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# Iron Acquisition by *Shigella dysenteriae* and *Shigella flexneri*

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# Iron Acquisition by *Shigella dysenteriae* and *Shigella flexneri*

**by**

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# Dedication

To Dad and Mark

## Acknowledgements

I would like to thank Shelley Payne for her patience, support and guidance over the last five years. I would also like to acknowledge the support and assistance of all of the members of the Payne lab. I am especially grateful to Erin Murphy and Alexandra Mey for many scientific discussions and excellent suggestions, and to Keren Hilgendorf for technical assistance.

# Iron Acquisition by *Shigella dysenteriae* and *Shigella flexneri*

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*Shigella dysenteriae* and *Shigella flexneri* are Gram-negative facultative intracellular pathogens that cause bacillary dysentery. Iron is an essential micronutrient for these pathogens and they have evolved several systems to obtain iron. The Feo and Sit systems are involved in acquisition of ferrous iron and the TonB-dependant systems transport ferric iron and heme. In *S. flexneri,* a strain with a defect in the Sit system exhibited decreased fitness in the intracellular environment compared to the wild type. Although the TonB, Sit and Feo iron transport systems are present in both *S. dysenteriae* and *S. flexneri*, *S. dysenteriae* strains with defects in iron transport exhibited different phenotypes than similar mutants in *S. flexneri.* In *S. dysenteriae*, the TonB-dependent iron transport systems were important for growth in low-iron environments, while the Feo system was important for growth when iron was abundant. The Feo system was the only system found to be important for competition with the wild type strain in vitro or in the intracellular environment. An *S. dysenteriae* strain with a mutation in *feoB* formed

smaller colonies than the wild type and overproduced enterobactin when grown in media containing supplemental iron, but exhibited no defects when grown in low-iron conditions. Despite these phenotypes, when grown in media containing abundant iron the *feoB* mutant did not appear to be starving for iron, indicating that the overproduction of enterobactin is not in response to iron starvation and suggesting that the Feo system plays a role in the regulation of enterobactin biosynthesis in *S. dysenteriae*. An *S. dysenteriae* mutant, defective in all three of the known iron transport systems, was able to grow well in media supplemented with iron, indicating the existence of an additional iron transport system. This system appears to transport free iron and is able to support growth to wild type levels in medium containing only 1 μM $FeSO_4$, suggesting that the system has a high affinity for iron. Taken together, the results of this study indicate that several redundant iron transport systems are employed by *S. dysenteriae*, highlighting the importance of iron acquisition to the survival and pathogenesis of this organism.

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# I. INTRODUCTION

## A. Pathogenesis of *Shigella* species

*Shigella* species are the etiological agents of bacillary dysentery, or shigellosis, a diarrheal disease responsible for over 1.1 million deaths each year, predominantly among children under 5 years of age in developing nations (2). *Shigella* are transmitted via the fecal oral route, often through contaminated food or water, and shigellosis is associated with poor sanitary conditions (95). *Shigella* are Gram-negative, facultative intracellular bacteria, and all four of the *Shigella* species, *Shigella flexneri*, *Shigella dysenteriae*, *Shigella sonnei* and *Shigella boydii* are associated with bacillary dysentery. *S. flexneri* is the predominant cause of endemic shigellosis and is responsible for 60% of cases in the developing world (2, 95). *S. dysenteriae*, which is considered a particular threat to human health because of its association with shigellosis epidemics, causes a severe form of dysentery, mediated by the production of Shiga toxin (1, 2). Fewer than 1% of reported cases of shigellosis occur in industrialized nations, where *S. sonnei* is the predominant species (95). Because of their prevalence, *S. flexneri* and *S. dysenteriae* are considered the most important *Shigella* species medically and have been the focus of a great deal of research (2, 95).

The *Shigella* species are closely related to *Escherichia coli* and it is generally agreed that they constitute a single species (22, 149, 150). Additionally, the *Shigella*

species do not form a single sub-group within *E. coli*, indicating that the *Shigella* do not have a single evolutionary origin and must be the result of convergent evolution (136, 149, 150). *Shigella* and enteroinvasive *E. coli*, which closely resembles *Shigella* in both mechanism of pathogenesis and disease produced, carry a large virulence plasmid that encodes several loci essential to pathogenesis (164-166), including genes that code for a type III secretion system and proteins required for the invasion of the colonic epithelium (118, 164, 165), spread between epithelial cells (13), macrophage apoptosis (205), induction of the host inflammatory response (164, 165) and actin-based motility (13).

Humans are the only natural host of *Shigella* and the infectious dose is very low, requiring as few as 100 organisms to cause disease in healthy adults (44). This low infectious dose is partly due to the ability of *Shigella* to survive the low pH of the human stomach (59). After passing through the stomach and small intestine, *Shigella* invade the basolateral side of the colonic epithelium by exploiting the action of M-cells, which sample the contents of the intestinal lumen, depositing the bacteria into the intraepithelial pocket, where the they are taken up by macrophages (130, 194) (Fig. 1). *Shigella* are able to escape the phagocytic vacuole and induce apoptosis of the macrophage, resulting in the induction of a host inflammatory response and the release of the bacteria into the basolateral side of the colonic epithelium (33, 80, 206). Once there, *Shigella* are able to induce their own uptake through the action of a type three secretion system and the invasion plasmid antigens (Ipa proteins) it secretes (117). The vacuole into which the *Shigella* are taken up is rapidly lysed and the bacteria released into the host cell cytoplasm where they are able to multiply (80, 108). *Shigella* polymerize host cell actin

at the bacterial pole, which facilitates spread to adjacent cells by generating a force that allows the bacteria to form membrane protrusions which are taken up by the neighboring cells, forming double-membraned vesicles (13, 58, 104, 126). The *Shigella* then lyse these vesicles, and the cycle of intracellular growth and intercellular spread is repeated.

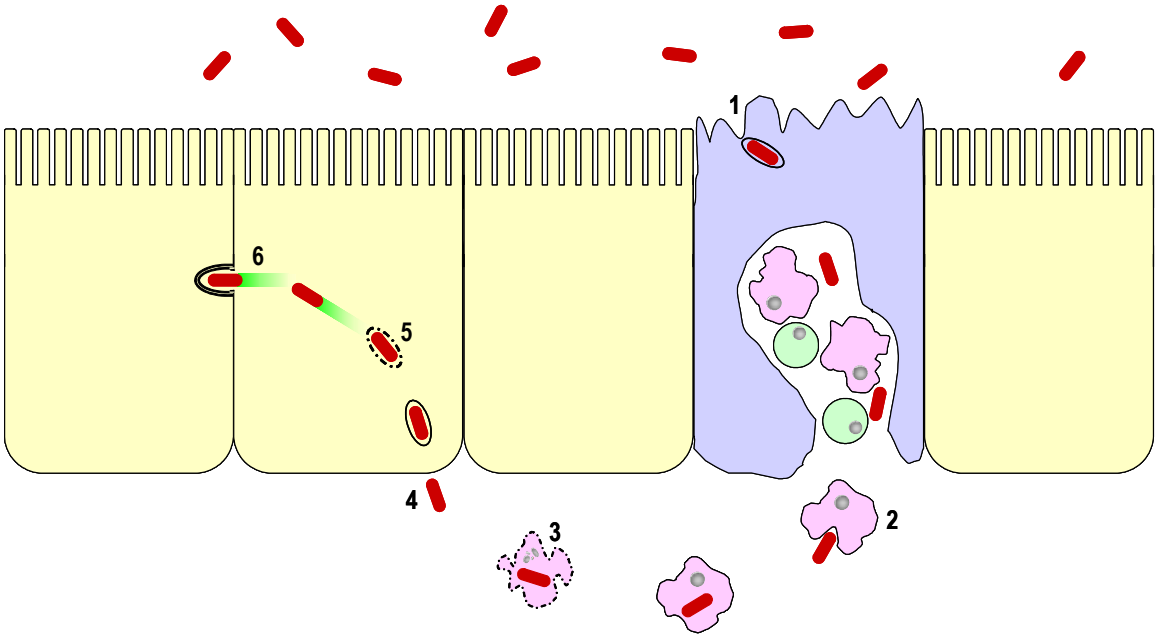



**Figure 1. Pathogenesis of *Shigella* species**

1. *Shigella* in the intestinal lumen are taken up by M cells (blue). 2. The bacteria are deposited in the intraepithelial pocket and taken up by macrophages (pink). 3. *Shigella* induce apoptosis of the macrophage. This results in a host inflammatory response. 4. *Shigella* induce their own uptake at the basolateral side of the colonic epithelial cell (yellow). This uptake is dependent upon a type three secretion system. 5. The bacteria lyse the vacuole and are released into the cell cytoplasm. 6. The bacteria polymerize actin permitting spread to adjacent cells.

## B. Differences Between *S. flexneri* and *S. dysenteriae*

Although *S. flexneri* and *S. dysenteriae* both cause the same disease, via the same mode of pathogenesis, the two species are different in many ways. The *S. dysenteriae* genome is smaller than the *S. flexneri* genome, with fewer open reading frames and more pseudogenes (199). The *S. dysenteriae* genome also harbors nearly twice as many insertion elements as the *S. flexneri* genome, with insertion elements comprising 12% of the genome (199). Insertion elements, phage genes and horizontally-transferred islands are found throughout both genomes (85, 195, 199) and some of these elements are associated with virulence factors. The *she* pathogenicity island, found in *S. flexneri* but not *S. dysenteriae*, encodes an enterotoxin thought to be responsible for the early symptoms of *S. flexneri* infection (153, 199). This island also encodes two serine proteases that are involved in virulence (3, 153, 199). *S. dysenteriae* makes Shiga toxin, the genes for which are encoded within a lambdoid prophage that is not found within the *S. flexneri* genome (114, 178).

Numerous differences in iron acquisition by the two species have also been observed. Both *S. flexneri* and *S. dysenteriae* secrete a siderophore, a low molecular weight, high-affinity iron binding molecule, when grown in iron-restricted media; however, *S .flexneri* makes the siderophore aerobactin, while *S. dysenteriae* produces enterobactin (141, 179). Although *S. flexneri* carries most of the genes for enterobactin biosynthesis, several mutations within the enterobactin biosynthetic genes prevent *S. flexneri* from making this siderophore (169, 170). The aerobactin genes in *S. flexneri* are

carried on the SHI-2 island, which is not found in *S. dysenteriae* (129, 188, 199). *S. dysenteriae* is able to use several iron sources that *S. flexneri* cannot. The siderophore salmochelin is taken up by the Iro system (71), the Shu system allows uptake of the iron-containing molecule, heme (122), and the Fec system transports citrate, an iron binding molecule (82). All of these systems are found only in *S. dysenteriae* (85, 122, 195, 199) and contribute to the ability of *S. dysenteriae* to obtain sufficient iron in a variety of environments.

## C. Iron Acquisition by *S. dysenteriae* and *S. flexneri*

Iron is the most abundant transition metal in living systems and is an essential micronutrient for nearly all organisms. Under physiological conditions, iron exists in two redox states, the reduced, ferrous ($Fe^{2+}$) form, and the oxidized, ferric ($Fe^{3+}$) form. While ferrous iron, which predominates in anaerobic environments or at acidic pH, is readily soluble in water, the ferric ion, which is the predominant form of iron in aerobic environments at neutral pH, is virtually insoluble. Because iron plays an important role in a variety of essential cellular functions including respiration, the tricarboxylic acid (TCA) cycle, gene regulation and DNA biosynthesis, the unavailability of iron at physiological pH poses an obstacle that aerobic bacteria must overcome (5). However, the need for iron must be balanced against the dangers associated with the presence of free iron, which is able to generate DNA-damaging hydroxyl radicals through the Fenton reaction within the cell (183).

For pathogenic bacteria, successful iron acquisition within the host is often essential for virulence. Humans have iron-, heme- and hemoglobin-binding proteins that reduce the availability of free iron within the body, thus restricting access to iron by pathogens (25). To facilitate iron acquisition in this hostile environment, bacteria have evolved iron transport systems that allow the uptake of iron even under conditions where free iron is scarce. In *S. flexneri* and *S. dysenteriae*, three types of iron acquisition system have been identified: the TonB-dependent iron transport systems, the Feo system and the Sit system (Fig. 2) (122, 141, 161).

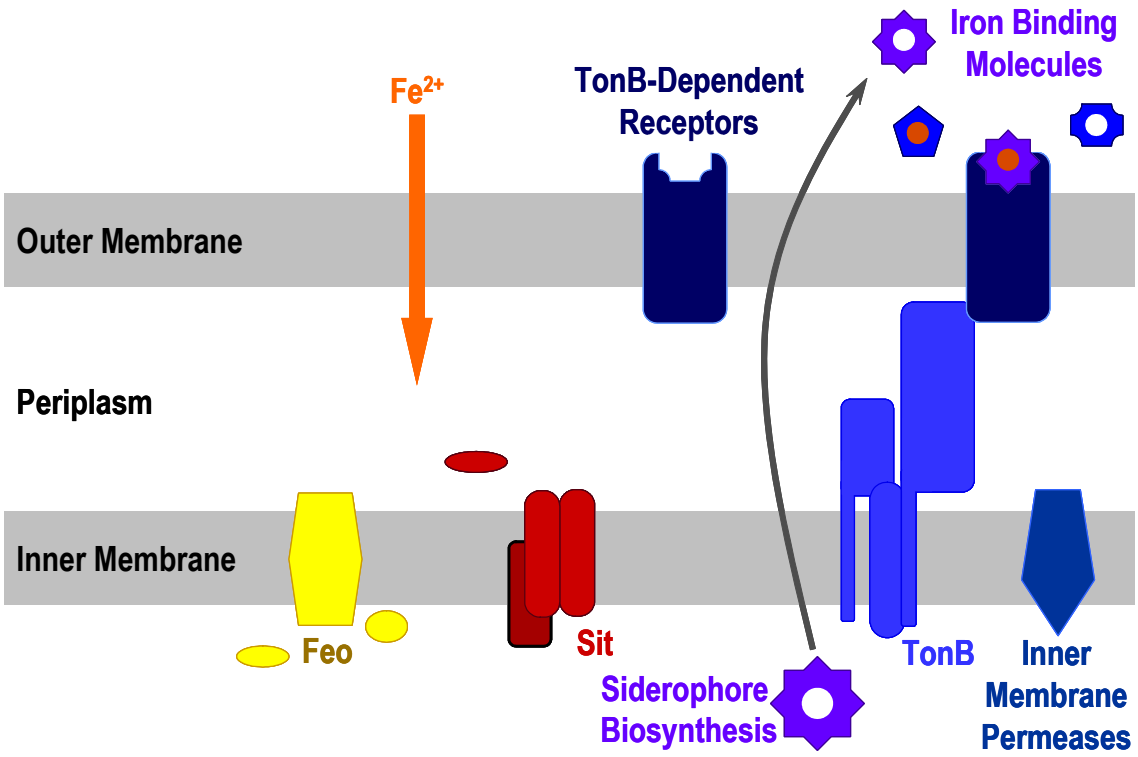



**Figure 2. *S. dysenteriae* and *S. flexneri* iron transport systems.**
*S. flexneri* synthesizes and secretes the siderophore aerobactin and *S. dysenteriae* synthesizes and secretes the siderophore enterobactin. Iron complexed to these siderophores and to other iron-binding molecules is taken up through the TonB-dependent iron transport systems (blue). Both *S. flexneri* and *S. dysenteriae* make use of the Feo ferrous iron transport system (yellow) and the Sit iron and manganese uptake system (red).

## 1. TONB-DEPENDENT IRON UPTAKE

The TonB-dependent iron transport systems allow the energy dependent acquisition of ferric iron complexed to iron-binding molecules (Fig. 2) (18). Siderophores are low molecular weight chelators with a high affinity for the ferric ion that are used by bacteria as a mechanism to solubilize ferric iron (98). *S. dysenteriae* synthesizes the catecholate siderophore enterobactin (179), while *S. flexneri* synthesizes aerobactin, a hydroxamate (141). In addition to transporting the endogenous siderophore, both *S. dysenteriae* and *S. flexneri* are able to scavenge exogenous siderophores, including several produced by fungi (141). TonB dependent systems in *S. dysenteriae* are also involved in the uptake of ferric dicitrate and heme (122, 123), an iron-containing molecule that is the most abundant source of iron within the human body.

Ferri-siderophore complexes are too large to pass through the pores in the outer membrane and must be actively transported into the cell. This process is dependent upon the TonB protein, which transduces energy from the inner membrane to a receptor in the outer membrane, providing energy for the transport of the ferri-siderophore across the outer membrane (64, 86). The outer membrane receptors, which consist of a β-barrel channel closed to the periplasm by a globular 'cork' domain, are highly specific for the cognate ferri-siderophore complex (24, 51, 52). Upon binding of the siderophore, the receptor undergoes a conformational change, exposing the N-terminal 'TonB box', a seven-amino acid region that interacts with the TonB protein and is essential for TonB-dependent transport (99, 171). The TonB protein forms a 1:7:2 complex with the

accessory proteins ExbB and ExbD in the inner membrane (78). Through the actions of ExbB and ExbD, energy from the proton motive force at the inner membrane is translated into a conformational change in TonB. This conformational energy is then transduced from the TonB protein to the outer membrane receptor, allowing translocation of the ferri-siderophore into the periplasm (79, 100). Once in the periplasm, the ferri-siderophore is bound by a specific periplasmic binding protein and transferred to an inner membrane permease (92, 107, 157). The permeases are ABC transporters composed of one or two integral membrane proteins and two ATP binding proteins at the cytoplasmic face of the inner membrane. These act as ATPases, supplying energy from the hydrolysis of ATP to provide energy for the transport of the ferri-siderophore across the inner membrane (92).

TonB-dependent iron transport systems play a role in the virulence of several Gram-negative pathogens. A *tonB* mutant in *Bordatella pertussis* exhibits decreased colonization in the mouse respiratory infection model. In *Vibrio cholerae*, strains with defects in heme uptake and the synthesis of the siderophore vibriobactin have defects in colonization of the infant mouse intestine compared to the wild type strain (75). Similarly, uropathogenic *E. coli* requires aerobactin and heme transport for full virulence in the mouse (182). In *S. flexneri*, the effects of a *tonB* mutation on virulence have not been shown; however, a strain with a defect in aerobactin biosynthesis exhibits a defect in plaque formation only when another iron transport system has also been inactivated (161).

**a) Biosynthesis and transport of the siderophore enterobactin**

The catecholate siderophore enterobactin, originally isolated from *E. coli* (135) and *S. enterica* serovar Typhimurium (145), is produced by all *E. coli* and *Salmonella* Typhimurium strains and by some *Shigella* isolates, including *S. dysenteriae* strains, but is not made by *S. flexneri* serotype 2A strains (141, 179). Biosynthesis requires six enzymes, designated *ent*, to convert chorismate, also the precursor for aromatic amino acid, folic acid and ubiquinone biosynthesis, to enterobactin (45, 189). The *ent* genes are found in three operons within a cluster of genes encoding enterobactin biosynthetic, export, uptake and breakdown proteins. The first three steps of enterobactin involve the conversion of chorismate to isochorismate to 2,3-dihydro-2,3-dihydroxybenzoic acid to 2,3-dihydroxybenzoic acid (DHBA) and are catalyzed by EntC (isochorismate synthase), EntB (2,3-dihydro-2,3-dihydroxybenzoate synthase) and EntA (2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase), respectively (45, 189, 200, 201) (Fig. 3). EntB is a bifunctional enzyme that also acts in the conversion of DHBA to enterobactin (57, 175), a process that is not as well understood as the synthesis of DHBA. The EntB, D, E and F proteins are believed to act as a complex to catalyze the addition of L-serine to DHBA to form 2,3-dyhydroxy-N-benzoly-L-serine (DBS) and the subsequent formation of a cyclic trimer of DBS – enterobactin (45, 61, 62) (Fig. 3). Once made, enterobactin is transported across the inner membrane by EntS, a major facilitator superfamily transporter (15, 35, 56, 172), and transport of enterobactin across the outer membrane requires the channel tunnel protein, TolC (15). Additional proteins, which interact with

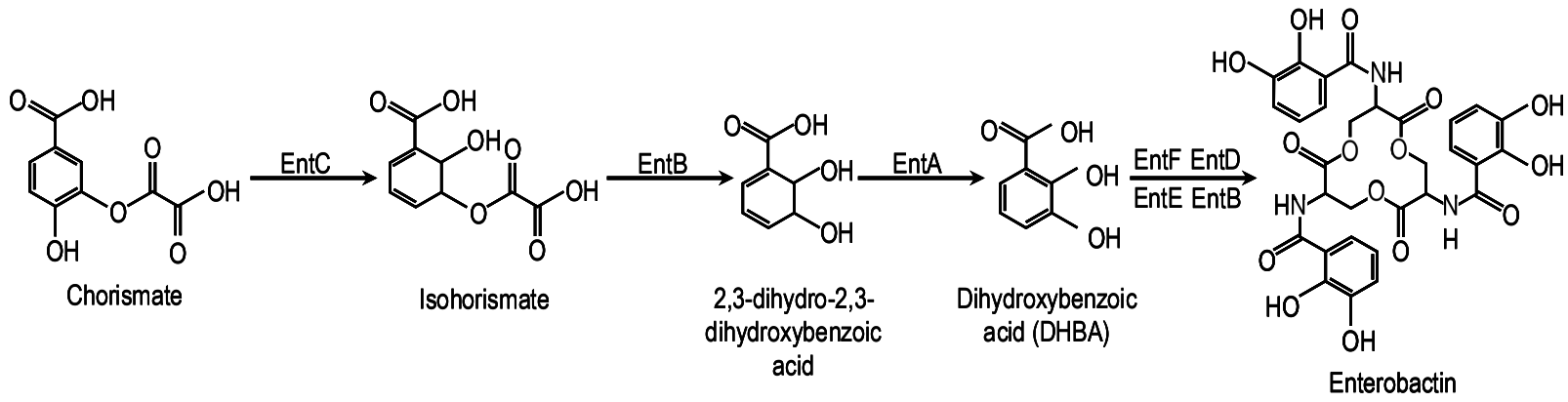



**Figure 3. Enterobactin biosynthesis.**

Biosynthesis of enterobactin occurs in two steps, the formation of DHBA from chorismate and the formation of enterobactin from DHBA. The formation of DHBA occurs in a stepwise manner and the conversion is catalyzed by EntC, EntB and EntA. The conversion of DBS to enterobactin is catalyzed by an enzyme complex composed of EntB, EntD, EntE and EntF. Adapted from Raymond et al. (154).

TolC, are likely involved in the export of enterobactin from the periplasm; however, these proteins have not been identified, possibly because of redundancy (15, 56, 60).

Ferri-enterobactin is taken up in a TonB-dependent manner, through the outer membrane receptor FepA (6, 144, 196), as described above. Upon entry to the periplasm, ferri-enterobactin is bound by FepB, the periplasmic binding protein of an inner membrane permease, and delivered to the cytoplasmic transmembrane proteins FepD and FepG (172). Energy for the transport of ferri-enterobactin across the inner membrane is by provided by ATP hydrolysis by FepC (172). In the cytoplasm, Fes, an enterobactin esterase, cleaves enterobactin to dihydroxybenzoylserine, releasing the bound iron (23). Fes may also facilitate the removal of iron from enterobactin by reducing the ferric ion, carried by enterobactin, to the ferrous ion, which is bound less strongly by enterobactin and which is the form of iron found in the cytoplasm (5, 45, 74).

**b) Biosynthesis and transport of the siderophore aerobactin**

*S. flexneri* 2A strains synthesize the siderophore aerobactin (103, 141, 142), which is also made by several pathogenic *E. coli* strains (20, 97, 110). The four aerobactin biosynthesis genes, *iucABCD*, encode proteins that catalyze the modification of lysine residues and the attachment of the modified lysines to citrate (28, 41, 197). Also in the aerobactin biosynthetic and transport operon is *iutA*, the gene encoding the TonB-dependent outer membrane receptor specific for aerobactin (28, 41). In *Shigella* and *E. coli*, this operon is carried on ColV plasmids (14, 20, 41) or is encoded within a pathogenicity island (110, 129, 151, 188). IucD catalyzes the conversion of lysine to $N^6$-

hydrolysine and the modification of lysine to $N^6$-acetyl-$N^6$-hydrolysine is completed by IucB (41) (Fig. 4). The aerobactin synthase complex, composed of IucA and IucC, is involved in the final steps of aerobactin biosynthesis, both of which are ATP-dependent (41, 63). IucA catalyzes the formation of $N^2$-citryl- $N^6$-acetyl-$N^6$-hydrolysine and IucC catalyzes the addition of the final modified lysine to form aerobactin (41, 63) (Fig. 4).

Ferri-aerobactin is transported across the outer membrane in a TonB-dependent manner, through the outer membrane receptor IutA (41, 197) and is transported across the inner membrane by the FhuBCD inner membrane permease, which transports hydroxamate siderophores from the periplasm to the cytoplasm (50, 197). After transport across the outer membrane, ferri-aerobactin is bound by the periplasmic binding protein, FhuD, and transferred to the FhuBC complex at the inner membrane (93, 94, 197). Transport into the cytoplasm is energy dependent, and is driven by the hydrolysis of ATP by FhuC (93, 107). Once in the cytoplasm, the iron is removed from aerobactin, possibly by reduction of the iron (36, 46), and the aerobactin molecule may be recycled (19, 36, 46).

Aerobactin plays a role in pathogenesis by *E. coli* (97) and is important for extracellular multiplication by *S. flexneri* (102); however, enterobactin does not appear to be as important as aerobactin in vivo (198). The fully deprotonated form of enterobactin has the highest affinity for $Fe^{3+}$ of any molecule tested (10) and at physiological pH, the affinity of enterobactin for ferric iron remains higher than that of aerobactin (72). However, the ability to make aerobactin provides a selective advantage to *E. coli* in the

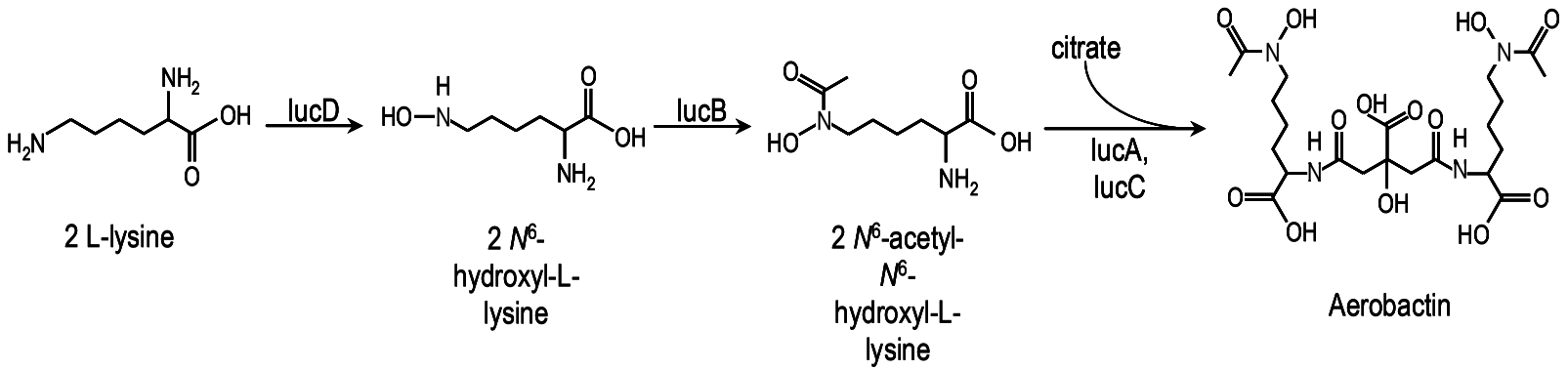



**Figure 4. Aerobactin biosynthesis.**

Biosynthesis of aerobactin from L-lysine and citrate occurs in a stepwise manner and is catalyzed by IucD, IucB, IucA and IucC.

host (198). Several theories have been proposed to account for the apparent disparity. Aerobactin, which is recycled (19), may be a more efficient method of iron uptake compared to enterobactin, which is broken down after one use (134). Enterobactin is readily adsorbed to albumin in serum, which reduces the ability of enterobactin to remove iron from transferrin in the host (91). Enterobactin also induces the formation of antibodies by the host (127, 128), something that aerobactin has not been observed to do (127). Additionally, enterobactin is bound by lipocalin 2, a protein secreted by the mammalian liver, spleen and macrophages during bacterial infections (54). The presence of enterobactin antibodies and enterobactin binding proteins in human serum reduce the effectiveness of enterobactin in the human host (54, 91, 127, 128), and may account for the observation that aerobactin production, but not enterobactin production, is a virulence associated trait in *E. coli* (198).

## 2. THE FEO IRON TRANSPORT SYSTEM

Both *S. flexneri* and *S. dysenteriae* encode a ferrous iron transport system, the Feo system. This system is widespread among bacteria, with nearly 50% of completely sequenced bacterial genomes encoding a homologue of FeoB (29); however, relatively little is known about iron acquisition through Feo. First identified in *E. coli* (66, 87), the *feo* operon in *E. coli* and *Shigella* includes three genes, *feoA*, *feoB*, and *feoC* (68). *feoA* encodes a small, positively charged protein that is probably cytosolic (29). The FeoA protein has not been extensively characterized, and its role in iron uptake by the Feo system is unclear. Mutation of *feoA* is associated with a reduction in ferrous iron uptake;

however, this effect may be due to polar effects on the expression of *feoB* (87). Additionally, the FeoA protein has amino acid similarity to the C-terminal domain of a group of iron-dependent repressors found in certain Gram-positive bacteria (29). The similarity is weak, but suggests that FeoA may have a regulatory role, possibly in modulating the activity of FeoB (29).

FeoB is the largest protein encoded by the *feo* operon and is the best characterized of the Feo proteins. The N-terminal domain is cytosolic, and includes a region with GTPase activity (109). The C-terminal portion of FeoB is predicted to be an integral membrane domain with eight transmembrane segments (87). Although there is little experimental evidence for the method by which iron is taken up by the Feo system, FeoB is presumed to act as a ferrous iron permease, with iron being transported through the C-terminal domain (87). The role of the N-terminal GTPase is less clear. It is possible that this region provides the energy for iron transport and the binding of GTP to this domain is essential for efficient iron transport; however, the rate of hydrolysis of GTP by FeoB appears to be too slow for such a role (109). Alternatively, the GTPase domain may play a regulatory role, modulating the rate of iron transport by the Feo system (29).

The *feoC* gene is much less widespread than *feoAB*, and is found only in the γ-proteobacteria. Like FeoA, the putative FeoC protein is small and poorly characterized. *feoC* is predicted to encode a 78 amino acid protein with an iron-sulfur cluster binding site (29). The structure of the *E. coli* FeoC protein indicates the presence of a winged-

helix motif at the N-terminus and suggests that FeoC may be a DNA-binding protein (29). The role of FeoC in iron transport, if any, is unknown.

Although the Feo iron acquisition system is poorly characterized, it has been shown to play a role in colonization of the gastrointestinal tract by several bacteria. In *Helicobacter pylori*, FeoB-dependent high-affinity iron transport was found to be essential for colonization of the mouse stomach (186). In *Salmonella enterica* serovar Typhimurium, *feoB* mutants were out-competed by the wild type strain in an intestinal colonization assay (185), and *E. coli feoB* mutants were unable to colonize the mouse intestine (176). In *S. flexneri*, a *feoB* single mutant exhibited no defects in growth in vitro or in the plaque assay, a measure of the ability of *Shigella* to invade, grow within and spread between epithelial cells; however, strains with mutations in both *feoB* and *sitA* grew poorly in vitro and formed smaller plaques than the wild type strain.

### 3. THE SIT IRON AND MANGANESE TRANSPORT SYSTEM

The Sit iron transport system in *Shigella* and homologous systems in enteroinvasive and uropathogenic *E. coli*, *S. enterica* and *Yersinia pestis* are ABC transporters implicated in the ATP-dependent transport of iron and manganese across the inner membrane (11, 12, 160, 204). The *S. flexneri* and the *S. enterica* serovar Typhimurium Sit systems are encoded by the *sitABCD* genes, which are induced in the eukaryotic intracellular environment(84, 160) and repressed by the presence of iron and manganese (84, 161, 204). The *S. flexneri* and *S. enterica* systems are each composed of four proteins that are 85 to 93% similar at the amino acid level. SitA is predicted to be a

periplasmic binding protein, while SitB is thought to be the ATPase and SitC and SitD are predicted to be inner membrane permeases (161, 195). In *Salmonella* Typhimurium, the Sit system is able to transport ferrous iron and manganese, and is inhibited by ferric iron; however, due to the low affinity of Sit for ferrous iron, this system is thought be involved mainly in the uptake of manganese in *Salmonella* Typhimurium (89). The affinity of the Sit system for iron in *S. flexneri* and *S. dysenteriae* is not known.

Like the TonB-dependent and Feo systems, the Sit iron transport system and its homologues have been implicated in virulence. In *Y. pestis* the Yfe system, encoded by the *yfe* operon, has homology to the Sit system in *S. flexneri*. *Y. pestis yfe* mutants are avirulent in intravenously infected mice and are attenuated for virulence in subcutaneous infections (11). In *Salmonella*, strains with mutations in the *sit* locus exhibit reduced virulence in mice and increased susceptibility to killing by macrophages (17, 84). In *S. flexneri* the Sit system does not appear to play as central a role in virulence. Although a *sitA* mutant has growth defects in vitro, the mutant exhibits defects in the plaque assay only when other iron transport systems are also inactivated, suggesting that redundancy among iron transport systems in *S. flexneri* protects against virulence defects in the *sitA* mutant (161).

## D. Regulation of Iron Acquisition by *S. dysenteriae* and *S. flexneri*

### 1. REGULATION BY FUR

The expression of iron uptake, iron storage and iron-containing proteins is tightly controlled by the ferric uptake regulator protein, Fur. The role, regulation and activity of Fur in *Shigella* are similar to those in *E. coli*, where Fur has been more fully characterized. Fur is considered the master regulator of bacterial iron homeostasis, responsible for the iron-dependent regulation of over 90 genes in *E. coli* (5, 67). The active Fur protein is a homodimer of two 17 kDa subunits (38). Each Fur monomer contains one zinc ion, which induces dimerization and stabilizes the quaternary structure of Fur, and a second metal ion at the iron site, which induces protein activity (124, 143). Several metal ions, including $Co^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Fe^{2+}$ and $Fe^{3+}$can bind to the iron site and activate Fur; however, only the ferrous ion is present in the cell at sufficient concentrations to allow physiologically relevant levels of Fur activation. Thus, ferrous iron is responsible for Fur activation in vivo (124).

Fur is a positive repressor of iron regulated genes. In the active, iron-bound form, the Fur dimer binds between the -10 and -35 sites in the promoter regions of Fur-repressed genes, preventing expression (26, 27). There is some debate about the sequence of the Fur-recognition motif and the manner in which Fur binds to DNA (42, 47, 48, 101, 131). The most recent suggestion is that Fur acts as a dimer of dimers, interacting with both strands of a 19 bp sequence ( $\begin{smallmatrix} 5' & \text{GATNATGATNATCAANATC} & 3' \\ 3' & \text{CTANTACTANTAGTTNTAG} & 5' \end{smallmatrix}$ ) of DNA (101). An alternative hypothesis describes the Fur box as a sequence of three or more

repeats of a 6 bp motif arranged as follows: NAT(A/T)AT NAT(A/T)AT AT(A/T)ATN (47). Promoters that include more than three repeats of the 6 bp motif allow for increased Fur binding (47, 48). Differentiating between these similar hypotheses will require further study and at present, the exact Fur recognition site and the number of Fur monomers in the active complex remain unclear.

Fur is an abundant protein, with approximately 5,000 copies per cell in exponentially growing *E. coli* and 10,000 copies per cell in *E. coli* at stationary phase (202). In *E. coli*, *fur* expression is weakly autoregulated (43). Additionally, expression of *fur* is induced by oxidative stress through SoxRS and OxyR, resulting in a two-fold increase in Fur levels (202). Oxidative damage is exacerbated by the presence of free iron and an increase in Fur levels would lead to decreased expression of iron uptake genes and would provide a sink for free iron in the cytoplasm, as the additional copies of Fur would bind available ferrous iron, thus reducing free iron levels and the associated oxidative damage (5).

## 2. POSITIVE REGULATION IN THE PRESENCE OF ABUNDANT IRON: THE ROLE OF THE SMALL RNA RYHB

Fur represses genes in *E. coli* when sufficient iron is present, and Fur-repressed genes become de-repressed when the external iron concentration drops below 5 to 10 μM, and the Fur protein is present predominantly in an inactive, iron-free form. Several *E. coli* genes also appear to be induced when iron is abundant, but lack an apparent Fur binding site (111, 140, 184). The small RNA RyhB has recently been identified as the

mediator of this positive regulation by Fur (111). Expression of *ryhB*, which is a negative regulator, is repressed by Fur when iron is abundant, such that RyhB-mediated repression occurs only when Fur is inactive. RyhB acts by facilitating degradation of target mRNA molecules and has been implicated in the regulation of 56 genes in *E. coli*, including genes encoding iron-binding TCA cycle enzymes, iron storage proteins and SodB, an iron-dependent superoxide dismutase (111, 112). Additionally, in *S. flexneri*, RyhB has a role in the regulation of acid resistance, essential to the low infectious dose of *Shigella* (137). In the presence of iron, Fur is able to act indirectly through RyhB to activate the expression of genes important for growth and survival when iron is abundant, more fully coordinating the iron-dependent regulation of gene expression.

## E. Purpose of This Study

The iron transport systems in *S. flexneri* have been the focus of several studies; however, much less is known about iron acquisition in *S. dysenteriae*. Although these two species are causative agents of the same disease, they exhibit many differences with respect to iron transport, and the knowledge gained from studies of iron acquisition in *S. flexneri* is often not applicable to *S. dysenteriae.* The purpose of this study was to identify and characterize the iron acquisition systems used by *S. dysenteriae* and to determine the role of these systems in virulence and to further characterize the iron acquisition systems in *S. flexneri*. Additionally, once the *S. dysenteriae* iron transport systems were identified, a comparison of the methods of iron uptake by *S. flexneri* and *S. dysenteriae* was undertaken to enable a better understanding of the role of each iron transport system in the survival and virulence of *Shigella* species.

# II. MATERIALS AND METHODS

## A. Bacterial Strains and Plasmids

Strains used in this work are listed in Table 1. Plasmids used in this study are listed in Table 2. DH5α was routinely used for the cloning of recombinant DNA. When restriction enzymes sensitive to DNA methylation by *dam* methylase were used, RM1602 was used for the cloning of recombinant DNA. Plasmids for which replication depended upon an exogenous copy of the *pir* gene were maintained in SY327 λ*pir*.

## B. Media and Growth Conditions

Bacterial strains were stored at -80˚C in tryptic soy broth (TSB) + 20% glycerol. *E. coli* strains were routinely grown in Luria-Bertani broth (LB broth) (10g tryptone, 5g yeast extract and 10g NaCl per liter) or on Luria-Bertani agar (LB agar). *S. flexneri* strains were grown in LB broth or on tryptic soy broth agar (TSB agar) plus 0.01% Congo red dye. M9 minimal medium and *Shigella* modified M9 minimal medium (MM9) were used as minimal media for *Shigella*. MM9 contained 30 mg/L monobasic potassium phosphate, 50 mg/L sodium chloride, 100 mg/L ammonium chloride 1.21 g/L

Table 1. Strains used in this study

| Strain | Relevant Characteristics | Ref. or Source |
|---|---|---|
| *E. coli* strains | | |
| DH5α | *endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169 deoR* [Φ80d*lac* Δ(*lacZ*)*M15*] | (162) |
| HB101 | F$^-$ Δ(*gpt-proA*)*62 leu supE44 ara-14 galK2 lacY1* Δ(*mcrC-mrr*) *rpsL20 xyl-5 mtl-1 recA13* | (162) |
| RM1602 | F$^-$ *dam-3* | R. Meyer, Univ. Texas, Austin |
| SY327 λ*pir* | Contains the prophage λ*pir* | (121) |
| CAG5052 | Hfr, *relA1, spoT1, metB1, btuB3191*::Tn*10* | (174) |
| CAG5052 *feoB*::kan | CAG5052 *feoB*::Tn*5* | S. Reeves, Univ. Texas, Austin |
| *S. flexneri* strains | | |
| SA100 | *S. flexneri* serotype 2A clinical isolate | (142, 161) |
| SM100 | SA100 spontaneous streptomycin resistance | (161) |
| SM166 | SM100 *sitA*::*cat* | (161) |
| SM190 | SM100 *feoB*::*dhfr* | (161) |
| SM193 | SM100 *sitA*::*cat*, *feoB*::*dhfr*, *iucD*::Tn*5* | (161) |
| SFZ011A | SM100 T7 RNA polymerase resistant | Z. Feng, Univ. Texas, Austin |
| JCS100 | SM100 T7 RNA polymerase resistant | J. Chang, Univ. Texas, Austin |
| JCS101 | SM100 T7 RNA polymerase resistant | J. Chang, Univ. Texas, Austin |
| JCS102 | SM100 T7 RNA polymerase resistant | J. Chang, University of Texas |
| JCS104 | SM100 T7 RNA polymerase resistant | J. Chang, Univ. Texas, Austin |
| NDS100 | SM100 *tonB*::*cat* | This work |

| Strain | Relevant Characteristics | Ref. or Source |
|---|---|---|
| *S. dysenteriae* strains | | |
| SDU378 | *S. dysenteriae* serotype 1 clinical isolate | (155) |
| SDU380 | SDU378 spontaneous pirazmonam resistance. Deletion encompassing *tonB*, *trp*, *opp*, *yciAB*. | (155) |
| 0-4576S1 | *S. dysenteriae* serotype 1. Antibiotic sensitive derivative of clinical isolate | (123) |
| 0-4576S1-G | 0-4576S1 spontaneous streptomycin resistance | This work |
| 0-4576S1 *feoB*::kan | 0-4576S1 *feoB*::Tn*5* transferred from *E. coli* CAG5052 *feoB*::kan | S. Reeves, Univ. Texas, Austin |
| NDS115 | 0-4576S1-G *tonB*::*cat* | This work |
| NDS116 | 0-4576S1-G *sitA*::*aph* | This work |
| NDS117 | NDS115 *sitA*::*aph* | This work |
| NDS118 | 0-4576S1-G *feoB*::*dhfr* | This work |
| NDS119 | NDS116 *feoB*::*dhfr* | This work |
| NDS120 | NDS115 *feoB*::*dhfr* | This work |
| NDS121 | NDS117 *feoB*::*dhfr* | This work |
| NDS122 | NDS116 *entB*::*tet* | This work |
| NDS123 | NDS116 *entB*::*tet* | This work |
| NDS124 | NDS115 *entB*::*tet* | This work |
| NDS125 | 0-4576S1-G *entB*::*tet* | This work |
| NDS126 | NDS125 *feoB*::*dhfr* | This work |
| NDS129 | O-4576S1-G *lacZ*::*cat* | This work |
| *V. cholerae* strains | | |
| 0395 | *V. cholerae* classical biotype | (116) |
| ARM575 | 0395 *feoB* | A. Mey, Univ. Texas, Austin |

Table 2. Plasmids used in this study

| Plasmid | Relevant Characteristics | Ref. or Source |
|---|---|---|
| pACYC184 | Cloning vector; $Tc^R$, $Cm^R$; p15A origin | (32) |
| pGEM-T Easy | Cloning vector; $Ap^R$; pMB1 origin | Promega |
| pWKS30 | Low copy-number cloning vector; $Ap^R$; pSC101 origin | (193) |
| pHM5 | Allelic exchange vector; $Ap^R$; carries *sacB*; | (159) |
| pQE2 | IPTG-inducible expression vector; $Ap^R$; Col E1 origin | Qiagen |
| pQF50 | Transcriptional fusion vector containing a promoterless *lacZ* gene; $Ap^R$; pMB1 origin | (49) |
| pRK2013 | Helper plasmid for mobilization of R2K transfer dependent plasmids, $Km^R$ | (53) |
| pAR1219 | T7 RNA polymerase gene under the control of the *lac* UV5 promoter; $Ap^R$; pMB1 origin | (40) |
| pMTL24Cm | Chloramphenicol resistance gene from pMA9 | S. Smith, Univ. Texas, Austin |
| pMTL24Tet | Tetracycline resistance gene from pACYC184 | S. Reeves, Univ. Texas, Austin |
| pUC4K | Kanamycin resistance gene | (187) |
| pANTfeo | Group II intron targeted to the *feoB* gene; $Cm^R$ | (88, 161, 203) |
| pKD46 | Red recombinase expression plasmid | (39) |
| pQE2-Kn | pQE2 carrying the kanamycin resistance gene from pUC4K in the *Xmn* I site | This work |
| pAMT11 | pWKS30 carrying *E. coli tonB* | A. Mey, Univ. Texas, Austin |
| pECS32 | *entC* promoter from SM301 fused to *lacZ* | M. Schmitt, Univ. Texas, Austin |
| pEFS34 | *entF* promoter from SM301 fused to *lacZ* | M. Schmitt, Univ. Texas, Austin |

| Plasmid | Relevant Characteristics | Ref. or Source |
|---|---|---|
| pRZ526 | Plasmid carrying *E. coli tonB* and *yciABC* | (148) |
| pYUKI | *E. coli tonB* in a low copy-number cloning vector | (155) |
| pNDS1 | Splice overlap PCR of *S. flexneri* SM100 *tonB* cloned into pHM5 | This work |
| pNDS2 | Chloramphenicol resistance gene from pMTL24Cam cloned into the *Sma* I site of pNDS1 | This work |
| pND14 | Splice overlap PCR of *S. dysenteriae* O-4576S1-G *tonB* cloned into pGEM-T Easy | This work |
| pND15 | Chloramphenicol resistance gene from pMTL24Cam cloned into the *Sma* I site of pND14 | This work |
| pNDS16 | *Eco*R V, *Xba* I fragment (*tonB::cat*) of pND15 cloned into pHM5 | This work |
| pND21 | PCR of *S. dysenteriae* O-4576S1-G *sitA* gene cloned into pGEM-T Easy | This work |
| pND22 | Kanamycin resistance gene from pUC4K cloned into the *Msc* I site of pND21 | This work |
| pNDS23 | *Eco*R V *Bgl* II fragment (*sitA*::*aph*) of pND22 cloned into pHM5 | This work |
| pND27 | PCR of *S. dysenteriae* O-4576S1-G *entB* gene cloned into pWKS30 | This work |
| pNDS28 | *Sal* I *Sac* I fragment (*entB*) of pND27 cloned into pHM5 | This work |
| pNDS29 | Tetracycline resistance gene from pMTL24Tc cloned into the *Nru* I site of pNDS28 | This work |
| pND34 | O-4576S1-G *tonB* gene cloned into pQE-2 | This work |
| pND35 | pND34 with the tetracycline resistance gene from pMTL24Tc cloned into the *bla* gene | This work |
| pND42 | Splice overlap of *S. dysenteriae* O-4576S1-G *lacZ* gene cloned into pGEM-T Easy | This work |
| pND43 | Chloramphenicol resistance gene from pMTL24Cam cloned into the *Sma* I site of pND42 | This work |

| Plasmid | Relevant Characteristics | Ref. or Source |
|---|---|---|
| pNDS44 | *Xba* I *Bgl* II fragment (*lacZ*::*cat*) of pND43 cloned into pHM5 | This work |
| pND54A | T7 promoter region of SM100 cloned into pGEM-T Easy. PCR product of SMP023 and SMP032; 513 bp fragment. | This work |
| pND56 | *Nco* I, *Hin*c II fragment of pND54A (T7 promoter region) cloned into the *Nco* I and *Sca* I sites of pACYC184. | This work |

Tris, 30 g/L PIPES, and 6 g/L sodium hydroxide. M9 contained 12.8 g/L dibasic sodium phosphate, 3 g/L monobasic potassium phosphate, 5 g/L sodium chloride, 1 g/L ammonium chloride, 2mM magnesium chloride and 0.1 mM calcium chloride. M9 and MM9 media were supplemented with 2 μg/mL nicotinic acid, 2 μg/mL tryptophan, 0.2 μg/mL thiamine, 0.0005% 2-deoxyadenosine, and 0.4% glucose. Where indicated, minimal media were treated with Chelex 100 ion exchange resin (Bio-Rad Laboratories, Hercules, CA) according to the manufacturers directions to remove contaminating iron. Antibiotics were used at the following concentrations (per milliliter): 250 μg of carbenicillin, 50 μg of kanamycin, 35 μg of chloramphenicol, 12.5 μg of tetracycline, 40 μg of trimethoprim, 25 μg of pirazmonam (kindly provided by S.J. Lucania, Bristol-Myers Squibb Co.), and 200 μg of streptomycin. Ethylenediamine-N,N'bis(o-hydroxyphenyl)acetic acid (EDDA) was deferrated as described by Rogers (156) and was added to LB broth, LB agar and TSB agar at concentrations of 10 μg/mL to 1 mg/mL to chelate iron where indicated. Strains with mutations in iron transport genes were routinely grown in media containing 40 μM $FeSO_4$. Expression from *lac* promoters was

routinely induced by the addition of 50 μM isopropyl-β-d-thiogalactosidase (IPTG) to LB broth or LB agar unless otherwise specified. Expression of T7 RNA polymerase was induced using 500 μM or 1 mM IPTG in LB broth or LB agar.

## C. Determination of Iron Concentration in Media

The concentration of free iron remaining in Chelex-treated media was determined using a modification of the ferrozine (monosodium salt hydrate of 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid) (Sigma-Aldrich, St. Louis, MO) method as detailed by Stookey (177). To 1 mL of medium or Chelex-treated water (control), 20 μL of the acid reagent solution (5.15 g/L ferrozine, 100g/L hydroxylamine hydrochloride (Sigma-Aldrich) in 6.2 M hydrochloric acid) was added. The solution was incubated in a boiling water bath for ten minutes. The solution was then cooled to room temperature and 20 μL of buffer solution (400 g/L ammonium acetate in 5.4 M sodium hydroxide) added. The solution was mixed thoroughly and the color allowed to develop for 1-5 minutes. The absorbance at 562 nm was measured and normalized to the absorbance of the Chelex treated water sample. To determine the amount of iron present in the solution, the normalized absorbance was compared to a standard curve prepared using solutions of 10, 5, 1, 0.5, 0.1 and 0.05 μM $FeSO_4$.

## D. Recombinant DNA Methods

Plasmids were isolated with the QIAprep Spin Miniprep kit (Qiagen, Santa Clarita, CA) as per the manufacturer's instructions. Isolation of DNA fragments from agarose gels was performed with the QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions. Chromosomal DNA was isolated by the CTAB method, as described by Ausubel et al. (9) or with the QIAGEN Genomic-tip System (Qiagen). Restriction digests and DNA ligations were performed as described by Sambrook and Russel (163) using enzymes purchased from New England Biolabs (Beverly, MA) or Promega (Madison, WI). DNA fragment sizes were estimated using *Hin*d III or *Bst*E II digested λDNA or *Hae* III digested ΦX DNA (New England Biolabs or Promega) as size markers.

## E. Polymerase Chain Reaction

All polymerase chain reactions (PCRs) were carried out with *Taq* polymerase (Qiagen) according to the manufacturer's instructions in an Applied Biosystems GeneAmp thermocycler or an MJ Research PTC-200 thermocycler. Unless otherwise specified, 100 µL PCR reactions contained template DNA, 5 units of Taq polymerase, 10 µM each primer and 250 µM each dNTP in Taq reaction buffer. For screening purposes, 20 µL reactions were used. In screening reactions, the concentrations of reagents were as above and 1 unit of Taq polymerase was used. Unless otherwise specified, the following

program was used for amplification of DNA: 5 minutes at 94˚C followed by 30 cycles of 30 seconds at 94˚C, annealing for 30 seconds at 50˚C and elongation at 72˚C for 1 minute per 1000 bp of expected product size. The reaction completed with a final extension of 7 minutes at 72˚C.

## F. Oligonucleotides

DNA primers for use in PCR and sequencing reactions were designed using Clone Manager Professional Software Suite version 8 (Scientific and Educational Software, Cary, NC) and purchased from Integrated DNA Technologies, Coralville, IA. Oligonucleotides were received lyophilized and were resuspended in distilled $H_2O$ to a concentration of 100 μM and stored at -20˚C. Primers used in this study are listed in Table 3.

## G. DNA Sequencing

Plasmid DNA and purified PCR products were routinely sequenced using the automated dye termination procedure and analyzed on an ABI 377A DNA sequencer by the Core Facility at the Institute for Cellular and Molecular Biology at the University of Texas at Austin.

Table 3. Primers used in this study

| **Oligonucleotide Name** | **Sequence** |
|---|---|
| 1.for | GATTTACCTCGCCGCTTCC |
| 2.for | TTGCGCCTGCTGATCTCG |
| 3.for | CGATGTCAAACCCGTAGAGTCG |
| 4.for | ACGCCCGACATCAAGTACG |
| 5.for | GGCATTGCGCATTGAAGGG |
| 6.for | AATGCGATGCGCAGATGG |
| 7.for | CCACCGAAATTCAGTAAGC |
| 8.for | TGCCCGATTGGTTCAGACG |
| 9.for | CATAGCAGCACACCACATCG |
| 10.for | AACCCGCACAGTCACTACG |
| 11.for | CGGGCATGGCTAAAGTACG |
| rev | GGCTTGACGCTGTTCTTCC |
| entB.1 | TAACTACGCCATGAGCTCGCCGG |
| entB.16 | TAAATGGCGTTTCTAGATCATGC |
| lacZ.1 | TCTAGACCTTTCGCGGTATGG |
| lacZ.2 | GCAGTTCAACCCGGGCTGTCTTCGGTATCG |
| lacZ.3 | CGAAGACAGCCCGGGTTGAACTGCACACC |

| Oligonucleotide Name | Sequence |
|---|---|
| lacZ.4 | TGCTGGAGATCTGCTTCCGTCAGCG |
| sitA.1 | AGACCGCATTCTGGAATTGG |
| sitA.2 | CCAGAACAGATCTCTTTCCGC |
| SMP023 | GTTATGCTAAAGAGGTCGAGG |
| SMP032 | CGCTTGGTGTCTGTAATTGG |
| tonB.2 | GCAGCCCCGGGACAACAGCACC |
| tonB.3 | GGTGCTGTTGTCCCGGGGCTGC |
| tonB.4 | CGGTTGCGGTCGACGATGTGG |
| tonB.5 | CTACAGCACGATATCAGGTCTGGG |
| tonB.dys.14 | GAGGAGATAACCGTTATGGCTAATGC |
| tonB.dys.15 | GGTCGGAAGCTTTTGACTTTATGC |
| tonB.dys.2 | CGTTATATTCGTGCGGTCTTAGTGC |
| tonB.dys.3 | GCTCACGCCATAACGGATTATATC |
| tonB.dys.4 | GCACGCTGTGTCTAGAAAGGGACG |
| tonB.dys.5 | GTGCGGTGATATCACCTATATCTGG |

## H. Transformation of Bacterial Strains

### 1. TRANSFORMATION OF $CaCl_2$-COMPETENT *E. COLI*

*E. coli* strains were made competent using $CaCl_2$ and plasmid DNA was introduced by heat shock. To prepare $CaCl_2$-competent *E. coli* cells, an overnight culture was diluted 1:100 into fresh LB broth and grown to mid-log phase (OD600 of 0.4-0.6). The culture was then chilled on ice for 10 to 60 minutes. The cells were pelleted by centrifugation at 6000 x g for 10 minutes at 4°C and resuspended in 10 ml ice-cold $CaCl_2$ solution (60mM $CaCl_2$, 15% Glycerol 10mM PIPES pH7). The centrifugation and resuspension were repeated. The cells were then harvested by centrifugation at 6000 x g for 10 minutes at 4°C and resuspended in 2.0 ml $CaCl_2$ solution. The cell suspension was divided into 150 μL aliquots and any unused aliquots stored at -80˚C. Competent cells were transformed by adding 100-200 ng plasmid DNA and incubating on ice for 30 minutes. The bacteria were then heat shocked at 42˚C for 2 minutes, chilled on ice for 2 minutes, added to 850 μL of LB broth and incubated at 37˚C for 1 hour. Transformed bacteria were selected for by plating on LB agar supplemented with the appropriate antibiotics.

## 2. ELECTROPORATION OF *SHIGELLA*

Plasmid DNA was introduced to *Shigella* strains by electroporation. *Shigella* strains were made electrocompetent as follows. Two mL cultures were grown to mid-log phase in LB broth at 30˚C in the presence of antibiotics where appropriate. Cultures were chilled on ice for 60 minutes and the bacteria harvested by centrifugation at 6000 x g for 10 minutes at 4˚C. The pellet was washed with 25 mL ice cold $dH_2O$ and centrifuged again as above. The bacteria were then resuspended in 10 mL of ice cold $dH_2O$ and centrifuged again. The bacteria were then resuspended in 5 mL of ice cold $dH_2O$, harvested by centrifugation as above and resuspended in 250 µL ice cold 10% glycerol. One hundred µL aliquots of electrocompetent cells were transformed as described by Sambrook and Russel (163).

## 3. CONJUGATION

Transfer of plasmids by conjugation was completed as follows. One mL overnight cultures of the donor strain, the recipient strain, and the mobilizing strain were washed in LB broth and each resuspended in 100 µL LB broth. Twenty µL of each strain were mixed and the mixture spotted onto an LB agar plate and incubated at 37˚C for 6-8 hours. The cells were swabbed off the plate and resuspended in 1 mL of LB broth. Transconjugants were isolated by plating the bacterial suspension on medium selective for the recipient strain and for the transferred plasmid or cosmid.

## I. Construction of Recombinant Plasmids

To construct pQE2-Kn, a *Hin*c II fragment of pUC4K, carrying the *aph* gene, was cloned into the *Xmn* I site of pQE2, disrupting the *bla* gene, creating a plasmid carrying kanamycin, but not carbenicillin, resistance.

To construct pQE2-Cm, a *Sma* I fragment of pMTL24Cam, carrying the *cat* gene, was cloned into the *Xmn* I site of pQE2, disrupting the *bla* gene, creating a plasmid carrying chloramphenicol, but not carbenicillin, resistance.

To construct pND34, the *tonB* gene was amplified from 0-4576S1-G using primers tonB.dys.14, which added a *Bse*R I site upstream of the *tonB* gene, and tonB.dys.15, which added a *Hin*d III site downstream of the *tonB* gene. The PCR product was cloned into the *Bse*R I and *Hin*d III sites of pQE2, such that the ribosome binding site of pQE2 remained intact but the His tag and multiple cloning site were removed. A *Sma* I fragment of pMTL24Tet, carrying the tetracycline resistance gene, was then inserted into the *Xmn* I site of pND34, disrupting the *bla* gene and creating pND35.

To construct pND54A and pND54B, the T7 promoter region of SM100 was amplified using primers SMP023 and SMP032. The PCR product was then cloned into pGEM-T Easy and the resulting plasmids screened by PCR to determine the orientation of the T7 promoter region relative to the plasmid backbone. In pND54A, the T7 promoters are in the same orientation as the *lacZ* gene and in pND54B, the T7 promoters are in the opposite orientation. To construct pND56A T7 promoter region was removed

from pND54A by digestion with *Nco* I and *Hin*c II and cloned into the *Nco* I and *Sca* I sites of pACYC184, disrupting the *cat* gene. The resulting plasmid was sequenced to confirm the orientation of the T7 promoter region relative to the plasmid backbone. The T7 promoters are in the same orientation as the *cat* gene.

## J. Construction of Mutant Strains

### 1. NDS100

The *S. flexneri tonB* mutant was constructed by allelic exchange. The *tonB* gene was amplified from SM100 by overlap extension PCR with primer pairs tonB.5-tonB.2 and tonB.3-tonB.4 to amplify overlapping fragments, followed by amplification of the overlap extension product with primer pair tonB.5-tonB.4 to create a final product containing a unique *Sma* I site within the *tonB* gene, an *Eco*R V site upstream and a *Sal* I site downstream of the *tonB* gene. The resulting PCR product was cloned into the *Sal* I and *Eco*R V sites of pHM5 creating the plasmid pNDS1. A *Sma* I fragment of pMTL24Cm, carrying the *cat* gene, was cloned into the *Sma* I site of pNDS1, and the resulting plasmid designated pNDS2. pNDS2 was transferred into SM100 by conjugation using MM294/pRK2013 as a helper strain. Primary integrants, resistant to streptomycin, carbenicillin and chloramphenicol were identified and were grown in the presence of sucrose to select for a secondary crossover even. The loss of the integrated

plasmid and the presence of the *cat* gene within the *tonB* gene were confirmed by PCR in sucrose-resistant, carbenicillin-sensitive, chloramphenicol-resistant isolates and the resulting *tonB*::*cat* strain designated NDS100.

**2. NDS115**

The *S. dysenteriae tonB* mutant was constructed by allelic exchange as described above. The *tonB* gene was amplified from 0-4576S1-G by overlap extension PCR with primer pairs tonB.dys.5-tonB.dys.2 and tonB.dys.3-tonB.dys.4 to amplify overlapping fragments, followed by amplification of the overlap extension product with primer pair tonB.dys.5-tonB.dys.4 to create the final product containing a unique *Sma* I site within the *tonB* gene and an *Eco*R V site upstream and *Bgl* II site downstream of the *tonB* gene. The PCR product was cloned into pGEM-T Easy, creating pND14. The *cat* gene was cloned into the *Sma* I site of pND14, and the resulting plasmid designated pND15. An *Eco*R V, *Bgl* II fragment, carrying *tonB*::*cat*, was excised from of pND15 and cloned into pHM5. The resulting plasmid was designated pNDS16. pNDS16 was transferred into 0-4576S1-G by conjugation as described above. The loss of the integrated plasmid and the presence of the *cat* gene within the *tonB* gene were confirmed by PCR in sucrose-resistant, carbenicillin-sensitive, chloramphenicol-resistant isolates and the resulting *tonB*::*cat* strain designated NDS115. The *tonB* gene and surrounding region were subsequently sequenced to confirm disruption of the *tonB* gene.

### 3. NDS116 AND NDS117

The *S. dysenteriae sitA* mutant was constructed by allelic exchange as described above. Briefly, the *sitA* gene was amplified from 0-4576S1-G by PCR using primers sitA.1 and sitA.2 to create a product containing an *Eco*R V site upstream and *Bgl* II site downstream of the *sitA* gene. The PCR product was cloned into pGEM-T Easy, creating pND21. A *Hin*c II fragment of pUC4K, carrying the *aph* gene, was cloned into the *Msc* I site of pND21, and the resulting plasmid designated pND22. An *Eco*R V *Bgl* II fragment, carrying *sitA*::*aph*, was excised from of pND22 and cloned into pHM5. The resulting plasmid was designated pNDS23. pNDS23 was transferred into 0-4576S1-G by conjugation as described above. The loss of the integrated plasmid and the presence of the *aph* gene within the *sitA* gene were confirmed by PCR in sucrose-resistant, carbenicillin-sensitive, kanamycin-resistant isolates and the resulting *sitA*::*aph* strain designated NDS116. NDS117 was constructed by allelic exchange of the cloned *sitA*::*aph* into NDS115 resulting in a *tonB*::*cat*, *sitA*::*aph* strain.

### 4. NDS118, NDS119, NDS120 AND NDS126

The *S. dysenteriae feoB*::*dhfr* mutant was constructed by targeting of a group II intron from plasmid pANTfeo to the *feoB* gene of 0-4576S1-G as described by Karberg et al. and Zhong et al. (88, 203). The same method was used to introduce a *feoB* mutation into NDS116 creating NDS119, a *sitA feoB* mutant, NDS115, creating NDS120, a *tonB feoB* mutant, and NDS125, creating NDS126, an *entB feoB* mutant.

**5. NDS121**

To construct the *S. dysenteriae tonB sitA feoB* mutant, *tonB* was expressed in NDS117 (*tonB*::*cat*, *sitA*::*aph*) from plasmid pND35 and the *feoB* gene was inactivated in this strain as described above. Once all three mutations were made and confirmed by PCR analysis, plasmid pND35 was cured by the introduction of pQE2, which has the same origin of replication as pND35, and selection for pQE2 in the absence of selection for pND35. The loss of pND35 was confirmed by PCR analysis and by analysis of the plasmid profile of NDS121/pQE2.

**6. NDS122**

The *S. dysenteriae tonB sitA entB* mutant was constructed by allelic exchange of *entB*::*tet* into NDS117. The *entB* gene was amplified from 0-4576S1-G by PCR with primers entB.1 and entB.16 to create a product containing a *Sac* I site downstream and a *Sal* I site upstream of the *entB* gene. The PCR product was cloned into pWKS30, creating pND27. A *Sal* I *Sac* I fragment, carrying *entB*, was excised from pND27 and cloned into pHM5. The resulting plasmid was designated pNDS28. A *Sma* I fragment from pMTL24Tet, carrying the tetracycline resistance gene, was cloned into the *Nru* I site of pNDS28, disrupting the *entB* gene and creating plasmid pNDS29. pNDS29 was transferred into NDS117 by conjugation as described above. The loss of the integrated plasmid and the presence of the *tet* gene within the *entB* gene were confirmed by PCR in sucrose-resistant, carbenicillin-sensitive, tetracycline-resistant isolates and the resulting *tonB*::*cat sitA*::*aph entB*::*tet* strain designated NDS122. NDS123 was constructed by

allelic exchange of *entB*::*tet* into NDS116 and NDS125 was constructed by allelic exchange of the clone of *entB*::*tet* into 0-4576-S1-G as described above.

**7. NDS129**

The *S. dysenteriae lacZ* mutant was constructed by allelic exchange as described above. The *lacZ* gene was amplified from 0-4576S1-G by overlap extension PCR with primer pairs lacZ.1-lacZ.2 and lacZ.3-lacZ.4 to amplify overlapping fragments, followed by amplification of the overlap extension product with primer pair lacZ.1-lacZ.4 to create a *lacZ*Δ product containing a unique *Sma* I site at the location of the deletion and an *Xba* I site upstream and *Bgl* II site downstream of the *lacZ* gene. The PCR product was cloned into pGEM-T Easy, creating pND42. A *Sma* I fragment of pMTL24Cm, carrying the *cat* gene, was cloned into the *Sma* I site of pND42, and the resulting plasmid designated pND43. An *Xba* I *Bgl* II fragment, carrying *lacZ*::*cat*, was excised from of pND43 and cloned into pHM5. The resulting plasmid was designated pNDS44. pNDS44 was transferred into 0-4576S1-G by conjugation as described above. The loss of the integrated plasmid and the presence of the *cat* gene within the *lacZ* gene were confirmed by failure to cleave 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) in the presence of IPTG and by PCR in sucrose-resistant, carbenicillin-sensitive, chloramphenicol-resistant isolates. The resulting *lacZ*::*cat* strain was designated NDS129.

## K. Tissue Culture

Monolayers of Henle cells (intestinal 407 cells; American Type Culture Collection, Manassas, Va.) were used in all experiments and were maintained in Henle medium, consisting of Eagle's minimum essential medium, 2 mM glutamine, and 10% fetal bovine serum in a 5% $CO_2$ atmosphere at 37°C.

### 1. INVASION ASSAYS

Invasion assays were completed as described by Hong et al. (81) with the following modification. Following infection, the cell monolayers were allowed to incubate 15 minutes and then the extracellular bacteria were removed from the monolayer by washing. Henle medium containing 20 μg/ml gentamicin was added to kill any remaining extracellular bacteria and the infected monolayers were incubated for an additional 15 to 105 minutes. To determine the percentage of Henle cells infected, 300 cells in each well were observed by microscopy after staining with Wright-Giemsa stain (Baxter Scientific Products, McGaw Park, Ill.), and those containing 3 or more bacteria were considered infected. To determine the number of intracellular bacteria, the intracellular bacteria were recovered as described by Hong et al. (73). The resulting lysate was plated on agar medium to determine bacterial CFU. The number of Henle cells harvested was determined with a hemocytometer prior to lysis. CFU recovered per infected Henle cell was calculated as number of CFU per (total number of Henle cells harvested × fraction of Henle cells infected).

### 2. PLAQUE ASSAYS

Plaque assays were performed as described by Oaks et al. (133) with the modifications by Hong et al. (81) and with the following modifications. Confluent Henle cell monolayers grown in 35 mm diameter plates (approximately $2\times10^6$ cells per plate) were infected with $2\times10^4$ bacteria. After 60 minutes of incubation, the medium overlaying the Henle cells was removed and replaced with fresh Henle medium plus 0.45% (wt/vol) glucose and 20μg of gentamicin per mL. The cells were incubated for 48 to 96 hours. The medium was then removed and the plaques were visualized by staining with Wright-Giemsa stain.

## L. Siderophore Assays

### 1. ARNOW ASSAYS

The presence of catecholate siderophores in culture supernatants was determined based on the method of Arnow (7). Briefly, bacteria were grown in MM9 overnight or for the indicated period of time. To 200 μL of culture supernatant, the following were added, in order, mixing well after each addition: 200 μL of 0.5 M HCl, 200 μL nitrite-molybdate reagent (10% (wt/vol) each sodium nitrite and sodium molybdate in water), 200 μL 1 M NaOH and 200 μL $H_2O$. Fresh MM9 was also assayed in this manner and used as the sample blank. The presence of catecholate siderophores within the culture

supernatant was indicated by a color change to yellow following addition of the nitrite-molybdate reagent and to red following addition of NaOH. This color change was quantified by determining the absorbance at 515 nm and was normalized to cell number by dividing by the optical density of the culture at 600 nm.

### 2. HYDROXAMATE ASSAYS

A modification of the method of Atkin et al. (8) was used to detect production of hydroxamate siderophores by *S. flexneri*. Briefly, equal volumes of culture supernatant and ferric perchlorate reagent (5 mM $Fe(ClO_4)_3$ in 0.1 M $HClO_4$) were mixed and the absorbance of the resulting solution at 500nm determined by spectrophotometer. The presence of hydroxamic acids was indicated by a color change following addition of ferric perchlorate. The quantity of hydroxamate present in the supernatant was normalized to cell number by dividing the absorbance of the supernatant-ferric perchlorate mixture at 500 nm by the absorbance of the culture at 600 nm.

## M. Utilization of Iron, Siderophores and Heme: Bioassays

Bacterial cultures were grown overnight and $1 \times 10^6$ to $1 \times 10^7$ CFU added to 20 mL warm LB agar containing 100 μg/mL (*S. flexneri*) or 1mg/mL (*S. dysenteriae*) EDDA. The agar was allowed to solidify and 5 μL of each of 200 μM ferrichrome, and 5 μM hemin were spotted on the surface of the plate. For siderophore utilization assays 5

μL of an overnight culture of the enterobactin-producing strain *S. dysenteriae* O-4576S1-G or the aerobactin producing strain SM100 was spotted onto the plate surface. For all bioassays, 20 μL of 40 μM $FeSO_4$ spotted onto a paper disk was used as a positive control. The plates were then incubated overnight at 37˚C and the zones of growth around each iron source measured. The ability of a strain to use a given iron source was indicated by growth of the seeded strain around the location on which that iron source was placed.

## N. Sensitivity to Metal Salts

Overnight cultures were diluted $1\times10^{-5}$ and 10 μL of each were dripped onto gradient plates containing 100 μM, 1 mM or 10 mM $AlCl_3$, $CoCl_2$, $CrCl_2$, $MnCl_2$, $NiCl_2$ or $ZnSO_4$. Forty μM $FeSO_4$ was added to the plates where indicated. Growth was assessed following incubation overnight at 37˚C.

## O. β-Galactosidase Assays

The method of Miller (119) was used to quantify β-galactosidase activity in bacterial cells. Overnight cultures were diluted approximately 1:100 into LB broth or a minimal medium and were grown at 37˚C for 3 to 8 hours. Cultures were cooled on ice

for 15 minutes to an hour to impede further growth. If grown in LB broth, the bacteria were first harvested and resuspended in an equal volume of Z buffer. The OD at 600 nm was determined and, if necessary, cultures were diluted such that the OD at 600 nm was between 0.3 and 0.7. Four-hundred μL were added to 400 μL of Z buffer and the cells permeabilized with 25 μL 0.1% SDS. The pre-reaction mix was incubated at 30˚C for 15 minutes. To the pre-reaction mix, 160 μL of an 4mg/mL solution of O-nitrophenyl β-D-galactopyranoside were added, the solution vortexed and the mixture incubated at 30˚C until a yellow color developed. The reaction was stopped with 400 μL of 1M $Na_2CO_3$ and the cellular debris pelleted by centrifugation. The OD at 420 nm and at 550 nm was measured and the units of β-galactosidase activity determine using the following calculation: Miller units = $(1000 \times (OD_{420} - 1.75 \times OD_{550}))/(t \times V \times OD_{600})$, where t is the reaction time in minutes and V is the volume of culture or resuspended cell pellet used in mL.

## P. Colony Size Assays

Overnight cultures were diluted and approximately 100 bacteria plated on LB agar containing iron or EDDA where indicated. Plates were incubated overnight at 37˚C. The size of 10 colonies per plate was measured by microscope and the mean colony size determined.

## Q. Southern Blot Analysis

Southern hybridization was completed as described by Sambrook and Russel (163). Briefly, SDU380 (pirazmonam resistant) and SDU378 (wild type) genomic DNA was isolated and digested with *Eco*R I, *Hin*d III and *Pvu* I. The digested DNA was separated by electrophoresis through a 0.8% agarose gel. The DNA was denatured and transferred from the agarose gel to a Hybond-ECL nitrocellulose membrane (Amersham Biosciences). The DNA was fixed to the membrane by UV irradiation. Probes were prepared and hybridization to probes was completed using the ECL Direct nucleic acid labeling and detection systems (Amersham Biosciences) per the manufacturer's instructions. DNA for probes for the *tonB* and *yciB* genes was generated by PCR with primers tonB.21 and tonB.22 (*tonB*) and 19545.for and 20063.rev (*yciB*). DNA for the *shuA* probe was made by digesting pShu262 with *Eco*R V.

## R. Frequency of Loss of the T7 Promoters and *sit* Locus

Serial dilutions were made from overnight cultures of SM166/pAR1219 and each dilution plated on both TSBA containing carbenicillin (viable count) and TSBA containing both carbenicillin and 500 μM IPTG. Colonies that grew in the presence of IPTG were then screened for resistance to chloramphenicol. The frequency of resistance to T7 RNA polymerase was calculated by dividing the number of colonies on the plates containing IPTG by the viable count. To calculate to frequency of loss of the *sit* operon,

the frequency of T7 RNA polymerase resistance was multiplied by the percentage of T7 RNA polymerase resistant colonies that were also chloramphenicol sensitive.

## S. Viability in the Presence of T7 RNA Polymerase Expression

Three mL of LB broth containing carbenicillin and 1mM IPTG were inoculated with approximately $1\times10^7$ bacteria from an overnight culture of SM100/pAR1219. Serial dilutions were plated on TSBA containing carbenicillin immediately following inoculation and hourly for 5 hours. The percent survival was calculated by dividing the number of colony forming units recovered at a given time by the number of colony forming units recovered at time 0.

## T. Competition Assays

An *S. dysenteriae lacZ* mutant, NDS129, was constructed as described above to facilitate identification of mutant and wild type bacteria in competition assays. *S. dysenteriae* mutant strains carry a wild type copy of the *lacZ* gene. NDS129 was used as the wild type strain in all *S. dysenteriae* competitions except those involving NDS118, where 0-4576S1-G was used as the wild type strain.

## 1. IN VITRO COMPETITION

From overnight cultures, $1\times10^7$ cfu of each of the wild type and mutant bacteria were mixed and added to 3mL of LB broth (*S. flexneri)* or MM9 broth (*S. dysenteriae*). The exact ratio of mutant to wild type bacteria in the inoculum was determined by plating dilutions of the inoculum and screening for antibiotic resistance (*S. flexneri* competitions and competitions involving NDS118) or for β-galactosidase activity (*S. dysenteriae* competitions). Colonies were screened for β-galactosidase activity by plating on LB agar containing 50 μM IPTG and 80 μg/mL X-gal. The resulting mixed cultures were then grown for 8 hours and the ratio of mutant to wild type bacteria determined again as described above. The competitive index was calculated as the ratio of mutant to wild type bacteria recovered divided by the ratio of mutant to wild type bacteria in the inoculum.

## 2. INTRACELLULAR COMPETITION

To determine the ability of mutant strains to compete with wild type strains in the intracellular environment, plaque assays were completed with a mixed inoculum. Henle cell monolayers were infected with approximately equal numbers of mutant and wild type bacteria and the exact ratio of mutant to wild type bacteria determined as above. Once the infected Henle cells had incubated for 48 hours, the intracellular bacteria were recovered as described above. Serial dilutions of the recovered intracellular bacteria were plated on TSB agar (and subsequently screened for antibiotic resistance) or LB agar containing 50 μM IPTG and 80 μg/mL X-gal (to screen for β-galactosidase activity) and

the ratio of mutant to wild type bacteria recovered was determined. The competitive index was calculated as above.

## U. Microarray Analysis

*S. flexneri* microarrays were constructed using 70-mer oligonucleotides as described by Oglesby et al. (137). Microarrays were to analyze both genomic DNA and RNA. For microarrays used to analyze RNA, the RNA was isolated and microarray analysis conducted as described by Oglesby et al. (137) with one exception: ribosomal cDNA was not removed from total cDNA samples. To analyze genomic DNA, chromosomal DNA was isolated from wild type and mutant strains and sheared to an average size of 500 bp by sonication. Fifteen μg of the sheared DNA was amplified by adding10 μg of pd(N)6 random hexamer primers (Amersham Biosciences) and incubating at 100˚C for 5 min. The mixture was then cooled on ice for 5 min and 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.2 mM dTTP, 0.3 mM amino allyl modified dUTP (Sigma-Aldrich), Klenow buffer to a final concentration of 1X and 40 units of Klenow exo$^-$ (New England Biolabs) were added. The reaction mixture was incubated at 37˚C for 2 hours and then 2.5 μmoles of EDTA, pH 8.0, added to stop the reaction. Amino allyl-dUTP incorporated into the DNA samples was coupled to reactive Cy3 (mutant strain) and Cy5 (wild type strains) dyes (Amersham Biosciences), the labeled samples incubated with the microarrays and the microarrays scanned as described by

Oglesby et al. (137). Analysis of microarrays was performed using the Genepix 5.0 software, and normalization of microarray data was carried out by the Longhorn Array Database.

# III. THE SIT SYSTEM AND THE SHI-4 ISLAND IN *S. FLEXNERI*.

## A. Introduction

Unlike the multisubunit *E. coli* RNA polymerase, T7 RNA polymerase is a single subunit polymerase (30) of approximately 99 kDa (125) with specificity for a 23 bp conserved promoter sequence (139, 158) that is not found in the *E. coli* K-12 genome (31). During infection of *E. coli* with bacteriophage T7, the *E. coli* RNA polymerase transcribes the early phage genes, which are carried on the first section of the phage chromosome to enter the bacterium (21). One of these genes, T7 gene 1, codes for the T7 RNA polymerase, which transcribes the intermediate and late T7 genes (30). Because of its efficiency of transcription, T7 RNA polymerase is often used experimentally in bacteria to regulate expression of genes cloned under the control of the T7 promoter. The products of genes cloned in this manner can reach levels as high as 50% of the total cellular protein within 3 hours when T7 RNA polymerase is present (180). T7 RNA polymerase is highly processive, such that transcripts several times the length of a plasmid carrying a T7 promoter have been observed (113, 180) and genes cloned upstream of the promoter on a plasmid are expressed as highly as genes cloned downstream (181). Such levels of transcription likely saturate the *E. coli* translational machinery and deplete ribonucleoside triphosphate stores (180), and continued expression of T7 RNA polymerase in the presence of a single T7 promoter is lethal to *E. coli* (181). For this reason, T7 RNA polymerase based exogenous expression systems

make use of inducible copies of the T7 RNA polymerase gene to allow the expression of the polymerase to be controlled. In *S. flexneri*, induction of T7 RNA polymerase in the absence of an exogenous T7 promoter led to decreased viability. Characterization of this phenotype indicated that the region involved in T7 RNA polymerase toxicity was carried on a pathogenicity-like island within the *S. flexneri* chromosome that also carried the Sit iron transport system.

First described in *E. coli* (16, 90), pathogenicity islands have since been identified in numerous Gram-negative and Gram-positive pathogenic bacteria, and are now defined by their genetic content and composition, their distribution and their location within the bacterial chromosome (for a review, see (168)). Pathogenicity islands are usually mosaic in nature; often including insertion sequence (IS) elements, phage remnants and cryptic or functional transposase and integrase genes, which lead to instability. Islands often differ from the surrounding sequence in GC content and codon usage, indicative of horizontal transfer, and most pathogenicity islands are found to have inserted at tRNA loci, flanked by small direct repeats. Pathogenicity islands that are not associated with tRNA genes are usually flanked by IS elements. In addition to pathogenicity islands, pathogenicity island-like regions also exist. These regions carry genes that contribute to pathogenesis but are not essential for virulence and may be found in related, non-pathogenic isolates.

Within the *S. flexneri* genome, six islands have been identified (129, 152, 153, 161, 188). The *mxi*/*spa* genes, located within an island on the virulence plasmid, encode a type III secretion system required for pathogenesis. Although not a true pathogenicity

island due to the extra-chromosomal location of this region, the *mxi*/*spa* island is homologous to other type III secretion systems encoded within chromosomal pathogenicity islands in other organisms (115, 173). The SHI-1 island, which is located downstream of the *pheV* tRNA gene, carries the *set1A* and *set1B* genes, which encode the two subunits of enterotoxin I, the *sigA* gene, which codes for an autotransported protease that has cytopathologic effects and is involved in intestinal fluid accumulation and *pic*, which encodes a mucinase with hemagglutinin activity (3, 4, 76, 153). The SHI-2 island is adjacent to the *selC* tRNA gene and contains genes encoding the virulence-enhancing aerobactin iron acquisition system and colicin V immunity (129, 188). A similar island, designated SHI-3, carries the aerobactin genes in *S. boydii* and is found between the *lysU* and *pheU* genes(151). The SHI-O island is found only in *S. flexneri* serotype 1 strains. This island is inserted at the *thrW* tRNA gene and encodes genes for O-antigen modifications (83). The *Shigella* resistance locus, an *S. flexneri* pathogenicity island-like region inserted at the *serX* tRNA gene, encodes resistance to several antibiotics and the ferric dicitrate uptake system (Fec) (105, 152). Finally, the *sit* iron transport genes appear to be part of an *ipaH*-associated island that we have designated SHI-4 (85, 161). This operon is also found within an island in *Shigella dysenteriae*, and it has homology to the *sit* genes found in *Salmonella enterica* serovar Typhimurium (154, 204). Thus, three *S. flexneri* islands, the *Shigella* resistance locus, the SHI-2 island, and the SHI-4 island, encode iron transport systems that contribute to the acquisition of this essential micronutrient (105, 129, 161, 188).

Because of the link between T7 RNA polymerase sensitivity and iron transport in *S. flexneri*, the SHI-4 island was studied further, and the sensitivity of *S. flexneri* strains carrying this island to T7 RNA polymerase was used as a tool to gain insight into the role of the Sit iron transport system in *S. flexneri*.

## B. Results

### 1. Expression of T7 RNA Polymerase is Toxic to *S. flexneri.*

In *S. flexneri*, expression of T7 RNA polymerase inhibits growth. To determine whether the effect of T7 RNA polymerase is bacteriostatic or bacteriocidal, the wild type strain, SM100, carrying pAR1219, a plasmid with the T7 RNA polymerase gene under the control of an inducible promoter (40), was cultured in broth and the number of viable bacteria determined hourly following induction of T7 RNA polymerase expression. After induction of T7 RNA polymerase there was an initial increase in number of cells followed by a decline in bacterial viability with less than 2% of the population surviving 4 hours after induction (Fig. 5). These data indicate that expression of T7 RNA polymerase is toxic to *S. flexneri*.

### 2. Genetic Basis of T7 RNA Polymerase Resistance.

*S. flexneri* resistant to T7 RNA polymerase expression were isolated and a region surrounding the *sit* locus was found to be absent from one of the resistant mutants, SFZ011A (Z. Feng, personal communication). The *sitABCD* operon is flanked by a large region that consists mainly of insertion elements and putative phage genes (160, 161, 195) that we have designated SHI-4. PCR was used to further define the boundaries of the deletion in SFZ011A and in other T7 RNA polymerase resistant strains, all of which had chromosomal deletions within the SHI-4 island (Fig. 6). Each of these deletions appeared to have been mediated by recombination between IS600 elements (Fig. 6). Three independent mutants contained a 32 kb deletion that involved the loss of both the

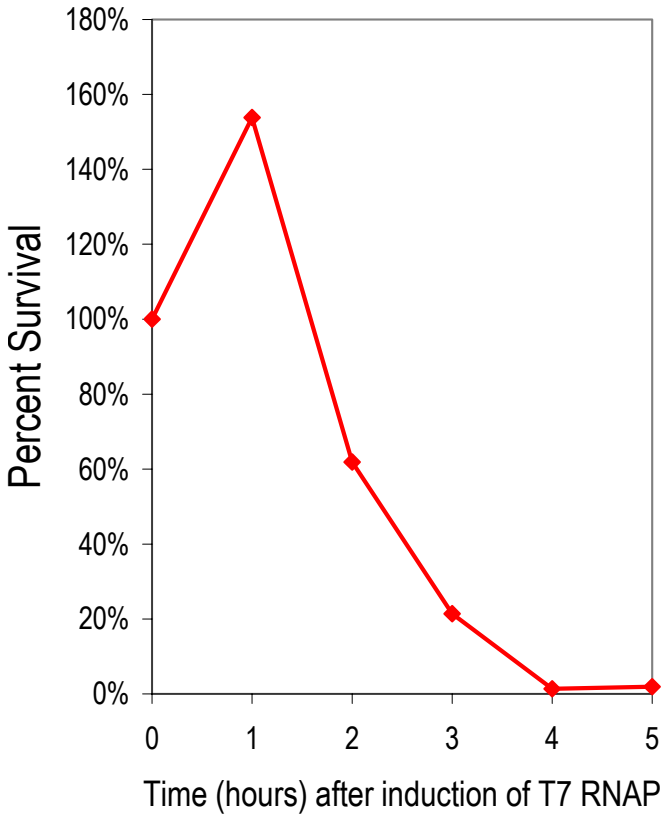



**Figure 5. Survival of SM100 declines following induction of T7 RNA polymerase.**

The number of colony forming units recovered at each time point following induction of T7 RNA polymerase expression is shown as a percentage of the number of colony forming units at the time of induction. Data from one representative experiment are shown.

*sit* operon and the T7 promoters, while JCS104 sustained a smaller deletion and lost the T7 promoters but retained the *sit* genes (Fig. 6).

A 513 bp region of the SM100 chromosome, cloned in pND56, was necessary and sufficient to confer sensitivity to T7 RNA polymerase (Fig. 7). Only clones pFZ70, pFZ74, pFZ76, and pND56, all of which contained this region, conferred sensitivity to T7 RNA polymerase (Fig. 6, 7). The cloned region includes three canonical T7 promoters (Z. Feng, personal communication) (Fig. 8). This region corresponds to one identified by Chen and Schneider in *S. flexneri* 2A strain 2457-T (NC_004741) as containing two T7-like promoter sequences (34), but is absent from the published sequence of *S. flexneri* 2A strain 301 (NC_004337) (85). The T7 promoters were located within a 370 bp region consisting of three 114 bp repeats that are 98% identical, each flanked by 7 bp direct repeats (Fig. 8). This T7 promoter region appears to be located in a larger region of putative phage genes and no open reading frames were identified immediately downstream of the T7 promoter sequences (Z. Feng, personal communication) (Fig. 6). pND56, which contains only the T7 promoter region, the 3' end of the putative phage integrase gene (*int*) upstream of the promoters and 63 bp of sequence downstream of the third repeat, conferred T7 RNA polymerase sensitivity on *E. coli* HB101, a strain that is not naturally sensitive, indicating that this region is necessary and sufficient to confer sensitivity to T7 RNA polymerase (Fig. 7).

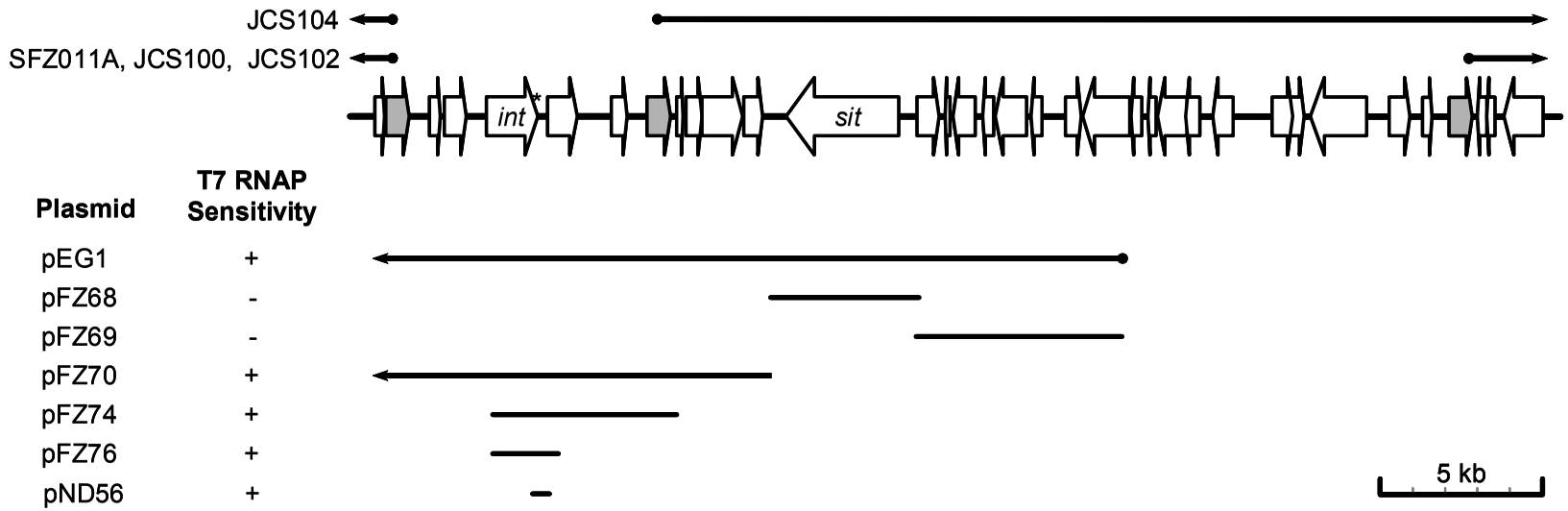





**Figure 6. Map of the T7 RNA polymerase sensitivity locus.**

The *sit* operon and *int* gene are indicated within the representative arrows. An asterisk denotes the location of the T7 promoter region. IS600 elements are shaded. Approximate endpoints are indicated by a dot (•) and an arrowhead indicates that the segment of DNA extends beyond the region shown in this figure. Regions deleted in spontaneous T7 RNA polymerase resistant mutants are shown above the mapline. Lines below the mapline represent the *S. flexneri* regions cloned into the indicated recombinant plasmids. The ability of each plasmid to restore sensitivity to T7 RNA polymerase is shown.

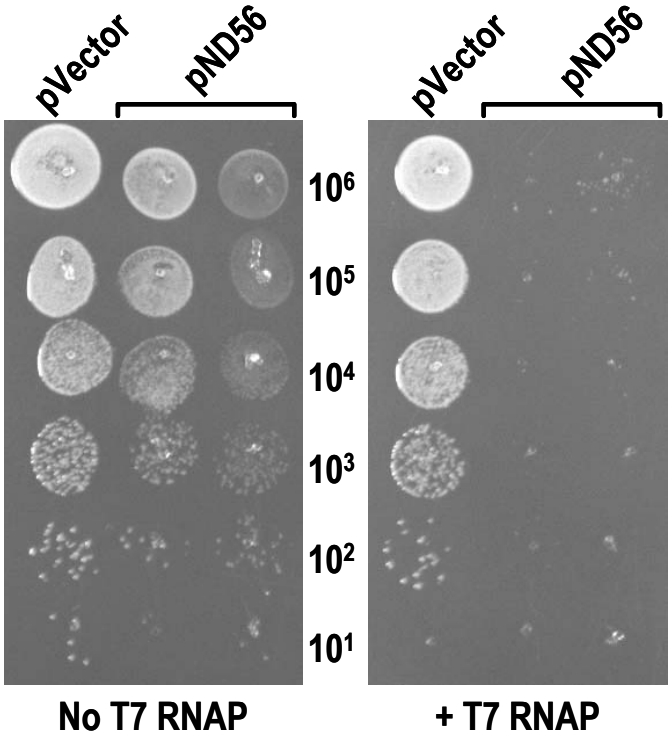
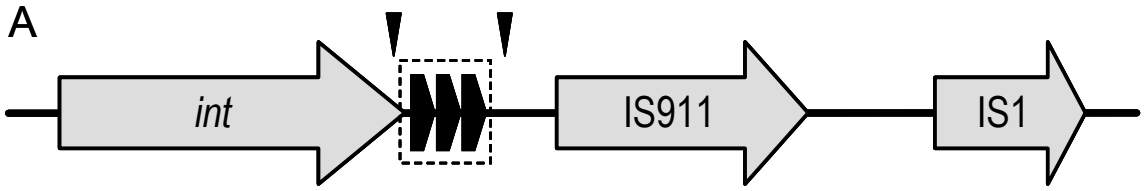



**Figure 7. pND56 confers sensitivity to T7 RNA polymerase on *E. coli* HB101.** Dilutions of HB101 carrying an inducible copy of T7 RNA polymerase (T7 RNAP) and either empty vector (pVector) or the T7 promoter region (pND56) were spotted in the absence (No T7 RNAP) and presence (+ T7 RNAP) of inducer and incubated overnight. Strains carrying pND56 failed to grow in the presence of T7 RNA polymerase.




B

```
  1  atgtgatggc tgatgtttat aaggagacct agaggagacc atagtctagg atgacactta
     >>..........integrase..........>>

 61  ctacagacct gtgtggtcat gtttttgact catagtttc tctacttgtc aagggataat

121  taatacgact cactataggg agaagggaca caaaaagttt tctcttctag gatgacactt

181  actacagacc tgtgtggtca tgtttttgac tcatagtttt ctctgcttgt caaggggtaa

241  ttaatgcgac tcactatagg gagaagggac acaaaaagtt ttctcttcta ggatgacact

301  tactacagac ctgtgtggtc atgtttttga ctcatggttt tctctacttg tcaaggataa

361  ttaatacgac tcactatagg gagaagggac acaaaaagtt ttctcttcta ggacaactaa
```

**Figure 8. Organization of the T7 promoter region in SM100.**

A map of the region surrounding the T7 promoters is shown (A). The integrase gene (*int*) and IS elements are indicated within the representative arrows. The T7 promoters and surrounding repeats are indicated by black arrows. The endpoints of the region cloned in pND56 are indicated by the black arrowheads. The sequence of the boxed area in (A) is shown in (B). The T7 promoter consensus sequences are in boldface type and the putative transcriptional start sites are indicated by an arrow. The long repeats are shaded and the short direct repeats flanking each long repeat are underlined.

### 3. PREVALENCE OF THE T7 PROMOTER REGION IN *SHIGELLA* AND *E. COLI* ISOLATES

Using primers designed to bind within the repetitive sequence that contains the T7 promoters, PCR was used to screen other *Shigella* isolates for the presence of this region. The T7 promoter region was identified in all *S. flexneri* serotype 2A strains tested (Table 4). Additionally, the promoters were found in every *S. flexneri* serotype 1A, 1B and 4B isolate tested, and in some of the serotype 2B, 3, 4A and 5 strains, but not in *S. flexneri* serotype 6, nor in the other *Shigella* species. Because *S. flexneri* is closely related to *E. coli*, several *E. coli* isolates were tested for the presence of the T7 promoter region; however, none of the isolates tested were found to carry the region (Table 4). Like *S. flexneri*, *Salmonella enterica* serovar Typhimurium has a Sit iron and manganese transport system, so *S. enterica* isolates were also tested for the T7 promoter region, but the region was not found (Table 4). The presence of one or more copies of the T7 promoter region appears to be unique to a subset of *S. flexneri* serotypes.

### 4. THE REGION ENCOMPASSING THE *SIT* LOCUS AND THE T7 PROMOTERS IS LOST AT HIGH FREQUENCY WHEN *S. FLEXNERI* IS GROWN UNDER NORMAL LABORATORY CONDITIONS.

Because the T7 promoters were linked to the *sit* locus and several T7 RNA polymerase resistant mutants had lost the *sit* genes, it was of interest to determine the frequency with which deletions that remove the T7 promoter region also remove this iron transport operon. A previously constructed *sitA* mutant, SM166, which contains a chloramphenicol acetyl transferase gene insertion in the *sitA* gene (161), was used for

Table 4. Distribution of the T7 promoter region in *Shigella*, *E. coli* and *S. enterica* isolates.

| Strain | No. positive for T7 promoter region/no. tested[a] |
|---|---|
| *Shigella* | |
| *S. boydii* | 0/3 |
| *S. dysenteriae* | 0/6 |
| *S. sonnei* | 0/5 |
| *S. flexneri* | |
| *S. flexneri* 1A | 5/5 |
| *S. flexneri* 1B | 5/5 |
| *S. flexneri* 2A | 6/6 |
| *S. flexneri* 2B | 1/5 |
| *S. flexneri* 3A | 1/5 |
| *S. flexneri* 3B | 3/5 |
| *S. flexneri* 3C | 1/5 |
| *S. flexneri* 4A | 2/3 |
| *S. flexneri* 4B | 5/5 |
| *S. flexneri* 5 | 2/5 |
| *S. flexneri* 6 | 0/5 |
| *E. coli* | |
| Enterohemorrhagic | 0/6 |
| Enteroinvasive | 0/5 |
| Enteropathogenic | 0/2 |
| K12 | 0/3 |
| *S. enterica* | |
| serovar Enteritidis | 0/2 |
| serovar Typhimurium | 0/3 |

[a] The presence of one or more copies of the T7 promoter region determined by PCR on cultures of each isolate

measuring loss of the *sit* region in T7 resistant isolates of *S. flexneri*. Use of this strain facilitated screening for loss of the *sit* locus and prevented selection for the Sit system from decreasing the apparent frequency of loss of the region. SM166/pAR1219 isolates resistant to T7 RNA polymerase expression were selected by growth in the presence of inducer and the frequency of loss of the *sit* operon determined by screening for loss of chloramphenicol resistance.

The frequency of spontaneous resistance to T7 RNA polymerase varied between $3.3\times10^{-3}$ and $1.4\times10^{-4}$ in SM166/pAR1219 (Table 5). Loss of sensitivity to T7 RNA polymerase was accompanied by loss of the *sit* genes approximately 40% of the time, yielding a frequency of loss for the *sit* locus of $4.6\times10^{-4}$ to $1.1\times10^{-4}$. Such a high frequency of loss is surprising given the ubiquitous nature of the *sit* locus in clinical isolates (161). The discrepancy between the frequency of loss of the *sit* operon in *S. flexneri* clinical isolates and *S. flexneri* grown under laboratory conditions suggests that selection must operate on this region of the *S. flexneri* genome in nature, preventing loss of the *sit* genes while simultaneously retaining the region required for T7 RNA polymerase sensitivity (161).

## 5. Loss of the Sit System Is Associated With a Decrease in Fitness in the Intracellular Environment.

To determine why the *sit* genes are present in all *S. flexneri* clinical isolates, despite a high frequency of loss when grown under normal laboratory conditions, competition assays were used. Growth of the *sitA* mutant strain relative to the growth of

Table 5. Frequency of loss of *sitA* in T7 RNA polymerase resistant isolates.

| | Replicate Number | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| Frequency of T7 RNA polymerase resistance | $1.9\times10^{-4}$ | $1.6\times10^{-3}$ | $3.3\times10^{-3}$ | $5.6\times10^{-4}$ | $1.8\times10^{-3}$ | $1.4\times10^{-4}$ |
| Percentage of resistant colonies lacking *sitA* | 59% | 28% | 12% | 23% | 26% | 76% |
| Frequency of *sitA* loss | $1.1\times10^{-4}$ | $4.4\times10^{-4}$ | $4.1\times10^{-4}$ | $1.3\times10^{-4}$ | $4.7\times10^{-4}$ | $1.1\times10^{-4}$ |

the wild type parent was assessed under different in vitro conditions in which selection for the *sit* locus may exist. The *sitA* strain was found to have no defect in growth compared to the wild type strain in aerobic or anaerobic conditions, at 30˚C or 37˚C and in media with or without supplemental iron (Fig. 9 and data not shown).

Competitive plaque assays were used to test the ability of the *sitA* mutant to compete with the wild type in the intracellular environment. Henle cell monolayers were inoculated with a mixture of equal numbers of wild type and *sitA S. flexneri* and the infected cells were incubated for 48 hours. The eukaryotic cells were then lysed and the bacteria recovered and quantified. The competitive index for *sitA* in the plaque assay was 0.6, indicating that the *sitA* mutant competed poorly with the wild type strain in the intracellular environment (Fig. 9). This nearly two-fold decrease in competitive index suggests a defect in intracellular growth, spread or survival that would provide selective pressure for maintenance of the *sit* genes and the potentially toxic region genetically linked to this locus.

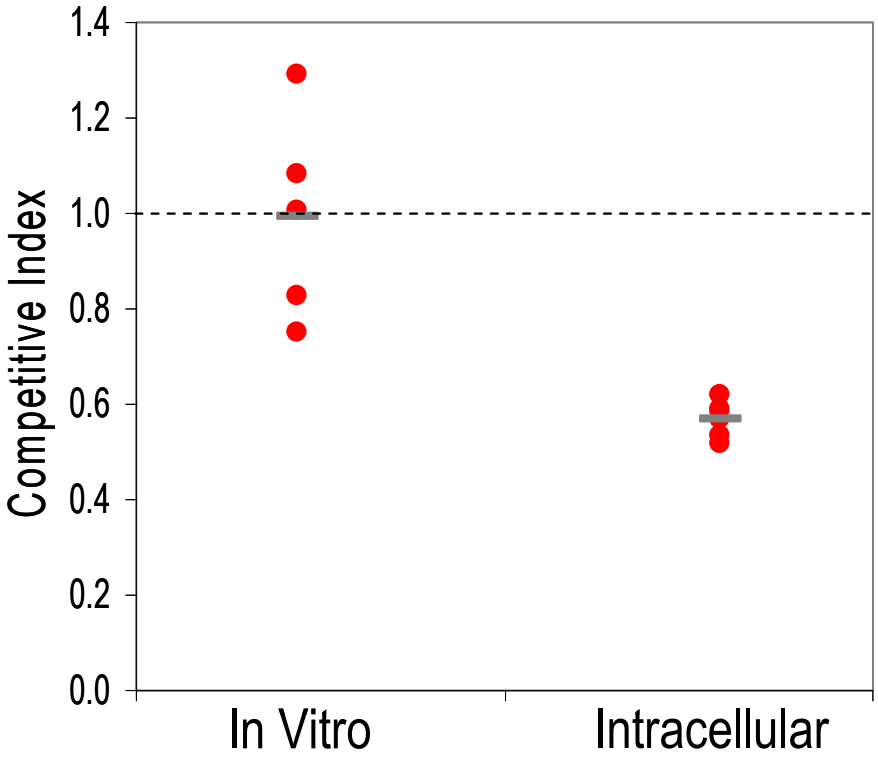



**Figure 9. An *S. flexneri sitA* mutant shows reduced fitness in the intracellular environment.**

The ability of the *sitA* mutant to compete with wild type *S. flexneri* was assessed in vitro (in LB broth) and in the intracellular environment (in plaque assays in Henle cell monolayers). Data from five (in vitro) or six (intracellular) experiments are shown. The means are indicated by gray bars.

## C. Discussion

Transcription from T7 promoters by exogenously expressed T7 RNA polymerase is known to be highly efficient such that transcription by the native RNA polymerase is often impaired and the translation apparatus overwhelmed when T7 RNA polymerase is expressed in the presence of T7 promoters (96, 180, 181). In systems designed to use T7 RNA polymerase for protein over-expression, the target protein can reach a concentration of more than 50% of the total cellular protein in under 3 hours, severely limiting the ability of the host bacterium to carry out normal cellular functions and leading to death if transcription is not halted (180). *S. flexneri* strain SM100 was found to harbor three canonical T7 promoters and induction of T7 RNA polymerase was lethal to over 97% of bacteria within 4 hours. These data are consistent with the hypothesis that in the presence of T7 RNA polymerase, expression from the T7 promoters is strongly induced, leading to bacterial death. No open reading frames were identified in the 475 bp between the transcriptional start site of the third T7 promoter and the start codon of the nearest open reading frame, an IS911 element, which was not required for toxicity. Additionally, pND56, a clone carrying only 97 bp of sequence downstream of the transcriptional start site of the third T7 promoter, was sufficient to confer sensitivity to T7 RNA polymerase. The possibility that a small RNA with biological activity is encoded downstream of the T7 promoters and that over-expression of that RNA is toxic cannot be eliminated. However, it is more likely that the composition of the sequence downstream of the

promoters is unimportant and that toxicity results from a combination of depletion of ribonucleoside triphosphates and interference with bacterial protein biosynthesis.

The T7 promoters are linked to genes encoding the Sit iron transport system. Homologues of the *sit* operon are found in all *Shigella* species, in all *S. enterica* serovars, in enteroinvasive *E. coli* and in *Yersinia pestis*, all facultative intracellular pathogens, but no homologues have been identified in closely related species such as enteropathogenic or enterohemorrhagic *E. coli* that do not invade eukaryotic cells (11, 12, 161, 204). Additionally, the *sit* operon appears to be encoded on an island of DNA that was mobile in origin, suggesting horizontal transfer of these genes; however, the location and composition of this island varies (161, 204). In *S. flexneri* the *sit* genes are contained within the SHI-4 island, which also harbors the T7 promoters. While the *sit* locus is conserved, the T7 promoter region was not found in the enteroinvasive *E. coli* or *S. flexneri* serotype 6 isolates tested, nor was the region found in *S. enterica* serovars Typhimurium or Enteritidis, both of which encode an iron transport system homologous to the *S. flexneri* Sit system. Additionally, the T7 promoter region was only identified in some of the *S. flexneri* serotype 2B, 3, 4A and 5 strains tested. The difference in distribution of the *sit* operon and the T7 promoter region may indicate that the promoters were acquired as part of a second horizontal transfer event subsequent to that in which the *sit* genes were acquired, resulting in an island within an island in some, but not all *S. flexneri* strains. More likely, *S. flexneri* may have originally acquired a segment of DNA containing both the *sit* genes and the T7 promoters, but the T7 promoters may have been subsequently lost from some strains. Deletions such as those sustained by SFZ011A,

JCS100 and JCS102, which remove the *sit* genes in addition to the T7 promoters, would have been selected against in nature; however, smaller deletions, such as that sustained by JCS104, that leave the *sit* operon intact, probably do not decrease fitness. Comparison of the sequence of the region surrounding the deletion in JCS104 to the sequence of *S. flexneri* strain 301 indicated that a similar event may have led to the excision of the T7 promoters and the surrounding sequence from the genome of that strain (Fig. 6 and data not shown).

The frequency with which T7 RNA polymerase resistant mutants arose within a population of *S. flexneri* SM100 was higher than expected and suggested that the region lay within a highly mutable area of the *S. flexneri* chromosome. IS elements allow for deletion by homologous recombination between direct repeats, which appears to be the mechanism by which resistance to T7 RNA polymerase was mediated in SM100. This instability is likely not confined to the region flanking the T7 promoter region, as IS elements are spread throughout the chromosome and the virulence plasmid (85, 195, 199). Instead, the frequency of deletion in this region probably reflects the overall plasticity of the *S. flexneri* genome, in which deletions and rearrangements mediated by homologous recombination between IS elements may be common. This effect would be tempered by selection against deleterious changes, such as the loss of the *sit* operon, which would be rapidly lost from the population. The high frequency at which the *sit* operon was lost in SM100 in the laboratory and the ubiquitousness of the Sit system in clinical isolates of *S. flexneri* and other enteric bacteria with intracellular lifestyles underscores the importance of Sit in the natural environment. Selection against loss of

the Sit system has likely driven both the retention of the *sit* operon, despite its location in a region of the *S. flexneri* genome in which deletions are common, and the maintenance of the potentially toxic T7 promoter region located near the *sit* locus in the same region of the chromosome.

Although the Sit system is induced in the intracellular environment in *S. flexneri* (160) and has been implicated in virulence in *S. enterica* serovar Typhimurium (84, 160), an *S. flexneri* strain with a mutation in *sitA* did not exhibit obvious defects in the invasion of, growth within or spread between human epithelial cells in culture, steps essential to pathogenesis by *Shigella* species (161). A defect in any of these steps or in the ability to grow in one of many conditions encountered in the human host or in the environment will greatly impact the fitness of the strain harboring the defect. A mixed inoculum plaque assay, in which the *sitA* mutant strain must compete with the wild type strain in vivo was developed to better represent the conditions under which loss of the *sit* operon may occur in nature and to increase the sensitivity of detection of any phenotypic changes resulting from inactivation of the Sit system. In this competitive plaque assay, the *sitA* mutant was out-competed by the wild type strain. This strongly suggests that the Sit system is important in the eukaryotic cell and is consistent with the finding that the *sit* operon is induced in the intracellular environment (161). While the other iron acquisition systems can partially compensate for the loss of *sit* in *S. flexneri*, the Sit iron transport system remains important in the intracellular environment and is required for bacteria to compete at wild type levels in the eukaryotic cell.

# IV. IRON ACQUISITION IN *S. FLEXNERI* AND *S. DYSENTERIAE*

## A. TonB-Dependent Iron Acquisition

*S. flexneri* and *S. dysenteriae* make use of two types of iron acquisition system; the TonB-dependent systems and the Sit and Feo iron transporters, which are TonB-independent. The TonB-dependent iron transport systems, which are involved in the transport of siderophores and heme, require the TonB protein (86, 122, 147). Therefore, the role of all of the TonB-dependent systems can be studied in strains with mutations in the *tonB* gene. Pirazmonam is an antibiotic that is transported in a TonB-dependent manner and spontaneous *tonB* mutants can be selected by resistance to pirazmonam (132). In *S. dysenteriae*, a *tonB* mutant, SDU380, was selected in such a manner (155). SDU380 does not make TonB and fails to form plaques in Henle cell monolayers, a measure of virulence-associated traits. However, because the mutation sustained by SDU380 is undefined and because no single *S. dysenteriae* TonB-dependent iron transport system required for plaque formation could be identified (155), *S. flexneri* and *S. dysenteriae* strains with defined mutations in *tonB* were constructed and characterized.

### 1. CHARACTERIZATION OF AN *S. FLEXNERI TONB* MUTANT

Although iron uptake by *S. flexneri* had been studied previously (102, 105, 141, 142, 161), the role of the TonB-dependent iron transport systems in growth and virulence had not been investigated. To allow for such study, a *tonB* mutant, NDS100, was

constructed and the ability of the mutant strain to grow and to obtain iron in a variety of conditions was examined. The effects of the *tonB* mutation on the virulence of *S. flexneri* were also investigated.

**a) Use of siderophores**

The TonB-dependent iron acquisition systems are high affinity iron transporters, using the energy of the proton motive force at the inner membrane to allow for uptake of iron across the outer membrane (86, 146). *S. flexneri* synthesizes and secretes the siderophore aerobactin (103, 141, 142), which is transported back into the cell in a TonB-dependent manner (20). *S. flexneri* is also able to use exogenous siderophores, including enterobactin and the fungal siderophore ferrichrome, the transport of which also requires TonB (141). To confirm that NDS100 had no residual TonB function, the ability of NDS100 to grow in the presence of various siderophores as the sole source of iron was tested. While the parent strain, SM100, grew using enterobactin, aerobactin and ferrichrome as the sole source of iron, NDS100 was only able to grow when $FeSO_4$ was supplied (Table 6), suggesting that NDS100 has lost all TonB function.

Table 6. Ability of various iron sources to stimulate the growth of an *S. flexneri tonB* mutant under iron-restricted conditions.

| | Zone of Growth[a] | |
|---|---|---|
| **Iron Source** | **Wild Type (SM100)** | ***tonB* (NDS100)** |
| Aerobactin | 21 mm | No Growth |
| Enterobactin | 25 mm | No Growth |
| Ferrichrome | 23 mm | No Growth |
| $FeSO_4$ | 27 mm | 16.5 mm |

[a] Bioassays were completed as described in Materials and Methods. The zone of growth is the diameter of the region of bacterial growth surrounding the specified iron source.

**b) The *tonB* mutant exhibits a growth defect compared to wild type in low iron conditions.**

To determine the ability of the *tonB* mutant to grow in low iron conditions, the growth of the mutant was compared to that of the wild type strain in LB broth containing 2 μg/ml EDDA, an iron chelator. Addition of this amount of EDDA did not cause any detectable defect in the growth of the wild type strain (data not show). Under these conditions, the *tonB* mutant strain exhibited a marked decrease in growth, reaching a final optical density (OD) approximately 10-fold lower than that of the wild type strain (Fig. 10). These data indicate that TonB is important for growth of *S. flexneri* in conditions where iron is limiting.

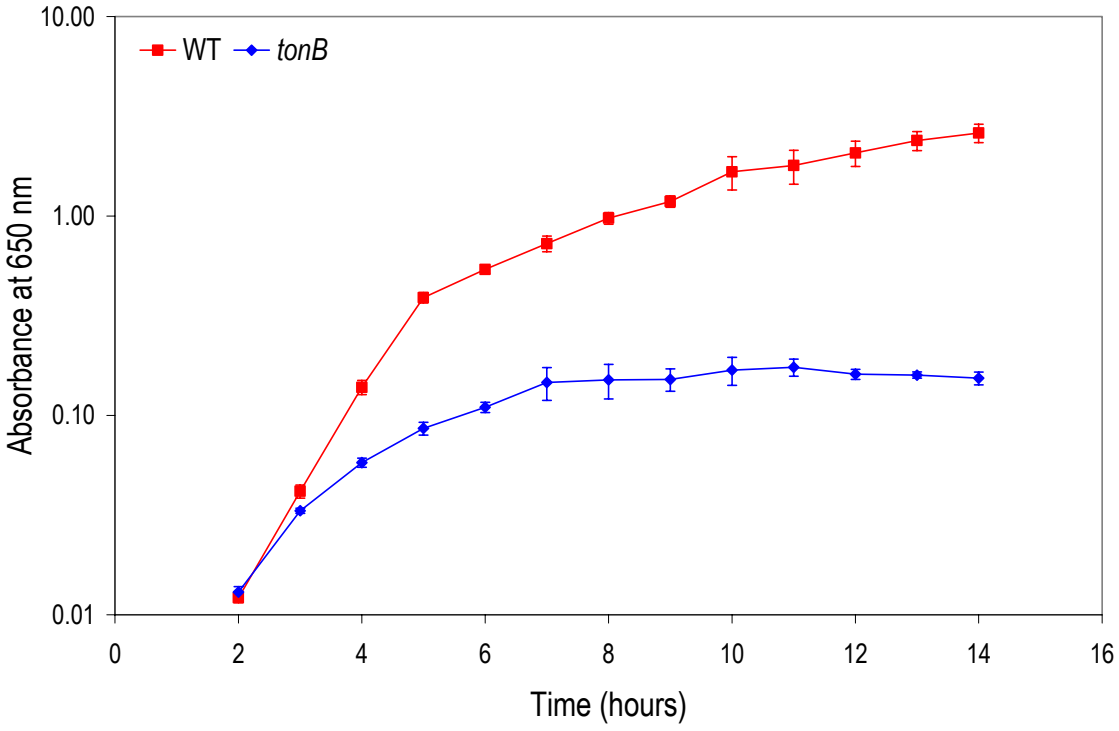



**Figure 10. An *S. flexneri tonB* mutant has a growth defect in iron-limiting conditions.**

Overnight cultures of *tonB* (NDS100) and wild type (SM100) *S. flexneri* were diluted approximately 1:1000 and grown in LB broth + 2 μg/ml EDDA. The optical density of each culture was measured every hour for 14 hours. The mean and standard deviation of three independent experiments are shown.

**c) TonB is not essential for the invasion of, growth within or spread between eukaryotic cells by *S. flexneri*.**

To successfully establish an infection in the human intestine, *S. flexneri* must first invade the intestinal epithelium. To measure the ability of the *S. flexneri tonB* mutant to gain access to the intracellular environment, an invasion assay was used. In this assay the percentage of invaded epithelial cells is calculated at intervals over a 2 hour time course. The *tonB* mutant displayed no defect in invasion compared to the wild type strain (Fig. 11), indicating that TonB is not required for invasion of epithelial cells by *S. flexneri*.

*Shigella* that have invaded the colonic epithelium must grow within the epithelial cells before spreading to the adjacent cells. By measuring the number of bacteria per invaded epithelial cell at intervals over a time course, it is possible to determine if the bacteria are multiplying successfully within the host cell. The *tonB* mutant showed no defect in intracellular growth (Fig. 12). The number of *tonB* bacteria per epithelial cell increased over time and the rate of increase and average number of bacteria per epithelial cell were similar to that of the wild type control. This is in contrast to the *S. dysenteriae* pirazmonam-resistant mutant, SDU380, which grows poorly in the intracellular environment (155).

The plaque assay is a measure of the ability of *Shigella* to invade, grow within and spread between eukaryotic cells, steps essential to the pathogenesis of *Shigella*. Strains that exhibit a defect in any one of these steps will form smaller or fewer plaques than the wild type strain. Such plaque defects are predictive of reduced virulence. The *S.*

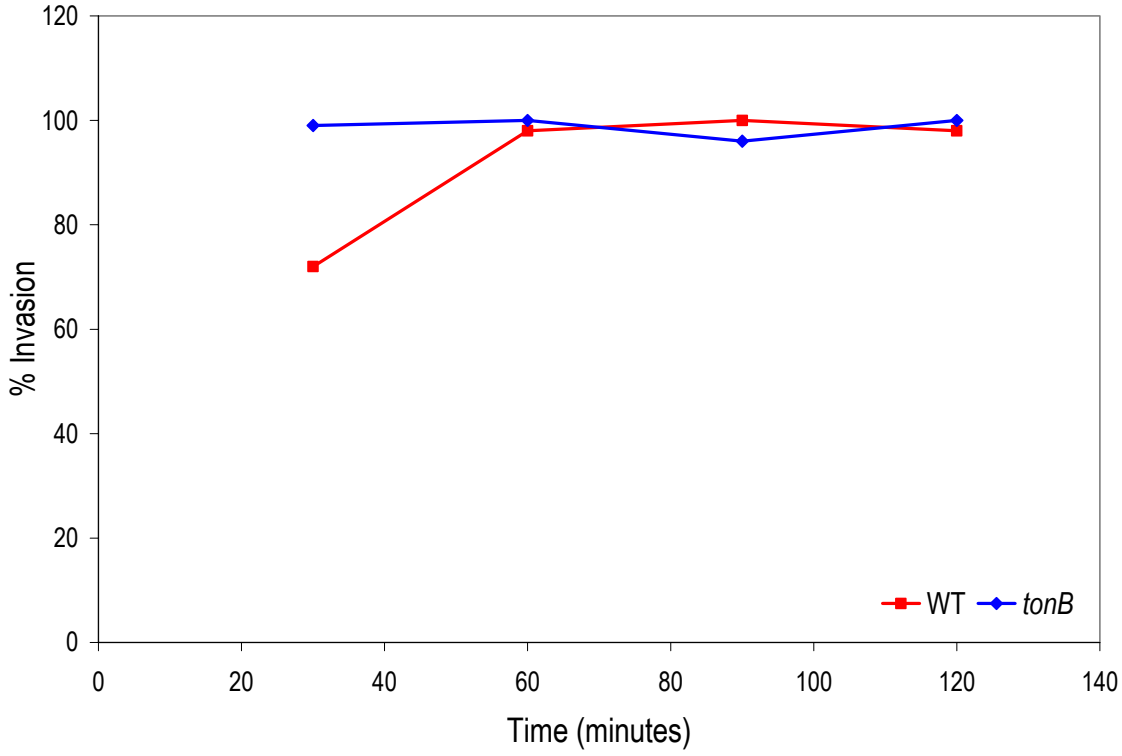



**Figure 11. An *S. flexneri tonB* mutant does not have a defect in epithelial cell invasion.**

Henle cell monolayers were inoculated with $2 \times 10^8$ bacteria and incubated at 37˚C for 15 minutes to allow for invasion before gentamicin was added to the medium to kill extracellular bacteria. After a total incubation time of 30, 60, 90 or 120 minutes, the infected monolayers were fixed and stained with Giemsa stain. The percentage of Henle cells invaded was determined by inspecting 300 Henle cells for the presence of intracellular bacteria. Henle cells containing 3 or more bacteria were considered invaded. Data from one representative experiment are shown.

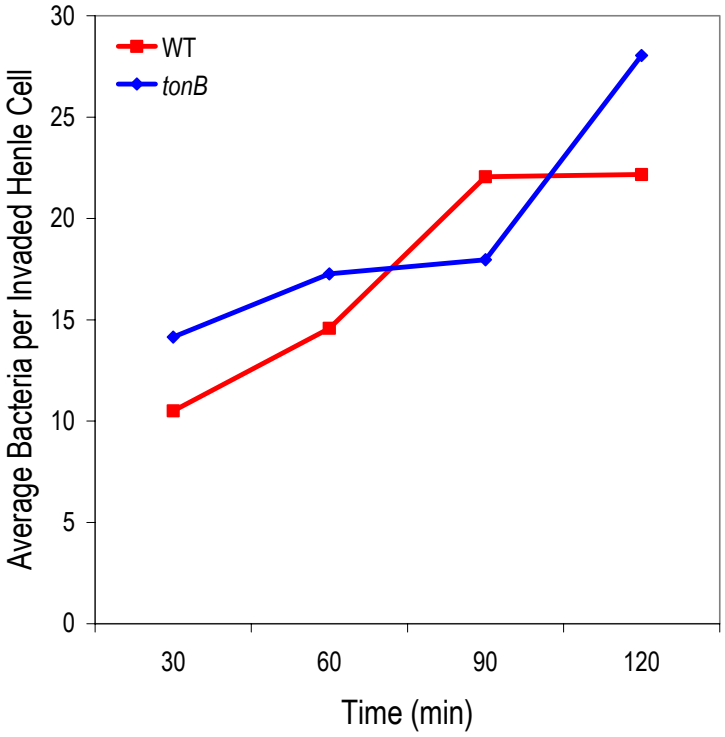



**Figure 12. An *S. flexneri tonB* mutant does not have a defect in intracellular growth.**

Henle cell monolayers were inoculated with $2 \times 10^8$ bacteria and incubated at 37˚C for 15 minutes to allow for invasion before gentamicin was added to the medium to kill extracellular bacteria. After a total incubation time of 30, 60, 90 or 120 minutes, the infected monolayers were detached by treatment with trypsin and the number of Henle cells present determined. The eukaryotic cells were then lysed with deoxycholate and the recovered intracellular bacteria were plated to determine the viable count. The number of bacteria per invaded Henle cell was determined by dividing the number of bacteria recovered by the number of Henle cells present and multiplying by the fraction of Henle cells that were invaded. Data from one representative experiment are shown.

*dysenteriae* pirazmonam-resistant mutant, SDU380, does not form plaques in Henle cell monolayers, possibly due to a defect in intracellular growth (155). The *S. flexneri tonB*

mutant exhibited no defects in the initial invasion of and growth within epithelial cells (Fig. 11, 12); however, the plaque assay measures these steps and the ability to spread to adjacent cells over a number of days, a time period more like that of a natural infection. In the plaque assay, the *tonB* mutant formed plaques similar in size and number to those formed by the wild type strain (Fig. 13). These data indicate that TonB is not essential for plaque formation by *S. flexneri*, and suggest that redundancy among the *S. flexneri* iron transport systems allows the *tonB* mutant to invade intestinal epithelial cells, grow within the eukaryotic cell and spread from cell-to-cell.

## 2. IN *S. DYSENTERIAE*, THE TONB- DEPENDENT IRON TRANSPORT SYSTEMS ARE IMPORTANT FOR GROWTH IN LOW-IRON CONDITIONS, BUT ARE NOT ESSENTIAL FOR PLAQUE FORMATION.

Because SDU380, a spontaneous *S. dysenteriae* pirazmonam-resistant mutant without TonB function, failed to form plaques in Henle cell monolayers but an *S. flexneri tonB* mutant, NDS100, exhibited no defect in plaque formation, an *S. dysenteriae* strain with a defined mutation in the *tonB* gene was constructed and characterized.

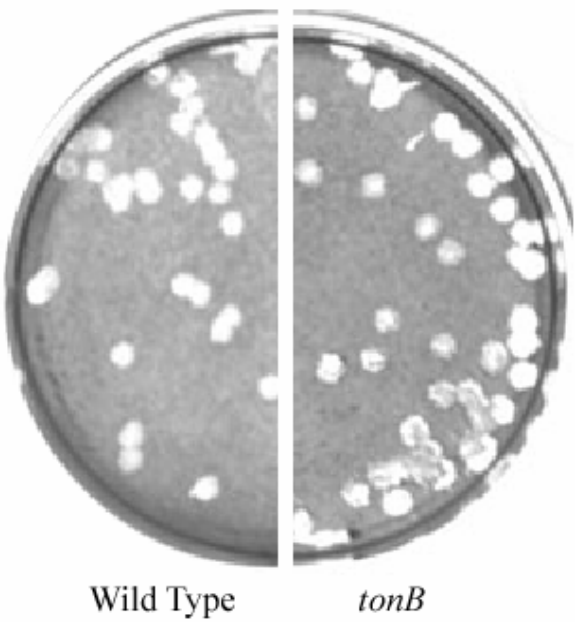



**Figure 13. The *S. flexneri tonB* mutant forms plaques in Henle cell monolayers.**

The *S. flexneri tonB* mutant formed plaques in Henle cell monolayers that were similar in size and number to those formed by the wild type strain. Confluent Henle cell monolayers (approximately $2\times10^6$ cells per 35 mm plate) were inoculated with $1 \times 10^4$ bacteria and incubated at 37˚C for 60 minutes to allow for invasion before gentamicin was added to the medium to kill extracellular bacteria. The infected monolayers were incubated at 37˚C for 4 days. Plaques were then visualized by staining with Giemsa stain.

**a) TonB is important for growth in low iron conditions in *S. dysenteriae*, but not when iron is abundant.**

In *S. dysenteriae*, TonB is required for high affinity iron uptake through several outer membrane receptors, including those that transport siderophores and heme. A mutation in the *tonB* gene prevents the bacterium from accessing many sources of iron, and *tonB* mutants often exhibit growth defects that likely result from iron starvation (55, 70, 190). An *S. dysenteriae tonB* mutant, NDS115, was found to grow well in conditions where iron was abundant (Fig. 14, 15), indicating that in such conditions, the TonB-dependent iron transport systems are not required. In low iron conditions, however, the *tonB* mutant exhibited a marked decrease in growth compared to the wild type strain. When an iron chelator was added to solid medium, the *tonB* strain formed smaller colonies than the wild type strain (Fig. 14), and when grown in broth in the presence of an iron chelator, the *tonB* mutant grew to a lower final OD than the wild type strain (Fig. 15). These data suggest that the TonB-dependent systems are important for growth in low iron conditions but are not required for growth when iron is abundant.

**b) TonB is not essential for plaque formation in *S. dysenteriae*.**

NDS115, an *S. dysenteriae tonB* mutant, formed plaques in Henle cell monolayers (Fig. 24). Although the plaques formed by NDS115 were slightly smaller than those formed by the wild type strain, these results, which contrast with those for SDU380, indicate that the TonB-dependent systems are important for plaque formation by *S.*

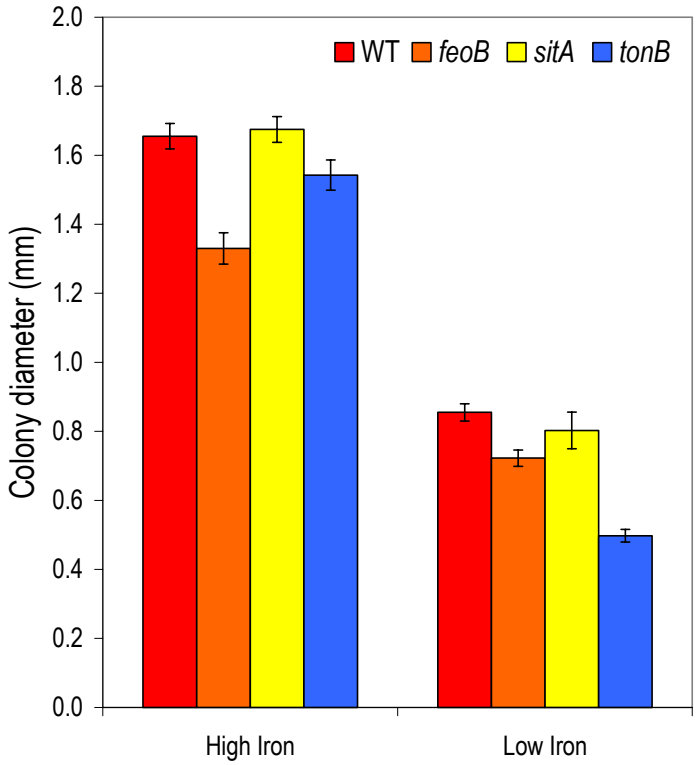



**Figure 14. Growth of *S. dysenteriae* iron transport mutants on solid media containing supplemental or limited iron.**

Overnight cultures were diluted and plated on medium containing 40 μM $FeSO_4$ (high iron) or 100 μg/ml EDDA (low iron) and were allowed to grow overnight at 37˚C. The average of the diameter of 10 colonies is shown and one standard deviation is indicated. A *tonB* mutant strain exhibited a growth defect when iron availability was limited, but grew as well as the wild type strain when the medium was supplemented with iron. A *feoB* mutant strain exhibited a growth defect when iron was abundant but not when iron was restricted.

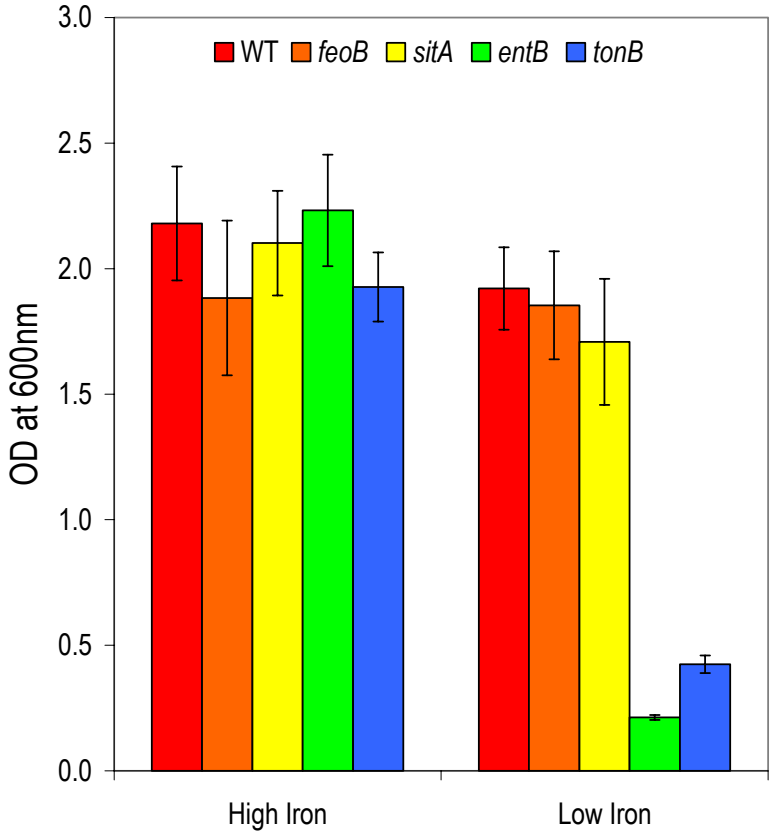
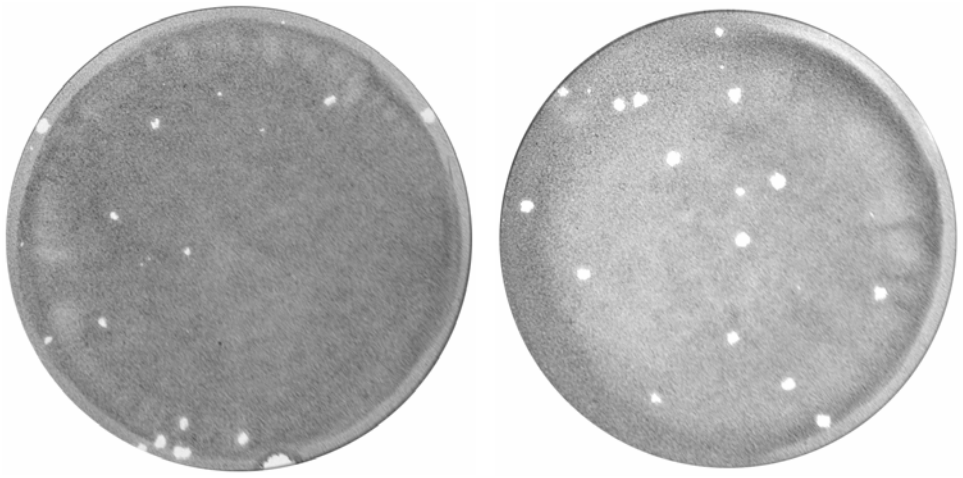



**Figure 15. Growth of *S. dysenteriae* iron transport mutants in LB broth containing supplemental or limited iron.**

Overnight cultures were diluted approximately 1:100 into LB broth + 40 μM $FeSO_4$ (high iron) or LB broth + 10 μg/ml EDDA (low iron) and allowed to grow for 8 hours. The OD of each culture at 600 nm was then determined. The mean of 3 independent experiments is shown and the standard deviation is indicated. The *tonB* and *entB* mutants display a growth defect when grown in medium containing an iron chelator. Such a defect is not observed when the *tonB* and *entB* strains are grown in medium containing supplemental iron.

*dysenteriae*, but are not required. Additionally, the number of plaques formed by NDS115 was similar to the number formed by the wild type strain, indicating that the *tonB* mutant was able to invade the epithelial cells at wild type levels, suggesting that TonB is important for intracellular growth or intercellular spread, but not for invasion of epithelial cells.



***tonB*** **(NDS115)**

**Wild Type (0-4576S1-G)**

**Figure 16. *tonB* is not required for plaque formation in *S. dysenteriae*.**
An *S. dysenteriae tonB* mutant, NDS115, formed smaller plaques than the wild type strain in Henle cell monolayers. Henle cell monolayers were inoculated with $1 \times 10^4$ bacteria and incubated at 37˚C for 60 minutes to allow for invasion before gentamicin was added to the medium to kill extracellular bacteria. The infected monolayers were incubated for 4 days. Plaques were then visualized by staining with Giemsa stain.

## B. Characterization of an Avirulent *S. dysenteriae* Mutant

SDU380 is a spontaneous *S. dysenteriae tonB* mutant that was previously isolated by selection for resistance to the antibiotic pirazmonam, which is transported in a TonB-dependent manner (132, 155). SDU380 does not produce TonB, and therefore, does not transport heme or siderophores, iron-binding molecules that require TonB for uptake, and exhibits a growth defect in low iron medium (155). Additionally, SDU380 does not form plaques in Henle cell monolayers, likely due to a defect in intracellular growth (155). This inability to grow intracellularly and spread from cell to cell would prevent establishment of an infection in the human colonic epithelium, and, therefore, suggests a defect in virulence. However, the ability of NDS100, an *S. flexneri tonB* mutant and NDS115, an *S. dysenteriae tonB* mutant, to form plaques in Henle cell monolayers indicates that *tonB* is not required for plaque formation and suggests than an additional gene or genes may be mutated in SDU380. To identify this virulence-associated gene, characterization of SDU380 was undertaken.

### 1. SDU380 HAS SUSTAINED MUTATIONS IN *TONB* AND IN AN ADDITIONAL GENE OR GENES.

A plasmid carrying *E. coli tonB*, pAMT11, restored the ability of SDU380 to grow using hemin, enterobactin, and ferrichrome as iron sources (Table 7). Growth of SDU380 in low iron conditions was also restored to wild type levels when *tonB* was supplied (Fig. 17). These data confirm that SDU380 lacks a functional copy of the *tonB* gene and that the iron utilization phenotype of SDU380 results from this mutation.

Table 7. Ability of various siderophores and of iron to stimulate the growth of SDU380 under iron-restricted conditions.

| Iron Source | Zone of Growth[a] | | | |
|---|---|---|---|---|
| | SDU378 (WT) | SDU380 ($Pir^R$) | SDU380/pWKS30 (vector) | SDU380/pAMT11 (*tonB*$^+$) |
| Enterobactin | +++ | - | - | +++ |
| Ferrichrome | +++ | - | - | +++ |
| Hemin | +++ | - | - | +++ |
| $FeSO_4$ | +++ | + | + | +++ |

[a] Bioassays were completed as described in Materials and Methods. - indicates no growth around the iron source, + indicates a small zone of growth was present around the iron source and +++ indicates that a large zone of growth was present around the iron source.

To confirm that the lack of TonB was not responsible for the failure of SDU380 to grow within and spread between epithelial cells, SDU380/pAMT11 was tested for the ability to form plaques in Henle cell monolayers. Despite the presence of a wild type copy of *tonB* carried on plasmid pAMT11, SDU380/pAMT11 did not form plaques (Fig. 18). These data indicate that the lack of TonB is not the sole reason for the failure of SDU380 to form plaques in epithelial cell monolayers and indicate that another gene or genes may be mutated.

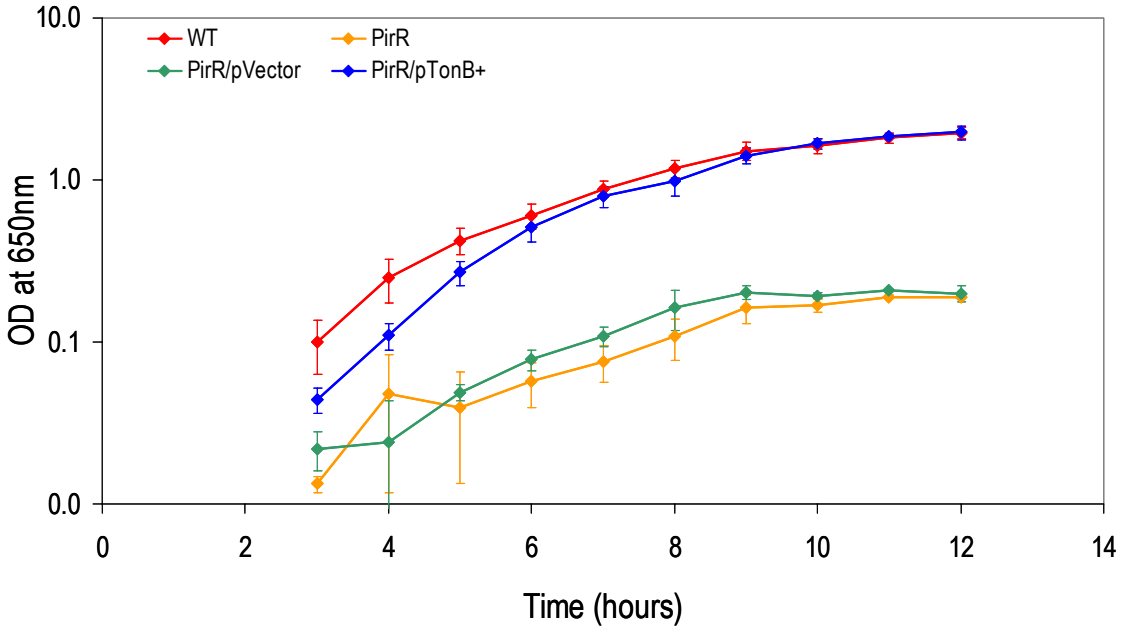



**Figure 17. An exogenous copy of *tonB* restores growth of SDU380 to wild type levels in iron-restricted medium.**

SDU380 exhibits a growth defect when iron is limited by the addition an iron chelator. When *tonB* is supplied on a plasmid, growth of SDU380 in low iron conditions is restored to the level of the wild type strain, SDU378. Overnight cultures of WT (SDU378), $pir^R$ mutant (SDU380), $pir^R$/vector (SDU380/pWKS30) and $pir^R$/$pTonB^+$ (SDU380/pAMT11) were diluted approximately 1:1000 and grown in LB broth + 10 µg/ml EDDA. The optical density of each culture was measured every hour for 12 hours. The mean and standard deviation of three independent experiments are shown.

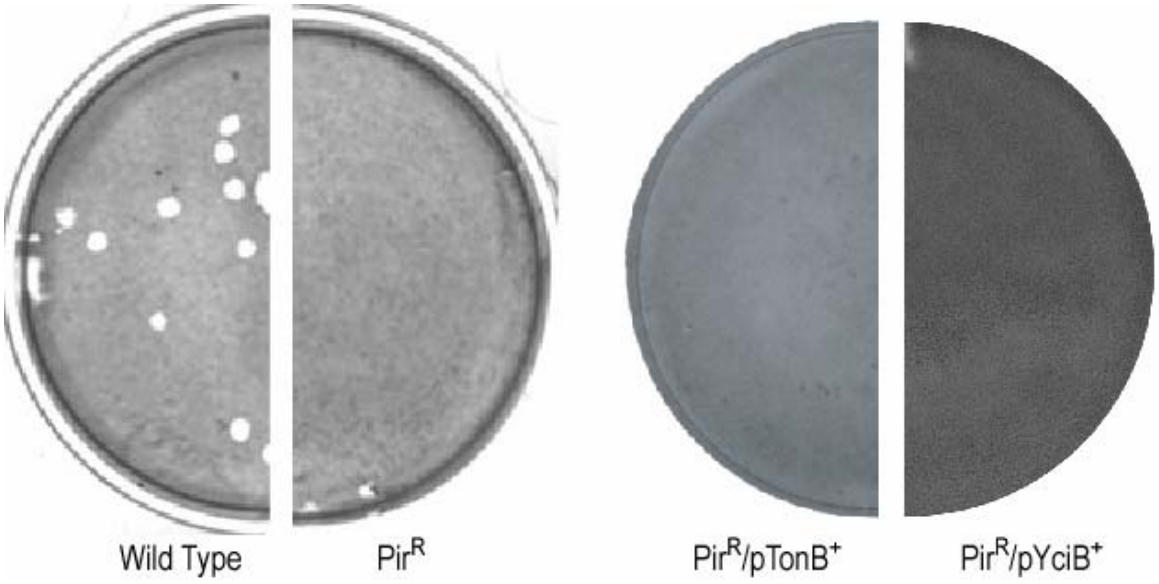



**Figure 18. TonB does not restore the ability to form plaques to SDU380.**
SDU380 carrying exogenous copies of the *tonB* gene (pAMT11) and the *tonB* and *yciABC* genes (pRZ526) did not form plaques in Henle cell monolayers. Henle cell monolayers were inoculated with $1 \times 10^4$ (SDU378) or $1 \times 10^5$ (SDU380) bacteria and incubated at 37˚C for 60 minutes to allow for invasion before gentamicin was added to the medium to kill extracellular bacteria. The infected monolayers were incubated for 4 days. Plaques were then visualized by staining with Giemsa stain.

## 2. SDU380 HAS SUSTAINED A LARGE DELETION FLANKING THE *TONB* GENE

To determine the exact nature of the mutation sustained by SDU380, attempts were made to amplify the region surrounding the *tonB* gene for sequence analysis. In the wild type strain, SDU378, this region could be amplified with no difficulty; however, in SDU380, the region surrounding the *tonB* gene could not be amplified by PCR (Fig. 19). This suggested that a deletion encompassing the *tonB* gene and an undetermined amount of flanking sequence may have been sustained by SDU380.

Southern blot analysis was used to confirm that SDU380 had sustained a deletion. Probes for *tonB* (Fig. 20) and for *yciAB* (data not shown) failed to anneal to the SDU380 genomic DNA, indicating that these genes, present in the wild type strain, were absent from the SDU380 genome and suggesting that a deletion had mediated the loss of TonB function in SDU380.

To determine the extent of the deletion, the genome of SDU380 was compared to that of the wild type strain by genomic hybridization to a microarray. In addition to the *tonB* gene and the *yciABC* operon, it was found that the *opp* operon, the *trp* operon, *cls* (the gene encoding cardiolipin synthase), *kch* (encoding a potassium channel protein), *ompW* and several additional loci of unknown function were absent from SDU380 (Fig. 21). Comparison of the genes found to be missing from SDU380 with the published sequence of *S. dysenteriae* 197 (NC_007606) indicated that a region of approximately 33 kb, flanked by IS1 insertion elements and encompassing the sequence from *oppA* to *yciO*, had been deleted (Fig. 21).

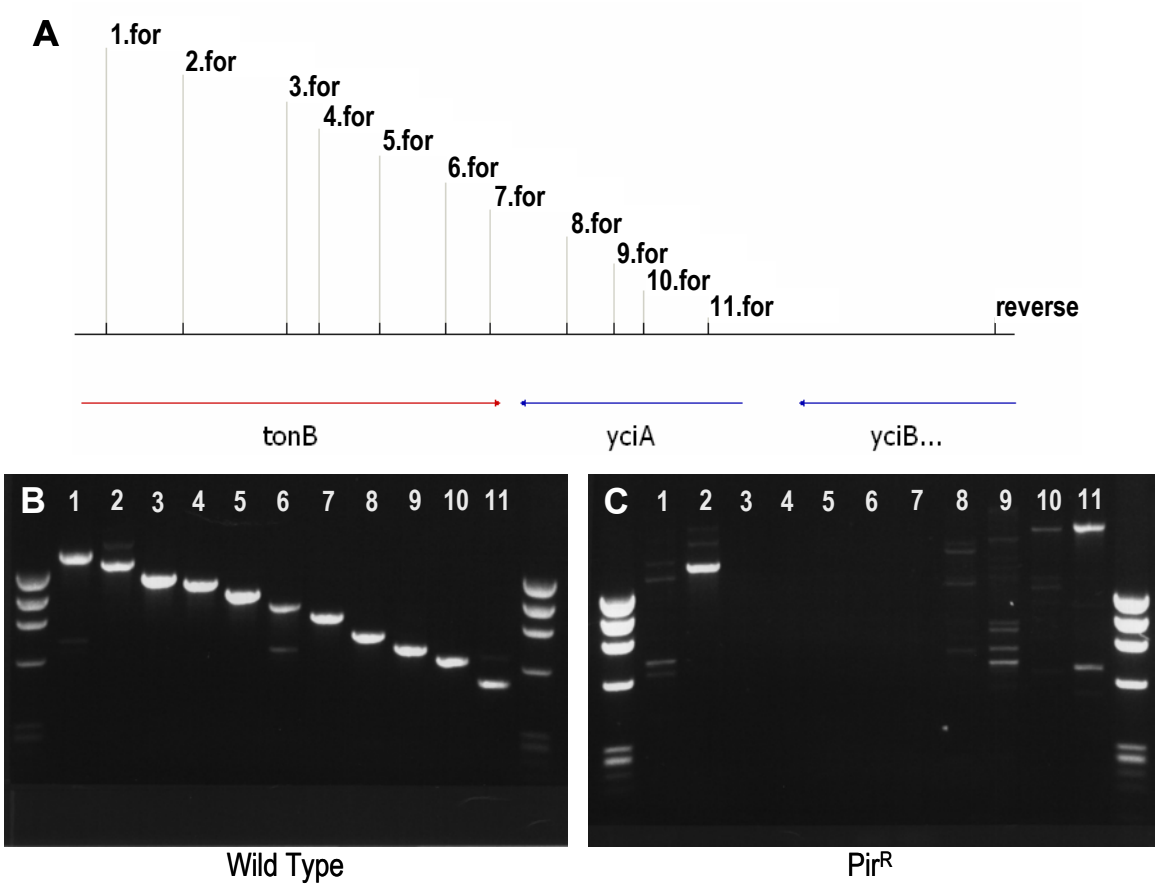



**Figure 19. SDU380 DNA cannot be amplified by primers designed to anneal within the *tonB* gene and to the surrounding sequence.**

A set of primers was designed to amplify fragments of decreasing length in the region surrounding the *tonB* gene in *S. dysenteriae*. The locations of the primers are shown in A. Following amplification of wild type or SDU380 DNA using one of the forward primers and the reverse primer detailed in A, the products were run on 1% agarose gels and visualized by staining with ethidium bromide. The forward primer used is indicated above each lane. SDU378 PCR products are shown in panel B and SDU380 products are shown in panel C.

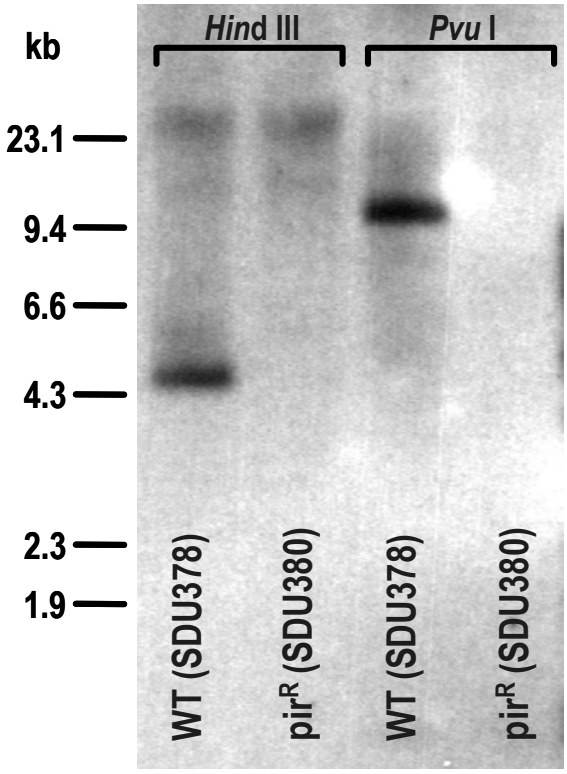



**Figure 20. The *tonB* gene is absent from the genome of SDU380.**
Genomic DNA was isolated from the wild type (SDU378) and pir$^R$ (SDU380) strains, digested with *Hin*d III and *Pvu* II, and subjected to Southern blot analysis using a probe with sequence internal to the *tonB* gene. The probe annealed to the SDU378 genome but did not anneal to the genome of SDU380. Sizes and locations of molecular weight markers are indicated on the left.

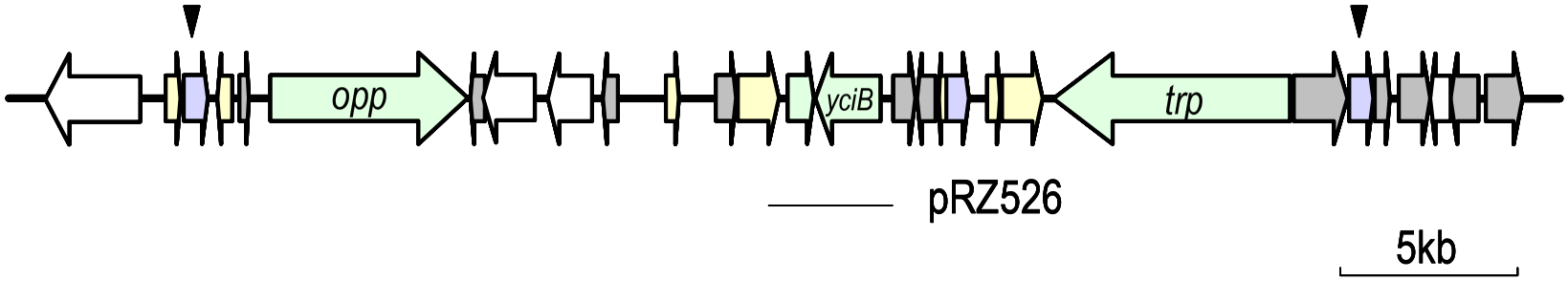





**Figure 21. Map of the region absent from SDU380.**

A map of the region deleted from SDU380 is shown. IS1 elements are indicated by the blue arrows and other insertion elements by the yellow arrows. Genes of unknown function and hypothetical or putative open reading fames are indicated by yellow arrows. The green arrows denote genes or operons that have been mutated in 0-4576S1-G. The *tonB* gene is indicated by the unlabeled green arrow and the *yciABC* operon is indicated by the designation *yciB*. The white arrows denote genes or operons of known function. Arrowheads indicate the approximate endpoints of the deletion in SDU380. The region cloned in plasmid pRZ526 is indicated below the mapline.

### 3. *YCIB* AND ONE OR MORE ADDITIONAL GENES ABSENT FROM SDU380 ARE REQUIRED FOR PLAQUE FORMATION IN *S. DYSENTERIAE*

Because SDU380 does not form plaques in Henle cell monolayers, one or more of the genes or operons in the deleted region may be important for plaque formation by *S. dysenteriae*. To identify that gene or genes, a cosmid library of *S. dysenteriae* chromosomal DNA was transferred into SDU380. The resulting transconjugants were screened for the ability to grow on medium containing ferrichrome, a fungal siderophore that requires TonB for transport, as the sole source of iron. SDU380 is resistant to multiple antibiotics, so the presence of a cosmid within SDU380 could not be selected for by antibiotic resistance; however, due to the absence of the *tonB* gene, SDU380 is unable to utilize siderophores as an iron source, so cosmids carrying *tonB* can be selected for by growth in the presence of a siderophore as the sole source of iron. Because the *tonB* gene is at the center of the deleted region, and because the cosmids selected would carry a large amount sequence flanking the *tonB* gene, this selection method made it possible to isolate cosmids likely to carry the gene of interest. Twenty cosmid-carrying isolates of SDU380 selected in this manner were tested for the ability to form plaques; however, none of the isolates formed plaques in Henle cell monolayers, suggesting that the gene or genes responsible for the failure of SDU380 to form plaques were not carried on the cosmids tested.

Due to the difficulty of performing genetics experiments in a strain that is resistant to multiple antibiotics, further experiments to determine the identity of the gene or genes responsible for the failure of SDU380 to form plaques were carried out using *S.*

*dysenteriae* type 1 strain 0-4576S1-G. Like SDU378 and SDU380, 0-4576S1-G is an *S. dysenteriae* type 1 strain derived from a clinical isolate; however, unlike SDU378 and SDU380, 0-4576S1-G is resistant to streptomycin, but is sensitive to other commonly used antibiotics. In this background, single mutations were made in the tryptophan biosynthetic operon and oligopeptide permease operon; however, neither of these strains exhibited defects in plaque formation, indicating that neither of these operons is essential for plaque formation by *S. dysenteriae* (K. Hilgendorf, personal communication).

One gene within the deleted region, *yciB* (also referred to as *ispA* or *ispZ*) has been implicated in virulence in *S. flexneri*, but the role of this gene in *S. dysenteriae* virulence is unknown (81, 106). In *S. flexneri*, *yciB* mutants exhibited septation defects in the intracellular environment and failed to polymerize actin, a requirement for intracellular spread (81, 106). A *yciB* mutant constructed in 0-4576S1-G, KHS103, failed to form plaques in Henle cell monolayers (Fig. 22). A plasmid carrying the *yciABC* operon and the *tonB* gene, pRZ526, complemented the plaque formation defect of KHS103; however, the plaques formed were smaller than those formed by the wild type strain (Fig. 22). This decreased plaque size may be due to altered expression or altered regulation of the plasmid-borne copy of the *yciABC* genes. In SDU380, pRZ526 did not restore the ability to form plaques, despite restoring the ability to grow using TonB-dependent iron sources as the sole source of iron (Fig. 18 and data not shown). These data suggest that *yciB* is not the only gene required for plaque formation that is mutated in or absent from SDU380.


**Figure 22. *yciB* is required for plaque formation in *S. dysenteriae*.**
An *S. dysenteriae yciB* mutant, KHS103, failed to form plaques in Henle cell monolayers. The ability of KHS103 to form plaques was restored when *yciB* was supplied on plasmid pRZ526. Henle cell monolayers were inoculated with $1 \times 10^4$ bacteria and incubated at 37˚C for 60 minutes to allow for invasion before gentamicin was added to the medium to kill extracellular bacteria. The infected monolayers were incubated for 4 days. Plaques were then visualized by staining with Giemsa stain.

## C. Identification and Characterization of the Iron Acquisition Systems Employed by *S. dysenteriae*

At the outset of this study, the genome of *S. dysenteriae* had not yet been completely sequenced; therefore, an analysis of the partially assembled genome sequence of *S. dysenteriae* M131649 (downloaded from the Wellcome Trust Sanger Institute at http://www.sanger.ac.uk/Projects/Escherichia_Shigella/) was used to identify possible iron transport systems. Putative iron acquisition systems were identified by homology to known iron transport systems in other organisms (Table 8), and mutations were made in the systems identified. In addition to the TonB-dependent iron acquisition systems, *S. dysenteriae* also has two TonB-independent iron transport systems, the Sit iron and manganese uptake system and the Feo ferrous iron acquisition system. A set of strains with mutations in one, two or three of the known *S. dysenteriae* iron transport systems was constructed and the mutants characterized. The sequence of the genome of an *S. dysenteriae* serotype 1 strain, 197 (NC_007606), has recently been published (199) and this sequence was analyzed to determine whether any other iron transport systems were identified; however, no additional iron transport systems beyond those identified by homology were annotated in this sequence.

Table 8. Iron acquisition systems identified in *S. dysenteriae* by homology to systems in *S. flexneri* and *E. coli*.

| Gene | Function | *E. coli* K12 | *S. dysenteriae* 197 | *S. flexneri* 301 | *S. flexneri* 2457T |
|---|---|---|---|---|---|
| TonB-dependent iron transport | | | | | |
| *tonB* | Energy transduction | annotated[a] | annotated | annotated | annotated |
| *exbB* | Energy transduction | annotated | annotated | annotated | annotated |
| *exbD* | Energy transduction | annotated | annotated | annotated | annotated |
| *cirA* | Catechol uptake | annotated | region of homology exists[b] | annotated | region of homology exists |
| *fecA* | Citrate uptake | annotated | annotated | - | - |
| *fepA* | Enterobactin uptake | annotated | annotated | annotated | annotated |
| *fhuA* | Ferrichrome uptake | annotated | annotated | annotated | annotated |
| *fhuE* | Coprogen, ferrioxamineB and rhodotorulic acid uptake | annotated | annotated | annotated | region of homology exists |
| *iroN* | Salmochelin and enterobactin uptake | - | annotated | - | - |
| *shuA* | Heme uptake | - | annotated | - | - |
| *fiu* | Ferric iron uptake | annotated | region of homology exists | - | - |
| *iutA* | Aerobactin uptake | - | - | annotated | annotated |
| Feo iron acquisition system | | | | | |
| *feoAB* | Ferrous iron acquisition | annotated | annotated | annotated | annotated |
| Sit iron and manganese uptake system | | | | | |
| *sitABCD* | Iron and manganese uptake | - | annotated | annotated | annotated |

[a] Genes annotated in the specified genome sequence.

[b] Genes which are not annotated in a given sequence, but for which there is a region of homology to the corresponding gene in the *E. coli* genome. May or may not encode a functional protein.

## 1. THE FEO SYSTEM PLAYS AN IMPORTANT ROLE IN *S. DYSENTERIAE* GROWTH AND VIRULENCE

### a) The Feo system is important for growth in high iron conditions.

The Feo system has been implicated in the acquisition of ferrous iron; however, the mechanism by which the Feo system participates in iron uptake is not known (29, 87, 186). In *S. dysenteriae*, a *feoB* mutant, NDS118, exhibited a growth defect in conditions where iron was abundant, forming smaller colonies than the wild type strain when grown on medium supplemented with 40 μM $FeSO_4$ (Fig. 14). This is in contrast to the growth of the *feoB* mutant in low iron conditions. When an iron chelator was added to the medium, the *feoB* mutant formed colonies similar in size to those formed by the wild type strain (Fig. 14). Because the *feo* operon is induced by growth in anaerobic conditions (87), the growth of the *feoB* mutant was also assessed following anaerobic growth and a similar trend was observed (data not shown). To determine if the decreased colony size observed when the *feoB* mutant was grown on media containing supplemental iron was as a result of iron toxicity or starvation, the sensitivity of the *feoB* mutant to hydrogen peroxide and cumene hydroperoxide was tested. The damage caused by these reactive oxygen species is exacerbated by the presence of free iron and the relative sensitivity of mutant strains to peroxides can be used to infer the amount of iron internal to the bacterium. The sensitivity of the *feoB* mutant to hydrogen peroxide and cumene hydroperoxide was very similar to that of the wild type strain, suggesting that the *feoB* mutant and the wild type strain contain similar amounts of internal iron (Fig. 23 and data not shown). These data indicate that the Feo iron transport system is important for

growth in conditions where iron is abundant but no obvious change in the level of free iron in the cell was detected by sensitivity to reactive oxygen species, suggesting that the decreased growth of the *feoB* mutant may not result from alterations in internal iron concentration.

**b) The Feo system is important for virulence in *S. dysenteriae*.**

The *feoB* mutant was tested for the ability to form plaques in Henle cell monolayers and was found to form plaques that were smaller in size than those formed by the wild type strain (Fig. 24). Because no decrease was seen in the number of plaques formed, the *feoB* mutant does not appear to have a defect in invasion; however, the decreased size of the plaques formed by the *feoB* mutant indicates a defect in intracellular growth or intercellular spread that would likely lead to reduced virulence in the human host. To better quantify this defect, competitive plaque assays were used. The *feoB* mutant exhibited reduced fitness compared to the wild type strain in the intracellular environment and when grown in medium containing supplemental iron (Fig. 25), suggesting that the Feo system is important for pathogenesis and that mutation of *feoB* is selected against in a variety of environments.

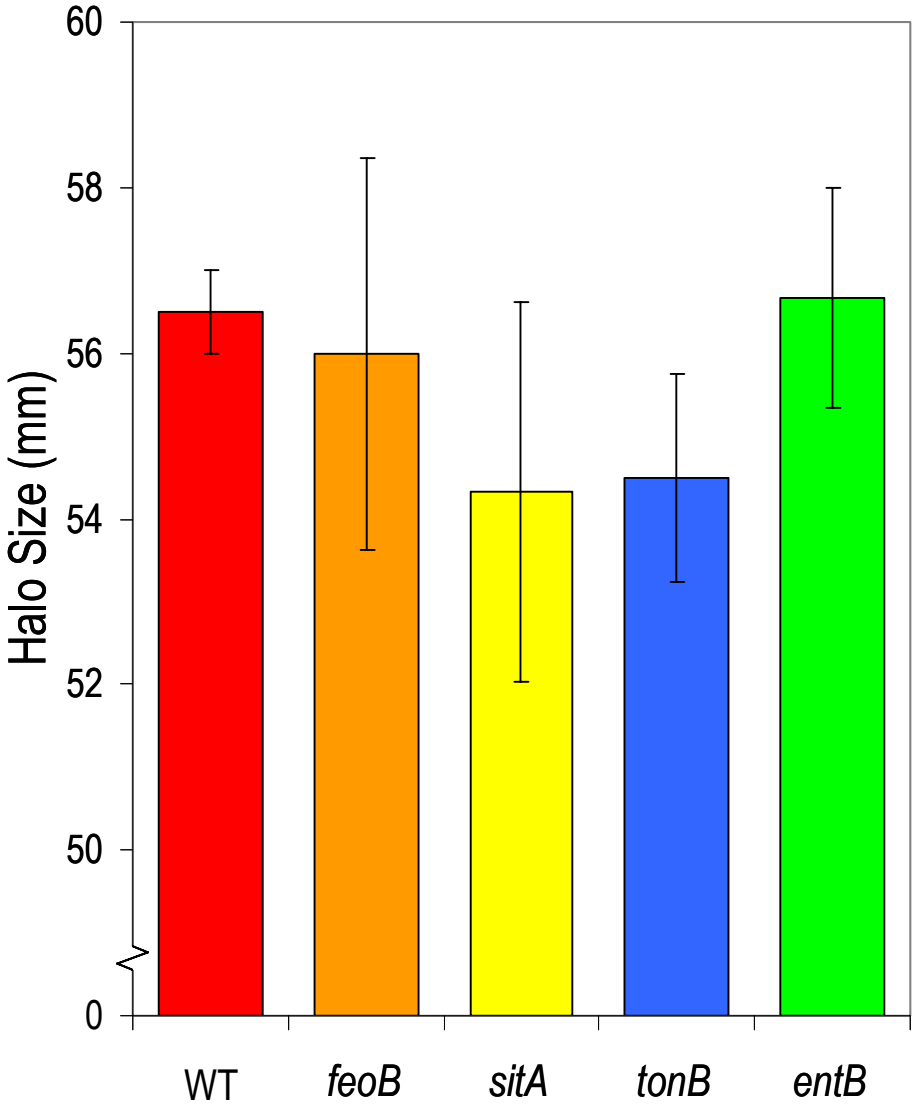



**Figure 23. Sensitivity of *S. dysenteriae* iron transport mutants to hydrogen peroxide.**

Overnight cultures were diluted approximately 1:1000 and added to warm LB agar containing 40 μM $FeSO_4$. The agar was allowed to solidify and 10 μL of 1M hydrogen peroxide was spotted on a paper disk on top of the plate. The plates were incubated overnight and the size of the clearing around the paper disk was measured. The means and standard deviations of three independent experiments are shown.

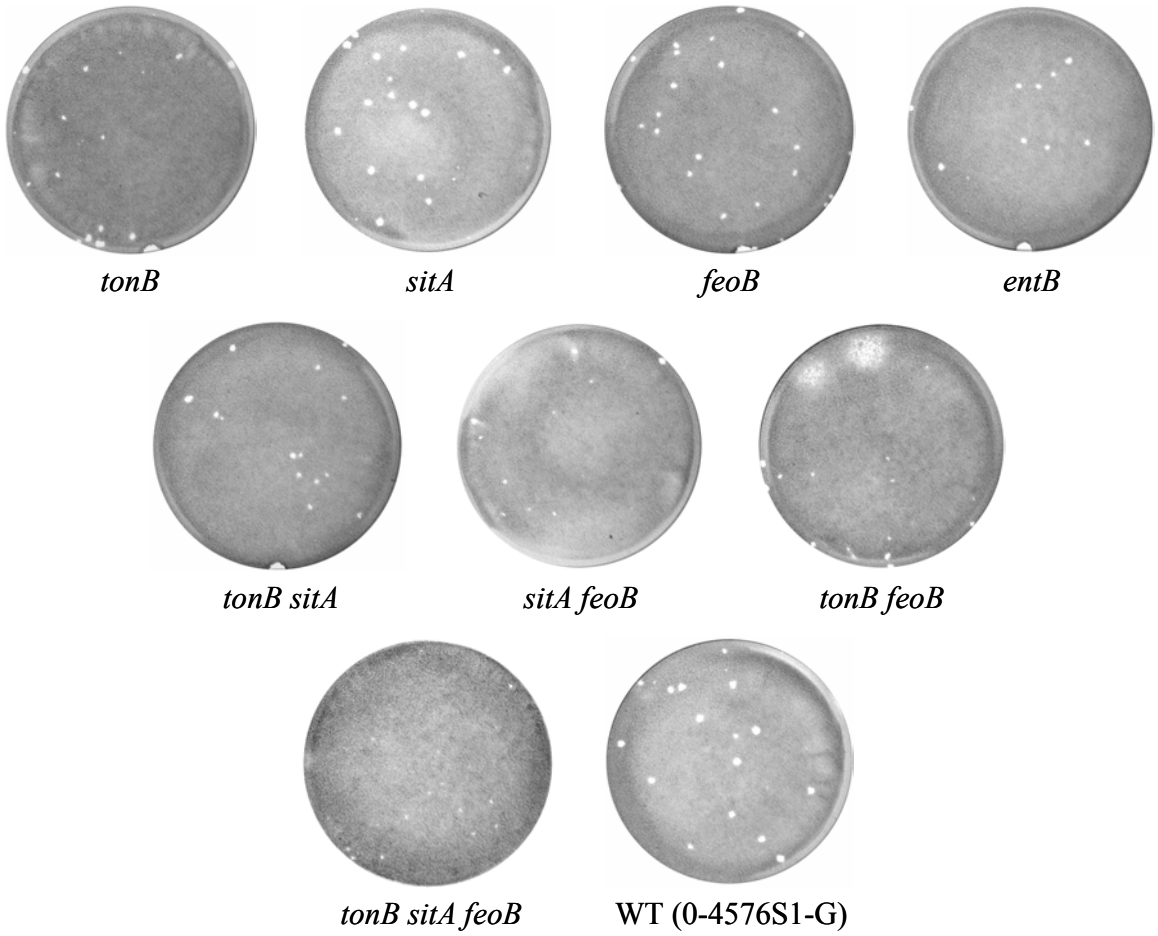



**Figure 24. *S. dysenteriae* iron transport mutants form plaques in Henle cell monolayers.**

Confluent Henle cell monolayers (approximately $2 \times 10^6$ cells per 35 mm plate) were inoculated with $1 \times 10^4$ bacteria and incubated at 37˚C for 60 minutes to allow for invasion before gentamicin was added to the medium to kill extracellular bacteria. The infected monolayers were incubated for 4 days. Plaques were visualized by staining with Giemsa stain. The *tonB, entB* and *feoB* mutants formed smaller plaques than the wild type and the *sitA* mutant formed plaques that were similar in size to those formed by the wild type strain. The double iron transport mutants formed small plaques as did the *tonB sitA feoB* triple mutant.

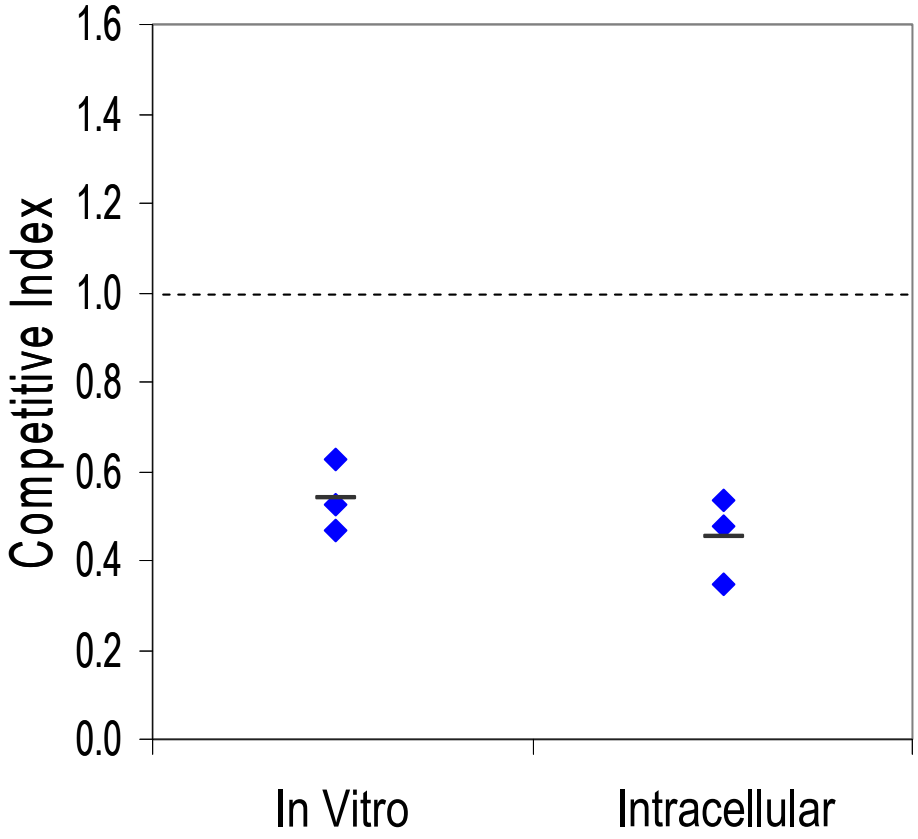



**Figure 25. The *feoB* mutant exhibits decreased fitness compared to the wild type strain.**

Approximately equal numbers of *feoB* and wild type bacteria were mixed, the ratio of *feoB* to wild type bacteria determined (ratio in), and the mixture grown in LB broth + 40 μM $FeSO_4$ for 8 hours (in vitro) or the mixture was used to inoculate a plaque assay (intracellular). The bacteria were then recovered, the ratio of *feoB* to wild type bacteria recovered determined (ratio out) and the competitive index calculated by dividing the ratio out by the ratio in. The *feoB* mutant exhibited decreased fitness in both the in vitro and intracellular environments. The results of three experiments are shown and the mean indicated by a gray bar.

**c) The *feoB* mutant overproduces enterobactin when grown in the presence of iron.**

When iron is abundant, the Fur protein represses the expression of genes whose products are involved in the acquisition of iron, including the enterobactin biosynthetic operon and the genes for uptake of ferri-enterobactin (69, 170). Because of this iron-dependent regulation, the production of enterobactin by bacteria can be used as an indicator of iron starvation, and enterobactin is seldom produced when strains are grown in media supplemented with iron.

When the *feoB* mutant was grown overnight in LB broth supplemented with 40 μM $FeSO_4$, a color change, characteristic of siderophore production, was observed in the medium (Fig. 26). To determine if the color change was as a result of enterobactin production, the amount of enterobactin present in the medium was quantified using the Arnow method (7) (Fig. 27). The *feoB* mutant produced far more enterobactin than the wild type, *tonB* or *sitA* strains when grown in medium containing iron. When the amount of available iron was limited by the addition of an iron chelator, the *feoB* mutant produced an amount of enterobactin similar to that produced by the wild type strain and the other iron transport mutant strains (Fig. 27). This effect was not limited to *feoB* strain NDS118; very similar results were observed when a second *S. dysenteriae feoB* mutant, 0-4576S1 *feoB*::kan, was tested under the same conditions (Fig. 28). In *S. flexneri*, no aerobactin production was observed in the *feoB* mutant in media containing supplemental iron, nor was production of vibriobactin, a catecholate siderophore produced by *V. cholerae* observed in the *V. cholerae feoB* mutant under similar conditions (Fig. 28 and data not shown). Additionally, an *E. coli feoB* mutant did not produce more enterobactin

than the wild type strains when grown in medium containing supplemental iron (Fig. 28). These results suggest that overproduction of enterobactin by a *feoB* mutant is limited to *S. dysenteriae* and is not a general phenomenon.

Expression of the enterobactin biosynthetic genes is normally repressed when iron is plentiful (69, 170). This repression is mediated by the Fur protein, which, when bound to iron, represses expression of many genes by interacting with the promoter region and inhibiting transcription initiation (65, 69, 167). In the *feoB* mutant, enterobactin was produced when the growth medium contained plentiful iron. Therefore it was of interest to determine if additional Fur-regulated genes were de-repressed, possibly indicating that the *feoB* mutant was starved for iron even when the metal was plentiful, or if the enterobactin biosynthetic genes were the only Fur-regulated genes expressed in the *feoB* mutant in the presence of iron. Microarray analysis was used to compare gene expression in the *feoB* and wild type strains during growth in medium containing 40 μM $FeSO_4$ (Table 9). Under the conditions tested, the *feoB* mutant expressed *entB*, and presumably all of the genes of the *entABCD* operon, at a higher level than the wild type; however, no other genes involved in iron acquisition were expressed more highly in the *feoB* mutant that in the wild type strain, suggesting that the overproduction of enterobactin by the *feoB* mutant is not an indication that the mutant is starving for iron.

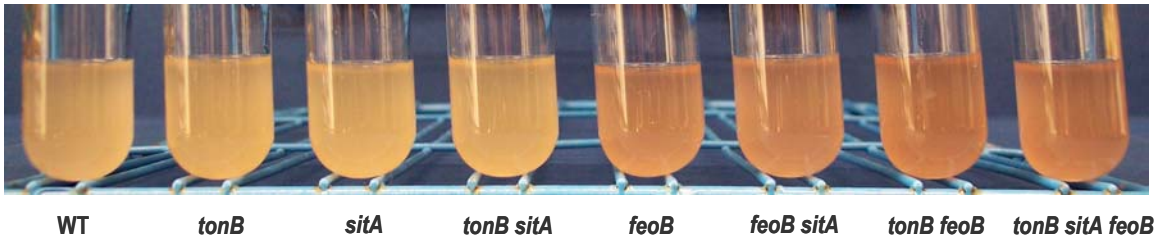



**Figure 26. A color change was observed following growth of *feoB* mutants in high iron medium.**

The strains indicated were grown overnight in LB broth + 40 μM $FeSO_4$. The color change seen in the *feoB* strains was not observed following growth of other iron transport mutants in the same medium. The effect was more noticeable when strains with mutations in both *feoB* and *tonB*, which prevents siderophore transport, were grown in high iron medium.

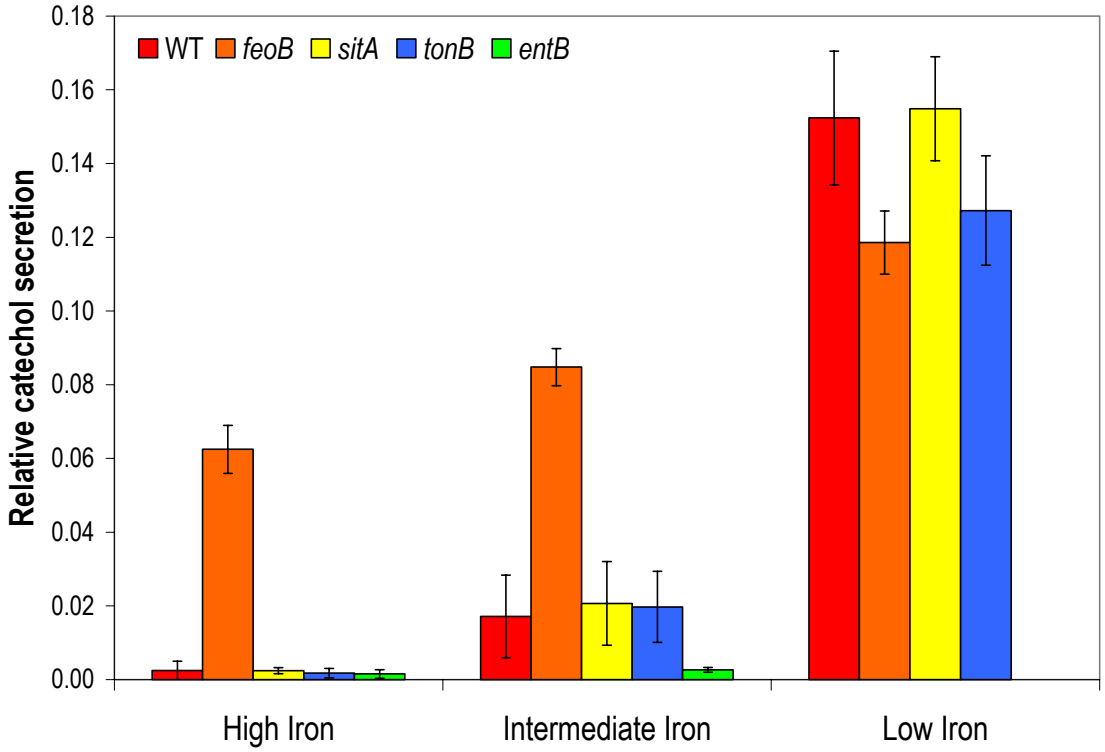



**Figure 27. The *feoB* mutant overproduces enterobactin when grown in medium containing added iron.**

The indicated strains were grown overnight and diluted 1:100 into MM9 to which 40 μM $FeSO_4$ (high iron), nothing (intermediate iron) or 100 μg/ml EDDA (low iron) was added. The cultures were then allowed to grow for 8 hours and the amount of catecholate siderophore present in the medium measured. In high iron conditions, the *feoB* mutant secreted large amounts of a catechol, likely enterobactin, into the medium. Under such conditions the wild type and the other iron transport mutants did not produce measurable amounts of enterobactin. In low iron conditions, all strains produced similar amounts of enterobactin.

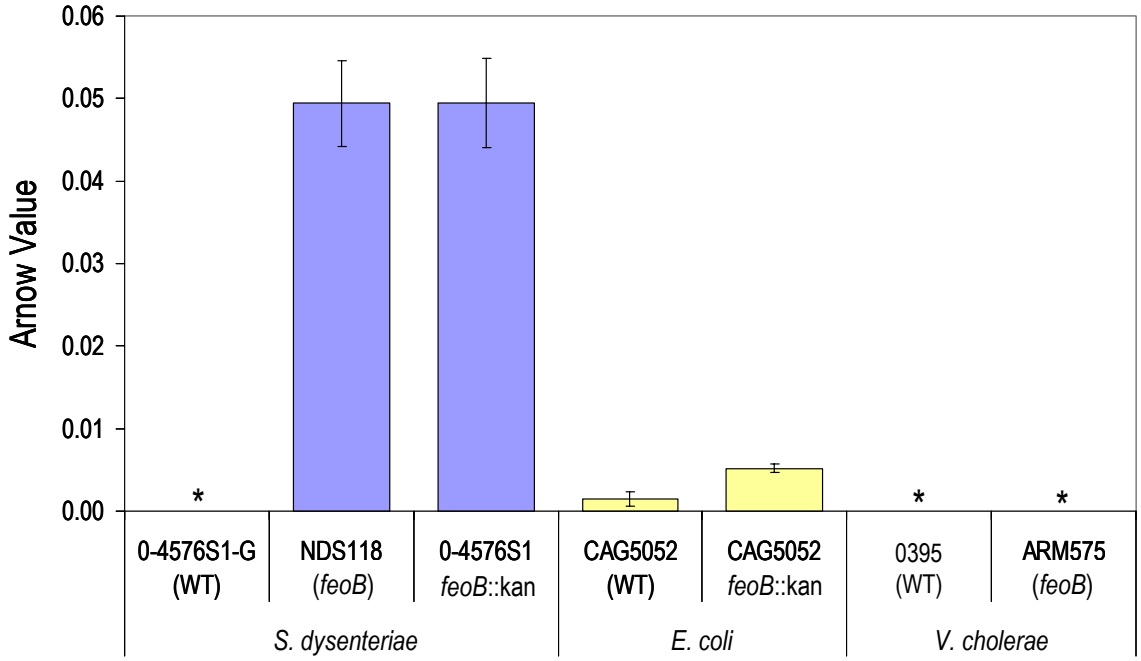



**Figure 28. *E. coli* and *V. cholerae feoB* mutants do not overproduce siderophore when grown in medium containing added iron.**

The indicated strains were grown overnight and diluted 1:100 into MM9 to which 40 μM $FeSO_4$ was added. The cultures were then allowed to grow for 6 hours and the amount of catecholate siderophore present in the medium measured. An asterisk indicates that no catechol was detectable. Both *S. dysenteriae feoB* mutants secreted large amounts of a catechol, likely enterobactin, into the medium. The *E. coli feoB* mutant produced very little catechol and the *V. cholerae feoB* did not produce a detectable amount of catechol under these conditions. An asterisk indicates that no catechol was detected.

Table 9. Genes expressed at a higher level in the *feoB* mutant (NDS118) than the wild type strain (0-4576S1-G) following growth in LB broth containing 40 μM $FeSO_4$.

| Gene Name | Fold Induction | Function |
|---|---|---|
| ECs1388 | 18 | putative transcriptional regulator |
| *gntY* | 5.6 | predicted gluconate transport associated protein |
| *ydjM* | 4.0 | predicted inner membrane protein |
| *gntT* | 2.9 | gluconate permease (high-affinity transport of gluconate) |
| *modC* | 2.9 | molybdate transporter subunit (ATP-binding component of ABC transporter) |
| *entB* | 2.8 | 2,3-dihydro-2,3-dihydroxybenzoate synthetase (enterobactin biosynthesis) |
| *fdnG* | 2.5 | formate dehydrogenase N alpha subunit (contains in-frame stop codon) |
| Z2346 | 2.4 | partial putative phage tail protein (prophage encoded) |
| *yjjN* | 2.3 | predicted oxidoreductase (truncated) |
| *ftsX* | 2.3 | integral membrane cell division membrane protein |
| *yihX* | 2.2 | putative phosphatase |
| Z3365 | 2.2 | putative host-nuclease inhibitor protein Gam (prophage encoded) |
| *ygiV* | 2.2 | predicted transcriptional regulator |
| *gapA* | 2.2 | glyceraldehyde-3-phosphate dehydrogenase A |
| *ykgE* | 2.1 | predicted oxidoreductase |
| *yaiN* | 2.1 | hypothetical protein |
| *pqqL* | 2.1 | putative zinc peptidase |
| *malT* | 2.1 | positive regulator of mal regulon (maltose utilization) |

Table 10. Genes expressed at a lower level in the *feoB* mutant (NDS118) than the wild type strain (0-4576S1-G) following growth in LB broth containing 40 μM $FeSO_4$.

| Gene Name | Fold Repression | Function |
|---|---|---|
| *lpdA* | 12 | lipoamide dehydrogenase (energy metabolism) |
| *yfgC* | 4.0 | putative zinc metalloprotease |
| *ugpA* | 3.8 | glycerol-3-phosphate transporter subunit (membrane component of ABC transporter) |
| *gspI* | 3.7 | putative export protein I (general secretory pathway) |
| *hrpA* | 3.3 | ATP-dependent helicase |
| *murG* | 3.0 | N-acetylglucosamine transferase (peptidoglycan biosynthesis) |
| *sgaU* | 2.8 | L-xylulose 5-phosphate 3-epimerase (contains in-frame stop codon) |
| *yidL* | 2.7 | putative ARAC-type regulatory protein |
| *yeaO* | 2.7 | hypothetical protein |
| *yidA* | 2.7 | hypothetical protein |
| *mutM* | 2.7 | formamidopyrimidine DNA glycosylase (DNA modification) |
| Z6078 | 2.5 | putative inhibitor of cell division encoded by cryptic phage |
| *yddA* | 2.4 | predicted multidrug transporter subunits (membrane component & ATP-binding component of ABC transporter) |
| *xylB* | 2.4 | xylulokinase (xylose utilization) |
| *mutY* | 2.4 | adenine glycosylase (DNA modification) |
| *adiY* | 2.4 | putative ARAC-type regulatory protein |
| b0332 | 2.4 | deacessioned gene between *prpB* and *prpC* |
| *yifA* | 2.4 | putative transcriptional regulator |
| *rplW* | 2.3 | 50S ribosomal subunit protein L23 |
| *katG* | 2.3 | Subunit of hydroperoxidase I (contains frameshift) |
| *rplD* | 2.2 | 50S ribosomal subunit protein L4 |
| *yagI* | 2.2 | putative transcriptional regulator encoded by prophage |
| *yehE* | 2.1 | hypothetical protein |
| *ygeR* | 2.1 | putative lipoprotein |
| b1500 | 2.1 | deacessioned gene between *ydeP* and *ydeO* |
| *ybgR* | 2.0 | putative Zn(II) transport protein |
| *yeaY* | 2.0 | putative outer membrane protein |

The microarray results were confirmed through the use of an *entC*-β-galactosidase transcriptional fusion, in which the *entC* promoter was fused to a promoterless copy of the *lacZ* gene. *entC* encodes an enterobactin biosynthetic protein (138). When strains were grown in medium containing 40 μM $FeSO_4$ expression from the *entC* promoter, as measured by β-galactosidase activity, was greater in the *feoB* mutant than in the wild type strain (Fig. 29). Expression from the *entC* promoter correlated well with enterobactin production as measured by the Arnow assay (7) (Fig. 29). These data indicate that the *feoB* mutant produces enterobactin when grown in media containing iron due to increased expression of the enterobactin biosynthetic genes. Because iron was abundant in the media, these results suggest that the production of enterobactin under these conditions is not due to a requirement for additional iron by the bacterium.

## 2. THE SIT SYSTEM IS NOT REQUIRED BY *S. DYSENTERIAE* FOR GROWTH IN VITRO OR IN THE INTRACELLULAR ENVIRONMENT.

Unlike the *S. dysenteriae tonB* and *feoB* mutants, which exhibited growth defects in low and high iron, respectively, the *S. dysenteriae sitA* mutant, NDS116, exhibited no defect in growth compared to the wild type strain in conditions where iron was abundant or where iron was restricted (Fig. 14, 15). This is in contrast to the *S. flexneri sitA* mutant, which grew poorly when iron was limited (161). Additionally, the *S. dysenteriae sitA* mutant formed plaques in Henle cell monolayers that were similar to the wild type strain in size and number (Fig. 24), indicating that the Sit system is not required for the invasion of, growth within or spread between eukaryotic cells.

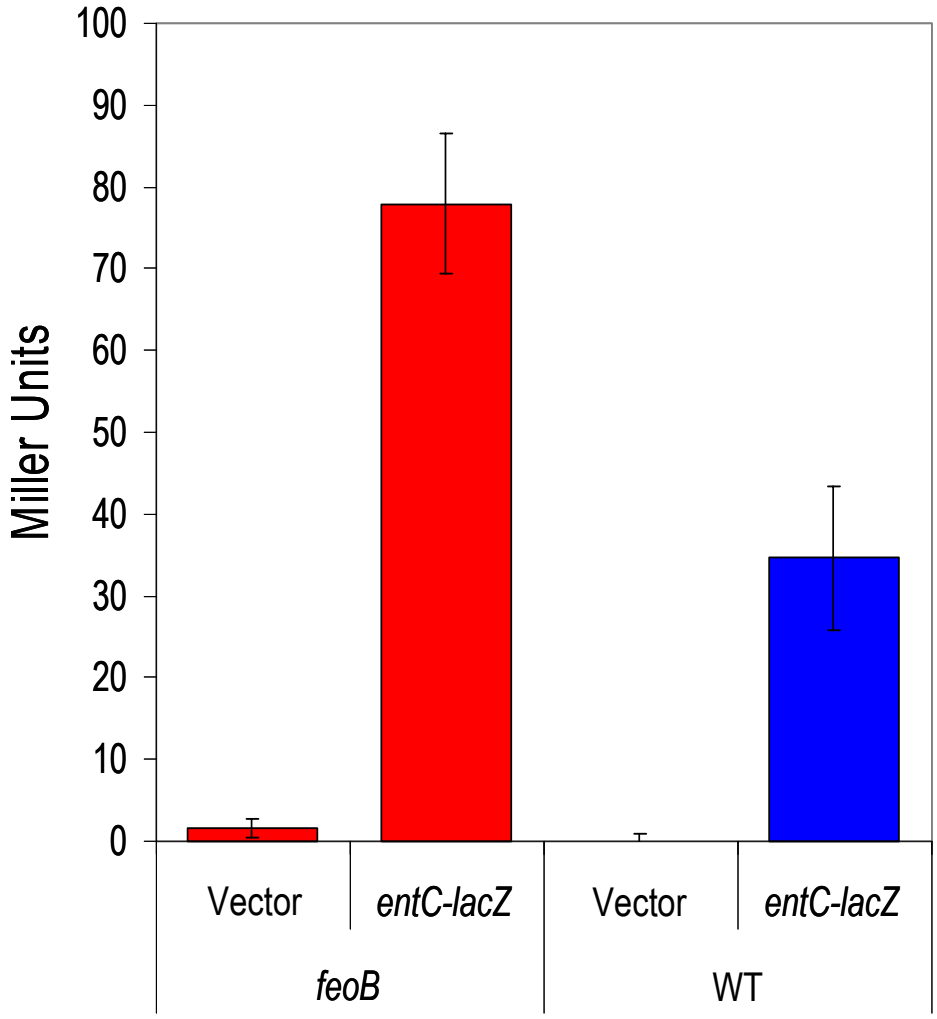



**Figure 29. Expression from the *entC* promoter is increased in the *feoB* mutant when grown in high iron conditions.**

The *feoB* mutant and wild type strain, each carrying an *entC-lacZ* promoter fusion (*entC-lacZ*) or a promoterless copy of the *lacZ* gene (Vector) were grown overnight and diluted 1:100 into MM9 with 40 μM $FeSO_4$. The cultures were then allowed to grow for 5 hours and the amount of β-galactosidase activity was measured. The mean β-galactosidase activity from 3 experiments is shown and one standard deviation is indicated. In the *feoB* mutant strain, the *entC* promoter was more active compared to the same promoter in the wild type strain, as measured by β-galactosidase activity.

To determine the relative fitness of the *S. dysenteriae sitA* mutant compared to the wild type strain, the *sitA* mutant was tested in intracellular and in vitro competition assays. The *S. dysenteriae sitA* mutant competed well with the wild type strain in both the in vitro and intracellular environments (Fig. 30). Taken together, these results suggest that the *S. dysenteriae* Sit system is not required for growth or fitness in vitro or in the intracellular environment.

## 3. *S. DYSENTERIAE* HAS ONE OR MORE CRYPTIC IRON TRANSPORT SYSTEMS.

### a) Strains with mutations in two iron transport systems exhibit only minor growth defects.

The defects in growth observed in the *tonB*, *sitA* and *feoB* mutants were mild. Therefore, strains with mutations in two iron transport systems were constructed and characterized. Like the single mutants, the double mutants exhibited growth defects in certain conditions, but none of the double mutant strains were severely impaired for growth. Like the *feoB* mutant, the *sitA feoB* and *tonB feoB* double mutants formed smaller colonies when grown on medium supplemented with iron and like the *tonB* mutant, the *tonB sitA* and the *tonB feoB* double mutants formed smaller colonies when grown on medium containing an iron chelator (Fig. 31). Additionally, the *sitA feoB* mutant exhibited a slight decrease in colony size compared to the wild type strain and to the *sitA* and *feoB* single mutants when grown on medium containing an iron chelator. None of the defects seen was severe. None of the double mutant strains formed smaller colonies on high iron medium than those formed by the *feoB* single mutant or smaller

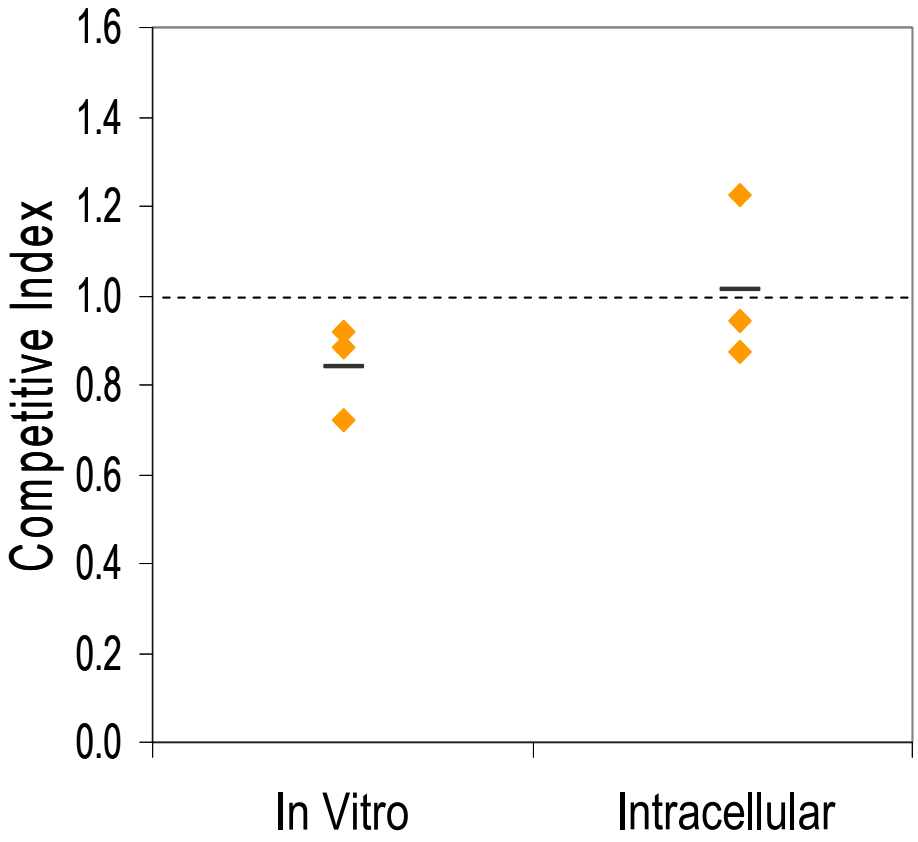



**Figure 30. An *S. dysenteriae sitA* mutant competes well with the wild type strain.**

Approximately equal numbers of *sitA* and wild type bacteria were mixed, the ratio of *sitA* to wild type bacteria determined (ratio in), and the mixture grown in LB broth + 40 μM $FeSO_4$ for 8 hours (in vitro) or the mixture was used to inoculate a plaque assay (intracellular). The bacteria were then recovered, the ratio of *sitA* to wild type bacteria recovered determined (ratio out) and the competitive index calculated by dividing the ratio out by the ratio in. The *S. dysenteriae sitA* mutant exhibited no decrease in fitness compared to the wild type strain in either the in vitro or intracellular environment. The results of three experiments are shown and the mean indicated by a gray bar.

colonies on low iron medium than those formed by the *tonB* single mutant (Fig. 31). The ability of the double mutants to grow in LB broth was also assessed, and similar trends were observed, although when grown in LB broth containing an iron chelator, the *tonB* single and double mutants exhibited a more severe growth restriction, and the *tonB* and *tonB sitA* mutant strains grew to a slightly lower final OD in LB broth containing supplemental iron (Fig. 32).

**b) Mutations in all three of the known *S. dysenteriae* iron transport systems can be made in a single strain.**

Because it was not known if an *S. dysenteriae* strain with mutations in all three of the known iron transport systems would be viable, an inducible copy of *tonB*, on plasmid pND35, was introduced to a strain with mutations in the *tonB* and *sitA* genes. NDS121, a strain with defects in all three of the known *S. dysenteriae* iron transport systems, Feo, Sit and TonB-dependent, was constructed by inactivating the *feoB* gene while inducing expression of *tonB*. Once the presence of all three mutations was confirmed, the inducer was withdrawn and NDS121 was found to be able to grow in media containing supplemental iron. To confirm that the exogenous copy of *tonB* was not contributing to the growth of the triple mutant, NDS121 was cured of pND35, the plasmid carrying *tonB*. The resulting strain grew well in media containing supplemental iron. When grown on medium containing 40 μM $FeSO_4$, the *tonB sitA feoB* triple mutant formed colonies slightly smaller in size than those formed by the *feoB* mutant (Fig. 31). When grown on medium containing an iron chelator, the triple mutant formed colonies similar in size to

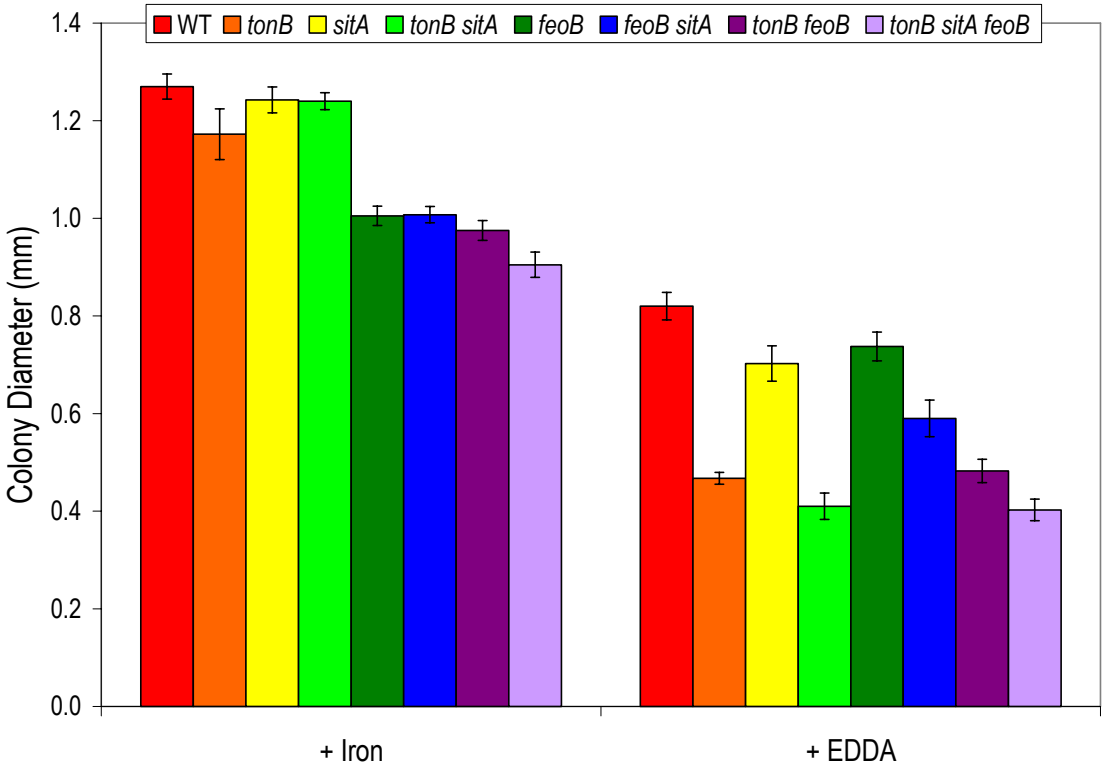



**Figure 31. Growth of *S. dysenteriae* iron transport mutants on media containing supplemental or limited iron.**

Overnight cultures were diluted and plated on medium containing 40 μM $FeSO_4$ (high iron) or 100 μg/ml EDDA (low iron) and were allowed to grow overnight at 37˚C. The average of the diameter of 10 colonies is shown and one standard deviation indicated. Strains with a mutation in the *tonB* gene exhibit a growth defect when iron availability is limited. Strains with mutations in the *feoB* gene exhibit a growth defect when iron is abundant. The *tonB feoB* double mutant and the *tonB sitA feoB* triple mutant exhibited growth defects in both low and high iron, similar to those displayed by the *tonB* single mutant on low iron and the *feoB* single mutant on high iron.

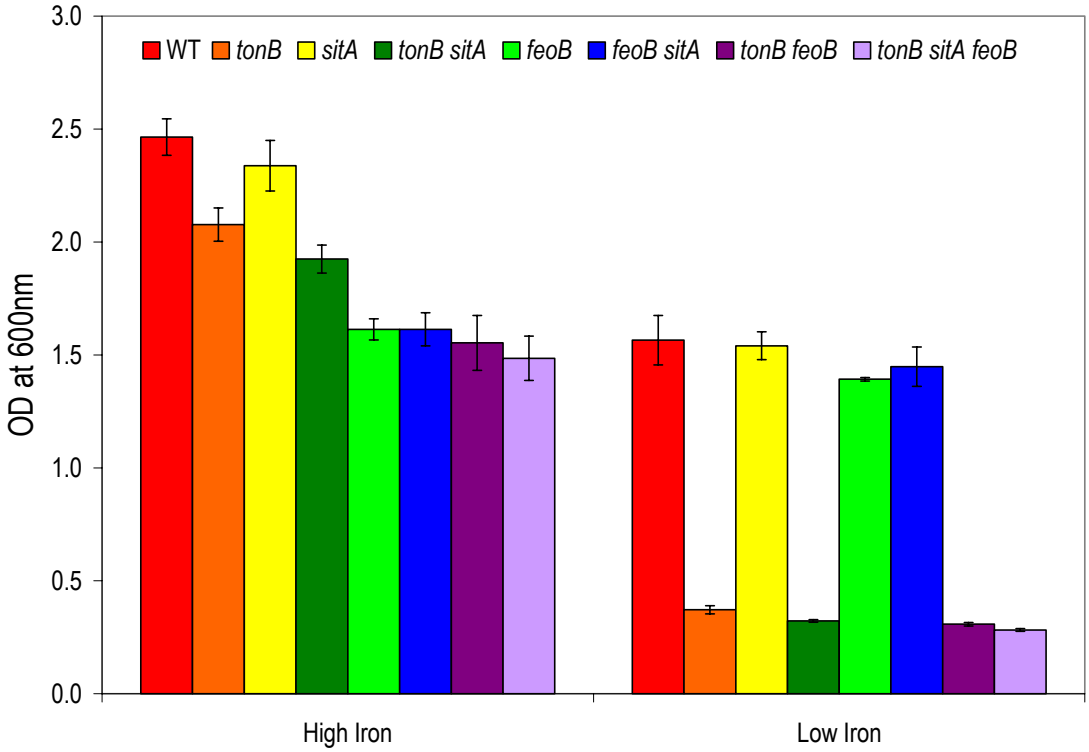



**Figure 32. Growth of *S. dysenteriae* iron transport mutants in LB broth containing supplemental or limited iron.**

Overnight cultures were diluted approximately 1:100 into LB broth + 40 μM $FeSO_4$ (high iron) or LB broth + 10 μg/ml EDDA (low iron) and allowed to grow for 8 hours. The OD of each culture at 600nm was then determined. The average of 3 independent experiments is shown and one standard deviation is indicated. Strains with a mutation in the *tonB* gene exhibit a growth defect when iron availability is limited. Strains with mutations in the *feoB* gene exhibit a growth defect when iron is abundant. The *tonB feoB* double mutant and the *tonB sitA feoB* triple mutant exhibited growth defects in both low and high iron similar to those displayed by the *tonB* single mutant in low iron and the *feoB* single mutant in high iron.

those formed by the *tonB* mutant (Fig. 31). In both conditions, the triple mutant grew nearly as well as the smallest of the single mutants, indicating that inactivation of all three of the known systems had little more effect than inactivation of a single iron transport system. Similar results were obtained when the triple mutant was grown in LB broth containing 40 μM $FeSO_4$ or 10 μg/ml EDDA (Fig. 32).

**c) The *S. dysenteriae tonB sitA feoB* triple mutant exhibits no growth defect compared to the wild type strain when grown in media containing 1 μM $FeSO_4$.**

To better characterize the ability of the triple mutant to grow in low iron conditions, the growth of the *tonB sitA feoB* mutant was assessed in minimal medium treated to remove all iron. Iron was then added back to the medium and the minimal amount of iron required for growth by the triple mutant was determined. At concentrations of iron greater than or equal to 1 μM, both the wild type and the triple mutant strains showed equivalent growth and final density was proportional to iron concentration (Fig. 33). At iron concentrations lower than 1 μM, the triple mutant showed reduced growth relative to wild type (Fig. 33). These data indicate that the triple mutant is able to obtain iron from media containing as little as 0.5 μM $FeSO_4$. The ability of the *tonB sitA feoB* mutant to grown in conditions in which the availability of iron is limited indicates that the *S. dysenteriae* triple mutant is able to obtain iron in the absence of any known iron transporter and suggests the existence of an additional iron acquisition system.

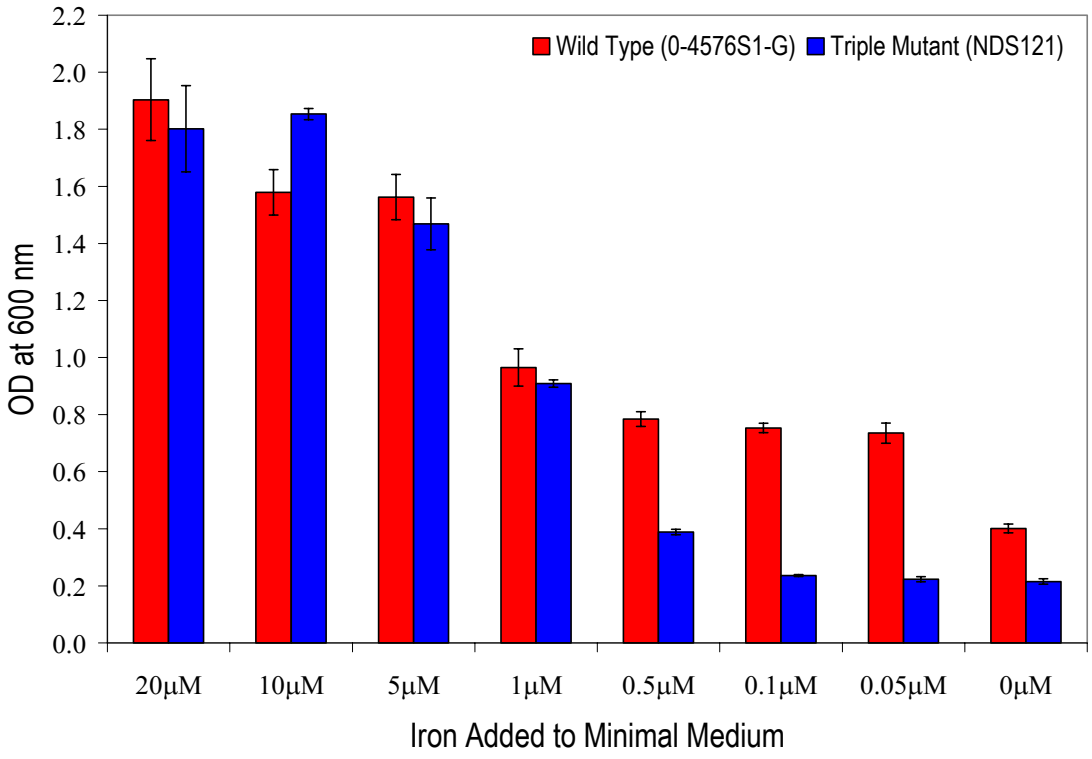



**Figure 33. An *S. dysenteriae tonB sitA feoB* mutant exhibits a growth defect compared to the wild type strain when grown in medium containing 0.5 µM $FeSO_4$ or less.**

Overnight cultures were diluted approximately 1:100 into Chelex-treated minimal medium to which varying concentrations of iron had been added and the cultures were allowed to grow overnight. The OD of each culture at 600 nm was then determined. The average of 3 independent experiments is shown and one standard deviation about the mean is indicated. The *tonB sitA feoB* triple mutant (NDS121) exhibited a defect in growth compared to the wild type strain in media to which 0.5 µM or less of $FeSO_4$ had been added.

**d) The *S. dysenteriae tonB sitA feoB* triple mutant is sensitive to chromium.**

Excess chromium inhibits growth of *E. coli tonB* mutants and *E. coli* strains with mutations in the genes for the synthesis and transport of enterobactin, likely through interference with iron acquisition by these strains, but has little effect on strains that are able to synthesize and transport enterobactin (190-192). The effects of chromium on *tonB* and siderophore mutants stem from the ability of $CrCl_3$ to interact with iron, forming complexes that make iron inaccessible to strains unable to make a siderophore (190). Because chromium inhibits growth indirectly, by making iron unavailable, chromium cannot be used to isolate strains with iron transport mutations; however, the response of a given strain to chromium can be used to infer whether that strain makes use of a siderophore-based iron acquisition system. When grown on medium containing $CrCl_3$, all of the *S. dysenteriae tonB* mutant strains, including the *tonB sitA feoB* triple mutant, exhibited a growth defect that could be reversed by the addition of $FeSO_4$ (Fig. Z). The *feoB*, *sitA* and *sitA feoB* strains were not affected by the addition of chromium to the medium. These results indicate that the iron transport system present in the *tonB sitA feoB* triple mutant is chromium sensitive and suggest that this system is not dependent upon a siderophore or other iron-chelating molecule.

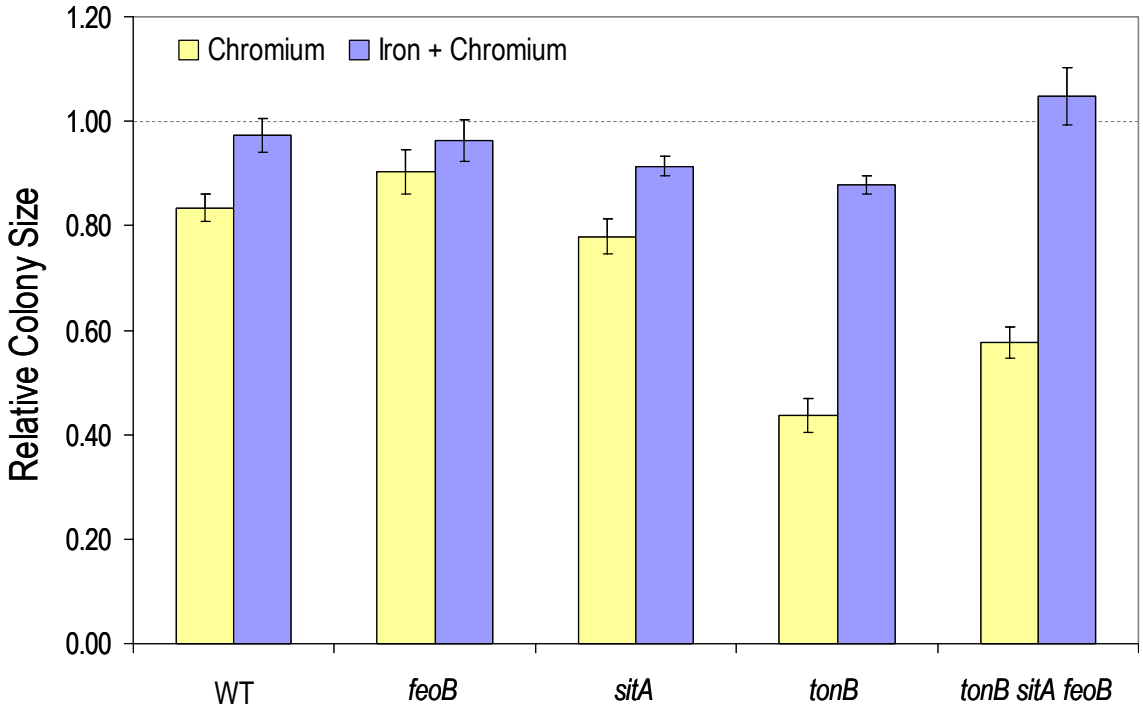



**Figure 34. Growth of *S. dysenteriae* iron transport mutant strains in the presence of chromium and chromium plus iron.**

Overnight cultures were diluted and plated on medium containing 100 μM $CrCl_3$ (Chromium) or 100 μM $CrCl_3$ + 40 μM $FeSO_4$ (Chromium + Iron) and were allowed to grow overnight at 37˚C. Colony sizes are reported relative to the average colony size for each strain on medium containing 40 μM $FeSO_4$, which was set to 1. The average of the relative diameter of 10 colonies is shown and one standard deviation indicated. Strains with a mutation in the *tonB* gene exhibit a growth defect when chromium was present in the medium. Addition of iron restored growth of the *tonB* mutant strains. The wild type strain and the *feoB* and *sitA* mutant strains exhibited only a minimal decrease in colony size in the presence of chromium.

## 4. THE IRON TRANSPORT SYSTEMS OF *S. DYSENTERIAE* ARE HIGHLY REDUNDANT.

### a) No single iron transport system is required for growth of *S. dysenteriae* in vitro.

As noted above, the single and double iron transport mutants exhibited only mild defects in growth in vitro (Fig. 31, 32), indicating that no single iron transport system is essential for growth of *S. dysenteriae* in the laboratory. The Feo system is more important for growth when iron is abundant, and the TonB-dependent iron uptake systems are more important for growth when iron concentrations are low, but growth data suggest that when one system is inactivated the remaining systems are able to compensate for the loss such that strains with a mutation in only one iron transport system are seldom unable to obtain sufficient iron for viability.

### b) *S. dysenteriae* single, double and triple iron transport mutants form plaques in Henle cell monolayers.

The *tonB*, *feoB*, and *entB* mutants all formed plaques in Henle cell monolayers that were only slightly smaller in size than those formed by the wild type strain, while the *sitA* mutant formed plaques that were similar in size to those formed by the wild type strain (Fig. 24). These minimal defects in plaque formation indicate that *S. dysenteriae* strains with a mutation in one iron transport system are still able to obtain the iron necessary for growth and spread and suggest that no one system is required for iron acquisition within the eukaryotic cell. Double mutants, with mutations in two iron

transport systems were also tested for the ability to form plaques. The *tonB sitA*, *tonB feoB* and *sitA feoB* mutants all formed plaques; however, the plaque size was reduced compared to those formed by the wild type and the single mutants (Fig. 24). Additionally, the *tonB sitA feoB* triple mutant formed small plaques in Henle cell monolayers (Fig. 24). The decrease in plaque size compared to the size of the plaques formed by the wild type strain is likely due to a decrease in intracellular growth; however, the ability of the double and triple mutant strains to form plaques of any size indicates that the TonB-dependent, Sit and Feo iron acquisition systems are not an absolute requirement for plaque formation by *S. dysenteriae*.

**c) Competition between *S. dysenteriae* iron transport mutants and the wild type strain in vitro and in the intracellular environment.**

To better quantify the defects in growth and in plaque formation exhibited by the *S. dysenteriae* iron transport mutants, in vitro and intracellular competition assays were used. In these experiments, mixtures of wild type and iron transport mutants were inoculated into broth or Henle cells, and the ratio of mutant to wild type bacteria determined after 8 hours of growth in broth or 72 hours of growth in Henle cells. In the in vitro condition, broth containing 40 μM $FeSO_4$ was used. As noted above, the *feoB* mutant strain exhibited decreased fitness compared to the wild type strain in both conditions (Fig. 25). Additionally, the *entB* strain showed no defect in growth compared to the wild type strain when grown in broth; however, the *entB* mutant exhibited reduced fitness compared to the wild type in the intracellular environment (Fig. 35). The *tonB*

and *sitA* strains competed well with the wild type strain in both the in vitro and intracellular environments (Fig. 30, 36). Although the *entB* and *feoB* strains exhibited reduced fitness in the intracellular environment, these defects were not severe. Together with the *tonB* and *sitA* competition data, these results suggest that *S. dysenteriae* strains with a defect in one iron transport system, while impaired, are not incapable of surviving in the presence of the wild type strain, and highlight the redundancy that exists among the iron transport systems employed by *S. dysenteriae*.

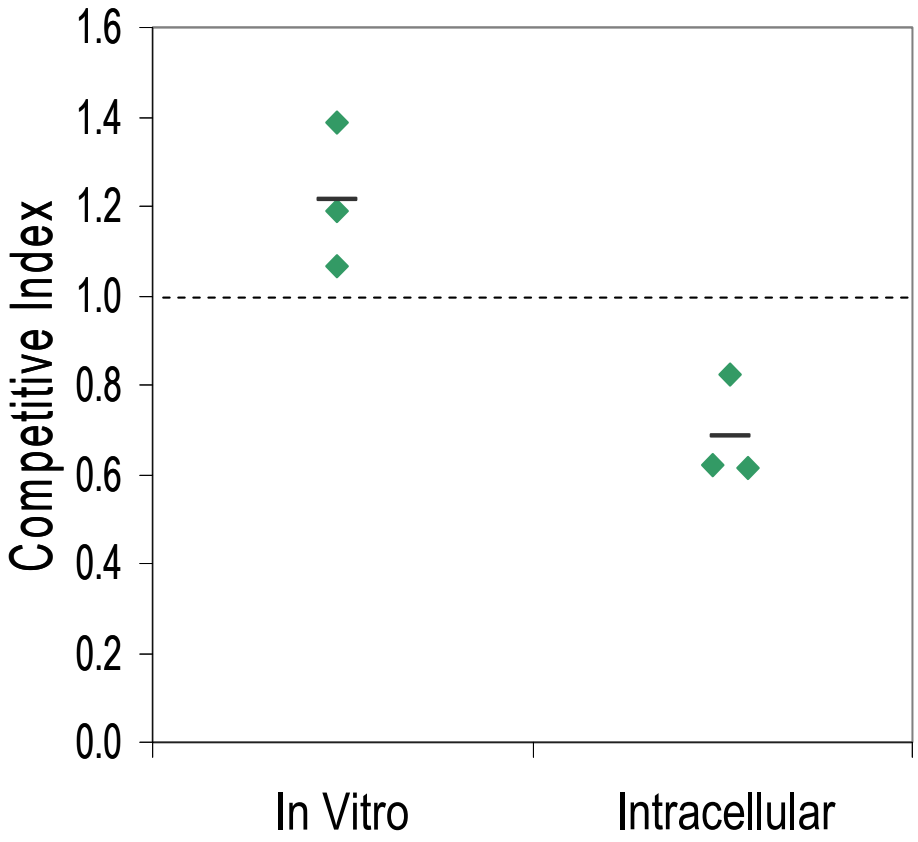



**Figure 35. An *S. dysenteriae entB* mutant exhibits decreased fitness in the intracellular environment.**

Approximately equal numbers of *entB* and wild type bacteria were mixed, the ratio of *entB* to wild type bacteria determined (ratio in), and the mixture grown in LB broth + 40 μM $FeSO_4$ for 8 hours (in vitro) or the mixture was used to inoculate a plaque assay (intracellular). The bacteria were then recovered, the ratio of *entB* to wild type bacteria recovered determined (ratio out) and the competitive index calculated by dividing the ratio out by the ratio in. The *entB* mutant exhibited no defect compared to the wild type strain in vitro, but showed decreased fitness in the intracellular environment. The results of three experiments are shown and the mean indicated by a gray bar.

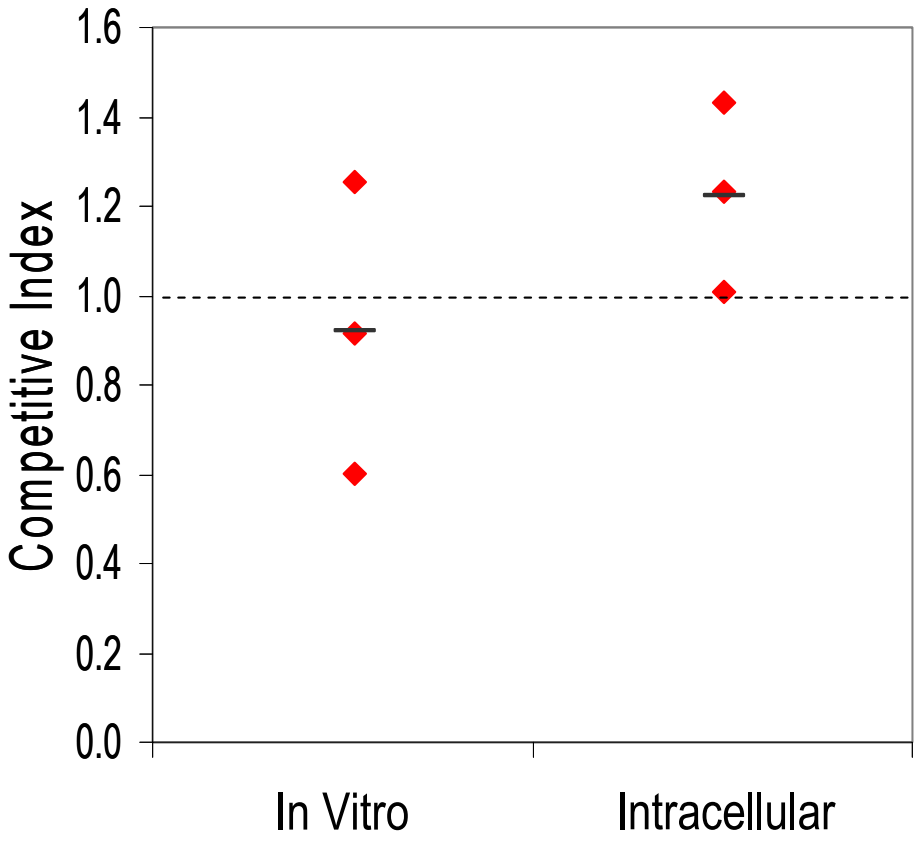



**Figure 36. An *S. dysenteriae tonB* mutant competes well with the wild type strain.**

Approximately equal numbers of *tonB* and wild type bacteria were mixed, the ratio of *tonB* to wild type bacteria determined (ratio in), and the mixture grown in LB broth + 40 μM $FeSO_4$ for 8 hours (in vitro) or the mixture was used to inoculate a plaque assay (intracellular). The bacteria were then recovered, the ratio of *tonB* to wild type bacteria recovered determined (ratio out) and the competitive index calculated by dividing the ratio out by the ratio in. The *tonB* mutant exhibited no decrease in fitness compared to the wild type strain in either the in vitro or the intracellular environment. The results of three experiments are shown and the mean indicated by a gray bar.

## B. Discussion

Iron is an essential micronutrient for nearly all bacteria, required for basic cellular functions. For this reason, bacteria have evolved numerous methods of iron acquisition, with a single species often employing several different mechanisms. For pathogenic bacteria, the ability to obtain iron while within the host is central to pathogenesis. In *S. flexneri* and *S. dysenteriae*, numerous systems facilitate the uptake of iron both in vitro and in the intracellular environment. These systems were investigated in the course of this study and many similarities between the two species were found; however, the iron transport systems of *S. flexneri* and *S. dysenteriae* are employed under different conditions and in different ways to meet the specific needs of these two closely related species. Additionally, in investigating the TonB-dependent iron transport systems in *S. flexneri* and *S. dysenteriae*, it was discovered that a gene required for plaque formation by *S. dysenteriae* may be located in the region surrounding the *tonB* gene. Characterization of this strain allowed identification of a gene that is required for plaque formation.

### 1. Plaque Formation in a Spontaneous Deletion Mutant of *S. dysenteriae*

Spontaneous deletions in the *tonB* region of the *E. coli* and *S. enterica* chromosomes have been documented frequently (37, 77, 120) and can be selected using the antibiotic pirazmonam, which is transported in a TonB-dependent manner through the outer membrane receptors Fiu and Cir (132). SDU380 is an *S. dysenteriae tonB* mutant strain that was generated in this manner and found to have a defect in plaque formation in

Henle cell monolayers (155). The defect in plaque formation is not due to the loss of the *tonB* gene, nor is it the result of the loss of the *opp* or *trp* operons. Additionally, although an *S. dysenteriae yciB* mutant failed to form plaques in Henle cell monolayers, the loss of the *yciABC* operon in SDU380 is also not the sole cause of the plaque formation defect, as complementation with the *yciABC* operon on a plasmid did not restore the ability to form plaques to this strain.

The gene or genes responsible for the failure of SDU380 to form plaques likely reside in the deleted region; however, repeated attempts to complement the plaque formation phenotype using partial sequences from the deleted region have failed. It also possible that SDU380 has sustained more than one mutation and that the failure to form plaques is as a result of a genomic change outside of the deleted region. Because of the size of the deletion and the number of genes lost, the combined effects of the loss of a number of genes, each with a small effect of plaque formation, not the loss of a single virulence-associated gene, are likely responsible for the failure of SUDU380 to form plaques.

The *tonB* region of the chromosome appears to have been lost from *S. dysenteriae* SDU378 by homologous recombination between two IS1 elements, a method similar to that which mediated the loss of the T7 promoter region from *S. flexneri* SM100. This method of gene loss is not restricted to this region of the chromosome as IS elements are found throughout the *S. dysenteriae* genome and have been implicated in genome rearrangement and gene loss in *Shigella* species (85, 195, 199), including in the loss of the *sit* genes and T7 promoter region from *S. flexneri*. These repetitive elements provide

a multitude of sites at which homologous recombination can occur, yielding a highly flexible genome and allowing rapid adaptation to the changing local environments that *S. dysenteriae* encounters.

## 2. *S. FLEXNERI* TONB IN VITRO AND IN THE INTRACELLULAR ENVIRONMENT.

Studies of iron transport mutants in *S. flexneri* have shown that the Sit, Feo and aerobactin-mediated iron acquisition systems are highly redundant and that the absence of any one of these systems causes only minimal defects in growth (102, 161). However, the presence of at least one iron transport system is essential for *S. flexneri* growth and pathogenesis (161). *S. flexneri* serotype 2A synthesizes only one siderophore, aerobactin, but is able to utilize several exogenous siderophores as iron sources. The role of these iron sources in the growth and pathogenesis of *S. flexneri* has not previously been studied. Because the TonB protein is essential to the function of all of the *S. flexneri* siderophore transport systems, inactivation of the *tonB* gene allows the study of *S. flexneri* in the absence of siderophore-mediated iron acquisition systems.

The *S. flexneri tonB* mutant, NDS100, exhibited a marked defect in growth in low iron conditions compared to the wild type strain, indicating an inability to extract iron from the growth medium, despite the presence of the Sit and Feo iron transport systems, both of which are TonB-independent. Despite this defect in growth in culture, the *S. flexneri tonB* mutant formed plaques that were similar to those formed by the wild type strain in both size and number. The TonB-dependent iron transport systems appear to be important for growth in low iron conditions, but, like the Sit and Feo systems (161), these

high-affinity systems are not essential for growth in the intracellular environment. These data are in agreement with previous findings that no single *S. flexneri* iron transport system is essential for iron acquisition in the epithelial cell and confirm the redundancy that exists among the *S. flexneri* iron uptake systems (161). This redundancy emphasizes the importance of iron acquisition within the human host by *S. flexneri*.

### 3. IRON TRANSPORT IN *S. DYSENTERIAE* AND *S. FLEXNERI* – SIMILARITIES AND DIFFERENCES

Although *S. flexneri* and *S. dysenteriae* are undeniably closely related and are etiological agents of the same disease, the differences between the two species, especially with respect to iron transport, are numerous. Both species make use of the Sit, Feo and TonB-dependent iron transport systems; however, many differences in the role of these systems were highlighted by this study. The role of the Sit system in the intracellular environment is one such difference. In *S. flexneri*, a mutation in *sitA* was associated with a two-fold decrease in fitness in the intracellular environment. A very similar mutation in *S. dysenteriae sitA* had no impact on the ability of the mutant strain to compete with the wild type strain in vitro or in the intracellular environment (Table 11). The reasons for this difference are not immediately clear. The sequence of the *sitABCD* genes in *S. flexneri* and *S. dysenteriae* is not identical; however, the few nucleotide differences that do exist result in synonymous or conservative amino acid changes and no changes were found in the known active sites of any of the proteins (data not shown). It is possible that differences in the regulation or expression of the *sit* genes have lead to differences in the role of the Sit system in these two organisms or that the sequence changes observed result

Table 11. Summary of findings.

| Iron Transport System | Experiment | Conclusions |
|---|---|---|
| TonB-Dependent Systems | Growth Assays (Colony Size and Broth) | TonB-dependent systems important in low iron condtions |
| | | TonB-dependent systems not important in high iron condtions |
| | Plaque Assays | TonB-dependent systems are important, but not essential, for intracellular growth or cell to cell spread of *S. dysenteriae* |
| | Competition Assys | Loss of the TonB-dependent systems does not decrease fitness in the intracellular environment |
| Sit System | Growth Assays (Colony Size and Broth) | The Sit system is not important for growth or survival of *S. dysenteriae* in any condition tested |
| | Plaque Assays | |
| | Competition Assys | |
| Feo System | Growth Assays (Colony Size and Broth) | The Feo system is important in high iron conditions |
| | | The Feo system is not important in low iron conditions |
| | Plaque Assays | The Feo system is important, but not essential, for intracellular growth or cell-to-cell spread of *S. dysenteriae* |
| | Competition Assays | The Feo system contributes to the fitness of *S. dysenteriae* |
| | Arnow Assay | The Feo system affects regulation of enterobactin biosynthesis |
| | Microarray Analysis | |
| | Promoter Reporter Assays | |
| Fourth Iron Transport System | Growth Assays (Colony Size and Broth) | An additional iron transport system exists in *S. dysenteriae* |
| | Plaque Assays | |
| | Competition Assays | |
| | Chromium Sensitivity Assay | The cryptic iron transport system is TonB-independent and does not require a high-affinity iron-binding molecule. |
| | Minimal Iron Requirement Assay (Growth in Chelex-treated M9) | The cryptic iron transport system has a high affinity for iron |

in a difference in affinity of Sit for iron. In *S. flexneri* the Sit system is believed to be mainly involved in the transport of iron (161), while in *S. enterica*, Sit is primarily implicated in manganese transport (17). Although the *S. dysenteriae* and *S. flexneri* Sit systems are much more similar to each other than to the *S. enterica* Sit system, it is possible that the *S. dysenteriae* Sit system may play a lesser role in the acquisition of iron than it does in *S. flexneri* and this difference may lead to the phenotypic differences observed between the two *sitA* mutants. Finally, it is possible that the functions of the Sit system in *S. dysenteriae* are identical to those in *S. flexneri*, but that differences in the genetic background, such as the presence of additional or more effective iron transport systems in *S. dysenteriae*, render the Sit system less important in *S. dysenteriae*, resulting in a milder phenotype for the *sitA* mutant in this species.

In addition to differences in the importance of the Sit system in the intracellular environment, several other important differences between *S. dysenteriae* and *S. flexneri* were identified. In *S. flexneri*, the Sit system was the most important iron transport system in conditions where iron is limited, while one of the TonB-dependent or Feo systems were important when iron concentrations were higher (161). This is in direct contrast to the findings in *S. dysenteriae*, in which the TonB-dependent iron transport systems were found to be the only iron transport systems required for growth in low iron conditions and the Feo system was the only iron acquisition system required for growth in the intracellular environment and when iron was abundant (Table 11). These data indicate that although the systems involved in iron uptake by *S. dysenteriae* and *S. flexneri* are nearly identical, the conditions in which these systems are critical vary

greatly and suggest that the same systems may be used by the two species to exploit different iron sources.

Differences in the use of siderophores, namely the production and transport of the hydroxamate siderophore aerobactin by *S. flexneri* but not *S. dysenteriae* and the production of the catecholate siderophore enterobactin by *S. dysenteriae* but not *S. flexneri* have been well documented (141, 142, 169, 170); however, the role of the Feo system in siderophore production was not previously recognized. In *S. dysenteriae*, mutations in *feoB* result in an overproduction of enterobactin in conditions where iron is abundant. This phenomenon is not observed in *S. flexneri*; the siderophore aerobactin was not detectable when an *S. flexneri feoB* mutant, SM190, was grown in the presence of iron, nor was increased siderophore production observed in a *V. cholerae feoB* mutant or an *E. coli feoB* mutant when grown in similar conditions. These results indicate that siderophore overproduction in *feoB* mutants in high iron environments is limited to *S. dysenteriae* strains and suggest that the Feo system plays a unique role in the regulation of *S. dysenteriae* enterobactin biosynthesis (Fig. 37 and Table 11).

It is possible that the production of enterobactin by the *S. dysenteriae feoB* mutant when iron is abundant is a response to a failure by the mutant to obtain sufficient iron for growth under these conditions. However, the *S. dysenteriae feoB* mutant and the wild type strain were equally sensitive to oxidative stress when grown in the presence of supplemental iron, indicating that the *feoB* mutant contains approximately the same amount of free iron as the wild type strain and suggesting that iron starvation is not the cause of the enterobactin overproduction. These data also indicate that the growth defect

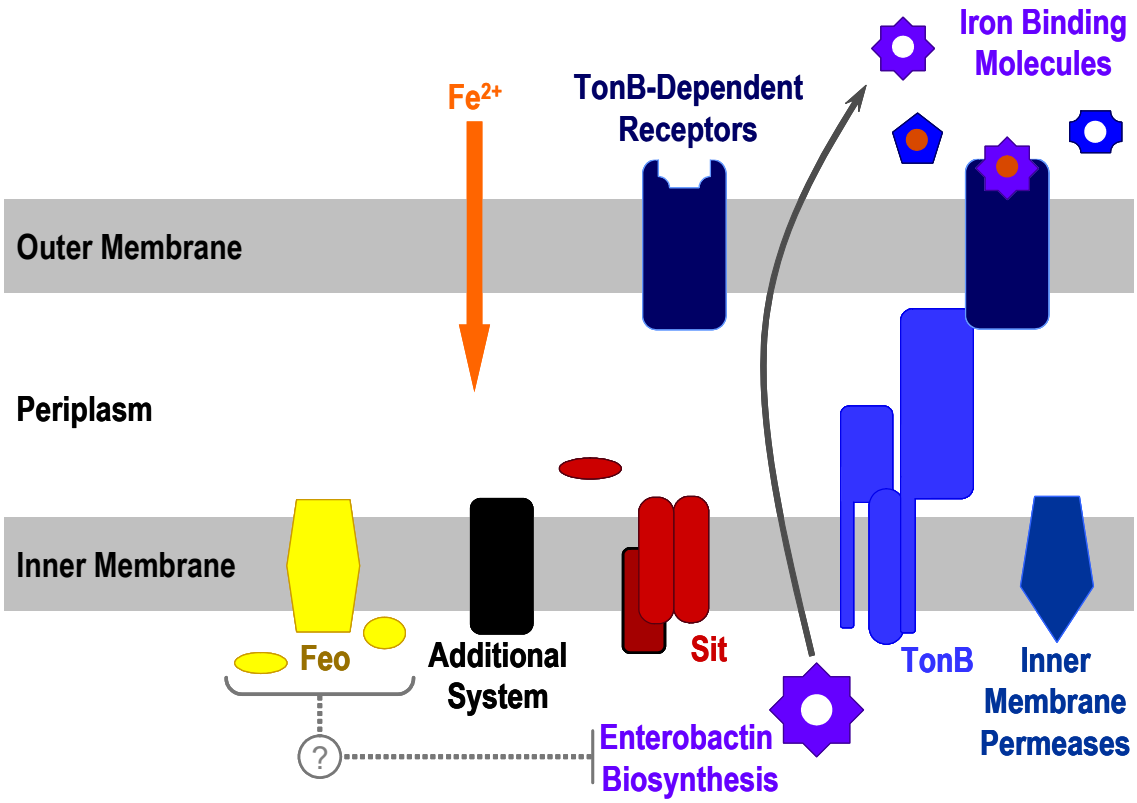



**Figure 37. *S. dysenteriae* makes use of the Feo, Sit and TonB-dependent iron transport systems and an additional iron uptake system.**

The iron acquisition systems employed by *S. dysenteriae* are shown. When iron is scarce, the siderophore enterobactin is secreted and iron complexed to enterobactin and other iron-binding molecules is taken up through the TonB-dependent iron transport systems (blue). In conditions where iron is abundant and in the intracellular environment, iron is primarily transported via the Feo system (yellow). The Feo system also plays a role in the regulation of enterobactin biosynthesis. This regulation may be direct or may require the action of one or more intermediates, indicated by the gray circle. The presence of an additional system was inferred experimentally; the identity of this system is not known. The Sit system (red) is not the primary iron acquisition system under any conditions, but acts in concert with the other iron transporters.

exhibited by the *feoB* mutant when grown on media containing supplemental iron is not due to iron toxicity or iron starvation. The growth defect and the overproduction of enterobactin occur under the same conditions; therefore the two phenotypes may have a common cause. It is also possible that the overproduction of enterobactin by the *feoB* mutant directly results in the growth defect; however, the mechanism by which enterobactin production would cause such a defect is unclear.

The weak induction of the enterobactin biosynthetic genes in the *S. dysenteriae feoB* mutant observed by microarray analysis and transcriptional fusions to the *lacZ* gene compared to the quantity of enterobactin produced by the *feoB* mutant suggests that regulation of enterobactin biosynthesis may occur at both the transcriptional and post-transcriptional levels. At the transcriptional level, the enterobactin biosynthetic operons are regulated by the global iron response regulator, Fur. Fur represses iron acquisition genes when bound to iron, but is unable to repress these genes when iron is scarce (67). Although it is possible that the overexpression of enterobactin by the *S. dysenteriae feoB* mutant is mediated by Fur, it is more likely that another regulatory mechanism is responsible for the overexpression, as other genes in the Fur regulon were not expressed more highly in the *feoB* mutant than in the wild type strain.

Regulation of enterobactin biosynthesis by Feo may be direct, possibly through the action of FeoC, a putative transcriptional repressor (29), or may be indirect, through one or more intermediates. Other iron acquisition systems may also be regulated in this way and additional study is required to further elucidate the mechanism and targets of Feo-dependent regulation.

The differences in the importance and role of the *S. flexneri* and *S. dysenteriae* Sit, Feo and TonB-dependent iron transport systems underlie a far greater difference between the two species. An *S. flexneri sitA feoB iucD* mutant, defective in the Sit and Feo systems and in siderophore biosynthesis, is unable to obtain iron and therefore fails to grow in the absence of exogenous siderophore (161). Growth of this mutant cannot be supported by supplementation with ferrous sulfate. This is in contrast to the *S. dysenteriae* triple mutant, NDS121, which is defective in the Sit, Feo and TonB-dependent iron transport systems, but grows well when supplied with ferrous sulfate. The ability of the *S. dysenteriae* triple mutant to obtain the iron necessary for growth suggests the presence of an additional iron transport system or systems in *S. dysenteriae* that are not found in *S. flexneri* (Fig. 37 and Table 11).

Chromium has been found to impair growth of strains unable to use siderophores as an iron source (191, 192). The additional iron transport system in *S. dysenteriae* is sensitive to chromium, which is believed to act by interacting with iron in the growth medium, making it unavailable to strains without a functional siderophore biosynthesis and transport system (190). The chromium sensitivity suggests that the system is able to transport free iron and does not make use of an iron binding molecule (Table 11). This additional system may be essential for growth in vitro, in the intracellular environment, or both, or may work in concert with the three known iron transport systems, with each system contributing in most growth conditions. The additional iron acquisition system operates even when iron is scarce, allowing growth to wild type levels when as little as 1 μM $FeSO_4$ is added to the growth medium. The ability of this system to support growth

at such low concentrations of iron suggests that it is specific for iron and is not a transport system specific for another metal that is able to transport iron with low affinity (Table 11). The additional *S. dysenteriae* iron transport system may also be present in *E. coli*. *E. coli* K12, which does not carry the *sit* genes, has only two known mechanisms of iron acquisition, the TonB dependent and Feo systems, but is able to grow when mutations are present in both the *tonB* and *feoB* genes, suggesting the presence of another iron transport system (87). *E. coli*, *S. dysenteriae* and *S. flexneri* are closely related, and such an iron transport system may have originally been present in all three genomes, having been subsequently lost from *S. flexneri*. Alternatively, the system may have been acquired by horizontal transfer by both *E. coli* and *S. dysenteriae* subsequent to the divergence of the three species.

Despite the many differences observed between *S. dysenteriae* and *S. flexneri*, one aspect of iron transport, redundancy among the iron acquisition systems, is very similar. In *S. dysenteriae*, as in *S. flexneri*, few defects in growth are observed when a single iron transport system is inactivated (161). It is only when two or even three systems are mutated that defects become apparent (161). This level of redundancy is indicative of the importance of iron to *Shigella* species, and is observed in both the in vitro and intracellular environments.

Taken together, the findings of this study suggest that the mechanisms and regulation of iron transport employed by *S. dysenteriae* differ greatly from those utilized by *S. flexneri*. Despite their close relationship, these two species share little in common with respect to iron acquisition, with the exception of the redundancy among uptake

systems exhibited by both organisms, particularly in the intracellular environment. This one commonality, despite the many differences, underscores the importance of iron acquisition by *Shigella* species, especially during pathogenesis.

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