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Salmonella typhimurium Encodes a Putative Iron Transport System within the Centisome 63 Pathogenicity Island

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Upon entry into the host, *Salmonella enterica* strains are presumed to encounter an iron-restricted environment. Consequently, these bacteria have evolved a variety of often-redundant high-affinity acquisition systems to obtain iron in this restricted environment. We have identified an iron transport system that is encoded within the centisome 63 pathogenicity island of *Salmonella typhimurium*. The nucleotide composition of this locus is significantly different from that of the rest of this pathogenicity island, suggesting a different ancestry and a mosaic structure for this region of the *S. typhimurium* chromosome. This locus, designated *sit*, consists of four open reading frames which encode polypeptides with extensive homology to the *yfe* ABC iron transport system of *Yersinia pestis*, as well as other ABC transporters. The *sitA* gene encodes a putative periplasmic binding protein, *sitB* encodes an ATP-binding protein, and *sitC* and *sitD* encode two putative permeases (integral membrane proteins). This operon is capable of complementing the growth defect of the enterobactin-deficient *Escherichia coli* strain SAB11 in iron-restricted minimal medium. Transcription of the *sit* operon is repressed under iron-rich growth conditions in a *fur*-dependent manner. Introduction of a *sitBCD* deletion into wild-type *S. typhimurium* resulted in no apparent growth defect in either nutrient-rich or minimal medium and no measurable virulence phenotype. These results further support the existence of redundant iron uptake systems in *S. enterica*.

It has long been recognized that virulence factors of bacterial pathogens are often encoded in mobile genetic elements such as plasmids, transposons, or bacteriophages. More recently, it has become apparent that virulence factors are frequently found in discrete contiguous regions of the chromosome termed pathogenicity islands (33, 43, 54). Pathogenicity islands often have a G+C content that is significantly different from the overall G+C content of the chromosome of the host organism. This observation, coupled with the frequent presence of sequences resembling transposable elements in the boundaries of pathogenicity islands, has led to the notion that these regions constitute genetic information that may have been acquired horizontally from a heterologous microorganism, a process that most likely contributed significantly to the speciation of different bacteria. It is also a common occurrence that genes located within a pathogenicity island encode functionally related proteins.

Salmonella spp. are enteropathogenic bacteria that have sustained, longstanding associations with their vertebrate hosts. As a consequence, these bacteria display very sophisticated means to interact with host cells, resulting in the stimulation of a variety of host cellular responses (27). These responses ultimately allow these bacteria to gain access to host cells and survive within the host's environment. The ability of *Salmonella* to stimulate host cellular responses is largely associated with a type III secretion system encoded within a pathogenicity island located at centisome 63 (26). This system directs the translocation of a number of bacterial proteins into the host cell, resulting in the stimulation of cellular responses such as membrane ruffling, activation of transcription factors, and, in some cells, programmed cell death (14, 35, 39). Ultimately, these responses allow *Salmonella* to initiate the processes that lead to the establishment of inflammatory diarrhea and the invasion of deeper tissues.

Upon entry into deeper tissues, *Salmonella* spp. encounter an iron-restricted environment. Consequently, similar to many other bacterial pathogens, *Salmonella* spp. have evolved a variety of high-affinity iron acquisition systems to obtain iron from this limiting environment. A number of iron uptake systems have been identified in *Salmonella* (7, 8, 10, 15, 22, 46, 47, 50, 51, 53). These include systems that make use of siderophores such as enterobactin or aerobactin to capture iron and specialized transport systems that mediate the uptake of the siderophore-iron(III) complexes. The activity of most of these specialized transport systems requires the function of the bacterial outer membrane protein TonB (42, 60). Another type of system identified in *Salmonella* is encoded by the *feoAB* locus and mediates the transport of iron(II) through the inner membrane (60). This system does not require siderophores, as iron (II) is soluble and therefore readily enters the periplasmic space by diffusion through the porins. *Salmonella* strains carrying mutations in known iron uptake systems are either minimally affected in virulence or not affected at all (10, 34, 40, 62). This is surprising, as *Salmonella* spp. are predicted to encounter iron-restricted environments in the course of their pathogenic cycle. The lack of strong phenotypes associated with mutations in iron uptake systems is therefore most likely due to the existence of several redundant systems that can mediate the uptake of this critical nutrient. Here, we report the identification and characterization of a novel iron uptake system encoded in the centisome 63 pathogenicity island of *Salmonella typhimurium*. This system belongs to the ABC family of transporters and complements the growth defect of an enterobactin-deficient mutant of *Escherichia coli* in iron-restricted medium.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Description, source and/or reference
E. coli strains		
DH5αMCR	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*)	GIBCO BRL
SM10 λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu (Kn^r) λ*pir*	48
BL21(DE3)	F⁻ *ompT hsdS*	59
SAB11	*proA leu lacYI* Ent⁻	Enterobactin-deficient strain (5)
S. typhimurium strains		
JF2043	*fur-1 zbf-5123*::Tn*10 iroA1*::MudJ	30
SL1344	Wild type	40
SB136	*invA*	29
SB801	Δ*sitBCD*::*aphT*	This study
SB804	*sitB*::*lacZ*	SL1344 derivative with pSB1000 integrated in the chromosome
SB930	*sitB*::*lacZ*	SL1344 derivative with pSB1005 integrated in the chromosome
SB833	*fur-1*	P22HT*int*[JF2043]→SL1344
SB835	*fur-1 sitB*::*lacZ*	SB833 derivative with pSB1005 integrated in the chromosome
SB836	*fur-1 iroA*::MudJ	P22HT*int*[JF2043]→SB833
Plasmids		
pSB377		R6K-derived replicon (41)
pGP704		48
pSB857	*sitABCD*	36
pSB992	Δ*sitBCD*::*aphT*	pSB377 backbone
pSB998	*sitABCD*	pSC101-derived replicon
pSB1000	*sitB*::*lacZ*	pSB377 backbone
pSB1005	*sitB*::*lacZ*	pGP704 backbone

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. All *S. typhimurium* strains were derived from the wild-type strain SL1344 (40). Clinical isolates from different *Salmonella enterica* serovars, *Shigella* spp., and *E. coli* were from the laboratory collection. The *sit* operon deletion mutant strain SB801 was constructed by deleting an *Hpa*I-to-*Nde*I DNA fragment (which encompasses the *sitB*, *sitC*, and *sitD* genes) and inserting a kanamycin resistance cassette lacking a transcription terminator (*aphT*) (29). The deletion construct was introduced into the chromosome by allelic exchange as previously described (41). The SL1344 derivative strain SB833, which carries the *fur*::Tn*10* allele from strain JF2043 (30), was constructed by P22HT*int*-mediated transduction (58). A reporter strain carrying a fusion of *sitB* to the promoterless *lacZ* gene was constructed as follows. A promoterless *lacZ* cassette was cloned into the *Eco*RV site of *sitB*, and the gene fusion was cloned into the R6K-derived suicide vector pGP704 (37). The resulting plasmid, pSB1005, was subsequently integrated into the chromosome of wild-type *S. typhimurium* SL1344 or its *fur*::Tn*10* derivative strain SB833 by conjugation and homologous recombination (41), yielding the reporter strains SB804 and SB835, respectively. The control reporter strain SB836 was constructed by moving the *iroA*::MudJ allele from strain JF2043 (30) into SB833 by P22HT*int*-mediated transduction. All *E. coli* and *S. typhimurium* strains were grown in Luria-Bertani medium at 37°C, and, when appropriate, antibiotics were added at the following concentrations: ampicillin, 100 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; streptomycin, 100 μg ml^{-1}; and tetracycline, 10 μg ml^{-1}. Iron-depleted conditions were created by using Curtiss's minimal salts (5 g of NH_4Cl, 1 g of NH_4NO_3, 3 g of Na_2SO_4, 9 g of K_2HPO_4, 3 g of KH_2PO_4, 96 mg of $MgSO_4 \cdot 7H_2O$, and 40 mg of histidine, per liter of medium) supplemented with a 150 mM concentration of the iron chelator 2,2′-dipyridyl (Sigma, St. Louis, Mo.). Iron-replete media were supplemented with 40 μM $FeSO_4$. Sodium citrate was added at a final concentration of 1 mM when required.

Recombinant DNA methods, DNA sequencing, and analyses. Recombinant DNA procedures were carried out by standard protocols (55). Chromosome walking of the *S. typhimurium* chromosome was carried out as previously described (36). Nucleotide sequence determination was carried out with both strands of the template DNA by the dideoxy termination method. DNA and protein sequences were analyzed with the Genetics Computer Group (GCG) package from the University of Wisconsin (19). The Blastp program was used for searching protein sequence databases (GenBank, EMBL, and Swissprot) (3). PCR amplification of the *sitA-fliA* intergenic region was carried out by standard procedures with primers complementary to of the 3′ end of *fliA* (5′-TGTGGG CACTGGCTTTCATA-3′) and the 5′ end of *sitA* (5′-CGTGCGGGTTCGGTT TAC-3′). The predicted size of the amplified fragment based on the *S. typhimurium* nucleotide sequence is 646 bp.

Southern and dot blot hybridizations. DNA samples were separated on a 1% agarose gel and transferred onto a Hybond-N nylon membrane (Amersham Life Science, Arlington Heights, Ill.). For dot blots, appropriate amounts of chromosomal DNA were spotted on Hybond-N nylon membranes. Southern and dot blot hybridizations were performed with an enhanced chemiluminescense-based kit (Amersham Life Science) according to the manufacturer's instructions. Fluorescence-labeled probes were generated by using a random-primed nonradioactive labeling reaction (Amersham Life Science).

Tissue culture cell invasion, macrophage cytotoxicity, and mouse infection. Bacterial internalization, macrophage cytotoxicity determination, and mouse infections were carried out as described elsewhere (13, 28).

β-Galactosidase assay. β-Galactosidase activity was measured by the Miller assay as previously described (55).

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been deposited in GenBank under accession number AF128999.

RESULTS

Identification of a putative iron transport system in the centisome 63 pathogenicity island of *S. typhimurium*. As part of our ongoing effort to characterize the centisome 63 pathogenicity island of *S. typhimurium*, we determined the nucleotide sequence of one of its border regions between the *avrA* and *fhlA* genes (36, 49). Plasmid pSB851, which harbors an insert that contains this entire region, was used as the source of DNA for nucleotide sequencing. This plasmid contains a segment of the centisome 63 pathogenicity island from wild-type *S. typhimurium* retrieved by chromosome walking (36). Four open reading frames (ORFs) apparently arranged in a single transcriptional unit were identified (Fig. 1). Putative ribosome-binding sites positioned at the appropriate distance from the initiation codons were found upstream of each of the four ORFs. A Blast search of the available databases revealed that the predicted polypeptides encoded by this operon exhibit extensive sequence similarity to ABC transport systems. The highest homologies are to the *yfe* ABC iron transport operon of *Yersinia pestis* (9), to the *mnt* manganese transport system of *Synechocystis* sp. strain 6803 (6), and to an ABC transporter of unknown function identified during sequencing of the *Haemophilus influenzae* genome (23). Significant similarity to a number of ABC transporters thought to mediate attachment of several gram-positive bacteria to host cells was also detected (45, 56). The arrangement of the *S. typhimurium* locus, which we have named *sit*, is characteristic of all binding-protein-dependent or ABC transport systems (11, 38) (Fig. 1). *sitA* encodes a 305-amino-acid polypeptide with closest similarity with the *Y. pestis*

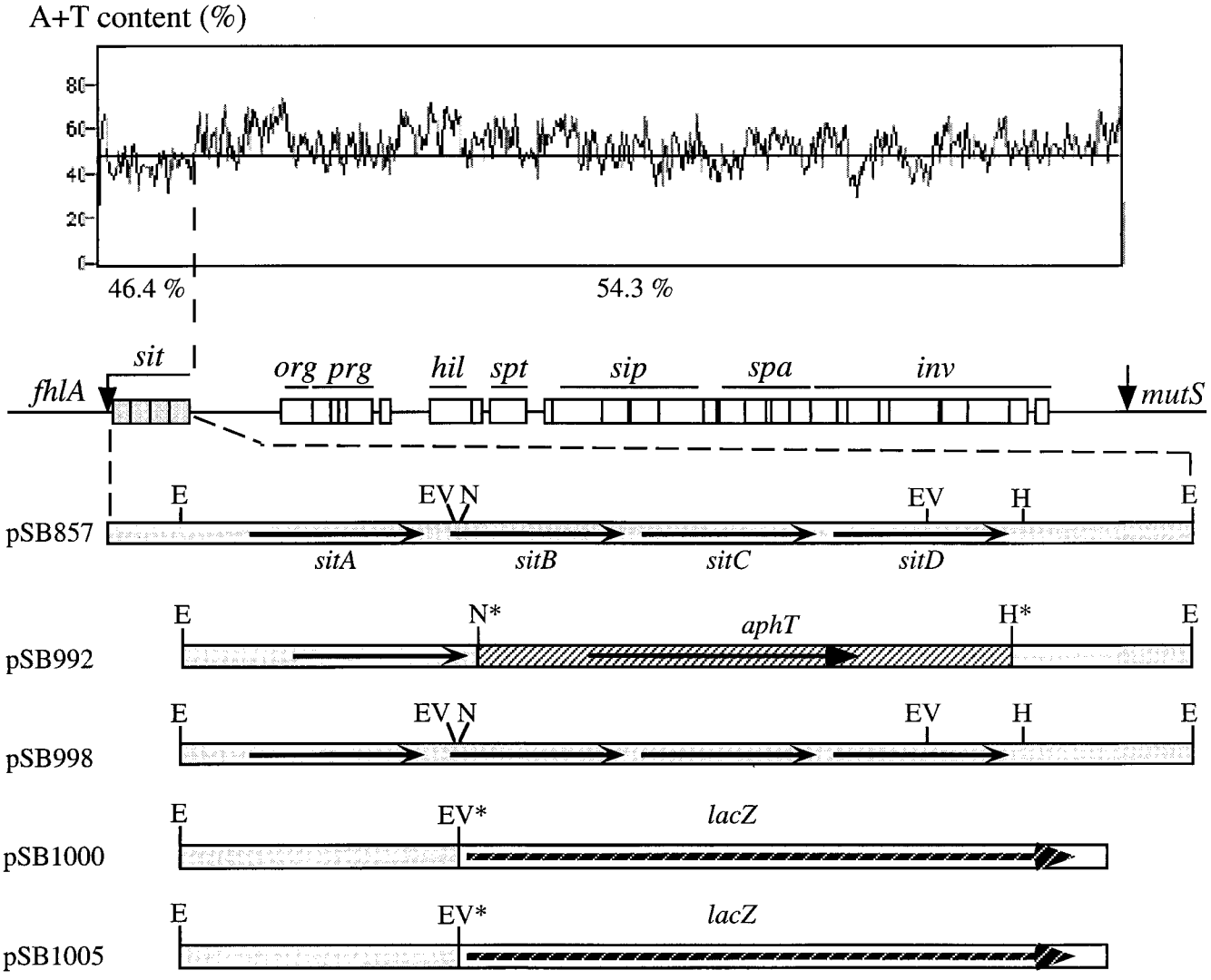

FIG. 1. A+T content and genetic organization of the *sit* locus encoded in the centisome 63 pathogenicity island of *S. typhimurium*. The region between *orgA* and *fhlA* was cloned by chromosomal walking, resulting in the plasmid pSB857. The nucleotide sequence of the region located immediately downstream of *fhlA* was determined. Four ORFs (*sitA*, *sitB*, *sitC*, and *sitD*) apparently arranged in a single transcriptional unit were identified. Abbreviations for restriction enzymes: E, *Eco*RI; EV, *Eco*RV; N, *NdeI*I; H, *Hpa*I. An asterisk indicates that the restriction site has been destroyed by cloning.

YfeA (9) and the cyanobacterium *Synechocystis* strain 6803 MntC (6) proteins (Fig. 2). YfeA and MntC are thought to function as periplasmic binding proteins in ABC transport systems involved in the transport of iron and manganese, respectively. In addition, SitA shows sequence similarity to EfaA and PsaA, which are putative adhesins from *Enterococcus faecalis* (45) and *Streptococcus pneumoniae* (56), respectively. The polypeptide encoded by *sitB* exhibits the signature motif (LSGGQKKRVFLARAI) of the ABC transporter family of proteins (25, 38). In addition, SitB displays a canonical nucleotide binding motif (GVNGSGKS), which is another characteristic feature of this protein family (17, 61). This region, generally referred to as Walker box A, is thought to form a flexible loop between a β-strand and an α-helix which interacts with one of the phosphate groups of the nucleotide. *sitC* and *sitD* encode polytopic integral membrane proteins which are predicted to function as permeases in this ABC system (25, 38, 57). Consistent with the homologies of the other polypeptides encoded in the *sit* locus, SitC and SitD are most closely related to the putative permeases of the *Yersinia* ABC iron transporter encoded by the *yfe* locus (data not shown).

Previous analysis of the nucleotide composition of the type III secretion genes encoded within the centisome 63 pathogenicity island has shown that the G+C content of this region is lower than the average for the *S. typhimurium* chromosome (29, 31, 32). This observation supports the hypothesis that this region of the *Salmonella* chromosome was acquired via horizontal gene transfer from a heterologous source. Analysis of the nucleotide composition of the *sit* operon shows a G+C content of 53.6%, which is significantly different from that of the rest of the centisome 63 pathogenicity island and is similar to the overall nucleotide composition of the *S. typhimurium* chromosome. In fact, there is a sharp transition in the nucleotide composition of the intergenic region that separates *sitD*, the last gene in the *sit* operon, and *avrA*, encoding a substrate of the type III secretion system (36). This observation suggests that the type III secretion system and the ABC transporter encoded by the *sit* operon have different ancestries and may have

FIG. 2. Sequence alignment of SitA with periplasmic binding proteins of binding-protein-dependent transport systems. Sequences were aligned by using the Pileup program of the GCG software package from the University of Wisconsin (19). Black boxes indicate identical amino acids, and shaded boxes indicate conservative substitutions.

been acquired from different sources. Thus, the centisome 63 pathogenicity island may be a mosaic of at least two different regions with different ancestries and different functions.

Distribution of the *sit* operon. To investigate the distribution of the *sit* operon among different strains of *S. enterica*, as well as other bacterial species, high-stringency dot blot DNA hybridization was performed with a 2-kb fragment encompassing a region between the 3′ end of *sitD* and the 5′ end of *sitC* as a probe. Chromosomal DNAs from 30 different *S. enterica* serovars, two pathogenic strains of *E. coli*, *E. coli* K-12, *Shigella sonnei*, and *Shigella flexneri* were used for the dot blotting. Under stringent conditions, the DNA probe hybridized strongly with all *S. enterica* isolates tested but did not hybridize with DNA samples from *Yersinia* and *E. coli* strains. A weak signal was detected in samples from *Shigella* spp. These results indicate that the *sit* operon is widely distributed among *S. enterica* serovars (Table 2). We also examined the location of the *sit* operon in representative serovars of *S. enterica* by PCR analysis with primers complementary to the *sit* locus and to the immediately adjacent gene *fhlA*, which constitutes one of the boundaries of SPI-1 (49). A fragment of ~650 bp was obtained from all of the strains tested, which include isolates of *S. typhimurium* (serogroup B), *Salmonella gallinarum* (serogroup D), *Salmonella pullorum* (serogroup D1), *Salmonella enteritidis* (serogroup D1), *Salmonella typhi* (serogroup D1), *Salmonella dublin* (serogroup D), *Salmonella nierstedten* (serogroup C4), *Salmonella thompson* (serogroup C1), *Salmonella duisburg* (serogroup E1), and *Salmonella choleraesuis* (serogroup C1). These results indicate that the *sit* locus is located in the same region of the chromosome in most likely all serovars of *S. enterica*.

The *S. typhimurium sit* operon allows utilization of chelated iron by an enterobactin-deficient *E. coli* strain. The close sequence similarity of the components of the ABC transporter encoded by the *sit* operon with similar systems involved in iron transport prompted us to test the possibility that this *Salmonella* system may be capable of transporting iron. To test this hypothesis, we introduced the plasmid pSB998, which contains the entire *sit* operon, into the enterobactin-deficient *E. coli* strain SAB11 (5, 9). This strain is incapable of growing in iron-limited media without the presence of exogenous siderophores (5, 9). As shown in Fig. 3, *E. coli* SAB11 expressing the *S. typhimurium sit* operon was able to grow in iron-deficient minimal medium. Growth was almost equivalent to that of the *ent*$^+$ parent strain HB101 (average colony sizes after 48 h, 1.1 ± 0.05 mm for SAB11 and 2.1 ± 0.04 mm for HB101). In contrast, the same strain carrying the vector plasmid (pWKS30) alone failed to form visible colonies after 48 h of incubation at 37°C in the same medium, although it was able to grow in the presence of an exogenous siderophore such as citrate (Fig. 3). These results demonstrate that the *sit* operon encodes an ABC

TABLE 1. Distribution of *sitCD* genes

Hybridization signal	Organism	Serotype
Positive	*S. enterica* serovar *agona*	B
	S. enterica serovar *anatum*	E1
	S. enterica serovar *arizona*	
	S. enterica serovar *bovis morbidificans*	C2
	S. enterica serovar *braenderup*	C1
	S. enterica serovar *Brandenburg*	B
	S. enterica serovar *bredeney*	B
	S. enterica serovar *choleraesuis*	C1
	S. enterica serovar *dublin*	D1
	S. enterica serovar *duisburg*	B
	S. enterica serovar *enteritidis*	D1
	S. enterica serovar *gallinarum*	D1
	S. enterica serovar *hadar*	C2
	S. enterica serovar *heidelberg*	B
	S. enterica serovar *infantis*	C1
	S. enterica serovar *java*	B
	S. enterica serovar *manhattan*	C2
	S. enterica serovar *montevideo*	C1
	S. enterica serovar *newport*	C2
	S. enterica serovar *nienstaedten*	C4
	S. enterica serovar *ohio*	C1
	S. enterica serovar *othmarschen*	C1
	S. enterica serovar *panama*	D1
	S. enterica serovar *pullorum*	D1
	S. enterica serovar *schwarzengrund*	B
	S. enterica serovar *tennessee*	C1
	S. enterica serovar *thompson*	C1
	S. enterica serovar *typhi*	D1
	S. enterica serovar *typhimurium*	B
	S. enterica serovar *virchov*	C1
Negative	*S. sonnei*	
	S. flexneri	
	Enteropathogenic *E. coli* (strain E2348)	
	Enteroinvasive *E. coli* (strain EIC32)	
	E. coli K-12	

system that is capable of transporting chelated iron to allow growth of an enterobactin-deficient strain of *E. coli* and suggest that this system may perform an equivalent function in *S. typhimurium*.

The expression of the *sit* operon is regulated by iron concentration. Binding-protein-dependent transport systems are often expressed only under certain conditions, such as with a specific nutrient limitation or in the presence of an appropriate substrate (16, 38). The sequence similarity of the predicted Sit proteins with components of iron transport systems, coupled to the ability of the *sit* genes to allow the utilization of chelated iron by an enterobactin-deficient strain of *E. coli*, prompted us to examine the effect of iron on the expression of the *sit* operon. To monitor *sit* gene expression, a transcriptional fusion of *sitB* to a promoterless β-galactosidase reporter gene was constructed and the resulting gene fusion was integrated into the chromosome of wild-type *S. typhimurium* by homologous recombination, resulting in strain SB804 (see Materials and Methods). The expression of the *sit* operon under iron-limiting and nonlimiting conditions was then monitored by measuring the levels of the β-galactosidase reporter enzyme. The expression of the *sit* operon was induced 18-fold when strain SB804 was grown under iron-limiting conditions (Fig. 4). The induction of *sit* gene expression was prevented by the addition Fe^{2+} but not by the addition of Ca^{2+}. No induction was observed when strain SB804 was grown in Luria-Bertani medium, which is rich in iron. These results demonstrate that the expression of the *sit* operon is regulated at the transcriptional level by the iron concentration in the medium and further support the hypothesis that this operon encodes an iron transport system in *S. typhimurium*.

Effect of a *fur* mutation on the expression of the *sit* operon. The expression of iron transport systems is negatively regulated by the transcriptional repressor Fur (16). When bound to iron, this protein is capable of binding to a consensus operator sequence located between the −10 and −35 promoter elements of iron-responsive genes, thereby repressing their transcription (18). We therefore examined the sequence upstream of the predicted ATG start codon of *sitA* for the presence of putative promoter elements and a Fur consensus binding site. Using the neural network algorithm (52), we identified putative −10 and −35 promoter elements in the region immediately upstream of *sitA*, the first gene in the *sit* operon (data not shown). Further analysis identified a 19-nucleotide sequence

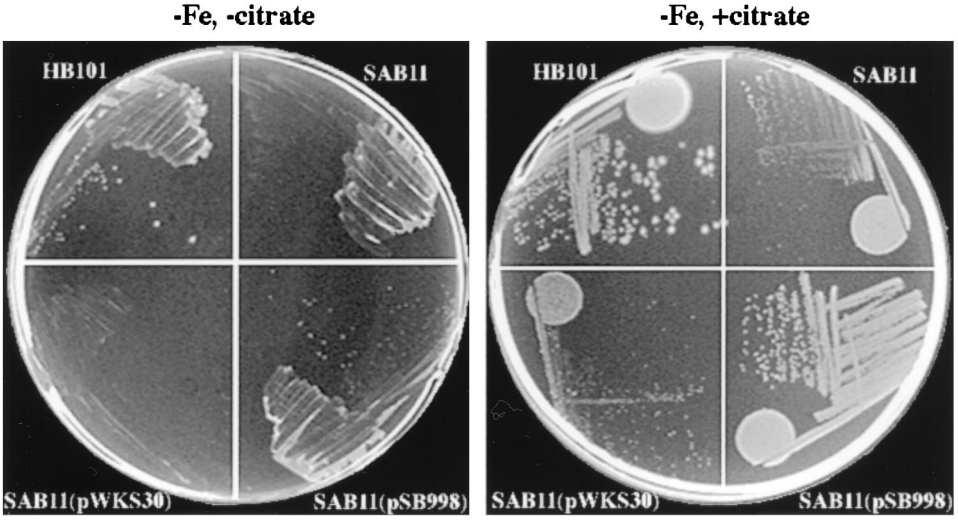

FIG. 3. Functional complementation of *E. coli* SAB11 by the *S. typhimurium sit* operon. The indicated strains were grown on Curtiss's minimal agar plates in the presence or absence of citrate. Growth plates after 3 days of incubation at 37°C are shown. The enterobactin-deficient *E. coli* SAB11 carried either no plasmid, pSB998 (which contains the *S. typhimurium sti* operon), or the plasmid vector pWKS30. The enterobactin-proficient HB101 strain was included as positive control.

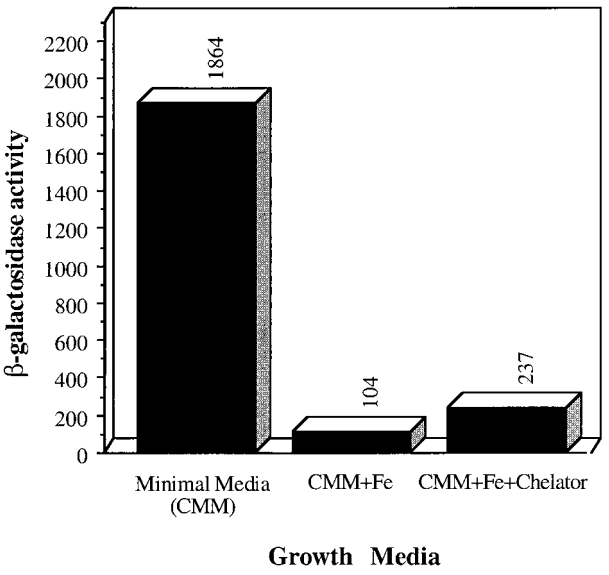

FIG. 4. Expression of the *sit* operon is regulated by iron concentration. The expression of the *sit* operon in bacteria grown under iron-restricted or iron-sufficient conditions as indicated was monitored. CMM, Curtiss's minimal medium. The iron chelator was 2,2′-dipyridyl (150 mM). β-Galactosidase activity is expressed in Miller units.

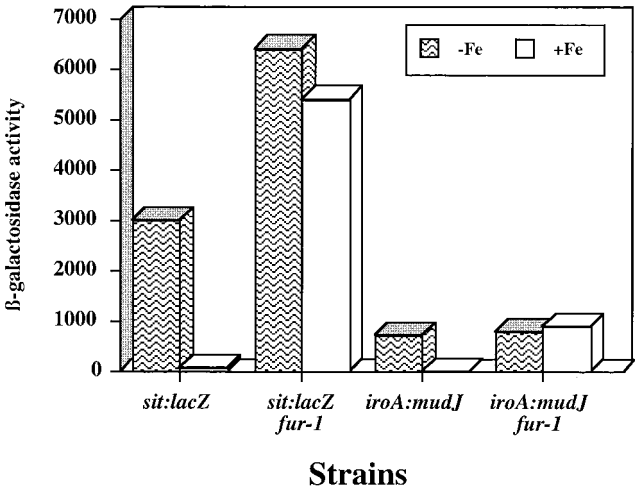

FIG. 6. Role of *fur* in expression of the *sit* operon. The influence of a *fur* mutation on the expression of the *sit* operon in bacteria grown under iron-restricted or iron-rich conditions as indicated in Materials and Methods was monitored. The *iroA* reporter gene (24) was used as a control for *fur*-regulated genes. The values are from one experiment and are equivalent to the results obtained in several repetitions of this experiment. β-Galactosidase activity is expressed in Miller units.

within the *sit* promoter region which resembles the Fur consensus binding site (Fig. 5).

We then examined the effect of *fur* on *sit* expression. For this purpose, a *sitB*::*lacZ* gene fusion was introduced into the chromosome of an *S. typhimurium fur* null mutant strain, resulting in strain SB835. The expression of the *sit* operon in strain SB835 was then tested under both iron-limiting and nonlimiting conditions. As shown in Fig. 6, a mutation in *fur* completely abolished the repression of the *sit* operon in the presence of iron. *iroA*, a previously described iron-regulated gene (24), showed similar derepression in the *fur* background strain. This result demonstrates that the iron-dependent repression of the *sit* operon is mediated by Fur.

Effect of *sit* mutations on phenotypes associated with the centisome 63 pathogenicity island. The association of an iron transport system with the centisome 63 pathogenicity island prompted us to investigate the role of this operon in the interaction of *Salmonella* with host cells. An *S. typhimurium* strain, SB801, carrying a deletion of the *sitB*, *sitC*, and *sitD* genes was constructed by allelic exchange as indicated in Materials and Methods. Strain SB801 was tested for its ability to enter cultured Henle-407 epithelial cells and for its ability to induce apoptosis in cultured macrophages. As shown in Fig. 7, the ability of the mutant strain to enter into Henle-407 cells and its toxic effect in macrophages were indistinguishable from those of the wild-type *S. typhimurium* strain SL1344. These results indicate that the iron transport system encoded by the *sit* operon is most likely not associated with these phenotypes. We then investigated the potential contribution of the *sit* operon to

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                1                    21
feoA      tgcTgATaga AACCATTcTC a
fhuA      tctTTATaAT AAtCATTcTC g
fbpA      .TAaacTGAT AAttATTtgC a
fepA      .TATTATGAT AACtATTtgC a
fur       cTATaATGAT AcgCATTaTC t
iucA      tgATaATGAg AAtCATTaTt g
sitA      catTTATcca tACCtTgtgC t
sodB      cTtTTgcGcc AcCgcTcaat t

Consensus -TATTATGAT AACCATT-TC -
          ||   |||| ||  |||| ||
de LORENZO'S -GATATTGAT AATCATTATC -
CONSENSUS
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FIG. 5. Location of a putative *fur* box in the −10 to −35 region upstream of *sitA*. Alignment with the *fur* box sequences was done with the GCG Pileup program. Uppercase letters indicate consensus residues. de Lorenzo's consensus sequence was previously published (18).

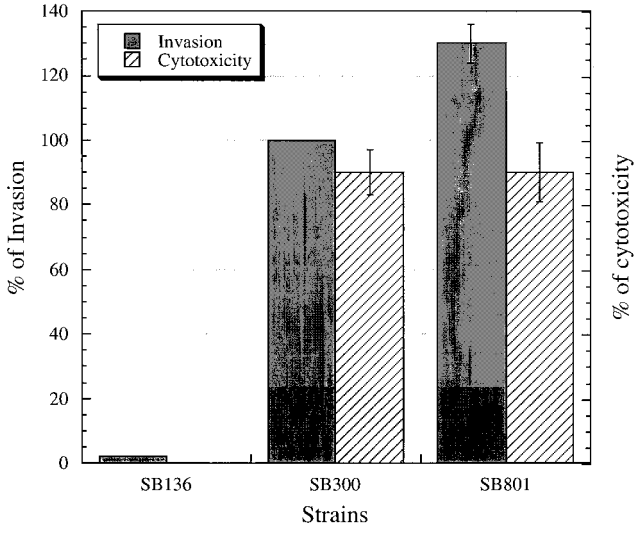

FIG. 7. Effect of *sit* on *S. typhimurium* entry into cultured epithelial cells and macrophage cytotoxicity. The internalization levels, measured by the gentamicin protection assay, were standardized by considering the levels for the wild-type *S. typhimurium* strain SL1344 to be 100% (the actual values in this case were 17% ± 0.8%). Macrophage J774 cytotoxicity is presented as the percentage of cells exhibiting cytotoxicity after 30 min of infection and was determined as described previously (13). Fewer than 1% of macrophages infected with *S. typhimurium* SB136 exhibited cytotoxicity.

S. typhimurium pathogenesis by examining the virulence of a *sitBCD* deletion mutant strain in a mouse model of infection. No difference in the virulences of the wild-type and Δ*sitBCD* strains was observed after oral infection of susceptible BALB/c mice (data not shown). These results are consistent with previous reports indicating that iron uptake systems are functionally redundant in *S. typhimurium* and that single mutations most often translate into either a weak or no virulence phenotype (10, 34, 40, 62).

DISCUSSION

We have described here an ABC transporter that is encoded within the centisome 63 pathogenicity island of *S. typhimurium*. The nucleotide composition of the 5 kb of DNA that comprises this locus (54% G+C) is significantly different from the nucleotide composition of the rest of the pathogenicity island (44% G+C), which encodes a type III secretion system. Our observation suggests that this pathogenicity island may be the result of independent events that allowed the acquisition of genetic material from different sources. The mosaic structure of this region is also supported by the unrelated functions encoded in these two loci. Our hybridization studies, although not exhaustive, clearly indicate that the *sit* locus is widely conserved among different *S. enterica* serotypes. This observation suggests that the acquisition of the *sit* region of the centisome 63 pathogenicity island must have occurred early in the evolution of *S. enterica*.

The ABC transporter encoded by the *sit* operon is most closely related to a *Y. pestis* iron transport system encoded by the *yfe* locus (9). Several ABC iron transporters have been described for gram-negative bacteria (38). Most of these systems are involved in the transport of a siderophore-iron complex across the inner membrane. Examples of these systems in *S. enterica* are the enterobactin and aerobactin iron uptake systems (21). However, more recently another family of ABC transport systems that mediate the transport of iron from the periplasm to the cytosol in a siderophore-independent manner has been recognized. These systems are thought to utilize several outer membrane proteins as iron receptors to capture iron from the medium into the periplasmic space. The *S. typhimurium sitABC* system is functionally more closely related to this family of iron transporters, which includes, in addition to the *Y. pestis yfeABC* system, the *Neisseria gonorrhoeae fbpABC, H. influenzae hitABC*, and *Serratia marcescens sfuABC* systems (1, 2, 4, 9, 12). The ability of the *S. typhimurium sit* operon to allow the utilization of chelated iron by an enterobactin-deficient strain of *E. coli* when grown in iron-deficient minimal medium supports this hypothesis.

The expression of iron uptake systems is stringently regulated by the concentration of iron in the growth medium (16). In most cases, control of gene expression is exerted through the function of the iron-sensitive transcriptional repressor protein Fur. Consistent with its involvement in iron uptake, the expression of the *sit* operon was strongly influenced by the levels of iron in the growth medium. When growth was under iron-limiting conditions, expression of the *sit* operon increased 18-fold. This induction of *sit* gene expression was readily prevented by the addition of Fe^{2+} to the growth medium but not by the addition of Ca^{2+}. Furthermore, repression by iron was completely abrogated by the introduction of a *fur* null mutation. These results demonstrate that the expression of the *sit* operon is regulated at the transcriptional level by the iron concentration in the medium, further supporting its involvement in iron uptake.

During the pathogenic cycle, *S. enterica* strains are thought to encounter iron-limited environments. It is therefore not surprising that these bacteria have evolved several iron uptake systems (8, 10, 15, 22, 46, 47, 50, 51, 53). Despite the expected importance of these systems for pathogenesis, the experimental demonstration of their involvement in *Salmonella* virulence has remained the subject of some controversy, as deficiency in any individual system has resulted in either a limited or no virulence phenotype (10, 34, 40, 62). Consistent with these results, the virulence of a strain carrying a deletion of the *sit* operon remained virtually indistinguishable from that of wild-type *S. typhimurium*. This is most likely a consequence of the redundancy of iron uptake systems in these bacteria rather than a reflection of the lack of importance of iron acquisition in *Salmonella* pathogenesis. Unambigous demonstration of the importance of iron in *Salmonella* virulence awaits the construction of a strain deficient in all described, and perhaps yet-to-be-discovered, iron uptake systems.

In summary, we have identified an ABC transporter encoded within the centisome 63 pathogenicity island of *S. typhimurium*. This transporter is most likely involved in iron uptake, since its expression is regulated by iron concentration and the Fur transcriptional repressor and it can confer the ability to grow in an iron-deficient minimal medium to an enterobactin-deficient strain of *E. coli*. Our results further support the existence of redundant iron uptake systems in *S. enterica* and provide evidence for a mosaic structure in the centisome 63 pathogenicity island.

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