INVESTIGATING ARGININE METABOLISM IN SALMONELLA TYPHIMURIUM

INVESTIGATING ADAPTIVE REGULATORY EVOLUTION OF INTRACELLULAR ARGININE METABOLISM IN SALMONELLA TYPHIMURIUM

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the Requirements for the Degree of Master of Science

McMaster University MASTER OF SCIENCE (2022) Hamilton, Ontario

(Biochemistry and Biomedical Sciences)

TITLE: Investigating Adaptive Regulatory Evolution of Intracellular Arginine Metabolism in *Salmonella* Typhimurium.

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NUMBER OF PAGES: xiii, 76

Lay Abstract

Salmonella enterica is an intestinal pathogen that survives within host immune cells and causes systemic disease. These bacteria replicate within antimicrobial cells by using sensory networks to detect harmful immune factors and respond adaptively by eliciting change in gene expression to defend against immune-based killing. The amino acid arginine is an important component of host immunity, as well as bacterial antimicrobial defenses; therefore, I hypothesized that bacterial metabolism might be adapted to the host immune cell environment in order to mitigate arginine-dependent antimicrobial activity. Here, I establish that arginine metabolism is controlled by intracellular-specific sensory networks, and demonstrate that this regulation is important for bacterial survival. This work provides evidence for the importance of this amino acid in *Salmonella* infection, which informs on our overall understanding of systemic disease.

Abstract

Salmonella enterica is a facultative intracellular pathogen capable of eliciting severe, systemic disease necessitating antibiotic intervention. Systemic infection is facilitated by intracellular replication within host immune cells, which is enabled by complex regulatory networks governed by two-component systems (TCSs). Intracellular-active TCSs sense antimicrobial chemical cues in the microenvironment and respond adaptively through transcriptional regulation to support intracellular survival. SsrA/SsrB and PhoQ/PhoP are two essential TCSs that elicit a robust defense against host immunity by regulating clusters of virulence genes and integrating novel targets to support regulon expansion and enhance pathogenicity. Metabolic adaptation is critical to bacterial survival and can initiate host-pathogen interactions that influence infection outcome. Further, mitigation of host immunity by manipulation of arginine metabolism has been documented in intracellular pathogens. Herein, I investigated TCS-mediated regulatory evolution pertaining to arginine metabolism, hypothesizing that adaptations to metabolic regulation might confer a fitness advantage to *Salmonella* replicating intracellularly. I explored intracellular regulation of *de novo* biosynthesis and extracellular import of arginine, establishing PhoP-mediated regulation of arginine transport. I determined that arginine transport contributes to bacterial fitness in macrophages and began to investigate the mechanism by which arginine importation enriches for intracellular replication. This work informs on evolutionary mechanisms that serve to enhance virulence in *Salmonella* and provides further insight into our understanding of the intracellular lifestyle of infection.

Acknowledgements

I would like to thank my supervisor, Dr. Brian Coombes, for the continuous support throughout my graduate studies. The Covid-19 pandemic brought on new challenges to graduate school, but you were always caring and helped me to adapt to a difficult situation. Thank you for pushing me to be a better student by teaching me how to think more critically and to be a stronger communicator in science. Thank you to my committee members Dr. Lori Burrows and Dr. Matthew Miller, you taught me to think outside the box and made me a better scientist for it.

Thank you to all of the members of the Coombes lab that I had the privilege to work alongside and learn from. Caressa Tsai, Elizabeth Chau, and Wael Elhenawy, you were there with me from the beginning when I was an undergraduate student; your passion and dedication to academia is what inspired me to pursue graduate studies. Caressa, thank you for your friendship over the last couple of years – you instilled a love for science and discovery in me that I will carry with me always. Thank you for being so supportive, believing in me, and encouraging me to pursue my goals no matter what. To Marie-Ange Massicotte, you have been the most wonderful friend to work alongside and I truly appreciate the help and support you have given me. I would also like to thank my undergraduate student, Lena Darwish, for helping me to grow as a leader. Your passion for science never ceases to amaze me and I have no doubt you will continue on to great things in graduate school.

I would also like to thank my family and friends for their unconditional support and encouragement. Mom, you taught me that I can achieve anything as long as I believe

in myself and these words have been a source of motivation for me over the last two years. To my fiancé, Luke, thank you for keeping me grounded during challenging times and encouraging me when I needed it most. You always know how to see the bright side of anything and put a smile on my face.

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List of Abbreviations and Symbols

Amp ..... ampicillin
Arginine ..... L-arginine
Art ..... arginine transport
AT ..... adenosine and thymine
BMM ..... bone marrow macrophage
Bp ..... base pair
cAMP ..... cationic antimicrobial peptide
CAAT ..... cationic amino acid transporter
CAT ..... chloramphenicol acetyltransferase
CFU ..... colony forming units
Cm ..... chloramphenicol
CO_2 ..... carbon dioxide
CRE ..... *cis*-regulatory evolution
DEPC ..... diethylpyrocarbonate
DMEM ..... Dulbecco's modified eagle medium
DNA ..... deoxyribonucleic acid
E. coli ..... *Escherichia coli*
EDTA ..... ethylenediaminetetraacetic acid
FBS ..... fetal bovine serum
Gm ..... gentamicin
H-NS ..... histone-like nucleoid structuring protein
HK ..... histidine kinase
INF-γ ..... interferon gamma
iNOS ..... inducible nitric oxide synthase
Kn ..... kanamycin
L-arginine ..... arginine
L-NIL ..... L-N^6-(1-Iminoethyl)-lysine
L-Sup ..... L929 fibroblast conditioned medium
LB ..... lysogeny broth
LPM ..... low phosphate, low magnesium media
LPS ..... lipopolysaccharide
$MgCl_2$ ..... magnesium chloride
Mg^{2+} ..... magnesium
MIC ..... minimum inhibitory concentration
MOI ..... multiplicity of infection
NADPH ..... nicotinamide adenine dinucleotide phosphate
NH_3 ..... ammonia
NHS ..... normal human serum
NO• ..... nitric oxide
NOS2 ..... nitric oxide synthase 2
$NOS2^-$ ..... nitric oxide synthase 2 knockout
O_2^- ..... superoxide

OD ..... optical density
OD_{600} ..... optical density at 600 nm
PAMP ..... pathogen-associated molecular pattern
PBS ..... phosphate-buffered saline
PCR ..... polymerase chain reaction
PRR ..... pattern recognition receptor
Rif ..... rifampicin
RLU ..... relative light unit
RNA ..... ribonucleic acid
RNS ..... reactive nitrogen species
ROS ..... reactive oxygen species
RPMI ..... Roswell park memorial institute medium
RR ..... response regulator
RT-qPCR ..... reverse transcriptase-quantitative polymerase chain reaction
S. Typhimurium ..... *Salmonella enterica* serovar Typhimurium
Salmonella ..... *Salmonella enterica* serovar Typhimurium
SCV ..... *Salmonella*-containing vacuole
SIF ..... *Salmonella*-induced filament
Sm ..... streptomycin
SPI ..... *Salmonella* pathogenicity island
T3SS ..... type three secretion system
TC incubator ..... 37°C, 5% CO_2 incubator
TCS ..... two-component system
TLR4 ..... toll-like receptor 4
Wt ..... wild-type
Δ ..... delta
γ ..... gamma

Declaration of Academic Achievement

Project design and conceptualization was led by myself (JNP), Dr. Brian Coombes and Dr. Caressa Tsai. JNP designed and performed experiments, and analysed data. Lena Darwish scarified mice and Marie-Ange Massicotte harvested bone marrow macrophage cells.

Introduction

Salmonella disease manifestation and current public health concerns

Salmonella is a bacterial genus belonging to the Enterobacteriaceae family, which encompasses Gram-negative pathogens capable of colonizing the gastrointestinal tract. The *Salmonella* genus split from *Escherichia coli* approximately 100 – 140 million years ago and has since diverged into two distinct species: *Salmonella bongori* and *Salmonella enterica* (Doolittle et al., 1996; Ochman & Wilson, 1987). While *S. bongori* is predominantly restricted to cold-blooded animals, *S. enterica* can infect both warm- and cold-blooded animals, as well as humans (Desai et al., 2013; Fookes et al., 2011). The latter species is further divided into six subspecies, including subsp. *enterica*, which accounts for the majority of human *Salmonella* infections and comprises both typhoidal and non-typhoidal serovars that are classified by antigenic determinants (Dekker & Frank, 2015; Desai et al., 2013). Non-typhoidal serovars largely elicit classical self-limiting gastrointestinal disease symptoms; however, in cases where individuals are immunocompromised or infected with a hypervirulent strain of *Salmonella*, intestinal disease can progress to become systemic (Uzzau et al., 2000; Watson & Holden, 2010). Such infections involve dissemination of bacteria throughout the circulatory system and critical organs, which can result in mortality if not properly treated.

Systemic salmonellosis is a major concern in underdeveloped regions where poor sanitary infrastructure leads to increased transmission of foodborne pathogens (Ashbolt, 2004). Perhaps the most disproportionately burdened geographic region is sub-Saharan Africa, where high incidence of immunocompromising conditions such as human

immunodeficiency virus, malaria, and malnutrition further increase susceptibility to severe infection (Stanaway et al., 2019; Uzzau et al., 2000). Here, the threat of invasive, non-typhoidal *Salmonella* is augmented by the emergence of multi-drug resistance (Akullian et al., 2018); however, such infections are not restricted to underdeveloped regions and represent a global public health concern. Antibiotic resistance has been documented across all classes of first-line anti-*Salmonella* therapies, including cephalosporins, fluoroquinolones, and macrolides (Brunelle et al., 2017; Gomes et al., 2017; Hakanen et al., 2001; Su et al., 2005). Multi-drug resistance has created complications in treating systemic infection by rendering traditional therapies ineffective and increasing morbidity and mortality rates. To combat this health crisis, we must gain further insight into the virulence mechanisms employed by *Salmonella* that permit progression to systemic disease so that we might uncover novel targets for anti-*Salmonella* therapies.

Salmonella undergoes two distinct pathogenic lifestyles to elicit systemic infection

Systemic disease is characterized by bacterial dissemination beyond the confines of the gastrointestinal niche. Over evolutionary time, *Salmonella* has acquired mechanisms that enable the pathogen to traverse the intestinal epithelial barrier and colonize host cells to facilitate widespread infection. Systemic disease progression has been studied extensively in mouse models using *Salmonella enterica* subsp. *enterica* serovar Typhimurium, herein referred to as *S.* Typhimurium. *S.* Typhimurium provides an optimal model for systemic, non-typhoidal infection, as this strain is amenable to genetic

manipulation and elicits systemic salmonellosis in murine models of *Salmonella* infection (Finlay & Brumell, 2000; Tsolis et al., 2011). Collectively, *S*. Typhimurium strains contain 21 virulence gene clusters that were acquired by *S*. Typhimurium over evolutionary timescales that have expanded the virulence potential of the pathogen (McClelland et al., 2001). Each gene cluster was integrated into the core genome during separate horizontal gene transfer events and is termed a *Salmonella*-pathogenicity island (SPI) (McClelland et al., 2001; Ochman et al., 2000). These genetic loci encode virulence factors that enhance niche-specific pathogenicity. During systemic infection, *Salmonella* adopts genetically distinct invasive and intracellular lifestyles, which are regulated by virulence networks encoded within SPI-1 and SPI-2, respectively (Gal-Mor & Finlay, 2006; Hansen-Wester & Hensel, 2001). SPI-1 and 2 both encode a type three secretion system (T3SS), associated effectors, chaperones, and other virulence factors; however, each SPI elicits distinct pathogenic activity that enhances bacterial fitness at different stages of infection (Gal-Mor & Finlay, 2006).

Within the intestinal lumen, *Salmonella* initiates the invasive lifestyle through SPI-1 activation. Upon contacting the apical surface of the epithelium, the SPI-1 T3SS secretes effectors, which induce cytoskeletal rearrangement of host cells to incite membrane ruffling that promotes bacterial internalization (Finlay et al., 1991; Galán, 1996). SPI-1 effector-mediated macropinocytosis is required at this stage of infection to enable the pathogen to invade and traverse the non-phagocytic epithelial barrier, and migrate to the lamina propria (Finlay et al., 1991). The lamina propria is populated by both innate and adaptive leukocytes that launch an immune response (Patel &

McCormick, 2014; Vazquez-Torres et al., 1999). Resident macrophages and neutrophils aim to neutralize the pathogen by means of phagosomal destruction; however, *Salmonella* is able to sense the changing microenvironment upon phagocytosis, thereby triggering transcription of SPI-2 genes and assembly of a second T3SS needed for intracellular survival (Hansen-Wester & Hensel, 2001). Secretion of SPI-2 effectors inhibits phagosomal maturation and induces structural modifications, which generate a vacuole that is conducive to bacterial replication called the *Salmonella*-containing vacuole (SCV) (Steele-Mortimer, 2008). The SCV differs from the canonical phagosome in that the compartment lacks endosomal maturation markers and resembles an early endosome (Smith et al., 2005; Steele-Mortimer et al., 1999). Within the SCV, *Salmonella* defends against bactericidal immune defenses and lysosomal destruction, and acquires adequate nutrients to support sustained replication and survival (Steele-Mortimer, 2008).

Salmonella overcomes challenging intracellular conditions to colonize host cells by sensing the microenvironment

While the SCV provides an enclosed compartment for bacteria to replicate within, host immune defenses can permeate this niche and pose a threat to intracellular survival. Antimicrobial immune factors encountered during the intracellular lifestyle include cationic antimicrobial peptides (cAMPs) and reactive nitrogen and oxygen species (RNS, ROS). cAMPs target bacteria by perturbing the negatively charged bacterial membrane to induce cell lysis, and can interfere with essential cellular processes by interacting with intracellular anionic molecules (Brown & Hancock, 2006; Hancock & Sahl, 2006). RNS

and ROS are generated by the host inducible nitric oxide synthase (iNOS) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase pathways, respectively, which produce nitric oxide (NO•) and superoxide (O_2^-) radicals that can diffuse across bacterial membranes and cause damage to enzymes and DNA (Fang, 2004; Pullan et al., 2007; Ren et al., 2008). In addition to immune factors, the acidic, nutrient and ion depleted SCV presents further challenges to intracellular survival. Disruptions in pH, nutrient, and ion balance interfere with homeostatic processes that are required for bacterial integrity and replication (Guan & Liu, 2020; Steele-Mortimer, 2008). To overcome the limitations of the intracellular environment, *Salmonella* employs regulatory networks that adaptively respond to microenvironmental cues.

The *Salmonella* genome encodes several two-component systems (TCSs) that allow the pathogen to sense chemical cues within the environment and respond accordingly at the transcriptional level (Wallis & Galyov, 2000). TCSs are found in multiple families of pathogenic bacteria and share a conserved activation mechanism that is coordinated between a membrane-bound sensor histidine kinase (HK) and a cellular response regulator (RR). The HK will activate by autophosphorylation of a conserved histidine residue upon sensing a change in a specific ligand and then transfer this phosphoryl group onto a conserved aspartate residue on the cognate RR, thereby activating transcription factor activity to elicit change in gene transcription. In the SCV, multiple TCSs work in a concerted fashion to respond to changes in the microenvironment including EnvZ/OmpR, PhoQ/PhoP, PmrA/PmrB, and SsrA/SsrB (Figure 1). In response to the microenvironmental shift upon phagocytosis, each TCS is

activated by system-specific ligands through direct binding, amino acid modifications, induction of conformational change, or a combination of these mechanisms. When *Salmonella* is internalized by immune cells, EnvZ/OmpR senses a microenvironmental shift characterized by increased acidity and osmolality (Chakraborty et al., 2015; Lee et al., 2000). Activation of this system leads to early induction of SPI-2 genes and triggers intracytosolic acidification of the bacteria, which supports effector secretion (Chakraborty et al., 2015; Lee et al., 2000). PhoQ/PhoP senses cAMPs, acidity, high osmolality, and magnesium (Mg^{2+}) limitation (Bader et al., 2005; Cho et al., 2006; Hicks et al., 2015). In response, the PhoP RR induces changes in gene expression that lead to the modification of the composition of outer membrane lipopolysaccharides (LPSs), which promotes resistance to cAMPs by inhibiting ionic membrane interactions and reducing immune detection by altering the pathogen-associated molecular patterns (PAMPs) of LPS that would typically be detected by pattern recognition receptors (PRRs) (Dalebroux & Miller, 2014). PhoQ/PhoP also contributes to RNS resistance by regulating Mg^{2+} homeostasis via an unknown mechanism (Bourret et al., 2017). PmrA/PmrB is indirectly activated by PhoQ/PhoP under Mg^{2+} limiting conditions, such that PhoP induces the expression of *pmrD*, enabling PmrD to phosphorylate the PmrA RR (Kato et al., 2003); however, this TCS can also be directly activated by PmrB when iron is present in high concentrations (Pescaretti et al., 2011). Similarly to PhoP, PmrA induces LPS modifications that aid in cAMP resistance and immune evasion (Farizano et al., 2012). Lastly, SsrA/SsrB senses increased acidity and responds by inducing SPI-2 gene expression, which elicits phagosomal modifications required to establish the SCV (Mulder et al., 2015; Walthers et

al., 2007). As such, SsrA/SsrB is integral to establishing the intracellular lifestyle; thus, it is unsurprising that *Salmonella* employs redundant regulatory mechanisms to ensure that *ssrA* and *ssrB* are expressed intracellularly via positive regulation by SsrB, OmpR, and PhoP (Figure 1) (Bijlsma & Groisman, 2005; Lee et al., 2000; Walthers et al., 2007). Together, these TCSs sense a broad range of intracellular-specific signals to allow *Salmonella* to adapt to the stringent antimicrobial conditions of the SCV.

TCSs contribute to regulatory evolution to enhance niche-specific pathogenicity

TCSs enable *Salmonella* to survive in different environments by regulating clusters of genes in response to niche-specific cues. This dynamic genetic response to varying infectious conditions is highly controlled so that virulence genes are preferentially active only under conditions where the expression of gene products is beneficial to bacterial fitness. *Salmonella* has undergone extensive regulatory evolution to coordinate this complex regulation of virulence genes. Further, TCSs are able to directly interact with *cis*-regulatory elements to induce or repress transcription, but many of these genes are also regulated by the histone-like nucleoid structuring (H-NS) silencing protein (Lucchini et al., 2006). H-NS silences genes when their expression would be disadvantageous to *Salmonella* and RRs often displace this protein when TCSs become activated (Lucchini et al., 2006). H-NS binds to curved regions of DNA that have high adenosine and thymine (AT) content in order to block RNA polymerase binding and inhibit gene expression (Lucchini et al., 2006). This method of gene silencing is particularly effective for silencing genes that are horizontally acquired, such as SPIs,

because the AT content in the gene promoters corresponds to H-NS binding (Lucchini et al., 2006). Integration of H-NS-mediated silencing into SPI-2 regulation has enabled the pathogen to enhance intracellular pathogenicity, while limiting the negative effects of SPI-2 expression during the non-intracellular lifestyle. Further, niche-specificity of SPI-2 expression is enhanced by H-NS silencing of both the *ssrA* and *ssrB* genes prior to commencing the intracellular lifestyle (Deiwick et al., 1999; Lucchini et al., 2006). Interestingly, silencing of the *ssrA* and *ssrB* promoters can also be reversed by ancestral transcription factors HilD and SlyA, indicating yet another layer of regulation governing SPI-2 genes (Banda et al., 2019).

In addition to enhancing control over existing TCS-regulated genes, regulatory evolution permits the expansion of TCS regulons. Such expansion allows for further genetic manipulation under niche-specific conditions to generate a more robust response and ostensibly increase fitness. Regulon expansion can be detected by comparing variation of gene targets in conserved TCSs across different families and species of bacteria. For example, many putative genes have been identified in the *S.* Typhimurium PhoQ/PhoP regulon that were not observed in *E. coli*, suggesting that PhoP has acquired novel gene targets since the divergence of *Salmonella* from *E. coli* (Monsieurs et al., 2005). While the same comparison cannot be made for the SsrA/SsrB TCS because it was newly acquired by *S. enterica*, our lab and others have determined that the SsrB regulon has also expanded to control genes located outside of the SPI-2 locus (Desai et al., 2019; Ilyas et al., 2018; Tomljenovic-Berube et al., 2010). This determination was made by comparing gene activity in *S. enterica* and *S. bongori* in the presence or absence of SsrB,

as only *S. enterica* horizontally acquired this TCS but both species share a common ancestral genome (Ilyas et al., 2018; Osborne et al., 2009). From this work, it was proposed that regulon expansion occurs through *cis*-regulatory evolution (CRE). CRE is a mechanism wherein gene promoters undergo random mutagenesis and under the selective pressure of the host environment, form regulatory nodes that permit novel input by regulatory factors (Carroll, 2005; Osborne et al., 2009). Analysis of putative, non-SPI-2 targets of SsrB uncovered trends that suggest regulon expansion might have occurred to enhance intracellular immune evasion (Ilyas et al., 2018; Osborne et al., 2009; Tomljenovic-Berube et al., 2010). Specifically, in the presence of SsrB, expression of motility and cellular invasion genes are repressed, while those encoding defenses against host immunity are induced (Ilyas et al., 2018). These trends support the hypothesis that *Salmonella* exploits regulon expansion to increase fitness. Although large-scale analyses of the PhoP and SsrB regulons has been conducted, it is likely that further investigation into the function of ancestral regulatory targets in the intracellular niche will uncover unknown virulence mechanisms that are important for survival during this stage of infection.

Salmonella exploits host nutrients during the intracellular lifestyle to increase fitness

Within the SCV, *Salmonella* experiences nutrient-limiting conditions that would ordinarily hinder bacterial replication and survival. To overcome this limitation, SPI-2 effectors facilitate host nutrient acquisition, which influences overall metabolic activity of the pathogen in turn (Liss et al., 2017; Noster et al., 2019). An essential SPI-2-induced

modification of the SCV involves the formation of microtubule projections that extend outwards from the SCV called *Salmonella*-induced filaments (SIFs) (Deiwick et al., 2006). SIFs enable the SCV to localize to the *trans*-golgi network, which permits vesicles intended for exocytosis to be redirected to fuse with SIFs and supply the SCV with nutrients and other cellular cargo (Deiwick et al., 2006; Knuff & Finlay, 2017). Likewise, endocytic vacuoles can also be redirected to the SIF network (Knuff & Finlay, 2017). This mode of nutrient acquisition is integral to intracellular fitness, as demonstrated by attenuation of metabolic activity and stress tolerance in *S.* Typhimurium unable to form stable SIFs (Liss et al., 2017; Noster et al., 2019). The formation and localization of SIFs necessitates interactions between SPI-2 effectors SseF and SseG, as well as the non-SPI-2 effector SifA, which is regulated by SsrA/SsrB (Beuzón et al., 2000; Deiwick et al., 2006). Such interactions between effectors encoded at diverse genetic loci demonstrates the adaptability that regulatory evolution has permitted to enhance intracellular fitness.

After host nutrients are released to the SCV, the pathogen imports and exploits these resources for energy or other cellular processes. At least seven host nutrients are required by *S.* Typhimurium to obtain wild-type levels of replication, which include glycerol, fatty acids, N-acetylglucosamine, gluconate, glucose, lactate, and L-arginine (arginine) (Steeb et al., 2013). Together, these metabolites additively contribute to intracellular fitness (Steeb et al., 2013). The current data suggests that *S.* Typhimurium preferentially imports these host nutrients in favour of *de novo* biosynthesis, as proteomic data shows the corresponding bacterial anabolic pathways are reduced intracellularly (Steeb et al., 2013). While it is likely that the pathogen preserves energy by exploiting

host metabolites, it is equally likely that bacterial nutrient sequestration impacts cellular processes in the host as well, which might impact infection outcome.

Arginine is an important factor of bacterial pathogenesis

Arginine is an important metabolite in the context of intracellular *Salmonella* infection for both host and bacterial cells. In macrophages, this amino acid is directly involved in modulating the inflammatory response to bacterial infection. Arginine is a substrate of the pro-inflammatory iNOS pathway that generates NO•, as well as the predominately anti-inflammatory arginase pathway that produces polyamines (Chang et al., 1998; Lahiri et al., 2008). When macrophages phagocytose bacteria, the cells employ cationic amino acid transporters (CAATs) to import extracellular arginine, which is channeled into NO• and polyamine production (Das et al., 2010; Rath et al., 2014). iNOS and arginase, which respectively contribute to cell-intrinsic pro-inflammatory M1 or anti-inflammatory M2 polarization, compete for arginine (Briken & Mosser, 2011; Rath et al., 2014). iNOS is induced by Toll-like receptor 4 (TLR4) signalling in response to bacterial LPS recognition, but is also influenced by interferon gamma (INF-γ) production characteristic of pathogen-induced inflammation (Deng et al., 2015; Ingram et al., 2017). While iNOS classically produces NO•, this pathway is also capable of generating O_2^- when arginine is depleted (Sun et al., 2010; Xia & Zweier, 1997). Despite the inflammatory nature of bacterial infection, M2 polarization also occurs (Brodsky, 2020). It was previously demonstrated that during the intracellular lifestyle, *Salmonella* was able to induce a shift towards arginine consumption by arginase in favour of polyamine

biosynthesis; however, it was unclear how this was achieved (Lahiri et al., 2008). More recently, it was determined that secretion of the SPI-2 effector SpeE induces M2 polarization, suggesting a potential connection between the processes governing intracellular arginine consumption (Stapels et al., 2018). It is important to note, however, that polyamines are not exclusively anti-inflammatory. Polyamines produced via arginine catabolism include putrescine, spermine, and spermidine. While putrescine and spermine favour M2 polarization, spermine supports M1 activity (Latour et al., 2020). Further, pathways governed by polyamine activities are complex and highly integrated with the abundance of interleukin cytokines that regulate the inflammatory state of macrophages (Latour et al., 2020). Thus, arginine availability and exploitation have a broad influence on the immune response to bacterial infection.

In *Salmonella*, arginine is a substrate for multiple pathways that protect against antimicrobial factors. Two infection-relevant pathways that consume arginine are the arginine deiminase and bacterial polyamine biosynthesis (Figure 2). Arginine deiminase catabolizes arginine to ornithine, adenosine triphosphate, ammonia (NH_3), and carbon dioxide (Figure 2); this pathway is involved in acid resistance in multiple pathogens, including *Salmonella* (Álvarez-Ordóñez et al., 2010; Kieboom & Abee, 2006; Lindgren et al., 2014). Bacteria continuously cycle arginine through this pathway by exporting ornithine in exchange for intracellular arginine via the arginine deiminase antiporter, while NH_3 accumulation raises the local pH to combat acid stress (Figure 2) (Lindgren et al., 2014). An alternative route for arginine consumption is the bacterial polyamine biosynthesis pathway, which generates multiple variants of polyamines presented in

Figure 2. Polyamines have many functions and have been implicated in stress responses, cell growth, and genetic regulation (Shah & Swiatlo, 2008). In *Salmonella*, the role of polyamines is incompletely understood; however, investigations in *E. coli* provide an excellent reference for *S.* Typhimurium, as these species share a highly conserved genome. Further, polyamine synthesis genes (*speABC*, *speDE*, *cadABC*), as well as polyamine transport (*potABCD*, *potFGHI*, *potE*) are present in both bacteria (Shah & Swiatlo, 2008). Of note, translation of H-NS is more efficient in the presence of polyamines in *E. coli* , providing evidence that these molecules might indirectly influence genetic regulation of virulence genes encoded in SPIs (Terui et al., 2007). Additionally, membrane permeability is reduced by putrescine and spermidine binding to OmpC and OmpF porins; these proteins are regulated by EnvZ/OmpR in *S.* Typhimurium, suggesting yet another potential link between TCS-regulated genes and polyamine function (Iyer et al., 2000). A study in *S.* Typhimurium provides further evidence for the integration of EnvZ/OmpR and polyamine activities, wherein the cadaverine synthesis enzyme, CadC, was shown to influence OmpR activity (Lee et al., 2007). This involvement of polyamines in intracellular infection-relevant processes highlights the importance of arginine, indicating that arginine availability is important for bacterial survival.

Purpose and Aims

Throughout the intracellular lifestyle, *S.* Typhimurium copes with stressful antimicrobial conditions by regulating transcriptional activity in response intracellular-specific chemical cues. Research on arginine has exposed this metabolite as an important

component in the establishment of bacterial pathogenesis in invasive species; further, multiple studies in *S.* Typhimurium have established metabolic profiles for arginine production and consumption that suggest that this component of metabolism has adapted to the intracellular niche (Steeb et al., 2013). The multifaceted role of this metabolite in both host and pathogen activities indicates that modulation of metabolic activity might be an evolved mechanism in response to the intracellular environment. Thus, I hypothesize that **the *Salmonella* genome has undergone regulatory evolution at loci involved in arginine metabolism to permit input by intracellular-active TCSs to confer a fitness advantage**. Herein, I investigate the two primary mechanisms governing arginine availability, including *de novo* biosynthesis and extracellular import. The aims of this study are to *(i)* determine if trends in arginine metabolism are influenced by intracellular-active TCS activity, *(ii)* investigate if metabolic activity impacts fitness outcomes in *S.* Typhimurium, and *(iii)* investigate the mechanism by which metabolic activity contributes to bacterial survival.

Results

Arginine biosynthesis gene expression is repressed in the presence of SsrB under infection-mimicking conditions

In previous work, our lab conducted comparative RNA sequencing to interrogate the SsrB regulon in *S.* Typhimurium (Ilyas et al., 2018; Tomljenovic-Berube et al., 2010). RNA was sequenced from SL1344 carrying a constitutively active variant of SsrB (SsrB+

(Δ*ssrAB* +pWSK129-*ssrAB*D56E)) or lacking *ssrA* and *ssrB* (SsrB- (Δ*ssrAB* + empty pWSK129 vector)) grown in intracellular-mimicking low phosphate, low Mg^{2+} (LPM) media buffered to a pH of 5.8 (Coombes et al., 2004). Putative SsrB gene targets were classified by functional grouping (Figure 3a) and within the metabolic data set, five arginine biosynthesis gene (*argA*, *argC*, *argG*, *argH*, *argI*) were significantly repressed in the presence of SsrB (Figure 3a) (Ilyas et al., 2018). The remaining genes in the pathway (*argB*, *argD*, *argE)* were not identified in the sequencing experiment (Figure 3b). I sought to reconfirm this repressive trend using RT-qPCR by replicating the conditions used for RNA-sequencing and elucidating mRNA expression patters between SsrB+ and SsrB-. While *argI* was the only gene that was significantly repressed in the presence of SsrB, all biosynthesis genes displayed a repressive trend (Figure 3c). I subsequently investigated if this mRNA expression pattern was present in *S.* Typhimurium lacking the constitutively active SsrB variant, with the aim to determine if wild-type SsrB activity was sufficient for observable repression. For this, I compared SsrB- to wild-type *S*. Typhimurium carrying an empty pWSK129 plasmid. In this comparison, SsrB-mediated repression was observed for all genes (Figure 3d). From these data, I reconfirmed the repressive trend in arginine biosynthesis that was uncovered in the RNA-sequencing experiment and, further, established that wild-type SsrB activity was sufficient for repression.

Biosynthesis gene promoter activity is dependent on growth state in vitro

Following reconfirmation of the RNA-sequencing data, I next sought to investigate if biosynthesis gene activity was influenced by SsrB at the promoter level. I

generated promoter-luciferase fusion constructs for each biosynthesis gene or operon (*argA*, *argBCH*, *argD*, *argE*, *argG*, *argI*, *argH*) and the biosynthesis regulator *argR* that represses biosynthesis upon allosteric activation by high levels of arginine (Tian & Maas, 1994). Each reporter construct was transformed into wild-type and Δ*ssrAB* backgrounds so that promoter activity could be compared. Using the SPI-2 gene *ssaB* as a positive control for SsrB activation, bacteria were grown in LPM media and luminescence was normalized to optical density (OD). P*ssaB-lux* activity showed induction of SsrB-mediated regulation at ~6 hours, peaking at ~10 hours (Figure 4). This expression pattern was not reflected in any of the biosynthesis genes or *argR* (Figure 4). Moreover, the biosynthesis promoters displayed growth-state dependence, such that activity was induced during exponential growth and plateaued upon entry into stationary phase (Figure 4; Supplementary Figure 1). While differences in luminescence signal were observed for *argBCH*, *argE*, *argG*, and *argI* during stationary phase, SsrB-mediated promoter regulation cannot by inferred from these data because arginine biosynthesis is highly induced in both wild type and Δ*ssrAB* to support growth and thus, detection of promoter repression would require high sensitivity which is not afforded by this assay. To ascertain if SsrB influences promoter activity of this biosynthetic pathway, infection-relevant gene activity must be isolated from that due to bacterial growth. To this end, a more representative model of intracellular infection is required.

Promoter regulation of the artPIQM operon is sensitive to intracellular-specific environmental cues

To begin investigating intracellular arginine import activity, I interrogated the arginine transport (Art) system encoded by the *artPIQM-artJ* operon. This system comprises two variants of arginine transporters that differ in their periplasmic binding protein (ArtI/ArtJ), which influences specificity of arginine analog import (Torres Montaguth et al., 2019). I was first interested in establishing if operon transcription was manipulated by cues present within the SCV. I generated a luciferase promoter reporter for the *artPIQM* operon and monitored promoter activity in wild-type *S.* Typhimurium cultured in modified infection-mimicking media and nutrient-rich LB. In acidic LPM media, P*artPIQM* was highly induced relative to the minimal baseline activity observed in LB (Figure 5a). Removal of stress conditions (acidity or Mg^{2+} limitation) enhanced promoter induction further, and removal of arginine from the media resulted in an expression pattern identical to the Mg^{2+} supplementation condition (Figure 5a). The peak in promoter activity at ~6-8 hours across LPM media conditions corresponded with exponential growth (Supplementary Figure 2) and therefore, it was difficult to ascertain if the intracellular chemical cues influence promoter activity in the initial stages of growth. However, significant differences in promoter expression upon entrance into stationary phase were observed when intracellular-specific environmental cues were removed (Figure 5b). Promoter activity was reduced only slightly below peak levels in standard LPM media, while the removal of stress conditions significantly reduced luminescence signal (Figure 5b). While I expected promoter activity to be reduced when arginine was

omitted from the media because production of arginine transporters would be futile in the absence of the transporter substrate, Mg^{2+}supplementation also greatly reduced promoter activity to levels that were non-significantly different from the former condition (Figure 5b). Taken together, these data demonstrate that arginine transport regulation is dynamic and sensitive to environmental cues that would be encountered within the SCV; thus, I hypothesized that the Art system might have acquired regulatory input by an intracellular-active TCS that is sensitive to Mg^{2+} limitation.

Arginine transport is transcriptionally regulated by PhoP in vitro

Previously, a comparative analysis of the PhoP regulon between *E. coli* and *S.* Typhimurium uncovered a putative connection between PhoP and the Art system in *S.* Typhimurium (Monsieurs et al., 2005). The PhoQ/PhoP TCS is responsive to both Mg^{2+} limitation and acidity; therefore, I postulated that PhoP-mediated regulation was probable. I sought to elucidate the role of PhoP in regulating arginine transport, while also probing for SsrB-mediated regulation due to the cross-talk that occurs between these systems during intracellular infection (Bijlsma & Groisman, 2005). For this, I monitored P*artPIQM* activity relative to wild-type levels in Δ*phoP* and Δ*ssrAB* backgrounds. No differences were observed in LB where these TCSs are largely inactive, while promoter activity was diminished in Δ*phoP* in standard LPM media after reaching stationary phase (Figure 6a; Supplementary Figure 2). To further interrogate chemical cues that might elicit PhoP-mediated regulation, the assay was repeated in the absence of chemical cues known to induce PhoP. In neutral LPM media or Mg^{2+} excess, promoter activity was

identical across all strains (Figure 6a). Consequently, these data provided evidence for PhoP-mediated regulation that is influenced by multiple chemical cues.

I next sought to investigate if this regulatory connection held in a macrophage model of intracellular infection. I infected the RAW264.7 macrophage-like cell line with the P*artPIQM* reporter strains and included positive controls for PhoP (P*phoP*, P*pagC*) and SsrB (P*ssrA*) activation. Colony forming units (CFUs) were plated at each time point to normalize luminescence signal and account for variability in intracellular fitness across mutant strains. At 0 and 12 hours following infection, all promoters were minimally induced (Figure 6b). After 24 hours, P*artPIQM* was significantly induced in wild type relative to both Δ*phoP* and Δ*ssrAB* (Figure 6b). Thus, PhoP-mediated regulation was replicated in an intracellular model of infection, providing further support for the initial hypothesis. While the Δ*ssrAB* reporter strain behaved differently during intracellular infection, this result was not surprising as LPM media encompasses only a subset of chemical cues that influence intracellular TCS activity. It is possible that regulatory input by SsrB is a result of cross-talk with the PhoQ/PhoP system, or is an indirect consequence of interrupted processes governed by SsrA/SsrB that feed into this regulatory system, which occur only under intracellular infection conditions. An investigation of promoter binding by these RRs will provide further insight into how *artPIQM* is controlled by these systems.

The Art system influences stress tolerance under infection-mimicking conditions

Input by PhoQ/PhoP and SsrA/SsrB TCSs suggested a potential virulence function for arginine transport in the intracellular lifestyle, and so, I sought to investigate if the Art system conferred a fitness advantage to *S*. Typhimurium. To isolate the impact of transport on bacterial fitness, I generated a marked deletion mutant for *artP* (Δ*artP*:: chloramphenicol acetyltransferase (CAT)), which encodes the ATPase subunit required for transporter activity. To establish if the transport mutant was growth defective under non-infectious conditions, I conducted growth curves in LB and standard LPM media. Δ*artP* growth was identical to wild type in both media conditions in the absence of antibiotic selection (Figure 7a); however, when the transport mutant was grown with chloramphenicol (Cm), I observed a prolonged lag phase in LPM media (Figure 7b). The mutant was marked with a CAT resistance cassette and was able to detoxify the antibiotic easily in nutrient rich media (Figure 7b); therefore, I hypothesized that this phenotype might be a result of reduced stress tolerance under stringent media conditions. To investigate if any intracellular-specific conditions influenced this growth phenotype, I repeated the growth curves in LPM media supplemented with phosphate or $MgCl_2$, or buffered to a neutral pH. I observed that elimination of acidity and phosphate supplementation did not impact bacterial growth (Supplementary Figure 3); however, the phenotype was reversed by Mg^{2+} supplementation in a dose-dependent manner (Figure 7c).

Mg^{2+} is involved in many process that contribute to antibiotic sensitivity by modulating bacterial stress tolerance, including membrane and ribosome stabilization, as

well as inhibition of membrane hyperpolarization (Ellis et al., 2019; Lee et al., 2019). Interference with any one of these processes in the transport mutant might explain why I observed a prolonged lag phase under Cm selection indicative of antibiotic sensitization. To probe for inhibition of Mg^{2+} homeostasis in the transport mutant, I investigated if membrane stabilization was impacted in Δ*artP*. Ellis et al. demonstrated that *S.* Typhimurium is sensitized to the intracellular-targeting antibiotic rifampicin (Rif) in LPM media due to increased membrane permeability at low concentrations of Mg^{2+} (Ellis et al., 2019). Therefore, to investigate if membrane permeability was augmented in the transport mutant in LPM media, I conducted minimum inhibitory concentration (MIC) assays with Rif in wild-type and Δ*artP* strains. The MIC for Rif was unchanged between strains in the presence or absence of Mg^{2+} supplementation in LPM media (Figure 8). Based on this result, elimination of arginine transport did not significantly reduce Mg^{2+}-dependent membrane integrity. Nevertheless, it is still possible that arginine transport influences a cellular process which protects against antibiotic assault that can be remedied by supplementing Mg^{2+}. Further analysis of Mg^{2+}-dependent processes is required to elucidate how elimination of the Art system impacts stress tolerance *in vitro*.

Arginine transport influences intracellular survival in RAW264.7 macrophages

Based on the growth curves for Δ*artP* in the absence of antibiotic stress, arginine transport did not influence overall growth relative to wild-type levels under stringent conditions (Figure 7a). Thus, I was confident that the transport mutant did not confer an inherent growth defect and so, I sought to investigate intracellular fitness of Δ*artP* in a

macrophage model of infection. To quantify fitness, I infected RAW264.7 macrophages with wild-type or Δ*artP* strains, and enumerated bacterial burdens. CFUs were consistent across both strains at the onset of infection (Figure 9); however, Δ*artP* was significantly attenuated for intracellular replication after 6 hours (Figure 9). This observation provided evidence for arginine import positively influencing intracellular fitness, although it was unclear if attenuated replication was a result of altered modulation of immunological host-pathogen interactions, or if arginine import was critical for standard replication.

Intracellular fitness of ΔartP is influenced by antimicrobial nitrosative species

Given that arginine is a substrate for iNOS-derived RNS and bacterial polyamines which can act as antioxidants and protect against RNS, I was interested in investigating a connection between arginine transport and tolerance to iNOS-derived species (Chang et al., 1998; Lahiri et al., 2008; Shah & Swiatlo, 2008). I hypothesized that intracellular fitness might be reduced when arginine import was obstructed due to *(i)* increased arginine availability in host cells to be directed to the iNOS pathway, and/or *(ii)* bacterial sensitization to nitrosative species resulting from reduced polyamine biosynthesis (Figure 10a). Modulation of these processes would impact the degree of nitrosative stress encountered by *Salmonella* or the ability for the bacteria to tolerate nitrosative species, respectively. To probe for a connection between nitrosative stress tolerance and bacterial fitness, I conducted an intramacrophage replication assay in the presence of the competitive iNOS inhibitor L-N^6-(1-iminoethyl)-lysine (L-NIL). When macrophages were exposed to 500 μM of L-NIL prior to bacterial infection, I observed a modest

reversal of intracellular attenuation in Δ*artP* after 6 hours (Figure 10b), which was consistent with the hypothesis. However, a comparison between initial Δ*artP* bacterial burdens and those at 6 hours showed minimal change in over the course of infection in L-NIL treated cells (Figure 10b). Interpretation of the results of this experiment were limited by potential off-target effects of L-NIL, which is an analog of L-arginine and thus, likely impacted arginase activity as well. To isolate for iNOS activity, I repeated the intramacrophage replication assay in bone marrow macrophages (BMMs) harvested from B6J NOS2 knockout ($NOS2^-$) mice, which do not produce the iNOS protein. As a control, I also infected BMMs harvested from unmodified B6J mice. Infection in control cells replicated the attenuated phenotype of Δ*artP* that I initially established in RAW264.7 cells (Figure 10c), while the mean CFU count for wild type and Δ*artP* was identical in $NOS2^-$ macrophages after 12 hours (Figure 10c). Notably, Δ*artP* burdens were greater in $NOS2^-$ cells relative to the control BMMs at the onset of infection (Figure 10c), indicating that loss of arginine transport might enhance bacterial invasion in the absence of iNOS. While statistically insignificant, this trend was also observed in L-NIL treated RAW264.7 macrophages (Figure 10b). Together, these data indicate that the Art system might have a role in modulating early iNOS-derived nitrosative stress, while contributing to long-term intracellular fitness by supporting bacterial replication.

Discussion

The emergence of multi-drug resistance has complicated the treatment of bacterial infections such as those caused by invasive *Salmonella*. Systemic infection necessitates survival within host cells and so, it is imperative that virulence mechanisms employed by the pathogen under intracellular conditions are well understood to support the discovery of novel antimicrobial targets. TCSs have become a major focus of infectious disease research over the last several decades owing to the critical role these systems play in enabling environmental adaptation to enhance pathogenicity. For this reason, our lab has an interest in the regulatory evolution of SsrA/SsrB and the associated fitness implications of regulon expansion (Ilyas et al., 2018; Tomljenovic-Berube et al., 2010). In previous work, we uncovered a connection between SsrB and the ancestral flagellin operon *flhDC*, wherein acquired repression by SsrB permitted suppression of inflammasome activation by limiting bacterial motility (Ilyas et al., 2018). Thus, I was interested in further exploring adaptive regulatory evolution of TCSs with the aim to uncover novel virulence factors. In this work, I interrogated the regulatory origin of intracellular expression patterns in genes encoding for arginine metabolism. I was interested in this component of intracellular metabolism due to growing evidence linking arginine exploitation to bacterial pathogenesis, coupled with preliminary data from our lab that suggested *de novo* biosynthesis might be under the regulatory control of SsrB (Gogoi et al., 2016; Ilyas et al., 2018; Xiong et al., 2016). Thus, I hypothesized that the *Salmonella* genome underwent regulatory evolution at loci involved in arginine

metabolism to permit input by intracellular-active TCSs as a means to confer a fitness advantage.

Studies of intracellular metabolism in *S.* Typhimurium have demonstrated general trends in arginine metabolism wherein biosynthesis is repressed, while transport is induced (Steeb et al., 2013). From preliminary RNA-sequencing data, I sought to reconfirm SsrB-mediated repression of biosynthetic genes. I reconfirmed this preliminary data by replicating the conditions of the RNA-sequencing experiment and elucidating mRNA expression of biosynthesis genes in the presence of the constitutively active variant of SsrB (Figure 3c). Further, an analysis of wild-type SsrB activity yielded similar results (Figure 3d), further supporting a regulatory connection with SsrB under infection-mimicking conditions. To elucidate this connection, I aimed to investigate promoter regulation of biosynthesis genes in the presence of SsrB; however, no conclusion could be drawn from this experiment regarding SsrB-mediated repression because promoter activity of biosynthetic genes was highly dependent on supporting bacterial growth (Figure 4; Supplementary Figure 1). A more sensitive assay that isolates for infection-relevant biosynthesis activity would aid in furthering our understanding of how SsrB provides regulatory input into biosynthetic gene expression at the promoter level.

With respect to arginine transport, I explored the regulation of the *artPIQM* operon because the Art system had yet to be studied in the context of intracellular infection, although it has been linked to virulence activity during biofilm formation (Mills et al., 2015). I demonstrated that transcriptional regulation of arginine transport was highly sensitive to arginine availability and chemical cues in infection-mimicking media

such that the *artPIQM* promoter was inactivated after reaching stationary growth phase when arginine, acidity, or Mg^{2+} limitation was removed (Figure 5). These observations led to an investigation of a regulatory connection between TCSs induced by Mg^{2+} and acidity, including SsrA/SsrB and PhoQ/PhoP. I established that P*artPIQM* was positively regulated by PhoP in infection-mimicking media (Figure 6a), which was recapitulated in an intramacrophage infection model (Figure 6b). Moreover, while I did not observe SsrB-mediated regulation in LPM media, promoter activity was significantly repressed in macrophages (Figure 6). LPM media provides only a small subset of extracellular chemical cues that are present in the SCV and feed into SsrA/SsrB activity, which might explain why the *artPIQM* promoter displayed differential regulation by SsrB between assays. Moreover, it is possible that intramacrophage repression is a result of cross-talk between with PhoQ/PhoP, or an indirect consequence of eliminating SsrA/SsrB. Although further work will be required to dissect how this operon might be regulated by both TCSs, I was able to provide strong evidence for regulatory evolution of arginine transport genes, which was consistent with the initial hypothesis.

Genes regulated by PhoQ/PhoP and SsrA/SsrB are conventionally involved in intracellular virulence and so, I hypothesized that arginine transport might confer a fitness advantage to *S*. Typhimurium. To gauge the importance of arginine import to overall growth *in vitro*, I monitored growth in nutrient-rich and infection-mimicking media, and did not observe any growth defects when the transport system was eliminated (Figure 7a). However, the Cm-resistant *artP* mutant exhibited a prolonged lag phase when Cm was supplemented into infection-mimicking media (Figure 7b), indicating sensitization to this

bacteriostatic antibiotic. I determined that this growth phenotype was dependent on Mg^{2+} availability in the media and that antibiotic resistance could be recovered by Mg^{2+} supplementation in a dose-dependent manner (Figure 7c). Mg^{2+} is known to be involved in bacterial antibiotic tolerance by acting as a membrane and ribosome stabilizer, as well as protects against membrane hyperpolarization (Ellis et al., 2019; Lee et al., 2019); however, in a strain carrying a CAT resistance cassette, as did the *artP* mutant, Cm sensitivity was unexpected. Moreover, under stringent conditions, *S.* Typhimurium generally responds to Mg^{2+} limitation by PhoP-mediated upregulation of importers to maintain Mg^{2+} homeostasis (Blanc-Potard & Groisman, 2020). Δ*artP* did not exhibit growth defects under Mg^{2+}-limiting conditions in the absence of Cm (Figure 7a), therefore I hypothesized that antibiotic supplementation might have exposed a function of arginine transport in regulating Mg^{2+} homeostasis that was augmented by additional stress of antibiotic exposure. When probing for increased membrane permeability in the transport mutant using a MIC assay for Rif, I did not observe increased antibiotic sensitization (Figure 8), which suggested that membrane integrity was not significantly impacted by elimination of arginine transport. Nevertheless, dissecting the relationship between arginine transport and Cm sensitization under Mg^{2+} limitation might provide valuable insight into how this system contributes to intracellular fitness where *Salmonella* is exposed to multiple sources of stress opposed by adaptive responses that establish Mg^{2+} homeostasis.

In the literature, it has been documented that arginine transport additively contributes to intracellular fitness in combination with six other host nutrients, though no

mechanism has been determined thus far (Steeb et al., 2013). I was able to reconfirm that arginine transport is required for wild-type fitness in a macrophage infection model (Figure 9), and began to investigate the role of the Art system in intracellular infection. I proposed that arginine transport might contribute to bacterial fitness by modulating substrate availability for production of iNOS-derived RNS species, or bacterial polyamine production to protect against such nitrosative species. Inhibition of iNOS in RAW264.7 macrophages by L-NIL supplementation modestly reversed attenuation in Δ*artP* relative to wild type (Figure 10b), although it was unclear if this was a result of enhanced fitness at the onset of infection or long-term survivability. Further investigation in NOS2 deficient BMMs suggested a role for arginine transport in modulating early iNOS stress, as macrophage colonization by Δ*artP* was increased in the absence of iNOS activity (Figure 10c). Moreover, Δ*artP* burdens were unchanged in both the control and $NOS2^{-}$ cells over the course of infection (Figure 10c), suggesting that arginine import contributes to bacterial replication after establishing the SCV. While further mechanistic investigation is required to elucidate the role of the Art system during early and long-term intracellular infection, these data uncovered a connection between intracellular arginine import and evasion of host immunity.

Future directions

The current work leaves several unanswered questions pertaining to the evolutionary regulation of arginine metabolism and how these metabolic processes contribute to intracellular fitness. Further investigation of the following questions will

provide a more comprehensive understanding of the role of arginine in *Salmonella* pathogenesis:

(1) The regulation of *de novo* biosynthesis during the intracellular lifestyle.

The investigation into biosynthetic gene expression in infection-mimicking media provided evidence for SsrB-mediated repression of this pathway. However, I was unable to further elucidate this connection at the promoter level due to limitations of bacterial growth requirements in the LPM media infection-mimicking model. Recapitulating the promoter-reporter assay for biosynthesis genes in a macrophage model of infection might allow for identification of differences in promoter activity between wild type and Δ*ssrAB*. Within this infectious environment, cellular activity would be directed towards survival rather than active natural bacterial replication, as in infection-mimicking media, which will likely provide a more accurate representation of transcriptional regulation that occurs during the intracellular lifestyle. Additionally, based on observations in this study that revealed dual input by PhoP and SsrB into arginine transport, it would be advantageous to also investigate possible PhoP-mediated regulation into this pathway. Determining if arginine biosynthesis genes have undergone regulatory evolution to permit input by these TCSs during infection will inform on a potential function of arginine biosynthesis in intracellular fitness.

If a regulatory connection is observed during intracellular infection, an investigation of the role of biosynthesis on intracellular fitness would be justified. Generation of a mutant lacking *argH* would allow for the elimination of *de novo* arginine biosynthesis, which could then be assayed for fitness relative to wild-type *S*.

Typhimurium by comparing bacterial burdens in a macrophage infection model. Further, an *in vivo* competitive indices infection directly competing these strains could provide a more direct quantification of fitness in a representative infection model.

(2) Determine if PhoP-mediated regulation of arginine transport is direct.

An analysis of the intergenic region upstream of the *artPIQM* operon uncovered a putative PhoP binding site (Table 1, Appendix). This sequence was identified by comparing sequences between *E. coli* and both *S. bongori* and Typhimurium to identify regions of variability between *E. coli* and *Salmonella* species to uncover nucleotides that had undergone mutagenesis. Further, an alignment of known PhoP binding sites for *phoP*, *mgtA*, and *pmrD* genes uncovered a high level of sequence similarity in the putative *artPIQM* binding site, which also displays similarity with the average binding motif for PhoP ((T/G)GTTTA-5nt-(T/G)GTTTA) underlined in Table 1 (Zwir et al., 2012). By mutating this putative binding site in a luciferase-fusion construct for the *artPIQM* promoter transformed into wild-type *S.* Typhimurium, this would provide insight into whether this putative binding site is required for PhoP-mediated regulation.

(3) Determine why Δ*artP* is sensitized to Cm under Mg^{2+} limiting conditions.

In the analysis of bacterial growth of Δ*artP*, I observed antibiotic sensitization in the Cm-resistant strain, which was reversed with Mg^{2+} supplementation. In order to rule out interference of Mg^{2+} homeostasis, it would be valuable to assay for Mg^{2+} flux and quantify intracellular Mg^{2+} ions in wild type and Δ*artP* grown in LPM media. To control for arginine transport activity, this should also be conducted with a strain carrying a Cm resistance cassette at a different locus, as well as a strain carrying a different antibiotic

resistance cassette within the transport operon to ensure that Cm sensitization is not a result of general stress tolerance in infection-mimicking media. Establishing if there is a connection between arginine transport and Mg^{2+} homeostasis would provide insight into how this system might influence intracellular survival.

Alternatively, it is also possible that Cm sensitization is a result of reduced arginine availability to be channeled into polyamine production. It has been demonstrated that polyamines have a protective role against multiple classes of antibiotics in *Pseudomonas aeruginosa*, including ribosome targeting molecules (Kwon & Lu, 2006). Moreover, polyamines and Mg^{2+} are able to interchangeably stabilize bacterial ribosomes (Dever & Ivanov, 2018). Thus, it is possible that Mg^{2+} supplementation was able to reverse Cm sensitization by substituting for polyamines in the arginine transport mutant, as Cm is a ribosomal inhibitor. A comparison of polyamine production in wild type and Δ*artP* in infection-mimicking media, as well as during intracellular infection would establish if imported arginine is channeled into polyamine biosynthesis. This could be accomplished using a fluorometric assay to quantify total polyamine abundance after exposing bacteria to infectious conditions. It would also be useful to assess if polyamine supplementation in infection-mimicking media is able to reverse Cm sensitization in Δ*artP* in order to establish if Mg^{2+} is dispensable. Together, this analysis would establish if transported arginine is directed to polyamine production to enhance stress tolerance *in vitro*, and might also provide insight into transport-directed activities that influence intracellular fitness.

(4) The role of arginine transport on intracellular bacterial fitness.

In this work, I reconfirmed that arginine transport contributes to intracellular fitness, demonstrating that Δ*artP* was attenuated for intracellular replication in a macrophage infection model. There are several mechanisms by which the import of arginine might influence bacterial replication, such that arginine could be directed to polyamine or protein synthesis in bacteria, or sequestration of host arginine might reduce NO• production. Moreover, it is possible that each of these factors contributes to intracellular viability, warranting an investigation into each process.

Concerning NO• evasion, investigations thus far have provided evidence for arginine import modulating early iNOS stress at either the host or bacterial level. To assess bacterial tolerance to RNS, growth curves of wild type and Δ*artP* in infection-mimicking media exposed to a NO• donor might reveal if removal of arginine import sensitizes *Salmonella* to RNS stress. Further, an analysis of RNS detoxification capability between these strains could likely provide a more sensitive assay. If increased RNS sensitivity was observed in the transport mutant *in vitro*, the question would still remain as to how this is occurring. Polyamines are known to provide a protective effect against redox species and so, it would be critical to also evaluate if polyamine synthesis correlated with RNS tolerance.

The impact of arginine transport on overall replication ability in Δ*artP* is another important consideration, which likely contributes to long-term fitness attenuation. To evaluate the extent to which arginine is directed to processes that support bacterial replication, integration of imported arginine into protein synthesis could be ascertained by infecting macrophages with wild type and Δ*artP*, and quantifying total protein

production. This would provide insight into the extent to which Δ*artP* is attenuated for replication solely due to amino acid deficiency. Together, these lines of investigation will provide further insight as to why arginine import is important and how the amino acid is exploited during *Salmonella* infection.

Conclusion

The intracellular lifestyle of *Salmonella* permits this pathogen to elicit severe, systemic infection that must be treated with effective antimicrobials. The development of complex virulence networks governed by TCSs enables *Salmonella* to incite a robust response against host immunity, but also engage in host-pathogen interactions that influence pathogenicity. Moreover, research on the influence of arginine on bacterial pathogenicity is a growing field due to the essentiality of this metabolite in regulating both host and bacterial processes under infectious conditions. Here, I demonstrated that *S.* Typhimurium has evolved regulatory input by the PhoQ/PhoP TCS into arginine transport. Further, I have provided preliminary evidence linking this regulatory connection to intracellular fitness. Although I have yet to determine how arginine import influences evasion of host nitrosative stress and long-term survivability, there are many mechanisms yet to be investigated. Overall, this work has provided further insight into TCS regulon expansion and cross-talk between systems, linking TCS-mediated regulation to yet another cellular process that enhances bacterial fitness.

Materials and Methods

Bacterial strains and conditions

All experiments were conducted with *S.* Typhimurium strain SL1344 or derivatives, listed in Table 2 of Appendix. Standard bacterial cultures were grown in LB media (10 g/L NaCl, 10 g/L Tryptone, 5 g/L yeast extract) supplemented with antibiotics as required for selection (ampicillin, 100 μg/mL; chloramphenicol, 34 μg/mL; kanamycin, 50 μg/mL; streptomycin, 50 μg/mL). For all experiments under intracellular-mimicking conditions, overnight bacterial cultures were subcultured in LPM media (5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 80 mM MES pH 5.8, 0.1% casamino acids, 0.3% (v/v) glycerol, 24 uM $MgCl_2$, 337 μM PO_4^{3-}). Modified LPM media was created by adding 10 mM $MgCl_2$ or 10 mM K_2PO_4 to standard LPM media, omitting L-arginine from casamino acid mixture, or substituting 100 mM Tris-HCl pH 7.4 for MES. All cultures were grown at 37°C.

Animal work

All animal procedures complied with the Canadian Council on Animal Care guidelines and followed protocols approved by the Animal Review Ethics Board at McMaster University under Animal Use Protocol #20-12-41. Six-to-eight-week-old female unmodified B6J and B6J NOS2 knockout mice were purchased from The Jackson Laboratory. Mice were housed in the Central Animal Facility at McMaster in a Biosafety Level 2 room.

Cloning and mutant generation

Primers used for cloning and mutant generation were synthesized by Sigma-Aldrich and can be found in Table 3 of Appendix. Polymerase chain reactions (PCR) were performed with Phire II, Phusion, or Taq DNA polymerase (Thermo Fisher). PCR products were resolved on 1% agarose gel for 18 minutes at 140 V and gel extracted using the QIAquick Gel Extraction Kit (QIAGEN). Plasmid transformations were performed on chemically component cells by heat shock at 42°C for 42 second, and electrocompetent cells were electroporated at -2.5 kV, 25 μF, 200 Ω. All genetic manipulations involving recombination of the SL1344 genome or plasmid cloning were first confirmed by colony PCR, and subsequently reconfirmed by DNA sequencing performed by Genewiz Incorporated.

Marked mutants were generated by lambda red recombination. A CAT cassette carried in the pKD3 plasmid was amplified with primers containing 90 base pair (bp) overhanging regions of homology to the gene of interested and purified by gel extraction. Overnight culture of wild-type SL1344 +pKD46 was subcultured at 1:50 in LB supplemented with 100 μg/mL ampicillin (Amp) and incubated at 30°C until reaching an OD of 0.2 – 0.3. The bacteria were induced with filter sterilized arabinose (1 M) and incubated at 30°C until reaching an OD of 0.4 – 0.5. Cells were centrifuged at 4000 rpm for 10 minutes at 4°C and resuspended in 50 mL of ice cold MilliQ water twice. After a third centrifugation, cells were resuspended in 300 μL of ice cold 10% glycerol. 150 μL of the electrocompetent cells were transformed with 10 μg of insert and recovered in 1 mL of SOC media (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM

$MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) for 3 hours at 37°C. Transformants were plated on LB agar, Cm-34 μg/mL for selection. Gene deletion was confirmed by colony PCR and DNA sequencing.

Luciferase promoter reporter clones were generated by amplifying 500 bp upstream of genes of interest with primers containing restriction enzyme sites; promoter regions were shortened if complete reading frames occurred within 500 bp region. Purified PCR products and pGEN-*luxCDABE* plasmid were then digested with restriction enzymes and ligated with T4 DNA ligase. Reporter constructs were transformed into *E. coli* Top 10 cells and plated on LB agar, Amp-200 μg/mL for plasmid selection. Clones were confirmed with colony PCR and DNA sequencing, and transformed into wild-type SL1344 and derivative strains.

RNA extraction and RT-qPCR

All RNA extraction was conducted following growth in standard LPM media. Overnight cultures were grown with 50 μg/mL streptomycin (Sm) and 50 μg/mL kanamycin (Kn), and subcultures were grown with only Kn-50 μg/mL for plasmid selection. Bacteria were subcultured into LB at 1:100 and incubated at 37°C for ~2.5 hours. Cultures were transferred into a secondary LPM subculture at 1:100 and were incubated at 37°C for ~5 hours until reaching an OD of 0.4 – 0.5. 5 mL of subculture was centrifuged at 4000 rpm for 10 minutes at 4°C. Cell pellets were resuspended in 1 mL of Trizol (Invitrogen) and stored overnight at -20°C. RNA was extracted by chloroform (BioShop) separation following the manufacturer's protocol, precipitated with 100% ice

cold isopropanol (BioShop), and washed with 75% ethanol (Sigma) in DEPC water. RNA samples were resuspended in DEPC water and treated with DNase I (Invitrogen) for 1 hour at 37°C. DNase activity was inactivated with 2.5 mM EDTA. cDNA was synthesized from purified RNA using qScript cDNA Supermix (Quantabio) and diluted by 1/10 in DEPC water. RT-qPCR was performed with Quanta Perfecta SYBR Green Supermix (Quantabio), and reactions were run using LightCycler 480 (Roche). Housekeeping gene *16S* was used for normalization between strains. Six biological replicates were run in technical duplicates for each gene analyzed.

In vitro transcriptional reporter assay

Luciferase promoter-reporter strains were grown overnight in LB supplemented with Sm-50 μg/mL and Amp-100 μg/mL for selection at 37°C. Strains were subcultured at 1:100 in LB supplemented with Amp-100 μg/mL for 2.5 hours and then subcultured into either LB or variations of LPM media with Amp-100 μg/mL at 1:100. 100 μL of culture was aliquoted into each well of a black, clear bottom 96-well plate. Luminescence was read at 450 nm and OD was measured at 600 nm using a Biotek Neo Synergy 2 while the plate was incubated at 37°C with double orbital shaking. Relative light units (RLU) were divided by OD for each time point to normalize promoter activity to bacterial growth.

Growth curves for transport mutant

Overnight cultures grown with antibiotic selection were subcultured at 1:100 in LB and incubated in a 37°C shaking incubator for 2.5 hours; for curves generated without selection, antibiotic was omitted from subculture, for selection wild type was grown with Sm-50 μg/mL and Δ*artP* was grown with Cm-34 μg/mL. Bacteria were subsequently subcultured in LB, standard LPM, or modified LPM with consistent antibiotic selection conditions. 100 μL of subculture was aliquoted into each well of a clear, 96-well plate in six replicates, across two biological replicates. OD was measured at 600 nm every 30 minutes for 24 hours using a Biotek Neo Synergy 2 while the plate was incubated at 37°C with double orbital shaking.

Minimal inhibitory concentration assay

Antibiotic was diluted to a working stock concentration of 256 μg/mL in media used for MIC assay, and serial diluted by 1:2 in a 96-well plate. Overnight bacterial cultures were diluted in media and 100 μL was added to each well for a final dilution of 1:10000. Plates were incubated overnight at 37°C. OD was measured at 600 nm using an EnVision plate reader at 0 and 18 hours, and growth was normalized by subtracting 0-hour values. MIC assays were conducted in technical duplicates and repeated twice.

Tissue culture infection conditions

RAW264.7 and BMM cells were stored in a humidified incubator at 37°C and 5% CO_2 (TC incubator). At ~16 hours prior to infecting, cells were seeded at 10^5 cells/well

and media was supplemented with 100 ng/mL LPS from *Salmonella* minnesota. Macrophages were infected with 5.0x10^6 cells/well for a multiplicity of infection (MOI) of 50. After adding bacteria, cultures were spun down at 500 xg for 2 minutes and incubated in TC incubator for 30 minutes. A gentamicin (Gm) protection assay was conducted by exposing infected cells to Gm-100 μg/mL for 30 minutes in TC incubator.

RAW264.7 intracellular replication assay

Macrophages were maintained in DMEM, 10% fetal bovine serum (FBS) media. Cells were seeded in a tissue culture treated, clear 96-well plate with 100 μg/mL LPS. For iNOS inhibition assay, macrophages were exposed to 500 μM L-N^6-(1-iminoethyl)-lysine (L-NIL) for 30 minutes prior to starting the infection (Sigma Aldrich). Bacterial strains were grown overnight in LB with antibiotic selection at 37°C. Cells were pelleted, washed once with phosphate-buffered saline (PBS), and resuspended in PBS at a final concentration of 5.0x10^7 cells/mL. Bacteria were opsonized with 20% normal human serum (NHS) for 30 minutes at 37°C, and 100 μL of cells were added to each well for a final concentration of 5.0x10^6 cells/well. Gm protection assay was conducted by aspirating supernatant from wells and adding 100 μL of fresh media supplemented with Gm-100 μg/mL. For 0-hour samples, wells were aspirated, gently washed twice with PBS, lysed with sterile MilliQ water, and serial diluted to plate on LB agar supplemented with antibiotic for selection (wild type = Sm-50 μg/mL, Δ*artP* = Sm-50 μg/mL, Cm-34 μg/mL). Remaining wells were aspirated, gently washed three times with PBS, and replenished with 200 μL of fresh media supplemented with Gm-10 μg/mL. Remaining

infected cells were incubated in TC incubator for six hours and were plated in the same manner as time zero cells.

Intramacrophage transcriptional reporter assay

RAW264.7 macrophages were maintained in RPMI media supplemented with 10% FBS. Macrophages were seeded into a tissue culture treated, black 96-well plate. Bacteria were washed with PBS, diluted to 5.0x10^7 cells/mL and opsonized with NHS for 30 minutes. 100 μL of cells were added to wells in triplicate. After spinning down cells and conducting a Gm protection assay, all wells were washed gently with PBS three times, and 200 μL of media was added. Luminescence was read at 450 nm using an EnVision plate reader for 0-hour samples and wells were washed twice with PBS before lysing with sterile MilliQ water. Bacteria were diluted and plated on LB-agar supplemented with Sm-50 μg/mL and Amp-100 μg/mL. Infected cells were incubated in TC incubator between time points. Cells were treated in the same manner for 12- and 24-hour time points. Promoter activity was normalized to bacterial burdens by dividing RLU by CFU.

Bone marrow intramacrophage assay

B6J and NOS2 knockout mice were sacrificed by cervical dislocation and BMMs were harvested from femur and tibia bones. Cells were maintained in RPMI media supplemented with 10% FBS, 10% L-Sup (L929 fibroblast conditioned medium) and 1% penicillin-streptomycin solution. On day 6, cells were exposed to ice cold PBS, scraped,

and seeded in a tissue culture treated, clear 96-well plate in RPMI supplemented with 10% FBS. Bacteria were grown overnight in LB with antibiotic selection at 37°C. Cells were washed with PBS and resuspended at a final concentration of 5.0x10^7 cells/mL in PBS, and 100 μL of bacteria was added to each well. After Gm protection assay, 0-hour wells were gently washed twice with PBS and lysed with sterile MilliQ water, and plated on LB-agar supplemented with antibiotic (wt = Sm-50 μg/mL, Δ*artP* = Sm-50 μg/mL, Cm-34 μg/mL). Media was replaced with 200 μL Gm-10 μg/mL and infected cells were stored in TC incubator between timepoints and lysed in the same manner.

Statistical analysis

Data were analyzed using GraphPad Prism Version 8.4.3 (GraphPad Inc.). Statistical tests and corrections are indicated in figure legends, and P values less than 0.05 were considered to be significant.

Figures and Figure Legends

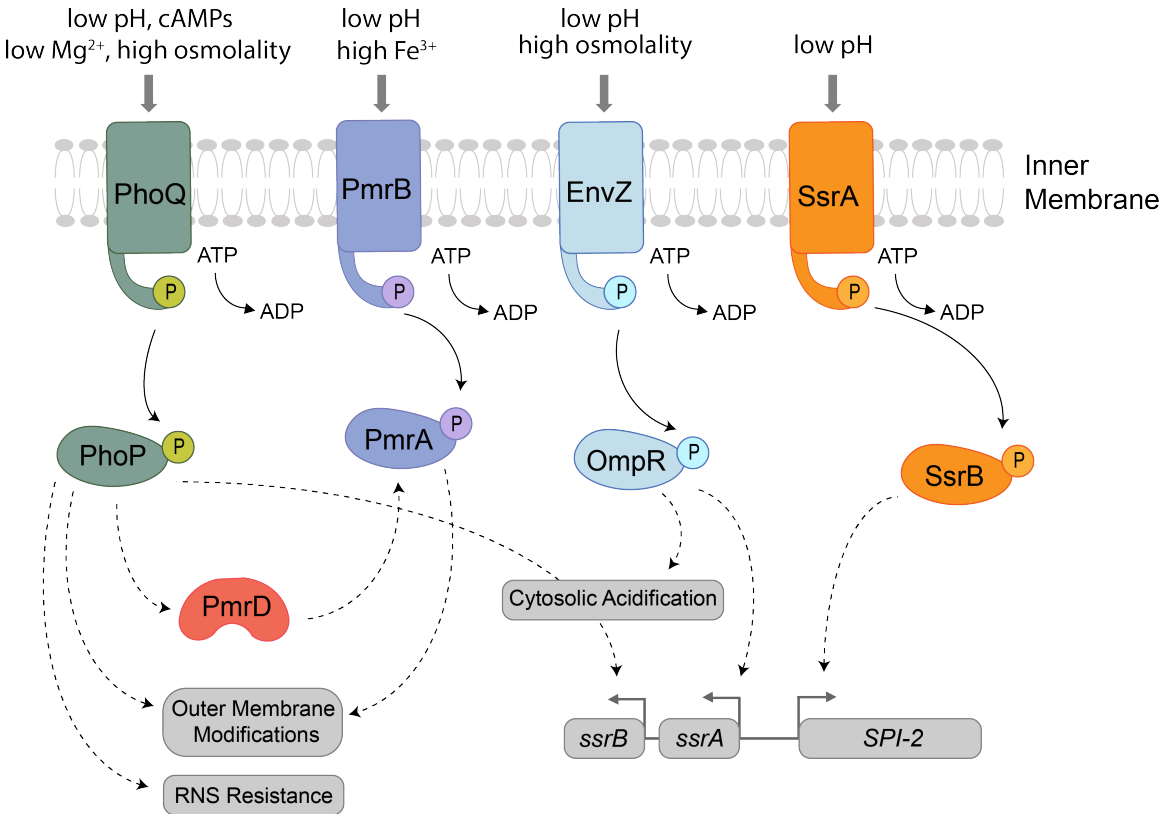

Figure 1. Intracellular-active two-component systems in *Salmonella* Typhimurium. Schematic depicting regulatory sensory systems PhoQ/PhoP, PmrA/PmrB, EnvZ/OmpR, and SsrA/SsrB under intracellular infectious conditions. Change in concentration of extracellular chemical cues induces autoactivation of membrane-bound sensor histidine kinases, which triggers a phosphorelay mechanism that activates the cognate response regulator of the two-component system. Activation and inter-system crosstalk elicit modifications of the outer membrane, RNS resistance, cytosolic acidification, and induction of *ssrA*, *ssrB*, and SPI-2 gene expression.

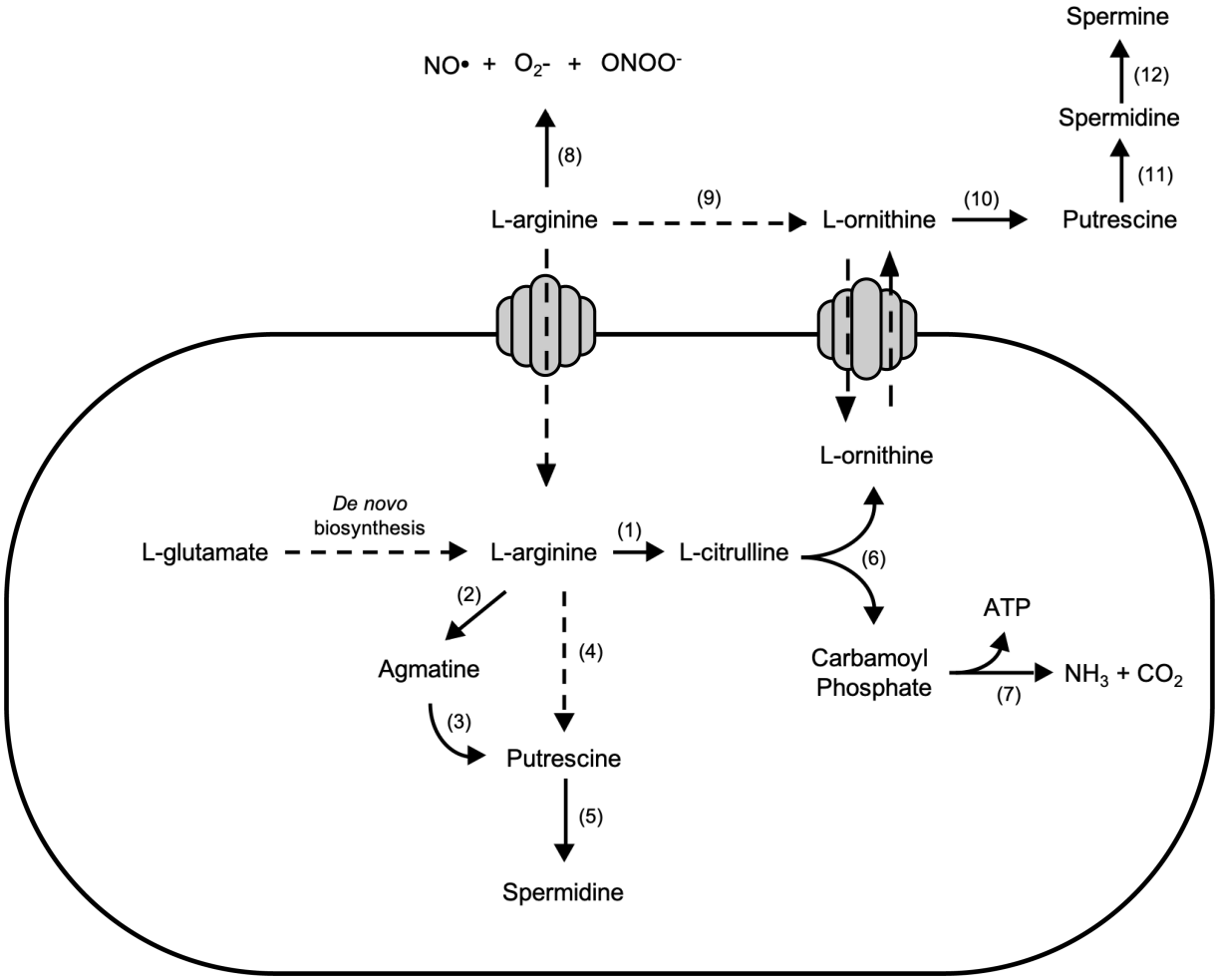

Figure 2. Bacterial and host processes governing arginine consumption during intracellular *Salmonella* infection. Schematic depicting arginine consumption in *S.* Typhimurium and host macrophage cells. Enzymes represented numerically (1-7 bacterial; 8-12 eukaryotic): (1) arginine deiminase; (2) biosynthetic arginine decarboxylase (SpeA)/ arginine decarboxylase (AdiA); (3) agmatinase (SpeB); (4) ornithine decarboxylase (SpeC/ SpeF); (5) spermidine N1-acetyltransferase (SpeG); (6) ornithine carbamoyltransferase; (7) carbamate kinase; (8) inducible nitric oxide synthase

(iNOS); (9) arginase 1/ arginase 2; (10) ornithine decarboxylase (11) spermidine synthase; (12) spermine synthase.

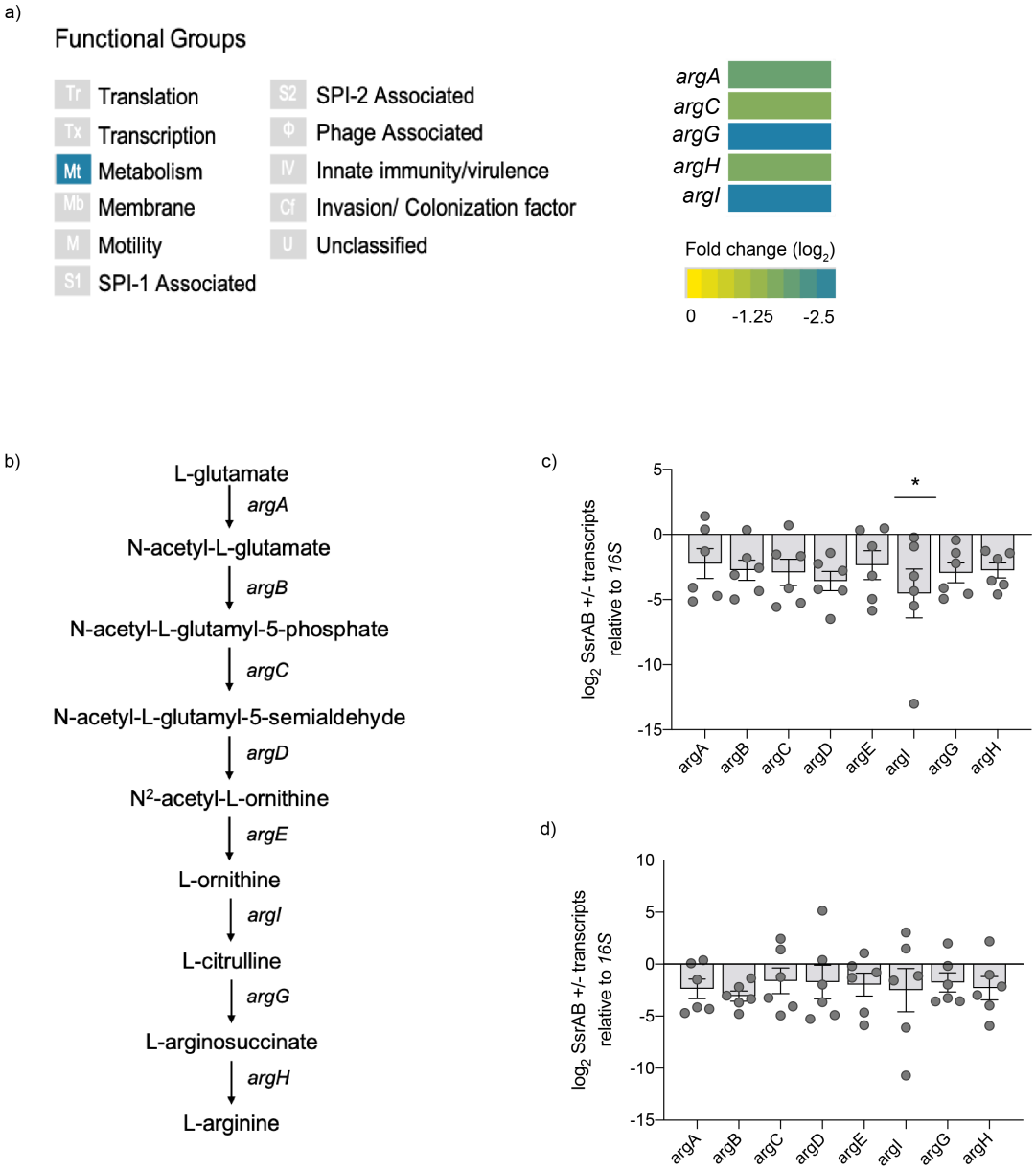

Figure 3. Arginine biosynthesis gene expression is repressed in the presence of SsrB under infection-mimicking conditions. a) Functional grouping of putative SsrB targets adapted from Ilyas et al. and heatmap of arginine biosynthesis genes identified within metabolism data set. Heatmap signifies $\log_2$ fold change in gene expression between

SsrB+ and SsrB- strains. b) *De novo* arginine biosynthesis pathway in *S*. Typhimurium. c) Expression of transcripts in SsrB+ relative to SsrB- quantified by RT-qPCR. Transcripts levels expressed as $\log_2$ fold change relative to *16S*. Bars signify the mean and error bars represent eh standard error of the mean. Groups were compared to a value of 0 by ordinary one-way ANOVA and corrected for multiple comparisons with Holm Sidak's test. *P<0.05. d) Expression of transcripts in wild type vs SsrB-.

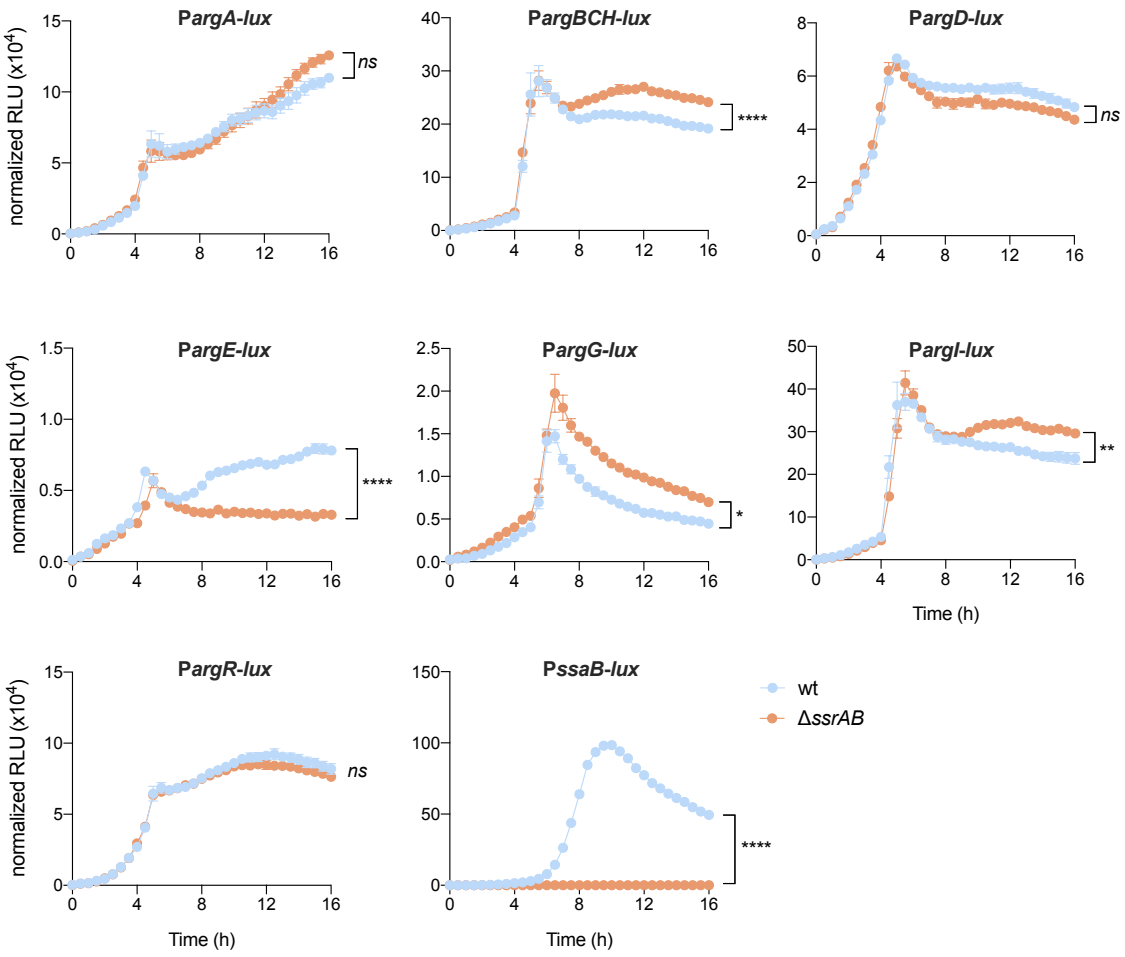

Figure 4. Biosynthesis gene promoter activity is dependent on growth state *in vitro*.

a) Promoter reporter assay of arginine biosynthesis genes in wild-type (wt) *S.* Typhimurium and Δ*ssrAB* backgrounds. Bacteria were subcultured in LPM pH 5.8 media and incubated in a double orbital shaking plate reader for 16 hours. Luminescence (RLU) and OD_{600} measurements were obtained every 30 minutes and RLU was normalized to bacterial growth by dividing by OD. Error bars represent the standard error of the mean.

Groups were compared by ordinary two-way ANOVA and corrected for multiple comparisons with Holm Sidak's test. **P<0.01, ***P<0.001, ****P<0.0001.

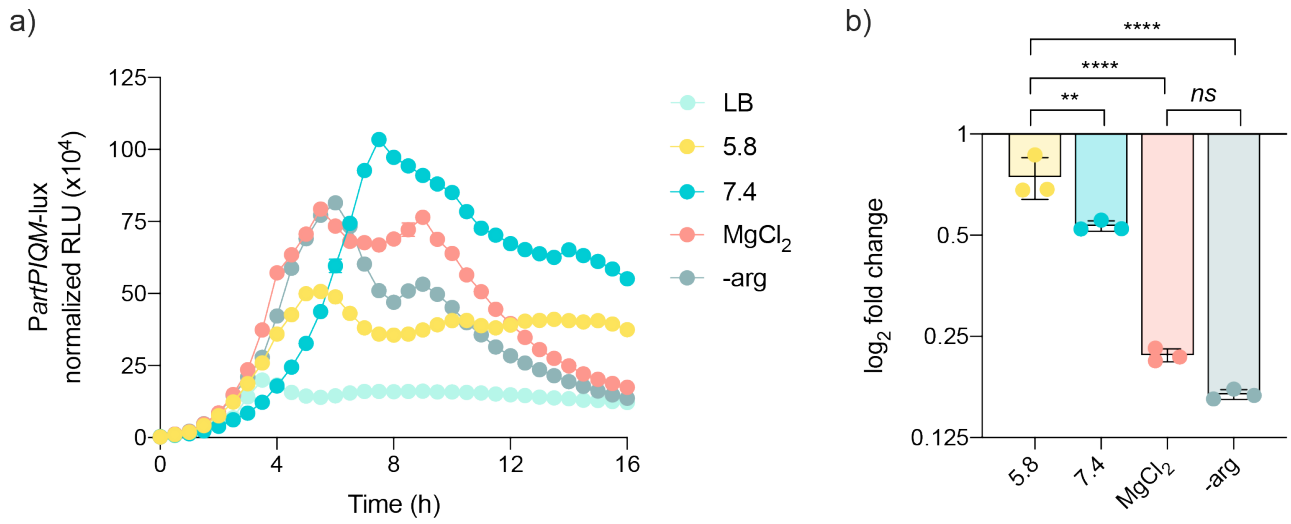

Figure 5. Promoter regulation of *artPIQM* operon is sensitive to infection-relevant environmental conditions. Transcriptional reporter assay for P*artPIQM-lux* in wild-type *S.* Typhimurium under variable media conditions. Bacteria were grown in LB or variations of LPM media: 5.8 = pH 5.8, 7.4 = pH 7.4, $MgCl_2$ = 10 mM supplementation, -arg = arginine omitted from media. a) Curves depicting promoter activity over time. Assay was conducted in technical quadruplicates and repeated three times. RLU and OD were monitored every 30 minutes for 16 hours. RLU was normalized to bacterial growth by dividing by OD measurements. Error bars represent standard error of the mean. b) Reduction of promoter activity after peak promoter activity signal in LPM media conditions represented by fold change in RLU normalized to OD. Dots represent average fold change per biological replicate, bars represent the mean, and error bars represent standard deviation. Groups were compared by ordinary one-way ANOVA and corrected

for multiple comparisons with Holm-Sidak's test. Significance not shown on graph: 7.4 vs $MgCl_2$***, 7.4 vs -arg***. **P<0.01, ***P<0.001, ****P,0.0001.

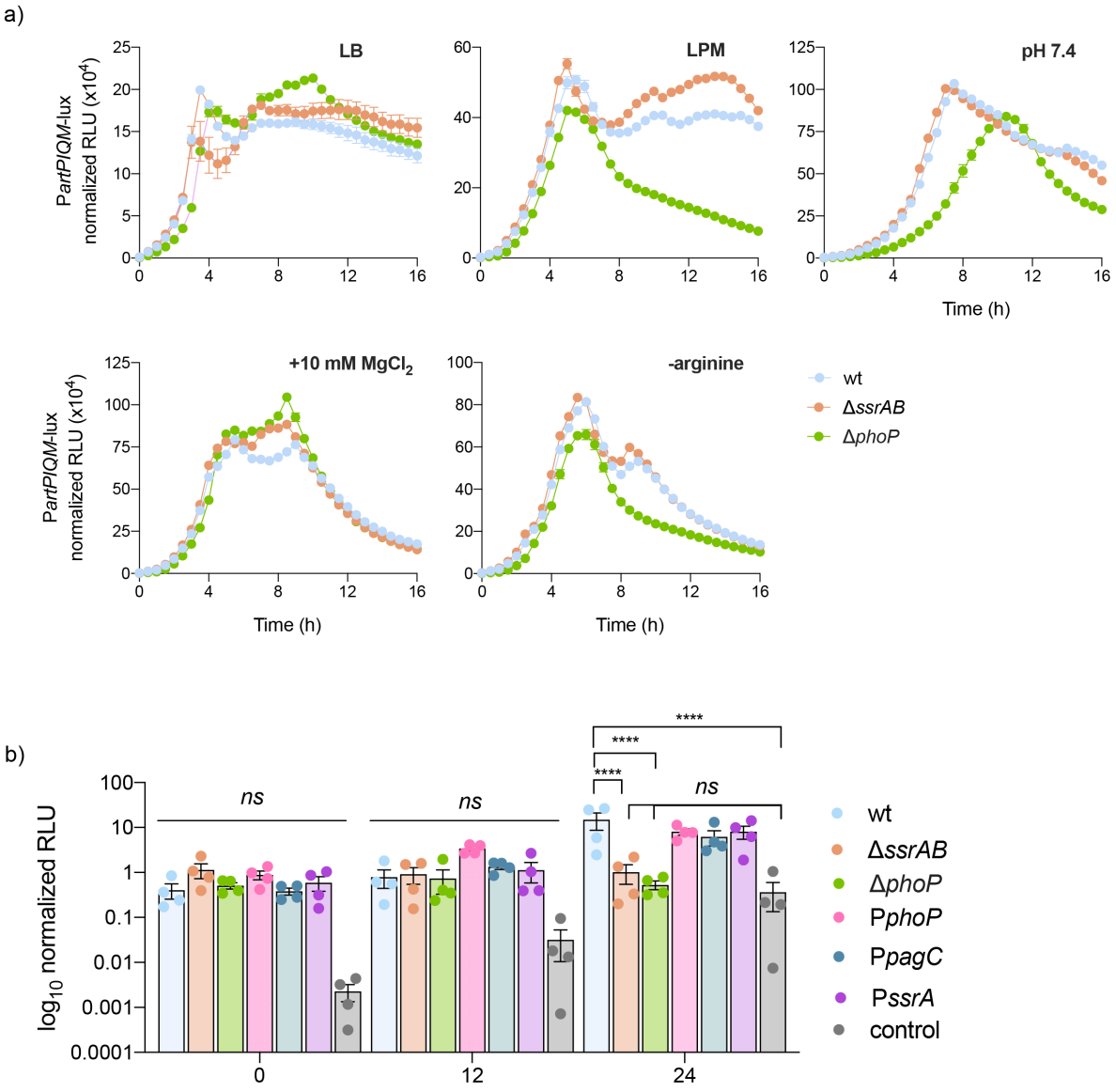

Figure 6. The *artPIQM* operon promoter is positively regulated by PhoP *in vitro.* a) Transcriptional reporter assay of P*artPIQM-lux* in wild-type (wt) *S.* Typhimurium, Δ*ssrAB,* and Δ*phoP* backgrounds. Bacteria were grown in LB, standard LPM (pH 5.8), or modified LPM. Samples were prepared in technical quadruplicates and assay was repeated three times. Luminescence (RLU) and OD were monitored every 30 minutes for

16 hours and RLU was normalized to bacterial growth. Error bars represent the standard error of the mean. b) Intramacrophage transcriptional reporter assay normalized to CFUs. RAW264.7 macrophages were infected with bacteria and RLU was monitored over 24 hours at 0, 12, and 24 hours, and normalized to bacterial burdens. Wt, Δ*ssrAB*, and Δ*phoP* carried P*artPIQM-lux* construct; all other promoter-reporters were monitored in wt background; control = wt, no luciferase plasmid. Dots represent average RLU/CFU of technical triplicates. Bars represent the mean and error bars represent the standard error of the mean. Groups were compared by two-way ANOVA and corrected for multiple comparisons with Tukey's test. Significance not shown on graph: wt vs P*pagC***; Δ*ssrAB* vs P*phoP**, P*ssrA**; Δ*phoP* vs P*phoP**, P*ssrA**; control vs P*phoP**, P*ssrA**. *P<0.05, **P<0.01, ***P<0.0001.

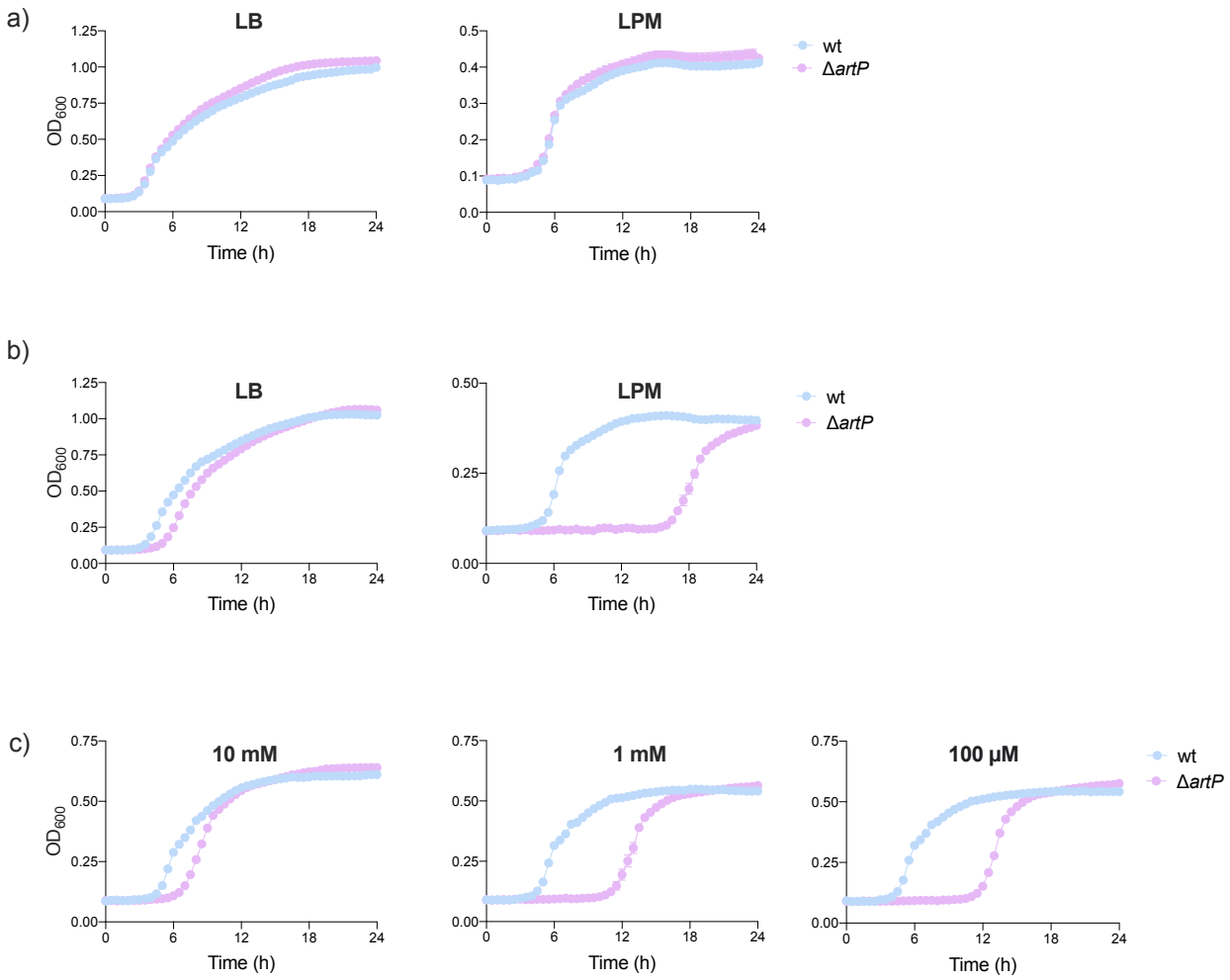

Figure 7. Stress tolerance of Δ*artP* is influenced by Mg^{2+} concentration under infection-mimicking conditions. Growth curves for wild-type (wt) *S.* Typhimurium and Δ*artP* strains in LB or low phosphate, low Mg^{2+} (LPM) media buffered to a pH of 5.8 quantified by OD measurements. Curves were conducted in 6 technical replicates and repeated twice. Bars represent standard error of the mean. a) No antibiotic selection. b) With antibiotic selection. c) With antibiotic selection in LPM media supplemented with $MgCl_2$ at varying concentrations.

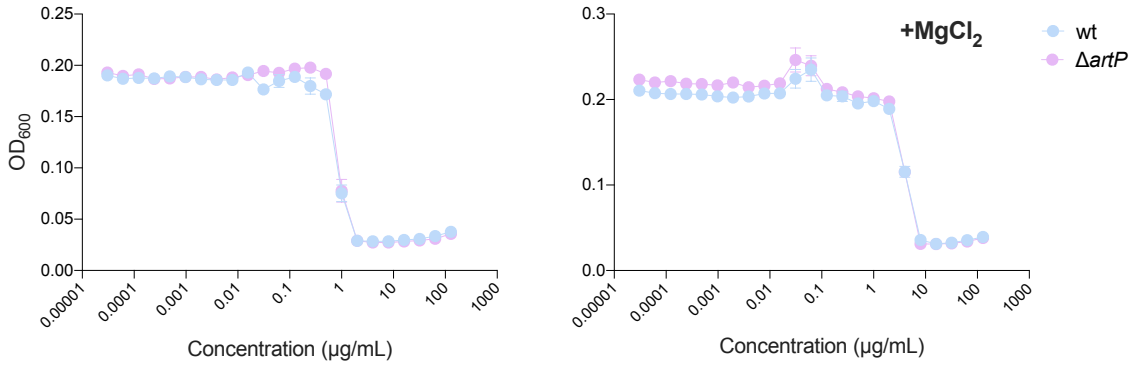

Figure 8. Inhibition of arginine transport does not impact membrane stability. MIC curves for wild-type (wt) *S.* Typhimurium and Δ*artP* treated with Rif. Strains were subcultured in standard, acidic LPM media or LPM supplemented with 10 mM $MgCl_2$. Bacterial growth was quantified by OD measured at 600 nm across an antibiotic concentration range of 0-128 µg/mL at 18 hours, normalized to growth at 0 hours. Bars represent the standard error of the mean.

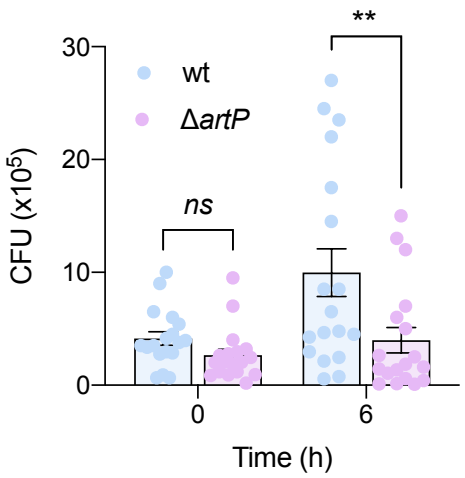

Figure 9. Inhibition of arginine transport attenuates *Salmonella* for intracellular survival in RAW264.7 macrophages. Intramacrophage replication assay comparing wild-type (wt) *S.* Typhimurium and Δ*artP* fitness. Bacterial burdens were enumerated at 0- and 6-hours following infection, as represented by CFUs. Dots represent technical replicates (n = 6) across three biological replicates. Groups were compared by two-way ANOVA and corrected for multiple comparisons with Holm Sidak's test. **P<0.01.

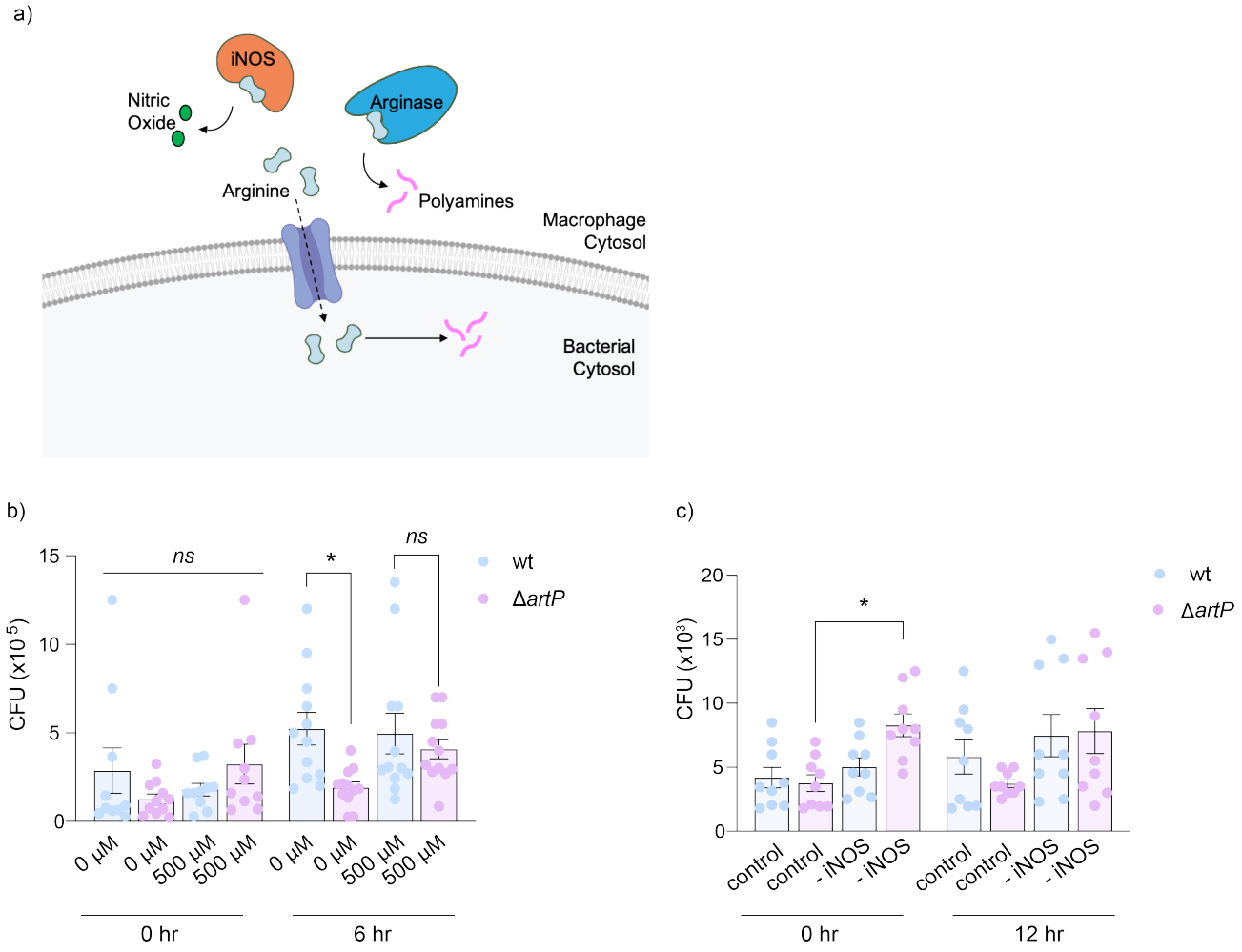

Figure 10. Intracellular fitness is impacted by nitrosative species in arginine transport mutant. a) Schematic of host arginine utilization during *Salmonella* infection depicting arginine catabolism in host macrophages and bacteria. b) Intramacrophage replication assay in RAW264.7 macrophages quantifying intracellular fitness of wild-type (wt) *S.* Typhimurium vs Δ*artP* strains treated with L-NIL. Bacterial burdens were enumerated and plotted as CFUs and 0 and 6 hours. Dots represent technical replicates across three biological replicates, bars represent the mean, and error bars represent the

standard error of the mean. Groups were compared by ordinary one-way ANOVA and corrected for multiple comparisons with Holm-Sidak's test. *P<0.05. c) Intramacrophage replication assay in BMMs harvested from B6J (control) or NOS2 knockout ($NOS2^-$) mice. Bacterial burdens were enumerated at 0 and 12 hours. Dots represent each technical replicate (n = 3) for three biological replicates, bars represent the mean, and error bars represent the standard error of the mean. Groups were compared by two-way ANOVA and corrected for multiple comparisons with Holm-Sidak's test. *P<0.05.

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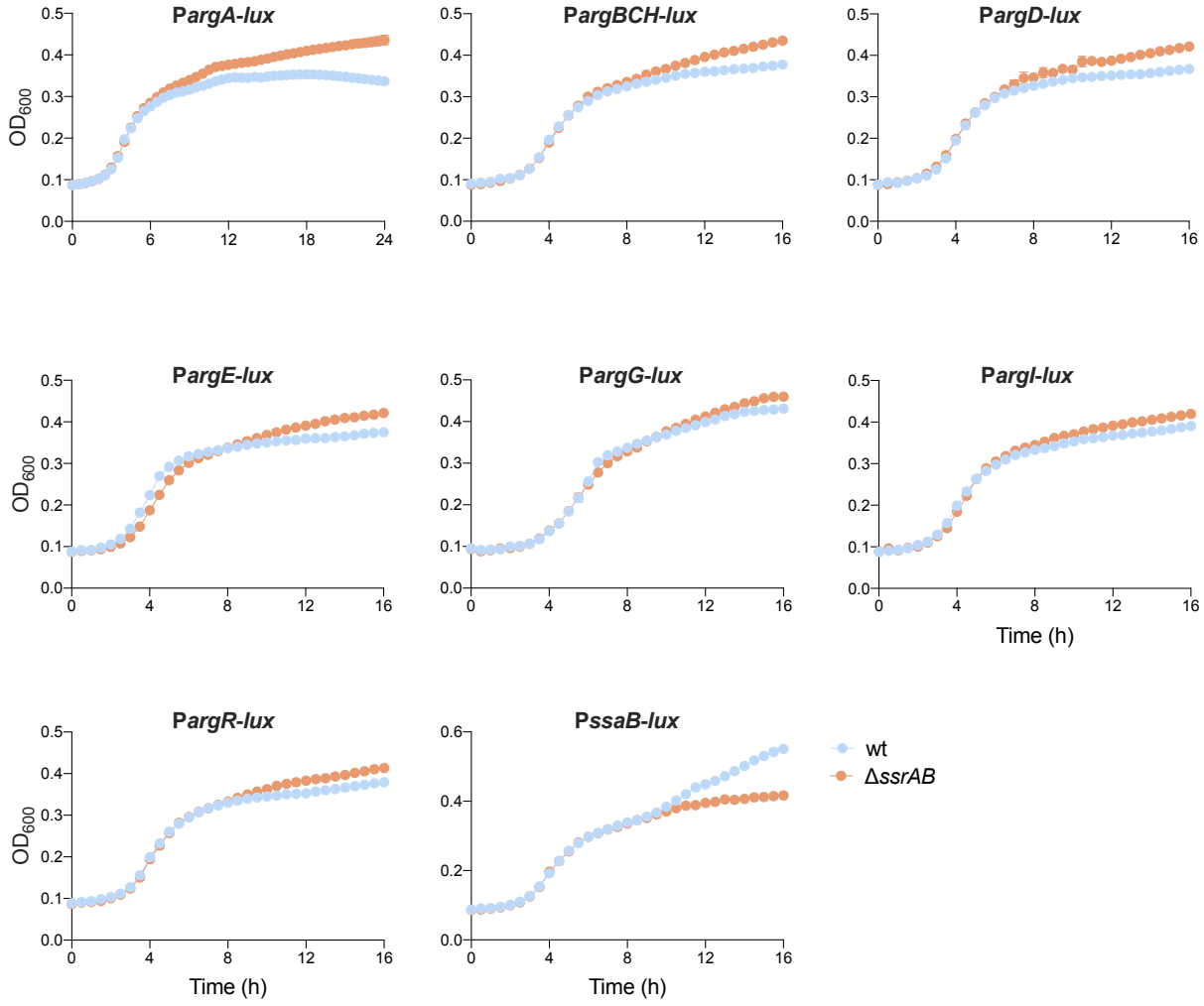
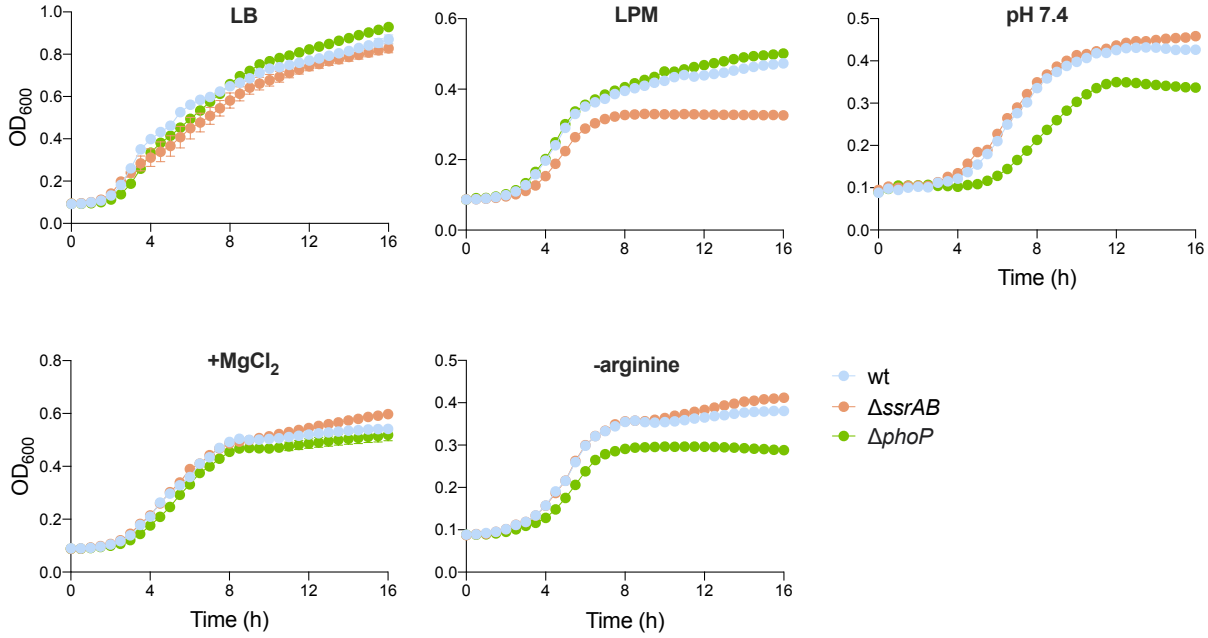
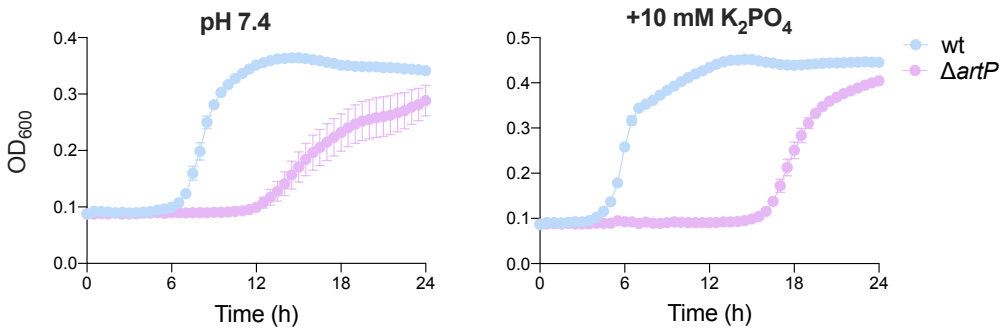
Appendix



Supplementary Figure 1. Growth curves for promoter-reporter strains carrying luciferase fusion constructs for biosynthesis genes. Bacterial growth of wild-type (wt) *S.* Typhimurium and Δ*ssrAB* during promoter-reporter assay in Figure 4. OD was monitored every 30 minutes for 16 hours. Error bars represent the standard error of the mean.



Supplementary Figure 2. Growth curves for promoter-reporter strains carrying luciferase fusion for P*artPIQM*. Bacterial growth of wild-type (wt) *S.* Typhimurium, Δ*ssrAB*, and Δ*phoP* during promoter-reporter assay in LPM media presented in Figure 6a. Strains were grown in LB, acidic LPM media (LPM), or modified LPM media. OD readings were obtained every 30 minutes for 16 hours. Error bars represent the standard error of the mean.



Supplementary Figure 3. Growth curves of wild type and Δ*artP* in modified LPM media. Bacterial growth in LPM media buffered to pH 7.4 or supplemented with excess phosphate. OD was monitored every 30 minutes for 24 hours. Growth is represented as an average from six technical replicates across two biological samples. Error bar represent the standard error of the mean.

Table 1. Alignment of PhoP binding sites in known targets of PhoQ/PhoP.

Gene	Sequence	% identity
artPIQM	GATATATTTAACTTTTA	
phoP	GGTTTATTAACTGTTTA	65.517
mgtA	GGTTTATCGTTGGTTTA	52.941
pmrD	TATTGCCGTTTTGTTTA	41.176

Table 2. Bacterial strains used in this study.

Strain	Plasmid	Resistance	Source
wt SL1344	NA	Streptomycin	BKC collection
Δ*ssrAB* unmarked	NA	Streptomycin	BKC collection
Δ*phoP* marked	NA	Streptomycin, Chloramphenicol	BKC collection
wt +pGEN-P*argA-lux*	pGEN-*luxCDABE*	Streptomycin, Ampicillin	This work
wt +pGEN-P*argBCH-lux*	pGEN-*luxCDABE*	Streptomycin, Ampicillin	This work
wt +pGEN-P*argD-lux*	pGEN-*luxCDABE*	Streptomycin, Ampicillin	This work
wt +pGEN-P*argE-lux*	pGEN-*luxCDABE*	Streptomycin, Ampicillin	This work
wt +pGEN-P*argG-lux*	pGEN-*luxCDABE*	Streptomycin, Ampicillin	This work
wt +pGEN-P*argI-lux*	pGEN-*luxCDABE*	Streptomycin, Ampicillin	BKC collection
wt +pGEN-P*argR-lux*	pGEN-*luxCDABE*	Streptomycin, Ampicillin	This work
Δ*ssrAB* +pGEN-P*argA-lux*	pGEN-*luxCDABE*	Streptomycin, Ampicillin	This work
Δ*ssrAB* +pGEN-P*argBCH-lux*	pGEN-*luxCDABE*	Streptomycin, Ampicillin	This work
Δ*ssrAB* +pGEN-P*argD-lux*	pGEN-*luxCDABE*	Streptomycin, Ampicillin	This work
Δ*ssrAB* +pGEN-P*argE-lux*	pGEN-*luxCDABE*	Streptomycin, Ampicillin	This work
Δ*ssrAB* +pGEN-P*argG-lux*	pGEN-*luxCDABE*	Streptomycin, Ampicillin	This work
Δ*ssrAB* +pGEN-P*argI-lux*	pGEN-*luxCDABE*	Streptomycin, Ampicillin	This work
Δ*ssrAB* +pGEN-P*argR-lux*	pGEN-*luxCDABE*	Streptomycin, Ampicillin	This work
wt +pGEN-P*ssaB-lux*	pGEN-*luxCDABE*	Streptomycin, Ampicillin	BKC collection
Δ*ssrAB* +pGEN-P*ssaB-lux*	pGEN-*luxCDABE*	Streptomycin, Ampicillin	BKC collection
Δ*artP* marked	NA	Streptomycin, Chloramphenicol	This work

wt +pGEN-P*artPIQM-lux*	pGEN-*luxCDABE*	Streptomycin, Ampicillin	This work
Δ*ssrAB* +pGEN-P*artPIQM-lux*	pGEN-*luxCDABE*	Streptomycin, Ampicillin	This work
Δ*phoP* +pGEN-P*artPIQM-lux*	pGEN-*luxCDABE*	Streptomycin, Ampicillin	This work
wt +pWSK-empty	pWSK129	Streptomycin, Kanamycin	BKC collection
Δ*ssrAB* +pWSK-empty	pWSK129	Streptomycin, Kanamycin	BKC collection
Δ*ssrAB* +pWSK-*ssrA-ssrBD56E*	pWSK129	Streptomycin, Kanamycin	BKC collection
wt +pGEN-P*ssrA-lux*	pGEN-*luxCDABE*	Streptomycin, Ampicillin	BKC collection
wt +pGEN-P*pagC-lux*	pGEN-*luxCDABE*	Streptomycin, Ampicillin	BKC collection
wt +pGEN-P*phoP-lux*	pGEN-*luxCDABE*	Streptomycin, Ampicillin	BKC collection

Table 3. Primers used in this study.

Primer	Sequence	Description
JP2-38	gcgattacaactccggaacc	*argA* RT-qPCR forward
JP2-39	aatatcgttgatggtggcgc	*argA* RT-qPCR reverse
JP2-50	aacaaaactctgctggcctg	*argB* RT-qPCR forward
JP2-51	ctgttacgccaatggagctg	*argB* RT-qPCR reverse
JP2-52	cattacctgccgcctgaaag	*argC* RT-qPCR forward
JP2-53	caagttgtcttcggttgcca	*argC* RT-qPCR reverse
JP2-40	tgatgaagtgcagtgtggga	*argD* RT-qPCR forward
JP2-41	attgccgccataggttgaac	*argD* RT-qPCR reverse
JP2-42	aatggactgcttaacgacgc	*argE* RT-qPCR forward
JP2-43	gcataaacggcgcttcagta	*argE* RT-qPCR reverse
JP1-69	atggtcgtcagctgggtaaa	*argG* RT-qPCR forward
JP1-70	tttctccatggtcagacgct	*argG* RT-qPCR reverse
JP1-65	tattgtcggttctgtggcct	*argH* RT-qPCR forward
JP1-66	acccactttgtcgatgagc	*argH* RT-qPCR reverse
JP1-55	tttgggtctctatgggcgaa	*argI* RT-qPCR forward
JP1-56	acttccatcccgccatgtaa	*argI* RT-qPCR reverse
CT5-19	aggccttcgggttgtaaagt	*16S* RT-qPCR forward
CT5-20	gactcaagcctgccagtttc	*16S* RT-qPCR reverse
JP2-72	gggggtaccctgcgcgccaatgcc	pGEN-P*argA-luxCDABE* forward

JP2-73	gggtacgtatgcgcgccccactag	pGEN-P*argA-luxCDABE* reverse
JP2-74	gggggtacctgtagagcggctttt	pGEN-P*argBCH-luxCDABE* forward
JP2-75	gggtacgtacgggttcctttatgc	pGEN-P*arBCH-luxCDABE* reverse
JP2-76	gggcccgggctgtgttttcagcggg	pGEN-P*argD-luxCDABE* forward
JP2-77	gggcccggggagatttccttaaag	pGEN-P*argD-luxCDABE* reverse
JP2-78	gggcccgggactccgccagaccgt	pGEN-P*argE-luxCDABE* forward
JP2-79	gggcccgggagaatagaaccctgc	pGEN-P*arE-luxCDABE* reverse
JP2-66	gggtacgtagcgggggccggattt	pGEN-P*argG-luxCDABE* forward
JP2-67	gggtacgtaagaatagaaccctgc	pGEN-P*arG-luxCDABE* reverse
JP2-82	gggggtaccttctgaacctgaagg	pGEN-P*argR-luxCDABE* forward
JP2-83	gggtacgtaaagtcacccgtaata	pGEN-P*argR-luxCDABE* reverse
JP4-2	gggggatccggagcaccgccagttgatga	pGEN-P*artPIQM-luxCDABE* reverse
JP3-80	gggtacgtatgacactcgtatacaggcag	pGEN-P*artPIQM-luxCDABE* forward
JP3-57	ttctgcgaaaggtactatctgcggcttcaatagctatcagactgcc tgtatacgagtgtcaatgagtattcaattaaacggcattaattggt gtaggctggagctgcttc	Δ*artP* forward lambda red
JP3-58	aggctaaagcctgcaattagcgcggcaatcagaacttttttcattgt cattgtcccgaatcttagtgagagagatagtttttgaacgcttcatat gaatatcctccttag	Δ*artP* reverse lambda red
JP3-59	gatatatttaacttttaacgcataa	Δ*artP* forward sequencing
JP3-60	tcggtcgcaaaacgaatggtctggg	Δ*artP* reverse sequencing