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Iron and pathogenesis of *Shigella*: Iron acquisition in the intracellular environment

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Abstract

Shigella species are able to grow in a variety of environments, including intracellularly in host epithelial cells. *Shigella* have a number of different iron transport systems that contribute to their ability to grow in these diverse environments. Siderophore iron uptake systems, heme transporters, and ferric and ferrous iron transport systems are present in these bacteria, and the genes encoding some of these systems appear to have spread among the *Shigella* species by horizontal transmission. Iron is not only essential for growth of *Shigella* but also plays an important role in regulation of metabolic processes and virulence determinants in *Shigella*. This regulation is mediated by the repressor protein Fur and the small RNA RyhB.

The *Shigella* species are the causative agents of bacillary dysentery. Following ingestion of even small numbers of the pathogens, these bacteria transit the acidic environment of the stomach and initiate infection of the colonic epithelium (Figure 1). The *Shigella* gain access to the intestinal tissue through M cells. Phagocytosis by macrophages results in apoptosis of the macrophage and release of the bacteria. The bacteria invade colonic epithelial cells from the basolateral side, lyse the resulting vacuole and multiply within the cytoplasm. *Shigella* then spread to adjacent cells via actin-based motility, producing ulcerative lesions. The resulting damage to the epithelium and intense inflammatory response are hallmarks of the disease (Phalipon & Sansonetti 2003).

Although there is not a small animal model of shigellosis, many aspects of *Shigella* pathogenesis can be studied in the laboratory using cultured epithelial cells. Invasion can be visualized in stained monolayers (Figure 2a) (Hale 1986), and intracellular multiplication and cell-to-cell spread can be assessed using a plaque assay (Oaks *et al.* 1985) (Figure 2b). By constructing defined mutations and measuring their effects in invasion and plaque assays, we can begin to define the roles of the *Shigella* iron transport systems in growth and pathogenesis of these organisms.

Analysis of iron uptake in *Shigella* species revealed that they have multiple iron transport systems (Payne & Mey 2004). The iron transport systems identified in these species to date are summarized in Table 1. These include the synthesis and transport of siderophores, ferrous iron transporters and uptake systems for heme. Interestingly, there are differences among the *Shigella* species regarding which iron transport systems are present. Both catechol and hydroxamate siderophores are produced by *Shigella*. However, strains vary in the siderophore synthesized. The differences in distribution of these transport systems among the *Shigella* species suggest horizontal transmission of the genes for these iron transport systems. Therefore, we cloned and characterized the genetic loci encoding these systems.

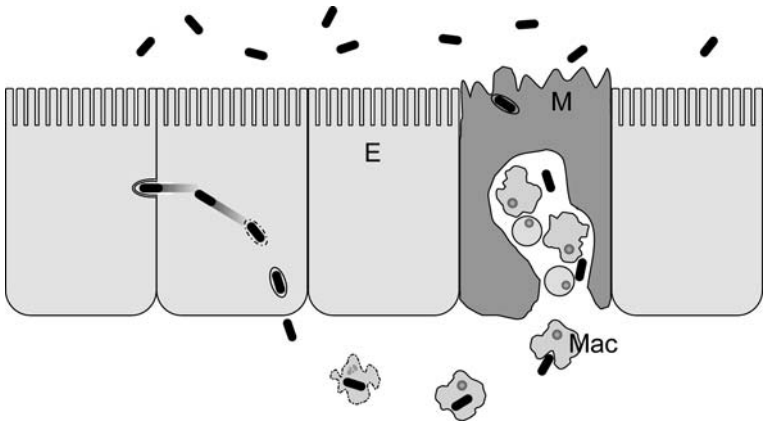


Figure 1. Invasion of colonic epithelial cells by *Shigella* species. The bacteria enter via the M cells (M), transcytose the epithelium and are released in the subepithelial spaces. If ingested by macrophages (Mac), shigellae escape the phagocyte after induction of apoptosis of the macrophage. The bacteria enter the epithelial cells (E), lyse the surrounding vesicle and multiply within the cytoplasm. *Shigella* spread to adjacent cells by actin polymerization at one pole of the bacterium.

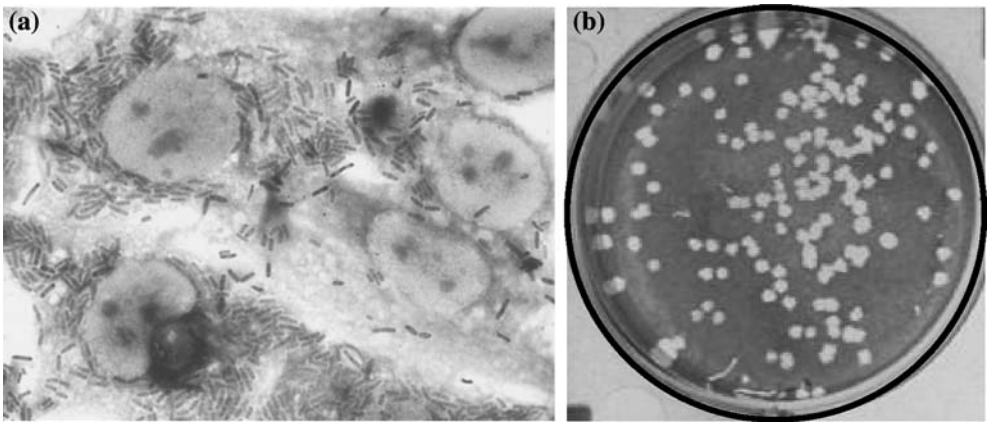


Figure 2. (a) In the invasion assay, subconfluent monolayers of cultured epithelial cells are infected with the *Shigella* strain. The bacteria are allowed to penetrate the cells and gentamicin is then added to kill any extracellular bacteria. After approximately 2 hours of incubation, the monolayers are fixed and stained to allow visualization of the intracellular bacteria. (b). The plaque assay is performed by infecting a confluent monolayer with a relatively small number of bacteria, which are allowed to invade as described by the invasion assay. After addition of the antibiotic to kill extracellular bacteria, the monolayers are incubated for 2–4 days. Wild type *Shigella* grows intracellularly and spreads to adjacent cells, resulting in cell death and formation of plaques in the monolayer. Plaques are visualized by fixing and staining the monolayers.

Siderophore synthesis and transport loci

Shigella dysenteriae produces both enterobactin, a siderophore common to many enteric bacteria, and salmochelins, catechols related to enterobactin that were first described in *Salmonella enterica* serovar Typhimurium (Reeves 2001; Hantke *et al*. 2003; Bister *et al*. 2004). *Shigella sonnei* strains also synthesize enterobactin, but this catechol is not typically produced by either *Shigella flexneri* or *Shigella boydii* (Perry & San Clemente 1979; Payne *et al*. 1983; Payne 1989). The enterobactin synthesis (*ent*) and transport (*fep*) genes are found in the latter two species, and the overall organization of the genes is the same as in Ent^+ *Shigella* isolates. However, in *S. flexneri*, and most likely in *S. boydii*, there are deletions, insertions or mutations in each of the operons, preventing expression of the genes or proper localization of the gene product (Schmitt & Payne 1988; Schmitt & Payne 1991).

The siderophore produced by *S. flexneri* and *S. boydii* is aerobactin, a hydroxamate. The genes for aerobactin synthesis *(iuc)* are found in many *Shigella* and pathogenic *E. coli* isolates, and some strains make both aerobactin and enterobactin. The aerobactin genes are conserved among the *Shigella*

Table 1. *Shigella* iron transport systems.

Iron transport system[a]	*Shigella* species			
	S. flexneri	*S. dysenteriae*	*S. sonnei*	*S. boydii*
Siderophore	Iuc	Ent, Iro	Iuc, Ent	Iuc
Xenosiderophore	Fhu	Fhu	Fhu	Fhu
Ferrous iron	Sit, Feo	Sit, Feo	Sit, Feo	Sit, Feo
Heme		Shu	Shu[b]	
Ferri-citrate	Fec[c]			

[a]Ent, enterobactin synthesis and transport; Iro, salmochelin synthesis and transport; Iuc, aerobactin synthesis; Iut, aerobactin transport; Fhu, ferrichrome transport; Sit, ferrous iron transporter with homology to the Salmonella Sit system; Feo, ferrous iron transport; Shu, heme transport; Fec, ferric dicitrate transport. [b]Uncharacterized heme transport system that is distinct from the *S. dysenteriae* Shu system. [c]Some strains have a ferric dicitrate transport system encoded within an antibiotic resistance island (Luck *et al.* 2001).

and *E. coli* strains, although there are some differences in the sequence of the receptor gene, *iutA*. There are also differences in the location of the genes and their flanking sequences. In *S. flexneri*, the *iuc* locus is found within an island, designated SHI-2, immediately downstream of *selC* (Figure 3a) (Moss *et al.* 1999; Vokes & Payne 1999). The island is approximately 30kb in size and, in addition to the aerobactin genes, contains a colicin immunity gene and a number of remnants of insertion sequences and phage-like genes. A P4 phage integrase-like gene (*int*) is found in the island, immediately adjacent to *selC*. The aerobactin sequences are also located within an island in *S. boydii*, but the location of the island, downstream of the *pheU* locus, is distinct from that in *S. flexneri* (Purdy & Payne 2001). The *S. boydii* island is 21 kb in size and includes a gene with homology to the P4-like integrase and other ORFs, in addition to the aerobactin genes, that are found in the SHI-2 island of *S. flexneri*, but other sequences are different between the two islands. This distribution is consistent with horizontal transmission of the aerobactin genes and with earlier observations that these genes could be plasmid or chromosomally encoded (Carbonetti & Williams 1984; Marolda *et al.* 1987).

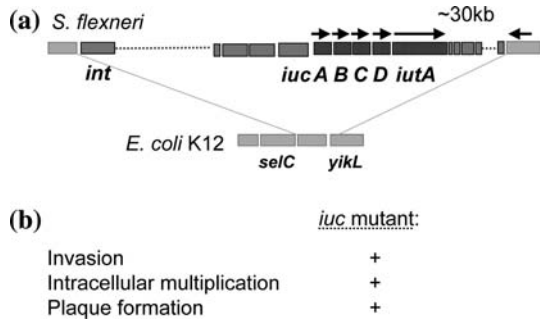


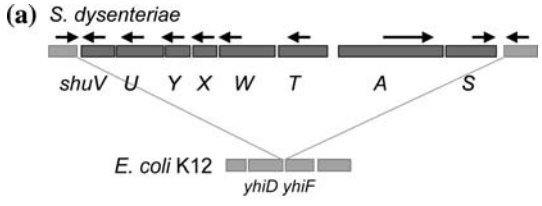
Figure 3. (a) Organization of the aerobactin genes in *S. flexneri*. The aerobactin biosynthesis genes (*iucABCD*) and the aerobactin outer membrane receptor (*iutA*) are located within the ~30 kb SHI-2 island. A gene with homology to the phage P4 integrase is located at the junction of the island with *selC*. Other sequences within the island are not shown. The location of the island relative to the *E. coli* K12 genome is indicated below the *S. flexneri* map. (b). Results of cell culture assays with an *iuc* mutant. + indicates the result was the same as the wild type strain.

Although there are differences in the specific siderophores synthesized by different *Shigella* strains, all the isolates we tested produced at least one siderophore. This suggests that the ability to produce a siderophore is important for survival of the bacteria in some environment.

To determine whether siderophore-mediated iron transport was important for growth in the intracellular environment, we constructed mutants that were defective in siderophore synthesis or transport and tested them for intracellular multiplication and plaque formation. Both aerobactin synthesis and transport mutants grew poorly in low iron medium *in vitro* but appeared to be normal for invasion, intracellular multiplication and cell-to-cell spread (Lawlor *et al.* 1987; Reeves *et al.* 2000) (Figure 3b). Thus, siderophore synthesis and transport may be important in the extracellular environments in the host or when the bacteria are between hosts.

Heme transport

The most abundant iron source in the host is heme and it is not surprising that many pathogens are able to use this iron source. *S. dysenteriae* type 1 has genes encoding an efficient heme uptake system that allows the bacteria to grow with heme in the absence of other added iron sources (Mills & Payne 1995) (Figure 4a). These genes comprise a 9.1 kb segment that appears to have inserted between two open reading frames that are contiguous in the *E. coli* K12 genome (Wyckoff *et al.* 1998). The *S. dysenteriae* heme transport system includes ShuA, the outer membrane heme receptor



(b)

	shuA	*entB,shuA*
Invasion	+	+
Intracellular multiplication	+	+
Plaque formation	+	+

Figure 4. (a) Organization of the heme transport genes in *S. dysenteriae* and *E. coli* O157:H7. The heme transport genes (*shu*) are located within a 9.1 kb segment of the chromosome. The location of the island relative to the *E. coli* K12 genome is indicated below the *S. dysenteriae* map. (b) Results of cell culture assays with a *shuA* mutant and an *entB shuA* double mutant. + indicates the result was the same as the wild type strain.

(Mills & Payne 1997). Mutations in *shuA* eliminate growth with heme as the sole iron source, but neither the *shuA* mutant nor a *shuA*, *entB* double mutant were defective for intracellular growth or plaque formation (Mills & Payne 1997; Reeves *et al*. 2000) (Figure 4b). Additional genes that map within this locus are *shuT*, *U* and *V* (Wyckoff *et al*. 1998), which encode proteins with homology to the heme periplasmic binding protein, cytoplasmic permease and ATP-binding protein of *Yersinia*, and *shuS*, *W*, *X*, *Y* whose functions are less well characterized. One or more of these may be required for metabolism or trafficking of the heme after it enters the cells, processes that are not understood. *shuS* maps downstream of *shuA* (Wyckoff *et al*. 1998) and is a heme binding protein (Wilks 2001) that is predicted to be a cytoplasmic protein. When heme is the sole iron source and its concentration is limiting, ShuS is required for efficient growth (Wyckoff *et al*. 2005); at 5 μM hemin, the *shuS* mutant failed to form colonies on agar medium lacking other iron sources (Figure 5a). Increasing the expression of the heme receptor, by introducing the cloned *shuA* gene into the *shuS* mutant enhanced growth, though not to the same extent as the wild type. Expressing *shuS* from a plasmid increased the colony size of both the wild type and the *shuS* mutant. The *shuS* mutant was able to grow at intermediate concentrations of heme (Wyckoff *et al*. 2005) but was inhibited when the heme concentration is high

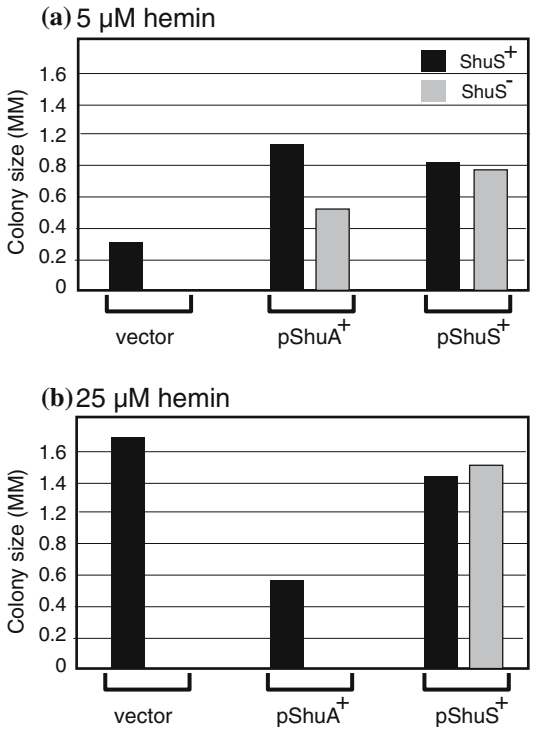


Figure 5. ShuS increases the efficiency of heme use and protects against heme toxicity. Wild type (black bars) or a *shuS* mutant (gray bars) containing the empty vector (vector), cloned *shuA* (pShuA$^+$) or cloned *shuS* (pShuS$^+$) were grown on agar medium containing (a) low (5μM) or (b) high (25μM) concentration of heme as the iron source. The colony size was measured after 24 hours incubation.

(> 25μM) (Figure 5b). This indicates that ShuS helps protect the cell against heme toxicity at high heme concentrations. Enhancement of growth at low heme concentrations and protection when the concentration is excessive suggest that ShuS has a role in intracellular binding and trafficking of heme in *Shigella*.

The *shu* heme transport locus is found at the same location in *E. coli* O157:H7 as in *S. dysenteriae* (Torres & Payne 1997) but is not found in the other *Shigella* species. Additionally, *shuA*, the gene encoding the heme receptor, was detected by hybridization in strains representing multiple phylogenetic groups of *E. coli* (Wyckoff *et al*. 1998). The distribution of *shu* locus is suggestive of horizontal transfer of the genes. Other strains that failed to hybridize to a *shuA* probe nevertheless use heme as an iron source, indicating that at least one other heme transport system is present in the *E. coli–Shigella* group.

Ferrous Iron transporters

Two additional systems characterized in *Shigella* species transport ferrous iron (Feo) or ferrous iron and manganese (Sit) (Runyen-Janecky *et al*. 2003). The Feo system is present in all *Shigella* species, and the *feo* genes are nearly identical to those described in *E. coli* by Hantke (Kammler *et al*. 1993). The Sit system has been shown to transport both ferrous iron and manganese in *Salmonella*, although it appears to transport primarily manganese under physiological conditions (Zhou *et al*. 1999; Kehres *et al*. 2002). Like the *Salmonella* genes, the *Shigella* locus is regulated by both iron and manganese, but in *Shigella*, Sit functions to provide iron to the cells in the absence of other iron transport systems (Runyen-Janecky *et al*. 2003). As was noted with siderophore systems, neither *feo* nor *sit* is essential for iron transport intracellularly (Runyen-Janecky *et al*. 2003).

Iron transport in the intracellular environment

No single iron transport system tested was essential for intracellular multiplication and cell-to-cell spread by *Shigella*. Single mutants defective in siderophore synthesis, Feo, or Sit mediated iron transport or heme utilization were indistinguishable from the wild type in invasion and plaque assays in epithelial cell monolayer. Therefore, double and triple mutants were constructed. Double mutants lacking any two of the iron utilization systems produced smaller plaques than the wild type, indicating a defect in the rate of intracellular multiplication and spread. The triple mutant defective in the siderophore, Sit and Feo systems was unable to multiply within the epithelial cell monolayer (Runyen-Janecky *et al*. 2003). This suggests that all three of these systems can function in the intracellular environment and that at least two iron transport systems are required for efficient intracellular growth and plaque formation.

We also measured the expression of *Shigella* iron transport genes when the bacteria were growing within epithelial cells. An aerobactin gene fusion was not induced in *S. flexneri* growing in HeLa cells, and synthesis of the aerobactin receptor was not detected by Western blot analysis of bacteria isolated from infected HeLa cells. (Headley *et al*. 1997). This indicates that the siderophore is not expressed in this assay. Fusions between *Shigella* promoters and a promoterless green fluorescent protein gene (*gfp*) were also constructed. Cultured epithelial cells were infected with wild type *Shigella* transformed with plasmids containing the fusions and sorted by fluorescence activated cell sorting (FACS) to isolate fusions that were more highly expressed in the intracellular environment of the host cell than in broth culture (Runyen-Janecky & Payne 2002) (Figure 6). As expected, aerobactin promoter fusions were not detected in this screen. However, among the promoters that were more active in the intracellular than the extracellular environment were *sitA*, encoding a ferrous iron transporter, *fhuA*, the receptor for the fungal siderophore ferrichrome, and *sufA*, which is involved in Fe-S cluster maturation. Further analysis showed that the *sitA* mutant grew at the same rate as the wild type *in vitro*, but the mutant was out-competed by the wild type in mixed infections of cultured epithelial cells. Expression of only a subset of the iron transport genes by bacteria in the intracellular environment may indicate that the various transporters respond to different levels of iron in the environment; some operons may require a lower concentration of iron for expression than others. It is also possible that factors in addition to iron are regulating the expression of these genes in the host cell cytoplasm.

Iron regulation of *Shigella* gene expression

The iron transport systems in *Shigella* are regulated by iron via the Fur protein. As was initially described in *E. coli*, Fur binds iron and the ferri-Fur complex binds to a specific sequence, called the Fur box, in the promoters of iron-regulated genes and blocks transcription. (Hantke 1981; Bagg & Neilands 1987; de Lorenzo *et al*. 1988). Genetic analysis of the individual iron transport systems and microarray analyses of *S. flexneri* wild type and *fur* mutant strains indicated that the known *Shigella* iron transport systems are regulated by Fur (Oglesby *et al*. 2005). These include the genes for aerobactin biosynthesis and transport (*iuc*, *iut*), transport of exogenous siderophores (*fhu*), TonB system (*tonB*, *exb*), and ferrous iron transport (*feo*, *sit*). The additional iron transport systems found in

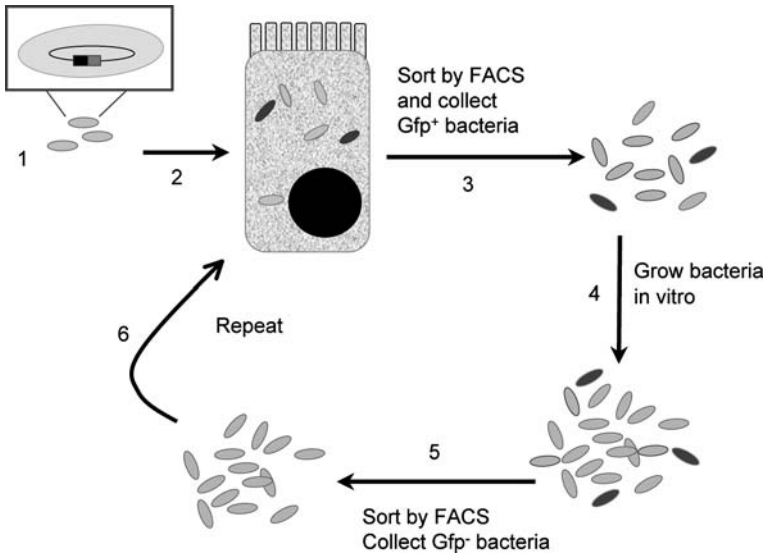


Figure 6. Detection of *Shigella* gene expression in the intracellular environment of the host. (1) Fragments of the *S. flexneri* chromosome (black box) were fused to a promoterless *gfp* (gray box) in a plasmid vector and introduced into wild type *S. flexneri*. (2) Cultured Henle cells were infected with shigellae containing a library of the promoter-*gfp* fusions. Following incubation to allow intracellular growth and gene expression, the infected cells were sorted by FACS and those containing Gfp^+ bacteria (promoter active in the intracellular environment) were collected and lysed to release the intracellular bacteria. The bacteria were grown *in vitro* and sorted to collect those that were Gfp^- (promoter not expressed *in vitro*). *Three rounds of selection were completed to enrich for promoter fusions that were expressed when the bacteria were growing inside host cells but were not expressed when the bacteria were growing extracellularly*.

S. dysenteriae, enterobactin (*ent*, *fep*) and heme transport (*shu*) were similarly regulated (Mills & Payne 1995; Schmitt & Payne, 1988).

In addition to the known iron transport genes, expression of several other groups of genes was regulated by iron and Fur in *Shigella* (Oglesby *et al*. 2005). These include oxidative stress genes (*dps*, *suf*), acid response genes (*gad*, *hde*, *yde*) and virulence factor genes (*ipa*, *mxi*, *spa*, *vir*).

To assess the role of virulence gene regulation by iron in *Shigella*, a *fur* mutant was compared to the wild type in the plaque assay (Figure 7). The *fur* mutant produced approximately 10-fold fewer plaques than the wild type. These few plaques, however, were the same size as wild type plaques suggesting a defect in invasion, consistent with reduced expression of the *ipa* genes, rather than a defect in intracellular multiplication or spread. A requirement for Fur in the expression of the virulence genes suggested that Fur might be repressing a negative regulator. RyhB, a small RNA known to be a Fur-regulated repressor (Masse & Gottesman 2002; Gottesman 2004), was evaluated for its role in virulence gene expression. A *fur*, *ryhB* double mutant was found to be as

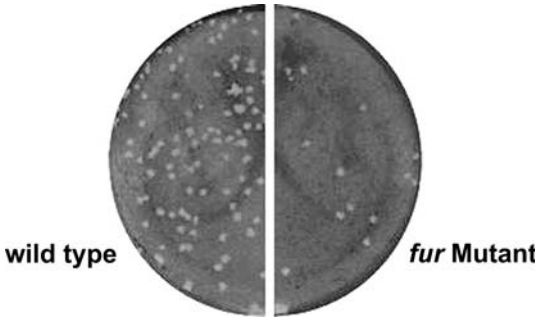


Figure 7. A *fur* mutant forms fewer plaques than wild type *Shigella*. Henle cell monolayers were infected with equal numbers of the wild type strain or a *fur* mutant and incubated for 2 days to allow plaque formation.

invasive and to have the same plaque forming efficiency as the wild type, indicating that Fur was influencing invasion through RyhB. Analysis of virulence gene expression showed that RyhB repressed expression of *virB* (Murphy & Payne (submitted)) (Figure 8), whose product is required for efficient expression of the *ipa*, *mxi*, *spa* virulence genes (Beloin *et al*. 2002).

Similarly, Fur and RyhB regulate acid response in *S. flexneri* (Oglesby *et al*. 2005). Shigellae are extremely resistant to acid, and this resistance is a

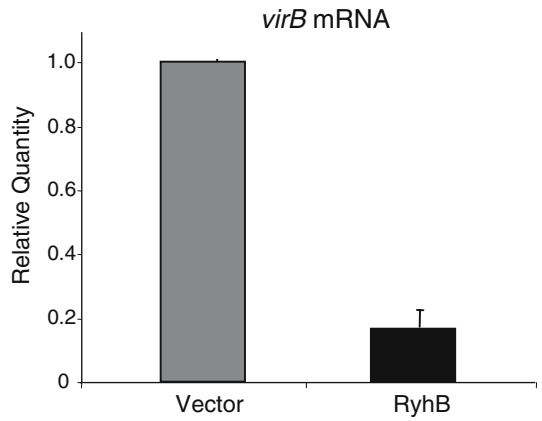


Figure 8. RyhB represses *virB* expression. *S. dysenteriae* containing the empty vector or a plasmid with *ryhB* cloned downstream of an inducible promoter was grown under inducing conditions. The relative amount of *virB* message was measured by real-time PCR.

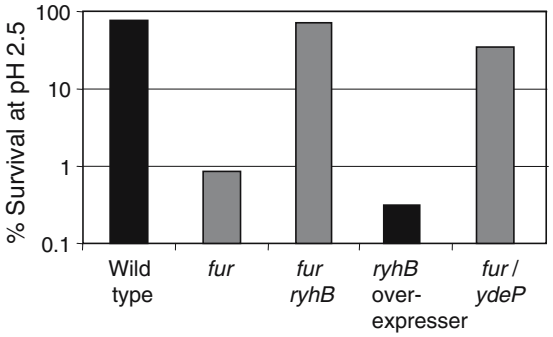


Figure 9. Fur is required for acid resistance in *S. flexneri*. Strains tested for glutamate-independent acid resistance at pH 2.5 included the wild type, a *fur* mutant, a *fur ryhB* double mutant, the wild type strain expressing *ryhB* from an inducible promoter, and the *fur* mutant expressing *ydeP* from a plasmid vector.

contributing factor to the very low infectious dose for this pathogen. A *fur* mutant was acid sensitive at pH 2.5, and the wild type strain became acid sensitive if it was grown in iron-limiting media. A mutation in *ryhB* suppressed the acid sensitivity of the *fur* mutant (Figure 9). Similarly, over-expression of *ryhB* increased acid sensitivity. Analysis of gene expression in the *fur* and *fur ryhB* mutants indicated that RyhB repressed *ydeP*, which encodes a putative oxidoreductase with homology to the alpha chain of formate dehydrogenase. Analysis of *ydeP* showed that this gene played a role in acid resistance. Expression of *ydeP* in the *fur* mutant restored acid resistance and a *ydeP* mutant

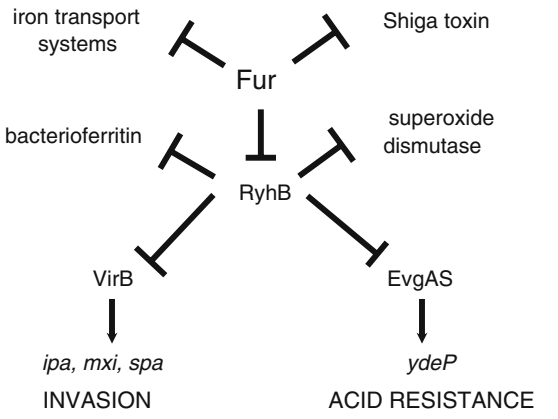


Figure 10. Regulation of gene expression by iron and Fur in *Shigella*. Genetic and microarrays analyses have shown that, in the presence of iron, Fur blocks transcription of a number of genes including those encoding iron transport systems, *stx* (Shiga toxin), and *ryhB*. RyhB represses genes involved in iron metabolism and two regulators important for virulence gene expression, *virB* and *evgAS*.

showed extreme acid sensitivity. RyhB-dependent repression of *ydeP* is likely indirect via repression of the two component regulatory system encoded by *evgAS* (Oglesby et al. 2005). The role of Fur and RyhB in regulating genes involved in virulence or survival in the host is summarized in Figure 10.

Thus iron plays a critical role, not only in cellular metabolism and growth, but also in allowing *Shigella* to sense and respond to the environment of the host.

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