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Identification of a new iron regulated locus of *Salmonella typhi*

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Abstract

In order to identify genes belonging to the Fur regulon of *Salmonella typhi* which are absent from *Escherichia coli* K-12, a plasmid gene bank consisting of 4000 independent clones was screened for Fur regulated promoters using the Fur titration assay (FURTA). DNA probes generated from FURTA positive plasmids were then used for hybridization with chromosomal DNA from *S. typhi*, *Salmonella typhimurium* and *E. coli*. Using these techniques we identified an iron regulated locus present in *S. typhi* and *S. typhimurium* but not in *E. coli*. Further cloning and nucleotide sequence analysis identified two open reading frames, termed *iroBC*, organized in a typical operon structure. The genes *iroBC* were located at 4 and 57 centisomes on the physical maps of *Salmonella typhi* and *S. typhimurium*, respectively. This region of the *S. typhimurium* chromosome contains a large DNA loop which is absent from the corresponding area of the *E. coli* chromosome. Finally, we developed a new method for generation of single copy transcriptional fusions. A suicide vector was constructed, which allows for the generation of chromosomal fusions to the promoterless *E. coli lacZYA* genes. By integration of this construct at the *iro* locus we could establish iron responsive expression of *iroBC*.

Keywords: Fur regulon; *Salmonella* loops; Pulsed field electrophoretic mapping; *Salmonella typhimurium*; *iro*; Transcriptional fusion; Suicide vector; β-Galactosidase

1. Introduction

Fur is a regulatory protein first described in *S. typhimurium*, which mediates gene regulation in response to iron availability (Ernst et al., 1978). The Fur regulon has been studied most extensively in *E. coli* where it contain some 36 genes (for a recent review see Braun and Hantke, 1991). Many of the Fur regulated genes found in *E. coli* have recently been shown also to be present in *S. typhimurium* (Tsolis et al., 1995). This is not surprising, since the *E. coli* and *Salmonella* lineages split only about 120–160 million years ago (Ochmann and Wilson, 1987). As a consequence, the gene order is highly conserved between these organisms and the divergence between homologous genes from *E. coli* K-12 and *S. typhimurium* ranges from 5% to 25% at the nucleotide level. However, both bacteria also contain Fur regulated genes which have no homologous counterpart in the other organism. For example, *S. typhimurium* possesses an outer membrane receptor for the siderophore ferrioxamine, which is not present in *E. coli* K-12 (Luckey et al., 1972; Reissbrodt and Rabsch, 1993). A *S. typhimurium* gene with homology to *foxA*, the outer membrane ferrioxamine receptor gene of *Yersinia enterocolitica* is a likely candidate for this function (Bäumler and Hantke, 1992; Tsolis et al., 1995). On the other hand, the genes *fecIR fecABCDE* which allow *E. coli* to utilize iron-citrate as an iron source are not present in *Salmonella* serotypes (Frost and Rosenberg, 1973; Staudenmaier et al., 1989). Direct comparison of the genetic maps of *E. coli* K-12 and *S. typhimurium* identified several long stretches of DNA, termed loops, which are present only in one species and most likely entered the genome by means of lateral transfer (Riley

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Abbreviations: ABC, ATP-binding cassette; ATCC, American type culture collection; ATP, adenosine triphosphate; bp, base pair(s); DNA, deoxyribonucleic acid; Fe(II), ferrous iron; Fur, ferric uptake regulator; FURTA, fur titration assay; kb, kilobase(s) or 1000 bp; SDS, sodium dodecyl sulfate.

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and Krawiec, 1987). Besides genes involved in iron utilization, *S. typhimurium*-specific loops also contain genes determining other metabolic pathways or surface antigens which are characteristic for *Salmonella* serotypes. For example, the large *S. typhimurium* loop spanning the region between 56 and 57 minutes (60 to 61 centisomes on the physical map), contains the genes for both citrate utilization, *tctABCD*, and the *hin fljAB* inversion system, which mediates flagellar antigen phase shifting (Riley and Krawiec, 1987). Characterization of genes present on *Salmonella* loops will improve our understanding of how horizontal gene transfer has contributed to the evolution of *Salmonella* serotypes.

During their separate evolution, *E. coli* and *Salmonella* serotypes obviously developed differences in their Fur regulons. In order to identify differences between the Fur regulons of *E. coli* and *S. typhi* we used a two step screen. In the first step, Fur regulated genes of *S. typhi* were identified in a genetic screen designated FURTA, for Fur titration assay (Stojiljkovic et al., 1994). In the second step, Fur regulated genes not present in *E. coli* K-12 were identified by hybridization. Using this approach we identified *iroBC*, an iron regulated operon of *S. typhi* which maps to the *Salmonella* loop containing *tct* and *hin fljB*.

2. Results and Discussion

2.1. Identification of FURTA positive plasmids from a S.typhi gene bank

Fur is a protein which regulates gene expression in response to changes in iron availability (Ernst et al., 1978; Hantke, 1981, 1984; Schäffer et al., 1985). If the intracellular iron concentration is high, Fur binds ferrous iron as a corepressor. The Fe(II)-Fur repressor complex recognizes a regulatory DNA sequence, designated Fur-box, which is located in the promoter region of Fur regulated genes (deLorenzo et al., 1987). In the absence of intracellular Fe(II), the Fur apoprotein fails to bind Fur-boxes, thus allowing expression of Fur repressed genes. These features of gene regulation by Fur form the basis for the FURTA, a genetic screen designed to identify Fur regulated genes or iron-binding proteins of Gram-negative bacteria (Stojiljkovic et al., 1994). In this assay, a plasmid is introduced into an *E. coli* strain (H1717, Table 1), which carries a Fur-regulated *lacZ* fusion (*fhuF*::*lacZ*) sensitive to changes in Fe(II)-Fur repressor concentration (Hantke, 1987). If a plasmid carries a Fur-box, introduction of the plasmid will result in competition between plasmid encoded and chromosomal Fur-boxes for the Fe(II)-Fur repressor. The plasmid encoded Fur-boxes are present in high copy number and will titrate the Fe(II)-Fur complex, thereby allowing expression of the reporter gene.

Chromosomal DNA of *S. typhi* AJB70 was digested with the restriction enzymes *Alu*I, *Dpn*I, *Hae*III, or *Rsa*I. Fragments between 0.5 and 2.5 kb were cloned into the vector pSUKS1 digested with *Eco*RV, and transformed into *E. coli* DH5α. A total of ca. 4000 independent colonies were pooled into four groups and screened by the FURTA. Assuming an average insert size of about 1.5 kb, the amount of DNA contained in this bank would exceed the size of the *S. typhi* genome (Liu and Sanderson, 1995). Initially, a total of 18 colonies were FURTA positive (red colonies on MacConkey agar plates containing 0.04 mM $FeSO_4$) and were further analyzed.

2.2. Hybridizational and sequence analysis of FURTA positive plasmids

To determine whether the DNA regions identified by the FURTA are also present in *S. typhimurium* and *E. coli*, hybridization of probes derived from inserts of FURTA positive clones with *Eco*RI-restricted chromosomal DNA was performed. Under the conditions used, a hybridization signal should be obtained if a DNA fragment showed more than 65% nucleotide sequence identity with a probe. As a control, a DNA probe containing the promoter region and the 5′-ends of *fes* and *fepA* of *S. typhimurium* (pFT17) and a DNA probe containing the 5′-ends of *p14* and *fepD* of *S. typhimurium* (pFT324) (Tsolis et al., 1995) were used for hybridization with chromosomal DNA of *S. typhi*, *S. typhimurium*, and *E. coli*. All probes except two hybridized with DNA from *E. coli* and both *Salmonella* serotypes. Probes derived from plasmids pTY1 and pTY7 hybridized only to DNA from *S. typhi* AJB70 and *S. typhimurium* ATCC14028 but not with DNA from *E. coli* K-12 (Fig. 1). The nucleotide sequence of these two FURTA positive plasmids was determined. Although the inserts of the two FURTA positive plasmids, pFT1 and pFT7, were different in size, both contained the promoter region and the 5′-end of an ORF, termed *iroB* . A potential Fur-binding site matching the consensus sequence (Braun and Hantke, 1991) in 15 of 18 bases was located upstream of *iroB* (Fig. 2). The fact that we identified only 18 FURTA positive plasmids, some of which may carry the same promoter or encode an iron-binding protein (rather than carry a Fur-box) indicated that our bank may not have been fully representative. However, we identified *iroB*, a gene also present in *S. typhimurium*, but which was not identified in a recent, more comprehensive study of the Fur regulon of this organism (Tsolis et al., 1995).

2.3. Cloning of cosmids containing iroB from a S.typhi gene bank

The region of the *S. typhi* chromosome encoding *iroB* was cloned from a cosmid bank constructed in pLAFRII

Table 1
Bacterial strains

Strain	Genotype	Source/Reference
Escherichia coli		
DH5α	*endA1hsdR17(rk−mk−) supE44t hi-1 recA1 gyrA relA1 Δ(lacZYA-argF) U169 deoR (ϕ80 dlac Δ(lacZ)M15)*	Lab collection
H1717	*araD139 Δ(argF-lac)U169 ralA1 rpsLflbB deoC ptsF rbsR fhuF*::λplacMu53	Hantke, 1987
S17-1 λ*pir*	*Prp thi recA hsdR*; chromosomal RP4-2(Tn*1*::ISR*1* *tet*::Mu Km::Tn7); λ*pir*	Miller and Mekalanos, 1988
Salmonella typhimurium		
14028	wild type strain (isolated from bovine septicemia)	ATCC
IR715	14028 Nal^r	Stojiljkovic et al., 1995
AJB27	IR715 *iroC*::*lacZYA*	This study
AJB20	IR715 *iroBC*::Km^r	This study
Salmonella typhi		
AJB70	wild type strain (clinical isolate)	Bäumler and Heffron, 1995
TY21a	*galE viaB rpoS*	Germanier and Fürer, 1975; Robbe-Saule et al., 1995
AJB22	TY21a Nal^r	This study
PTY011	AJB22 *iroB*::pTY1-1	This study
AJB58	AJB22 *iroC*::*lacZYA*	This study

and propagated in *E. coli* DH5α. The cosmid bank was spread on plates. From these, 600 colonies were picked and grown up individually overnight. Cosmid DNA was prepared from 30 pools, each containing 20 over night cultures, digested with *Eco*RI, separated on an agarose gel and hybridized with probe pTY1. The DNA of two pools hybridized with probe pTY1. The 40 cosmids included in these two pools were then isolated individually, digested with *Pst*I and the hybridization was repeated with probe pTY1. Each pool contained one cosmid, termed pTY908 and pTY2117, respectively, which gave a hybridization signal corresponding to a 4-kb *Pst*I DNA fragment (data not shown). Several subclones of these cosmids were created by cloning *Eco*RI, *Pst*I, or *Cla*I restriction fragments into pBluescript SK+ (Fig. 3).

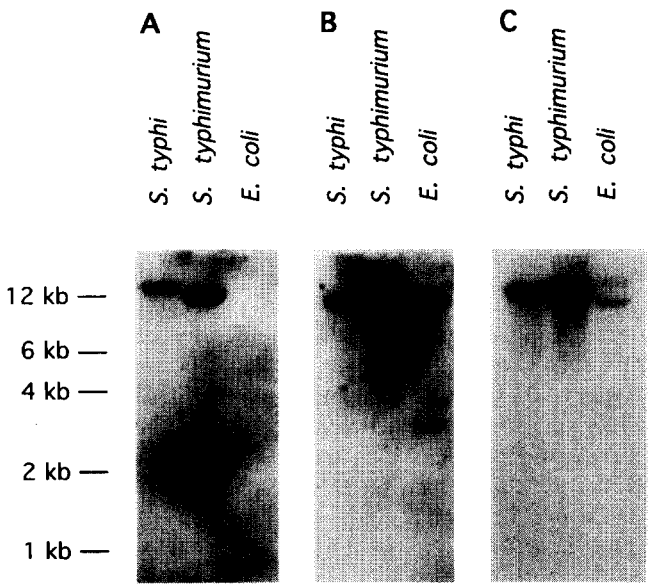

Fig. 1. Southern hybridization of *Eco*RI restricted chromosomal DNA of *S. typhi*, *S. typhimurium*, and *E. coli* with DNA probes specific to *iroB* (**A**), *fes fepA* (**B**), or *p14 fepD* (**C**). Positions and sizes of bands from standard DNA are indicated. Labeling of DNA probes, hybridization, and immunological detection were performed using the DNA labeling and detection kit (non-radioactive) from Boehringer-Mannheim. Hybridization to the blot was performed at 60°C in solutions without formamide. Subsequently, two 15-min washes were performed under nonstringent conditions at room temperature in 2×SSC, 0.1% SDS.

2.4. *Sequence analysis of* iroBC

The nucleotide sequence of a 6000-bp DNA region was determined and deposited under accession No. U62129 at GenBank. The region contained two complete open reading frames, designated *iroBC*, respectively. The nucleotide sequences were compared to SWISS-PROT, PIR(R), and GenPept at the National Center for Biotechnology Information (NCBI) using the program blastX, and to GenBank and EMBL using the program blastN (Altschul et al., 1990). IroB is homologous to RhlB (23% identity and 53% overall similarity), a rhamnosyl-transferase of *Pseudomonas aeruginosa* (Ochsner et al., 1994). IroC shares homology with a large number of proteins belonging to the family of ATP-binding cassette (ABC) transporters (Fath and Kolter, 1993). Typical ATP-binding motifs (A site: 377-GSGKST-382; B site: 502-LLILD-506) are present in the IroC primary

Fur box consensus sequence:		GATAATGAT A ATCATTATC	
iroB	1,004	GATAtTGgT A ATtATTATC	1,022

Fig. 2. Fur-binding site (Fur-box) located in the *iroB* promoter region. The consensus sequence of the *E. coli* Fur-box is given above (Braun and Hantke, 1991). Numbers indicate the position of the Fur-box in the nucleotide sequence deposited at GenBank (U62129). Sequencing was performed using the ALF automated sequencer (Pharmacia).

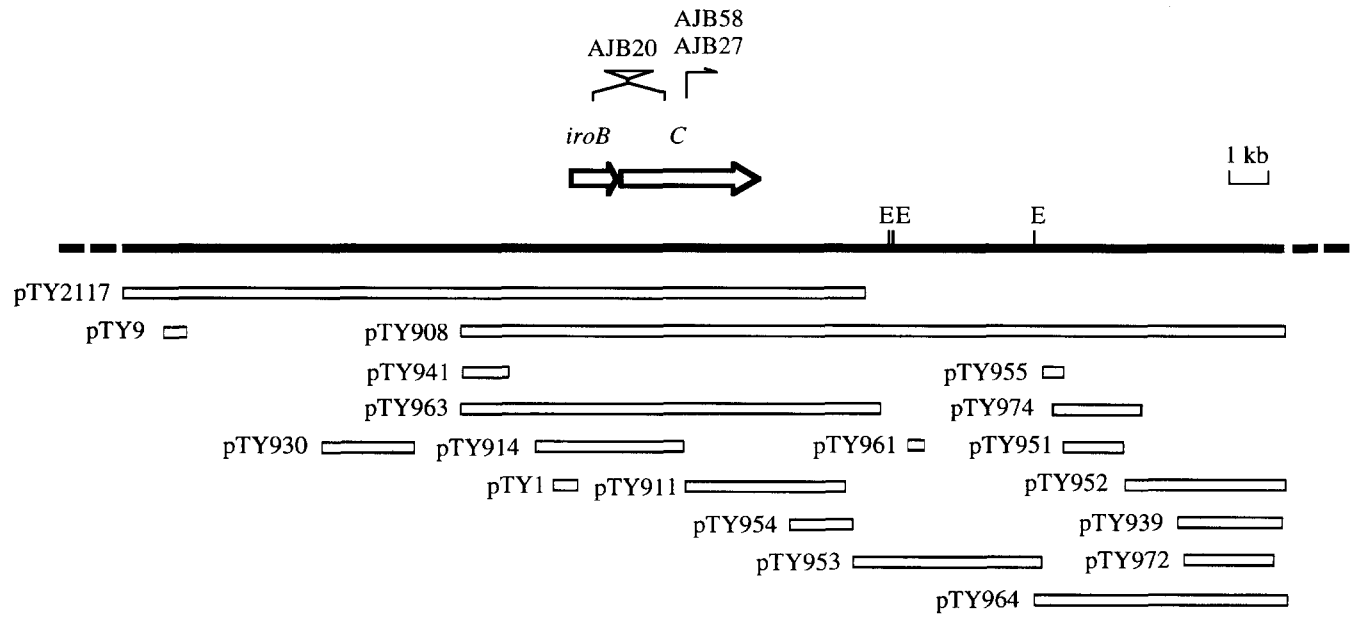

Fig. 3. Restriction map of a DNA region located at about 4 centisomes on the *S. typhi* chromosome. Positions and sizes of inserts carried in cosmids (pTY908, pTY2117) or plasmids are given below. Arrows above the map indicate positions and orientation of open reading frames identified by sequence analysis. The location of the fusion between *iroBC* and *lacZYA* in mutants AJB58 and AJB27 is indicated. The position of the deletion in mutant AJB20 is indicated. E, *Eco*RI.

structure (Walker et al., 1982). The strongest homology to IroC is found with the eukaryotic multidrug resistance P-glycoprotein encoded by atpgp1 of *Arabidopsis thaliana* (30% identity) (Dudler and Hertig, 1992). This homology suggest that IroC may also function as an ABC Exporter. Homologies to ABC exporters or glycosyl-transferases have so far not been described for any known proteins involved in iron utilization.

2.5. *Mapping of* iroB *in* S. typhi *and* S. typhimurium

Pulsed Field Gel Electrophoresis was used to determine the location of *iroB* on the *S. typhi* and the *S. typhimurium* chromosomes. Macrorestriction maps of the *S. typhi* and the *S. typhimurium* genomes with the enzymes *Bln*I and *Xba*I have been published recently (Liu and Sanderson, 1992, 1995; Wong and McClelland, 1992). In order to map *iroB* on the *S. typhi* chromosome we cloned the insert of pTY1 into the suicide vector pMAP (Tsolis et al., 1995), which contains *Xba*I and *Bln*I restriction sites for physical mapping and can be propagated in the *E. coli* host S17-1λpir. The resulting construct, pTY1-1, was conjugated into *S. typhi* AJB22. This vector will integrate into the chromosome by homologous recombination between its internal *iroB* fragment and the chromosomal *iroB* allele, thereby introducing new *Xba*I and *Bln*I restriction sites into the genome. After a restriction digest of chromosomal DNA from a *iroB*::pMAP1 mutant (PTY011), the fragment in which the suicide vector is inserted will therefore disappear while two new, smaller fragments can be detected after separation with pulsed field gel electrophoresis and hybridization with a pTY1 derived DNA probe (Table 2). Using these techniques, *iroB* was mapped at 4 centisomes on the *S. typhi* chromosome and between 60.5 and 62 centisomes on the *S. typhimurium* physical map. These are corresponding genomic regions and the different map positions are due to chromosomal inversions which occurred after the lineages of *S. typhi* and *S. typhimurium* split (Liu and Sanderson, 1995). During a comparison with the genetic map of *E. coli* K-12 and *S. typhimurium*, a large chromosomal region only present in *S. typhimurium* was found between 56 and 57 minutes on the genetic map (Riley and Krawiec, 1987), which corresponds to 60 to 61 centisomes on the physical map (Sanderson et al., 1995). Both mapping and hybridizational analysis indicated that *iroB* may be located in this DNA region, close to the *tct* operon. Recently, Foster and Hall identified Fur regulated mud*J* insertions which mapped close to *tct* in *S. typhimurium* and the corresponding locus was designated *iroA* (Foster and Hall, 1992; Foster et al., 1994; Sanderson et al., 1995). Based on the similar map position it is likely that the genes *iroBC* are part of the *iroA* locus. However, since no sequence data are available, it is unclear whether the transposon insertions identified by Foster and coworkers were actually located in *iroBC* or in other genes positioned at this area of the chromosome.

2.6. *Iron responsiveness of the* iroB *promoter*

To generate transcriptional fusions to a reporter gene, a suicide vector carrying the promoterless *lacZYA* genes

Table 2
Pulsed field gel electrophoretic mapping of the *iro* locus

Organism	Size of restriction fragment hybridizing with *sil*-specific DNA probe in kb (designation of fragment[a,c])			Calculated map position in centisomes (kb)
	*Xba*I	*Bln*I	*Xba*I-*Bln*I	
S. typhimurium ATCC14028	675 (C)	190 (F1)	110	60.5 – 62[b] (2900 – 2975)
*S. typhi*AJB22	323 (A)	500 (C)	110	4[c]
S. typhi PTY011	290 + 29	440 + 62	62 + 29	(200)

Preparation of agarose-embedded chromosomal DNA for pulsed field gel electrophoresis was performed as previously described (Liu and Sanderson, 1992; Bäumler et al., 1994) according to [a]Liu and Sanderson (1992)), [b]Sanderson et al. (1995) and [c]Liu and Sanderson (1995).

was constructed. For this purpose, a 5-kb *Bam*HI-*Stu*I fragment of plasmid pRS528 (Simons et al., 1987) containing *lacZYA* from *E. coli* was cloned into *Bam*HI-*Eco*RV-restricted pSUSK1 (Ondraczek et al., 1992) to give rise to pSULac. The *Xba*I-*Sal*I-restricted insert of pSULac was cloned into *Xba*I-*Sal*I-restricted suicide plasmid pEP185.2 (Kinder et al., 1993) to give rise to pFUSE (Fig. 4). We then introduced the *Xba*I-*Eco*RV-restricted insert of pTY914 into *Sma*I-*Xba*I-restricted pFUSE to give rise to pTY949. This suicide vector was conjugated into *S. typhi* (AJB22) and *S. typhimurium* (IR715) and exconjugants carrying pTY949 inserted into their chromosome were selected. The insert of pTY949 allows integration of this suicide plasmid into the *Salmonella* chromosome at the *iro* locus by homologous recombination, thereby creating a transcriptional fusion between *iroBC* and *lacZYA*. The resulting *S. typhi* and *S. typhimurium* strains carrying *iroBC*::*lacZYA* transcriptional fusions were designated AJB58 and AJB27, respectively (Fig. 3), and the insertion of pTY949 was confirmed by Southern hybridization. In these strains, expression of β-galactosidase was quantified under iron-deficient and iron-sufficient growth conditions (Table 3). In both *Salmonella* serotypes, expression of the reporter gene was strongly increased during growth under iron-limiting conditions. These data thus confirmed iron regulated expression of *iroB*, which was indicated by the presence of a typical Fur DNA-binding site upstream of *iroB* (Fig. 2) and by detection of pTY1 and pTY7 in the FURTA. Under iron limitation, the *iroBC*::*lacZYA* fusions were expressed at similar levels as the *S. typhimurium iroA*::mud*J* fusions described by Foster et al., 1994.

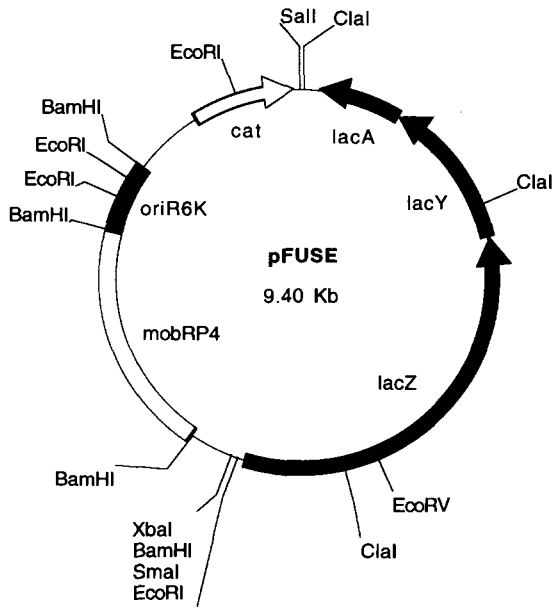

Fig. 4. Restriction map of the suicide vector pFUSE. The promoterless *lacZYA* genes from plasmid pRS528 (Simons et al., 1987) were cloned into *Xba*I-*Sal*I restricted pEP185.2 (Kinder et al., 1993) to give rise to pFUSE. This low copy number vector allows for simple construction of transcriptional fusions by directional cloning of part of a gene into the unique *Sma*I and *Xba*I sites of the vector and subsequent integration of the resulting construct into the chromosome by homologous recombination.

2.7. Mutational analysis

For mutational analysis of *iroBC*, a 1.3-kb deletion was created in pTY914 by digestion with *Bgl*II and a 1.6-kb *Bam*HI DNA fragment containing a kanamycin resistance cassette (KIXX, Pharmacia) was introduced to give rise to pTY1000. The *Xba*I-*Kpn*I-restricted insert of pTY1000 was isolated and introduced into the suicide vector pEP185.2 (Kinder et al., 1993) to give rise to pTY1001. Plasmid pTY1001 was conjugated into *S.*

Table 3
Expression of a *iroBC*::*lacZYA* fusion under iron-rich and iron-restricted growth conditions

Organism	Strain	β-Galactosidase units	
		+Fe[a]	–Fe[b]
S. typhi	AJB58	4.9	218
S. typhimurium	AJB27	22.3	446

In order to create iron-limiting[b] or iron-sufficient[a] growth conditions, 0.2 mM 2,2′dipyridyl or 0.04 mM $FeSO_4$ were added, respectively. The quantitative determination of β-galactosidase units has been published elsewhere (Miller, 1972).

typhimurium strain IR715 and a chloramphenicol sensitive (vector) kanamycin resistant exconjugant isolated (AJB20). Inactivation of *iroBC* in AJB20 was confirmed by Southern hybridization. The *S. typhimurium* mutant AJB20 had the same LD_{50} (Reed and Muench, 1938) as its parent IR715 (6×10^5) after oral infection of mice. Thus, these data suggest that the genes *iroBC* are not necessary for iron utilization during systemic infection of mice.

3. Conclusions

(1) In this report we identified and characterized the regulation of two genes, *iroBC*, which are present in *S. typhimurium* and *S. typhi* but are absent from the *E. coli* K-12 genome. Both the map position of *iroB* (between 60 and 61 centisomes on the *S. typhimurium* physical map), and hybridization analysis indicated that this operon is located on the same *Salmonella* loop as *tct* and *hin fljB*. Iron responsiveness of the *iroBC* promoter and the map position suggest that these genes may be part of the *iroA* locus described in *S. typhimurium*.

(2) We describe a new suicide vector, designated pFUSE, which allows for simple construction of transcriptional fusions to a reporter gene. Using this vector, we were able to confirm iron responsiveness of the promoter of the *iroBC* operon.

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