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Contribution of the *Shigella flexneri* Sit, Iuc, and Feo Iron Acquisition Systems to Iron Acquisition In Vitro and in Cultured Cells

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Shigella flexneri **possesses multiple iron acquisition systems, including proteins involved in the synthesis and uptake of siderophores and the Feo system for ferrous iron utilization. We identified an additional *S. flexneri* putative iron transport gene, *sitA*, in a screen for *S. flexneri* genes that are induced in the eukaryotic intracellular environment. *sitA* was present in all *Shigella* species and in most enteroinvasive *Escherichia coli* strains but not in any other *E. coli* isolates tested. The *sit* locus consists of four genes encoding a potential ABC transport system. The deduced amino acid sequence of the *S. flexneri sit* locus was homologous to the *Salmonella enterica* serovar Typhimurium Sit and *Yersinia pestis* Yfe systems, which mediate both manganese and iron transport. The *S. flexneri sit* promoter was repressed by either iron or manganese, and the iron repression was partially dependent upon Fur. A *sitA*::*cam* mutation was constructed in *S. flexneri*. The *sitA* mutant showed reduced growth, relative to the wild type, in Luria broth containing an iron chelator but formed wild-type plaques on Henle cell monolayers, indicating that the *sitA* mutant was able to acquire iron and/or manganese in the host cell. However, mutants defective in two of these iron acquisition systems (*sitA iucD*, *sitA feoB*, and *feoB iucD*) formed slightly smaller plaques on Henle cell monolayers. A strain carrying mutations in *sitA*, *feoB*, and *iucD* did not form plaques on Henle cell monolayers.**

Shigella flexneri, a facultative intracellular bacterium that causes bacterial dysentery in humans, requires iron for growth (12, 33, 49). This pathogen encounters a wide range of environmental conditions throughout its life cycle, which includes exposure to the external environment, transit through the human gastrointestinal tract, and growth within colonic epithelial cells. In each of these environments, iron availability is limited and the potential sources of iron vary. In aerobic external environments, iron is present in the ferric (Fe^{3+}) form, mainly as insoluble iron hydroxide complexes. In the human host, extracellular iron is sequestered in high-affinity iron binding proteins such as lactoferrin, while the iron in the intracellular environment is in heme, ferritin, and other iron-containing proteins.

Shigellae possess numerous systems for iron acquisition. They synthesize and secrete low-molecular-weight, high-affinity ferric iron chelators called siderophores that can either solubilize ferric iron from insoluble complexes or remove iron from some binding proteins and deliver the iron to the bacterial cell. *Shigella* isolates synthesize and use the hydroxamate siderophore aerobactin or the catechol siderophore enterobactin (40, 41). Additionally, shigellae can use the fungal siderophore ferrichrome (40). There are specific receptors in the bacterial outer membrane that bind each ferrisiderophore or other iron complexes with high affinity. In *Shigella*, these receptors include IutA for aerobactin (28), FepA for enterobactin (41, 50), and the heme receptor ShuA (36). The receptor for ferrichrome is likely to be the *Shigella* homologue of the *Escherichia coli* FhuA receptor (5, 47). Transport of these iron-containing ligands through the outer membrane receptors and into the periplasm is an energy-dependent process which requires the TonB-ExbBD system (37).

Periplasmic binding protein-dependent ABC transport systems transport periplasmic iron complexes into the cytoplasm (4). Each system consists of a periplasmic ligand-binding protein, two cytoplasmic membrane permeases, and two subunits of a peripheral cytoplasmic membrane protein with ATP binding motifs. The transported iron ligand binds to the periplasmic binding protein and is transferred to cytoplasmic membrane permeases. Transport through the cytoplasmic membrane requires ATP hydrolysis by the associated ATPase. Unlike the outer membrane receptors, transport through this system is less ligand specific. For example, both ferriferrichrome and ferriaerobactin are transported though the FhuBCD system (25).

Preliminary analysis of the *S. flexneri* genome shows that, in addition to genes encoding high-affinity ferric transport systems, *S. flexneri* has *feoAB*, which are homologous to *E. coli feoAB* (F. R. Blattner, unpublished observations). *E. coli* FeoB, a large cytoplasmic membrane protein with homology to ATPases, mediates ferrous iron uptake (19). However, it is not known whether the 9-kDa FeoA protein is expressed or what role it plays in ferrous iron transport. Unlike ferric iron, which is poorly soluble, ferrous iron is relatively soluble but is primarily found under anaerobic conditions or at nonphysiological pH.

We recently identified another putative iron transport gene, *sitA*, in a screen for *S. flexneri* genes that are induced in the intracellular environment (47). *S. flexneri* SitA is homologous to *Salmonella enterica* serovar Typhimurium SitA, which is

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Reference or source
E. coli strains		
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169 deoR* [Φ80d*lac*Δ(*lacZ*)*M15*]	48
SM10 λ*pir*	*pir*R6K	52
HB101	F$^-$ Δ(*gpt-proA*)*62 leu supE44 ara-14 galK2 lacY1* Δ(*mcrC-mrr*) *rpsL20 xyl-5 mtl-1 recA13*	48
1017	HB101 *ent*::Tn*5*	7
S. flexneri strains		
SA100	*S. flexneri* wild-type serotype 2a	41
SM100	SA100 Str^r	S. Seliger
SA240	SA100 *iucD*::Tn*5*	27
SM166	SM100 *sitA*::*cam*	This study
SA167	SA240 (*iuc*::Tn*5*) *sitA*::*cam*	This study
SA190	SA100 *feoB*::*dhfr*	This study
SM191	SM166 (*sitA*::*cam*) *feoB*::*dhfr*	This study
SA192	SA190 (*feoB*::*dhfr*) *iucD*::Tn*5*	This study
SM193	SA191 (*sitA*::*cam feoB*::*dhfr*) *iucD*::Tn*5*	This study
SA514	SA100 Cam^r	17
SA101	SA100 derivative with deletion in virulence plasmid	6
SA211	SA101 *fur*::Tn*5*	51
Plasmids		
pEG2	pLR29 carrying the *sitA* promoter	47
pHM5	Allelic-exchange vector	46
pLAFR1	Cosmid vector	10
pLR29	pGTXN3 with RP4 mobilization region from pGP704 (35)	47
pWKS30	Low-copy-number cloning vector	54
pEG1	pLAFR1 carrying SA100 *sit* operon	This study
pSIT1	pLAFR1 carrying *S. dysenteriae sit* operon	42
pKL971	pLAFR1 carrying SA100 *iuc* operon	32

encoded in a four-member operon that mediates manganese and iron transport (18, 22, 56). In *S. enterica* serovar Typhimurium, *sitA* expression is repressed in the presence of either iron or manganese (56). The iron repression is mediated by the global transcriptional regulator Fur (56). When bound to ferrous iron, Fur binds to Fur boxes in iron-regulated promoters, thereby repressing transcription (9, 51). The *S. enterica* serovar Typhimurium *sitA* promoter also has a putative MntR binding site, and thus the manganese repression of *sitA* has been proposed elsewhere to be mediated by the manganese-binding transcriptional regulator MntR (21, 22). This report describes the characterization of the *S. flexneri sit* operon and the role of the Sit system and other iron acquisition systems in the ability of *S. flexneri* to productively infect eukaryotic cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this work are listed in Table 1. *E. coli* strains were routinely grown in Luria broth (L broth) or Luria agar (L agar). Minimal medium was Tris-buffered T medium without added iron (47). *S. flexneri* strains were grown in L broth or on tryptic soy broth agar plus 0.01% Congo red dye at 37°C. Ethylene diamino-*o*-dihydroxyphenyl acetic acid (EDDA) was deferrated (45) and added to the medium to chelate available iron. Antibiotics were used at the following concentrations (per milliliter): 125 μg of carbenicillin, 25 μg of kanamycin, 15 μg of chloramphenicol, 25 μg of trimethoprim, and 200 μg of streptomycin.

Recombinant DNA and PCR methods. Plasmids were isolated with the QIAprep Spin Miniprep kit (Qiagen, Santa Clarita, Calif.). Isolation of DNA fragments from agarose gels was performed with the QIAquick gel extraction kit (Qiagen). Chromosomal DNA was isolated by the method of Marmur (31).

All PCRs were carried out with either *Taq* (Qiagen) or *Pfu* (Stratagene Cloning Systems, La Jolla, Calif.) polymerase according to the manufacturer's instructions. *Taq* was used for all PCRs unless the fragments were to be cloned or sequenced, in which case *Pfu* was used. Primers for detection of the *sitA* gene were sit3 (5′ATGCTCTTGGGGTGCTTGGC3′) and sit4 (5′TTCCAGATTCA TACCATTGGCG3′). Other primers for individual PCRs are listed in the appropriate sections below.

Sequence analysis of the *S. flexneri sitA* operon. The nucleotide sequence of part of a *Shigella dysenteriae* O-4576 cosmid that contained the *sit* locus (pSIT1) had previously been determined (42). Based on this sequence, PCR primers were designed to amplify the *sit* locus from *S. flexneri* SA100 in five overlapping fragments. These primers were SA100.1 (CATGAACGACGAACAGATAGC) and SA100.2 (GCTTGGTTATGGATGAGACTT), SA100.3 (TCGCATTTCA GGCAAGTGC) and SA100.4 (ATCTTTCCGCTGGTCAGACG), SA100.5 (TGGTTGGGGTAACGGTTC) and SA100.6 (TAGGACGATGGTCTGAAT G), SA100.7 (GGGCTGTTTATGGTGTCATTGAAC) and SA100.8 (AAAGC GTTGTGTCAGGAG), and SA100.9 (CGCTGAAAGCAGTAGGTATC) and SA100.10 (TTTTGACGACAGGGACCAG).

***sitA-gfp* expression studies.** Strains containing *gfp* were grown in low-salt Luria-Bertani (LB) broth or LB agar, which contains 5 g of NaCl per liter (24). *sitA* expression was measured by use of the plasmid-borne *sitA-gfp* fusion pEG2 (47). Bacteria containing pEG2 were grown in LB broth containing 1 to 32 μg of the iron chelator EDDA per ml to late log phase. One milliliter of each culture was pelleted and resuspended in 4% paraformaldehyde for 10 min and then washed twice and resuspended in low-salt phosphate-buffered saline (47). Green fluorescent protein levels were quantified with a FACSCaliber (Becton Dickinson) fluorescence-activated cell sorter with an excitation at 488 nm. FACSCaliber settings were as follows: forward scatter, E01; side scatter, 505; and relative fluorescence between 515 and 545 nm, 798.

Construction of mutations in *S. flexneri*. The *sitA*::*cam* mutant SM166 was constructed by allelic exchange. For construction of the *sitA*::*cam* plasmid, the *sitA* gene was amplified from SA514 chromosomal DNA with primers sitABfor (5′CTCTTGAAGCACTGAAGGAG3′) and sitABrev (5′CGCACAAATCCCA TAATC3′) and was cloned into *Sma*I-digested pWKS30 (54) to generate pLR61. A 1.6-kb fragment containing a chloramphenicol resistance gene (*cam*) was isolated from pMA9 (17) by digestion with *Hin*cII and inserted into the *Msc*I site in *sitA*. The gene with the *cam* resistance cassette was excised as a *Eco*RV-*Xba*I fragment and ligated into pHM5 digested with *Eco*RV-*Xba*I to generate pLR64. Allelic exchange was done in SM100 as described previously (46).

The *feoB*::*dhfr* mutant SA190 was constructed by targeting of a group II intron to the *feoB* gene as described elsewhere (20, 55). Double and triple mutants

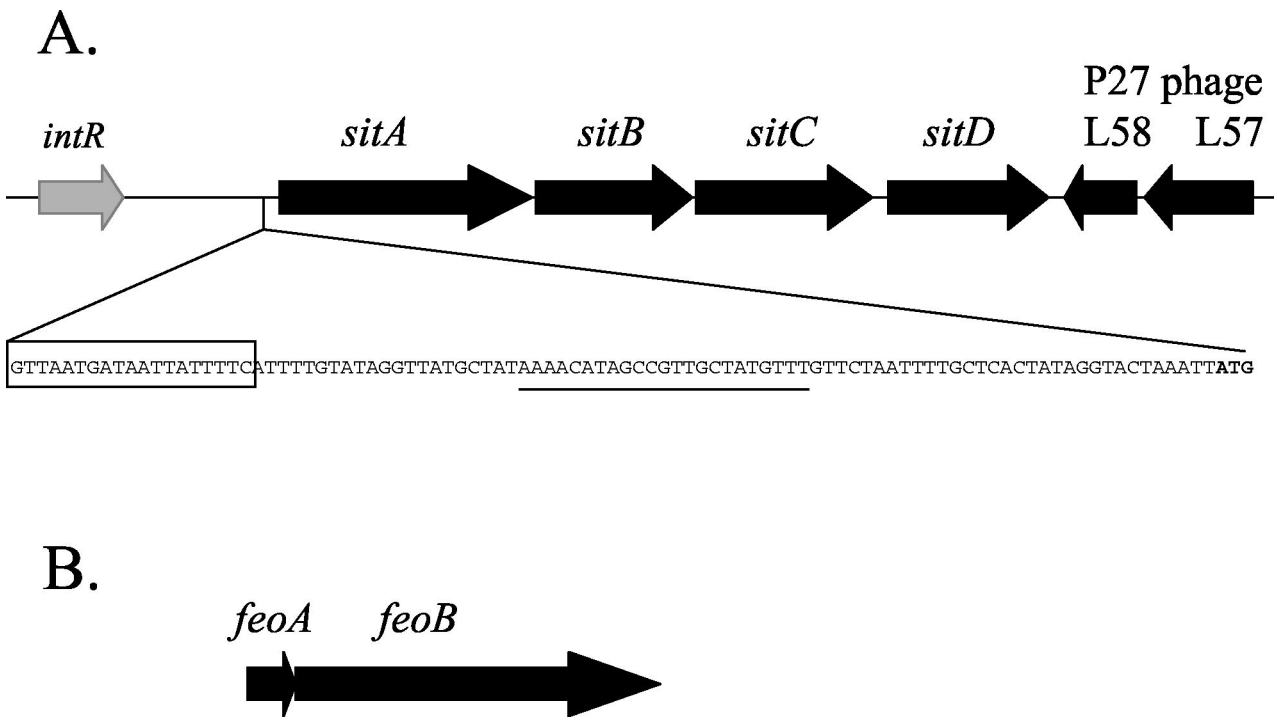

FIG. 1. Map of the *S. flexneri sit* and *feo* loci. The ORFs of the *sit* (A) and *feo* (B) loci are indicated above the representative arrows. The nucleotide sequence of the putative *sitA* promoter is shown. The putative Fur and MntR binding sites are boxed and underlined, respectively, and the SitA start codon is in boldface.

(Table 1) were constructed by P1 transduction of mutations into various backgrounds (34).

All mutations were confirmed by PCR analysis.

Screening of chromosomal library for *sit* operon. A cosmid library of *S. flexneri* chromosomal DNA (K. Lawlor, unpublished data) in *E. coli* HB101 (48) was screened by colony hybridization for clones that hybridize to the *S. flexneri sitA* gene. Probe labeling, hybridization, and detection were performed as described for the Genius II system (Boehringer Mannheim). Cosmids that hybridized with the *sitA* probe were further screened for downstream *sit* genes by PCR with primers SA100.9 and SA100.10.

Tissue culture cell invasion and plaque assays. Monolayers of Henle cells (intestine 407 cells; American Type Culture Collection, Manassas, Va.) were used in all experiments and were maintained in Henle medium, which consists of minimum essential medium, 10% Tryptose phosphate broth, 2 mM glutamine, minimum essential medium nonessential amino acid solution (Life Technologies, Grand Island, N.Y.), and 10% fetal bovine serum (Life Technologies) in a 5% CO_2 atmosphere at 37°C. Plaque assays were done as described previously (38) with the modifications described in the work of Hong et al. (16), and plaques were scored after 3 to 4 days.

Nucleotide sequence accession number. The nucleotide sequence of each fragment was determined by the Molecular Biology Sequencing Facility at the University of Texas at Austin and was submitted to the GenBank database under the accession number AY126440.

RESULTS

Sequencing the *sit* operon from *S. flexneri* SA100. The *S. flexneri sitA* gene was previously identified in a screen for *Shigella* genes that were induced in the eukaryotic cytoplasm (47). The nucleotide sequence of the *S. flexneri* SA100 *sit* operon and flanking regions was determined and was used to search the National Center for Biotechnology Information nonredundant database for similarities. A map of this region is shown in Fig. 1A. The DNA sequence revealed four closely spaced open reading frames (ORFs) that encode proteins homologous to several periplasmic binding protein-dependent ABC transport systems, including the SitABCD system of *S. enterica* serovar Typhimurium (18, 56) and the YfeABCD system of *Yersinia pestis* (3) (Table 2). Based on homologies to the *S. enterica* serovar Typhimurium Sit and *Y. pestis* Yfe systems, *S. flexneri* SitA is predicted to be a periplasmic binding protein. The second ORF, SitB, is predicted to be an ATP binding protein and contains the canonical Walker boxes. SitC and SitD are predicted to be inner membrane permeases based on putative membrane-spanning domains and their homologies with the *S. enterica* serovar Typhimurium SitCD and *Y. pestis* YfeCD inner membrane permeases (2, 3, 18, 22, 56). All four ORFs are predicted to be in one operon since there is no significant space between the ORFs.

The *S. enterica* serovar Typhimurium *sit* genes are located

TABLE 2. Selected homologies of *S. flexneri* SitABCD to other proteins

Species (locus)	% Identity at amino acid level to *Shigella flexneri*[a]			
	SitA	SitB	SitC	SitD
Salmonella enterica serovar Typhimurium (Sit)	77	77	75	64
Sinorhizobium meliloti (Sit)	70	70	68	55
Haemophilus influenzae (Yfe)	67	60	55	46
Yersinia pestis (Yfe)	64	61	59	47
Pasteurella multocida (Yfe)	62	58	53	46
Agrobacterium tumefaciens (Sit)	65	67	65	49
Synechocystis sp. (Mnt)	55	59	42	40

[a] Homologies were calculated with the BLAST algorithm (National Center for Biotechnology Information).

TABLE 3. Distribution of *sitA* in *Shigella* spp. and pathogenic *E. coli* strains

Strain	No. *sitA* positive/no. tested[a]
Shigella	
S. flexneri	13/13
S. dysenteriae	5/5
S. boydii	6/6
S. sonnei	1/1
E. coli	
Enteroinvasive	6/7
Enteropathogenic	0/13
Enterohemorrhagic	0/8
Enterotoxigenic	0/1
K1 (meningitis)	0/6
K-12	0/2

[a] The presence of *sitA* was determined by PCR on cultures of each isolate.

within the SPI1 pathogenicity island (18, 56). The *S. flexneri* genes also appear to be in a pathogenicity island, but the *S. flexneri* island has no homology with the *Salmonella* island other than *sit* genes. We determined the DNA sequence of the regions flanking the *sit* genes in *S. flexneri* and found that a region upstream of *sit* is 97% identical to the 3′ end of an integrase gene (*intR*) from a cryptic RAC prophage. This prophage is located at 31 min on the *E. coli* K-12 chromosome (Fig. 1A). Analysis of the sequence of the *S. flexneri* chromosome shows that the RAC prophage is deleted in the *S. flexneri* chromosome and that the *sit* genes are not located in the region where the prophage is found in *E. coli* (Blattner, unpublished). The nucleotide sequence downstream of the *S. flexneri sit* operon is 85% identical to the 3′ end of P27 phage (Fig. 1A).

The *sit* genes and downstream P27-like phage genes were also found in *S. dysenteriae* (42). The 300-bp region immediately 5′ to the *sit* genes is almost identical in *S. flexneri* and *S. dysenteriae* but shows no homology with any other nucleotide sequences in the nonredundant database (data not shown). However, the nucleotide sequences further upstream of the *sit* genes in these *Shigella* species differ: the *S. flexneri* sequence is homologous to the *intR* integrase in the *E. coli* K-12 chromosome, while the *S. dysenteriae* sequence is homologous to a region containing *ompN* and *ynaF* in the *E. coli* K-12 chromosome. The presence of phage-like elements near the *sit* genes and the difference in the sequences flanking the *sit* genes in *S. flexneri* and *S. dysenteriae* are consistent with the *Shigella sit* genes being present on a pathogenicity island-like element and with the *S. flexneri* and *S. dysenteriae sit* genes having been acquired independently.

Presence of the *sit* locus in other *Shigella* and *E. coli* isolates. To determine the phylogenetic distribution of the *sit* locus among other *Shigella* and pathogenic *E. coli* isolates, we did PCR analysis with primers within the *sitA* gene (Table 3). *sitA* was present in all of the tested isolates of *Shigella*, which represented all the *Shigella* species. Because the *E. coli* and *S. flexneri* chromosomes are highly similar, numerous *E. coli* isolates were also tested for the presence of the *sitA* gene. Six of seven enteroinvasive isolates tested contained the *sitA* gene (Table 3). However, none of the enteropathogenic, enterohemorrhagic, uropathogenic, or meningitis-causing *E. coli* isolates tested had *sitA*, nor did *E. coli* W3110 or DH5α, two nonpathogenic *E. coli* K-12 strains (Table 3). The presence of the *sitA* gene in enteroinvasive enteric pathogens, but not in other pathogenic and nonpathogenic *E. coli* strains, suggests that SitA may contribute to growth in the intracellular environment.

The *sit* genes promote growth of an iron transport mutant. To determine whether the *sit* genes could promote growth of an iron transport mutant, the *sit* loci from *S. dysenteriae* and *S. flexneri* were transferred into *E. coli* 1017, an *ent* mutant defective in the synthesis of enterobactin. This strain grows at the same rate as the wild type in media containing high levels of iron, but its growth is inhibited in the presence of the iron chelator EDDA. *E. coli* 1017 strains carrying the *S. flexneri sit* genes (pEG1), the *S. dysenteriae sit* genes (pSIT1), or the cloning vector alone (pLAFR1) were compared for growth in L broth containing increasing amounts of EDDA. *E. coli* 1017/pLAFR1 was inhibited by relatively low amounts of the chelator (MIC, 6.25 μg of EDDA/ml). The inhibition was reversed by addition of equimolar amounts of iron (data not shown). The presence of either *sit* clone allowed the *ent* mutant to grow at much higher concentrations of the chelator; the MIC of EDDA for either 1017/pEG1 or 1017/pSIT1 was 200 μg/ml. The ability of the *sit* genes to promote the growth of an iron transport mutant in low-iron medium suggests that *sit* genes encode a functional iron uptake system.

The *sit* genes were also tested for growth promotion of 1017 in Tris-buffered minimal medium without added iron. The *ent* mutant failed to grow in this medium, and the presence of the *sit* genes had no effect. The addition of small amounts of iron (0.5 to 1 μM) allowed growth of all three strains (data not shown). It is likely that low-affinity iron transport systems present in 1017 are as efficient as the *sit* genes in promoting growth in medium with minimal iron levels, but the *sit* genes provide an advantage in iron acquisition in the presence of an exogenous iron chelator.

Effect of *sitA*, *iucD*, and *feoB* mutations on growth of *S. flexneri* in media containing EDDA. To further determine whether SitA enhanced growth in iron-depleted media, we constructed a *sitA* mutant, SM166, in which the wild-type *sitA* allele was disrupted with the *cam* gene. Growth of the *sitA* mutant in L broth containing >125 μg of EDDA/ml was decreased relative to the wild type after 8 h (Fig. 2A). There was no difference in optical density between the wild type and the *sitA* mutant after 24 h of growth or in cultures grown with EDDA concentrations of <125 μg/ml (data not shown).

We also examined the growth defect conferred by the *sitA*::*cam* mutation in *S. flexneri* strains containing mutations in other iron transport systems. *S. flexneri* expresses the aerobactin siderophore synthesis and uptake system (IucABCD and IutA) (28). We also identified the *feoAB* genes, which encode a ferrous iron transport system homologous to the *E. coli* Feo system, in isolates from all four *Shigella* species (data not shown and Fig. 1B). *S. flexneri* mutants defective in either FeoB or IucD grew less well than the parent, but better than the *sitA* mutant, after 8 h of growth in L broth containing >125 μg of EDDA/ml (Fig. 2A). Double mutants that had the *sitA* mutation in combination with another iron acquisition mutation grew similarly to the single *sitA* mutant, probably because the growth rate was already very low in the *sitA* background (Fig.

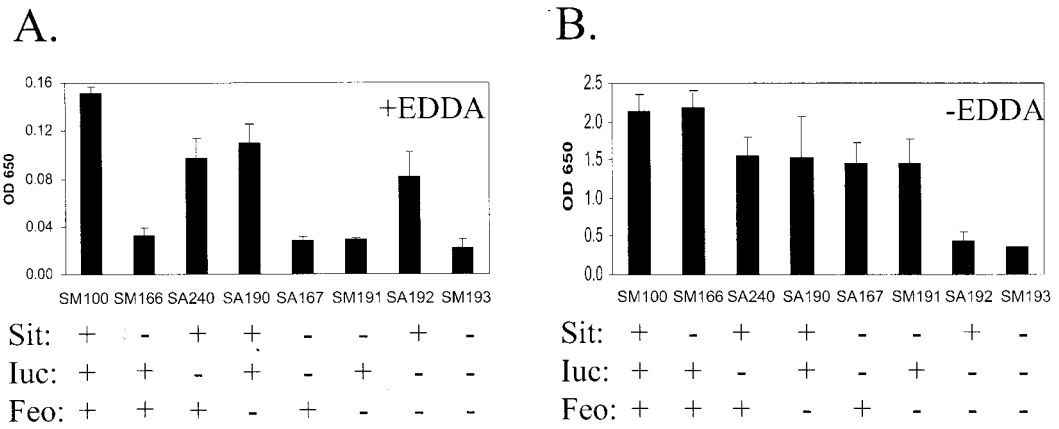

FIG. 2. The *S. flexneri sitA* mutants have slightly reduced growth in L broth plus EDDA. Overnight cultures of each strain were subcultured 1:1,000 into L broth containing 250 μg of EDDA/ml (A) or without EDDA (B) and grown at 37°C. The optical densities of the cultures were measured after 8 h. The data presented are the means of three experiments, and the standard deviations of the means are indicated. The presence of mutations in *sitA*, *iucD*, and *feoB* or the wild-type allele is indicated by − or +, respectively.

2A). With the exception of the strains containing both *feoB* and *iucD* mutations, all strains grew to similar optical densities in L broth not containing EDDA (Fig. 2B). The reason for the poor growth in vitro of the *feoB iucD* double mutant is not known. The addition of relatively high levels of exogenous iron (40 μM $FeSO_4$) partially restored growth, but growth at wild-type levels was observed only when aerobactin was added to the medium (data not shown). Since aerobactin is not known to transport anything other than iron, there is no obvious reason for the failure of added iron to compensate for the mutations.

Because the *S. enterica* serovar Typhimurium Sit and the *Y. pestis* Yfe systems transport both iron and manganese, the *S. flexneri sit* mutant could have reduced capacity to acquire manganese as well as iron. EDDA has a much higher affinity for iron than for manganese (29), but it is possible that manganese was also chelated at the EDDA concentrations used in the growth experiments. To determine whether the decreased growth of the *sitA* mutant in the presence of EDDA was due to depletion of iron or manganese, each of the metals was added to the broth cultures containing EDDA (Fig. 3). Addition of $FeCl_3$ to the cultures stimulated the growth of the *sitA* mutant to wild-type levels, and growth of both the wild-type and mutant strains was comparable to that in cultures without EDDA (Fig. 3). Addition of manganese also abolished the difference in growth between the wild type and the mutant, although growth was not restored to the levels without EDDA (Fig. 3). This suggests that both the parent and the mutant are iron starved. Further, the *sitA* mutant may also be slightly starved for manganese, since the addition of manganese restores the *sitA* mutant's growth to the level of the parent.

Effect of iron transport mutations on plaque formation in Henle cells. Expression of *S. flexneri sitA* was induced in the eukaryotic intracellular environment (47), suggesting that SitA may be required for survival or growth in this environment. Therefore, we tested the *S. flexneri sitA* mutant for growth in the Henle cell intracellular environment by examining its ability to form plaques on a Henle cell monolayer. The *sitA* mutant SM166 formed plaques of the same number and size as those of the parental strain SM100 (Fig. 4), indicating that either SitA function is not required for growth in the eukaryotic cell or another system, e.g., the aerobactin (Iuc) or ferrous (Feo) iron transport system, can compensate for the lack of SitA. Like the *sitA* mutant, the single mutants with mutations in *iucD* (SA240) and *feoB* (SA190) formed plaques of the same number and size as those of the wild-type strain SM100 (Fig. 4). To determine whether the presence of one or more of these iron transport systems compensates for the loss of a single system, we assessed the ability of double mutants to form plaques on Henle cell monolayers. The double mutants with mutations in *iucD sitA* (SA167), *sitA feoB* (SM191), and *feoB iucD* (SA192) formed plaques that were slightly smaller than those of the wild type (Fig. 4), suggesting that the lack of any two of these three iron acquisition systems reduces the efficiency of iron trans-

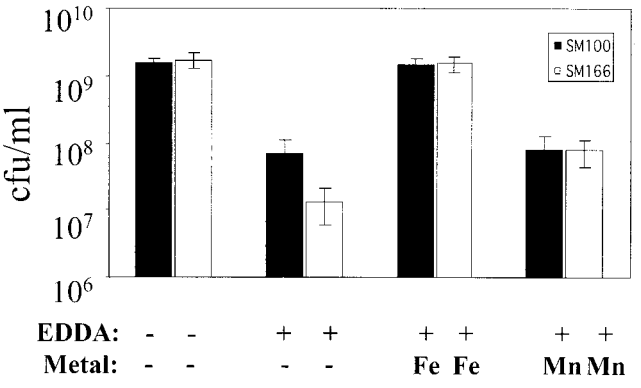

FIG. 3. Addition of either iron or manganese restores *sitA* mutant growth to parental levels in L broth containing EDDA. Overnight cultures of each strain were subcultured 1:1,000 (approximately 10^6 bacteria/ml) into L broth, L broth containing 125 μg of EDDA/ml (350 μM), L broth containing 125 μg of EDDA/ml and 350 μM ferric chloride (Fe), or L broth containing 125 μg of EDDA/ml and 350 μM manganese chloride (Mn). After 8 h of growth at 37°C, the number of bacteria per milliliter of cultures was determined by viable plate counts. The data presented are the means of three experiments, and the standard deviations of the means are indicated.

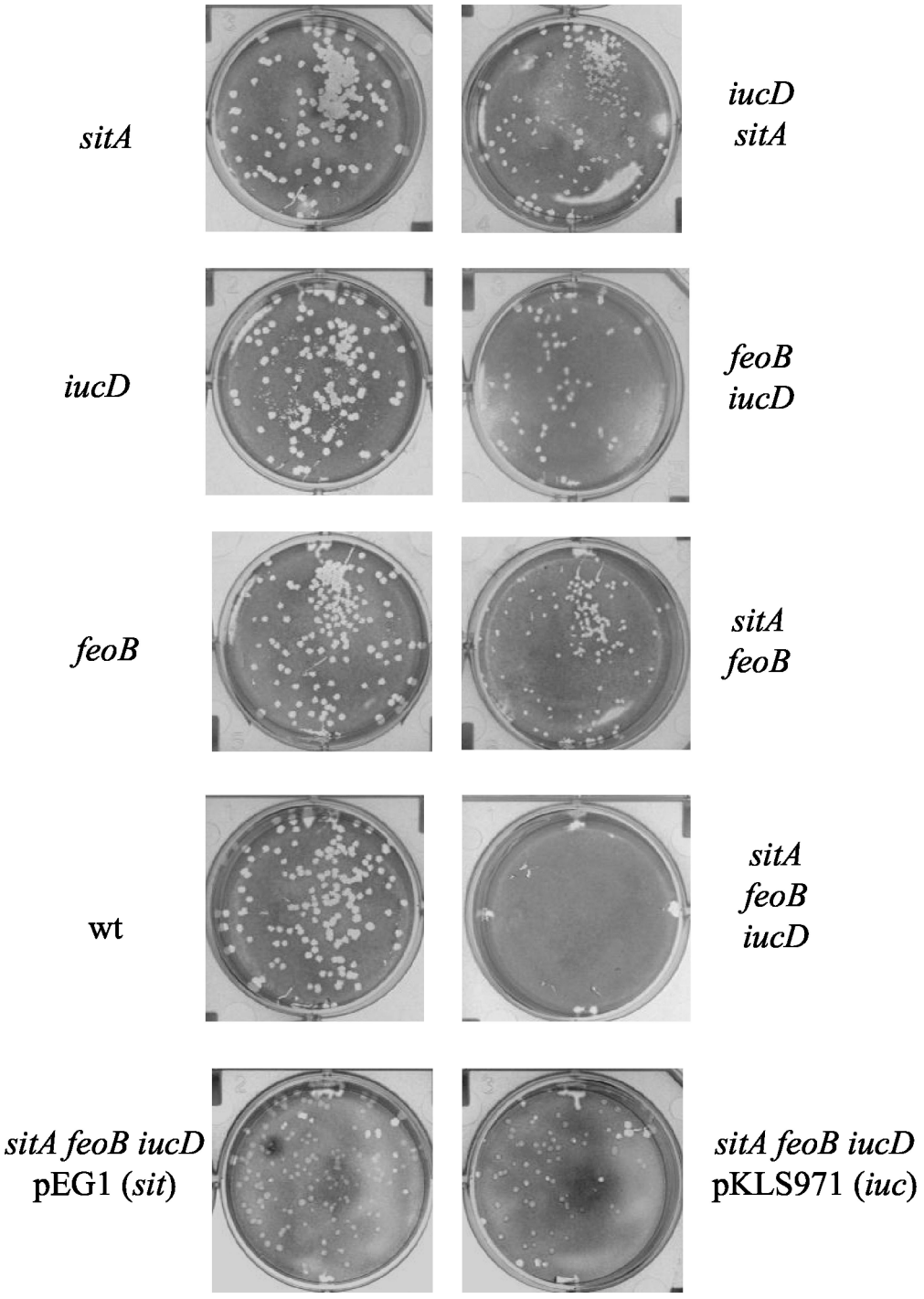

FIG. 4. *S. flexneri* iron acquisition mutants in Henle cell plaque assays. Confluent Henle cell monolayers were infected with 10^4 bacteria per 35-mm-diameter plate, and the plaques were photographed after 3 days. pEG1 and pKLS971 carry the *S. flexneri sit* and *iuc* operons, respectively.

port into the cell when *S. flexneri* is in the eukaryotic cytoplasm (Fig. 4).

Because the double mutants had smaller plaques than did the parental strains, the extent of the plaque formation defect in the triple mutant (*sitA feoB iucD*) was determined. The triple mutant (SM193) was constructed by transducing the *iucD* mutation into the *sitA feoB* mutant SM191. The triple mutant grew poorly on solid media, even when supplemented with 40 μM ferrous sulfate. Growth of the triple mutant, like that of the *feoB iucD* double mutant, was enhanced by addition of aerobactin to the plates, as the *iucD* mutation affects synthesis of aerobactin but not uptake. The triple mutant SM193 did not form plaques on Henle cell monolayers (Fig. 4), suggesting that the lack of these three iron acquisition systems

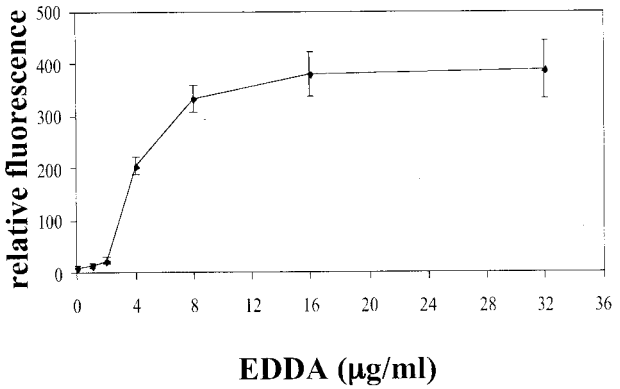

FIG. 5. Induction of the *sitA* promoter in LB broth containing EDDA. SM100 carrying pEG2 (*sitA-gfp*) was grown in LB broth containing EDDA as indicated, and the fluorescence was quantitated by fluorescence-activated cell sorting after 6 h. Ten thousand bacterial cells were assayed for each experimental condition. The data presented are the means of three experiments, and the standard deviations of the means are indicated.

eliminates the ability of *Shigella* to acquire iron and grow in the Henle cell. Addition of cosmids encoding either the *sit* (pEG1) or the *iuc* (pKLS971) operon to the triple mutant restored the small plaque phenotype formation on Henle cell monolayers that was observed for the double mutants (Fig. 4).

Regulation of the *sit* operon. Because the *S. flexneri sitA* gene is induced in the eukaryotic intracellular environment and functions with the Iuc and Feo systems to allow intracellular growth, it was of interest to determine the environmental stimuli that activate *sit* expression. To approach this issue, we examined the regulation of the *S. flexneri sit* operon in vitro using the *sitA-gfp* transcriptional fusion on pEG2. Since SitABCD have homology to iron transport proteins that are repressed in iron-replete conditions, we measured regulation of *S. flexneri sitA* by iron. *S. flexneri* containing pEG2 was grown in L broth containing increasing levels of the iron chelator EDDA. *gfp* expression controlled by the *sitA* promoter increased as the concentration of the iron chelator increased (Fig. 5). At the concentrations used in this experiment, EDDA should specifically chelate ferric iron (29). The addition of $FeCl_3$ to L broth containing EDDA repressed expression of *sitA-gfp* in *S. flexneri* to levels similar to those in L broth cultures without EDDA (Fig. 6), confirming that EDDA was reducing iron availability. These data are consistent with iron-mediated repression of the *S. flexneri sitA* promoter.

Because the *sitA* promoter contains a putative binding site for the iron-responsive transcriptional repressor Fur (Fig. 1A), we determined the expression of the *sitA-gfp* fusion in an *S. flexneri* Fur mutant (SA211). In iron-replete media, expression of the *sitA-gfp* fusion was greater in the *fur* mutant SA211 than in the parent strain SA101 (Fig. 6), suggesting that iron repression of *sitA* expression is mediated by Fur. However, *sitA-gfp* expression in the Fur mutant SA211 increased twofold in the presence of the iron chelator EDDA, relative to expression in the strain grown without EDDA (Fig. 6). This is not due to residual Fur activity in the mutant, as this mutation has previously been shown to eliminate iron regulation of a known, Fur-regulated *S. flexneri* gene (*entB*) (51). This suggested that there was an additional, unidentified, Fur-independent component of iron regulation of *sitA* expression. This twofold increase in the presence of EDDA was eliminated by addition of excess $FeCl_3$ to the EDDA-containing cultures, confirming that the regulation was indeed iron mediated.

The *S. enterica* serovar Typhimurium *sit* genes are repressed by manganese, and the *sit* promoter has a putative binding site for the manganese-responsive MntR repressor (21). The *Y. pestis yfe* operon, which is homologous to the *sit* operon, also has been shown elsewhere to be repressed by Mn^{2+} (3). Because the *S. flexneri sit* promoter contains a putative MntR binding site (Fig. 1A), we tested whether $MnCl_2$ would repress expression of the *S. flexneri sit* operon in the Fur deletion mutant. This genetic background was chosen to eliminate possible repression due to Mn^{2+} binding to Fur, which may allow Fur-dependent repression (13). *gfp* expression driven by the *sitA* promoter decreased to basal levels in the *fur* mutant, as well as in the parent strain, when $MnCl_2$ was added to the cultures (Fig. 6), consistent with Fur-independent manganese repression.

DISCUSSION

Shigella species express numerous iron acquisition systems, reflecting the importance of obtaining iron. The presence of systems for acquisition of different iron sources may also reflect the multiple environments in which *Shigella* lives. In the work described here, we have identified another probable iron acquisition system (SitABCD), which has homology to periplasmic binding protein-dependent ABC transport systems. The highest homology was to the *S. enterica* serovar Typhimurium SitABCD system. The *Salmonella* system can transport both ferrous iron and manganese, although the relevance of iron transport is uncertain, since the concentration of iron

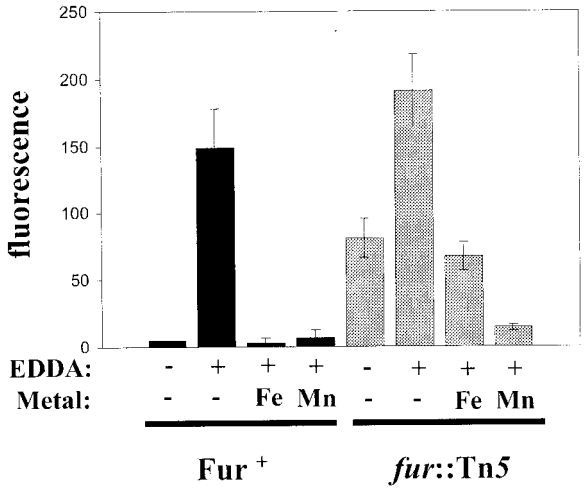

FIG. 6. Induction of the *sitA* promoter. SA211 (*fur*::Tn*5*) or the parent strain SA101 (*fur*$^+$) carrying pEG2 (*sitA-gfp*) was grown in LB broth with or without 16 μg of EDDA/ml (45 μM), and the fluorescence was quantitated by fluorescence-activated cell sorting after 5 h. Where indicated, 500 μM ferric chloride (Fe) or 100 μM manganese chloride (Mn) was added to EDDA cultures. Ten thousand bacterial cells were assayed for each experimental condition. The data presented are the means of three experiments, and the standard deviations of the means are indicated.

required for transport by the *Salmonella* system is significantly greater than physiological concentrations (22). *Y. pestis* also encodes Sit homologues (YfeABCD), and as in *Salmonella*, this system has been shown to transport both iron and manganese; however, unlike *Salmonella*, the iron species transported by *Y. pestis* Yfe was ferric iron (2). It is possible that differences in amino acid sequence among the *S. enterica* serovar Typhimurium Sit, *Y. pestis* Yfe, and *S. flexneri* Sit systems result in altered affinities for each of the cations, such that the physiological role of each system may be unique. The *Salmonella* Sit system may transport primarily manganese, while *S. flexneri* SitA may transport both manganese and iron. We found that, while manganese did not restore growth to the same level as did iron, addition of manganese did abolish the small growth defect of the *S. flexneri* SitA mutant relative to the wild type in L broth with EDDA. This indicates that the *S. flexneri* Sit system contributes to manganese, as well as iron, acquisition. Manganese requirements and uptake systems have not been characterized for *S. flexneri*, but the amount of manganese needed is apparently small, as it is not necessary to add manganese to defined media for maximal growth of *S. flexneri* (data not shown). If the *S. flexneri* Sit system does transport manganese, it is unlikely to be the only manganese transporter present in these bacteria, since *S. flexneri* has an *mntH* gene encoding a protein homologous to the *E. coli* and *S. enterica* serovar Typhimurium NRAMP homologue MntH (23, 30; Blattner, unpublished). Thus, it is likely that there is functional redundancy in manganese, as well as iron, transport.

Several lines of evidence suggest that the *Shigella* Sit system mediates iron acquisition. First, although the *sitA* mutation alone showed no defect in plaque formation, a *sitA* mutation in combination with other iron acquisition mutations showed additive effects in plaque assays. Second, addition of iron to L broth containing EDDA eliminated growth differences between the *sit* mutant and the parent strain in L broth containing EDDA. Finally, both the *S. flexneri* and the *S. dysenteriae sitABCD* genes (42) restored growth to an *E. coli* enterobactin synthesis mutant in iron-restricted media. Likewise, both the *S. enterica* serovar Typhimurium *sit* and *Y. pestis yfe* operons complemented growth defects of enterobactin-deficient *E. coli* in iron-restricted media (3, 56).

The importance of iron transport when *S. flexneri* is in the eukaryotic cytoplasm is supported by the study by Reeves et al. (43) that showed that an *S. dysenteriae tonB* mutant, which eliminates all high-affinity iron transport, was defective in intracellular growth in Henle cells. The *S. flexneri* triple mutant SM193 was unable to form plaques on Henle cells and is defective in iron acquisition via the aerobactin, Feo, and Sit systems. The aerobactin system is TonB dependent (37), but the FeoB system is TonB independent (19). The TonB dependency of the Sit system is unclear, since the mechanism for entry of its ligands into the periplasm in *S. flexneri* has not been identified.

In the eukaryotic cell, free iron is not abundant, since iron, unless tightly complexed, can catalyze formation of toxic hydroxyl radicals. Thus, iron is bound in ferritin, heme proteins, and other proteins. The iron sources used by some intracellular pathogens have been identified. Based on the observation that apolactoferrin and lactoferrin saturated with either manganese or zinc inhibit growth of *Legionella pneumophila*, this bacterium is predicted to use lactoferrin as an intracellular iron source while in the phagosome (11). The Feo system is also important for *L. pneumophila* intracellular growth, since a *feoB* mutant shows decreased replication in amoebae and human U937 cell macrophages (44). Additionally, a mutation in an *L. pneumophila* gene with homology to the aerobactin synthetase genes *iucA* and *iucC* impairs intracellular growth (15), suggesting that siderophore is important. Likewise, the *Brucella abortus* siderophore dihydroxybenzoic acid enhances intracellular survival of the bacteria in murine macrophages (39). Transferrin may be a source of iron for the obligate intracellular pathogens *Chlamydia pneumoniae* (1) and *Mycobacterium tuberculosis* (8). Finally, intracellular replication of *Neisseria meningitidis* requires TonB-dependent acquisition of an unidentified iron source, which was shown previously not to be transferrin, lactoferrin, or hemoglobin (26).

It has been difficult to assess the intracellular iron sources for *Shigella* species since they have numerous iron acquisition systems for multiple iron sources, and single mutations in genes encoding these systems have had no effect on intracellular growth (27, 36, 43). Elimination of one *Shigella* iron acquisition system may be compensated for by increased uptake of iron through another system. However, mutations in more than one iron acquisition system decreased the ability of *S. flexneri* to form plaques on Henle cell monolayers (Fig. 4). This may indicate that more than one iron transport system can access the same intracellular iron source, or there may be several iron sources in the Henle cell cytosol available to *Shigella*. The fact that the *S. flexneri iucD feoB* double mutant forms smaller plaques than do the parental strains suggests that both ferric and ferrous iron sources are available in the cell. It is not known whether the iron ligand for *S. flexneri* SitA is ferric or ferrous iron.

Although all of the *S. flexneri* strains carrying mutations in more than one iron transport system formed smaller plaques on Henle cell monolayers, there are examples in other *Shigella* spp. where elimination of two iron acquisition systems does not affect plaque formation. For example, an *S. dysenteriae* mutant defective in both heme and siderophore iron uptake formed wild-type plaques (43). In the case of the heme receptor, it is possible that, although there is heme in the eukaryotic cell, it may not be accessible to the pathogen. This is true for *Neisseria gonorrhoeae* heme synthesis mutants, which are defective in intracellular growth (53).

Expression of the *S. flexneri sit* operon is regulated in response to multiple signals. Expression of *sit* is repressed by manganese (Fig. 6), likely via the manganese-responsive repressor MntR. The *sit* promoter has a region homologous to the MntR binding site (21). As expected for an iron acquisition system, the *sit* genes are repressed in iron-replete media. A significant portion of this regulation can be attributed to Fur, since the repression is abolished in a Fur mutant (Fig. 6). The regulation of *sit* expression also has a Fur-independent component, since *sit* expression increases in a Fur mutant upon iron depletion of the culture. This would not be expected if Fur were the sole effector of iron-mediated repression. The Fur-independent effects may result from iron binding to MntR, albeit with lower affinity than manganese, and thus causing iron-mediated repression in the absence of Fur. Kehres et al. (21) suggested this type of regulation for the *mntH* promoter,

which is repressed primarily by iron via Fur and by manganese via MntR. Alternatively, an unidentified regulator may mediate Fur-independent iron regulation.

sit expression increases in the eukaryotic intracellular environment (47). This induction may simply be a reflection of a possible low iron and manganese concentration in the eukaryotic cytoplasm. Other *S. flexneri* Fur-regulated, iron-repressed genes show increased expression in Henle cells, including *fhuA* and *sufA* (47). However, since the *S. dysenteriae shuA* and the *S. flexneri iuc* promoters, which are also Fur regulated, were not induced when the bacteria were in Henle cells (14, 43), it is possible that repression of different promoters may be sensitive to different levels of iron. Alternatively, increased expression of *S. flexneri sitA* in the eukaryotic cytoplasm may be in response to additional unknown signals. Another signal that we have examined for potentially regulating *sitA* expression is oxidative stress, but we found that *sitA* expression is not significantly affected by hydrogen peroxide (data not shown). Like the *S. flexneri sitA* gene, the *Salmonella sitA* gene is also expressed in eukaryotic cells (murine hepatocytes) (18). However, while *S. flexneri* multiplies in the cytoplasm, *Salmonella* resides in the vacuole of the eukaryotic cell, and so the signals may be slightly different.

The *S. enterica* serovar Typhimurium *sit* genes are on the SPI1 pathogenicity island (18, 56). Pathogenicity islands are characterized by features including: presence in pathogenic but not nonpathogenic isolates; presence of mobile elements, integrases, insertion sequence elements, and phage genes; association with a tRNA gene; and acquisition via horizontal transfer. Among strains of the related genera *Escherichia* and *Shigella*, the *sit* operon was found exclusively in enteroinvasive strains, which replicate inside host cells. The *S. flexneri sit* operon is flanked by phage genes: part of a RAC prophage integrase gene is located upstream of the *sit* operon. Two ORFs with homology to P27 phage genes are located downstream of the *sit* operon. Furthermore, the regions upstream of *sit* operons are different in *S. flexneri* and *S. dysenteriae*, suggesting a difference in composition or location of the islands. Thus, in *Shigella*, the *sit* genes also appear to be in pathogenicity islands. The acquisition and maintenance of the *sit* genes by pathogens that invade eukaryotic cells suggest a possible role for the *sit* genes in growth in the intracellular environment. *Salmonella sit* mutants are slightly attenuated for virulence (18), and in *Y. pestis yfe* mutants are significantly attenuated for intravenous infection (2). Although the *S. flexneri sitA* mutant was defective in plaque formation only when other iron transport systems were eliminated, it is possible that the Sit system may be important in other stages of the natural infection process.

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