PERIPLASMIC DETERMINANTS OF VIRULENCE IN *SALMONELLA ENTERICA*

BY

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DISSERTATION

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Abstract

Salmonella enterica serovar Typhimurium is a facultative intracellular pathogen that is capable of causing systemic infection in mammals. Its normal course of infection brings this organism into the diverse environments of the stomach, the small intestine, the large intestine, and in the case of susceptible hosts, the phagosomal compartments of macrophages. Not only does *S. typhimurium* induce the expression of myriad virulence factors in order to successfully establish an infection, this organism must adjust its metabolism to the changing conditions present in the host in order to grow. *S. typhimurium* has a flexible respiratory chain that is capable of utilizing numerous terminal electron acceptors. I present evidence that although S. typhimurium passes through the microaerobic, shifting to anaerobic, conditions of the intestine, and it is capable of respiring anaerobically, it only grows in the presence of oxygen in the mouse host. When *Salmonella* passes from the gut to the systemic environment, it encounters oxidative stress brought on by the respiratory burst of phagocytes. Superoxide is the reactive oxygen species generated in macrophages in response to phagocytosis. *S. typhimurium* encodes a superoxide dismutase, SodCI, that is important for resistance to phagocytic superoxide. Currently it is not known how phagocytic superoxide kills or damages microorganisms taken up by macrophages. However, I have shown that phagocytic superoxide does not damage the DNA of *S. typhimurium*, and that SodCI protects an extracytoplasmic target from this exogenous superoxide. Superoxide can only directly damage a class of enzymes with a solvent exposed [4Fe-4S] cluster, and this type

of enzyme is not known to be transported out of the cytoplasm. However, if an enzyme of this type were to be exported, it would be transported via the Twin Arginine Transport (Tat) system, which translocates folded proteins into the periplasm. This transport system is important for virulence in mice, but it is not needed for most growth conditions in the laboratory. I found that SodCI does not protect a Tat substrate, but I did find that strains with mutations that inactivate this transport system are attenuated because of mislocalization of three proteins involved in cell septation: AmiA, AmiC, and SufI.

Table of Contents

Chapter 1: Introduction

1.1 Overview of *Salmonella*

Salmonella are widespread members of the γ-Proteobacter Enterobacteriaceae that are estimated to cause 1.4 million cases of food borne illness annually in the United States (268), and *Salmonella enterica* serovar Typhimurium is one of the most commonly recovered serovars. In humans, infection with *Salmonella enterica* serovar Typhimurium usually presents as a self-limiting gastroenteritis, but in children, the elderly or immunocompromised individuals, more serious extra-intestinal infections are commonly observed. *Salmonella enterica* serovar Typhimurium, commonly referred to as *S. typhimurium*, is a facultative intracellular pathogen that replicates within macrophages.

1.2 Course of infection

Infection is normally initiated through the oral consumption of contaminated food or water (134). Bacteria must survive the acidic environment of the stomach and travel to the small intestine to begin colonization of epithelial cells and Peyer's patches (58). Colonization of Peyer's patches occurs through invasion of the overlying M cells (205), whose function is to sample lumenal antigens for presentation to lymphocytes in the mucosal associated lymphatic tissue (200), and through invasion of non-phagocytic epithelial cells. This process requires the type three secretion system (T3SS) encoded on *Salmonella* pathogenicity island 1 (SPI1) (278). SPI1 encodes all of the proteins of the needle complex as well as several regulators and secreted effectors (88). The secreted

effector proteins delivered into host cells by the SPI1 T3SS cause membrane ruffling and actin rearrangements that cause the bacterium to be engulfed (203, 414).

S. typhimurium gains access to Peyer's patches, where it replicates and eventually destroys the tissue. This is a SPI1 mediated action (69, 205) and is important for systemic dissemination. In caspase-1 deficient mice *S. typhimurium* does not destroy Peyer's patches and has a greatly increased LD_{50} (279). *S. typhimurium* gains access to the $CD18^{+}$ phagocytes, including macrophages, that underlie Peyer's patches. This leads to dissemination to the spleen, liver, and bone marrow (58, 389). *S. typhimurium* replicates in the macrophages of these organs (57, 75, 328, 335), and this ability to survive and replicate in macrophages is required for *S. typhimurium* to cause systemic infection (46, 131). Survival in macrophages requires the type three secretion system encoded on *Salmonella* pathogenicity island 2 (185, 349) and several additional virulence regulons€ (127, 210, 244).

1.3 Macrophage response to bacteria

Macrophages are normally an inhospitable environment for bacterial growth. After engulfing a bacterium in a phagosome, the compartment becomes acidified and fuses with vesicles that deliver degradative enzymes, cationic antimicrobial peptides, defensins, and the enzyme complexes that produce reactive oxygen and nitrogen species (148, 201, 239). A highly regulated series of events causes the phagosome to mature into a degradative compartment (29). Initially the membrane of the phagosome contains receptors and membrane proteins that resemble the cell membrane of the phagocyte. A progression of regulatory proteins are recruited to and are directed away from the

phagosome membrane, causing the phagosome to fuse with vesicles of the endocytic pathway (227, 393). Early endosomes, then late endosomes, and ultimately lysosomes fuse with the phagosome to form a phagolysosome, in which the bacteria are digested (99, 378). These endocytic vesicles can be distinguished by molecular markers present in their membranes such as the Rab family of GTPases (378). Characteristic markers include Rab5 for early endosomes; Rab7, Rab9, and mannose-6-phosphate receptor for late endosomes; and LAMP-1 and LAMP-2 for phagolysosomes. Rab proteins play a role in membrane targeting specificity (411). Endosomes that contain non-functional Rab proteins fail to fuse with other vesicles (65, 129, 142, 286, 358). Mannose-6-phosphate receptor binds to lysosomal hydrolases and delivers them to late endosomes (101, 168). In model systems, phagosomes rapidly mature from early phagosomes to phagolysosomes (92).

Several enzyme complexes are also delivered to maturing phagosomes. Vacuolar ATPase (v-ATPase) is recruited to the phagosome and is responsible for vacuole acidification by pumping protons into this space (255, 290, 370, 371). Acidification is important for proper activity of lysosomal acid hydrolases (410), and artificially raising the pH of phagosomes has been shown to interfere with fusion with late endosomes (71) and lysosomes (393). Phagocytes lacking a fully functional v-ATPase fail to degrade engulfed bacteria (371). Enzyme complexes that generate reactive oxygen and nitrogen species in the phagosome are also delivered. The components of the NADPH oxidase complex are recruited to the maturing phagosome membrane and expose bacteria to superoxide and hydrogen peroxide (11, 70, 94, 95). Generation of superoxide is an important antimicrobial function of phagocytes (11, 280). In humans, lack of NADPH

oxidase activity due to mutation of one of the subunits results in chronic granulomatous disease, which causes individuals to be highly susceptible to infections, including Salmonellosis (357). Inducible nitric oxide synthase creates nitric oxide from L-arginine (291). Nitric oxide alone is not very toxic (44), but when combined with superoxide it forms highly bactericidal peroxynitrite (218, 415).

1.4 *Salmonella* in macrophages

In contrast, several intracellular bacterial pathogens, including *Salmonella*, can modify the macrophage vacuole following phagocytosis (1). The unique compartment that *S. typhimurium* creates is referred to as a *Salmonella*-containing vacuole (SCV). SCVs interact with early endosomes but avoid many components of late endosomes and lysosomes (146, 150, 317, 363). *S. typhimurium* alters the Rab proteins present on the surface of the SCV (179, 285, 355, 356), and it has been demonstrated that SPI-2 secreted effectors (see below) can interfere with Rab/Rab-interacting protein interactions to prevent fusion with lysosomes (178, 261). This unique compartment is characterized by acidification to a pH less than 5.0 (112, 318) and some membrane markers common to late endosomes and lysosomes (317), but evidence from several groups indicates that the *Salmonella*-containing vacuole is reduced in acquired lysosomal contents, such as acid hydrolases and cathepsins (47, 150, 179, 385). The mature SCV has been shown to form tubular membrane projections called *Salmonella*-induced filaments that are required for bacterial growth (147). Recruitment of NADPH oxidase and iNOS to the SCV are also altered (61, 144, 391). However, studies with NADPH oxidase and iNOS deficient mice

demonstrated that these enzymes are still important in controlling the growth of *S. typhimurium in vivo* (264, 274, 350, 390).

Salmonella produces virulence factors in order to create this specialized compartment within macrophages (196), and it alters the regulation of numerous genes to survive and replicate (122, 165). The effectors of the type III secretion system encoded on pathogenicity island 2 (SPI2) are incompletely characterized, but SPI2 is known to be required for survival in macrophages (68, 183). Three effectors, SifA, SifB and SseJ, localize to the membrane of the *Salmonella*-containing vacuole (270). SifA promotes *Salmonella*-induced filaments by associating with host cell microtubules and is involved in maintaining the phagosomal membrane (31, 43, 364). It interacts with Rab7, preventing association with lysosomes (178). SifB and SseJ associate with *Salmonella*-induced filaments, but their function in virulence is not clear (138). SspH2 and SseI localize to the actin cytoskeleton of the host cell, presumably to alter actin associated with the phagosome (270). SpiC is an effector encoded on SPI2 that interferes with endosome fusion with the SCV by targeting a mammalian regulatory protein (351, 385). It has been proposed that *S. typhimurium* is able to interfere with the timely localization of NADPH oxidase and iNOS to the phagosomal membrane in a SPI2 dependent manner (61, 144, 391), but the effectors which might be involved are not currently known.

The SPI2 type III secretion system (TTSS) is controlled by several regulators. SsrAB is encoded on SPI2 (68) and induces production of the TTS needle complex at low pH (93, 271). Expression of *ssrAB* is in turn positively regulated by OmpR (235). SlyA is a positive regulator of SPI2 type III secretion that responds to low osmolarity like

OmpR. Null mutants of *slyA* secrete decreased levels of known SPI2 effectors and are impaired in formation of *Salmonella*-induced filaments (244).

The PhoPQ two-component regulatory system is required for virulence in macrophages and mice (166, 276). It responds to low Mg^{++} concentration (145), to low pH (4, 262), and to antimicrobial peptides found in the intracellular environment (12, 13). PhoPQ activates expression of another two-component regulator, PmrAB (172). PmrAB regulates a set of genes that confers resistance to antimicrobial peptides (173). This involves modification of lipid A, resulting in more positively charged LPS that is presumably less reactive with cationic peptides (167, 171). It has also been suggested that PhoPQ plays a role in the formation of the SCV because *phoP* null mutants reside in phagosomes that do not exclude late endosomal and lysosomal markers normally excluded by wild type *S. typhimurium* (150).

1.5 Oxidative stress

Reactive oxygen species cause characteristic damage to biological molecules, and aerobic organisms have multiple layers of regulation to adapt to and defend themselves from this process. Generation of reactive oxygen species as a byproduct of aerobic growth is a constant occurrence that all aerobic organisms must manage (365). Respiratory chain dehydrogenases contain flavins that can participate in single electron transfers to oxygen. The resulting superoxide undergoes spontaneous or enzyme-mediated dismutation to hydrogen peroxide. In *E. coli*, the primary metabolic source of hydrogen peroxide is not currently known, but evidence suggests it is not a respiratory

chain enzyme (347). Aerobic organisms produce enzymes that scavenge reactive oxygen species:

$O_2^{\cdot -} + O_2^{\cdot -} + 2\ H^+ \rightarrow H_2O_2 + O_2$	superoxide dismutase
$H_2O_2 + H_2O_2 \rightarrow O_2 + 2\ H_2O$	catalase
$H^+ + NADH + H_2O_2 \rightarrow NAD^+ + 2\ H_2O$	alkylhydroperoxide reductase

1.5.1 Damage caused by reactive oxygen species

The dangers that hydrogen peroxide and superoxide pose to organisms is illustrated by work done with mutants unable to detoxify these substances. *E. coli* mutants lacking cytosolic superoxide dismutase are unable to synthesize branched chain, aromatic, or sulfur-containing amino acids, and they grow poorly on nonfermentable carbon sources (55, 192). The reason for sulfur-containing amino acid auxotrophy is still unresolved, but it has been observed that sulfite is found in the growth medium of cytosolic SOD mutants, suggesting that sulfur metabolism is affected (20, 22, 260). A mechanism to account for branch chain amino acid auxotrophy and the inability to grow on nonfermentable carbon sources in SOD mutants has been proposed. Superoxide can damage enzymes which contain a [4Fe-4S] cluster that is involved in dehydratase reactions (135). Dihydroxy acid dehydratase, an intermediate in the branched chain amino acid biosynthetic pathway, 6-phosphogluconate dehydratase in the Entner-Doudoroff pathway, and aconitase and fumarase of the tricarboxylic acid pathway are examples of this type of enzyme. The damage that results in aromatic amino acid auxotrophy is less straightforward. In this case, superoxide oxidizes the 1,2-dihydroxyethyl thiamine pyrophosphate intermediate of the transketolase reaction. Since

erythrose-4-P is required for aromatic amino acid synthesis, damage to the transketolase intermediate causes aromatic amino acid auxotrophy (21).

Another observed phenotype of cytosolic SOD deficient *E. coli* strains is increased DNA damage (125). However this is not due to a direct effect of superoxide. Superoxide does damage the labile [4Fe-4S] cluster of dehydratases, and this results in the release of the solvent-exposed iron atom. This increase in intracellular free iron is associated with the observed increase in mutant frequency of SOD mutants (213). When reduced iron reacts with hydrogen peroxide, hydroxyl radicals are produced via the Fenton reaction:

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\cdot}OH$ Fenton reaction

Hydroxyl radicals can oxidize most biological molecules, and when they are formed near DNA they are able to cause damage to the bases, damage to deoxyribose, and damage to the sugar-phosphate bonds, causing strand breaks (191, 194, 313).

1.5.2 Superoxide dismutases

S. typhimurium has four superoxide dismutases. Three, *sodA*, *sodB*, and *sodCII*, are chromosomally encoded orthologs of the *E. coli* superoxide dismutases *sodA*, *sodB*, and *sodC*. The fourth, *sodCI*, is a paralog of *sodCII*, encoded on the Gifsy2 prophage (123, 132). All superoxide dismutases convert superoxide to hydrogen peroxide and molecular oxygen, but these enzymes differ in their regulation, physical properties and location in the cell. SodA is a manganese cofactored enzyme located in the cytoplasm. It is positively regulated by SoxRS (6, 163, 407), and it is negatively regulated under anaerobiosis by FNR, ArcAB, and Fur (180). SodB contains iron in its active site and is

also found in the cytoplasm (164). It is positively regulated by Fur via the small RNA RyhB and the RNA chaperone Hfq (114, 120, 152, 263, 392). SodCI and SodCII are both periplasmic Cu/Zn-SODs. RpoS induces *sodCII* in stationary phase, and *sodCII* is repressed under anaerobic conditions by FNR (7, 158, 160). Expression of *sodCI* is positively regulated by the two-component regulator PhoPQ and is induced in stationary phase independently of RpoS (158).

Periplasmic superoxide dismutases have been shown to be important for virulence in *S. typhimurium* (123, 126, 187). SodCI and SodCII have similar specific activity *in vitro*. However, only SodCI contributes to virulence (221). Some differences exist between expression of *sodCI* and *sodCII* in the host, but this is not the main reason SodCI, but not SodCII, contributes to virulence (158, 216). When the *sodCI* open reading frame was placed under the *sodCII* native promoter, it was able to complement a *sodCI* deletion (221). It was concluded that physical differences between SodCI and SodCII beyond enzymatic activity were responsible. One striking physical difference between SodCI and SodCII is that SodCI is not released from the periplasm by osmotic shock. SodCI remains in the soluble fraction as opposed to the membrane fraction following French press lysis, indicating that SodCI is not membrane bound. This characteristic is unique among periplasmic proteins , and it has been termed “tethering” (216). SodCI is also more protease resistant than SodCII (222). Both of these properties may be important in the intracellular environment of the host. Macrophages produce antimicrobial peptides that may transiently increases the permeability of the outer membrane of *S. typhimuruim*. *In vitro* treatment with antimicrobial peptides causes the release of periplasmic proteins, including SodCII, but not SodCI because it is tethered to

peptidoglycan (B. Kim, personal communication). Furthermore, in a transgenic mouse strain that cannot produce the antimicrobial peptide CRAMP, SodCII can contribute to virulence (216). The proposed model is that in the intracellular environment of a macrophage, *Salmonella* encounters antimicrobial peptides that damage the outer membrane, exposing periplasmic proteins to macrophage proteases. Since SodCII is neither tethered nor protease resistant, it is degraded and unable to function during infection. SodCI remains tethered inside the periplasm and is protease resistant, so it is able to contribute to virulence.

1.5.3 Regulators involved in aerobic growth

OxyR is a regulator that responds to elevated concentrations of H_2O_2 (64). Transcription of *oxyR* is growth phase regulated, positively by Crp and adenylate cyclase and negatively by RpoS (159), but transcription is not induced by hydrogen peroxide (366). It is also represses its own transcription. OxyR forms a tetramer in solution and binds DNA in this configuration (380). OxyR has two cysteines on the surface of the protein that are normally reduced, but exposure to a minimum concentration of 0.1 µM H_2O_2 causes disulfide bond formation. Oxidation of these cysteines activates OxyR, which increases transcription of the *oxyR* regulon (10). Genes activated by OxyR are involved in antioxidant defense and include *dps*, a non-specific DNA-binding protein; *katG* and *ahpCF*, which encode hydrogen peroxide scavengers; and *fur*, a transcriptional repressor that responds to the Fe^{2+}concentration in the cell (207). OxyR also activates synthesis of *oxyS*, a small noncoding RNA that also acts as a regulator (5). Microarray analysis has revealed several previously unidentified members of the OxyR regulon

(413). OxyR is an important regulator in responding to hydrogen peroxide *in vitro*. However, in *S. typhimurium* OxyR is not important for virulence (305).

SoxRS is a regulatory system that responds to superoxide generating compounds *in vitro* (163), but it does not respond to superoxide (169) . SoxR is a transcriptional regulator that is produced constitutively at low levels. In structure, it contains a [2Fe-2S] cluster near the carboxyl terminus and forms a homodimer (186). In its inactive form the [2Fe-2S] cluster of SoxR is reduced, but upon interaction with redox-cycling drugs, the cluster is oxidized, and SoxR becomes activated (105, 107, 169). Activated SoxR induces transcription of *soxS*, and SoxS acts as the transcriptional activator of the SoxRS regulon. This regulon includes *sodA*, *fur*, *fumC*, *acnA*, and *nfo*. (309). In addition to redox-cycling drugs, SoxR responds to NO in *E. coli* (106). However, the SoxRS regulator is not required for *S. typhimurium* virulence in mice (124).

Other regulators are also involved in surviving oxidative stress. RpoS, or σ^S, is the master regulator of the stationary phase response (229), but it also regulates gene expression in response to high osmolarity, heat shock, starvation, acid stress, and oxidative stress (15, 115, 229, 283, 284). Mutants of *rpoS* exhibit dramatically increased sensitivity to hydrogen peroxide (229, 265). RpoS positively regulates *xthA*, *katE*, *katG* and *sodCII* (*sodC* in *E. coli*). In addition, RpoS regulation of some genes involved in oxidative stress overlaps with SoxRS and OxyR regulation. RpoS regulates *ahpCF* and *dps*, which are part of the OxyR regulon, and it regulates *fumC* and *acnA*, which are regulated by SoxRS (367).

1.6 Bacterial response to DNA damage

Like all organisms, *Salmonella* and *E. coli* must accurately replicate their DNA. A wide variety of chemical agents are capable of damaging DNA, and many different damaged products are possible. These lesions can be mutagenic or lead to blocked replication. Therefore, DNA repair systems are essential for survival in environments that promote DNA damage.

1.6.1 Repair of DNA damage

Salmonella and *E. coli* have DNA repair systems that recognize different types of DNA damage. UV radiation causes pyrimidine dimers, such as cyclobutane pyrimidine dimers, that can be repaired by DNA photolyase or by nucleotide excision repair (140). Photolyases execute the simplest and most direct type of repair, using the energy of visible light to break the inappropriate bonds (339). Nucleotide excision repair is a repair process that recognizes distortions in the DNA helix. These could be pyrimidine dimers caused by UV exposure or bulky additions covalently linked to purines by agents such as mitomycin C (139). Incisions are made 5' and 3' of the lesion by the UvrABC endonuclease, and a 12-13 nucleotide segment is released. The single-strand gap is then filled in by DNA polymerase I (319, 338). Reactive oxygen can also cause DNA damage. Hydroxyl radicals are the principal species responsible for oxidative DNA damage, and the most common stable lesions are abasic sites, single-strand breaks, and modified bases (39). Most of this damage is repaired by the base excision repair pathway (BER). However, since single-strand breaks can lead to replication fork collapse (233), recombinational repair is also important for surviving oxidative DNA damage.

Base excision repair removes DNA lesions by the sequential action of four enzymes: a DNA N-glycosylase, a 5' abasic-site (ABS) endonuclease, DNA polymerase I, and DNA ligase (52, 97). DNA glycosylases remove the damaged base by cleaving the glycosylic bond and at the same time cleave the phosphodiester bond 3' to the resulting abasic site (230). *E. coli* and *S. typhimurium* have several DNA glycosylases that are part of the base excision repair system, and a subset are involved in oxidative DNA damage repair. Formamidopyrimidine DNA glycosylase (Fpg), encoded by *fpg*, recognizes and removes oxidized purines (36) including 8-oxoguanine (67), which is used as a diagnostic indicator of oxidative DNA damage (342). Endonuclease III, encoded by *nth* (82, 110, 151, 209, 368), and endonuclease VIII, encoded by *nei* (109, 204), remove oxidized pyrimidines. Next a 5' AP endonuclease cleaves the DNA strand, leaving a single base gap (326, 327). If the original lesion is an abasic site, a glycosylase does not act, and an ABS-endonuclease and a deoxyribophosphodiesterase are needed to create the single base gap (137). If the original lesion is a single strand break, the activity of the AP endonuclease is sufficient to create a gap. *E. coli* and *S. typhimurium* have two AP endonucleases, exonuclease III (403) and endonuclease IV (81), encoded by *xthA* and *nfo*, respectively. Finally, the single base gap is filled by polymerase I and sealed by DNA ligase (100). Mutants deficient in base excision repair are very sensitive to exogenous hydrogen peroxide *in vitro* (81, 96, 372, 412) and display decreased virulence in murine macrophages and in mice (372).

Recombinational repair mutants have also been shown to be highly sensitive to reactive oxygen species (48, 56, 96, 193). Oxidative DNA damage can take the form of abasic sites or other blocks to replication. Unrepaired, these lesions result in replication

fork collapse, which must be repaired through recombination (72, 79). The function of recombinational repair is to restart stalled or collapsed replication forks by bypassing a damaged section of DNA. This involves pairing of homologous DNA strands, strand exchange, and resolution of branched DNA intermediates. Recombinational repair of DNA damage has been reviewed extensively (104, 220, 225, 273, 288).

In recombinational repair, RecA forms a filament on single-strand DNA and promotes homologous sequence pairing and strand exchange. Regions of single-stranded DNA are rapidly coated with ssDNA-binding protein (SSB), but this does not interfere with RecA assembly on single-strand DNA to form a filament (287). RecFOR recognizes single-stranded gaps in DNA and promotes RecA polymerization and displacement of SSB (282). If the substrate DNA is a double-strand break, the RecBCD pathway initiates recombination. Double strand breaks can only be repaired by homologous recombination, and this requires RecA to nucleate on a ssDNA strand. The enzyme complex of RecBCD provides this substrate by recognizing a double strand end and degrading the DNA until it encounters a Chi site (5'-GCTGGTGG-3'), upon which the 5’ end is degraded much faster than the 3’ end, creating a single-stranded 3’ end (9). RecBCD also promotes formation of RecA filament on ssDNA, displacing SSB. After daughter strands are homologously paired, strand exchange occurs. Resolution of Holliday junctions, the four DNA strand intermediates created during strand exchange, is facilitated by the RuvABC resolvasome or the RecG helicase (249, 250), although their functions are not completely redundant (111, 251, 402).

1.6.2 SOS

At times DNA repair pathways cannot keep pace with DNA damage, and lesions accumulate. The SOS response describes a large set of genes transcribed in response to DNA damage (315). Transcription of more than 50 genes is activated by SOS inducing conditions in *E. coli* (78, 130, 141, 211, 241, 299). The two proteins that regulate SOS are LexA and RecA. LexA is a repressor that binds to a consensus sequence called an SOS box (40, 247, 396). RecA is activated when some threshold level of accumulated DNA damage is reached. RecA is then able to act as a coprotease to facilitate autocleavage of LexA (245). Cleavage of LexA results in derepression of genes in the SOS regulon, although many SOS-induced genes are expressed at a significant, basal level in the absence of induction. Evidence suggests that two conditions must be met in order for RecA to be activated to act as a coprotease of LexA. One is that RecA must form filaments coating regions of single-stranded DNA (ssDNA) (189, 246), and the other is that DNA replication forks must be stalled (336, 341). Induction of transcription of individual SOS genes ranges from 2 fold to more than 100 fold (225). The level to which genes in the SOS regulon are induced is largely a function of the strength of LexA binding to an SOS box, but other factors may play a role. The strength of a given promotor and the location and number of LexA binding sites also influences the level of induction. LexA binds with variable affinity to the promoters of genes it regulates, depending on the sequence of the SOS box, and this allows certain genes to be activated before others in this system. As the concentration of intact LexA decreases, promoters with lower affinity for LexA become derepressed, while promoters with the highest affinity for LexA are the last to be induced. For example LexA has relatively high

affinity for the SOS box in the *sulA* promotor, and as such it is among the last to be induced. SulA is a cell division inhibitor, and transcriptional activation of this gene results in inhibition of septation until the conditions inducing SOS have been resolved. LexA is also an autoregulator, and it has a very low affinity for the SOS box in its own promoter. Expression of *lexA* is among the first to be induced. Eventually the inducing signal disappears, LexA cleavage ceases, and the system is turned off.

1.7 Transport of polypeptides across the cytoplasmic membrane

Gram-negative bacteria are characterized as having a large cellular compartment called the periplasm which lies between the cytoplasmic membrane and the outer membrane. Redox reactions, solute transport, detoxifying reactions, LPS and peptidoglycan assembly, and many other reactions occur beyond the cytoplasmic membrane. However, all protein synthesis occurs in the cytoplasm. Cell envelpoe proteins must be exported to the appropriate location in the inner membrane, periplasm or outer membrane in order to perform their appropriate physiological functions. Estimates of the proportion of open reading frames of *E. coli* that encode proteins destined for export from the cytoplasm range from 10% to 30% (223, 304). The Sec pathway and the Twin-Arginine transport (Tat) pathway are two separate export systems that move proteins across the inner membrane. Proteins destined for transport to the periplasm by either Sec or Tat contain an amino-terminal signal sequence, but the signal peptide for the two systems is different. However, the most striking difference between these systems is that proteins transited via the Sec pathway are unfolded, and the Tat system transports

fully folded proteins, frequently cofactor-containing proteins, that can also cross the inner membrane bound in complex with other proteins.

1.7.1 The Sec pathway

Most proteins targeted for export to the periplasm in *E. coli* and *Salmonella* cross the inner membrane via the Sec pathway (314). The general scheme is that a pre-protein with the appropriate N-terminal signal sequence is targeted to the translocase, interacts with the motor protein SecA, and is translocated through the SecYEG channel. A functional Sec pathway is required for viability (300). Much of the genetic work on the Sec pathway was achieved with conditional mutants (87).

The structure of the Sec signal sequence is complex. There is no consensus sequence, but Sec signal peptides have three distinct features. The N domain of the signal sequence, at the N-terminal end of the polypeptide, contains 1-3 positively charged amino acids. It is followed by 10-15 hydrophobic amino acids, called the H domain, and ends with the C domain, which contains polar amino acids and is the site of cleavage of the signal peptide from the mature protein (394). Polypeptides containing this type of signal sequence can be exported either post-translationally or co-translationally via the Sec translocon to ultimately reach the periplasm (217). The protein-conducting-channel of the Sec pathway consists of SecY, SecE, and SecG, which operate in the inner membrane as a heterotrimer (41). The energy required to export proteins is provided by ATP hydrolysis and by the proton motive force (62, 113, 344).

Most proteins leaving the cytoplasm follow the post-translational path. Presecretory proteins are fully translated and interact with chaperones that prevent them

from folding and keep them ready for transport (306). SecB is an export-dedicated chaperone (128) that binds to long stretches of the pre-protein, but it is not required for all proteins transported via Sec (74). This suggests that other chaperones can keep presecretory proteins competent for export. The preprotein-SecB complex binds SecA, which is an ATPase peripherally associated with the inner membrane and the Sec translocon. SecA acts as a motor protein that drives movement of polypeptides across the inner membrane through SecYEG (98).

The co-translational transport path of *E. coli* is similar to protein transport in the endoplasmic reticulum of eukaryotes (reviewed in (85)). In eukaryotes, proteins destined for secretion are conducted to the rough endoplasmic reticulum by the signal recognition particle (SRP). Briefly, SRP recognizes a secretion signal in an emerging polypeptide and binds the exposed end of the peptide along with the ribosome, halting translation until SRP binds the SRP receptor in the endoplasmic reticulum membrane. The SRP-SRP receptor disassociate from the ribosome at the same time proteins of the translocon, also located in the membrane, associate with it. Translation resumes and the polypeptide crosses the endoplasmic reticulum membrane (reviewed in (397)). This pathway differs slightly in *E. coli*. The components are similar, but translation does not halt (352). The *E. coli* pathway can involve a signal recognition particle (202, 310, 324) composed of 4.5S RNA (311, 369) and the protein Ffh (254). This SRP recognizes the signal sequence of a polypeptide emerging from a ribosome, and the association is stronger with increasing hydrophobicity of the signal sequence. In many cases inner membrane proteins do not have a signal sequence. Instead SRP interacts with the hydrophobic transmembrane region of the polypeptide (217, 237, 257, 387, 388).

FtsY is the SRP receptor in *E. coli* (28). Inner membrane proteins and some other proteins are targeted to the Sec aparatus as nascent polypeptide chains still associated with ribosomes. When the emerging peptide is hydrophobic, it can interact with the signal recognition particle (SRP). YidC is an essential protein that aids the folding and membrane insertion of some SRP-dependent proteins (337).

Soluble periplasmic proteins and outer membrane lipoproteins have their signal peptides removed prior to achieving their mature structure by a signal peptidase (86, 89, 416). Signal peptidase I, encoded by *lep*, cleaves non-lipoproteins, and signal peptidase II, encoded by *lspA*, cleaves pro-lipoproteins (24, 195, 379). Signal peptidase I and II are membrane-bound proteins with their active sites at the membrane, on the periplasmic side. As a pre-protein emerges from either the Sec or Tat machinery, the signal peptide is inserted into the inner membrane with the N-terminus facing the cytoplasm. A signal peptidase can gain access to the cleavage site and release the mature protein (303).

1.7.2 Twin-arginine translocation pathway

An alternative protein export pathway was proposed for proteins that contain cofactors, such as flavins, Fe-S clusters, or molybdopterins. These proteins have unusually long signal sequences with a twin-arginine consensus sequence (S/TRRXFLK) present (24, 60). Proteins from thylakoids, as well as bacteria (348), involved in redox reactions were observed to have this conserved N-terminal sequence that was infrequently found in other types of proteins (34, 35). Transport was dependent on ΔpH, but not ATP hydrolosis (73), and prefolded proteins were targeted to this system (80, 340). It was also proposed that multi-subunit protein complexes in which only one

subunit contains a twin-arginine signal sequence are secreted as a complex (24). This was demonstrated to occur for the large and small subunits of hydrogenase 2 (329). The Tat transport system is present in diverse organisms, having been identified in chloroplasts, gram-positive and gram-negative bacteria, and halophilic archaea (102, 103, 404, 408).

The gene locus responsible for this type of transport was identified in *E. coli* by screening for mutants capable of anaerobic respiration on nitrate or fumarate but incapable of anaerobic respiration on DMSO. These mutants were not rescued by a plasmid carrying the structural genes for DMSO reductase. DMSO reductase contains a twin-arginine signal sequence, but neither fumarate reductase nor nitrate reductase encoded by *narGHI* or *narZYV* do (400). In *E. coli* the components required for a fully functional Tat translocon are TatA, TatB, and TatC (35, 340, 400). The genes *tatA, tatB, tatC,* and *tatD* are in an operon, while *tatE* is transcribed alone from another chromosomal location. These genes are constitutively expressed (199). TatD is a nuclease with no demonstrated effect on protein secretion (401). It appears that *tatE* is a paralog of *tatA* (408), but it is only a minor contributor to Tat secretion (340).

1.7.3 Tat translocon

TatA, TatB, and TatE are grouped into a protein family (348, 408). The common structural feature of these proteins is the N-terminal transmembrane α-helix which anchors these proteins to the cytoplasmic membrane followed by a stretch of sequence that codes for an amphipathic α-helix and a C-terminal tail of varying length (26, 340). TatA forms multimeric ring structures with varying numbers of TatA subunits, creating

the protein translocation channel. The range of observed pore sized was from 30 Å-70 Å (157, 228, 234). TatC has six transmembrane domains, and both the N-terminus and the C-terminus are located in the cytoplasm (17, 214). TatC and TatB form complexes in a 1:1 ratio with variable numbers of TatBC subunits (37, 301). Export substrates bind the TatBC complex, and the consensus sequence of the leader peptide interacts with TatC. This then interacts with the TatA complex to form the translocon (2, 234, 266). When not transporting substrates, the TAT export system sits in the inner membrane of *E. coli* as complexes of TatA alone and complexes of TatB and TatC (266, 281). The actual mechanism of moving the bound substrate across the membrane is not yet completely understood.

1.7.4 Tat substrates

The number of Tat substrates in a given organism is usually estimated by genome analysis with an algorithm designed to look for sequences characteristic of known Tat signal peptides. Two such programs currently in use are TatFind (331) and TatP (19). The TAT system has been found in many species, and it is used to a greater or lesser degree depending on the species. Examples include the halophilic archaeon *Halobacterium* sp. strain NRC-1, which exports 64 of about 80 total exported proteins via Tat, and *Streptomyces coelicolor*, which exports 145 proteins via Tat. In both *S. typhimurium* and *E. coli* about 33 proteins are predicted to be transported through the Tat system (103). Most, but not all, proteins exported by the Tat system in *E. coli* contain redox cofactors and participate in the electron transport pathway. Cofactors commonly found in Tat-targeted proteins are iron-sulfur clusters and molybdopterin cofactors (24,

25). Anaerobic respiratory chain proteins are a sizable fraction of predicted Tat exported proteins in *S. typhimurium*, but another important group includes proteins involved in cell septation. N-acetylmuramyl-L-alanine amidases are enzymes that remove cross-links in peptidoglycan and are involved in separating daughter cells. Two of the three enzymes of this type in *E. coli* are Tat substrates (27, 182).

1.7.5 Physiological effects

In *E. coli* mutants with disruptions to *tatA/E*, *tatB* or *tatC* display numerous physiological defects. One defect is in motility. In *E. coli* O157:H7 *tat* mutants do not express flagellin, the protein that forms the flagellar filament, and as a result they are non-motile. (312). Another defect is that *E. coli tat* mutants form biofilms poorly on abiotic surfaces (177, 197). More interestingly, *tat* mutants of *E. coli* are defective in cell division and display decreased resistance to detergents and lysozyme (198, 362). These strains form chains up to 15 cells long that are arrested at a late stage of cell division; they have the appearance of a strand of beads. When sensitivity to detergent was tested, survival of *tat* mutants was greatly decreased in rich media supplemented with SDS. Normally *E. coli* is not sensitive to lysozyme in the absence of EDTA, but *tat* mutants were found to be lysed by lysozyme alone. In addition, these strains are highly resistant to infection by P1. It has been suggested that, taken together, these characteristics are indicative of outer membrane defects (198, 362). This presumptive outer membrane defect is due to mislocalization of two Tat substrates, the cell wall amidases AmiA and AmiC. Both the long chain formation and SDS sensitivity seen for *tatC* mutants were abolished by overproducing the Sec-exported amidase, AmiB (198).

1.7.6 The role of Tat transport in pathogenesis

The Tat transport system is present in numerous pathogens (103, 408), but currently the role of Tat in virulence has only been studied in a limited number of organisms. In a few cases, virulence factors important for survival of pathogens in their respective hosts have been identified. The contribution of Tat transport to virulence is direct. Secretion of virulence determinants like phospholipase toxins in *Pseudomonas aeruginosa* (298, 395), *Pseudomonas syringae* (42) and *Legionella pneumophila* (333), as well as Shiga toxin 1 in enterohemorrhagic *E. coli* (312) rely on the Tat system to transport these proteins to the periplasm where they can interact with the type II secretion aparatus. In other organisms in which *tat* mutants are attenuated, no Tat exported virulence factors are known, so the role of this transport system in virulence is likely indirect. *Pseudomonas syringae* pv. tomato DC3000 *tatC* mutants display a slight decrease in type III secretion and are attenuated for virulence in plants (42). A *tatC* mutant of *Yersinia pseudotuberculosis* is non-motile and is highly attenuated in mice when administered both orally and intraperitoneally, but there is no data to indicate why. Type III secretion was not shown to be affected in this mutant, and only a slight sensitivity to low pH was observed (232). Similarly, when the effect of *tatBC* mutations on *Salmonella enteriditis* virulence was examined, it was found that this organism had many of the physiological defects observed for *E. coli tat* mutants. These strains form long chains of cells, are sensitive to SDS, and impaired in motility. They were also shown to be impaired for survival in polarized epithelial cells and in chickens. However, the mechanism by which loss of the Tat secretion system impaired virulence in this organism was not determined (274).

1.8 The scope of this thesis

In the systemic phase of infection, *S. typhimurium* encounters superoxide when residing in macrophages (11, 280). Production of superoxide is an important antimicrobial activity of macrophages, but the bacterial target it damages is not yet known. Chapter 3 of this thesis attempts to identify this target, and the conclusion is that it is a periplasmic target that is currently unidentified. Since the only known bacterial targets that are directly damaged by superoxide are [4Fe-4S] containing enzymes (135), and since periplasmic proteins containing redox cofactors must be exported via the Tat pathway (24, 25), we examined the possibility that the target damaged by phagocytic superoxide is exported by Tat. This turned out to not be true, but we learned a great deal about the role this protein export system plays in virulence. Many of the proteins exported by Tat are redox proteins involved in anaerobic respiration (400). In chapter 4 we show that *S. typhimurium* does not utilize anaerobic respiration, but it does aerobically respire in the murine host. In chapter 5 we show that the Tat system is important for virulence because of three proteins involved in cell septation that are exported by this system.

Chapter 2: Materials and methods

2.1 Protocols

2.1.1 Bacterial strains and growth conditions

Bacterial strains and plasmids are described in Table 2.1. All *Salmonella enterica* serovar Typhimurium strains used in this study are isogenic derivatives of strain 14028 (American Type Culture Collection) and were constructed using P22 HT105/1 *int*-201 (P22) mediated transduction (259). Deletion of various genes and concomitant insertion of an antibiotic resistance cassette was carried out using lambda Red-mediated recombination (90) as described in (117). Primers were purchased from IDT Inc. The endpoints of each deletion are indicated in Table 2.1. In all cases, the appropriate insertion of the antibiotic resistance marker was checked by PCR analysis. The constructs resulting from this procedure were moved into a clean wild type background (14028) by P22 transduction. Antibiotic resistance cassettes were removed using the temperature sensitive plasmid pCP20 carrying the FLP recombinase (63).

Luria-Bertani (LB) medium was used in all experiments for growth of bacteria, except where noted. Bacterial strains were routinely grown at 37^{o}C except for strains containing the temperature sensitive plasmids, pCP20 or pKD46 (90), which were grown at 30^{o}C. Antibiotics were used at the following concentrations: 20 μg/ml chloramphenicol; 50 μg/ml kanamycin; and 12 μg/ml tetracycline.

Anaerobic growth media was a modified Neidhardt Supplemented MOPS Defined Medium (294), called EZ. Plates were made with either 0.4% glucose or 0.4% glycerol

and supplemented with 1μM Na_2SeO_3 and 1μM $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$. If alternative electron acceptors were used, 0.4% nitrate, 0.4% fumarate or 0.4% DMSO were added to plates. Plates were incubated at 37° in an anaerobic glove box.

2.1.2 Assay of β-galactosidase activity

β-galactosidase assays were performed using a microtiter plate assay as previously described (353) on strains grown under the indicated conditions. β-galactosidase activity units are defined as (μmol of ONP formed min^{-1}) x 10^6/ (OD_{600} x ml of cell suspension) and are reported as mean ± standard deviation where n = 4. For log phase cultures, bacteria were grown overnight in LB, diluted 1/100 in the indicated medium and upon reaching OD_{600} of 0.2, diluted 1/4 and grown to OD_{600} of 0.2-0.3. Cultures grown in standard SPI1 inducing conditions were initially inoculated into LB (0.5% NaCl), grown for 8-12 hours, then subcultured 1/100 and grown statically for 18-22 hours in 3 ml LB with 1% NaCl (high salt LB, HSLB) in a 13 x 100 mm tubes. LB, or LB without NaCl (NSLB) were used where indicated.

Bunny et al. (50) recombined the uninducible *lexA3* allele from *E. coli* into the *Salmonella* Typhimurium genome linked to a Cm marker. We moved this allele into our strain background via P22 HT105/1 *int*-201 (P22) mediated transduction (259). LB cultures of each strain were grown to mid-log phase and nalidixic acid was added to a final concentration of 10 μg/ml. After 3 hours incubation at 37°C, the β-galactosidase activity produced from the fusion in each strain was measured using a microtiter plate assay as previously described (354). β-galactosidase activity units are defined as (μmol

of ONP formed min^{-1}) x 10^6/(OD_{600} x ml of cell suspension) and are reported as mean ± standard deviation where n = 4.

2.1.3 Competition assays

BALB/c mice (BALB/cAnNHsd) were purchased from Harlan Sprague Dawley, Inc. C57BL/6 and congenic phox-/- (B6.129S6-*Cybb*^{tm1Din}/J) and iNOS2-/- (B6.129P2-*Nos2*^{tm1Lau}/J) mice were from Jax Mice. Bacterial strains were grown overnight (16 h) in LB medium. Cultures of the two strains of interest were mixed 1:1 and the mixture was washed, and diluted in sterile 0.15 M NaCl. For competition assays, female mice were inoculated intraperitoneally (i.p.) in groups of 4 to 6 with the mixture of the two bacterial strains (approximately 500 total bacteria). Inocula were plated on LB and then replica plated onto the appropriate selective media to determine the total number and percentage of the two strains used for the infection. Mice were sacrificed after 4 to 5 days of infection and their spleens were removed. The spleens were homogenized, diluted and plated on LB medium. The resulting colonies were replica plated onto the selective medium to determine the relative percent of each strain recovered. The competitive index (CI) was calculated as follows: (percent strain A recovered/percent strain B recovered)/(percent strain A inoculated/ percent strain B inoculated). The CI of each set of assays was analyzed statistically using the Student's t test. In most cases, the strains were rebuilt by P22 transduction, and the mouse assay was repeated to ensure that the virulence phenotypes were the result of the designated mutations. All animal work was reviewed and approved by the University of Illinois IACUC and performed under protocols 04137 and 07070.

Growth and dilution of bacteria for *in vitro* competitions were performed as above, but 0.1 ml of the bacterial mixture was introduced into 5 ml of LB in a 50 ml flask. If detergents or toxic drugs were part of the experiment, they were added at this time. Flasks were incubated at 37º on a platform shaker rotating at 225 RPM for 18hrs. Cells were diluted and plated on LB medium, and the resulting colonies were replica plated onto the selective medium to determine the relative percent of each strain recovered. Competitive index was calculated as above and the Student's t-test was used for statistical analyses.

2.1.4 Sensitivity to external superoxide and H_2O_2 *in vitro*

To test sensitivity to external superoxide, cultures grown overnight in LB medium were diluted 1/100 in LB and grown in highly aerated flasks to OD_{600} of 0.2. Cells were washed and diluted to approximately 10^6 cfu/ml in PBS pH 7.4 containing 250 µM hypoxanthine. Samples were split and either remained untreated or xanthine oxidase (Sigma) was added to 0.1 unit/ml. The samples were incubated at 37° C with aeration. At the given time points, cells were diluted in sterile 150 mM NaCl and plated for colony forming units. Samples with xanthine oxidase were compared to untreated samples at each time point.

Strains were assessed for H_2O_2 sensitivity as previously described (193). Overnight LB cultures were diluted in LB medium with 0.2% glucose to OD_{600} of 0.01 and grown in highly aerated flasks to OD_{600} of 0.2. Cells were diluted 1/100 in LB with 0.2% glucose, split, and one part was exposed to 2.5 mM H_2O_2 for 20 min at 37°C.

Samples were immediately diluted in sterile 150 mM NaCl and plated for colony forming units. Samples with H_2O_2 were compared to untreated samples.

2.1.5 cAMP assay

Translocation of SlrP by the SPI1 TTSS was assayed by using a SlrP-CyaA fusion protein. Strains weregrown under SPI1-inducing conditions, grown with aeration in LB for 8hrs then subcultured 1/100 into high salt LB (10g/L) and grown standing overnight, and used to infect RAW264.7 macrophages at a multiplicity of infection of 10 for 1 h. Infected macrophages were then washed three times with PBS. The cells were lysed with 200 µl of 0.1M HCl and heated for 10 min at 95°C. The levels of cAMP were assayed by using a Direct cAMP kit (Assay Designs, Ann Arbor, Mich.). The protein content of each sample was determined by a BCA assay (Pierce, Rockford, Ill.). The protein concentrations were calculated by using KC4 software. All cAMP assays were performed in triplicate and repeated at least two times; the results of a representative experiment are described below.

To assay SPI2-dependent TTS, cultures of serovar Typhimurium strains producing SspH2-CyaA fusions grown under SPI2-inducing conditions, grown overnight in LB with aeration then diluted 1 to 100 in 2 ml of N minimal medium with 0.2% glycerol and 8 mM MgCl2 and grown 16 h with aeration, were opsonized with 50% mouse serum (Equitech-Bio, Kerrville, Tex.) for 20 min at 37°C. The opsonized bacteria were then used to infect RAW264.7 cells at a 10:1 ratio. After 1 h, the macrophages were washed three times with PBS and 1 ml of RPMI 1640 containing 10% fetal bovine serum, and 6.25 µg of gentamicin per ml was added. The infection was allowed to

proceed for 5 h. The macrophages were washed, and the cAMP levels were assayed as described above (118).

2.1.6 Fluorescence microscopy

For each sample, 30µl of cell culture was loaded onto a cover glass precoated with 0.1% polylysine and incubated at room temperature for 5–10 min. The excess liquid was then blotted away, and the cells were allowed to dry. The cover glass was then mounted on glass slides with 20% glycerol. Microscopy images were collected with an Applied Precision assembled DeltaVision epifluorescence microscope containing an Olympus Plan Apo x100 oil objective with a numerical aperture of 1.42 and a working distance of 0.15 mm, and the images were processed with the SoftWoRX (Issaquah, WA) Explorer Suite program.

2.1.7 Motility assay

Strains were tested for ability to swim on 0.3% agar Luria-Bertani (LB) plates supplemented with 0.5% glucose. Overnight cultures were grown at 37° C with aeration in LB. Bacteria were inoculated into fresh motility agar by transferring 2µl of overnight culture with a pipette tip and grown for 3 hours at 37° C.

2.2 Tables

Table 2.1 *Salmonella typhimurium* strains and plasmids used in this thesis.

Strain	**Genotype**[a]	**Deletion End Points**[b]	**Source or Reference**[c]
14028	Wild type serovar Typhimurium		ATCC[d]
JS452	Δ*sodB102*::Km	1509486-1509923	(221)
JS454	Δ*sodCII-103*::Cm	1516106-1516488	(221)
JS456	Δ*sodCI-1*::*aph* Δ*sodCII-103*::Cm		(221)
JS472	Δ*sodCI-1*::*aph*		(221)
JS830	Δ*sodA112*::Cm	4266594-4266789	
JS831	Δ*sodA112*::Cm Δ*sodB102*::Km		
JS832	Δ*sodA112*::Cm Δ*sodB102* Δ*sodCI-1*::*aph*		
JS833	Δ*xthA51*::Cm	1380972- 1381714	
JS834	Δ*nfo1*::Km	2302687-2303476	
JS835	Δ*xthA51*::Cm Δ*nfo1*::Km		
JS836	Δ*xthA51*::Cm Δ*nfo1* Δ*sodB102*::Km		
JS837	Δ*xthA51*::Cm Δ*nfo1* Δ*sodCI-1*::*aph*		
JS838	Δ*ruvAB*::Cm	1989088-1990664	
JS839	Δ*ruvAB*::Cm Δ*sodCI-1*::*aph*		
JS840	Δ*ruvAB*::Cm Δ*sodB102*::Km		
JS841	Δ*lexA33*:: [Cm *lexA3*(Ind^-)](sw)		

Table 2.1 (Continued)

Strain	Genotype[a]	Deletion End Points[b]	Source or Reference[c]
JS842	Δ*sodCI-1*::*aph* Δ*lexA33*:: [Cm *lexA3*(Ind$^-$)](sw)		
JS843	Δ*recA711*::Tc	2974870-2975903	
JS844	Δ*recA711*::Tc Δ*sodB102*::Km		
JS845	Δ*recA711*::Tc Δ*sodCI-1*::*aph*		
JS846	Φ(*sulA*$^+$-*lac*$^+$)*111*		
JS847	Φ(*sulA*$^+$-*lac*$^+$)*111* Δ*recA711*::Tc		
JS848	Φ(*sulA*$^+$-*lac*$^+$)*111* Δ*lexA33*:: [Cam *lexA3*(Ind$^-$)](sw)		
BMC1232	*ΔmoaDE*::Cm	872647-873334	
BMC1231	*ΔnrfA*::Cm	4516122-4517515	
BMC1258	*ΔfrdA*::Kn	4583987-4585757	
BMC1257	*ΔmoaDE ΔnrfA ΔfrdA*		
BMC1317	*Δfnr1*::Kn	1754380-1755116	
BMC1451	*ΔnrdDG*::Cm	4691991-4694687	
BMC1461	*ΔnuoA-N*::Cm	2424362-2439467	
BMC1450	*Δndh*::Cm	1294478-1295743	
BMC1316	*ΔcyoABCD*::Cm	494284-498135	
BMC1364	*ΔcydAB*::Cm	809541-812206	
BMC1201	*ΔtatC*::Kn	4181325-4182076	
BMC1649	*ΔsodCI ΔtatC*		
BMC1444	*ΔtatABC*::Cm	4180512-4182076	
BMC1209	*ΔybiP*::Cm	900151-901691	
BMC1207	*ΔfdnG*::Kn	1650441-1653513	
BMC1208	*ΔfdoG*::Kn	4244757-4247803	
BMC1250	*ΔfdnG ΔfdoG*		

Table 2.1 (Continued)

Strain	Genotype[a]	Deletion End Points[b]	Source or Reference[c]
BMC1217	*ΔycbK*::Cm	1087124-1087659	
BMC1323	*ΔhyaAB*::Cm	1884828-1887733	
BMC1321	*ΔhybABC*::Cm	3313762-3308790	
BMC1322	*ΔhydBC*::Kn	1614904-1611999	
BMC1333	*ΔhyaAB ΔhybABC ΔhydBC*		
BMC1215	*ΔttrA*::Cm	1466373-1469408	
BMC1219	*ΔydcG*::Cm	1709852-1711470	
BMC1211	*ΔamiA*Cm	2560337-2561281	
BMC1214	*ΔamiC*::Kn	3141365-3142605	
BMC1367	*ΔsufI*::Cm	3334472-3335887	
BMC1229	*ΔamiA ΔamiC*		
BMC1475	*ΔamiA ΔamiC ΔsufI*		
BMC1266	*ΔthiP*::Cm	124115-125693	
BMC1267	*ΔfhuD*::Cm	226783-227649	
BMC1352	*ΔwcaM*::Cm	2180008-2181429	
BMC1353	*ΔpSLT46*::Cm	37534-38262	
JS481	*ΔSPI1-2916*::Kn		(116)
BMC1949	*ΔSPI1-2916 ΔtatC*		
JS749	attλ::pDX1::hilA'-lacZ		(242)
BMC1643	*ΔtatC* attλ::pDX1::hilA'-lacZ		
BMC1673	*ΔamiA ΔamiC ΔsufI* attλ::pDX1::hilA'-lacZ		
pSG161	*bla* P_{LAC} sspH2'-cyaA Pacyc184 ori		(118)
BMC1780	*ΔtatC* pSG161		

Table 2.1 (Continued)

Strain	Genotype[a]	Deletion End Points[b]	Source or Reference[c]
JS326	*dsbA100::*Cm		(118)
JS362	*dsbA100::*Cm pSG161		(118)

[a] All strains are isogenic derivatives of 14028. [b] Numbers indicate the base pairs that are deleted or cloned (inclusive) as defined in the *S. enterica* serovar Typhimurium LT2 genome sequence in the National Center for Biotechnology Information Database. [c] This study, unless otherwise noted. [d] ATCC, American Type Culture Collection.

Chapter 3: Phagocytic superoxide specifically damages an extracytoplasmic target to inhibit or kill *Salmonella*

The phagocytic oxidative burst is a primary effector of innate immunity that protects against bacterial infection. However, the mechanism by which reactive oxygen species (ROS) kill or inhibit bacteria is not known. It is often assumed that DNA is a primary target of oxidative damage, consistent with known effects of endogenously produced ROS in the bacterial cytoplasm. But most studies fail to distinguish between effects of host derived ROS versus damage caused by endogenous bacterial sources. We took advantage of both the ability of *Salmonella enterica* serovar Typhimurium to survive in macrophages and the genetic tractability of the system to test the hypothesis that phagocytic superoxide damages cytoplasmic targets including DNA. SodCI is a periplasmic Cu-Zn superoxide dismutase (SOD) that contributes to the survival of *Salmonella* Typhimurium in macrophages. Through competitive virulence assays, we asked if *sodCI* has a genetic interaction with various cytoplasmic systems. We found that SodCI acts independently of cytoplasmic SODs, SodA and SodB. In addition, SodCI acts independently of the base excision repair system and RuvAB, involved in DNA repair. Although *sodCI* did show genetic interaction with *recA*, this was apparently independent of recombination and is presumably due to the pleiotropic effects of a *recA* mutation. Taken together, these results suggest that bacterial inhibition by phagocytic superoxide is primarily the result of damage to an extracytoplasmic target.

3.1 Introduction

Macrophages normally kill bacteria by a coordinated delivery of toxic substances following phagocytosis. Phagosomes fuse with various membrane vesicles that deliver, for example, hydrolytic degradative enzymes and antimicrobial peptides. The NADPH-dependent oxidase (Phox), which produces superoxide, assembles in the phagosomal membrane (293). Activated macrophages also produce nitric oxide, generated from arginine and oxygen by the inducible nitric oxide synthase (iNOS; (275)). Other reactive oxygen species (ROS) and reactive nitrogen species (RNS) can result (275, 365). The phagocytic oxidative burst is a fundamental aspect of innate immunity, yet the mechanism by which these reactive species kill bacteria is not well understood.

Salmonella enterica serovar Typhimurium is a facultative intracellular pathogen that is capable of causing systemic infection in humans and mice, the common animal model (277). Systemic infection by serovar Typhimurium requires survival within macrophages (131, 328, 335). When engulfed by macrophages, serovar Typhimurium produces a series of virulence factors that allow the bacterium to delay or prevent the delivery of the antibacterial enzymes to the phagosome, survive the various killing mechanisms, and propagate in a unique compartment called the *Salmonella* containing vacuole (243). Although *Salmonella* inhibits delivery of Phox, host production of ROS is clearly important for controlling infection (264, 405). The fact that *Salmonella* survive in the macrophage despite being exposed to ROS, along with the genetic tractability of both the bacterium and host, provides a powerful set of tools to address how the effectors lead to bacterial inhibition or death.

The NADPH-dependent oxidase complex in phagocytes generates superoxide from the univalent reduction of molecular oxygen (293). At neutral pH, superoxide is charged and cannot penetrate membranes. However, the pKa of superoxide is approximately 4.8. Therefore, in the acidified phagosome, phagocytic superoxide could potentially be protonated, allowing flux into the bacterial cytoplasm (219, 275). Superoxide is also produced endogenously in the bacteria by the inadvertent transfer of an electron to O_2 from flavoproteins, particularly the respiratory NADH dehydrogenase II (269). *E. coli* and *Salmonella* detoxify this endogenous superoxide using two cytoplasmic superoxide dismutases, SodA and SodB (365). Results from several groups examining the phenotypes of *E. coli sodAB* double mutants provide the framework for a model of endogenous superoxide toxicity . Superoxide directly inactivates a set of dehydratases containing exposed [4Fe-4S] clusters and damages additional specific enzymes blocking several metabolic pathways. Mutants devoid of cytoplasmic superoxide dismutase (SOD) are auxotrophic for branched chain amino acids, sulfur-containing amino acids, and aromatic amino acids, and, due to defects in aconitase and fumarase, can grow only on fermentable carbon sources (190). Damage to iron-sulfur clusters also causes the release of iron. Superoxide rapidly dismutes, either enzymatically or spontaneously, to form hydrogen peroxide (H_2O_2), which is reduced by the free iron to form hydroxyl radical (HO•) via the Fenton reaction. HO• is highly reactive and oxidation of biological molecules is diffusion limited. Because of the apparent propensity of positively charged iron to associate with the negatively charged phosphodiester backbone of DNA, H_2O_2 mediated cell death results from DNA damage

(190). Reduction of Fe^{3+} to Fe^{2+} by an unknown reductant allows the production of HO• to continue in the cell (190).

Many investigators have assumed that phagocytic superoxide kills by initiating DNA damage via the same mechanism described for endogenously produced oxygen radicals (45, 48, 49, 54, 175, 297, 372, 373). We would argue that much of these data are based on experiments that fail to separate the effects of phagocytic and endogenously produced reactive oxygen species or other general defects in bacterial metabolism. For example, Buchmeier *et al*. (49) have previously shown that *recA* mutants of serovar Typhimurium are 3-4 logs less virulent than an isogenic wild type strain. The *recA* mutant was also sensitive to killing by tissue culture macrophages. It was presumed that this virulence defect is due to increased DNA damage in the *recA* mutant mediated by ROS produced by macrophages. However, *recA* mutants are generally defective and grow poorly compared to the wild type (225). Thus, it is not clear whether this virulence defect is due to specific sensitivity to phagocytic ROS, or is a nonspecific effect caused by the altered growth rate of the mutant. Similarly, Suvarnapunya *et al*. (372) examined the effects of deleting components of the base excision repair system (BER) on *Salmonella* virulence. They showed that BER mutants were decreased in the ability to proliferate in tissue culture macrophages and were attenuated in mouse competition assays. Again, these results, per se, do not distinguish between a defect that is the direct result of phagocytic oxidative damage and oxidative damage mediated by endogenous sources of oxygen radicals. One must distinguish between endogenous and phagocytic superoxide when making conclusions about the mechanism of killing by the phagocytic oxidative burst.

The periplasmic copper-zinc cofactored superoxide dismutase, SodCI, is needed for full virulence of serovar Typhimurium (123, 126, 187). Our strain of serovar Typhimurium also produces a second periplasmic SOD, SodCII, but this enzyme does not contribute to virulence . Published data (91) and our data presented below indicate that SodCI specifically protects against phagocytic superoxide during infection. Based on this starting premise, and taking advantage of the genetic power of our system, we provide evidence that phagocytic superoxide primarily damages an extracytoplasmic target to inhibit or kill *Salmonella*. These results indicate that we need to reevaluate the dogma that DNA is a primary bacterial target of the phagocytic oxidative burst.

3.2 Results

3.2.1 SodCI protects specifically against phagocytic superoxide

The oxidative burst of phagocytes is a critical innate immune effector used to kill or inhibit invading bacteria, but the mechanism of action is unknown. We have taken advantage of the genetic power of *Salmonella* in the context of the mouse infection model to test the hypothesis that phagocytic superoxide damages a cytoplasmic target. The mouse competition assay, in which mutant and wild type are mixed one to one and the mixture is used to infect a group of animals, provides a sensitive measure of the virulence potential of a given strain. Moreover, it allows us to assay the relative virulence of different strains and is, therefore, amenable to genetic analysis (30, 32). We can also use the competition assay to compare relative virulence in different mouse backgrounds, even though different strains of mice may differ in absolute sensitivity to *Salmonella*.

The periplasmic superoxide dismutase, SodCI, specifically protects serovar Typhimurium against phagocytic superoxide. This conclusion is based on the following evidence. Complete deletion of the *sodCI* gene ((187, 222); Tables 3.1 and 3.2) or point mutations that block SodCI enzymatic activity attenuate virulence 8-17 fold in a competition assay after intraperitoneal infection (222). This attenuation is dependent on the production of phagocytic superoxide; in *phox-/-* knockout mice, a *sodCI* mutant had no phenotype (Table 3.2). This is consistent with previous results in cultured macrophages (91). Moreover, in vitro, a strain deleted for both *sodCI* and *sodCII* shows no growth defect. The most sensitive measure of this is an *in vitro* competition assay, in which mutant and wild type are mixed one to one and grown overnight in a flask under highly aerated conditions. The *sodC* mutant and wild type strain competed evenly in this assay (Table 3.3). Thus, the *sodCI* mutant is specifically sensitive to phagocytic superoxide; there is neither a general growth defect nor apparent sensitivity to endogenous superoxide.

The defect conferred in the animal by loss of SodCI is the direct result of superoxide. Superoxide spontaneously dismutes to hydrogen peroxide, which can subsequently be converted into additional downstream reactive oxygen species. SodCI simply enhances the rate of dismutation and lowers the steady state concentration of superoxide. Thus, equal amounts of hydrogen peroxide and downstream reactive oxygen species are expected whether SodCI is present or not. Since superoxide and nitric oxide can react to form peroxynitrite, it is possible that the protective role of SodCI in the host is to prevent the formation of this highly reactive antimicrobial substance. To test this, we performed a competition assay in iNOS deficient mice. The data show that the *sodCI*

mutant was 8-fold attenuated in this background compared to the wild type strain and this defect was not significantly different than that observed in the parent C57BL/6 mice (Table 3.2). Since the *sodCI* mutant still shows a virulence defect in iNOS mice, the role of SodCI in the host is apparently not to protect against peroxynitrite. This is consistent with published data suggesting a temporal separation between the effects of superoxide and nitric oxide in protecting against *Salmonella* infection (264). Taken together, these data show that SodCI directly protects the bacterial cell against phagocytic superoxide.

3.2.2 SodCI acts independently of cytoplasmic SODs

Since the only biological molecules in bacteria known to be damaged by superoxide are located in the cytoplasm, we tested the hypothesis that SodCI protects a cytoplasmic target. In this series of experiments, we take advantage of the genetic concept of "synthetic phenotypes" (170). If two gene products contribute independently to the same process, then the combination of the two mutations should give a phenotype that is more severe than what would be predicted by the simple combination of the two individual mutations. Following this rationale, we conducted a series of competitive virulence assays involving *sodCI* mutants.

It is established that SodA and SodB prevent DNA damage (212) and inactivation of sensitive enzymes (190, 365) caused by superoxide in the cytoplasm. We tested the effects of deleting *sodA* and *sodB* in the mouse competition assay. As reported previously (384), deletion of *sodA* did not significantly affect virulence (Table 3.1). In contrast, deletion of *sodB* conferred a mild but significant virulence defect; 4 fold in a competition assay (Table 3.1). These data suggest that SodB contributes the majority of

the cytoplasmic superoxide dismutase activity during growth of *Salmonella* in the animal. We then tested the effect of deleting *sodA* in the *sodB* background by directly competing a *sodA sodB* double mutant against the *sodB* single mutant. The *sodA sodB* double mutant was highly attenuated (Table 3.1). This is an example of a synthetic phenotype and provides proof of principle for both our rationale and the use of the animal competition assay for this analysis. Because SodA and SodB both protect the cytoplasm from damage by superoxide, in the absence of SodB, the further loss of SodA has a dramatic effect, 300-fold attenuation, whereas in the wild-type background, loss of SodA has a negligible effect on virulence. As striking as this phenotype is, we cannot simply ascribe the virulence defect to phagocyte derived superoxide. Indeed, in an in vitro competition assay in LB broth, the *sodA sodB* mutant was highly compromised in its ability to grow aerobically compared to the wild-type (Table 3.3). Moreover, the *sodA sodB* mutant remained attenuated in *phox-/-* knockout mice (Table 3.2). In other words, phagocytic superoxide is not required to "attenuate" the *sodA sodB* double mutant.

If the role of SodCI is to protect the cytoplasm from phagocytic superoxide, then a *sodCI* mutation should be synthetic with mutations in *sodA* and *sodB*. This was not observed. In the *sodA sodB* background, a *sodCI* mutation was 5-fold attenuated, which was not significantly different than the 8-fold attenuation observed in the wild type background (Table 3.1). Thus, SodCI acts independently of the cytoplasmic superoxide dismutases. This strongly suggests that SodCI protects an extracytoplasmic target.

3.2.3 SodCI acts independently of base excision repair

Because superoxide can potentiate DNA damage, we further examined the possibility that SodCI protects DNA from phagocyte derived superoxide. The base excision repair system (BER) is critical for the repair of DNA damage mediated by ROS (231). The *xthA* gene product, ExoIII, and the *nfo* gene product, EndoIV, remove oxidatively damaged bases (96) or other fragments left after oxidative DNA damage, or the deoxyribose moieties that result from removal of damaged bases by other N-glycosylases (295). An *xthA nfo* double deletion mutant was constructed via lambda Red-mediated recombination (90). To further confirm that these mutations had the appropriate phenotype, we showed that the *xthA nfo* mutations were synthetically lethal with *recA* (398). Using P22 lysates grown on a Δ*recA*::Tc strain or a strain containing an unrelated Tc insertion, we transduced the *xthA nfo* mutant and an isogenic wild type strain selecting for tetracycline resistance. In the case of the wild type recipient, we obtained approximately equal numbers (>250) of tetracycline resistant colonies using the two lysates. In contrast, the unrelated Tc marker could be transduced into the *xthA nfo* strain at approximately the same frequency as into the wild type. However, we never obtained any transductants of the Δ*recA*::Tc into the *xthA nfo* strain, consistent with this being a lethal combination.

The *xthA*/*nfo* mutant was 4-fold attenuated in an i.p. competition assay (Table 3.4). This result is consistent with the data of Suvarnapunya *et al*. (372). However, the *xthA/nfo* mutant was 2-fold attenuated in an in vitro competition assay in Luria-Bertani broth (Table 3.3), and, therefore, the attenuation does not prove that phagocytic oxygen radicals mediate this apparent DNA damage. We then tested the phenotype conferred by

the *sodCI* deletion in an *xthA*/*nfo* background. The result was not significantly different than the phenotype conferred by deletion of *sodCI* in the wild type background (Table 3.4). As a control for this experiment, we tested the phenotype conferred by the *sodB* deletion in the *xthA/nfo* background. As expected, the combination of these mutations conferred a synthetic phenotype. The *sodB* mutation conferred 15-fold attenuation in the *xthA/nfo* background (Table 3.4). Genetically, this shows that XthA, Nfo, and SodB participate in the same process, protecting the DNA from oxidative damage. In contrast, SodCI acts independently of XthA/Nfo, strongly arguing that attenuation in the *sodCI* mutant is not the result of oxidative DNA damage.

3.2.4 SodCI acts independently of recombinational repair but is affected by mutations in *recA*

Double strand breaks are another possible form of damage caused indirectly by superoxide via hydroxyl radical formation (212). This type of lesion is repaired by the *recBCD* recombination pathway, which requires RecA, RecBCD, and the helicase RecG or the RuvABC resolvasome (225). Buchmeier et al. reported that *recA* and *recBC* mutants were attenuated in mice and exhibited decreased survival in the J774.16 macrophage cell line (49). This defect was partially suppressed in the D9 variant of macrophage cell line J774.16, which is deficient for NADPH oxidase. Since this suggested that recombinational repair may be necessary to protect DNA from phagocytic superoxide, we asked genetically if SodCI participates with this repair pathway to protect DNA. In the mouse competition assay, the *recA* deletion mutant was 56-fold attenuated (Table 3.5). Again, this defect cannot simply be attributed to phagocytic ROS effects; the

recA strain was 62-fold decreased in competitive index *in vitro* (Table 3.3). As expected, a *sodB recA* double mutant showed a synthetic phenotype in the mouse competition assay; deletion of *sodB* conferred 244-fold attenuation in the *recA* background compared to 4-fold in a wild type background (Table 3.5). We then tested the effect of deleting *sodCI* in a *recA* background. Surprisingly, *sodCI* showed a slight synthetic interaction with *recA*, 32-fold attenuated compared to 8-fold attenuated in the wild type background. Although much less dramatic than the synthetic interaction between *sodB* and *recA*, this result was reproducible and statistically significant.

Given the apparent synthetic interaction between *sodCI* and *recA*, we tested other members of the recombinational repair pathway. Deletion of *recBCD* attenuated *Salmonella* in the mouse competition assay to such a level that it was difficult to accurately measure additional defects and we could draw no conclusions from double mutants (data not shown). We then asked genetically if SodCI participates with RuvAB to protect DNA. In i.p. competition assays the *ruvAB* mutant was 150-fold attenuated compared to the wild-type strain (Table 3.5). This mutant was also 10-fold attenuated when competed against the wild type strain in vitro (Table 3.3). As a control, we examined the effect of loss of SodB in the *ruvAB* background. As expected, *sodB* was synthetic with *ruvAB*, conferring a 36-fold defect in this background (Table 3.5). We then tested the phenotype of a *sodCI* mutant in the *ruvAB* background. In this case *sodCI* was 5-fold attenuated in the *ruvAB* background, indicating that SodCI acts independently of RuvAB (Table 3.5). Thus, in contrast to the results obtained above, these results suggest that SodCI acts independently of recombinational repair.

Taken together, the data above suggest that the synthetic phenotype observed in the *recA sodCI* double mutant is not a result of the defect in recombinational repair. The RecA protein also plays a regulatory role in the cell, indirectly controlling gene expression, mainly via LexA and the SOS response. It was possible that the synthetic phenotype was the result of the lack of SOS induction rather than the loss of recombinational repair. Therefore, we tested the role of SOS in resistance to phagocytic superoxide. We introduced a non-inducible *lexA* allele (*lexA3*; (50)) into serovar Typhimurium. To confirm the presence and phenotype of this allele, we created an *sulA*$^{+}$-*lac*$^{+}$ transcriptional fusion (117) and transduced this fusion construct into isogenic wild type and *lexA3* strains. As a control, we transduced the Δ*recA*::Tc allele into the wild type background. As shown in Table 3.6, the *sulA* fusion in the wild type background was highly induced in response to nalidixic acid treatment, whereas, as expected, neither the *recA* or *lexA3* strains were capable of inducing the SOS-dependent fusion.

The *lexA3* strain was fully virulent in a competition assay (Table 3.5). These data suggest that *Salmonella* is not overcoming significant DNA damage in order to survive in the host. If the synthetic phenotype observed between *sodCI* and *recA* in the animal was due to the inability of the *recA* strain to induce SOS, then *sodCI* should also show a synthetic phenotype in the *lexA* non-inducible strains. This was not observed. As shown in Table 3.5, *sodCI* conferred its normal phenotype in the *lexA3* background. Thus, the increased sensitivity to phagocytic superoxide observed in a *recA* mutant is apparently independent of both SOS induction and recombinational repair.

3.2.5 Sensitivity to in vitro generated superoxide and hydrogen peroxide

The data above suggest that the most vulnerable target of phagocytic superoxide is extracytoplasmic. But identifying the target(s) is complicated by the fact that *sodC* mutants of *Salmonella* show no significant in vitro phenotypes. As shown in Figure 1A, there was no significant difference in sensitivity to 250 μM Xanthine/Xanthine oxidase of the wild type and isogenic mutants devoid of periplasmic SODs or cytoplasmic SODs. We have performed similar experiments using this and other superoxide-generating systems under a variety of conditions and have never observed a reproducible difference between the wild type and *sodCI sodCII* double mutant. This should not be surprising. It is estimated that NADPH oxidase is capable of producing a steady state superoxide concentration of 100 μM in a phagosome (219). Using published data (33, 296), one can calculate that in vitro systems such as that used above are capable of generating only <1 μM of superoxide for a few minutes. When higher concentrations (2.5 mM) of hydrogen peroxide are used, the *sodA sodB* double mutant does show increased sensitivity (Figure 1B), as previously reported (55). The *sodCI sodCII* mutant behaves identically to wild type, again distinguishing loss of cytoplasmic and periplasmic SOD activity.

3.3 Discussion

The phagocytic NADPH oxidase plays a central role in the antimicrobial arsenal of the innate immune response. This is evidenced by the increased susceptibility of both humans and mice that lack the NADPH oxidase to a variety of bacterial infections, including *Salmonella* (264, 390, 405). The periplasmic superoxide dismutase SodCI in *Salmonella* specifically protects the bacterium from phagocytic superoxide. Starting with

this premise, and taking advantage of the genetic power of a competition assay, we tested the common assumption that the primary target of the phagocytic oxidative burst is the bacterial DNA or other cytoplasmic target. Our results show that SodCI acts independently of SodA and SodB, strongly suggesting that phagocytic superoxide is not gaining access to the cytoplasm. Furthermore, we could exclude DNA as a target of phagocytic superoxide based on the lack of genetic interaction between *sodCI* and mutants of BER, *xthA nfo*, and homologous DNA repair, *ruvAB*. These data provide evidence that phagocytic superoxide specifically damages an extracytoplasmic target to inhibit or kill *Salmonella*. To our knowledge, this is the first evidence that such an extracytoplasmic target exists, although it could be inferred by the periplasmic localization of the superoxide dismutase.

SodCI is protecting against direct damage by superoxide, given that the periplasmic superoxide dismutase will decrease the steady state concentration of superoxide but not ultimately change the yield of hydrogen peroxide or other downstream reactive oxygen species. Our results do not address the potential damage to cytoplasmic targets, including DNA, caused by these downstream reactive oxygen species. However, in vivo evidence for such damage is surprisingly limited, and our results suggest that simply showing that mutants that are sensitive to cytoplasmic oxidative stress are attenuated in the host is not necessarily meaningful. Indeed, several studies suggest that the cytoplasm is not under increased oxidative stress during infection. Serovar Typhimurium strains mutant in *soxS* (124) or *oxyR* (376), whose products regulate adaptation to cytoplasmic oxidative stress (365), are fully virulent in an animal model, as are mutants incapable of inducing LexA-dependent SOS (Table 3.5). Strains lacking

catalase (*katE katG*; (48)) or alkyl hydroperoxide reductase (*ahpCF*; (376)) are also fully virulent, suggesting that there is enough redundancy in these peroxide scavenging systems to the keep the cytoplasmic H_2O_2 level below 5 µM during growth in the host (307). Schloss-Silverman *et al*. measured mutant frequency and plasmid nicking in *E. coli* and serovar Typhimurium recovered from the J774 macrophage line. *E. coli* showed increased DNA damage, but serovar Typhimurium did not (345). Moreover, it is well established that cytoplasmic superoxide or hydrogen peroxide damage key enzymes in metabolic pathways at concentrations below that required to cause significant DNA damage (190), leading to, for example, aromatic amino acid auxotrophy and blocks in the TCA cycle, both of which are known to significantly attenuate *Salmonella* (188, 409). Taken together, these results suggest that those *Salmonella* cells that survive in the host are not experiencing cytoplasmic oxidative stress or significant oxidative DNA damage.

Our results seem to exclude any cytoplasmic damage from phagocytic superoxide, yet, in contrast to the results with base excision repair and *ruvAB*, we observed a genetic interaction between periplasmic superoxide dismutase and RecA. It must be noted that RecA has multiple roles in the cell in addition to its direct action in recombination. Most importantly, RecA controls the expression of more than 40 genes. Most of these are under the control of the LexA repressor, but we could show that the genetic interaction with SodCI is not dependent on the LexA-dependent SOS response. However, RecA is also known to regulate a number of genes/proteins independently of LexA. These include: 2-keto-4-hydroxygluterate aldolase, required for recovery of a respiratory block induced by UV irradiation (59); DinY (308); and the universal stress proteins UspA, UspC, UspD, and UspE (226), which have multiple roles in the cell, including effects on

extracytoplasmic processes (289). Lesca et al. (240) noted 14 proteins by two-dimensional gel electrophoresis that were induced in a RecA-dependent fashion but independent of LexA. The simplest explanation of our results is that in the *recA* mutant pleiotropic effects result in increased sensitivity to extracytoplasmic superoxide.

Salmonella is one among a small number of organisms that can survive in macrophages. At least part of the *Salmonella* survival mechanism is the redirection of the phagocytic oxidase via the SPI2 type three secretion system, reducing the amount of enzyme delivered to the *Salmonella* containing vacuole (144, 351, 385). In contrast, the vast majority of bacterial species engulfed by a macrophage are efficiently killed. The fact that *sodCI* mutants are significantly attenuated despite the prowess of *Salmonella* to survive in macrophages suggests that the extracytoplasmic target of superoxide is the most vulnerable target to the phagocytic oxidative burst. There is no reason to think that our results do not apply to bacterial killing in general. Even for bacteria that do not interfere with normal phagocytic function, extracytoplasmic damage by superoxide could be the primary vulnerability to the oxidative burst in macrophages.

Segal and colleagues have proposed that, in neutrophils, the role of superoxide production by the phagocyte oxidase is not to kill bacteria per se, but rather to deliver electrons into the phagosome. This stimulates an influx of K^+ and a rise in the pH to ~7.5, which are required for the activity of granule proteases, proposed to be the ultimate cause of bacterial death (321, 322). One could invoke similar arguments to explain our results, but they do not withstand criticism. *Salmonella* grows in macrophages rather than neutrophils (328, 335) and the overall killing mechanisms differ between the two phagocytic cell types (292). For example, the pH of the SCV in macrophages is known

to be between 4 and 5 (318) and macrophage proteases are active under acidic conditions. More importantly, if the sole function of producing superoxide is to introduce electrons into the phagosome, then the periplasmic superoxide dismutase, SodCI, should be irrelevant. Dismutation of superoxide to H_2O_2 does not change the electron flux.

We currently do not know what the extracytoplasmic target of superoxide could be. The known biological molecules damaged directly by superoxide are quite limited and include certain dehydratases containing solvent exposed iron sulfur clusters (190). No enzymes of this class are known to be localized to the periplasm. Superoxide does not damage proteins per se, nor is it expected to react with bacterial membranes (190). Other cytoplasmic enzyme targets exist, but the mechanism of damage is not clear (190). Identification of the target will have to await further investigation.

3.4 Figures and Tables

Table 3.1 Competition assays with SOD mutants in BALB/c mice.

Strain A	**Strain B**	**Median CI**	**# of Mice**	***p*[a]**	**Fold Attenuated[b]**
sodCI	wt	0.13 †	21	<0.0005	8
sodA	wt	1.11 §	11	NS	*
sodB	wt	0.24 ‡	10	<0.0005	4
sodA sodB	wt	0.0013	9	<0.0005	770
sodA sodB	*sodB*	0.0029[c]	10	<0.0005	345
sodCI sodA sodB	*sodA sodB*	0.20[d]	5	<0.0005	5

[a] Student's t-test comparing CI versus inoculums; NS or *, Not significant; [b] Reciprocal of median CI; [c] Significantly different (p<0.0005) versus §; [d] Not significantly different (p>0.05) versus †.

Table 3.2 Competition assays in phox-/- and iNOS2 -/- mice.

Mouse Genotype	Strain A	Strain B	Median CI	# of Mice	p[a]	Fold Attenuated[b]
C57BL/6	*sodCI*	wt	0.18 ¶	21	<0.0005	5
C57BL/6 phox-/-	*sodCI*	wt	1.33[c]	5	NS	*
C57BL/6 iNOS2-/-	*sodCI*	wt	0.13[d]	6	<0.0005	8
C57BL/6	*sodAB*	wt	0.0018	6	<0.0005	556
C57BL/6 phox-/-	*sodAB*	wt	0.012	6	<0.0005	83

[a] Student's t-test comparing CI versus inoculums; NS or *, Not significant; [b]Reciprocal of median CI; [c] Significantly different (p<0.0005) versus ¶; [d] Not significantly different (p>0.05) versus ¶.

Table 3.3 Competition assays in aerated LB cultures.

Strain A	Strain B	Median CI	# Samples	p[a]	Fold Attenuated[b]
sodCI sodCII	wt	0.96	5	NS	*
sodA sodB	wt	0.0002	5	<0.0005	5000
xthA nfo	wt	0.49	5	0.0047	2
ruvAB	wt	0.10	5	<0.0005	10
recA	wt	0.016	5	<0.0005	62

[a] Student's t-test comparing CI versus inoculums; NS or *, Not significant; [b]Reciprocal of median CI.

Table 3.4 Competition assays with BER mutants in BALB/c mice.

Strain A	Strain B	Median CI	# Mice	p[a]	Fold Attenuated[b]
xthA nfo	wt	0.24	9	0.0065	4
sodCI xthA nfo	*xthA nfo*	0.16[c]	4	0.0006	6
sodB xthA nfo	*xthA nfo*	0.065[d]	9	<0.0005	15

[a] Student's t-test comparing CI versus inoculums; NS or *, Not significant; [b]Reciprocal of median CI; [c] Not significantly different (p>0.05) versus †, Table 1; [d] Significantly different (p<0.03) versus ‡, Table 1.

Table 3.5 Competition assays with recombination deficient strains in BALB/c mice.

Strain A	Strain B	Median CI	# of Mice	p[a]	Fold Attenuated[b]
recA	wt	0.019	13	<0.0005	56
sodB recA	*recA*	0.0041[c]	3	<0.0005	244
sodCI recA	*recA*	0.031[d]	12	<0.0005	32
ruvAB	wt	0.0064	4	<0.0005	156
sodB ruvAB	*ruvAB*	0.028[e]	5	0.0005	36
sodCI ruvAB	*ruvAB*	0.19[f]	9	<0.0005	5
lexA3	*wt*	1.42	4	NS	*
sodCI lexA3	*lexA3*	0.22[f]	4	0.0029	5

[a] Student's t-test comparing CI versus inoculums; NS or *, Not significant; [b]Reciprocal of median CI; [c] Significantly different (p=0.001) versus ‡, Table 1; [d] Significantly different (p=0.0008) versus †, Table 1; [e] Significantly different (p=0.05) versus ‡, Table 1;[f] Not significantly different (p>0.05) versus †, Table 1.

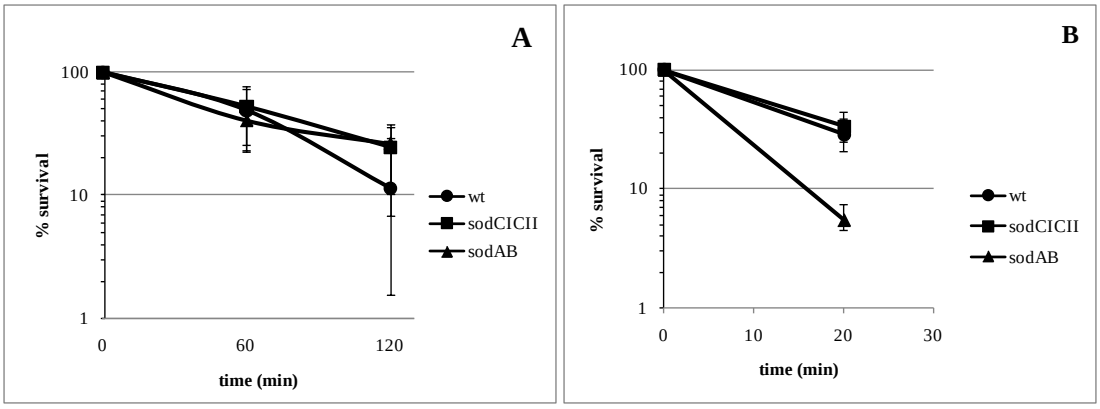

Figure 3.1 In vitro sensitivity to reactive oxygen species.

Effect of SOD mutations on survival when exposed to exogenously supplied reactive oxygen species. A) Following treatment with 0.25mM hypoxanthine and 0.1u/ml xanthine oxidase, the change in viable count is compared to untreated samples. B) Survival following exposure to 2.5mM H_2O_2 is determined by viable count of treated samples compared to viable count of untreated samples. The mean and range are plotted for both.

Table 3.6 Confirmation of *lexA3* uninducible allele.

Strain	**β-gal Activity**	
	-Nal	**+Nal**
WT	51.3 ± 0.74	1491.8 ± 30
recA	47.3 ± 0.23	20.7 ± 0.65
lexA3	44.2 ± 0.83	31.2 ± 1.6

β-galactosidase activity units are defined as (μmol of ONP formed/min) x 10^6/ (OD_{600} x ml of cell suspension) and are reported as mean ± standard deviation where number of measurements, n=4.

Chapter 4: The role of respiration in *Salmonella typhimurium* virulence in mice

The mammalian intestine is the focus of infection by *Salmonella typhimurium*. Gastroenteritis can progress to a more serious systemic infection in susceptible hosts. The environment *S. typhimurium* experiences in the small intestine has not been characterized in great detail. *S. typhimurium* is a facultative anaerobe that can respire on a variety of terminal electron acceptors in addition to oxygen. We asked what role the anaerobic respiratory chain plays in the pathogenesis of *Salmonella* in mice. We found that mutants that could not respire anaerobically were fully virulent, even in the small intestine. Furthermore aerobic respiratory chain mutants *cyoABCD* and *cydAB* were attenuated both in the small intestine and systemically. Together these results indicate that *Salmonella typhimurium* grows in aerobic environments in the murine host.

4.1 Introduction

Salmonella typhimurium is a facultative intracellular pathogen that is a leading cause of food borne illness. It causes infections ranging from self-limiting gastroenteritis to acute systemic infection in susceptible hosts (58, 268). Following ingestion, *S. typhimurium* travels through the small intestine until it reaches the distal ileum, where environmental signals trigger the expression of virulence factors that facilitate invasion of the intestinal mucosa (119). Following invasion, *S. typhimurium* is taken up by macrophages residing in the mucosal-associated lymphatic tissue of the small intestine (58, 205). Infected macrophages disseminate bacteria throughout the body (51), and *S.*

typhimurium mutants that cannot survive in macrophages fail to establish systemic infections (131, 328, 335).

The molecular mechanisms S. typhimurium employs to breach the intestinal epithelia have been studied, but the environmental conditions the bacterium encountered are not as well characterized. Measurements in live mice have shown that the concentration of oxygen decreases as distance from the stomach increases, indicating that microaerobic conditions are likely found in the distal small intestine (181). This is consistent with evidence that low oxygen concentrations are a signal for invasion (14, 236). Like *E. coli*, *S. typhimurium* has a flexible respiratory chain utilizing several terminal electron acceptors besides oxygen. In addition to those acceptors utilized by *E. coli*, *S. typhimurium* is capable of reducing tetrathionate and thiosulfate (153, 184). Anaerobic respiration has been assumed to be important for survival in the intestine due to low oxygen concentrations (121, 359), but this has not been definitively demonstrated.

The two major regulators that control metabolic changes that occur as cells sense decreasing oxygen concentrations are FNR and ArcBA. FNR is a global regulator important for transitioning to anaerobic conditions (16, 174, 208, 215, 224, 256), and it positively regulates several terminal oxidases of the anaerobic respiratory chain (399). ArcBA is a two-component regulator that is activated in the transition to microaerobic conditions (3, 18, 153, 248, 330) and is involved in the transcriptional regulation of many genes, including the high oxygen affinity terminal oxidase encoded by *cydAB* (84, 383). ArcBA and FNR work separately and coordinately to alter gene expression in order to adapt to low oxygen conditions (256).

In both aerobic and anaerobic respiration, dehydrogenases transfer electrons from their substrate to the quinone pool, which in turn transfers electrons to a terminal electron acceptor via a terminal oxidoreductase. Two regulators, FNR and ArcAB, play important roles in the regulation of respiratory chain components. FNR is an oxygen sensor that regulates the switch between aerobic and anaerobic metabolism (215, 302). It is only active under anaerobic conditions (16). ArcBA is a two-component regulator that senses the redox state of the quinone pool (155). It becomes activated under microaerobic conditions and stays active under anaerobic conditions (154). *Salmonella* and *E. coli* have two NADH-quinone oxidoreductases. NADH dehydrogenase I, encoded by the *nuo* locus, is a proton pump that couples the free energy released in the transfer of electrons from NADH to the quinone pool to proton translocation across the inner membrane (153). The *nuo* operon is negatively regulated by ArcBA and positively regulated by NarXL, in the presence of nitrate (38). NADH dehydrogenase I is active under aerobic conditions, but it is also used for respiration on some alternative electron acceptors (53, 381). NADH dehydrogenase II expression is positively regulated by Arc under aerobic conditions (382), and FNR represses transcription of *ndh* under anaerobic conditions (162, 361). Quinone synthesis is regulated in response to available terminal electron acceptors. Ubiquinone synthesis is highest in the presence of oxygen (156), menaquinone synthesis is negatively regulated in the presence of oxygen (23, 386), and demethylmenaquinone is expressed under anaerobic conditions in the presence of nitrate (386). Terminal oxidoreductases transfer electrons from the quinone pool to an electron acceptor. In *Salmonella* and *E. coli* two types of quinol oxidases transfer electrons to oxygen, quinol oxidase bd_3 encoded by *cydAB*, and quinol oxidase bo_3 encoded by

cyoABCD. Expression of these enzymes is regulated in response to the availability of oxygen by FNR and ArcBA (76, 77). Quinol oxidase bo3 has a lower affinity for oxygen and is maximally expressed under aerobic conditions (83, 325), while quinol oxidase bd3 has a higher affinity for oxygen and is maximally expressed under microaerophilic conditions (84, 383).

Previously published reports do not give a clear indication of the growth conditions *S. typhimurium* faces in the host. Fink et. al. showed that *fnr* mutants were attenuated both orally and in macrophages (133), but ArcA was shown to not be needed for virulence of *Salmonella* in oral infection of mice (253). Here we examine the role of both anaerobic and aerobic respiration in virulence of *S. typhimurium* in mice. We show that a mutant deficient in anaerobic respiration, *moaDE nrfA frdA*, retains full virulence in both the oral and systemic phases of infection. Similarly, an obligately aerobic mutant, *nrdDG*, retains full virulence in both the oral and systemic phases of infection. We also show that a *fnr* mutant is fully virulent both orally and systemically, in contrast to published reports. Furthermore, mutations in the aerobic respiratory chain components cytochrome oxidases bo3 and bd3, encoded by *cyoABCD* and *cydAB*, are attenuated. This suggests that in the normal course of infection in mice, *S. typhimurium* only grows in aerobic environments.

4.2 Results

4.2.1 Anaerobic respiration is not required for *S. typhimurium* infection of the mouse

Salmonella typhimurium is capable of utilizing several compounds for anaerobic respiration (153). Enzymes involved in reduction of thiosulfate, TMAO, DMSO, tetrathionate and nitrate utilize a molybdenum-containing cofactor, and they are therefore inactive in a strain that does not produce molybdopterin (8, 267). By deleting genes that encode nitrite reductase and fumarate reductase, along with genes in the molybdopterin biosynthesis pathway, we constructed a mutant that could not perform anaerobic respiration, *moaDE nrfA frdA*. The inability of this strain to grow anaerobically on glycerol with the above listed terminal electron acceptors was confirmed (Figure 4.1). Competitive virulence assays were preformed in Balb/c mice both orally and intraperitoneally. The mutant deficient in anaerobic respiration did not display a virulence defect either in the small intestine or in the spleen (Table 4.1). This suggests that *S. typhimurium* is not utilizing anaerobic respiration in the murine host.

We then asked if other mutations that affected growth under anaerobic conditions were required for virulence. FNR is a global regulator that controls the transition to anaerobic metabolism (161, 208). A *fnr* mutant was constructed, and its effect on a gene known to be FNR-regulated was tested (Figure 4.2) The growth of this strain was tested in an *in vitro* competition assay against the wild type strain. Our *fnr* mutant did not have a growth defect under aerobic *in vitro* conditions, but a growth defect was observed under anaerobic conditions (Table 4.2). When this competition was performed in Balb/c mice intraperitoneally, the *fnr* mutation did not confer a survival disadvantage in the spleen.

An oral infection showed loss of FNR did confer a slight virulence defect in the small intestine (Table 4.1). Since the *fnr* mutant showed a large competitive disadvantage under *in vitro* anaerobic conditions in rich media, but it was only slightly attenuated in oral inoculation of mice, this raised the possibility that *Salmonella* is not growing in an anaerobic environment in the small intestine.

4.2.2 Salmonella does not grow in an anaerobic environment in the host

To ask if *Salmonella* is growing in an anaerobic environment in the small intestine, an obligately aerobic mutant was constructed. Ribonucleotide reductase is required for the last step of deoxynucleotide biosynthesis. The enzyme encoded by *nrdDG* only functions anaerobically, leaving the enzyme encoded by *nrdAB* to function aerobically (136, 149). Table 4.2 shows that a *nrdDG* mutant has no phenotype aerobically, but it does not grow anaerobically on rich media. Published data shows that purine or pyrimidine auxotrophs of *Salmonella* cannot survive in the host, indicating that *de novo* synthesis is required for survival (131). Therefore if a *nrdDG* mutant does not have a virulence defect in the host, *Salmonella* is not growing in an anaerobic environment.

We asked what effect loss of *nrdDG* would confer on *S. typhimurium* in both orally and intraperitoneally administered competitive virulence assays. In both cases the *nrdDG* mutant was fully virulent, even in the large intestine, which is anaerobic (Table 4.3). This suggests that *S. typhimurium* is not growing in anaerobic environments in the host and that bacteria not associated with aerobic host tissue are not replicating.

4.2.3 Aerobic respiratory chain components are required for full virulence

The above data indicate that *Salmonella* is not growing anaerobically in the murine host, so we attempted to confirm that it is growing aerobically. If *S. typhimurium* is growing aerobically in the host, loss of components of the aerobic respiratory chain would negatively impact virulence. We asked if this is occurring in both the gut and in the systemic phase of infection.

NADH dehydrogenase I is encoded by *nuoA-N*, and NADH dehydrogenase II is encoded by *ndh* (153). Both are used under aerobic conditions, but NADH dehydrogenase II predominates in fully aerobic conditions (360, 361). Table 4.4 shows that a *nuoA-N* mutant has a growth defect compared to wild type when grown aerobically, but not anaerobically. Similarly an *ndh* mutant has a substantial growth defect compared to wild type when grown aerobically but not when grown anaerobically. We then asked what the effects of these mutations would be in the host. Table 4.5 shows that in orally administered competitive virulence assay a *nuoA-N* mutant is attenuated 10-fold compared to wild type in the small intestine, while an *ndh* mutant is 4-fold attenuated. This suggests that both NADH dehydrogenase I and II are important for survival in the small intestine.

We asked what the effect of loss of the aerobic quinol oxidases would be. Quinol oxidase bd_3 encoded by *cydAB*, and quinol oxidase bo_3 encoded by *cyoABCD*, were deleted, and the mutations were tested by *in vitro* and *in vivo* competition assays. Both the *cyoABCD* and *cydAB* mutants displayed growth defects in aerobic *in vitro* competitions but not in anaerobic competitions (Table 4.4). This is consistent with the role these enzymes play in aerobic respiration. In *in vivo* competitions in mice the

cyoABCD mutant did not have an appreciable virulence defect in th intestine when administered orally, but it did display a defect systemically (Table 4.5). The *cydAB* mutant was significantly attenuated in the small intestine. This is consistent with the idea that microaerobic conditions may exist in the small intestine (181). However, it is puzzling that both the bo and bd oxidases contribute to virulence systemically, since they are regulated differently, have different affinities for oxygen, and contribute to the ΔpH gradient in diffrerent ways.

4.3 Discussion

In this study we conducted experiments designed to identify components of the *Salmonella typhimurium* respiratory chain required for virulence both in the gut and in the systemic phase of infection. We used competitive virulence assays to assess the effect of disruption of terminal oxidoreductases and found that those specific for terminal electron acceptors other than oxygen were dispensable for virulence in the small intestine and spleen. We also found that an obligately aerobic mutant did not have a virulence defect in the small intestine. When considered with the phenotypes of cytochrome oxidase mutants, this strongly suggested that *S. typhimuruim* replicates in the presence of oxygen in the small intestine. The lack of a phenotype in the large intestine is more surprising since it is presumed to be anaerobic (181). This is likely because *Salmonella* is not replicating in the large intestine, possibly because of the large number of anaerobic bacteria already residing there (343).

Salmonella typhimurium is a common food-borne pathogen of mammals, but the metabolic requirements of the bacterium in the host are not currently well understood.

Recent studies have made direct measurements of the physical environment of the murine gastrointestinal tract that shed light on environmental signals that may be important for virulence. Direct measurements of oxygen concentration in live mice confirms that oxygen concentrations decrease along the length of the intestine, with microaerobic conditions in the small intestine (181). This is consistent with previous reports that the full TCA cycle, not the reductive path, is needed for virulence (377), because this occurs under aerobic conditions (153). Respiratory hydrogenases have been shown to be important for virulence in *S. typhimurium*(258), but since one of these hydrogenases is active under aerobic conditions, that observation is consistent with the data presented here. Winter *et. al.* showed that gut inflammation caused by infection with *S. typhimurium* provided tetrathionate, which could be utilized as a terminal electron acceptor in the cecum. However this only affected survival of bacteria associated with tissue below the mucus layer, because there was no significant difference in virulence between a tetrathionate reductase mutant and wild type when total numbers recovered from the organ were examined. A tetrathionate reductase mutant was not attenuated in an oral competition assay recovered from the spleen (406). This could be because tetrathionate reduction was shown to affect colonization of the cecum, which is distal to the Peyer's patches where invasion occurs.

Work has been done in *E. coli* to identify the growth substrates this organism uses in the host intestine (206). Jones et. al. found that aerobic respiration was important for colonization of the intestine by *E. coli*, but this group focused on the cecum, which is distal to the primary site of invasion by *S. typhimurium* (58). They also detected nitrate in cecal mucus which served as a substrate for anaerobic respiration. Similarly, both

ArcA and FNR were shown to be needed for efficient colonization. This is in contrast to the findings presented here where in *S. typhimurium* infection neither anaerobic respiration nor regulators of anaerobic respiration were needed for growth in the host.

Our findings that aerobic, but not anaerobic, respiration is important for the survival of *S. typhimurium* in the mouse intestine raise the question of if other organisms present in the mouse small intestine are growing aerobically. At present the normal flora is still not very well characterized. A fluorescence based *in situ* hybridization study found that few bacterial species were present in the ileum of the healthy mouse intestine, and that these organisms were not associated with the mucosal tissue. In addition this groups finds suggest that there is not a defined, stable bacterial population in the healthy mouse ileum, in contrast to the populations found in the large bowel (374). Culturable bacteria were examined in another study, and *Lactobacillus* species and *E. coli* were recovered from the small intestine but not in high numbers (316). These organisms are facultative anaerobes, like *Salmoenlla*, and an investigation of the similarities and differences in the metabolisms of these organisms may reveal much about growth substrates available in the small intestine.

4.4 Figures and Tables

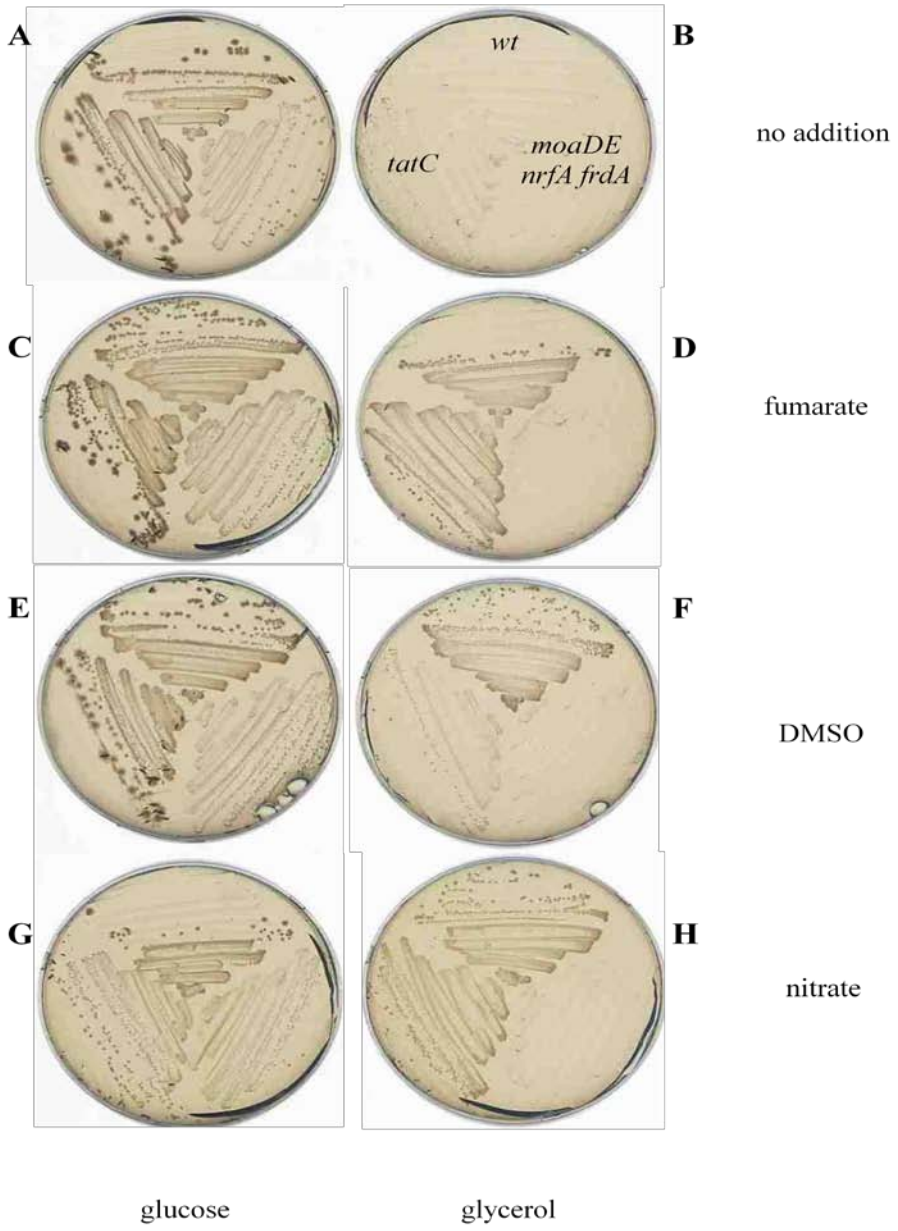

Figure 4.1 Growth on anaerobic media with terminal electron acceptors. The strains streaked are wild type, *tatC* and *moaDE nrfA frdA*. The pattern labeled on plate (B) is the same on all plates. Plates are EZ minimal media, with either 0.4% glucose or 0.4% glycerol and the indicated terminal electron acceptor added, incubated at 37°. The *moaDE nrfA frdA* strain cannot perform respiration on any electron acceptor provided.

Table 4.1 Effects of anaerobic respiratory chain mutations in competition assays against wild type in Balb/c mice.

Relevant genotype	Method of Inoculation	Organ	Median CI	Number of mice	P[a]
moaDE nrfA frdA	oral	spleen	1.30	5	NS
	oral	small intestine	4.07	5	NS
	I.P.	spleen	1.96	10	NS
fnr	oral	spleen	1.29	11	NS
	oral	small intestine	0.54	11	0.029
	I.P.	spleen	1.22	8	NS

[a]Student's t-test comparing CI versus inoculums.

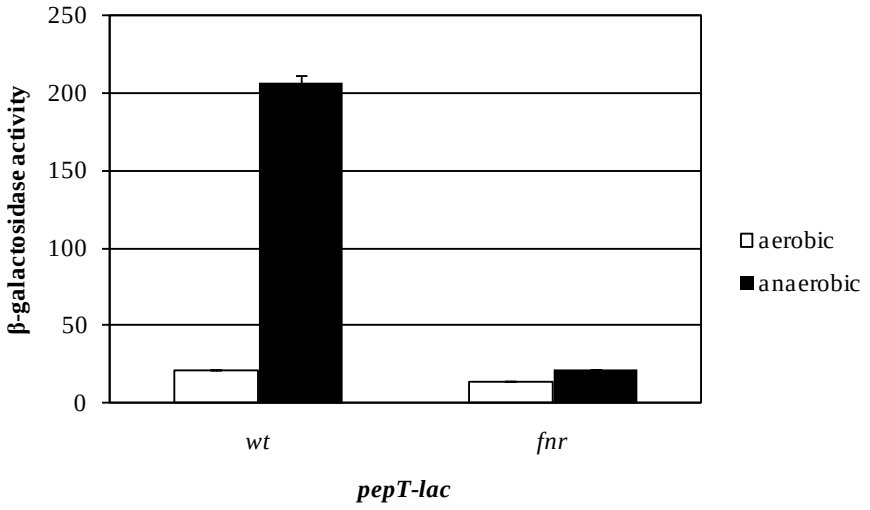

Figure 4.2 β-galactosidase activity of pepT-lac fusion strains. Cells were assayed after growth in LB under aerobic or anaerobic conditions for the effect of *fnr*::Cm mutation on *pepT* expression. β-galactosidase activity units are defined as (μmol of ONP formed/min) x 10^6/ (OD_{600} x ml of cell suspension) and are reported as mean ± standard deviation where number of measurements, n=4.

Table 4.2 Competitions *in vitro* between mutations that affect anaerobic growth and wild type.

Relevant genotype	**Environmental Conditions**	**Culture Medium**	**Median CI**	**Number of Flasks**	**P[a]**
fnr	Aerobic	LB	0.89	4	NS
fnr	Anaerobic	LB	0.011	4	<0.0005
nrdDG	Aerobic	LB	1.6	4	0.006
nrdDG	Anaerobic	LB	<0.00019*	4	<0.0005

[a]Student's t-test comparing CI versus inoculums *****If no mutants were recovered, the competitive index calculation was performed as if one mutant colony were recovered.

Table 4.3 *In vivo* competitions between an obligately aerobic mutant and wild type.

Relevant genotype	**Method of Inoculation**	**Organ**	**Median CI**	**Number of Mice**	**P[a]**
nrdDG	oral	spleen	2.65	5	0.003
	oral	small intestine	0.85	6	NS
	oral	cecum	1.24	6	NS
	oral	large intestine	0.75	7	NS
	I.P.	spleen	1.28	9	NS

[a]Student's t-test comparing CI versus inoculums.

Table 4.4 *In vitro* competitions between aerobic respiratory chain mutants and wild type.

Relevant genotype	**Environmental Conditions**	**Culture Medium**	**Median CI**	**Number of Flasks**	**P[a]**
nuoA-N	Aerobic	LB	0.32	4	<0.0005
	Anaerobic	LB	1.15	4	NS
ndh	Aerobic	LB	0.050	4	<0.0005
	Anaerobic	LB	0.93	4	NS
cyoABCD	Aerobic	LB	0.45	4	0.0009
	Anaerobic	LB	1.05	4	NS
cydAB	Aerobic	LB	0.0014	4	<0.0005
	Anaerobic	LB	0.41	4	0.02

[a]Student's t-test comparing CI versus inoculums.

Table 4.5 Effects of aerobic respiratory chain mutations in competition assays against wild type in Balb/c mice.

Relevant genotype	**Method of Inoculation**	**Organ**	**Median CI**	**Number of mice**	**P[a]**
nuoA-N	oral	spleen	0.11	7	0.010
	oral	small intestine	0.093	6	<0.0005
	I.P.	spleen	0.008	3	<0.0005
ndh	oral	spleen	0.15	6	NS
	oral	small intestine	0.25	4	NS
	I.P.	spleen	1.29	7	NS
cyoABCD	oral	spleen	0.15	4	0.042
	oral	small intestine	0.74	7	NS
	I.P.	spleen	0.097	4	<0.0005
cydAB	oral	spleen	0.0009	7	0.023
	oral	small intestine	0.043	4	<0.0005
	I.P.	spleen	0.050	5	<0.0005

[a]Student's t-test comparing CI versus inoculums.

Chapter 5: The role of the twin-arginine translocation (Tat) pathway in *Salmonella typhimurium* virulence in mice

The twin-arginine translocation (Tat) pathway allows folded proteins to be transported across the cytoplasmic membrane. Several of the known transport substrates are involved in anaerobic respiration, but other substrates have widely varying functions. Tat secretion plays a role in the virulence of many pathogens. However with the exception of Shiga toxin 1 in *E. coli* O157:H7, there has been little to link specific Tat substrates with a virulence phenotype. A bioinformatics survey of *Salmonella typhimurium* predicts that several proteins are exported via the Tat system. Through competitive virulence assays, we have determined that a mutant deficient for Tat mediated protein export is highly attenuated. We further examined the meaning of this result by knocking out every predicted Tat exported substrate and determining the virulence phenotype. As has been shown previously, the Tat exported NiFe hydrogenases are important for the oral phase of infection. Our results suggest that several Tat exported substrates have a role in systemic infection; however, changes to the cell envelope caused by loss of the Tat substrates SufI, AmiA, and AmiC apparently are the main cause of the observed virulence defect.

5.1 Introduction

Most proteins targeted for export to the cell envelope in *E. coli* and *Salmonella* cross the inner membrane as unfolded polypeptides via the Sec pathway (314). however,

proteins that contain cofactors, such as flavin or molybdopterin, are folded and assembled in the cytoplasm and then cross the inner membrane via the Twin Arginine Transport (Tat) system (348, 400). Substrates for this transport system have unusually long N-terminal signal sequences that contain a twin-arginine consensus sequence (S/TRRXFLK) (24, 60). In *E. coli*, the components required for a functional Tat translocase are TatA, TatB, and TatC (35, 340, 400). Substrates to be transported associate with complexes of TatBC, which then interact with the translocation channel formed by multiple subunits of TatA (2, 37, 157, 228, 234, 301).

The physiological consequences of mutations in the Tat transport system have been most extensively studied in *E. coli*. Since several anaerobic respiratory chain proteins are transported via the Tat system, anaerobic respiration utilizing certain electron acceptors is disrupted (103, 238). Also, Tat mutants have been observed to have impaired motility, septation defects and are sensitive to detergents and bile (198, 323, 362). In *E. coli* O157:H7, deletion of the Tat transport aparatus decreased secretion of flagellin and Shiga toxin 1, two known virulence factors (312). In other pathogens, the Tat system also plays a role in virulence. Mutants of *tatC* in *P. aeruginosa* were impaired in secretion of the virulence factor phospholipase C and were shown to be attenuated in respiratory infections (298), while in *Y. pseudotuberculosis tatC* mutants were attenuated in oral infections of mice, but no specific virulence factor was implicated (232). The plant pathogen *A. tumefaciens* was shown to be highly attenuated, but again, no molecular basis for this loss of virulence was described (108).

Salmonella typhimurium is a facultative intracellular pathogen that gains access to its host through contaminated food or water (134). It causes gastroenteritis or systemic

infection, depending on the susceptibility of the host (328, 335). The normal course of infection brings *S. typhimurium* into contact with the acidic environment of the stomach, then bile and decreased oxygen concentrations in the small intestine, and eventually the low pH and antimicrobial products present inside phagocytes (58, 334). Two type III secretion systems, encoded on *Salmonella* Pathogenicity Island 1 (SPI1) (332) and on *Salmonella* Pathogenicity Island 2 (SPI2) (349), are major virulence factors employed by this organism. The SPI1 type III secretion system facilitates invasion of M cells and non-phagocytic epithelial cells in the small intestine (88, 143, 252), while the SPI2 type III secretion system is required for growth and survival in macrophages (68, 183). Mutants that cannot survive in macrophages are unable to mount a systemic infection (131).

The Tat system of *S. typhimurium* has been shown to be important for virulence in mice, but numerous substrates are predicted to be transported in this organism (103), and the substrate or substrates responsible for the virulence were not identified (323). In this study we show that the virulence defect of a *tatC* mutant of *S. typhimurium* is due to outer membrane defects associated with failure to transport three Tat substrates: AmiA, AmiC, and SufI. Loss of all three is required to see the effect.

5.2 Results

5.2.1 *S. typhimurium* tatC mutants display pleiotropic morphological defects

We began this study by asking if the characteristics of a *tatC* mutant of *S. typhimurium* resembled those recorded for *E. coli*. Physiological changes have been observed in Tat mutants of *E. coli*, including SDS sensitivity, decreased motility, and decreased septation (198, 362). In addition, since the Tat system is required for export of

several cofactor-containing anaerobic respiratory chain proteins, *E. coli* Tat mutants are unable to grow anaerobically on non-fermentable carbon sources in the presence of the terminal electron acceptors TMAO, DMSO, or nitrite (103). We confirmed that disruption of *tatC* in *S. typhimurium* abolishes anaerobic growth in the presence of DMSO with glycerol as a carbon source (Figure 5.1). This is not a general growth defect under anaerobic conditions, since the *tatC* mutant grows well on glucose on these plates, and it is able to utilize fumarate as a terminal electron acceptor when grown on glycerol. Next we asked if motility was affected in this mutant. As has been observed previously (362), we saw that a *tatC* mutant is less motile than wild type (Figure 5.3). Less flagellin was detected on the surface of tatC mutants (323). The *tatC* mutant generated for this study displays the growth defects expected of this mutant.

We examined the effect of loss of Tat on the cell envelope of *S. typhimurium*. As has been observed previously in *S. enteriditis* (274) and *E. coli* (362), Tat mutants of *S. typhimurium* form chains of unseparated daughter cells and are sensitive to SDS. Figure 5.3 shows that a *tatC* mutant of *S. typhimurium* forms chains of unseparated cells. However, as was observed by others in *Salmonella*, the elongated cell phenotype we see here is different from chains of *E. coli*. Our cells do not have the indentions of incomplete septation seen in *E. coli*, which resemble a string of beads. This may represent some difference between these two organisms in the machinery of septation, but this is only speculation.

We also tested our *tatC* mutant for sensitivity to detergents. Figure 5.4 shows that *tatC* mutants are very sensitive to SDS compared to wild type. Approximately 130-fold more wild type are recovered than *tatC* when grown in 5% SDS. We also observed that

Tat mutants are sensitive to bile. When grown in aerated LB supplemented with 1% bile salts, 25-fold fewer *tatC* mutants are recovered compared to wild type after 18 hours growth. This confirms that the defect in the cell envelope observed in *E. coli* also occurs in *S. typhimurium*.

5.2.2 The Twin-Arginine transport pathway does not export the target damaged by phagocytic superoxide

In Chapter 3 of this work we showed that the periplasmic superoxide dismutase SodCI does not protect a cytoplasmic target from phagocytic superoxide. Published data indicates that the only known targets that can be directly damaged by superoxide are dehydratases, [4Fe-4S] cluster-containing proteins (135). Although no dehydratases are known to be exported to the periplasm, if an enzyme of this type were to be transported to the periplasm, it would have to exit the cytoplasm via the Tat pathway. Therefore we asked if the target of phagocytic superoxide is transported via the Tat pathway.

Since we assume that the function of SodCI is to protect an extracytoplasmic target from phagocytic superoxide, under conditions where the target cannot reach the periplasm, deletion of *sodCI* should confer no further virulence defect. If the target of phagocytic superoxide is exported via the Tat pathway, a *tatC* mutation should be epistatic to a *sodCI* mutation. In Table 5.1 the virulence defect confered by a *sodCI* mutation in both the wild type background and in the *tatC* background. The *sodCI* mutation confers the same virulence defect in both backgrounds, therefore *tatC* is not epistatic to *sodCI*, and the target of phagocytic superoxide is not transported via the twin-

arginine translocation pathway. However, we wanted to know why the Tat pathway is required for virulence.

5.2.3 A functional Tat secretion aparatus is required for virulence of *S. typhimurium*

It has been shown that *tatC* mutants of *S. typhimurium* are attenuated in both oral and I.P competition assays and showed impaired survival in J774 macrophages (323). To confirm this we tested our Tat mutants in IP competitions and oral competition assays in Balb/c mice. We found that a *tatC* mutant is highly attenuated in both oral and IP competition assays (Table 5.2). This result is not surprising since this mutation causes the mislocalization of many proteins, over thirty predicted for *S. typhimurium* (103). Since the tatC mutation results in several changes to cellular physiology, our next question was if the observed virulence defect is due to one of the general defects observed. Motility is affected in *tatC* mutants, but production of flagella has been shown to not be important for *S. typhimurium* virulence in the mouse model, so this is unlikely to be the cause of the virulence defect of *tatC* mutants (346). Similarly, in Chapter 4 of this work we presented evidence that *S. typhimurium* does not utilize anaerobic respiration in the murine host, so the impaired anaerobic respiration characteristic of Tat mutants is not likely to cause its observed virulence defect. In addition to detergent sensitivity, problems in cell division have been observed in *E. coli*, presumably due to mislocalization of the cell wall amidases AmiA and AmiC (198), and the Tat substrate SufI has also been shown to play a role in cell division (320, 375). Figure 5.2 shows that an *amiA amiC* double mutant has a slight septation defect compared to wild type,

similar to a *tatC* mutant. Table 5.3 shows that an *amiA amiC* mutant does not have a virulence defect in the systemic phase of infection. This suggests that impaired septation, in itself, does not have a significant impact on virulence. The pronounced virulence defect of a *tatC* mutant did not seem to correlate to the known physiological defects of the mutant.

5.2.4 A subset of Tat secreted proteins contribute to virulence of *S. typhimurium*

Genome-wide surveys have been performed for several organisms using available Tat signal sequence prediction programs, such as TatP. The analysis done for *S. typhimurium* has revealed approximately 30 putative Tat substrates (103). We generated mutations for most of the Tat substrates predicted in *Salmonella* and then asked what each individual predicted Tat exported protein contributed to virulence. We do not include here Tat substrates involved in anaerobic respiration because that topic is covered in chapter 4 of this thesis. The contribution of hydrogenase mutants to virulence has already been examined (258). These mutants are moderately attenuated, but they do not account for the full virulence defect of a *tatC* mutant (Table 5.2, 5.3). No one target displayed a large virulence defect when deleted. Indeed, some deletions appear to increase virulence (Table 5.3). It seemed possible that multiple deletions would have to be combined in order to approach the virulence defect observed in a *tatC* mutant, so we began constructing strains with multiple mutations based on perceived common function.

5.2.5 Three Tat substrates contribute to the cell envelope defect observed in Tat mutants

Since *amiA amiC* mutations in *E. coli* cause pleiotropic cell envelope defects (198, 362), we revisited this mutant. AmiA and AmiC, two *N*-acetylmuramyl-L-alanine amidases involved in septation, are exported to the periplasm via the Tat system (27, 182), and SufI is a cell-division protein that localizes to the septal ring (320, 375). We asked how an *amiA amiC sufI* would survive in the presence of SDS in *in vitro* competitions. Figure 5.4 shows that the triple mutant behaves more like a *tatC* mutant than like an *amiA amiC* mutant in high concentrations of SDS.

We then tested the virulence of an *amiA amiC sufI* mutant in mice. Individually these mutations did not confer a large virulence phenotype. Since these three Tat-exported proteins play a role in cell division, we asked if we could observe a synthetic phenotype in the triple mutant. The *amiA amiC* double mutant competes evenly with wild type in I.P. competition assays , and the *sufI* mutant may be 2-fold down in an I.P. competition (Table 5.3). Table 5.4 shows that the triple mutant *amiA amiC sufI* is about 60-fold down compared to wild type in an I.P. competition assay. This is a synthetic genetic interaction, and this phenotype is approaching that observed for a *tatC* mutant, which is approximately 220-fold down compared to wild type. To confirm that the majority of the *tatC* phenotype is due to mislocalization of AmiA, AmiC, and SufI, we asked what the effect of a *tatC* mutation would be in the *amiA amiC sufI* background. In the I.P. competition of *amiA amiC sufI tatC* versus *amiA amiC sufI*, the quadruple mutant is 4-fold down compared to the triple mutant. This indicates that most of the *tatC* virulence phenotype in systemic infection is due to loss of AmiA, AmiC, and SufI.

5.2.6 Loss of the Tat secretion aparatus affects type III secretion

In the plant pathogen *Pseudomonas syringae*, a *tatC* mutant decreased the translocation of a type III secreted effector by about 30% (42). Since type III secretion is important for *S. typhimurium* survival in the host, both in the intestine and systemically (185, 278, 349), we asked what effect a *tatC* mutation has on type III secretion. We can address the effect of a *tatC* deletion on SPI1 type III secretion, involved in invasion in the intestine, by examining the level of *hilA* transcription. The level of expression of SPI1 type III secretion system genes is directly dependent on the level of *hilA* expression (119), and deletion of *hilA* is equivalent to deletion of all of SPI1 (116). Figure 5.5 shows that deletion of *tatC* decreases *hilA* expression nearly 4-fold, and the triple mutant *amiA amiC sufI* decreases *hilA* expression approximately 2-fold. This level of effect on *hilA* expression may be enough to impact type III secretion and virulence, since deletion of *fliZ*, a regulator of SPI1 type III secretion, has a 4-fold effect on *hilA* expression and the mutant is approximately 60-fold down in oral competition assays in mice (66).

To test directly if a *tatC* mutation has an effect on SPI1 type III secretion, the effect of deletion of the entier SPI1 island on virulence was tested. If loss of a functional Tat secretion system negatively affects the SPI1 type III secretion system, deletion of SPI1 should have a reduced phenotype or no phenotype in the *tatC* background. In Table 5.5 we look at the effect of a *spi1* mutation on virulence in oral infections. As expected, a *spi1* mutant is about 4-fold down in oral competitions in the small intestine. We then asked if a *tatC* mutation is epistatic to the *spi1* mutation. We see that *tatC* is epistatic to the *spi1* mutation, because the *spi1* mutation no longer has a phenotype in the *tatC* background, compared to the phenotype observed in the wild type background. We see a

similar result with *spi1* in the *amiAC sufI* background, but the result is not as striking. This correlates with the intermediate effect we see on *hilA* expression in Figure 5.5.

We decided to look at SPI2 type III secretion as well, but instead of monitoring transcriptional fusions, we directly measured secretion of a tagged effector protein. We used a fusion of an N-terminal portion of SspH2, a known SPI2 type III secreted effector (272), to the catalytic domain of CyaA. The adenylate cyclase toxin Cya of *Bordetella perusssis* converts ATP to cAMP in the presence of calmodulin in host cells (176). cAMP is only produced if the fusion protein is translocated into host cells. We infected J774 macrophages with strains expressing this fusion and measured the level of cAMP after 6 hours. Figure 5.6 shows that translocation of the SspH2-CyaA fusion protein is decreased in the *tatC* background, but it is not abolished, since more cAMP is detected in this strain than in the negative controls. DsbA is required for type III secretion systems in *S. typhimurium* to be functional, so secretion is abolished in a *dsbA* mutant (118), and the LacZ-CyaA fusion protein is not competent for type III secretion.

5.3 Discussion

The Twin Arginine Transport system moves folded proteins across the cytoplasmic membrane (348, 400). It is present in the genomes of diverse bacteria (102, 103, 404, 408), and the number of substrates exported by this system varies greatly depending on the organism. In *Salmonella* and *E. coli* approximately 30 proteins are exported via Tat (102), and many of these proteins contain redox cofactors (24, 25). Mutants deficient in Tat transport are sensitive to detergents, have difficulty with cell division, and are decreased in motility (198, 323, 362). The Tat system has been shown

to be important for virulence in several organisms, and where a molecular mechanism has been offered, it is due to mislocalization of a secreted toxin (42, 298, 312, 333). Since *Salmonella* is not known to secrete toxins, the reason the Tat pathway is important for virulence in this organisms is less obvious.

It was shown for *E. coli* that an *amiA amiC* double mutant has a leaky outer membrane (362). This leads to sensitivity to detergents and is associated with septation that is arrested at a late stage. This differs from what was observed here in *S. typhimurium*. In *E. coli* the *amiA amiC* mutant showed SDS sensitivity comparable to a *tatC* mutant. However, in the current work, *sufI* also had to be deleted before comparable SDS sensitivity was achieved. In addition, Figure 5.2 shows that all of the mutants produced smooth elongated cells, unlike *E. coli*. The lack of indentation at the expected septum may indicate a difference in the cell division process between *Salmonella* and *E. coli*.

The mere observation that a mutant lacking the Tat pathway is attenuated is not interesting, per se, but there are over 4000 open reading frames in *S. typhimurium*, and it is not easy to evaluate each of these genes individually for their contribution to virulence in a host. Indeed, as we saw in Tables 5.3 and 5.4, individual deletions often cannot fully reveal the contribution of a gene to virulence. We were afforded an opportunity to observe the virulence defect of a mutant with a loss of function of a diverse set of proteins because they are transported by Tat. If we had not known the total virulence defect to expect, we would not have continued combining mutations looking for a synthetic genetic interaction. The motiviation for this investigation was to attempt to

identify the periplasmic target that is damaged by phagocytic superoxide, but when this failed, we were able to gain new insight into a fascinating protein transport pathway.

5.4 Figures and Tables

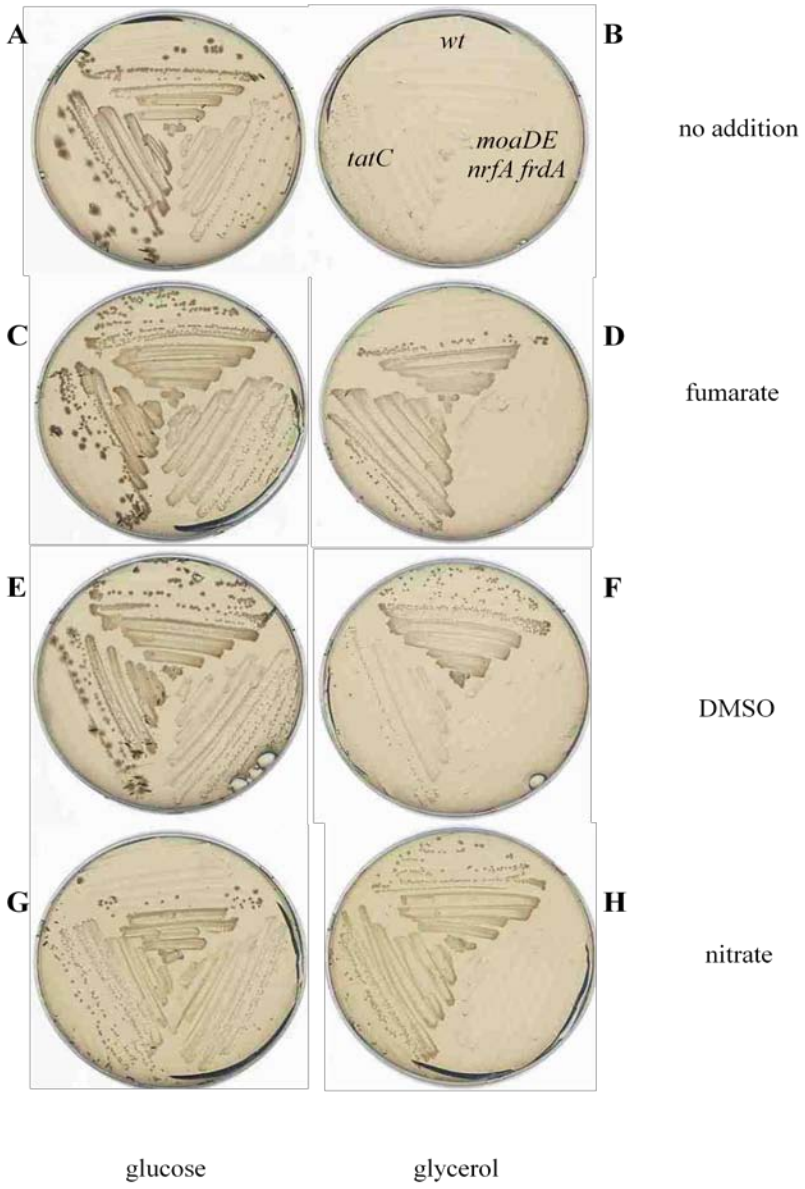

Figure 5.1 Growth of Tat mutants on anaerobic media. The strains streaked are wild type, *tatC* and *moaDE nrfA frdA*. The pattern labeled on plate (B) is the same on all plates. Plates are EZ minimal media, with either 0.4% glucose or 0.4% glycerol and the indicated terminal electron acceptor added, incubated at 37°. The *moaDE nrfA frdA* strain cannot perform respiration on any electron acceptor provided.

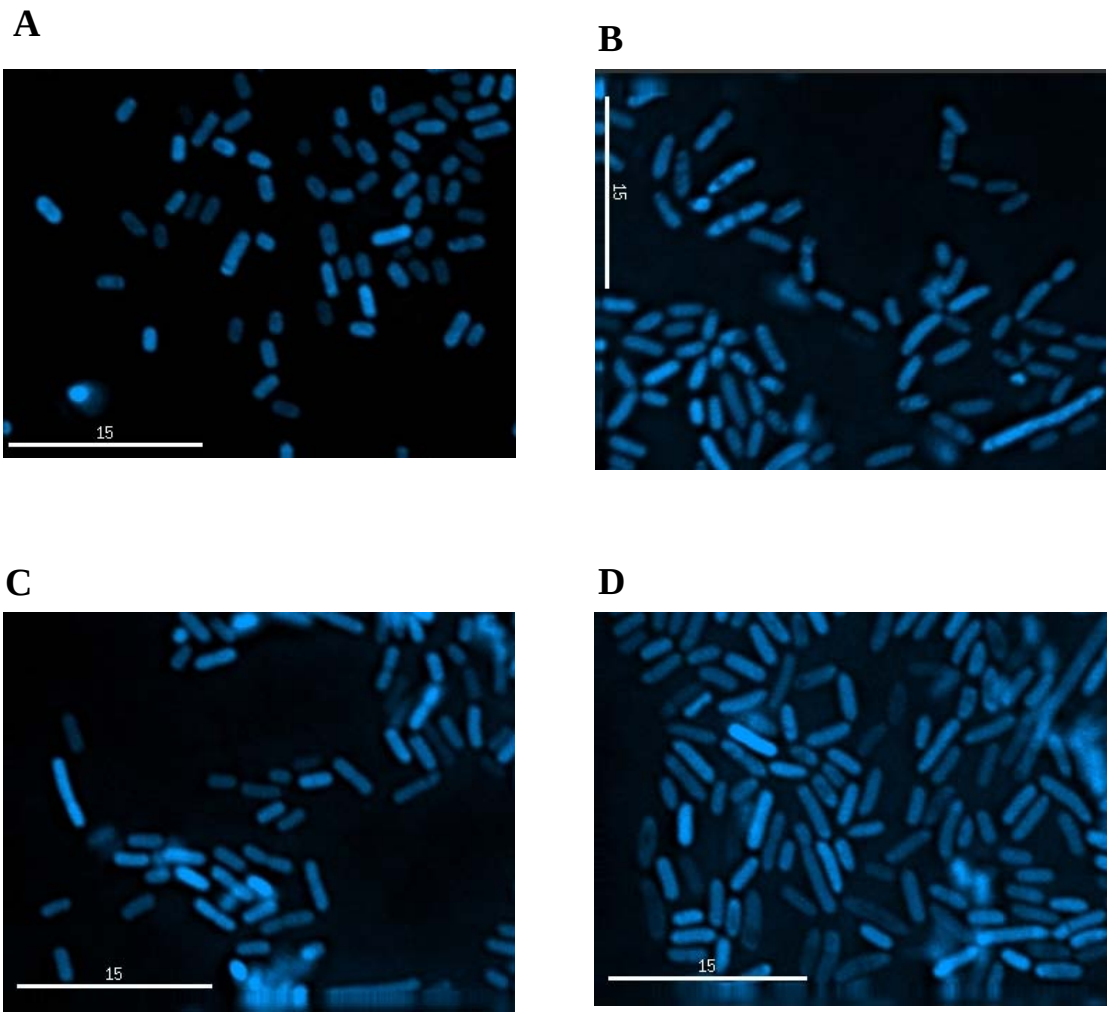

Figure 5.2 Confocal microscopy of Tat mutants expressing CFP from a plasmid. All of the mutants tested (B), (C), and (D) had an elongated cell phenotype. (A) wild type (B) *tatC* (C) *amiA amiC* (D) *amiA amiC sufI*.

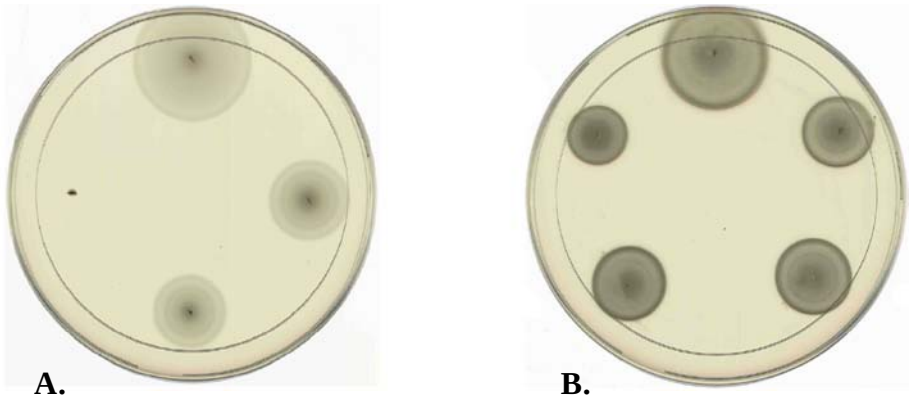

Figure 5.3 Motility of Tat mutants in LB + 0.3% agar. The plates were grown at 37° for 3 hours and then scanned. The strains tested are listed clock-wise from the top. A. wild type, *tatC, tatABC, flhDC*. B. wild type, *amiA amiC sufI, amiA amiC, sufI, tatC*. All of the mutants tested, except *flhDC*, are similarly impared in swimming motility.

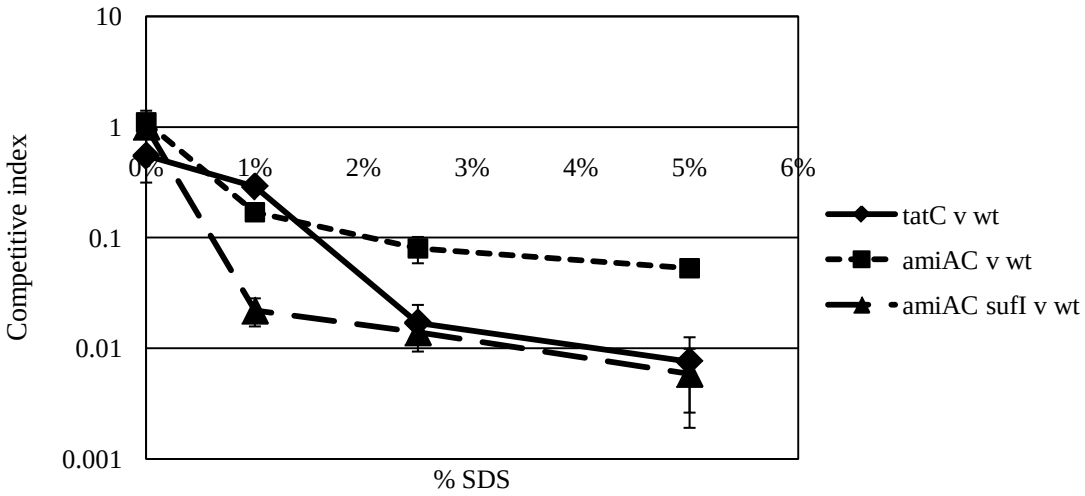

Figure 5.4 SDS sensitivity of Tat mutants in aerobic LB. Mixed cultures were grown for 18 hours shaking in the presence of SDS as indicated. Competitive index is calculated as % mutant output/ % mutant input.

Table 5.1 The *Salmonella* mutant, *sodCI*, acts genetically independently of *tatC* in mice.

Strain A	**Strain B**	**Median CI**	**Number of mice**	**P[a]**
sodCI	wt	0.13	16	<0.0005
sodCI tatC	*tatC*	0.17	5	<0.0005

[a] Student's t-test comparing CI versus inoculums.

Table 5.2 Tat mutants are attenuated in mice.

Relevant genotype	**Method of Delivery**	**Organ**	**Median CI**	**Number of mice**	**P[a]**
tatC	oral	small intestine	0.0065	11	<0.0005
	oral	spleen	0.0077	16	<0.0005
	I.P.	spleen	0.0045	5	<0.0005

[a] Student's t-test comparing CI versus inoculums.

Table 5.3 Virulence characteristics of mutations of specific Tat substrates.

Relevant genotype	Median CI	Number of mice	P[a]
ybiP	1.07	5	NS
fdnG fdoG	3.84	9	0.036
ycbK	0.32	4	0.018
hyaB hybC hydB	0.2	9	0.039
ttrA	0.27	4	0.011
ydcG	1.73	9	0.0048
amiA amiC	1.09	11	NS
thiP	0.42	3	NS
fhuD	1.72	3	NS
wcaM	2.23	5	NS
sufI	0.40	3	NS
pSLT46	0.73	3	NS

[a] Student's t-test comparing CI versus inoculums. NS=not significant. All mutants are competed against wild type.

Table 5.4 Intraperitoneal infection of Balb/c mice with mutants that impare septation.

Strain A	Strain B	Median CI	Number of Mice	P[a]
amiA amiC sufI	wt	0.016	5	0.0029
amiA amiC sufI tatC	*amiAC sufI*	0.26	9	0.036

[a] Student's t-test comparing CI versus inoculums.

Table 5.5 Oral infection of Balb/c mice with SPI1 mutants.

Strain A	**Strain B**	**Median CI**	**Number of Mice**	**P[a]**
spi1	wt	0.27	8	0.039
spi1 tatC	*tatC*	0.92	4	NS
spi1 amiAC sufI	*amiAC sufI*	0.50	4	NS

[a] Student's t-test comparing CI versus inoculums.

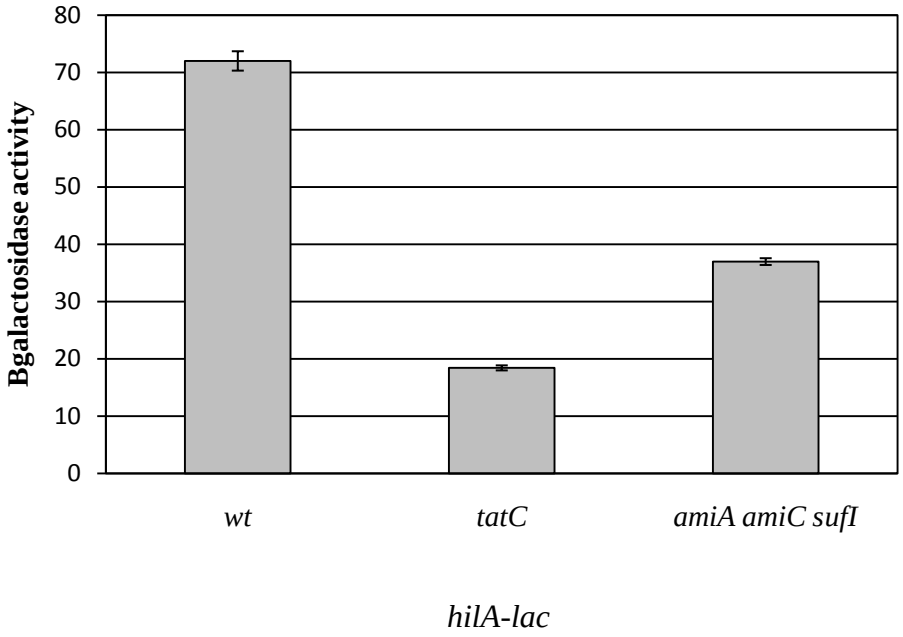

Figure 5.5 Loss of *tatC* decreases β-galactosidase activity of a *hilA-lac* fusion. β-galactosidase activity units are defined as (μmol of ONP formed/min) x 10^6/ (OD_{600} x ml of cell suspension) and are reported as mean ± standard deviation where number of measurements, n=4.

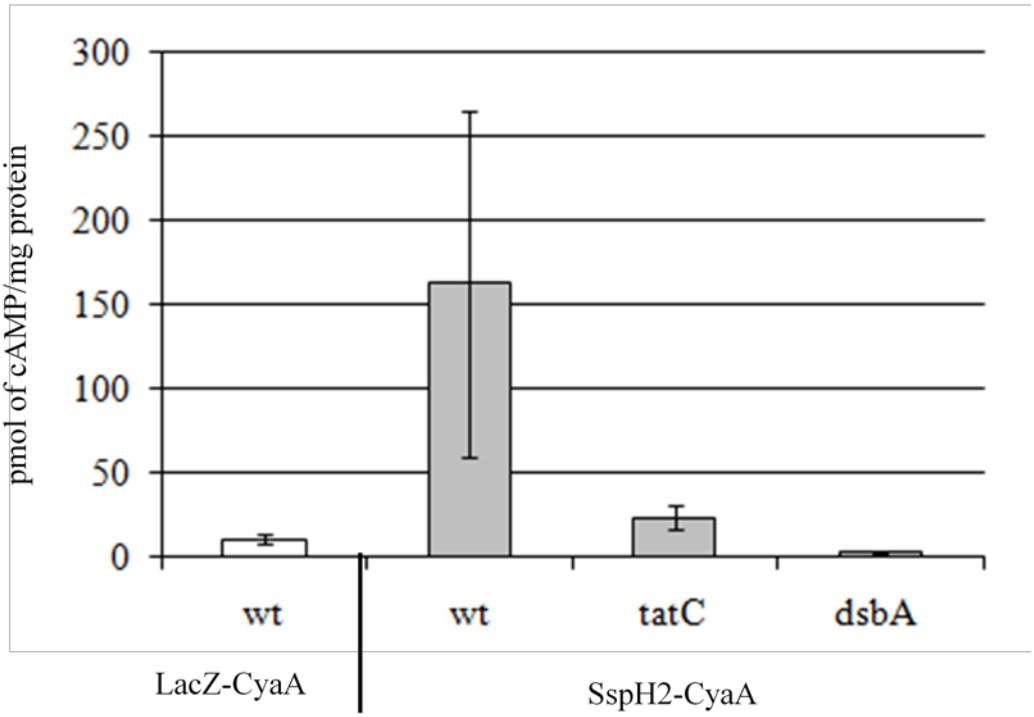

Figure 5.6 Effect of tatC mutation on translocation of a SPI2 type III secreted effector SspH2. Strains were grown in SPI2 inducing conditions and then assays as described in chapter 2. The LacZ-CyaA fusion is a negative control for the type III secretion assay.

Chapter 6: Conclusions and future work

In this work I characterized the virulence properties of several periplasmic proteins. I showed that the periplasmic superoxide dismutase, SodCI, protects a periplasmic target from phagocytic superoxide. Although bacterial DNA has been presumed to be damaged by the oxidative burst of phagocytes, I have shown that this is not the case for *Salmonella typhimurium* in mice. I also characterized the virulence defect of a mutant that lacked a functional Twin Arginine Translocation (Tat) pathway. In doing so I found that the Tat pathway is important in the host because it translocates three proteins, AmiA, AmiC, and SufI, that affect cell septation and envelope integrity. I also found that anaerobic respiration is not important for virulence in mice, and that aerobic respiration is important.

The effect the Tat translocation pathway has on the integrity of the cell envelope is very interesting. Under normal laboratory growth conditions, mutations in Tat structural genes or in genes that encode Tat-exported proteins involved in septation do not have much effect on growth. However, in the presence of detergent or very low osmolartiy these mutations negatively impact growth. This may reveal more about the host environments where *Salmonella* resides. In addition, it offers a new opportunity to study the effect of envelope stress with different environmental conditions.

More work should be done to identify the conditions present in the host that influence bacterial metabolism. In chapter 4, I examined the importance of aerobic and anaerobic respiration in the mouse intestine for *Salmonella* virulence. While this is interesting, this could be expanded to include an exmination of pathways in central

metabolism that would identify the carbon sources *Salmonella* utilizes in the different host environments. A better understanding of bacterial metabolism in the host would have a positive impact on understanding host-pathogen interactions.

Since host superoxide production is a very important to the control of bacterial growth in the host, gaining insight into the molecular mechanism of bacterial killing would be useful. Host-produced superoxide is effective in killing many bacterial species, so the target that is damaged should be common to most bacteria. This is probably why bacterial DNA was presumed to be the target damaged, and for most bacteria, this may be true. The ability of *Salmonella* to modulate the normal processing of engulphed bacteria within macrophages offers an opportunity to examine the damage caused by the host with a bacterial species that can survive the encounter well enough to be studied.

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Author's biography

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