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Genetics and environmental regulation of *Shigella* iron transport systems

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Abstract *Shigella* spp. have transport systems for both ferric and ferrous iron. The iron can be taken up as free iron or complexed to a variety of carriers. All *Shigella* species have both the Feo and Sit systems for acquisition of ferrous iron, and all have at least one siderophore-mediated system for transport of ferric iron. Several of the transport systems, including Sit, Iuc/IutA (aerobactin synthesis and transport), Fec (ferric di-citrate uptake), and Shu (heme transport) are encoded within pathogenicity islands. The presence and the genomic locations of these islands vary considerably among the *Shigella* species, and even between isolates of the same species. The expression of the iron transport systems is influenced by the concentration of iron and by environmental conditions including the level of oxygen. ArcA and FNR regulate iron transport gene expression as a function of oxygen tension, with the *sit* and *iuc* promoters being highly expressed in aerobic conditions, while the *feo* ferrous iron transporter promoter is most active under anaerobic conditions. The effects of oxygen are also seen in infection of cultured cells by *Shigella flexneri*; the Sit and Iuc systems support plaque formation under aerobic conditions, whereas Feo allows plaque formation anaerobically.

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The *Shigella* species are close genetic relatives of *Escherichia coli* and may be considered as subspecies, although they were historically categorized as a different genus and form a distinct group with respect to pathogenicity. These enteric pathogens are divided into four species (*S. dysenteriae*, *S. flexneri*, *S. sonnei*, *S. boydii*) based on serological and biochemical differences, but all the *Shigella* species are capable of invading human colonic epithelial cells, rapidly lysing the endocytic vesicle and multiplying intracellularly before moving directly into adjacent cells (Fig. 1). Shigellae are highly infectious, and the destruction of intestinal epithelial cells and resultant inflammatory response are responsible for the pathology of shigellosis, also termed bacillary dysentery (Sansonetti 2006).

The intracellular environment inhabited by shigellae is quite different from that encountered outside the epithelial cell or outside the human host. Temperature, oxygen tension, nutrient availability and the form and amount of available iron are distinctly different in these environments. To better understand how *Shigella* acquires iron from its diverse environments, we have characterized the iron transport systems of *Shigella* spp. and measured their expression, regulation and function in vitro and in the eukaryotic cell cytoplasm.

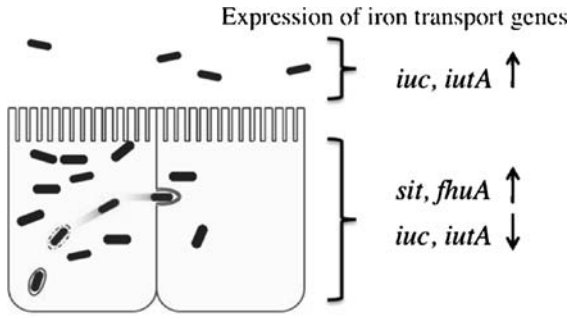

Fig. 1 Invasion and expression of iron transport genes in *Shigella.* Shigellae invade human colonic epithelial cells, lyse the endocytic vesicle and multiply within the host cell cytoplasm. The bacteria spread into adjacent cells via actin polymerization. The siderophore synthesis and transport genes (*iuc, iut*) are expressed in the extracellular environment, but are repressed in bacteria growing intracellularly, while the *sit* and *fhuA* genes are induced in the intracellular environment

Shigella iron transport systems

The *Shigella* species have a common set of iron transport systems, with additional systems being present in some strains (Table 1). The systems that are common to all the strains tested are the ferrous iron transporters Sit and Feo (Runyen-Janecky et al. 2003). Sit, which has homology to a ferrous iron and manganese ABC transport system in *Salmonella enterica* Typhimurium (Janakiraman and Slauch 2000; Kehres et al. 2002), is found in all *Shigella* as well as enteroinvasive and some other pathogenic *E. coli*, but is not present in *E. coli* K12 or other non-pathogenic enterics (Runyen-Janecky et al. 2003). In contrast, the Feo transport system is widely distributed among bacteria, and the *Shigella* genes have a high degree of homology with the *feoABC* genes present in *E. coli* (Kammler et al. 1993). Given its ubiquity and ability to transport ferrous iron, the Feo system was likely the first iron transport system to arise in the ancestor of the enteric bacteria. The appearance of oxygen and, subsequently, ferric iron necessitated specific transporters for this less soluble form of iron.

Shigella species can transport ferric iron using siderophore-mediated transport systems. These ferric transporters use a low-molecular-weight, high-affinity iron chelator that promotes the uptake of ferric iron, even at very low concentrations (Neilands 1984). The transport systems for the ferric siderophore include an outer membrane receptor, a periplasmic binding protein and a cytoplasmic membrane permease. Interestingly, there is no single siderophore that is common to all the *Shigella* spp. *S. dysenteriae*, *S. sonnei* and some strains of *S. boydii* produce enterobactin (Ent), the catechol type siderophore produced by most *E. coli* and *Salmonella* strains (Payne 1980; Payne et al. 1983; Perry and San Clemente 1979). *S. dysenteriae* type 1 also produces salmochelins, which are catechol-type siderophores related to enterobactin (Hantke et al.

Table 1 Iron transport systems in *Shigella* species[a]

Species	Ferrous transporters[b]	Ferric transporters[c]	Heme transporters[d]
S. boydii	Feo, Sit	Aerobactin	Uncharacterized
		Enterobactin (some strains)	
S. dysenteriae type 1	Feo, Sit	Enterobactin	Shu
		Iro (Salmochelin)	
S. flexneri	Feo, Sit	Aerobactin	Uncharacterized
		Ferric di-citrate	
S. sonnei	Feo, Sit	Enterobactin	Shu
		Aerobactin (some strains)	
		Ferric di-citrate	

[a] The presence the iron transport systems was determined experimentally as indicated in the references noted and by analysis of the following sequenced genomes: *S. boydii* 227 (Yang et al. 2005), *S. dysenteriae* 197 (Yang et al. 2005), *S. flexneri* 2457T (Wei et al. 2003) and *S. sonnei* 046 (Yang et al. 2005)

[b] Runyen-Janecky et al. (2003)

[c] Aerobactin (Lawlor and Payne 1984; Payne 1980), Enterobactin (Payne 1980; Payne et al. 2006; Perry and San Clemente 1979), Iro (Hantke et al. 2003; Reeves 2001), Fec (Luck et al. 2001)

[d] Wyckoff et al. (1998)

2003; Payne et al. 2006). However, many *S. flexneri* and *S. boydii* have lost the ability to produce and use this compound. The genes are present within the genome, but point mutations, deletions and insertions have rendered the system inoperative in these pathogens (Schmitt and Payne 1988, 1991). Instead, these species produce aerobactin (Iuc), a hydroxamate siderophore that is transported by a distinct siderophore transport system (Iut) (Lawlor and Payne 1984; Payne 1980). Some *Shigella* isolates produce both siderophores (Payne et al. 2006). Although aerobactin production has been associated with virulence in some pathogenic *E. coli*, there does not appear to be an advantage of aerobactin over enterobactin production among *Shigella* strains. Both enterobactin producers and aerobactin producers are fully virulent, and they invade and grow within cultured epithelial cells in the laboratory with equal efficiency. All clinical isolates that we have tested produce at least one siderophore. Thus, siderophore production, but not the specific type of siderophore, appears to be important. In addition to their own siderophore, *Shigella* species, like other enterics, have a transport system for ferrichrome (Fhu), a fungal hydroxamate siderophore (Payne 1980).

Ferric iron can also be transported as ferric dicitrate. The *fec* locus has been identified in some strains of *S. flexneri* (Luck et al. 2001), and the genes are present in the sequenced genome of *S. sonnei* (ERIC database). *S. dysenteriae* type 1 has genes with homology to the *E. coli fec* genes, but two of the genes, *fecR* and *fecB*, are pseudogenes. Thus, a functional ferric dicitrate system is not commonly found in *Shigella*.

Heme, which is the most abundant iron source in the human host, is used by some, but not all *Shigella* spp. S. *dysenteriae* type 1 has an efficient heme transport system consisting of an outer membrane receptor (ShuA), a periplasmic binding protein (ShuT) and a cytoplasmic membrane permease (ShuUV) (Mills and Payne 1997; Wyckoff et al. 1998). ShuS, a cytoplasmic protein, is required for efficient use of heme at low heme concentrations and for protection against heme toxicity (Wyckoff et al. 2005). ShuW, X and Y are encoded within the *shu* locus, but their functions are unknown. No heme oxygenase or other protein for removing iron from the imported heme has yet been identified in *Shigella*. The *shu* genes are present in enterohemorrhagic *E. coli*, in other pathogenic *E. coli* and in *S. sonnei* (Wyckoff et al. 1998). The heme transport locus in *E. coli* O157:H7 was analyzed and found to be essentially identical to the *S. dysenteriae shu* locus (Torres and Payne 1997). Some *S. boydii* and *S. flexneri* strains also use hemin for growth in low iron, but the genes encoding the transport system do not appear to be closely related to the *shu* genes.

Genetics of *Shigella* iron transport systems

Analysis of the genomic sequences (Darling et al. 2007; Glasner et al. 2008) in and around the loci encoding the iron transport systems suggests that horizontal transmission of these genes is common (Fig. 2). Only the *feo* and *ent/fep* genes appear to share a common ancestry with *E. coli* K-12. The other *Shigella* iron transport systems, Iuc, Sit, Iro, Fec and Shu, are all encoded within pathogenicity islands. These appear as insertions, often at tRNA genes, within the *E. coli/Shigella* genomic backbone and are usually associated with insertion sequences, phage genes, and pseudogenes.

Aerobactin biosynthesis and transport genes, *iucABCD iutA*

The aerobactin genes are found within pathogenicity islands in both *S. flexneri* and *S. boydii* (Fig. 2). In *S. flexneri*, the aerobactin operon is encoded within an island located adjacent to the tRNA gene *selC* (Moss et al. 1999; Vokes et al. 1999) (Fig. 2), a common site of pathogenicity islands in *E. coli* and *Shigella*. However, the aerobactin genes are absent from the *S. dysenteriae* and O157:H7 islands. In *S. boydii*, the *iuc/iutA* sequences are highly homologous to the corresponding *S. flexneri* DNA sequences, but the flanking DNA and the location of the island are different (Purdy and Payne 2001) (Fig. 2). In *S. boydii* 0- 1392 the island is also linked to a phage integrase gene, but the island maps at the *pheU* locus, rather than *selC*. In other *S. boydii* strains, the island is at yet another location (Purdy and Payne 2001).

Shu heme transport locus

The *S. dysenteriae shu* locus is located between two ORFs that are contiguous in the *E. coli* K12 genome (Fig. 2) (Wyckoff et al. 1998). There are no insertion

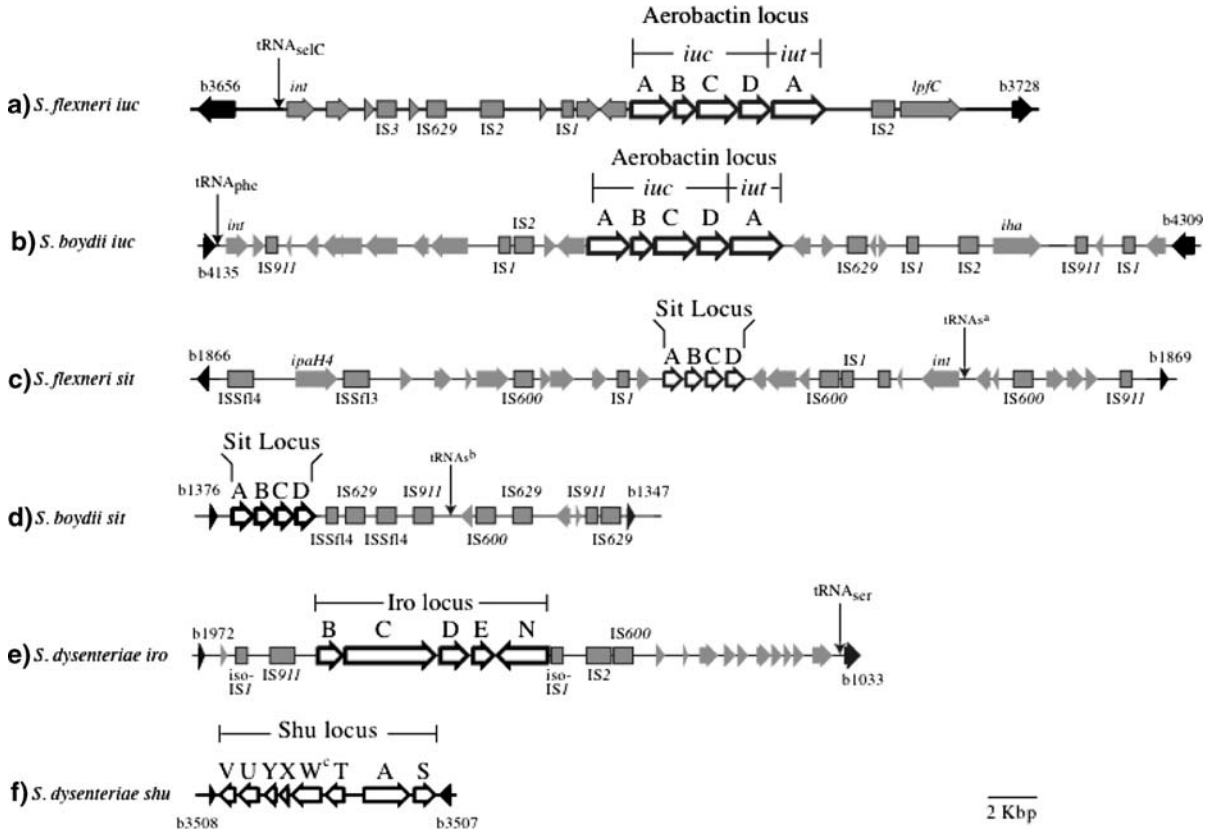

Fig. 2 Genetics of iron transport systems of *Shigella* spp. Partial maps of the pathogenicity islands which encode **a** *S. flexneri* aerobactin (Iuc, Iut) system, **b** *S. boydii* aerobactin system, **c** *S. flexneri* Sit system, **d** *S. boydii* Sit system, **e** *S. dysenteriae* salmochelin (Iro) system, and **f** *S. dysenteriae* heme (Shu) transporter. Unfilled arrows show the iron transport genes; gray arrows are other open reading frames; gray boxes are insertion sequences; and black arrows are the sequences with homology to *E. coli* K-12 at the junctions of the islands. [a] tRNA genes in *S. boydii* Sit locus are gly, thr, arg and ile in that order. [b] tRNA genes in *S. flexneri* Sit locus are, thr, arg and met in that order. [c] the *shuW* gene in *S. dysenteriae* contains a premature stop codon

sequences or phage-like genes associated with *shu*, nor is it associated with a tRNA gene. In *E. coli* O157:H7, the location of the *shu* locus is the same as in *S. dysenteriae*, and the DNA sequence of the locus is almost identical (Torres and Payne 1997). Thus, the heme transport genes may have been acquired by a mechanism other than phage or plasmid transfer, or the genes may have been acquired in the distant past, and any associated insertion elements or phage genes have subsequently been lost.

Ferric dicitrate (*fec*) genes

In one strain of *S. flexneri* 2a, the *fec* genes are located within a large island that encodes several antibiotic resistance determinants (Luck et al. 2001). This island also includes a phage-like integrase and is located at the *serX* tRNA gene. In *S. sonnei*, the *fec* genes are flanked by insertion sequences and phage-like genes, but do not appear to be part of a larger island associated with antibiotic resistance elements as in *S. flexneri* (Darling et al. 2007; Glasner et al. 2008; Yang et al. 2005). The overall organization and location of the *S. dysenteriae fec* genes is the same as in *S. sonnei*, but the presence of a stop codon in *fecR* and a frameshift mutation in *fecB* indicates that the system is unlikely to be functional in *S. dysenteriae.*

Salmochelin synthesis and transport, *iro* genes

The *iro* locus, which encodes an enterobactin modification system to produce salmochelin as well as

transport proteins for this siderophore, is found in *S. dysenteriae* and appears to be part of an island (Reeves 2001) (Fig. 2). These genes are not found in the other *Shigella* species.

Sit A, B, C, D ferrous iron transport system

The Sit system, which permits transport of both iron and manganese, is found in all *Shigella* strains that have been tested and in most enteroinvasive *E. coli*, which also cause dysentery (Runyen-Janecky et al. 2003). In *S. flexneri* 2a, the genes are flanked by multiple copies of insertion sequences and phage-like genes, including an integrase (Fig. 2). There is considerable diversity in the Sit islands, and even in closely related *S. flexneri* 2a strains (2457T and 301) the islands are at different locations and there are differences in sequences flanking the *sit* operon.

Role of iron transport systems in virulence

Our initial studies to identify iron transport systems involved in virulence were done using *S. flexneri* 2a strains SA100 and 2457T. These strains have a relatively small number of iron transport systems, making it possible to carry out mutational analysis of each system alone or in combination with one or more other iron transporters. These strains synthesize aerobactin for ferric iron acquisition and use the Feo and Sit transporters for ferrous iron uptake. Mutants lacking one, two or all three systems were constructed and tested for growth in vitro and in cultured epithelial cells (Runyen-Janecky et al. 2003). Mutants with defects in a single iron transport system grew under typical, iron-sufficient laboratory conditions, indicating some overlap in the ability of these systems to provide iron in vitro. The aerobactin synthesis mutant showed a significant growth defect in low iron media, but the *sit* and *feo* single mutants did not, suggesting that iron is predominantly in the ferric form under these conditions. The double mutants also grew on iron deficient medium, although the *iuc*, *feo* mutant, which only expresses the Sit system, produced much smaller colonies. The triple mutant (*feo*, *sit*, *iuc*) failed to grow in vitro, even when high levels of iron were added to the medium, showing that no other iron transport systems are present in this strain. The mutant did, however, grow in the presence of exogenously added aerobactin, which it can transport but not synthesize.

The iron transport mutants were also tested in virulence and intracellular growth assays (Table 2). In plaque assays, in which virulent *Shigella* invade an epithelial cell monolayer and spread intercellularly, giving rise to clear plaques within the monolayer, neither the *iuc* mutant, nor any of the other single iron transport mutants were defective (Nassif et al. 1987; Runyen-Janecky et al. 2003). However, the aerobactin mutants (*iuc)* were attenuated for infection of embryonated chicken eggs (Lawlor et al. 1987) or rabbit ligated ileal loops (Nassif et al. 1987). In both cases, the *iuc* mutant showed less growth in the extracellular spaces of the host. These results indicate that aerobactin may play a role in growth in the extracellular environment, but neither the siderophore nor either of the ferrous iron transporters is essential for intracellular growth and direct cell-to-cell spread. The triple mutant failed to form plaques within Henle cells, indicating that at least one iron transport system is required for intracellular growth and that no other, undetected, iron transport systems are functioning in vivo in this strain (Runyen-Janecky et al. 2003). A mutation in *fur*, encoding a transcriptional regulator of iron homeostasis, resulted in fewer plaques (Boulette

Table 2 Effect of mutations in iron transport genes on aerobic and anaerobic plaque formation by *S. flexneri* [a]

S. flexneri mutant	Aerobic plaque formation	Anaerobic plaque formation
Wild type SA100	+	+
iuc	+	+
feo	+	+
sit	+	+
iuc, feo	+	+/−, very small plaques
iuc, sit	−	+
feo, sit	+	−
iuc, feo, sit	−	−
fur	+, but fewer plaques	+, but fewer plaques

[a] Ability to form plaques was measured in Henle cell monolayers as described previously. Infected cultures were incubated aerobically or anaerobically for 3 days and the size and number of plaques determined (Boulette 2007; Boulette and Payne 2007; Runyen-Janecky et al. 2003)

and Payne 2007). This strain has reduced invasion of the monolayers due to de-repression of all the iron transport systems and of *ryhB*, a small RNA that regulates virulence gene expression (Murphy and Payne 2007).

Regulation of expression of *S. flexneri* iron transport genes

To better understand the roles of the *Shigella* iron transport systems in the host cell cytoplasm, we looked at the expression of the iron transport genes in *Shigella* growing intracellularly (Fig. 1). In a screen of promoter fusions to green fluorescent protein, the *sit* and *fhuA* (ferrichrome receptor) promoters were more highly expressed intracellularly than extracellularly (Runyen-Janecky and Payne 2002). Intracellular induction of the *feo* and *iuc* operons was not detected in this screen. These results were confirmed by Lucchini et al. (2005) using microarray transcriptional profiling. Additional analysis of the *iuc* promoter and levels of the IutA outer membrane receptor for aerobactin showed that the aerobactin genes were expressed at a lower level in the intracellular environment compared to extracellular bacteria (Headley et al. 1997). All the *Shigella* iron transport systems are repressed by iron via *fur*. Their promoters contain the consensus Fur binding site, or Fur box, and in the presence of iron, Fur binds to the Fur box and represses transcription. Induction of some transporters in the intracellular environment while others are repressed suggests that environmental factors in addition to iron are involved in regulation of iron transport gene expression.

In *E. coli*, *feo* is also regulated by FNR, which activates expression of genes in anaerobic environments (Kammler et al. 1993). Because the Feo system transports ferrous iron, its increased expression in low oxygen environments would enhance iron acquisition under conditions where the ferrous form would be expected to predominate. We tested the effects of oxygen on expression of the *S. flexneri* iron transport systems and on invasion and plaque formation (Boulette and Payne 2007) (Table 3). Using both transcriptional profiling and promoter fusions to *gfp*, we observed increased expression of the *S. flexneri feo* genes under anaerobic conditions. In contrast, the *sit* and *iuc* genes were induced aerobically. Aerobic induction of *sit* was unexpected since *sit* appears to be a ferrous iron transporter, and under aerobic conditions at neutral pH, the iron will be predominantly in the ferric form.

Because the colonic epithelium where *Shigella* infection occurs is an anaerobic environment, we modified the plaque assay to compare the effects of aerobiosis vs. anaerobiosis on function of the iron transport systems during intracellular growth of the bacteria. Epithelial cell monolayers were infected with the wild type or *S. flexneri* iron transport mutants and incubated either aerobically or anaerobically (Table 2). The triple mutant (*iuc, feo, sit*) was unable to plaque anaerobically or aerobically while the wild type formed plaques under both conditions (Boulette and Payne 2007). The *iuc, sit* double mutant, which has only the Feo system, formed plaques when the cultured cells were incubated anaerobically, but not aerobically, indicating that the *feo* system is functional only under anaerobic conditions. Conversely, the double mutants expressing

Table 3 Effect of oxygen and the oxygen-dependent regulators ArcA and FNR on expression of *S. flexneri* iron transport genes

S. flexneri iron transport system genes	Effect of O_2 on gene expression[a]	Effect of ArcA[b]	Effect of FNR[c]
iuc, iutA	↑	Represses anaerobically	None detected
sit	↑	Induces aerobically	Induces anaerobically
feo	↓	Induces anaerobically	Induces anaerobically
fhuA	↑	Induces anaerobically	Induces anaerobically
fur	↓	Represses anaerobically	None detected

[a] Gene expression was determined in microarrays and qPCR and by measuring activity of promoter fusions to *gfp*

[b] Effect of ArcA was determined by comparing gene expression both aerobically and anaerobically in an *arcA* mutant and wild type

[c] Effect of FNR was determined by comparing gene expression both aerobically and anaerobically in an *fnr* mutant and wild type

either the *sit* or *iuc* systems formed wild-type plaques under aerobic, but not anaerobic, conditions. Thus, the plaque formation results were consistent with the in vitro gene expression data; the *feo* genes, which are induced anaerobically, supported intracellular growth and plaque formation under anaerobic conditions, while the *sit* and *iuc* genes, which were induced aerobically, permitted the bacteria to form plaques under aerobic conditions (Tables 2 and 3).

There are two transcriptional regulators, ArcA and FNR that have been shown to regulate gene expression in response to oxygen in *E. coli*. Their roles in regulating the *S. flexneri* iron transport systems were determined by constructing mutants defective in one or both of these environmental response regulators and measuring expression of the iron transport genes in the wild type and mutant backgrounds (Boulette 2007; Boulette and Payne 2007). *feo* and *fhuA* expression were reduced under anaerobic conditions in the *fnr* and *arcA* single mutants and were further reduced in the double mutant. This indicates that both FNR and ArcA induce expression of *feo* and *fhuA* under anaerobic conditions, and the effects of these transcription factors are additive. Analysis of the *feo* and *fhu* promoters revealed the presence of potential binding sites for both FNR and ArcA. Although FNR has been shown to regulate *feo* expression in *E. coli* (Kammler et al. 1993), little is known about ArcA regulation of iron transport genes. Therefore, the putative ArcA binding sites were mutated to determine whether they were required for induction by ArcA.

Altering the putative ArcA binding sites in the *feo* and *fhuA* promoters resulted in loss of ArcA induction of expression. The direct binding of ArcA to the *S. flexneri feo* promoter at the predicted ArcA binding site was confirmed by showing a shift in electrophoretic mobility for the wild type *feo* promoter but not the promoter with the altered ArcA box (Boulette 2007; Boulette and Payne 2007). The *iuc* operon was also regulated by ArcA, but in this case, expression of *iuc* was reduced anaerobically by ArcA, consistent with the aerobic induction of expression of *iuc* seen in microarrays. As in the case of *feo*, ArcA directly binds the *iuc* promoter in gel-shift assays (Boulette 2007).

Although *iuc* and *sit* are both induced under aerobic conditions, the mechanisms of induction are different. Sit expression was not induced aerobically in an *arcA* mutant indicating that ArcA is needed for aerobic induction, rather than acting as a repressor under anaerobic conditions. The effect of ArcA may be indirect since no binding of ArcA to the *sit* promoter could be detected (Boulette 2007).

Oxygen not only affects the expression of the individual iron transport systems, but also it regulates iron uptake through regulation of Fur, which in turn affects the levels of transcription of each system. *fur* expression was elevated anaerobically in an *arcA* mutant, but not in the wild type. Two putative, overlapping ArcA boxes were identified in the *fur* promoter, and mutating these resulted in loss of ArcA repression. The effect of ArcA on *fur* is direct, and binding of ArcA to the promoter was demonstrated by gel shift (Boulette 2007).

It is likely that additional levels of regulation exist for controlling expression of the iron transport systems in *Shigella.* These bacteria must be able to adapt to changes in their environment as they move from cell to cell and from the intracellular to the extracellular environment. Not only will the concentration of iron change, but the ratio of ferric to ferrous iron and of free to complexed iron will vary. The bacteria must obtain sufficient iron to survive and grow with optimal efficiency but must also avoid importing too much iron and risking iron toxicity. Linking expression of the iron transport systems to the level of iron, as well as to environmental conditions such as oxygen that will control the form of available iron, allows the bacteria to express the appropriate iron transport system in a given situation.

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