gene is essential because mutants cannot synthesize phospholipids. This target was hypothesized for several reasons. First, Mtl-1P intoxicated cells upregulate *gpsA* and downregulate members of the GlpR regulon (G3P is an inducer of GlpR) (unpublished

transcriptomic study). Second, MtlD and GpsA are structurally similar ([224,398]). Third, the inactivation of GpsA is consistent with the defects in acetate incorporation observed by Jensen et al. [132]. The essentiality of *gpsA* can be bypassed by addition of glycerol to the media, due to the *glpK*-dependent formation of G3P. I tested this hypothesis by supplementing intoxicated cells with glycerol and measuring their recovery rate. No change was observed (unpublished data). These results suggest that GpsA is not the target of Mtl-1P in the cell. Alternatively, multiple targets could be affected, and alleviation of one target may not be sufficient to see a change in toxicity phenotypes. Another possibility is that a substrate of GlpK (i.e. ATP) or GpsA (DHAP, NAD^+) are depleted in the cell. Glucose-6-phosphate, fructose-6-phosphate, and mannose-6-phosphate intoxicated cells all undergo cell lysis concomitant with the accumulation of the toxic intermediate methylglyoxal [414] and methylglyoxal also uses DHAP as a substrate. The glycolytic intermediate DHAP is also upstream of 2PG and 3PG, both depleted in early intoxicated cells (unpublished data). Although not investigated in this thesis, it is interesting to note that intoxicated cells appear to activate cell envelope stress responses, which could be secondary to defects in lipid synthesis (manuscript in preparation).

The mechanism of recovery is unknown. We have generated two hypotheses by which Mtl-1P could be processed by the cell: conversion and efflux. Bacteria use efflux pumps to remove many toxic compounds, including antibiotics [415]. We hypothesized that Mtl-1P could be removed from the cell in a *tolC*-dependent manner, but a *tolC* mutation had no effect on the recovery rate of a *mtlD* mutant (unpublished data). This result was consistent with the absence of Mtl-1P in the supernatant of recovered cells (unpublished

data). A pathway in which bacteria could convert Mtl-1P to another intermediate is, to our knowledge, unknown. It is somewhat odd that bacteria encode this secondary hypothetical pathway that is apparently conserved. In *E. coli* under anaerobic conditions, Mtl-1P is formed when fed glucose and this does not require intact *mtlD* [376,416-418]. It is thought this may be a pathway used to dump excess reducing power. If this is true, the cell may also have evolved a pathway to process this toxic intermediate rather than allow Mtl-1P to accumulate while waiting for aerobic conditions. Identifying this pathway will provide new insights into the bacterial sugar metabolism.

Mannose-6-phosphate reductase, used in plants for the synthesis of mannitol, converts mannose-6-phosphate to Mtl-1P [419] and putative homologs can be found in *mtlD* encoding bacteria. If a similar reaction occurred in bacteria, the hypothesis that Mtl-1P is converted to mannose-6-phosphate could be tested by measuring recovery rates in a *mtlD manA* double mutant (which would be unable to process mannose-6-phosphate). Two phosphatases, *hxpA* and *hxpB*, have been shown to convert Mtl-1P to mannitol in vitro [420]. Phosphatases may play a role in managing Mtl-1P concentrations during intoxication, though it is unknown how intracellular mannitol may be processed. Another possibility is that the recovery mechanism involves the conversion of Mtl-1P to a ribose sugar or linked to nucleosides, as suggested by the earlier studies discussed above.

To identify the mechanisms of toxicity and recovery, we propose three large data collection experiments. One, identification of Mtl-1P direct targets in the cell using affinity purification. Two, a thorough quantification of the cellular components of intoxicated cells (e.g. ATP levels, NAD/NADH ratios, lipid content, metabolites) that

may reveal which physiological dysbiosis is occurring. Third, a selection experiment (Tn-seq or Barseq) to identify mutations conferring resistance and hyper-susceptibility to intoxication. Here we may use different concentrations of mannitol to test in both recovery permissive and non-permissive conditions. These data sets would provide new insights into the underlying mechanism of toxicity and recovery and, if successful, would develop an experimental approach in elucidating other mechanisms of sugar-phosphate toxicity that remain un-investigated.

6.7 Conclusions and final thoughts

In this thesis, I lay out a collection of studies performed in collaboration with other researchers on the topic of SdiA-mediated eavesdropping and mannitol sensitivity. In both fields, new insights were gained. More importantly, new questions were generated by the results of each study which may guide future researchers in their attempts to understand the biology of these two systems.

Bibliography

1. Schuster, M., D. Joseph Sexton, S.P. Diggle, and E. Peter Greenberg, *Acyl-Homoserine Lactone Quorum Sensing: From Evolution to Application.* Annual Review of Microbiology, 2013. **67**(Volume 67, 2013): p. 43-63.
2. Duddy, O.P. and B.L. Bassler, *Quorum sensing across bacterial and viral domains.* PLOS Pathogens, 2021. **17**(1): p. e1009074.
3. Mukherjee, S. and B.L. Bassler, *Bacterial quorum sensing in complex and dynamically changing environments.* Nat Rev Microbiol, 2019. **17**(6): p. 371-382.
4. Papenfort, K. and B.L. Bassler, *Quorum sensing signal-response systems in Gram-negative bacteria.* Nature reviews. Microbiology, 2016. **14**(9): p. 576-588.
5. Azimi, S., A.D. Klementiev, M. Whiteley, and S.P. Diggle, *Bacterial Quorum Sensing During Infection.* Annual Review of Microbiology, 2020. **74**(1): p. 201-219.
6. Grandclément, C., M. Tannières, S. Moréra, Y. Dessaux, and D. Faure, *Quorum quenching: role in nature and applied developments.* FEMS Microbiology Reviews, 2015. **40**(1): p. 86-116.
7. Kaplan, H.B. and E.P. Greenberg, *Diffusion of autoinducer is involved in regulation of the Vibrio fischeri luminescence system.* Journal of Bacteriology, 1985. **163**(3): p. 1210-1214.
8. Yates, E.A., B. Philipp, C. Buckley, S. Atkinson, S.R. Chhabra, R.E. Sockett, M. Goldner, Y. Dessaux, M. Camara, H. Smith, and P. Williams, *N-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of Yersinia pseudotuberculosis and Pseudomonas aeruginosa.* Infect Immun, 2002. **70**(10): p. 5635-46.
9. Dong, Y.H., L.H. Wang, J.L. Xu, H.B. Zhang, X.F. Zhang, and L.H. Zhang, *Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase.* Nature, 2001. **411**(6839): p. 813-7.
10. Nealson, K.H. and J.W. Hastings, *Bacterial bioluminescence: its control and ecological significance.* Microbiological reviews, 1979. **43**(4): p. 496-518.
11. Rutherford, S.T. and B.L. Bassler, *Bacterial quorum sensing: its role in virulence and possibilities for its control.* Cold Spring Harbor perspectives in medicine, 2012. **2**(11): p. a012427.
12. Hwang, I., A.J. Smyth, Z.-Q. Luo, and S.K. Farrand, *Modulating quorum sensing by antiactivation: TraM interacts with TraR to inhibit activation of Ti plasmid conjugal transfer genes.* Molecular Microbiology, 1999. **34**(2): p. 282-294.

13. Lee, M.S. and D.A. Morrison, *Identification of a new regulator in Streptococcus pneumoniae linking quorum sensing to competence for genetic transformation.* Journal of Bacteriology, 1999. **181**(16): p. 5004-5016.
14. Silpe, J.E., A.A. Bridges, X. Huang, D.R. Coronado, O.P. Duddy, and B.L. Bassler, *Separating Functions of the Phage-Encoded Quorum-Sensing-Activated Antirepressor Qtip.* Cell Host Microbe, 2020.
15. Silpe, J.E. and B.L. Bassler, *Phage-Encoded LuxR-Type Receptors Responsive to Host-Produced Bacterial Quorum-Sensing Autoinducers.* MBio, 2019. **10**(2).
16. Høyland-Kroghsbo, N.M., R.B. Mærkedahl, and S.L. Svenningsen, *A Quorum-Sensing-Induced Bacteriophage Defense Mechanism.* mBio, 2013. **4**(1): p. e00362-12.
17. Fuqua, W.C., S.C. Winans, and E.P. Greenberg, *Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators.* Journal of bacteriology, 1994. **176**(2): p. 269-275.
18. Redfield, R.J., *Is quorum sensing a side effect of diffusion sensing?* Trends in microbiology, 2002. **10**(8): p. 365-370.
19. Hense, B.A., C. Kuttler, J. Müller, M. Rothballer, A. Hartmann, and J.-U. Kreft, *Does efficiency sensing unify diffusion and quorum sensing?* Nature Reviews Microbiology, 2007. **5**(3): p. 230-239.
20. West, S.A., K. Winzer, A. Gardner, and S.P. Diggle, *Quorum sensing and the confusion about diffusion.* Trends in microbiology, 2012. **20**(12): p. 586-594.
21. Wang, M., A.L. Schaefer, A.A. Dandekar, and E.P. Greenberg, *Quorum sensing and policing of Pseudomonas aeruginosa social cheaters.* Proceedings of the National Academy of Sciences, 2015. **112**(7): p. 2187-2191.
22. Czárán, T. and R.F. Hoekstra, *Microbial communication, cooperation and cheating: quorum sensing drives the evolution of cooperation in bacteria.* PloS one, 2009. **4**(8): p. e6655-e6655.
23. Brown, S.P. and R.A. Johnstone, *Cooperation in the dark: signalling and collective action in quorum-sensing bacteria.* Proceedings of the Royal Society of London. Series B: Biological Sciences, 2001. **268**(1470): p. 961-965.
24. Hudaiberdiev, S., K.S. Choudhary, R. Vera Alvarez, Z. Gelencsér, B. Ligeti, D. Lamba, and S. Pongor, *Census of solo LuxR genes in prokaryotic genomes.* Frontiers in Cellular and Infection Microbiology, 2015. **5**(20).
25. Venturi, V. and B.M.M. Ahmer, *Editorial: LuxR Solos are Becoming Major Players in Cell–Cell Communication in Bacteria.* Frontiers in Cellular and Infection Microbiology, 2015. **5**(89).
26. Lintz, M.J., K.-I. Oinuma, C.L. Wysoczynski, E.P. Greenberg, and M.E.A. Churchill, *Crystal structure of QscR, a Pseudomonas aeruginosa quorum sensing signal receptor.* Proceedings of the National Academy of Sciences, 2011. **108**(38): p. 15763-15768.
27. Lee, J.-H., Y. Lequette, and E.P. Greenberg, *Activity of purified QscR, a Pseudomonas aeruginosa orphan quorum-sensing transcription factor.* Molecular Microbiology, 2006. **59**(2): p. 602-609.

28. Ding, F., K.-I. Oinuma, N.E. Smalley, A.L. Schaefer, O. Hamwy, E.P. Greenberg, and A.A. Dandekar, *The Pseudomonas aeruginosa Orphan Quorum Sensing Signal Receptor QscR Regulates Global Quorum Sensing Gene Expression by Activating a Single Linked Operon.* mBio, 2018. **9**(4): p. e01274-18.
29. Sabag-Daigle, A. and B.M.M. Ahmer, *ExpI and PhzI Are Descendants of the Long Lost Cognate Signal Synthase for SdiA.* PLOS ONE, 2012. **7**(10): p. e47720.
30. Winfield, M.D. and E.A. Groisman, *Evolution and Ecology of Salmonella.* EcoSal Plus, 2004. **1**(1): p. 10.1128/ecosalplus.6.4.6.
31. Sanderson, K.E. and S. Nair, *Taxonomy and species concepts in the genus Salmonella.* Salmonella in domestic animals, 2013. **2**: p. 1-19.
32. Lamas, A., J.M. Miranda, P. Regal, B. Vázquez, C.M. Franco, and A. Cepeda, *A comprehensive review of non-enterica subspecies of Salmonella enterica.* Microbiological Research, 2018. **206**: p. 60-73.
33. Schikora, A., A.V. Garcia, and H. Hirt, *Plants as alternative hosts for Salmonella.* Trends in Plant Science, 2012. **17**(5): p. 245-249.
34. Silva, C., E. Calva, and S. Maloy, *One Health and Food-Borne Disease: Salmonella Transmission between Humans, Animals, and Plants.* Microbiol Spectr, 2014. **2**(1): p. Oh-0020-2013.
35. Back, D.-S., G.-W. Shin, M. Wendt, and G.-J. Heo, *Prevalence of Salmonella spp. in pet turtles and their environment.* Laboratory Animal Research, 2016. **32**(3): p. 166-170.
36. Hidalgo-Vila, J., C. Díaz-Paniagua, C. de Frutos-Escobar, C. Jiménez-Martínez, and N. Pérez-Santigosa, *Salmonella in free living terrestrial and aquatic turtles.* Veterinary Microbiology, 2007. **119**(2): p. 311-315.
37. Wales, A.D., J.J. Carrique-Mas, M. Rankin, B. Bell, B.B. Thind, and R.H. Davies, *Review of the Carriage of Zoonotic Bacteria by Arthropods, with Special Reference to Salmonella in Mites, Flies and Litter Beetles.* Zoonoses and Public Health, 2010. **57**(5): p. 299-314.
38. Rosenau, M.J., *Investigation of a Pathogenic Microbe (B. Typhi Murium-Danyz) Applied to the Destruction of Rats*. 1901: US Government Printing Office.
39. Blackwell, J.M., T. Goswami, C.A. Evans, D. Sibthorpe, N. Papo, J.K. White, S. Searle, E.N. Miller, C.S. Peacock, H. Mohammed, and M. Ibrahim, *SLC11A1 (formerly NRAMP1) and disease resistance.* Cell Microbiol, 2001. **3**(12): p. 773-84.
40. Cunrath, O. and D. Bumann, *Host resistance factor SLC11A1 restricts Salmonella growth through magnesium deprivation.* Science, 2019. **366**(6468): p. 995-999.
41. Fritsche, G., M. Nairz, S.J. Libby, F.C. Fang, and G. Weiss, *Slc11a1 (Nramp1) impairs growth of Salmonella enterica serovar typhimurium in macrophages via stimulation of lipocalin-2 expression.* Journal of Leukocyte Biology, 2012. **92**(2): p. 353-359.
42. Giacomodonato, M.N., S. Uzzau, D. Bacciu, R. Caccuri, S.H. Sarnacki, S. Rubino, and M.C. Cerquetti, *SipA, SopA, SopB, SopD and SopE2 effector proteins of Salmonella enterica serovar Typhimurium are synthesized at late stages of infection in mice.* Microbiology, 2007. **153**(4): p. 1221-1228.

43. Miller, S.I., A.M. Kukral, and J.J. Mekalanos, *A two-component regulatory system (phoP phoQ) controls Salmonella typhimurium virulence.* Proceedings of the National Academy of Sciences, 1989. **86**(13): p. 5054-5058.
44. Buchmeier, N.A. and F. Heffron, *Intracellular survival of wild-type Salmonella typhimurium and macrophage-sensitive mutants in diverse populations of macrophages.* Infect Immun, 1989. **57**(1): p. 1-7.
45. Shippy, D.C., N.M. Eakley, C.T. Lauhon, P.N. Bochsler, and A.A. Fadl, *Virulence characteristics of Salmonella following deletion of genes encoding the tRNA modification enzymes GidA and MnmE.* Microbial Pathogenesis, 2013. **57**: p. 1-9.
46. Killar, L.M. and T.K. Eisenstein, *Immunity to Salmonella typhimurium infection in C3H/HeJ and C3H/HeNCrlBR mice: studies with an aromatic-dependent live S. typhimurium strain as a vaccine.* Infection and Immunity, 1985. **47**(3): p. 605-612.
47. Eisenstein, T.K., L.W. Deakins, L. Killar, P.H. Saluk, and B.M. Sultzer, *Dissociation of innate susceptibility to Salmonella infection and endotoxin responsiveness in C3HeB/FeJ mice and other strains in the C3H lineage.* Infection and Immunity, 1982. **36**(2): p. 696-703.
48. Velazquez, E.M., H. Nguyen, K.T. Heasley, C.H. Saechao, L.M. Gil, A.W.L. Rogers, B.M. Miller, M.R. Rolston, C.A. Lopez, Y. Litvak, M.J. Liou, F. Faber, D.N. Bronner, C.R. Tiffany, M.X. Byndloss, A.J. Byndloss, and A.J. Baumler, *Endogenous Enterobacteriaceae underlie variation in susceptibility to Salmonella infection.* Nat Microbiol, 2019. **4**(6): p. 1057-1064.
49. Woelfel, S., M.S. Silva, and B. Stecher, *Intestinal colonization resistance in the context of environmental, host, and microbial determinants.* Cell Host & Microbe, 2024. **32**(6): p. 820-836.
50. Caballero-Flores, G., J.M. Pickard, and G. Núñez, *Microbiota-mediated colonization resistance: mechanisms and regulation.* Nature Reviews Microbiology, 2023. **21**(6): p. 347-360.
51. Rogers, A.W.L., R.M. Tsolis, and A.J. Bäumler, *Salmonella versus the Microbiome.* Microbiology and Molecular Biology Reviews, 2021. **85**(1): p. 10.1128/mmbr.00027-19.
52. Stecher, B., R. Robbiani, A.W. Walker, A.M. Westendorf, M. Barthel, M. Kremer, S. Chaffron, A.J. Macpherson, J. Buer, J. Parkhill, G. Dougan, C. von Mering, and W.-D. Hardt, *Salmonella enterica Serovar Typhimurium Exploits Inflammation to Compete with the Intestinal Microbiota.* PLOS Biology, 2007. **5**(10): p. e244.
53. Hapfelmeier, S. and W.-D. Hardt, *A mouse model for S. typhimurium-induced enterocolitis.* Trends in Microbiology, 2005. **13**(10): p. 497-503.
54. Bohnhoff, M., B.L. Drake, and C.P. Miller, *Effect of streptomycin on susceptibility of intestinal tract to experimental Salmonella infection.* Proceedings of the Society for Experimental Biology and Medicine, 1954. **86**(1): p. 132-137.

55. Bohnhoff, M. and C.P. Miller, *Enhanced Susceptibility to Salmonella Infection in Streptomycin-Treated Mice.* The Journal of Infectious Diseases, 1962. **111**(2): p. 117-127.
56. Leleiwi, I., J. Rodriguez-Ramos, M. Shaffer, A. Sabag-Daigle, K. Kokkinias, R.M. Flynn, R.A. Daly, L.F.M. Kop, L.M. Solden, B.M.M. Ahmer, M.A. Borton, and K.C. Wrighton, *Exposing new taxonomic variation with inflammation — a murine model-specific genome database for gut microbiome researchers.* Microbiome, 2023. **11**(1): p. 114.
57. Wotzka, S.Y., M. Kreuzer, L. Maier, M. Arnoldini, B.D. Nguyen, A.O. Brachmann, D.L. Berthold, M. Zünd, A. Hausmann, E. Bakkeren, D. Hoces, E. Gül, M. Beutler, T. Dolowschiak, M. Zimmermann, T. Fuhrer, K. Moor, U. Sauer, A. Typas, J. Piel, M. Diard, A.J. Macpherson, B. Stecher, S. Sunagawa, E. Slack, and W.-D. Hardt, *Escherichia coli limits Salmonella Typhimurium infections after diet shifts and fat-mediated microbiota perturbation in mice.* Nature Microbiology, 2019. **4**(12): p. 2164-2174.
58. Stecher, B., *Establishing causality in Salmonella-microbiota-host interaction: The use of gnotobiotic mouse models and synthetic microbial communities.* International Journal of Medical Microbiology, 2021. **311**(3): p. 151484.
59. Alugupalli, K.R., S. Kothari, M.P. Cravens, J.A. Walker, D.T. Dougharty, G.S. Dickinson, L.A. Gatto, A.J. Bäumler, T. Wangdi, D.R. Miller, F. Pardo-Manuel de Villena, and L.D. Siracusa, *Identification of collaborative cross mouse strains permissive to Salmonella enterica serovar Typhi infection.* Scientific Reports, 2023. **13**(1): p. 393.
60. Lam, L.H. and D.M. Monack, *Intraspecies Competition for Niches in the Distal Gut Dictate Transmission during Persistent Salmonella Infection.* PLOS Pathogens, 2014. **10**(12): p. e1004527.
61. Ruby, T., L. McLaughlin, S. Gopinath, and D. Monack, *Salmonella's long-term relationship with its host.* FEMS Microbiology Reviews, 2012. **36**(3): p. 600-615.
62. Crawford, R.W., R. Rosales-Reyes, M.d.l.L. Ramirez-Aguilar, O. Chapa-Azuela, C. Alpuche-Aranda, and J.S. Gunn, *Gallstones play a significant role in Salmonella spp. gallbladder colonization and carriage.* Proceedings of the National Academy of Sciences, 2010. **107**(9): p. 4353-4358.
63. Prouty, A.M., W.H. Schwesinger, and J.S. Gunn, *Biofilm Formation and Interaction with the Surfaces of Gallstones by Salmonella spp.* Infection and Immunity, 2002. **70**(5): p. 2640-2649.
64. Ochman, H. and E.A. Groisman, *The origin and evolution of species differences in Escherichia coli and Salmonella typhimurium.* Exs, 1994. **69**: p. 479-93.
65. McQuiston, J.R., S. Herrera-Leon, B.C. Wertheim, J. Doyle, P.I. Fields, R.V. Tauxe, and J.M. Logsdon, *Molecular Phylogeny of the Salmonellae: Relationships among Salmonella Species and Subspecies Determined from Four Housekeeping Genes and Evidence of Lateral Gene Transfer Events.* Journal of Bacteriology, 2008. **190**(21): p. 7060-7067.

66. Fàbrega, A. and J. Vila, *Salmonella enterica Serovar Typhimurium Skills To Succeed in the Host: Virulence and Regulation.* Clinical Microbiology Reviews, 2013. **26**(2): p. 308-341.
67. Müller, S.I., M. Valdebenito, and K. Hantke, *Salmochelin, the long-overlooked catecholate siderophore of Salmonella.* BioMetals, 2009. **22**(4): p. 691-695.
68. Saphra, I. and J.W. Winter, *Clinical manifestations of salmonellosis in man; an evaluation of 7779 human infections identified at the New York Salmonella Center.* N Engl J Med, 1957. **256**(24): p. 1128-34.
69. Buchwald, D.S. and M.J. Blaser, *A review of human salmonellosis: II. Duration of excretion following infection with nontyphi Salmonella.* Rev Infect Dis, 1984. **6**(3): p. 345-56.
70. Wotzka, S.Y., B.D. Nguyen, and W.-D. Hardt, *Salmonella Typhimurium Diarrhea Reveals Basic Principles of Enteropathogen Infection and Disease-Promoted DNA Exchange.* Cell Host & Microbe, 2017. **21**(4): p. 443-454.
71. Rivera-Chávez, F. and A.J. Bäumler, *The pyromaniac inside you: Salmonella metabolism in the host gut.* Annual review of microbiology, 2015. **69**: p. 31-48.
72. Thiennimitr, P., S.E. Winter, and A.J. Baumler, *Salmonella, the host and its microbiota.* Curr Opin Microbiol, 2012. **15**(1): p. 108-14.
73. Grzymajlo, K., *The Game for Three: Salmonella-Host-Microbiota Interaction Models.* Front Microbiol, 2022. **13**: p. 854112.
74. Galán, J.E., *Salmonella Typhimurium and inflammation: a pathogen-centric affair.* Nature Reviews Microbiology, 2021. **19**(11): p. 716-725.
75. dos Santos, A.M.P., R.G. Ferrari, and C.A. Conte-Junior, *Virulence Factors in Salmonella Typhimurium: The Sagacity of a Bacterium.* Current Microbiology, 2019. **76**(6): p. 762-773.
76. Teunis, P.F.M., F. Kasuga, A. Fazil, I.D. Ogden, O. Rotariu, and N.J.C. Strachan, *Dose–response modeling of Salmonella using outbreak data.* International Journal of Food Microbiology, 2010. **144**(2): p. 243-249.
77. Giannella, R.A., S.A. Broitman, and N. Zamcheck, *Gastric acid barrier to ingested microorganisms in man: studies in vivo and in vitro.* Gut, 1972. **13**(4): p. 251-256.
78. Gül, E., U. Enz, L. Maurer, A. Abi Younes, S.A. Fattinger, B.D. Nguyen, A. Hausmann, M. Furter, M. Barthel, M.E. Sellin, and W.-D. Hardt, *Intraluminal neutrophils limit epithelium damage by reducing pathogen assault on intestinal epithelial cells during Salmonella gut infection.* PLOS Pathogens, 2023. **19**(6): p. e1011235.
79. Hapfelmeier, S., B.r. Stecher, M. Barthel, M. Kremer, A.J. Muller, M. Heikenwalder, T. Stallmach, M. Hensel, K. Pfeffer, and S. Akira, *The Salmonella pathogenicity island (SPI)-2 and SPI-1 type III secretion systems allow Salmonella serovar typhimurium to trigger colitis via MyD88-dependent and MyD88-independent mechanisms.* The Journal of Immunology, 2005. **174**(3): p. 1675-1685.
80. Coburn, B., Y. Li, D. Owen, B.A. Vallance, and B.B. Finlay, *Salmonella enterica Serovar Typhimurium Pathogenicity Island 2 Is Necessary for*

Complete Virulence in a Mouse Model of Infectious Enterocolitis. Infection and Immunity, 2005. **73**(6): p. 3219-3227.
81. Hume, P.J., V. Singh, A.C. Davidson, and V. Koronakis, *Swiss Army Pathogen: The Salmonella Entry Toolkit.* Frontiers in Cellular and Infection Microbiology, 2017. **7**(348).
82. Zhou, D., L.-M. Chen, L. Hernandez, S.B. Shears, and J.E. Galán, *A Salmonella inositol polyphosphatase acts in conjunction with other bacterial effectors to promote host cell actin cytoskeleton rearrangements and bacterial internalization.* Molecular Microbiology, 2001. **39**(2): p. 248-260.
83. Mallo, G.V., M. Espina, A.C. Smith, M.R. Terebiznik, A. Alemán, B.B. Finlay, L.E. Rameh, S. Grinstein, and J.H. Brumell, *SopB promotes phosphatidylinositol 3-phosphate formation on Salmonella vacuoles by recruiting Rab5 and Vps34.* The Journal of cell biology, 2008. **182**(4): p. 741-752.
84. Bakshi, C.S., V.P. Singh, M.W. Wood, P.W. Jones, T.S. Wallis, and E.E. Galyov, *Identification of SopE2, a Salmonella Secreted Protein Which Is Highly Homologous to SopE and Involved in Bacterial Invasion of Epithelial Cells.* Journal of Bacteriology, 2000. **182**(8): p. 2341-2344.
85. Bosco, E.E., J.C. Mulloy, and Y. Zheng, *Rac1 GTPase: A "Rac" of All Trades.* Cellular and Molecular Life Sciences, 2008. **66**(3): p. 370.
86. Etienne-Manneville, S., *Cdc42-the centre of polarity.* Journal of cell science, 2004. **117**(8): p. 1291-1300.
87. Binder, H.J., *Mechanisms of Diarrhea in Inflammatory Bowel Diseases.* Annals of the New York Academy of Sciences, 2009. **1165**(1): p. 285-293.
88. Zhang, S., R.A. Kingsley, R.L. Santos, H. Andrews-Polymenis, M. Raffatellu, J. Figueiredo, J. Nunes, R.M. Tsolis, L.G. Adams, and A.J. Bäumler, *Molecular Pathogenesis of Salmonella enterica Serotype Typhimurium-Induced Diarrhea.* Infection and Immunity, 2003. **71**(1): p. 1-12.
89. Norris, F.A., M.P. Wilson, T.S. Wallis, E.E. Galyov, and P.W. Majerus, *SopB, a protein required for virulence of Salmonella dublin, is an inositol phosphate phosphatase.* Proceedings of the National Academy of Sciences, 1998. **95**(24): p. 14057-14059.
90. Bertelsen, L.S., G. Paesold, S.L. Marcus, B.B. Finlay, L. Eckmann, and K.E. Barrett, *Modulation of chloride secretory responses and barrier function of intestinal epithelial cells by the Salmonella effector protein SigD.* American Journal of Physiology-Cell Physiology, 2004. **287**(4): p. C939-C948.
91. Marchelletta, R.R., M.G. Gareau, D.F. McCole, S. Okamoto, E. Roel, R. Klinkenberg, D.G. Guiney, J. Fierer, and K.E. Barrett, *Altered expression and localization of ion transporters contribute to diarrhea in mice with Salmonella-induced enteritis.* Gastroenterology, 2013. **145**(6): p. 1358-1368. e4.
92. Wallis, T.S., A.T.M. Vaughan, G.J. Clarke, G.-M. Qi, K.J. Worton, D.C.A. Candy, M.P. Osborne, and J. Stephen, *The role of leucocytes in the induction of fluid secretion by Salmonella typhimurium.* Journal of Medical Microbiology, 1990. **31**(1): p. 27-35.

93. Vanden Broeck, D., C. Horvath, and M.J.S. De Wolf, *Vibrio cholerae: Cholera toxin.* The International Journal of Biochemistry & Cell Biology, 2007. **39**(10): p. 1771-1775.
94. Marchelletta, R.R., M.G. Gareau, S. Okamoto, D.G. Guiney, K.E. Barrett, and J. Fierer, *Salmonella-induced Diarrhea Occurs in the Absence of IL-8 Receptor (CXCR2)-Dependent Neutrophilic Inflammation.* The Journal of Infectious Diseases, 2014. **212**(1): p. 128-136.
95. Woo, H., S. Okamoto, D. Guiney, J.S. Gunn, and J. Fierer, *A Model of Salmonella Colitis with Features of Diarrhea in SLC11A1 Wild-Type Mice.* PLOS ONE, 2008. **3**(2): p. e1603.
96. Zaffiri, L., J. Gardner, and L.H. Toledo-Pereyra, *History of antibiotics. From salvarsan to cephalosporins.* Journal of Investigative Surgery, 2012. **25**(2): p. 67-77.
97. Hutchings, M.I., A.W. Truman, and B. Wilkinson, *Antibiotics: past, present and future.* Current Opinion in Microbiology, 2019. **51**: p. 72-80.
98. Naghavi, M., S.E. Vollset, K.S. Ikuta, L.R. Swetschinski, A.P. Gray, E.E. Wool, G. Robles Aguilar, T. Mestrovic, G. Smith, C. Han, R.L. Hsu, J. Chalek, D.T. Araki, E. Chung, C. Raggi, A. Gershberg Hayoon, N. Davis Weaver, P.A. Lindstedt, A.E. Smith, U. Altay, N.V. Bhattacharjee, K. Giannakis, F. Fell, B. McManigal, N. Ekapirat, J.A. Mendes, T. Runghien, O. Srimokla, A. Abdelkader, S. Abd-Elsalam, R.G. Aboagye, H. Abolhassani, H. Abualruz, U. Abubakar, H.J. Abukhadijah, S. Aburuz, A. Abu-Zaid, S. Achalapong, I.Y. Addo, V. Adekanmbi, T.E. Adeyeoluwa, Q.E.S. Adnani, L.A. Adzigbli, M.S. Afzal, S. Afzal, A. Agodi, A.J. Ahlstrom, A. Ahmad, S. Ahmad, T. Ahmad, A. Ahmadi, A. Ahmed, H. Ahmed, I. Ahmed, M. Ahmed, S. Ahmed, S.A. Ahmed, M.A. Akkaif, S. Al Awaidy, Y. Al Thaher, S.O. Alalalmeh, M.T. AlBataineh, W.A. Aldhaleei, A.A.S. Al-Gheethi, N.B. Alhaji, A. Ali, L. Ali, S.S. Ali, W. Ali, K. Allel, S. Al-Marwani, A. Alrawashdeh, A. Altaf, A.B. Al-Tammemi, J.A. Al-Tawfiq, K.H. Alzoubi, W.A. Al-Zyoud, B. Amos, J.H. Amuasi, R. Ancuceanu, J.R. Andrews, A. Anil, I.A. Anuoluwa, S. Anvari, A.E. Anyasodor, G.L.C. Apostol, J. Arabloo, M. Arafat, A.Y. Aravkin, D. Areda, A. Aremu, A.A. Artamonov, E.A. Ashley, M.O. Asika, S.S. Athari, M.M.d.W. Atout, T. Awoke, S. Azadnajafabad, J.M. Azam, S. Aziz, A.Y. Azzam, M. Babaei, F.-X. Babin, M. Badar, A.A. Baig, M. Bajcetic, S. Baker, M. Bardhan, H.J. Barqawi, Z. Basharat, A. Basiru, M. Bastard, S. Basu, N.S. Bayleyegn, M.A. Belete, O.O. Bello, A. Beloukas, J.A. Berkley, A.S. Bhagavathula, S. Bhaskar, S.S. Bhuyan, J.A. Bielicki, N.I. Briko, C.S. Brown, A.J. Browne, D. Buonsenso, Y. Bustanji, C.G. Carvalheiro, C.A. Castañeda-Orjuela, M. Cenderadewi, J. Chadwick, S. Chakraborty, R.M. Chandika, S. Chandy, V. Chansamouth, V.K. Chattu, A.A. Chaudhary, P.R. Ching, H. Chopra, F.R. Chowdhury, D.-T. Chu, M. Chutiyami, N. Cruz-Martins, A.G. da Silva, O. Dadras, X. Dai, S.D. Darcho, S. Das, F.P. De la Hoz, D.M. Dekker, K. Dhama, D. Diaz, B.F.R. Dickson, S.G. Djorie, M. Dodangeh, S. Dohare, K.G. Dokova, O.P. Doshi, R.K. Dowou, H.L. Dsouza, S.J. Dunachie, A.M. Dziedzic, T. Eckmanns, A. Ed-Dra, A. Eftekharimehrabad, T.C. Ekundayo,

I. El Sayed, M. Elhadi, W. El-Huneidi, C. Elias, S.J. Ellis, R. Elsheikh, I. Elsohaby, C. Eltaha, B. Eshrati, M. Eslami, D.W. Eyre, A.O. Fadaka, A.F. Fagbamigbe, A. Fahim, A. Fakhri-Demeshghieh, F.O. Fasina, M.M. Fasina, A. Fatehizadeh, N.A. Feasey, A. Feizkhah, G. Fekadu, F. Fischer, I. Fitriana, K.M. Forrest, C. Fortuna Rodrigues, J.E. Fuller, M.A. Gadanya, M. Gajdács, A.P. Gandhi, E.E. Garcia-Gallo, D.O. Garrett, R.K. Gautam, M.W. Gebregergis, M. Gebrehiwot, T.G. Gebremeskel, C. Geffers, L. Georgalis, R.M. Ghazy, M. Golechha, D. Golinelli, M. Gordon, S. Gulati, R.D. Gupta, S. Gupta, V.K. Gupta, A.D. Habteyohannes, S. Haller, H. Harapan, M.L. Harrison, A.I. Hasaballah, I. Hasan, R.S. Hasan, H. Hasani, A.H. Haselbeck, M.S. Hasnain, I.I. Hassan, S. Hassan, M.S. Hassan Zadeh Tabatabaei, K. Hayat, J. He, O.E. Hegazi, M. Heidari, K. Hezam, R. Holla, M. Holm, H. Hopkins, M.M. Hossain, M. Hosseinzadeh, S. Hostiuc, N.R. Hussein, L.D. Huy, E.D. Ibáñez-Prada, A. Ikiroma, I.M. Ilic, S.M.S. Islam, F. Ismail, N.E. Ismail, C.D. Iwu, C.J. Iwu-Jaja, A. Jafarzadeh, F. Jaiteh, R. Jalilzadeh Yengejeh, R.D.G. Jamora, J. Javidnia, T. Jawaid, A.W.J. Jenney, H.J. Jeon, M. Jokar, N. Jomehzadeh, T. Joo, N. Joseph, Z. Kamal, K.K. Kanmodi, R.S. Kantar, J.A. Kapisi, I.M. Karaye, Y.S. Khader, H. Khajuria, N. Khalid, F. Khamesipour, A. Khan, M.J. Khan, M.T. Khan, V. Khanal, F.F. Khidri, J. Khubchandani, S. Khusuwan, M.S. Kim, A. Kisa, V.A. Korshunov, F. Krapp, R. Krumkamp, M. Kuddus, M. Kulimbet, D. Kumar, E.A.P. Kumaran, A. Kuttikkattu, H.H. Kyu, I. Landires, B.K. Lawal, T.T.T. Le, I.M. Lederer, M. Lee, S.W. Lee, A. Lepape, T.L. Lerango, V.S. Ligade, C. Lim, S.S. Lim, L.W. Limenh, C. Liu, X. Liu, X. Liu, M.J. Loftus, H.I. M Amin, K.L. Maass, S.B. Maharaj, M.A. Mahmoud, P. Maikanti-Charalampous, O.M. Makram, K. Malhotra, A.A. Malik, G.D. Mandilara, F. Marks, B.A. Martinez-Guerra, M. Martorell, H. Masoumi-Asl, A.G. Mathioudakis, J. May, T.A. McHugh, J. Meiring, H.N. Meles, A. Melese, E.B. Melese, G. Minervini, N.S. Mohamed, S. Mohammed, S. Mohan, A.H. Mokdad, L. Monasta, A. Moodi Ghalibaf, C.E. Moore, Y. Moradi, E. Mossialos, V. Mougin, G.D. Mukoro, F. Mulita, B. Muller-Pebody, E. Murillo-Zamora, S. Musa, P. Musicha, L.A. Musila, S. Muthupandian, A.J. Nagarajan, P. Naghavi, F. Nainu, T.S. Nair, H.H.R. Najmuldeen, Z.S. Natto, J. Nauman, B.P. Nayak, G.T. Nchanji, P. Ndishimye, I. Negoi, R.I. Negoi, S.A. Nejadghaderi, Q.P. Nguyen, E.A. Noman, D.C. Nwakanma, S. O'Brien, T.J. Ochoa, I.A. Odetokun, O.A. Ogundijo, T.R. Ojo-Akosile, S.R. Okeke, O.C. Okonji, A.T. Olagunju, A. Olivas-Martinez, A.A. Olorukooba, P. Olwoch, K.I. Onyedibe, E. Ortiz-Brizuela, O. Osuolale, P. Ounchanum, O.T. Oyeyemi, M.P. P A, J.L. Paredes, R.R. Parikh, J. Patel, S. Patil, S. Pawar, A.Y. Peleg, P. Peprah, J. Perdigão, C. Perrone, I.-R. Petcu, K. Phommasone, Z.Z. Piracha, D. Poddighe, A.J. Pollard, R. Poluru, A. Ponce-De-Leon, J. Puvvula, F.N. Qamar, N.H. Qasim, C.D. Rafai, P. Raghav, L. Rahbarnia, F. Rahim, V. Rahimi-Movaghar, M. Rahman, M.A. Rahman, H. Ramadan, S.K. Ramasamy, P.S. Ramesh, P.W. Ramteke, R.K. Rana, U. Rani, M.-M. Rashidi, D. Rathish, S. Rattanavong, S. Rawaf, E.M.M. Redwan, L.F. Reyes, T. Roberts, J.V. Robotham, V.D. Rosenthal, A.G. Ross, N. Roy, K.E. Rudd, C.J. Sabet, B.A.

Saddik, M.R. Saeb, U. Saeed, S. Saeedi Moghaddam, W. Saengchan, M. Safaei, A. Saghazadeh, N. Saheb Sharif-Askari, A. Sahebkar, S.S. Sahoo, M. Sahu, M. Saki, N. Salam, Z. Saleem, M.A. Saleh, Y.L. Samodra, A.M. Samy, A. Saravanan, M. Satpathy, A.E. Schumacher, M. Sedighi, S. Seekaew, M. Shafie, P.A. Shah, S. Shahid, M.J. Shahwan, S. Shakoor, N. Shalev, M.A. Shamim, M.A. Shamshirgaran, A. Shamsi, A. Sharifan, R.P. Shastry, M. Shetty, A. Shittu, S. Shrestha, E.E. Siddig, T. Sideroglou, J. Sifuentes-Osornio, L.M.L.R. Silva, E.A.F. Simões, A.J.H. Simpson, A. Singh, S. Singh, R. Sinto, S.S.M. Soliman, S. Soraneh, N. Stoesser, T.Z. Stoeva, C.K. Swain, L. Szarpak, S.S. T Y, S. Tabatabai, C. Tabche, Z.M.-A. Taha, K.-K. Tan, N. Tasak, N.Y. Tat, A. Thaiprakong, P. Thangaraju, C.C. Tigoi, K. Tiwari, M.R. Tovani-Palone, T.H. Tran, M. Tumurkhuu, P. Turner, A.J. Udoakang, A. Udoh, N. Ullah, S. Ullah, A.G. Vaithinathan, M. Valenti, T. Vos, H.T.L. Vu, Y. Waheed, A.S. Walker, J.L. Walson, T. Wangrangsimakul, K.G. Weerakoon, H.F.L. Wertheim, P.C.M. Williams, A.A. Wolde, T.M. Wozniak, F. Wu, Z. Wu, M.K.K. Yadav, S. Yaghoubi, Z.S. Yahaya, A. Yarahmadi, S. Yezli, Y.E. Yismaw, D.K. Yon, C.-W. Yuan, H. Yusuf, F. Zakham, G. Zamagni, H. Zhang, Z.-J. Zhang, M. Zielińska, A. Zumla, S.e.H.H. Zyoud, S.H. Zyoud, S.I. Hay, A. Stergachis, B. Sartorius, B.S. Cooper, C. Dolecek and C.J.L. Murray, *Global burden of bacterial antimicrobial resistance 1990–2021: a systematic analysis with forecasts to 2050.* The Lancet, 2024. **404**(10459): p. 1199-1226.

99. Smits, W.K., D. Lyras, D.B. Lacy, M.H. Wilcox, and E.J. Kuijper, *Clostridium difficile infection.* Nature Reviews Disease Primers, 2016. **2**(1): p. 16020.
100. Aserkoff, B. and J.V. Bennett, *Effect of antibiotic therapy in acute salmonellosis on the fecal excretion of salmonellae.* New England Journal of Medicine, 1969. **281**(12): p. 636-640.
101. Wiström, J., M. Jertborn, E. Ekwall, K. Norlin, B. Söderquist, A. Strömberg, R. Lundholm, H. Hogevik, L. Lagergren, and G. Englund, *Empiric treatment of acute diarrheal disease with norfloxacin: a randomized, placebo-controlled study.* Annals of internal medicine, 1992. **117**(3): p. 202-208.
102. Onwuezobe, I.A., P.O. Oshun, and C.C. Odigwe, *Antimicrobials for treating symptomatic non-typhoidal Salmonella infection.* Cochrane Database of Systematic Reviews, 2012(11).
103. Nelson, J.D., H. Kusmiesz, L.H. Jackson, and E. Woodman, *Treatment of Salmonella gastroenteritis with ampicillin, amoxicillin, or placebo.* Pediatrics, 1980. **65**(6): p. 1125-1130.
104. Dolowschiak, T., A.A. Mueller, L.J. Pisan, R. Feigelman, B. Felmy, M.E. Sellin, S. Namineni, B.D. Nguyen, S.Y. Wotzka, and M. Heikenwalder, *IFN-γ hinders recovery from mucosal inflammation during antibiotic therapy for Salmonella gut infection.* Cell host & microbe, 2016. **20**(2): p. 238-249.
105. Kotloff, K.L., *Bacterial diarrhoea.* Current opinion in pediatrics, 2022. **34**(2): p. 147-155.

106. Chen, H.-M., Y. Wang, L.-H. Su, and C.-H. Chiu, *Nontyphoid Salmonella infection: microbiology, clinical features, and antimicrobial therapy.* Pediatrics & Neonatology, 2013. **54**(3): p. 147-152.
107. Marchello, C.S., A.P. Dale, S. Pisharody, M.P. Rubach, and J.A. Crump, *A systematic review and meta-analysis of the prevalence of community-onset bloodstream infections among hospitalized patients in Africa and Asia.* Antimicrobial agents and chemotherapy, 2019. **64**(1): p. 10.1128/aac. 01974-19.
108. Marks, F., V. von Kalckreuth, P. Aaby, Y. Adu-Sarkodie, M.A. El Tayeb, M. Ali, A. Aseffa, S. Baker, H.M. Biggs, and M. Bjerregaard-Andersen, *Incidence of invasive salmonella disease in sub-Saharan Africa: a multicentre population-based surveillance study.* The Lancet Global Health, 2017. **5**(3): p. e310-e323.
109. Klemm, E.J., S. Shakoor, A.J. Page, F.N. Qamar, K. Judge, D.K. Saeed, V.K. Wong, T.J. Dallman, S. Nair, and S. Baker, *Emergence of an extensively drug-resistant Salmonella enterica serovar Typhi clone harboring a promiscuous plasmid encoding resistance to fluoroquinolones and third-generation cephalosporins.* MBio, 2018. **9**(1): p. 10.1128/mbio. 00105-18.
110. Deutscher, J., C. Francke, and P.W. Postma, *How Phosphotransferase System-Related Protein Phosphorylation Regulates Carbohydrate Metabolism in Bacteria.* Microbiology and Molecular Biology Reviews, 2006. **70**(4): p. 939-1031.
111. Boulanger, E.F., A. Sabag-Daigle, P. Thirugnanasambantham, V. Gopalan, and B.M.M. Ahmer, *Sugar-Phosphate Toxicities.* Microbiology and Molecular Biology Reviews, 2021. **85**(4): p. e00123-21.
112. Sabag-Daigle, A., E.F. Boulanger, P. Thirugnanasambantham, J.D. Law, A.J. Bogard, E.J. Behrman, V. Gopalan, and B.M.M. Ahmer, *Identification of Small-Molecule Inhibitors of the Salmonella FraB Deglycase Using a Live-Cell Assay.* Microbiology Spectrum, 2023. **11**(2): p. e04606-22.
113. Ali, M.M., D.L. Newsom, J.F. González, A. Sabag-Daigle, C. Stahl, B. Steidley, J. Dubena, J.L. Dyszel, J.N. Smith, Y. Dieye, R. Arsenescu, P.N. Boyaka, S. Krakowka, T. Romeo, E.J. Behrman, P. White, and B.M.M. Ahmer, *Fructose-Asparagine Is a Primary Nutrient during Growth of Salmonella in the Inflamed Intestine.* PLOS Pathogens, 2014. **10**(6): p. e1004209.
114. Patel, T.K. and J.D. Williamson, *Mannitol in Plants, Fungi, and Plant–Fungal Interactions.* Trends in Plant Science, 2016. **21**(6): p. 486-497.
115. Nasrallah, S.M. and F.L. Iber, *Mannitol absorption and metabolism in man.* Am J Med Sci, 1969. **258**(2): p. 80-8.
116. Deis, R.C. and M.W. Kearsley, *Sorbitol and Mannitol*, in *Sweeteners and Sugar Alternatives in Food Technology*. 2012. p. 331-346.
117. Warren, S.E. and R.C. Blantz, *Mannitol.* Archives of internal medicine, 1981. **141**(4): p. 493-497.
118. Nissenson, A.R., R.E. Weston, and C.R. Kleeman, *Mannitol.* West J Med, 1979. **131**(4): p. 277-84.

119. Anderson, S.D., E. Daviskas, J.D. Brannan, and H.K. Chan, *Repurposing excipients as active inhalation agents: The mannitol story.* Advanced Drug Delivery Reviews, 2018. **133**: p. 45-56.
120. Blanchard, A.C. and V.J. Waters, *Opportunistic Pathogens in Cystic Fibrosis: Epidemiology and Pathogenesis of Lung Infection.* Journal of the Pediatric Infectious Diseases Society, 2022. **11**(Supplement_2): p. S3-S12.
121. Nguyen, T., T. Kim, H.M. Ta, W.S. Yeo, J. Choi, P. Mizar, S.S. Lee, T. Bae, A.K. Chaurasia, and K.K. Kim, *Targeting mannitol metabolism as an alternative antimicrobial strategy based on the structure-function study of mannitol-1-phosphate dehydrogenase in Staphylococcus aureus.* MBio, 2019. **10**(4): p. e02660-18.
122. Kenny, J.G., J. Moran, S.L. Kolar, A. Ulanov, Z. Li, L.N. Shaw, E. Josefsson, and M.J. Horsburgh, *Mannitol Utilisation is Required for Protection of Staphylococcus aureus from Human Skin Antimicrobial Fatty Acids.* PLOS ONE, 2013. **8**(7): p. e67698.
123. Timm, M.R., S.K. Russell, and S.J. Hultgren, *Urinary tract infections: pathogenesis, host susceptibility and emerging therapeutics.* Nature Reviews Microbiology, 2024.
124. Lee, C.A. and M.H. Saier, Jr., *Mannitol-specific enzyme II of the bacterial phosphotransferase system. III. The nucleotide sequence of the permease gene.* J Biol Chem, 1983. **258**(17): p. 10761-7.
125. Lengeler, J., *Mutations affecting transport of the hexitols D-mannitol, D-glucitol, and galactitol in Escherichia coli K-12: isolation and mapping.* Journal of Bacteriology, 1975. **124**(1): p. 26-38.
126. Choe, M., H. Min, Y.H. Park, Y.R. Kim, J.S. Woo, and Y.J. Seok, *Structural insight into glucose repression of the mannitol operon.* Sci Rep, 2019. **9**(1): p. 13930.
127. Choe, M., Y.H. Park, C.R. Lee, Y.R. Kim, and Y.J. Seok, *The general PTS component HPr determines the preference for glucose over mannitol.* Sci Rep, 2017. **7**: p. 43431.
128. Joyet, P., M. Derkaoui, H. Bouraoui, and J. Deutscher, *PTS-Mediated Regulation of the Transcription Activator MtlR from Different Species: Surprising Differences despite Strong Sequence Conservation.* J Mol Microbiol Biotechnol, 2015. **25**(2-3): p. 94-105.
129. Tan, K., S. Clancy, M. Borovilos, M. Zhou, S. Hörer, S. Moy, L.L. Volkart, J. Sassoon, U. Baumann, and A. Joachimiak, *The mannitol operon repressor MtlR belongs to a new class of transcription regulators in bacteria.* J Biol Chem, 2009. **284**(52): p. 36670-36679.
130. Figge, R.M., T.M. Ramseier, and M.H. Saier, *The mannitol repressor (MtlR) of Escherichia coli.* Journal of Bacteriology, 1994. **176**(3): p. 840-847.
131. Boulanger, E.F., A. Sabag-Daigle, M. Baniasad, K. Kokkinias, A. Schwieters, K.C. Wrighton, V.H. Wysocki, and B.M.M. Ahmer, *Sugar-Phosphate Toxicities Attenuate Salmonella Fitness in the Gut.* Journal of Bacteriology, 2022. **0**(0): p. e00344-22.

132. Jensen, P., C. Parkes, and D. Berkowitz, *Mannitol Sensitivity.* Journal of Bacteriology, 1972. **111**(2): p. 351-355.
133. Edwards, K.G., H.J. Blumenthal, M. Khan, and M.E. Slodki, *Intracellular mannitol, a product of glucose metabolism in staphylococci.* Journal of Bacteriology, 1981. **146**(3): p. 1020-1029.
134. Berkowitz, D., *D-Mannitol utilization in Salmonella typhimurium.* J Bacteriol, 1971. **105**(1): p. 232-40.
135. Whiteley, M., S.P. Diggle, and E.P. Greenberg, *Progress in and promise of bacterial quorum sensing research.* Nature, 2017. **551**(7680): p. 313-320.
136. Aframian, N. and A. Eldar, *A Bacterial Tower of Babel: Quorum-Sensing Signaling Diversity and Its Evolution.* Annual Review of Microbiology, 2020. **74**(1): p. 587-606.
137. Laue, B.E., Y. Jiang, S.R. Chhabra, S. Jacob, G.S.A.B. Stewart, A. Hardman, J.A. Downie, F. O'Gara, and P. Williams, *The biocontrol strain Pseudomonas fluorescens F113 produces the Rhizobium small bacteriocin, N-(3-hydroxy-7-cis-tetradecenoyl)homoserine lactone, via HdtS, a putative novel N-acylhomoserine lactone synthaseThe GenBank accession number for the sequence determined in this work is AF286536.* Microbiology, 2000. **146**(10): p. 2469-2480.
138. Michael, B., J.N. Smith, S. Swift, F. Heffron, and B.M.M. Ahmer, *SdiA of Salmonella enterica Is a LuxR Homolog That Detects Mixed Microbial Communities.* Journal of Bacteriology, 2001. **183**(19): p. 5733-5742.
139. Ahmer, B.M.M., J. van Reeuwijk, C.D. Timmers, P.J. Valentine, and F. Heffron, *Salmonella typhimurium Encodes an SdiA Homolog, a Putative Quorum Sensor of the LuxR Family, That Regulates Genes on the Virulence Plasmid.* Journal of Bacteriology, 1998. **180**(5): p. 1185-1193.
140. Visick, K.L. and C. Fuqua, *Decoding Microbial Chatter: Cell-Cell Communication in Bacteria.* Journal of Bacteriology, 2005. **187**(16): p. 5507-5519.
141. Zhang, L.-H. and Y.-H. Dong, *Quorum sensing and signal interference: diverse implications.* Molecular Microbiology, 2004. **53**(6): p. 1563-1571.
142. Fathpour, H., G. Emtiazi, and E. Ghasemi, *Cockroaches as reservoirs and vectors of drug resistant Salmonella spp.* Iranian Biomedical Journal, 2003. **7**(1): p. 35-38.
143. Noel, J.T.J., J; Smith, JN; Fatica, M; Schneider, KR; Ahmer, BM; Teplitski, M, *Salmonella SdiA Recognizes N-acyl Homoserine Lactone Signals from Pectobacterium carotovorum in Vitro, but Not in a Bacterial Soft Rot.* Molecular Plant-Microbe Interactions, 2010. **23**(3): p. 273-282.
144. Dyszel, J.L., J.N. Smith, D.E. Lucas, J.A. Soares, M.C. Swearingen, M.A. Vross, G.M. Young, and B.M.M. Ahmer, *Salmonella enterica serovar Typhimurium can detect acyl homoserine lactone production by Yersinia enterocolitica in mice.* Journal of bacteriology, 2010. **192**(1): p. 29-37.
145. Smith, J.N., J.L. Dyszel, J.A. Soares, C.D. Ellermeier, C. Altier, S.D. Lawhon, L.G. Adams, V. Konjufca, R. Curtiss, III, J.M. Slauch, and B.M.M. Ahmer, *SdiA, an N-Acylhomoserine Lactone Receptor, Becomes Active during the Transit of*

Salmonella enterica through the Gastrointestinal Tract of Turtles. PLOS ONE, 2008. **3**(7): p. e2826.
146. Smith, J.N. and B.M.M. Ahmer, *Detection of Other Microbial Species by Salmonella: Expression of the SdiA Regulon.* Journal of Bacteriology, 2003. **185**(4): p. 1357-1366.
147. Pompeani, A.J., J.J. Irgon, M.F. Berger, M.L. Bulyk, N.S. Wingreen, and B.L. Bassler, *The Vibrio harveyi master quorum-sensing regulator, LuxR, a TetR-type protein is both an activator and a repressor: DNA recognition and binding specificity at target promoters.* Mol Microbiol, 2008. **70**(1): p. 76-88.
148. Egland, K.A. and E.P. Greenberg, *Quorum sensing in Vibrio fischeri: elements of the luxI promoter.* Molecular Microbiology, 1999. **31**(4): p. 1197-1204.
149. Castang, S., S. Reverchon, P. Gouet, and W. Nasser, *Direct Evidence for the Modulation of the Activity of the Erwinia chrysanthemi Quorum-sensing Regulator ExpR by Acylhomoserine Lactone Pheromone.* Journal of Biological Chemistry, 2006. **281**(40): p. 29972-29987.
150. White, C.E. and S.C. Winans, *Identification of amino acid residues of the Agrobacterium tumefaciens quorum-sensing regulator TraR that are critical for positive control of transcription.* Molecular Microbiology, 2005. **55**(5): p. 1473-1486.
151. Yamamoto, K., K. Yata, N. Fujita, and A. Ishihama, *Novel mode of transcription regulation by SdiA, an Escherichia coli homologue of the quorum-sensing regulator.* Mol Microbiol, 2001. **41**(5): p. 1187-98.
152. Schuster, M. and E.P. Greenberg, *LuxR-Type Proteins in Pseudomonas aeruginosa Quorum Sensing: Distinct Mechanisms with Global Implications*, in *Chemical Communication among Bacteria*. 2008, American Society of Microbiology.
153. Zhu, J. and S.C. Winans, *The quorum-sensing transcriptional regulator TraR requires its cognate signaling ligand for protein folding, protease resistance, and dimerization.* Proceedings of the National Academy of Sciences, 2001. **98**(4): p. 1507-1512.
154. Urbanowski, M.L., C.P. Lostroh, and E.P. Greenberg, *Reversible acyl-homoserine lactone binding to purified Vibrio fischeri LuxR protein.* J Bacteriol, 2004. **186**(3): p. 631-7.
155. Nguyen, Y., N.X. Nguyen, J.L. Rogers, J. Liao, J.B. MacMillan, Y. Jiang, and V. Sperandio, *Structural and mechanistic roles of novel chemical ligands on the SdiA quorum-sensing transcription regulator.* MBio, 2015. **6**(2).
156. Kim, T., T. Duong, C.-a. Wu, J. Choi, N. Lan, S.W. Kang, N.K. Lokanath, D. Shin, H.-Y. Hwang, and K.K. Kim, *Structural insights into the molecular mechanism of Escherichia coli SdiA, a quorum-sensing receptor.* Acta Crystallographica Section D: Biological Crystallography, 2014. **70**(3): p. 694-707.
157. Sabag-Daigle, A., J.L. Dyszel, J.F. Gonzalez, M.M. Ali, and B.M.M. Ahmer, *Identification of sdiA-regulated genes in a mouse commensal strain of Enterobacter cloacae.* Frontiers in Cellular and Infection Microbiology, 2015. **5**(47).

158. Dyszel, J.L., J.A. Soares, M.C. Swearingen, A. Lindsay, J.N. Smith, and B.M.M. Ahmer, *E. coli K-12 and EHEC Genes Regulated by SdiA.* PLOS ONE, 2010. **5**(1): p. e8946.
159. Yao, Y., M.A. Martinez-Yamout, T.J. Dickerson, A.P. Brogan, P.E. Wright, and H.J. Dyson, *Structure of the Escherichia coli Quorum Sensing Protein SdiA: Activation of the Folding Switch by Acyl Homoserine Lactones.* Journal of Molecular Biology, 2006. **355**(2): p. 262-273.
160. Abed, N., O. Grépinet, S. Canepa, G.A. Hurtado-Escobar, N. Guichard, A. Wiedemann, P. Velge, and I. Virlogeux-Payant, *Direct regulation of the pefI-srgC operon encoding the Rck invasin by the quorum-sensing regulator SdiA in Salmonella Typhimurium.* Molecular Microbiology, 2014. **94**(2): p. 254-271.
161. Camilli, A., D.T. Beattie, and J.J. Mekalanos, *Use of genetic recombination as a reporter of gene expression.* Proceedings of the National Academy of Sciences, 1994. **91**(7): p. 2634-2638.
162. Slauch, J.M. and A. Camilli, *[5] IVET and RIVET: Use of gene fusions to identify bacterial virulence factors specifically induced in host tissues*, in *Methods in Enzymology*. 2000, Academic Press. p. 73-96.
163. Falcão, D.P., M.T. Shimizu, and L.R. Trabulsi, *Kinetics of infection induced byYersinia.* Current Microbiology, 1984. **11**(5): p. 303-308.
164. Carter, P.B., *Pathogenecity of Yersinia enterocolitica for mice.* Infection and Immunity, 1975. **11**(1): p. 164-170.
165. Habyarimana, F., M.C. Swearingen, G.M. Young, S. Seveau, and B.M.M. Ahmer, *Yersinia enterocolitica Inhibits Salmonella enterica Serovar Typhimurium and Listeria monocytogenes Cellular Uptake.* Infection and Immunity, 2014. **82**(1): p. 174-183.
166. Habyarimana, F., A. Sabag-Daigle, and B.M.M. Ahmer, *The SdiA-regulated gene srgE encodes a type III secreted effector.* Journal of bacteriology, 2014. **196**(12): p. 2301-2312.
167. Rosselin, M., I. Virlogeux-Payant, C. Roy, E. Bottreau, P.-Y. Sizaret, L. Mijouin, P. Germon, E. Caron, P. Velge, and A. Wiedemann, *Rck of Salmonella enterica, subspecies enterica serovar Enteritidis, mediates Zipper-like internalization.* Cell research, 2010. **20**(6): p. 647.
168. Ritchie, A.J., A.O. Yam, K.M. Tanabe, S.A. Rice, and M.A. Cooley, *Modification of in vivo and in vitro T-and B-cell-mediated immune responses by the Pseudomonas aeruginosa quorum-sensing molecule N-(3-oxododecanoyl)-L-homoserine lactone.* Infection and immunity, 2003. **71**(8): p. 4421-4431.
169. Tateda, K., Y. Ishii, M. Horikawa, T. Matsumoto, S. Miyairi, J.C. Pechere, T.J. Standiford, M. Ishiguro, and K. Yamaguchi, *The Pseudomonas aeruginosa autoinducer N-3-oxododecanoyl homoserine lactone accelerates apoptosis in macrophages and neutrophils.* Infection and immunity, 2003. **71**(10): p. 5785-5793.
170. Telford, G., D. Wheeler, P. Williams, P. Tomkins, P. Appleby, H. Sewell, G.S. Stewart, B.W. Bycroft, and D.I. Pritchard, *The Pseudomonas aeruginosaquorum-*

sensing signal moleculen-(3-oxododecanoyl)-l-homoserine lactone has immunomodulatory activity. Infection and immunity, 1998. **66**(1): p. 36-42.
171. Oliveira, R.A., V. Cabral, I. Torcato, and K.B. Xavier, *Deciphering the quorum-sensing lexicon of the gut microbiota.* Cell Host & Microbe, 2023. **31**(4): p. 500-512.
172. Swearingen, M.C., A. Sabag-Daigle, and B.M.M. Ahmer, *Are there acyl-homoserine lactones within mammalian intestines?* Journal of bacteriology, 2013. **195**(2): p. 173-179.
173. Grellier, N., M.T. Suzuki, L. Brot, A.M.S. Rodrigues, L. Humbert, K. Escoubeyrou, D. Rainteau, J.-P. Grill, R. Lami, and P. Seksik, *Impact of IBD-Associated Dysbiosis on Bacterial Quorum Sensing Mediated by Acyl-Homoserine Lactone in Human Gut Microbiota.* International Journal of Molecular Sciences, 2022. **23**(23): p. 15404.
174. Xue, J., L. Chi, P. Tu, Y. Lai, C.-W. Liu, H. Ru, and K. Lu, *Detection of gut microbiota and pathogen produced N-acyl homoserine in host circulation and tissues.* npj Biofilms and Microbiomes, 2021. **7**(1): p. 53.
175. Landman, C., J.P. Grill, J.M. Mallet, P. Marteau, L. Humbert, E. Le Balc'h, M.A. Maubert, K. Perez, W. Chaara, L. Brot, L. Beaugerie, H. Sokol, S. Thenet, D. Rainteau, P. Seksik, and E. Quévrain, *Inter-kingdom effect on epithelial cells of the N-Acyl homoserine lactone 3-oxo-C12:2, a major quorum-sensing molecule from gut microbiota.* PLoS One, 2018. **13**(8): p. e0202587.
176. Styles, M.J. and H.E. Blackwell, *Non-native autoinducer analogs capable of modulating the SdiA quorum sensing receptor in Salmonella enterica serovar Typhimurium.* Beilstein journal of organic chemistry, 2018. **14**(1): p. 2651-2664.
177. Sabag-Daigle, A., J.A. Soares, J.N. Smith, M.E. Elmasry, and B.M.M. Ahmer, *The Acyl Homoserine Lactone Receptor, SdiA, of Escherichia coli and Salmonella enterica Serovar Typhimurium Does Not Respond to Indole.* Applied and Environmental Microbiology, 2012. **78**(15): p. 5424-5431.
178. Argüello, H., J. Estellé, S. Zaldívar-López, Á. Jiménez-Marín, A. Carvajal, M.A. López-Bascón, F. Crispie, O. O'Sullivan, P.D. Cotter, F. Priego-Capote, L. Morera, and J.J. Garrido, *Early Salmonella Typhimurium infection in pigs disrupts Microbiome composition and functionality principally at the ileum mucosa.* Scientific Reports, 2018. **8**(1): p. 7788.
179. Borton, M.A., A. Sabag-Daigle, J. Wu, L.M. Solden, B.S. O'Banion, R.A. Daly, R.A. Wolfe, J.F. Gonzalez, V.H. Wysocki, and B.M. Ahmer, *Chemical and pathogen-induced inflammation disrupt the murine intestinal microbiome.* Microbiome, 2017. **5**: p. 1-15.
180. Stecher, B., L. Maier, and W.-D. Hardt, *Blooming; in the gut: how dysbiosis might contribute to pathogen evolution.* Nature Reviews Microbiology, 2013. **11**: p. 277.
181. Sheng, H., Y.N. Nguyen, C.J. Hovde, and V. Sperandio, *SdiA Aids Enterohemorrhagic Escherichia coli Carriage by Cattle Fed a Forage or Grain Diet.* Infection and Immunity, 2013. **81**(9): p. 3472-3478.

182. Sperandio, V., *SdiA sensing of acyl-homoserine lactones by enterohemorrhagic E. coli (EHEC) serotype O157:H7 in the bovine rumen.* Gut Microbes, 2010. **1**(6): p. 432-5.
183. Hughes, D.T., D.A. Terekhova, L. Liou, C.J. Hovde, J.W. Sahl, A.V. Patankar, J.E. Gonzalez, T.S. Edrington, D.A. Rasko, and V. Sperandio, *Chemical sensing in mammalian host-bacterial commensal associations.* Proc Natl Acad Sci U S A, 2010. **107**(21): p. 9831-6.
184. Sharma, V.K. and S.M.D. Bearson, *Evaluation of the impact of quorum sensing transcriptional regulator SdiA on long-term persistence and fecal shedding of Escherichia coli O157:H7 in weaned calves.* Microbial Pathogenesis, 2013. **57**: p. 21-26.
185. Edrington, T.S., R.L. Farrow, V. Sperandio, D.T. Hughes, T.E. Lawrence, T.R. Callaway, R.C. Anderson, and D.J. Nisbet, *Acyl-Homoserine-Lactone Autoinducer in the Gastrointesinal Tract of Feedlot Cattle and Correlation to Season, E. Coli O157:H7 Prevalence, and Diet.* Current Microbiology, 2009. **58**(3): p. 227-232.
186. Kuschke, S.G., *What lives on and in the sea turtle? A literature review of sea turtle bacterial microbiota.* Animal Microbiome, 2022. **4**(1): p. 52.
187. Blazar, J., M. Allard, and E.K. Lienau, *Insects as vectors of foodborne pathogenic bacteria.* Terrestrial Arthropod Reviews, 2011. **4**(1): p. 5-16.
188. Engel, P. and N.A. Moran, *The gut microbiota of insects – diversity in structure and function.* FEMS Microbiology Reviews, 2013. **37**(5): p. 699-735.
189. de Freitas, L.L., D.G. Carneiro, G.S. Oliveira, and M.C.D. Vanetti, *N-acyl-homoserine lactone produced by Rahnella inusitata isolated from the gut of Galleria mellonella influences Salmonella phenotypes.* Brazilian Journal of Microbiology, 2022.
190. Luiz de Freitas, L., F. Pereira da Silva, K.M. Fernandes, D.G. Carneiro, L. Licursi de Oliveira, G.F. Martins, and M.C. Dantas Vanetti, *The virulence of Salmonella Enteritidis in Galleria mellonella is improved by N-dodecanoyl-homoserine lactone.* Microbial Pathogenesis, 2021. **152**: p. 104730.
191. Hartmann, A., M. Rothballer, B.A. Hense, and P. Schröder, *Bacterial quorum sensing compounds are important modulators of microbe-plant interactions.* Frontiers in Plant Science, 2014. **5**(131).
192. Cellini, A., I. Donati, L. Fiorentini, E. Vandelle, A. Polverari, V. Venturi, G. Buriani, J.L. Vanneste, and F. Spinelli, *N-Acyl Homoserine Lactones and Lux Solos Regulate Social Behaviour and Virulence of Pseudomonas syringae pv. actinidiae.* Microb Ecol, 2020. **79**(2): p. 383-396.
193. Susanne B. von Bodman, W. Dietz Bauer, and D.L. Coplin, *QUORUM SENSING IN PLANT-PATHOGENIC BACTERIA.* Annual Review of Phytopathology, 2003. **41**(1): p. 455-482.
194. González, J.E. and M.M. Marketon, *Quorum Sensing in Nitrogen-Fixing Rhizobia.* Microbiology and Molecular Biology Reviews, 2003. **67**(4): p. 574-592.

195. Plitnick, J., F.F.V. Chevance, A. Stringer, K.T. Hughes, and J.T. Wade, *Regulatory Crosstalk between Motility and Interbacterial Communication in Salmonella Typhimurium.* J Bacteriol, 2020.
196. Turnbull, A.L., W. Kim, and M.G. Surette, *Transcriptional regulation of sdiA by cAMP-receptor protein, LeuO, and environmental signals in Salmonella enterica serovar Typhimurium.* Canadian Journal of Microbiology, 2011. **58**(1): p. 10-22.
197. Shankar, M., P. Ponraj, D. Illakkiam, J. Rajendhran, and P. Gunasekaran, *Inactivation of the Transcriptional Regulator-Encoding Gene sdiA Enhances Rice Root Colonization and Biofilm Formation in Enterobacter cloacae.* Journal of Bacteriology, 2013. **195**(1): p. 39-45.
198. Starr, M.P. and A.K. Chatterjee, *The genus Erwinia: enterobacteria pathogenic to plants and animals.* Annual Reviews in Microbiology, 1972. **26**(1): p. 389-426.
199. Walterson, A.M. and J. Stavrinides, *Pantoea: insights into a highly versatile and diverse genus within the Enterobacteriaceae.* FEMS Microbiology Reviews, 2015. **39**(6): p. 968-984.
200. Shanna, S., T.F. Stark, W.G. Beattie, and R.E. Moses, *Multiple control elements for the uvrC gene unit of Escherichia coli.* Nucleic Acids Research, 1986. **14**(5): p. 2301-2318.
201. Konigsberg, W. and G.N. Godson, *Evidence for use of rare codons in the dnaG gene and other regulatory genes of Escherichia coli.* Proceedings of the National Academy of Sciences, 1983. **80**(3): p. 687-691.
202. Wang, X.D., P.A. de Boer, and L.I. Rothfield, *A factor that positively regulates cell division by activating transcription of the major cluster of essential cell division genes of Escherichia coli.* The EMBO journal, 1991. **10**(11): p. 3363-3372.
203. Sitnikov, D.M., J.B. Schineller, and T.O. Baldwin, *Control of cell division in Escherichia coli: regulation of transcription of ftsQA involves both rpoS and SdiA-mediated autoinduction.* Proceedings of the National Academy of Sciences, 1996. **93**(1): p. 336-341.
204. Kanamaru, K., K. Kanamaru, I. Tatsuno, T. Tobe, and C. Sasakawa, *SdiA, an Escherichia coli homologue of quorum-sensing regulators, controls the expression of virulence factors in enterohaemorrhagic Escherichia coli O157:H7.* Molecular Microbiology, 2000. **38**(4): p. 805-816.
205. Pacheco, T., A.É.I. Gomes, N.M.G. Siqueira, L. Assoni, M. Darrieux, H. Venter, and L.F.C. Ferraz, *SdiA, a Quorum-Sensing Regulator, Suppresses Fimbriae Expression, Biofilm Formation, and Quorum-Sensing Signaling Molecules Production in Klebsiella pneumoniae.* Frontiers in Microbiology, 2021. **12**.
206. Wei, Y., J.-M. Lee, D.R. Smulski, and R.A. LaRossa, *Global Impact of sdiA Amplification Revealed by Comprehensive Gene Expression Profiling of Escherichia coli.* Journal of Bacteriology, 2001. **183**(7): p. 2265-2272.
207. Pos, K.M., *Drug transport mechanism of the AcrB efflux pump.* Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics, 2009. **1794**(5): p. 782-793.

208. Rahmati, S., S. Yang, A.L. Davidson, and E.L. Zechiedrich, *Control of the AcrAB multidrug efflux pump by quorum-sensing regulator SdiA.* Mol Microbiol, 2002. **43**(3): p. 677-85.
209. Tavio, M.M., V.D. Aquili, A. Fabrega, J. Vila, and J.B. Poveda, *Overexpression of the quorum-sensing regulator sdiA and soxS is involved in low-level multidrug resistance induced in Escherichia coli AG100 by haloperidol, diazepam and NaCl.* Int J Antimicrob Agents, 2012. **39**(1): p. 91-3.
210. Tavio, M.M., V.D. Aquili, J. Vila, and J.B. Poveda, *Resistance to ceftazidime in Escherichia coli associated with AcrR, MarR and PBP3 mutations and overexpression of sdiA.* J Med Microbiol, 2014. **63**(Pt 1): p. 56-65.
211. Cheng, C., X. Yan, B. Liu, T. Jiang, Z. Zhou, F. Guo, Q. Zhang, C. Li, and T. Fang, *SdiA Enhanced the Drug Resistance of Cronobacter sakazakii and Suppressed Its Motility, Adhesion and Biofilm Formation.* Frontiers in microbiology, 2022. **13**: p. 901912-901912.
212. Lee, J., A. Jayaraman, and T.K. Wood, *Indole is an inter-species biofilm signal mediated by SdiA.* BMC Microbiol, 2007. **7**: p. 42.
213. Lee, J., X.-S. Zhang, M. Hegde, W.E. Bentley, A. Jayaraman, and T.K. Wood, *Indole cell signaling occurs primarily at low temperatures in Escherichia coli.* The Isme Journal, 2008. **2**: p. 1007.
214. Karlin, D.A., A.J. Mastromarino, R.D. Jones, J.R. Stroehlein, and O. Lorentz, *Fecal skatole and indole and breath methane and hydrogen in patients with large bowel polyps or cancer.* Journal of Cancer Research and Clinical Oncology, 1985. **109**(2): p. 135-141.
215. Zuccato, E., M. Venturi, G. Di Leo, L. Colombo, C. Bertolo, S.B. Doldi, and E. Mussini, *Role of bile acids and metabolic activity of colonic bacteria in increased risk of colon cancer after cholecystectomy.* Digestive Diseases and Sciences, 1993. **38**(3): p. 514-519.
216. Cao, Y., L. Li, Y. Zhang, F. Liu, X. Xiao, X. Li, and Y. Yu, *Evaluation of Cronobacter sakazakii biofilm formation after sdiA knockout in different osmotic pressure conditions.* Food Research International, 2022. **151**: p. 110886.
217. Wood, T.K., A.F. González Barrios, M. Herzberg, and J. Lee, *Motility influences biofilm architecture in Escherichia coli.* Applied Microbiology and Biotechnology, 2006. **72**(2): p. 361-367.
218. Simm, R., I. Ahmad, M. Rhen, S. Le Guyon, and U. Römling, *Regulation of Biofilm Formation in Salmonella Enterica Serovar Typhimurium.* Future Microbiology, 2014. **9**(11): p. 1261-1282.
219. Wang, F., L. Deng, F. Huang, Z. Wang, Q. Lu, and C. Xu, *Flagellar Motility Is Critical for Salmonella enterica Serovar Typhimurium Biofilm Development.* Frontiers in Microbiology, 2020. **11**.
220. Van Houdt, R., A. Aertsen, P. Moons, K. Vanoirbeek, and C.W. Michiels, *N-acyl-l-homoserine lactone signal interception by Escherichia coli.* FEMS Microbiology Letters, 2006. **256**(1): p. 83-89.

221. Pedersen, K., A.-M. Lassen-Nielsen, S. Nordentoft, and A.S. Hammer, *Serovars of Salmonella from captive reptiles.* Zoonoses and Public Health, 2009. **56**(5): p. 238-242.
222. Kidgell, C., U. Reichard, J. Wain, B. Linz, M. Torpdahl, G. Dougan, and M. Achtman, *Salmonella typhi, the causative agent of typhoid fever, is approximately 50,000 years old.* Infection, Genetics and Evolution, 2002. **2**(1): p. 39-45.
223. Zimmermann, L., A. Stephens, S.Z. Nam, D. Rau, J. Kübler, M. Lozajic, F. Gabler, J. Söding, A.N. Lupas, and V. Alva, *A Completely Reimplemented MPI Bioinformatics Toolkit with a New HHpred Server at its Core.* J Mol Biol, 2018. **430**(15): p. 2237-2243.
224. van Kempen, M., S.S. Kim, C. Tumescheit, M. Mirdita, J. Lee, C.L.M. Gilchrist, J. Söding, and M. Steinegger, *Fast and accurate protein structure search with Foldseek.* Nature Biotechnology, 2023.
225. Kröger, C., A. Colgan, S. Srikumar, K. Händler, Sathesh K. Sivasankaran, Disa L. Hammarlöf, R. Canals, Joe E. Grissom, T. Conway, K. Hokamp, and Jay C.D. Hinton, *An Infection-Relevant Transcriptomic Compendium for Salmonella enterica Serovar Typhimurium.* Cell Host & Microbe, 2013. **14**(6): p. 683-695.
226. Chaudhuri, R.R., E. Morgan, S.E. Peters, S.J. Pleasance, D.L. Hudson, H.M. Davies, J. Wang, P.M. van Diemen, A.M. Buckley, and A.J. Bowen, *Comprehensive assignment of roles for Salmonella typhimurium genes in intestinal colonization of food-producing animals.* PLoS genetics, 2013. **9**(4).
227. Wang, Q., S. Mariconda, A. Suzuki, M. McClelland, and R.M. Harshey, *Uncovering a Large Set of Genes That Affect Surface Motility in Salmonella enterica Serovar Typhimurium.* Journal of Bacteriology, 2006. **188**(22): p. 7981-7984.
228. Knudsen, G.M., M.-B. Nielsen, T. Grassby, V. Danino-Appleton, L.E. Thomsen, I.J. Colquhoun, T.F. Brocklehurst, J.E. Olsen, and J.C.D. Hinton, *A third mode of surface-associated growth: immobilization of Salmonella enterica serovar Typhimurium modulates the RpoS-directed transcriptional programme.* Environmental Microbiology, 2012. **14**(8): p. 1855-1875.
229. Haznedaroglu, B., S. Porwollik, M. McClelland, P. Cheng, and B. Ahmer, *Survival and Fitness of Random Generated Salmonella typhimurium Transposon Library under Long Term Environmental Stress: From in vitro to in silico.* Transport and Pathogenicity of Salmonella enterica Subspecies in Groundwater: In vitro, in, 2009: p. 172.
230. Adler, B.A., A.E. Kazakov, C. Zhong, H. Liu, E. Kutter, L.M. Lui, T.N. Nielsen, H. Carion, A.M. Deutschbauer, V.K. Mutalik, and A.P. Arkin, *The genetic basis of phage susceptibility, cross-resistance and host-range in Salmonella.* Microbiology (Reading), 2021. **167**(12).
231. Chiba, S., K. Ito, and Y. Akiyama, *The Escherichia coli plasma membrane contains two PHB (prohibitin homology) domain protein complexes of opposite orientations.* Mol Microbiol, 2006. **60**(2): p. 448-57.
232. Buss, K., R. Müller, C. Dahm, N. Gaitatzis, E. Skrzypczak-Pietraszek, S. Lohmann, M. Gassen, and E. Leistner, *Clustering of isochorismate synthase*

genes menF and entC and channeling of isochorismate in Escherichia coli. Biochim Biophys Acta, 2001. **1522**(3): p. 151-7.
233. Dahm, C., R. Müller, G. Schulte, K. Schmidt, and E. Leistner, *The role of isochorismate hydroxymutase genes entC and menF in enterobactin and menaquinone biosynthesis in Escherichia coli.* Biochimica et Biophysica Acta (BBA) - General Subjects, 1998. **1425**(2): p. 377-386.
234. Mambu, J., I. Virlogeux-Payant, S. Holbert, O. Grépinet, P. Velge, and A. Wiedemann, *An Updated View on the Rck Invasin of Salmonella: Still Much to Discover.* Frontiers in Cellular and Infection Microbiology, 2017. **7**(500).
235. Bouwman, C.W., M. Kohli, A. Killoran, G.A. Touchie, R.J. Kadner, and N.L. Martin, *Characterization of SrgA, a Salmonella enterica Serovar Typhimurium Virulence Plasmid-Encoded Paralogue of the Disulfide Oxidoreductase DsbA, Essential for Biogenesis of Plasmid-Encoded Fimbriae.* Journal of Bacteriology, 2003. **185**(3): p. 991-1000.
236. Wallar, L.E., A.M. Bysice, and B.K. Coombes, *The non-motile phenotype of Salmonella hha ydgT mutants is mediated through PefI-SrgD.* BMC Microbiology, 2011. **11**(1): p. 141.
237. Wozniak, C.E., C. Lee, and K.T. Hughes, *T-POP Array Identifies EcnR and PefI-SrgD as Novel Regulators of Flagellar Gene Expression.* Journal of Bacteriology, 2009. **191**(5): p. 1498-1508.
238. Cirillo, D.M., E.J. Heffernan, L. Wu, J. Harwood, J. Fierer, and D.G. Guiney, *Identification of a domain in Rck, a product of the Salmonella typhimurium virulence plasmid, required for both serum resistance and cell invasion.* Infect Immun, 1996. **64**(6): p. 2019-23.
239. Wiedemann, A., L. Mijouin, M.A. Ayoub, E. Barilleau, S. Canepa, A.P. Teixeira-Gomes, Y.L. Vern, M. Rosselin, E. Reiter, and P. Velge, *Identification of the epidermal growth factor receptor as the receptor for Salmonella Rck–dependent invasion.* The FASEB Journal, 2016. **30**(12): p. 4180-4191.
240. Koczerka, M., I. Lantier, M. Morillon, J. Deperne, C.D. Clamagirand, I. Virlogeux-Payant, and O. Grépinet, *From intestine to beyond: Salmonella entry factors display distinct transcription pattern upon infection in murine models.* Open Biology, 2024. **14**(1): p. 230312.
241. Personnic, N., K. Bärlocher, I. Finsel, and H. Hilbi, *Subversion of Retrograde Trafficking by Translocated Pathogen Effectors.* Trends Microbiol, 2016. **24**(6): p. 450-462.
242. Finsel, I., C. Ragaz, C. Hoffmann, Christopher F. Harrison, S. Weber, Vanessa A. van Rahden, L. Johannes, and H. Hilbi, *The Legionella Effector RidL Inhibits Retrograde Trafficking to Promote Intracellular Replication.* Cell Host & Microbe, 2013. **14**(1): p. 38-50.
243. McGourty, K., T.L. Thurston, S.A. Matthews, L. Pinaud, L.J. Mota, and D.W. Holden, *Salmonella Inhibits Retrograde Trafficking of Mannose-6-Phosphate Receptors and Lysosome Function.* Science, 2012. **338**(6109): p. 963-967.
244. Steele-Mortimer, O., *The Salmonella-containing vacuole—Moving with the times.* Current Opinion in Microbiology, 2008. **11**(1): p. 38-45.

245. Bakowski, M.A., V. Braun, and J.H. Brumell, *Salmonella-Containing Vacuoles: Directing Traffic and Nesting to Grow.* Traffic, 2008. **9**(12): p. 2022-2031.
246. Srikumar, S., C. Kröger, M. Hébrard, A. Colgan, S.V. Owen, S.K. Sivasankaran, A.D.S. Cameron, K. Hokamp, and J.C.D. Hinton, *RNA-seq Brings New Insights to the Intra-Macrophage Transcriptome of Salmonella Typhimurium.* PLOS Pathogens, 2015. **11**(11): p. e1005262.
247. Skunca, N., M. Bošnjak, A. Križko, P. Panov, S. Džeroski, T. Smuc, and F. Supek, *Phyletic profiling with cliques of orthologs is enhanced by signatures of paralogy relationships.* PLoS Comput Biol, 2013. **9**(1): p. e1002852.
248. Kanjee, U. and W.A. Houry, *Mechanisms of Acid Resistance in Escherichia coli.* Annual Review of Microbiology, 2013. **67**(1): p. 65-81.
249. Ma, X., S. Zhang, Z. Xu, H. Li, Q. Xiao, F. Qiu, W. Zhang, Y. Long, D. Zheng, B. Huang, C. Chen, and Y. Lu, *SdiA Improves the Acid Tolerance of E. coli by Regulating GadW and GadY Expression.* Front Microbiol, 2020. **11**: p. 1078.
250. Sharma, V.K., S.M.D. Bearson, and B.L. Bearson, *Evaluation of the effects of sdiA, a luxR homologue, on adherence and motility of Escherichia coli O157:H7.* Microbiology, 2010. **156**(5): p. 1303-1312.
251. Suzuki, K., X. Wang, T. Weilbacher, A.-K. Pernestig, Ö. Melefors, D. Georgellis, P. Babitzke, and T. Romeo, *Regulatory Circuitry of the CsrA/CsrB and BarA/UvrY Systems of Escherichia coli.* Journal of Bacteriology, 2002. **184**(18): p. 5130-5140.
252. Ghosh, D., K. Roy, K.E. Williamson, S. Srinivasiah, K.E. Wommack, and M. Radosevich, *Acyl-Homoserine Lactones Can Induce Virus Production in Lysogenic Bacteria: an Alternative Paradigm for Prophage Induction.* Applied and Environmental Microbiology, 2009. **75**(22): p. 7142-7152.
253. Kolenda, R., M. Ugorski, and K. Grzymajlo, *Everything You Always Wanted to Know About Salmonella Type 1 Fimbriae, but Were Afraid to Ask.* Frontiers in Microbiology, 2019. **10**(1017).
254. Clegg, S., L.S. Hancox, and K.S. Yeh, *Salmonella typhimurium fimbrial phase variation and FimA expression.* Journal of Bacteriology, 1996. **178**(2): p. 542-545.
255. Abraham, J.M., C.S. Freitag, J.R. Clements, and B.I. Eisenstein, *An invertible element of DNA controls phase variation of type 1 fimbriae of Escherichia coli.* Proceedings of the National Academy of Sciences, 1985. **82**(17): p. 5724-5727.
256. Schwieters, A. and B.M.M. Ahmer, *Identification of new SdiA regulon members of Escherichia coli, Enterobacter cloacae, and Salmonella enterica serovars Typhimurium and Typhi.* Microbiology Spectrum, 2024. **0**(0): p. e01929-24.
257. Bassler, B.L. and R. Losick, *Bacterially speaking.* Cell, 2006. **125**(2): p. 237-246.
258. Nealson, K.H., T. Platt, and J.W. Hastings, *Cellular Control of the Synthesis and Activity of the Bacterial Luminescent System.* Journal of Bacteriology, 1970. **104**(1): p. 313-322.
259. Jimenez, P.N., G. Koch, J.A. Thompson, K.B. Xavier, R.H. Cool, and W.J. Quax, *The Multiple Signaling Systems Regulating Virulence in Pseudomonas aeruginosa.* Microbiology and Molecular Biology Reviews, 2012. **76**(1): p. 46-65.

260. Soares, J.A. and B.M.M. Ahmer, *Detection of acyl-homoserine lactones by Escherichia and Salmonella.* Current Opinion in Microbiology, 2011. **14**(2): p. 188-193.
261. Ahmer, B.M.M., *Cell-to-cell signalling in Escherichia coli and Salmonella enterica.* Molecular Microbiology, 2004. **52**(4): p. 933-945.
262. Goodier, R.I. and B.M.M. Ahmer, *SirA Orthologs Affect both Motility and Virulence.* Journal of Bacteriology, 2001. **183**(7): p. 2249-2258.
263. Miki, T., N. Okada, and H. Danbara, *Two periplasmic disulfide oxidoreductases, DsbA and SrgA, target outer membrane protein SpiA, a component of the Salmonella pathogenicity island 2 type III secretion system.* Journal of Biological Chemistry, 2004. **279**(33): p. 34631-34642.
264. Nicholson, B. and D. Low, *DNA methylation-dependent regulation of Pef expression in Salmonella typhimurium.* Molecular Microbiology, 2000. **35**(4): p. 728-742.
265. Mambu, J., E. Barilleau, L. Fragnet-Trapp, Y. Le Vern, M. Olivier, G. Sadrin, O. Grépinet, F. Taieb, P. Velge, and A. Wiedemann, *Rck of Salmonella Typhimurium Delays the Host Cell Cycle to Facilitate Bacterial Invasion.* Frontiers in Cellular and Infection Microbiology, 2020. **10**(656).
266. Mijouin, L., M. Rosselin, E. Bottreau, J. Pizarro-Cerda, P. Cossart, P. Velge, and A. Wiedemann, *Salmonella enteritidis Rck-mediated invasion requires activation of Rac1, which is dependent on the class I PI 3-kinases-Akt signaling pathway.* Faseb j, 2012. **26**(4): p. 1569-81.
267. Wiedemann, A., M. Rosselin, L. Mijouin, E. Bottreau, and P. Velge, *Involvement of c-Src Tyrosine Kinase Upstream of Class I Phosphatidylinositol (PI) 3-Kinases in Salmonella Enteritidis Rck Protein-mediated Invasion.* Journal of Biological Chemistry, 2012. **287**(37): p. 31148-31154.
268. Retchless, A.C. and J.G. Lawrence, *Temporal Fragmentation of Speciation in Bacteria.* Science, 2007. **317**(5841): p. 1093-1096.
269. Dougan, G. and S. Baker, *Salmonella enterica serovar Typhi and the pathogenesis of typhoid fever.* Annu Rev Microbiol, 2014. **68**: p. 317-36.
270. Wang, B.X., D.S. Butler, M. Hamblin, and D.M. Monack, *One species, different diseases: the unique molecular mechanisms that underlie the pathogenesis of typhoidal Salmonella infections.* Curr Opin Microbiol, 2023. **72**: p. 102262.
271. Meiring, J.E., F. Khanam, B. Basnyat, R.C. Charles, J.A. Crump, F. Debellut, K.E. Holt, S. Kariuki, E. Mugisha, K.M. Neuzil, C.M. Parry, V.E. Pitzer, A.J. Pollard, F. Qadri, and M.A. Gordon, *Typhoid fever.* Nat Rev Dis Primers, 2023. **9**(1): p. 71.
272. Gonzalez-Escobedo, G., J.M. Marshall, and J.S. Gunn, *Chronic and acute infection of the gall bladder by Salmonella Typhi: understanding the carrier state.* Nat Rev Microbiol, 2011. **9**(1): p. 9-14.
273. Dillon, S.C., E. Espinosa, K. Hokamp, D.W. Ussery, J. Casadesús, and C.J. Dorman, *LeuO is a global regulator of gene expression in Salmonella enterica serovar Typhimurium.* Molecular Microbiology, 2012. **85**(6): p. 1072-1089.

274. Winson, M.K., S. Swift, L. Fish, J.P. Throup, F. Jørgensen, S.R. Chhabra, B.W. Bycroft, P. Williams, and G.S.A.B. Stewart, *Construction and analysis of luxCDABE-based plasmid sensors for investigating N-acyl homoserine lactone-mediated quorum sensing.* FEMS Microbiology Letters, 1998. **163**(2): p. 185-192.
275. Käll, L., A. Krogh, and E.L. Sonnhammer, *A combined transmembrane topology and signal peptide prediction method.* J Mol Biol, 2004. **338**(5): p. 1027-36.
276. Schroder, W., M. Burger, C. Edwards, M. Douglas, D. Innes, I.R. Beacham, and D.M. Burns, *The Escherichia coli orthologue of the Salmonella ushB gene (ushBc) produces neither UDP-sugar hydrolase activity nor detectable protein, but has an identical sequence to that of Escherichia coli cdh.* FEMS Microbiology Letters, 2001. **203**(1): p. 63-68.
277. Kwon, O., M.E. Hudspeth, and R. Meganathan, *Anaerobic biosynthesis of enterobactin Escherichia coli: regulation of entC gene expression and evidence against its involvement in menaquinone (vitamin K2) biosynthesis.* J Bacteriol, 1996. **178**(11): p. 3252-9.
278. Koczerka, M., P.-E. Douarre, F. Kempf, S. Holbert, M.-Y. Mistou, O. Grépinet, I. Virlogeux-Payant, and C.A. Cuomo, *The Invasin and Complement-Resistance Protein Rck of Salmonella is More Widely Distributed than Previously Expected.* Microbiology Spectrum, 2021. **9**(2): p. e01457-21.
279. Gonnet, P., K.E. Rudd, and F. Lisacek, *Fine-tuning the prediction of sequences cleaved by signal peptidase II: a curated set of proven and predicted lipoproteins of Escherichia coli K-12.* Proteomics, 2004. **4**(6): p. 1597-613.
280. Kroner, G.M., M.B. Wolfe, and P.L. Freddolino, *Escherichia coli Lrp Regulates One-Third of the Genome via Direct, Cooperative, and Indirect Routes.* J Bacteriol, 2019. **201**(3).
281. Stirling, D., C. Hulton, L. Waddell, S. Park, G. Stewart, I. Booth, and C. Higgins, *Molecular characterization of the proU loci of Salmonella typhimurium and Escherichia coli encoding osmoregulated glycine betaine transport systems.* Molecular microbiology, 1989. **3**(8): p. 1025-1038.
282. Scheu, P.D., J. Witan, M. Rauschmeier, S. Graf, Y.F. Liao, A. Ebert-Jung, T. Basché, W. Erker, and G. Unden, *CitA/CitB two-component system regulating citrate fermentation in Escherichia coli and its relation to the DcuS/DcuR system in vivo.* J Bacteriol, 2012. **194**(3): p. 636-45.
283. Stoyanov, J.V., J.L. Hobman, and N.L. Brown, *CueR (YbbI) of Escherichia coli is a MerR family regulator controlling expression of the copper exporter CopA.* Molecular Microbiology, 2001. **39**(2): p. 502-512.
284. Helmuth, R., R. Stephan, C. Bunge, B. Hoog, A. Steinbeck, and E. Bulling, *Epidemiology of virulence-associated plasmids and outer membrane protein patterns within seven common Salmonella serotypes.* Infection and Immunity, 1985. **48**(1): p. 175-182.
285. Ahmer, B.M.M., M. Tran, and F. Heffron, *The Virulence Plasmid of Salmonella typhimurium Is Self-Transmissible.* Journal of Bacteriology, 1999. **181**(4): p. 1364-1368.

286. García-Quintanilla, M. and J. Casadesús, *Virulence plasmid interchange between strains ATCC 14028, LT2, and SL1344 of Salmonella enterica serovar Typhimurium.* Plasmid, 2011. **65**(2): p. 169-175.
287. García-Quintanilla, M., F. Ramos-Morales, and J. Casadesús, *Conjugal Transfer of the Salmonella enterica Virulence Plasmid in the Mouse Intestine.* Journal of Bacteriology, 2008. **190**(6): p. 1922-1927.
288. Gunn, J.S. and S.I. Miller, *PhoP-PhoQ activates transcription of pmrAB, encoding a two-component regulatory system involved in Salmonella typhimurium antimicrobial peptide resistance.* Journal of Bacteriology, 1996. **178**(23): p. 6857-6864.
289. Lu, Y., J. Zeng, B. Wu, S. E, L. Wang, R. Cai, N. Zhang, Y. Li, X. Huang, B. Huang, and C. Chen, *Quorum Sensing N-acyl Homoserine Lactones-SdiA Suppresses Escherichia coli-Pseudomonas aeruginosa Conjugation through Inhibiting traI Expression.* Frontiers in Cellular and Infection Microbiology, 2017. **7**(7).
290. Håvarstein, L.S., G. Coomaraswamy, and D.A. Morrison, *An unmodified heptadecapeptide pheromone induces competence for genetic transformation in Streptococcus pneumoniae.* Proceedings of the National Academy of Sciences, 1995. **92**(24): p. 11140-11144.
291. Parsek, M.R. and E.P. Greenberg, *Sociomicrobiology: the connections between quorum sensing and biofilms.* Trends in Microbiology, 2005. **13**(1): p. 27-33.
292. Silpe, J.E. and B.L. Bassler, *A Host-Produced Quorum-Sensing Autoinducer Controls a Phage Lysis-Lysogeny Decision.* Cell, 2019. **176**(1-2): p. 268-280.e13.
293. Garcia-Lara, J., L.H. Shang, and L.I. Rothfield, *An extracellular factor regulates expression of sdiA, a transcriptional activator of cell division genes in Escherichia coli.* J Bacteriol, 1996. **178**(10): p. 2742-8.
294. Serna, A., E. Espinosa, E.M. Camacho, and J. Casadesús, *Regulation of Bacterial Conjugation in Microaerobiosis by Host-Encoded Functions ArcAB and SdhABCD.* Genetics, 2010. **184**(4): p. 947-958.
295. Dixon, M.H., K.N. Cowles, S.C. Zaacks, I.N. Marciniak, and J.D. Barak, *Xanthomonas Infection Transforms the Apoplast into an Accessible and Habitable Niche for Salmonella enterica.* Applied and Environmental Microbiology, 2022. **88**(22): p. e01330-22.
296. Quinones-Olvera, N., S.V. Owen, L.M. McCully, M.G. Marin, E.A. Rand, A.C. Fan, O.J. Martins Dosumu, K. Paul, C.E. Sanchez Castaño, R. Petherbridge, J.S. Paull, and M. Baym, *Diverse and abundant phages exploit conjugative plasmids.* Nature Communications, 2024. **15**(1): p. 3197.
297. Pearson, J.P., K.M. Gray, L. Passador, K.D. Tucker, A. Eberhard, B.H. Iglewski, and E.P. Greenberg, *Structure of the autoinducer required for expression of Pseudomonas aeruginosa virulence genes.* Proceedings of the National Academy of Sciences, 1994. **91**(1): p. 197-201.
298. Maloy, S.R., V.J. Stewart, R. Taylor, and S.I. Miller, *Genetic analysis of pathogenic bacteria.* Trends in Microbiology, 1996. **4**(12): p. 504.

299. O'Callaghan, D. and A. Charbit, *High efficiency transformation of Salmonella typhimurium and Salmonella typhi by electroporation.* Molecular and General Genetics MGG, 1990. **223**(1): p. 156-158.
300. Cianfanelli, F.R., O. Cunrath, and D. Bumann, *Efficient dual-negative selection for bacterial genome editing.* BMC Microbiology, 2020. **20**(1): p. 129.
301. Gibson, D.G., L. Young, R.-Y. Chuang, J.C. Venter, C.A. Hutchison, and H.O. Smith, *Enzymatic assembly of DNA molecules up to several hundred kilobases.* Nature Methods, 2009. **6**(5): p. 343-345.
302. Datsenko, K.A. and B.L. Wanner, *One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products.* Proceedings of the National Academy of Sciences, 2000. **97**(12): p. 6640-6645.
303. Andrews, S., *FASTQC. A quality control tool for high throughput sequence data*. 2010.
304. Bolger, A.M., M. Lohse, and B. Usadel, *Trimmomatic: a flexible trimmer for Illumina sequence data.* Bioinformatics, 2014. **30**(15): p. 2114-2120.
305. Langmead, B. and S.L. Salzberg, *Fast gapped-read alignment with Bowtie 2.* Nature methods, 2012. **9**(4): p. 357-359.
306. Pertea, M., G.M. Pertea, C.M. Antonescu, T.-C. Chang, J.T. Mendell, and S.L. Salzberg, *StringTie enables improved reconstruction of a transcriptome from RNA-seq reads.* Nature Biotechnology, 2015. **33**(3): p. 290-295.
307. Love, M.I., W. Huber, and S. Anders, *Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2.* Genome Biology, 2014. **15**(12): p. 550.
308. Council, N.R., *Guide for the Care and Use of Laboratory Animals: Eighth Edition*. 2011, Washington, DC: The National Academies Press. 246.
309. Harms, A., M. Liesch, J. Körner, M. Québatte, P. Engel, and C. Dehio, *A bacterial toxin-antitoxin module is the origin of inter-bacterial and inter-kingdom effectors of Bartonella.* PLOS Genetics, 2017. **13**(10): p. e1007077.
310. Ellermeier, C.D., A. Janakiraman, and J.M. Slauch, *Construction of targeted single copy lac fusions using lambda Red and FLP-mediated site-specific recombination in bacteria.* Gene, 2002. **290**(1-2): p. 153-61.
311. Santiviago, C.A., M.M. Reynolds, S. Porwollik, S.-H. Choi, F. Long, H.L. Andrews-Polymenis, and M. McClelland, *Analysis of Pools of Targeted Salmonella Deletion Mutants Identifies Novel Genes Affecting Fitness during Competitive Infection in Mice.* PLOS Pathogens, 2009. **5**(7): p. e1000477.
312. Guzman, L.M., D. Belin, M.J. Carson, and J. Beckwith, *Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter.* Journal of Bacteriology, 1995. **177**(14): p. 4121-4130.
313. Dyszel, J.L., *Phenotypes of the LuxR homolog, SdiA, in Salmonella and Escherichia coli*. 2009, The Ohio State University: United States -- Ohio. p. 171.
314. Lucas, D.E., *Coordinated Regulation of Salmonella Virulence Genes by the BarA/SirA Two-Component System and the Csr Global Regulatory System*. 2013, The Ohio State University: United States -- Ohio. p. 219.

315. Teplitski, M., A. Al-Agely, and B.M.M. Ahmer, *Contribution of the SirA regulon to biofilm formation in Salmonella enterica serovar Typhimurium.* Microbiology, 2006. **152**(11): p. 3411-3424.
316. Jacobsen, C.S. and T.B. Bech, *Soil survival of Salmonella and transfer to freshwater and fresh produce.* Food Research International, 2012. **45**(2): p. 557-566.
317. Tatfeng, Y.M., M.U. Usuanlele, A. Orukpe, A.K. Digban, M. Okodua, F. Oviasogie, and A.A. Turay, *Mechanical transmission of pathogenic organisms: the role of cockroaches.* J Vector Borne Dis, 2005. **42**(4): p. 129-34.
318. Letellier, A., S. Messier, J. Pare, J. Menard, and S. Quessy, *Distribution of Salmonella in swine herds in Quebec.* Vet Microbiol, 1999. **67**(4): p. 299-306.
319. Skov, M.N., J.J. Madsen, C. Rahbek, J. Lodal, J.B. Jespersen, J.C. Jørgensen, H.H. Dietz, M. Chriél, and D.L. Baggesen, *Transmission of Salmonella between wildlife and meat-production animals in Denmark.* Journal of Applied Microbiology, 2008. **105**(5): p. 1558-1568.
320. Tizard, I.R., N.A. Fish, and J. Harmeson, *Free flying sparrows as carriers of salmonellosis.* The Canadian veterinary journal = La revue veterinaire canadienne, 1979. **20**(5): p. 143-144.
321. Scheelings, T.F., D. Lightfoot, and P. Holz, *PREVALENCE OF SALMONELLA IN AUSTRALIAN REPTILES.* Journal of Wildlife Diseases, 2011. **47**(1): p. 1-11.
322. Mermin, J., L. Hutwagner, D. Vugia, S. Shallow, P. Daily, J. Bender, J. Koehler, R. Marcus, F.J. Angulo, and f.t.E.I.P.F.W. Group, *Reptiles, Amphibians, and Human Salmonella Infection: A Population-Based, Case-Control Study.* Clinical Infectious Diseases, 2004. **38**(Supplement_3): p. S253-S261.
323. Trautwein, M.D., B.M. Wiegmann, R. Beutel, K.M. Kjer, and D.K. Yeates, *Advances in Insect Phylogeny at the Dawn of the Postgenomic Era.* Annual Review of Entomology, 2012. **57**(Volume 57, 2012): p. 449-468.
324. Thomson, J.L., K.M. Yeater, L. Zurek, and D. Nayduch, *Abundance and Accumulation of Escherichia coli and Salmonella Typhimurium Procured by Male and Female House Flies (Diptera: Muscidae) Exposed to Cattle Manure.* Annals of the Entomological Society of America, 2016. **110**(1): p. 37-44.
325. Pava-Ripoll, M., R.E.G. Pearson, A.K. Miller, B.D. Tall, C.E. Keys, and G.C. Ziobro, *Ingested Salmonella enterica, Cronobacter sakazakii, Escherichia coli O157:H7, and Listeria monocytogenes: transmission dynamics from adult house flies to their eggs and first filial (F1) generation adults.* BMC Microbiology, 2015. **15**(1): p. 150.
326. Olafson, P.U., K.H. Lohmeyer, T.S. Edrington, and G.H. Loneragan, *Survival and Fate of Salmonella enterica serovar Montevideo in Adult Horn Flies (Diptera: Muscidae).* Journal of Medical Entomology, 2014. **51**(5): p. 993-1001.
327. Holt, P.S., C.J. Geden, R.W. Moore, and R.K. Gast, *Isolation of Salmonella enterica Serovar Enteritidis from Houseflies (Musca domestica) Found in Rooms Containing Salmonella Serovar Enteritidis-Challenged Hens.* Applied and Environmental Microbiology, 2007. **73**(19): p. 6030-6035.

328. Mian, L.S., H. Maag, and J.V. Tacal, *Isolation of Salmonella from muscoid flies at commercial animal establishments in San Bernardino County, California.* J Vector Ecol, 2002. **27**(1): p. 82-5.
329. Olsen, A.R. and T.S. Hammack, *Isolation of Salmonella spp. from the housefly, Musca domestica L., and the dump fly, Hydrotaea aenescens (Wiedemann) (Diptera: Muscidae), at caged-layer houses.* J Food Prot, 2000. **63**(7): p. 958-60.
330. Greenberg, B., J.A. Kowalski, and M.J. Klowden, *Factors Affecting the Transmission of Salmonella by Flies: Natural Resistance to Colonization and Bacterial Interference.* Infection and Immunity, 1970. **2**(6): p. 800-809.
331. Alam, M.J. and L. Zurek, *Association of Escherichia coli O157:H7 with houseflies on a cattle farm.* Applied and environmental microbiology, 2004. **70**(12): p. 7578-7580.
332. Sulaiman, S., M.Z. Othman, and A.H. Aziz, *Isolations of enteric pathogens from synanthropic flies trapped in downtown Kuala Lumpur.* Journal of vector ecology : journal of the Society for Vector Ecology, 2000. **25**(1): p. 90-93.
333. Greenberg, B., *Experimental Transmission of Salmonella typhimurium by Houseflies to Man.* American Journal of Hygiene, 1964. **80**(2): p. 149-56.
334. Hinnebusch, B.J., C.O. Jarrett, and D.M. Bland, *"Fleaing" the Plague: Adaptations of Yersinia pestis to Its Insect Vector That Lead to Transmission.* Annu Rev Microbiol, 2017. **71**: p. 215-232.
335. Chifanzwa, R., *House fly (Musca domestica L.) temporal and spatial immune response to Streptococcus pyogenes and Salmonella typhimurium: role of pathogen density in bacterial fate, persistence and transmission.* 2011.
336. Howard, L.O., *A Contribution to the Study of the Insect Fauna of Human Excrement:(With Especial Reference to the Spread of Typhoid Fever by Flies.)*. 1900: The Academy.
337. Faichnie, N., *Fly-Borne Enteric Fever: the Source of Infection.* Journal of the Royal Army Medical Corps, 1909. **13**(5): p. 580-584.
338. Greenberg, B. and M. Klowden, *Enteric bacterial interactions in insects.* Am J Clin Nutr, 1972. **25**(12): p. 1459-66.
339. Kobayashi, M., T. Sasaki, N. Saito, K. Tamura, K. Suzuki, H. Watanabe, and N. Agui, *Houseflies: not simple mechanical vectors of enterohemorrhagic Escherichia coli O157:H7.* The American Journal of Tropical Medicine and Hygiene, 1999. **61**(4): p. 625-629.
340. Kobayashi, M., N. Agui, and T. Sasaki, *Epidemiological Potential of Excretion and Regurgitation by Musca domestica (Diptera: Muscidae) in the Dissemination of Escherichia coli O157: H7 to Food.* Journal of Medical Entomology, 2000. **37**(6): p. 945-949.
341. Chiang, S.L., J.J. Mekalanos, and D.W. Holden, *In vivo genetic analysis of bacterial virulence.* Annu Rev Microbiol, 1999. **53**: p. 129-54.
342. Merighi, M., C.D. Ellermeier, J.M. Slauch, and J.S. Gunn, *Resolvase-In Vivo Expression Technology Analysis of the Salmonella enterica Serovar Typhimurium PhoP and PmrA Regulons in BALB/c Mice.* Journal of Bacteriology, 2005. **187**(21): p. 7407-7416.

343. Sperandio, V. and Y. Nguyen, *Enterohemorrhagic E. coli (EHEC) pathogenesis.* Frontiers in Cellular and Infection Microbiology, 2012. **2**(90).
344. Feng, Y., J. Liu, Y.-G. Li, F.-L. Cao, R.N. Johnston, J. Zhou, G.-R. Liu, and S.-L. Liu, *Inheritance of the Salmonella virulence plasmids: Mostly vertical and rarely horizontal.* Infection, Genetics and Evolution, 2012. **12**(5): p. 1058-1063.
345. Ducarmon, Q.R., R.D. Zwittink, B.V.H. Hornung, W. van Schaik, V.B. Young, and E.J. Kuijper, *Gut Microbiota and Colonization Resistance against Bacterial Enteric Infection.* Microbiol Mol Biol Rev, 2019. **83**(3).
346. Fàbrega, A. and J. Vila, *Yersinia enterocolitica: pathogenesis, virulence and antimicrobial resistance.* Enferm Infecc Microbiol Clin, 2012. **30**(1): p. 24-32.
347. Brugiroux, S., M. Beutler, C. Pfann, D. Garzetti, H.-J. Ruscheweyh, D. Ring, M. Diehl, S. Herp, Y. Lötscher, S. Hussain, B. Bunk, R. Pukall, D.H. Huson, P.C. Münch, A.C. McHardy, K.D. McCoy, A.J. Macpherson, A. Loy, T. Clavel, D. Berry, and B. Stecher, *Genome-guided design of a defined mouse microbiota that confers colonization resistance against Salmonella enterica serovar Typhimurium.* Nature Microbiology, 2016. **2**(2): p. 16215.
348. Boos, W. and H. Shuman, *Maltose/Maltodextrin System of Escherichia coli: Transport, Metabolism, and Regulation.* Microbiology and Molecular Biology Reviews, 1998. **62**(1): p. 204-229.
349. Dippel, R. and W. Boos, *The Maltodextrin System of Escherichia coli: Metabolism and Transport.* Journal of Bacteriology, 2005. **187**(24): p. 8322-8331.
350. Bassford, P.J., T.J. Silhavy, and J.R. Beckwith, *Use of gene fusion to study secretion of maltose-binding protein into Escherichia coli periplasm.* Journal of Bacteriology, 1979. **139**(1): p. 19-31.
351. Jones, S.A., M. Jorgensen, F.Z. Chowdhury, R. Rodgers, J. Hartline, M.P. Leatham, C. Struve, K.A. Krogfelt, P.S. Cohen, and T. Conway, *Glycogen and Maltose Utilization by Escherichia coli O157:H7 in the Mouse Intestine.* Infection and Immunity, 2008. **76**(6): p. 2531-2540.
352. Lång, H., G. Jonson, J. Holmgren, and E.T. Palva, *The maltose regulon of Vibrio cholerae affects production and secretion of virulence factors.* Infection and Immunity, 1994. **62**(11): p. 4781-4788.
353. Miller, J., *A short course in bacterial genetics: a laboratory manual and handbood for escherichia coli and related bacteria.* A short course in bacterial genetics: a laboratory manual and handbood for escherichia coli and related bacteria., 1992: p. 143-149.
354. Lazarus, J.E., A.R. Warr, C.J. Kuehl, R.T. Giorgio, B.M. Davis, and M.K. Waldor, *A New Suite of Allelic-Exchange Vectors for the Scarless Modification of Proteobacterial Genomes.* Appl Environ Microbiol, 2019. **85**(16).
355. Kinder, S.A., J.L. Badger, G.O. Bryant, J.C. Pepe, and V.L. Miller, *Cloning of the YenI restriction endonuclease and methyltransferase from Yersinia enterocolitica serotype O8 and construction of a transformable R−M+ mutant.* Gene, 1993. **136**(1): p. 271-275.

356. Tsolis, R.e.M., L.G. Adams, T.A. Ficht, and A.J. Bäumler, *Contribution of Salmonella typhimurium virulence factors to diarrheal disease in calves.* Infection and immunity, 1999. **67**(9): p. 4879-4885.
357. Winter, S.E., M.G. Winter, M.N. Xavier, P. Thiennimitr, V. Poon, A.M. Keestra, R.C. Laughlin, G. Gomez, J. Wu, and S.D. Lawhon, *Host-derived nitrate boosts growth of E. coli in the inflamed gut.* science, 2013. **339**(6120): p. 708-711.
358. Winter, S.E., P. Thiennimitr, M.G. Winter, B.P. Butler, D.L. Huseby, R.W. Crawford, J.M. Russell, C.L. Bevins, L.G. Adams, R.M. Tsolis, J.R. Roth, and A.J. Bäumler, *Gut inflammation provides a respiratory electron acceptor for Salmonella.* Nature, 2010. **467**(7314): p. 426-429.
359. Hohmann, E.L., *Nontyphoidal Salmonella: Gastrointestinal infection and asymptomatic carriage*, in *UpToDate*, S.B. Calderwood, Editor. 2024 Accessed on June 30, 2024.
360. Pulford, C.V., B.M. Perez-Sepulveda, R. Canals, J.A. Bevington, R.J. Bengtsson, N. Wenner, E.V. Rodwell, B. Kumwenda, X. Zhu, R.J. Bennett, G.E. Stenhouse, P. Malaka De Silva, H.J. Webster, J.A. Bengoechea, A. Dumigan, A. Tran-Dien, R. Prakash, H.C. Banda, L. Alufandika, M.P. Mautanga, A. Bowers-Barnard, A.Y. Beliavskaia, A.V. Predeus, W.P.M. Rowe, A.C. Darby, N. Hall, F.-X. Weill, M.A. Gordon, N.A. Feasey, K.S. Baker, and J.C.D. Hinton, *Stepwise evolution of Salmonella Typhimurium ST313 causing bloodstream infection in Africa.* Nature Microbiology, 2021. **6**(3): p. 327-338.
361. Fierer, J., *Invasive non-typhoidal Salmonella (iNTS) infections.* Clinical Infectious Diseases, 2022. **75**(4): p. 732-738.
362. Park, S.E., D.T. Pham, G.D. Pak, U. Panzner, L.M.C. Espinoza, V. Von Kalckreuth, J. Im, O.D. Mogeni, H. Schütt-Gerowitt, and J.A. Crump, *The genomic epidemiology of multi-drug resistant invasive non-typhoidal Salmonella in selected sub-Saharan African countries.* BMJ Global Health, 2021. **6**(8): p. e005659.
363. Hoffman, S.A., M.J. Sikorski, and M.M. Levine, *Chronic Salmonella Typhi carriage at sites other than the gallbladder.* PLoS Neglected Tropical Diseases, 2023. **17**(3): p. e0011168.
364. Gunn, J.S., J.M. Marshall, S. Baker, S. Dongol, R.C. Charles, and E.T. Ryan, *Salmonella chronic carriage: epidemiology, diagnosis, and gallbladder persistence.* Trends in microbiology, 2014. **22**(11): p. 648-655.
365. Harrell, J.E., M.M. Hahn, S.J. D'Souza, E.M. Vasicek, J.L. Sandala, J.S. Gunn, and J.B. McLachlan, *Salmonella biofilm formation, chronic infection, and immunity within the intestine and hepatobiliary tract.* Frontiers in cellular and infection microbiology, 2021. **10**: p. 624622.
366. Ferreccio, C., J. Glenn Morris, C. Valdivieso, I. Prenzel, V. Sotomayor, G.L. Drusano, and M.M. Levine, *Efficacy of ciprofloxacin in the treatment of chronic typhoid carriers.* The Journal of infectious diseases, 1988. **157**(6): p. 1235-1239.
367. Antillón, M., J.L. Warren, F.W. Crawford, D.M. Weinberger, E. Kürüm, G.D. Pak, F. Marks, and V.E. Pitzer, *The burden of typhoid fever in low-and middle-*

income countries: a meta-regression approach. PLoS neglected tropical diseases, 2017. **11**(2): p. e0005376.
368. Mogasale, V., B. Maskery, R.L. Ochiai, J.S. Lee, V.V. Mogasale, E. Ramani, Y.E. Kim, J.K. Park, and T.F. Wierzba, *Burden of typhoid fever in low-income and middle-income countries: a systematic, literature-based update with risk-factor adjustment.* The Lancet Global Health, 2014. **2**(10): p. e570-e580.
369. Control, C.f.D. and Prevention, *Antibiotic resistance threats in the United States, 2019*. 2019: US Department of Health and Human Services, Centres for Disease Control and ….
370. Tacconelli, E., *Global Priority List of Antibiotic-Resistant Bacteria to Guide Research, Discovery, and Development.* 2017.
371. Kurahashi, K. and A.J. Wahba, *Interference with growth of certain Escherichia coli mutants by galactose.* Biochimica et biophysica acta, 1958. **30**(2): p. 298-302.
372. Fukasawa, T. and H. Nikaido, *Galactose-sensitive mutants of Salmonella.* Nature, 1959. **184**(4693): p. 1168-1169.
373. Yarmolinsky, M.B., H. Wiesmeyer, H.M. Kalckar, and E. Jordan, *Hereditary defects in galactose metabolism in Escherichia coli mutants, II. Galactose-induced sensitivity.* Proceedings of the National Academy of Sciences, 1959. **45**(12): p. 1786-1791.
374. Englesberg, E., R. Anderson, R. Weinberg, N. Lee, P. Hoffee, G. Huttenhauer, and H. Boyer, *L-Arabinose-sensitive, L-ribulose 5-phosphate 4-epimerase-deficient mutants of Escherichia coli.* Journal of bacteriology, 1962. **84**(1): p. 137-146.
375. Englesberg, E., *Inhibition of the growth of Salmonella typhosa by L-rhamnose.* Journal of Bacteriology, 1960. **79**(1): p. 58-64.
376. Helle, K.B. and L. Klungsøyr, *Mannitol 1-phosphate formation in Escherichia coli during glucose utilization.* Biochimica et Biophysica Acta, 1962. **65**(3): p. 461-471.
377. Upadhyay, R.S., M. Meena, V. Prasad, A. Zehra, and V.K. Gupta, *Mannitol metabolism during pathogenic fungal–host interactions under stressed conditions.* Frontiers in Microbiology, 2015. **6**.
378. Novotny, M.J., J. Reizer, F. Esch, and M.H. Saier, Jr., *Purification and properties of D-mannitol-1-phosphate dehydrogenase and D-glucitol-6-phosphate dehydrogenase from Escherichia coli.* J Bacteriol, 1984. **159**(3): p. 986-90.
379. Teschner, W., M.C. Serre, and J.R. Garel, *Enzymatic properties, renaturation and metabolic role of mannitol-1-phosphate dehydrogenase from Escherichia coli.* Biochimie, 1990. **72**(1): p. 33-40.
380. Murphey, W.H. and E.D. Rosenblum, *MANNITOL CATABOLISM BY STAPHYLOCOCCUS AUREUS.* Arch Biochem Biophys, 1964. **107**: p. 292-7.
381. Solomon, E. and E.C.C. Lin, *Mutations Affecting the Dissimilation of Mannitol by Escherichia coli K-12.* Journal of Bacteriology, 1972. **111**(2): p. 566-574.
382. Jacobs, M.A., A. Alwood, I. Thaipisuttikul, D. Spencer, E. Haugen, S. Ernst, O. Will, R. Kaul, C. Raymond, R. Levy, L. Chun-Rong, D. Guenthner, D. Bovee, M.V. Olson, and C. Manoil, *Comprehensive transposon mutant library of*

Pseudomonas aeruginosa. Proceedings of the National Academy of Sciences, 2003. **100**(24): p. 14339-14344.
383. Govoni, G., S. Vidal, S. Gauthier, E. Skamene, D. Malo, and P. Gros, *The Bcg/Ity/Lsh locus: genetic transfer of resistance to infections in C57BL/6J mice transgenic for the Nramp1 Gly169 allele.* Infection and Immunity, 1996. **64**(8): p. 2923-2929.
384. Vidal, S., P. Gros, and E. Skamene, *Natural resistance to infection with intracellular parasites: molecular genetics identifies Nramp1 as the Bcg/Ity/Lsh locus.* Journal of Leukocyte Biology, 1995. **58**(4): p. 382-390.
385. Vidal, S., M.L. Tremblay, G. Govoni, S. Gauthier, G. Sebastiani, D. Malo, E. Skamene, M. Olivier, S. Jothy, and P. Gros, *The Ity/Lsh/Bcg locus: natural resistance to infection with intracellular parasites is abrogated by disruption of the Nramp1 gene.* The Journal of experimental medicine, 1995. **182**(3): p. 655-666.
386. Brook, I., *Inoculum Effect.* Reviews of Infectious Diseases, 1989. **11**(3): p. 361-368.
387. Loffredo, M.R., F. Savini, S. Bobone, B. Casciaro, H. Franzyk, M.L. Mangoni, and L. Stella, *Inoculum effect of antimicrobial peptides.* Proceedings of the National Academy of Sciences, 2021. **118**(21): p. e2014364118.
388. Smith, K.P. and J.E. Kirby, *The Inoculum Effect in the Era of Multidrug Resistance: Minor Differences in Inoculum Have Dramatic Effect on MIC Determination.* Antimicrobial Agents and Chemotherapy, 2018. **62**(8): p. 10.1128/aac.00433-18.
389. Kebriaei, R., J.C. Abdul-Mutakabbir, K.C. Stamper, K.L. Lev, and M.J. Rybak, *Targeting Dalbavancin Inoculum Effect: Adjunctive Single Dose of Daptomycin.* Infect Dis Ther, 2023. **12**(10): p. 2485-2494.
390. Kebriaei, R., S.A. Rice, K.V. Singh, K.C. Stamper, A.Q. Dinh, R. Rios, L. Diaz, B.E. Murray, J.M. Munita, T.T. Tran, C.A. Arias, and M.J. Rybak, *Influence of Inoculum Effect on the Efficacy of Daptomycin Monotherapy and in Combination with β-Lactams against Daptomycin-Susceptible Enterococcus faecium Harboring LiaSR Substitutions.* Antimicrob Agents Chemother, 2018. **62**(8).
391. Lin, Y.H., T. Dharmaraj, Q. Chen, A. Echterhof, R. Manasherob, L.J. Zhang, C. de Leeuw, N.A. Peterson, W. Stannard, Z. Li, M. Hajfathalian, A. Hargil, H.A. Martinez, J. Pourtois, T.H.W. Chang, F.G. Blankenberg, D. Amanatullah, O. Chaudhuri, and P.L. Bollyky, *Optimized Dosing and Delivery of Bacteriophage Therapy for Wound Infections.* bioRxiv, 2024.
392. Sabag-Daigle, A., H.M. Blunk, A. Sengupta, J. Wu, A.J. Bogard, M.M. Ali, C. Stahl, V.H. Wysocki, V. Gopalan, E.J. Behrman, and B.M.M. Ahmer, *A metabolic intermediate of the fructose-asparagine utilization pathway inhibits growth of a Salmonella fraB mutant.* Scientific Reports, 2016. **6**(1): p. 28117.
393. Mitosch, K., M. Beyß, P. Phapale, B. Drotleff, K. Nöh, T. Alexandrov, K.R. Patil, and A. Typas, *A pathogen-specific isotope tracing approach reveals metabolic activities and fluxes of intracellular Salmonella.* PLOS Biology, 2023. **21**(8): p. e3002198.

394. Steeb, B., B. Claudi, N.A. Burton, P. Tienz, A. Schmidt, H. Farhan, A. Mazé, and D. Bumann, *Parallel Exploitation of Diverse Host Nutrients Enhances Salmonella Virulence.* PLOS Pathogens, 2013. **9**(4): p. e1003301.
395. Lee, J.Y., C.R. Tiffany, S.P. Mahan, M. Kellom, A.W.L. Rogers, H. Nguyen, E.T. Stevens, H.L.P. Masson, K. Yamazaki, M.L. Marco, E.A. Eloe-Fadrosh, P.J. Turnbaugh, and A.J. Bäumler, *High fat intake sustains sorbitol intolerance after antibiotic-mediated Clostridia depletion from the gut microbiota.* Cell, 2024. **187**(5): p. 1191-1205.e15.
396. Corazza, G.R., A. Strocchi, R. Rossi, D. Sirola, and G. Gasbarrini, *Sorbitol malabsorption in normal volunteers and in patients with coeliac disease.* Gut, 1988. **29**(1): p. 44-48.
397. Reele, S.B. and D.J. Chodos, *Sorbitol induced diarrheal illness model.* International Journal of Clinical Pharmacology Therapy and Toxicology, 1985. **23**(8): p. 403-405.
398. Kavanagh, K.L., M. Klimacek, B. Nidetzky, and D.K. Wilson, *Crystal structure of Pseudomonas fluorescens mannitol 2-dehydrogenase binary and ternary complexes: specificity and catalytic mechanism.* Journal of Biological Chemistry, 2002. **277**(45): p. 43433-43442.
399. Diaz-Tang, G., E.M. Meneses, K. Patel, S. Mirkin, L. García-Diéguez, C. Pajon, I. Barraza, V. Patel, H. Ghali, A.P. Tracey, C.A. Blanar, A.J. Lopatkin, and R.P. Smith, *Growth productivity as a determinant of the inoculum effect for bactericidal antibiotics.* Science Advances, 2022. **8**(50): p. eadd0924.
400. Sévin, D.C., T. Fuhrer, N. Zamboni, and U. Sauer, *Nontargeted in vitro metabolomics for high-throughput identification of novel enzymes in Escherichia coli.* Nature Methods, 2017. **14**(2): p. 187-194.
401. Murray, G.L., S.R. Attridge, and R. Morona, *Altering the length of the lipopolysaccharide O antigen has an impact on the interaction of Salmonella enterica serovar Typhimurium with macrophages and complement.* J Bacteriol, 2006. **188**(7): p. 2735-9.
402. Richardson, E.J., B. Limaye, H. Inamdar, A. Datta, K.S. Manjari, G.D. Pullinger, N.R. Thomson, R.R. Joshi, M. Watson, and M.P. Stevens, *Genome Sequences of Salmonella enterica Serovar Typhimurium, Choleraesuis, Dublin, and Gallinarum Strains of Well- Defined Virulence in Food-Producing Animals.* Journal of Bacteriology, 2011. **193**(12): p. 3162-3163.
403. Kingsley, R.A., C.L. Msefula, N.R. Thomson, S. Kariuki, K.E. Holt, M.A. Gordon, D. Harris, L. Clarke, S. Whitehead, V. Sangal, K. Marsh, M. Achtman, M.E. Molyneux, M. Cormican, J. Parkhill, C.A. MacLennan, R.S. Heyderman, and G. Dougan, *Epidemic multiple drug resistant Salmonella Typhimurium causing invasive disease in sub-Saharan Africa have a distinct genotype.* Genome Res, 2009. **19**(12): p. 2279-87.
404. Swearingen, M.C., S. Porwollik, P.T. Desai, M. McClelland, and B.M.M. Ahmer, *Virulence of 32 Salmonella Strains in Mice.* PLOS ONE, 2012. **7**(4): p. e36043.

405. Wang, R.F. and S.R. Kushner, *Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in Escherichia coli.* Gene, 1991. **100**: p. 195-9.
406. Shimada, T., K. Shimada, M. Matsui, Y. Kitai, J. Igarashi, H. Suga, and A. Ishihama, *Roles of cell division control factor SdiA: recognition of quorum sensing signals and modulation of transcription regulation targets.* Genes to Cells, 2014. **19**(5): p. 405-418.
407. Hillyer, J.F., *Insect immunology and hematopoiesis.* Dev Comp Immunol, 2016. **58**: p. 102-18.
408. van den Berg, D.F., A.R. Costa, J.Q. Esser, I. Stanciu, J.Q. Geissler, A.D. Zoumaro-Djayoon, P.-J. Haas, and S.J.J. Brouns, *Bacterial homologs of innate eukaryotic antiviral defenses with anti-phage activity highlight shared evolutionary roots of viral defenses.* Cell Host & Microbe, 2024. **32**(8): p. 1427-1443.e8.
409. Maestri, A., B.J. Pons, E. Pursey, C.E. Chong, S. Gandon, R. Custodio, A. Olina, A. Agapov, M.A.W. Chisnall, A. Grasso, S. Paterson, M.D. Szczelkun, K.S. Baker, S. van Houte, A. Chevallereau, and E.R. Westra, *The bacterial defense system MADS interacts with CRISPR-Cas to limit phage infection and escape.* Cell Host & Microbe, 2024. **32**(8): p. 1412-1426.e11.
410. Sather, L.M., M. Zamani, Z. Muhammed, J.V.S. Kearsley, G.T. Fisher, K.M. Jones, and T.M. Finan, *A broadly distributed predicted helicase/nuclease confers phage resistance via abortive infection.* Cell Host & Microbe, 2023. **31**(3): p. 343-355.e5.
411. Bernal, V., S. Castaño-Cerezo, and M. Cánovas, *Acetate metabolism regulation in Escherichia coli: carbon overflow, pathogenicity, and beyond.* Applied Microbiology and Biotechnology, 2016. **100**(21): p. 8985-9001.
412. Rosenberg, H. and C.M. Hardy, *Conversion of D-mannitol to D-ribose: a newly discovered pathway in Escherichia coli.* Journal of Bacteriology, 1984. **158**(1): p. 69-72.
413. Scher, B. and V. Ginsburg, *Isolation of adenosine S'-diphosphate D-mannitol from Salmonella typhimurium.* Journal of Biological Chemistry, 1968. **243**(9): p. 2385–9.
414. Kadner, R.J., G.P. Murphy, and C.M. Stephens, *Two mechanisms for growth inhibition by elevated transport of sugar phosphates in Escherichia coli.* Microbiology, 1992. **138**(10): p. 2007-2014.
415. Nové, M., A. Kincses, J. Molnár, L. Amaral, and G. Spengler, *The Role of Efflux Pumps and Environmental pH in Bacterial Multidrug Resistance.* In Vivo, 2020. **34**(1): p. 65-71.
416. Wolfe, J.B. and N.O. Kaplan, *d-MANNITOL 1-PHOSPHATE DEHYDROGENASE FROM ESCHERICHIA COLI.* Journal of Biological Chemistry, 1956. **218**(2): p. 849-869.
417. Rosenberg, H., L.M. Russell, P.A. Jacomb, and K. Chegwidden, *Phosphate exchange in the pit transport system in Escherichia coli.* Journal of Bacteriology, 1982. **149**(1): p. 123-130.

418. Rosenberg, H., S.M. Pearce, C.M. Hardy, and P.A. Jacomb, *Rapid turnover of mannitol-1-phosphate in Escherichia coli.* Journal of Bacteriology, 1984. **158**(1): p. 63-68.
419. Loescher, W.H., R.H. Tyson, J.D. Everard, R.J. Redgwell, and R.L. Bieleski, *Mannitol Synthesis in Higher Plants 1: Evidence for the Role and Characterization of a NADPH-Dependent Mannose 6-Phosphate Reductase.* Plant Physiology, 1992. **98**(4): p. 1396-1402.
420. Kuznetsova, E., M. Proudfoot, C.F. Gonzalez, G. Brown, M.V. Omelchenko, I. Borozan, L. Carmel, Y.I. Wolf, H. Mori, A.V. Savchenko, C.H. Arrowsmith, E.V. Koonin, A.M. Edwards, and A.F. Yakunin, *Genome-wide analysis of substrate specificities of the Escherichia coli haloacid dehalogenase-like phosphatase family.* J Biol Chem, 2006. **281**(47): p. 36149-61.