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Contribution of TonB- and Feo-Mediated Iron Uptake to Growth of *Salmonella typhimurium* in the Mouse

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We examined the role of iron(II) and iron(III) uptake, mediated by FeoB and TonB, respectively, in infection of the mouse by *Salmonella typhimurium*. The *S. typhimurium feoB* gene, encoding a homolog of an *Escherichia coli* cytoplasmic membrane iron(II) permease, was cloned, and a mutant was generated by allelic exchange. In addition, an *S. typhimurium tonB* mutant was constructed. Together these two mutations inactivate all known iron uptake systems of *S. typhimurium*. We examined the abilities of these mutants to grow in vitro and in different compartments of the host. Mutants in *feoB* were outcompeted by the wild type during mixed colonization of the mouse intestine, but the *feoB* mutation did not attenuate *S. typhimurium* for oral or intraperitoneal infection of mice. The *tonB* mutation attenuated *S. typhimurium* for infection of mice by the intragastric route but not the intraperitoneal route, and the mutant was recovered in lower numbers from the Peyer's patches and mesenteric lymph nodes than the wild type. These results indicate that TonB-mediated iron uptake contributes to colonization of the Peyer's patches and mesenteric lymph nodes but not the liver and spleen of the mouse. The *tonB feoB* double mutant, given intraperitoneally, was able to infect the liver and spleen at wild-type doses, indicating that additional iron acquisition systems are used during growth at systemic sites of infection.

Pathogenic bacteria are able to grow in various niches in the host. These niches differ in the types of available sources of iron, an essential nutrient for bacterial growth. For example, under anaerobic conditions found in the intestine, soluble iron(II) may be available for bacterial growth. An additional iron source is provided by host compounds such as heme originating from dead epithelial cells which are constantly shed from the tips of villi. Once bacteria manage to penetrate the intestinal barrier, they face components of the host's iron-withholding defense (37). In the blood and extracellular fluid, iron is withheld from invading microorganisms by the high-affinity iron(III)-binding proteins lactoferrin and transferrin. In response to infection, the host reduces the serum iron to about 30% of its normal levels, making it even harder for microbes to obtain this nutrient (37). Some pathogens are able to grow in an intracellular location, where they again encounter dramatically different conditions. It is not known what iron sources are available to bacteria growing intracellularly.

How do bacteria obtain iron under all of these different conditions? Because it passes through all of these compartments during murine typhoid, *Salmonella typhimurium* is a good model with which to address this question. Several genes involved in iron uptake, such as genes encoding functions involved in biosynthesis and uptake of the siderophore enterobactin, have been identified in *S. typhimurium* (36). In addition to enterobactin, *S. typhimurium* is able to utilize a variety of other siderophores which it does not produce, including ferrioxamine and ferrichrome (26). The outer membrane receptor genes for these siderophores, *foxA* and *sidK*, have been identified recently (36). The receptor for enterobactin, as well as the other *S. typhimurium* siderophore receptors, belongs to a family of proteins whose transport activity depends on the function of TonB (2, 18, 32). These TonB-dependent uptake systems have in common that they mediate uptake of iron(III) complexes. Recently, a new type of iron uptake system has been found in *Escherichia coli*, a close relative of *S. typhimurium*. This system, encoded by the *feoAB* genes, pumps iron(II) through the cytoplasmic membrane (22). Since iron(II) is readily soluble and can enter the periplasmic space by diffusion through porins, specialized outer membrane receptors are not necessary for its uptake.

To assess the roles of these iron uptake systems, we constructed *S. typhimurium* strains carrying mutations in *tonB* and *feoB*. The effects of these mutations on bacterial growth were tested, singly and in combination, by using in vitro assays and the murine model of typhoid fever.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The strains used are listed in Table 1. Plasmid pIRS618 contains a 3.5-kb *Sac*I-*Eco*RI fragment carrying the *tonB* gene of *S. typhimurium* ATCC 14028 as determined by sequence analysis. It was obtained during an attempt to clone *S. typhimurium feoB* by complementation of *E. coli* H5128 (*aroB tonB feoB*) for growth on nutrient broth (NB) agar (22), using the vector pSUKS1(29). Bacteria were cultured aerobically at 37°C. Media used were Luria-Bertani broth (LB) (27) and NB (8 g of Nutrient Broth powder [Difco] and 5 g of NaCl per liter). Solid media contained 15 g of agar per liter. Antibiotics, when required, were included in the culture medium or plates at the following concentrations: tetracycline, 10 mg/liter; kanamycin, 100 mg/liter; chloramphenicol, 20 mg/liter; and carbenicillin, 100 mg/liter. To create iron-limiting growth conditions, 0.2 mM 2,2′-dipyridyl was added to solid or liquid media. Growth in liquid media was quantified by measuring the optical density of cultures at 578 nm. Bacterial cultures used to infect mice were grown for 16 h with aeration in LB.

Utilization of ferrioxamine was detected by an agar diffusion assay (2). The strain to be tested was poured in 3 ml of 2% Noble agar onto an NB-dipyridyl (NBD) agar plate. Ferrioxamine solution was prepared by dissolving Desferal (Ciba-Geigy) in distilled water at a concentration of 1 mg/ml and adding 20 μl of $FeCl_3$ (1 mg/ml) per ml of Desferal solution. Filter paper disks impregnated with ferrioxamine (3 μl of 1-mg/ml ferrioxamine solution) were laid onto the top agar,

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Source or reference
S. typhimurium		
ATCC 14028	Wild type	ATCC
IR715	Nalidixic acid resistant Derivative of ATCC 14028	33
AR1258	14028 *entB*::Mud*J*	36
AIR15	IR715, *feoB*::*tet*	This study
AIR17	AR1258, *feoB*::*tet*	This study
AIR36	IR715, *tonB*::*km*	This study
AIR62	AIR15, *tonB*::*km*	This study
CL1509	14028 *aroA*::Tn*10*	10
AIR51	AIR36, *aroA*::Tn*10*	This study
AJB29	*fhuB*::Mud*J*, *aroA*::Tn*10*	36
S. typhi AJB70	Clinical isolate	4
E. coli		
S17-1 λ*pir*	*prp thi recA hsdR*; chromosomal RP4-2 (Tn*1*::ISR*1 tet*::Mu Km::Tn*7*); λ*pir*	31
DH5α	*endA1hsdR17* ($r_K^- m_K^-$) *supE44t hi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*) *U169 deoR* [φ80 *dlac* Δ(*lacZ*)*M15*]	16
CC118	*araD139* Δ(*ara, leu*) Δ*lacY74 phoA20 galE galK thi rpsE rpoB argF*(Am)	R. Taylor
H5128	*recA1 aroB tsx malT tonB feoB*::Tn*5*	K. Hantke (22)
Plasmids		
pEP185.2	pGP704, pBluescript MCS, *cat*	J. Pepe (23)
pBluescript KS	ColE1, *bla*	Stratagene
pUH18	pT7-6, *E. coli feoB*	K. Hantke (22)
pIRS618	pSUKS1, *S. typhimurium tonB*	This study

and after incubation overnight at 37°C, the zone growth around the filter disk was measured.

Genetic techniques. P22 HT*int* was used for generalized transduction of transposon insertions into a different genetic background. Transductants were routinely streaked on Evans blue-uranine plates to detect phage contamination before use in further experiments (7). Quantification of β-galactosidase activity was performed as described elsewhere (27).

Recombinant DNA techniques. Plasmid DNA was isolated by using ion-exchange columns from Qiagen. Standard methods were used for restriction endonuclease analyses, ligation, and transformation of plasmid DNA (27). Sequencing was performed with an ALF automated sequencer (Pharmacia).

Construction of an *S. typhimurium feoB* mutant by allelic exchange. A fragment of *Salmonella typhi feoB* gene was amplified by using the primers 5′-TCC GAATTCTGGCAAGACAACG-3′ and 5′-CCGAATTCAGCGCCTGCATCA GACG-3′ (22). Primers used for amplification of an 895-bp fragment of *feoB* from *S. typhimurium* were 5′-TCACCGTCGAGCGTAAAGAGG-3′ and 5′-G CCAATCCATTGAATGCCATG-3′. This product was cloned into pBluescriptKS (Stratagene). To create a construct for allelic exchange, the 2-kb pAK1900 tetracycline resistance cassette (29a) was introduced into an internal *Hin*dIII restriction site, at the position of the *S. typhimurium feoB* gene corresponding to nucleotide 979 of the *E. coli feo* sequence (22). The fragment of the *S. typhimurium feoB* gene containing the tetracycline resistance gene was excised from pBluescript by using the enzymes *Xba*I and *Kpn*I and inserted into the suicide vector pEP185.2 (23). The resulting construct was propagated in the host strain S17-1λ*pir* and introduced into *S. typhimurium* IR715 (Nal^r) by conjugation. Exconjugants were plated on agar containing nalidixic acid and tetracycline to select for recipients of the suicide vector. These were then restreaked on plates containing chloramphenicol to test for loss of the suicide vector and stable integration of the Tet cassette into the *feoB* gene. Recombinants resistant to tetracycline but sensitive to chloramphenicol originate from allelic exchange between the chromosomal *feoB* and the mutated copy carried on pEP185.2.

Construction of an *S. typhimurium tonB* mutant by allelic exchange. Two fragments of the *S. typhimurium tonB* gene were amplified by PCR using primers designed from the published sequence (19): 5′-GGAATTCGGTGAGCGTGC CCACTG-3′, 5′-GGTCGACCTAAACCTCGCCCGCTCCC-3′, 5′-CGGGATC CCGACGATTATGACTTCAATGACCCTTG-3′, and 5′-GGAATTCCATGC GGTGCTGCTTGTTGG-3′. Restriction sites for *Eco*RI, *Sal*I, and *Bam*HI were incorporated into the primers for subsequent cloning steps. The 5′ fragment, containing bp 105 to 547 of *S. typhimurium tonB*, was flanked by sites for *Bam*HI and *Eco*RI, and the 3′ fragment, containing bp 566 to 939, was flanked by *Eco*RI and *Sal*I sites. These two fragments were digested with the enzymes *Eco*RI, *Sal*I, and *Bam*HI and cloned together into *Bam*HI-*Sal*I-digested pBluescript KS, to yield pTB1. Thus, pTB1 contains a fragment of *tonB* with an *Eco*RI site introduced into an internal deletion from bp 547 to 566. The correct orientation of these fragments was confirmed by sequence analysis (data not shown). The 1.3-kb kanamycin resistance cassette KSAC (Pharmacia) was introduced into this internal *Eco*RI site. The entire insert containing KSAC cassette flanked by *tonB* sequence was excised by using the enzymes *Xba*I and *Kpn*I and inserted into the suicide vector pEP185.2 (23) to yield pTB3. This construct was propagated in the host strain S17-1λ*pir* and introduced into *S. typhimurium* IR715 by conjugation. Exconjugants were selected on plates containing nalidixic acid and kanamycin and then restreaked on plates containing chloramphenicol to test for loss of the suicide vector. Recombinants resistant to kanamycin but sensitive to chloramphenicol originate from allelic exchange between the chromosomal *feoB* and the mutated copy on pTB3.

Southern hybridization. Southern transfer of DNA onto a nylon membrane was performed as previously described (1). Labeling of DNA probes, hybridization, and immunological detection were performed with a Renaissance DNA labeling and detection kit (nonradioactive) from New England Nuclear.

Tissue culture. HEp-2 cells were obtained from the American Type Culture Collection and maintained in Eagle's minimal essential medium with Earle's balanced salt solution (EMEM; Bio Whitaker), 1 mM L-glutamine, and 10% fetal calf serum. Intracellular growth was assayed essentially as described by Leung and Finlay (25). Briefly, 10 μl of a standing overnight culture was added to each well of a 24-well microtiter plate containing a monolayer of HEp-2 cells. Bacteria were allowed to invade for 1 h, and then the wells were rinsed five times with 1 ml of phosphate-buffered saline (PBS). Cells were overlaid with fresh medium containing 12 μg of gentamicin per ml to kill extracellular bacteria. At this point (t = 0 h) and 18 h, cells were lysed in 0.5% sodium deoxycholate and plated to determine the number of intracellular bacteria. The fold growth was determined as the ratio of bacterial numbers per well at 18 h to the number present at 0 h. Throughout this assay, bacteria are iron stressed, as indicated by induction of an iron-regulated fusion to *lacZ* after overnight growth in LB and after transfer into EMEM (data not shown). Furthermore, since EMEM used to culture the cells does not contain iron salts, the HEp-2 cells are continually cultured in low-iron medium.

Mouse experiments. (i) LD_{50} and in vivo stability of mutations. Female BALB/c ByJ mice (6 to 8 weeks old) obtained from Jackson Laboratories (Bar Harbor, Maine) and housed under specific-pathogen-free conditions were used for all experiments. Bacteria were grown aerobically for 16 h in LB prior to infection. Bacteria grown under these conditions are iron starved, as indicated by induction of an iron-repressible transcriptional fusion to *lacZ* in *S. typhimurium* (data not shown). Virulence of mutants was tested by the 50% lethal dose (LD_{50}) assay. A volume of 0.2 ml of each of serial 10-fold dilutions of cultures in LB (ranging from 10^9 to 10^4 CFU/ml) was administered intragastrically (i.g.) to groups of four mice. Dilution of bacteria in physiological saline, which is comparatively iron poor, prior to i.g. infection had no effect on the ability of mutants to kill mice compared with the wild type. For intraperitoneal (i.p.) infection, dilutions ranging from 10 to 1,000 CFU/ml were made in PBS, and 0.1 ml was injected. Mortality was recorded at 28 days postinfection, and the LD_{50} for each route of infection was calculated by the method of Reed and Muench (30).

Stability of mutations or plasmids in vivo was assessed by determining colony counts in Peyer's patches, mesenteric lymph nodes, livers, and spleens of moribund infected mice. Liver and spleen samples were each homogenized in 1 ml of PBS, using a Stomacher (Tekmar, Cincinnati, Ohio), and 0.1 ml of diluted homogenate was plated on agar containing appropriate antibiotics as well as plates without antibiotics. Viable counts on the plates were compared to determine stability of the mutations tested. Plasmid stability was tested by plating in duplicate on agar with and without the antibiotic used to maintain the plasmid.

(ii) Determination of bacterial counts in organs. Groups of 10 mice were infected i.g. with a 1:1 mixture of wild-type and mutant *S. typhimurium* containing approximately 10^7 bacteria. At 5 days postinfection, mice were sacrificed, and internal organs (the three Peyer's patches proximal to the ileum, mesenteric lymph node, spleen, and liver) were collected and homogenized in 5 ml of PBS, using a Stomacher (Tekmar). Dilutions were plated on LB plates containing the appropriate antibiotics, and the significance of differences observed was determined with the Wilcoxon signed rank test for a paired experiment. For colonization studies, groups of 10 mice were infected i.g. with a 1:1 mixture of wild-type and mutant *S. typhimurium* containing approximately 10^7 bacteria, and two fecal pellets were collected daily for enumeration of bacterial counts. Fecal pellets were weighed, homogenized in 1 ml of PBS, and plated on appropriate antibiotic plates to determine CFU per milligram of feces for each strain.

(iii) Growth in serum. BALB/c mice (retired breeders) were anesthetized, and blood was collected from the axillary vessels. Blood from 15 mice was pooled, allowed to coagulate for 1 h at room temperature, and then kept overnight on ice. Serum was filtered and used immediately for bacterial growth experiments. To assess bacterial growth, overnight cultures of strains to be tested were washed in PBS and then diluted to 10^6 CFU/ml in PBS. Ten microliters was added to duplicate wells containing 0.5 ml of serum in a 24-well microtiter plate. To assess growth, 20 μl was removed at appropriate time points, diluted, and plated on antibiotic plates for enumeration of bacteria.

Nucleotide sequence accession number. The partial sequence of *S. typhimurium, feoB* has been assigned GenBank accession number U70434.

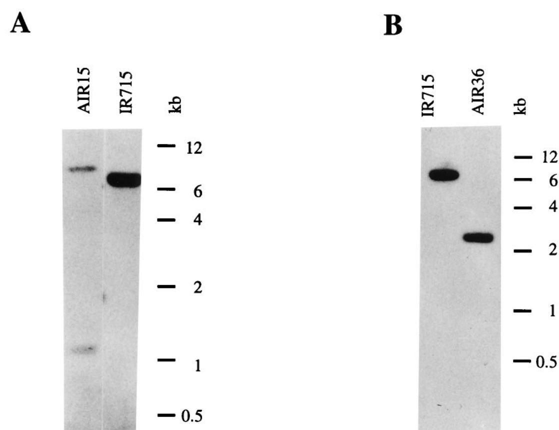

FIG. 1. Southern blots showing the disruption of *S. typhimurium tonB* and *feoB* genes. (A) Chromosomal DNA of AIR15 (containing a 2-kb tetracycline resistance element inserted in *feoB*) and IR715 (parent strain) digested with *Eco*RI-*Hin*dIII and probed with an 895-bp fragment of *S. typhimurium feoB*. (B) Chromosomal DNA of AIR36 (containing a 1.3-kb kanamycin resistance gene in *tonB*) and IR715 digested with *Eco*RI and probed with a fragment containing bp 106 to 547 of *S. typhimurium tonB.*

RESULTS

Cloning of the *S. typhimurium feoB* homolog and construction of a *feoB* mutant. In *E. coli*, *feoAB* has been shown to encode a transport system which mediates uptake of ferrous ions (22). To examine the role of iron(II) uptake during *S. typhimurium* infection of mice, we cloned a fragment of the *S. typhimurium feoB* homolog and used this fragment to construct a defined mutant in the *S. typhimurium feoB* gene. To this end, we attempted to amplify a fragment of *feoB* from *S. typhimurium* IR715 (33), using primers designed from the *E. coli feoB* sequence (22). We were unable to amplify a fragment of *feoB* from *S. typhimurium* but could amplify an 1.0-kb product from *S. typhi* AJB70. The *S. typhi* PCR product was cloned in the vector pCRII (Invitrogen) and was confirmed by sequencing to contain an *S. typhi feoB* homolog (data not shown). Using this sequence, we designed new primers and amplified an 895-bp DNA fragment from *S. typhimurium*. This product was cloned into pCRII. Sequence analysis of this construct indicated that it contained a fragment of the *S. typhimurium feoB* homolog corresponding to bp 79 to 952 of the *E. coli feoB* gene (22). The deduced amino acid sequence of the *S. typhimurium* FeoB protein possessed 87% identity and 96% similarity at the amino acid level to the *E. coli* protein (22) (data not shown).

An *S. typhimurium feoB* mutant was constructed by insertion of the 2-kb pAK1900 tetracycline resistance element as described in Materials and Methods and designated AIR15. To confirm the disruption of *feoB* in AIR15, DNA from this strain was analyzed by Southern blotting. Chromosomal DNA of AIR15 and IR715 (parent strain) was digested with *Eco*RI-*Hin*dIII and probed with an 895-bp fragment of *S. typhimurium feoB* spanning the predicted insertion site. The probe hybridized to a 7-kb band in the parent strain. Because the tetracycline resistance element contains an *Eco*RI site, insertion should result in generation of two bands which hybridize to the probe. The combined size of these fragments should be the sum of the 7 kb of the band in the parent strain and the 2 kb of the cassette. In the mutant strain AIR15, bands of 1.2 and 7.8 kb were detected by the *feoB* probe, which is consistent with insertion of the tetracycline resistance element into *feoB* (Fig. 1A).

Although mutations in the *feo* locus of *E. coli* do not affect the bacterial growth rate, they cause constitutive expression of iron uptake genes, even under iron-replete conditions (20). This observation indicated that Feo participates in iron uptake, but in the *feo* mutant, the expression of other iron uptake systems is upregulated to compensate for loss of Feo function. The *S. typhimurium feoB* mutant was therefore tested for constitutive expression of enterobactin synthesis genes by introducing an *entB*::*lacZ* fusion from strain AR1258 (36) to create AIR17. Levels of expression of the *entB*::*lacZ* fusion were compared in both AR1258 (*entB*::*lacZ*) and AIR17 (*feoB entB*::*lacZ*) by measuring β-galactosidase levels after growth under both iron-replete and iron-deficient conditions (Table 2). While the *entB*::*lacZ* fusion was expressed only under iron-deficient conditions in AR1258, similar levels of β-galactosidase were found under both growth conditions in AIR17. Iron regulation of the *entB*::*lacZ* fusion in *S. typhimurium* AIR17 could be restored by introduction of the *E. coli feoB* gene on plasmid pUH18 (Table 2). This result showed that as in *E. coli*, disruption of *feoB* results in derepression of iron uptake genes in *S. typhimurium* under iron sufficiency.

Construction and characterization of an *S. typhimurium tonB* mutant. TonB-mediated iron uptake mechanisms contribute to the virulence of pathogens such as *Vibrio cholerae*, *Vibrio anguillarum*, and *Haemophilus influenzae* (12, 15, 21). TonB has been shown to be required for assimilation of iron(III) from a variety of compounds, including siderophores and host iron proteins (8, 35). Thus, mutation of *S. typhimurium tonB* should lead to inactivation of a wide range of potential iron(III) uptake mechanisms. To examine the role of iron(III) uptake on growth of *S. typhimurium* in its host, we constructed an *S. typhimurium tonB* mutant (AIR36) by insertion of the 1.3-kb KSAC kanamycin resistance cassette between bp 547 and 566 of its open reading frame by allelic exchange. Chromosomal DNA isolated from AIR36 and its parent IR715 was digested with *Eco*RI, and a Southern blot of this DNA was probed with a fragment containing bp 106 to 547 of the *S. typhimurium tonB* open reading frame (Fig. 1B). The probe hybridized to a 6-kb fragment in the parent strain. Because KSAC is flanked by *Eco*RI sites, the *tonB* probe was expected to detect only the portion of this 6-kb fragment that is located upstream of the insertion site in AIR36. The *tonB* probe hybridized to a 2-kb *Eco*RI fragment in AIR36, which is consistent with insertion of KSAC into the *tonB* gene.

We tested the effect of the *tonB* mutation on the ability of *S. typhimurium* to utilize ferrioxamine B as an iron source. In other enteric bacteria, uptake of ferrioxamines has been shown to be *tonB* dependent (2), and we have found that *S. typhimurium* possesses a TonB-dependent receptor for ferrioxamine B (36). Since *S. typhimurium* is able to utilize ferrioxamines

TABLE 2. Effect of a mutation in *feoB* on the expression of an iron-regulated gene

Strain	Relevant genotype	β-Galactosidase activity (Miller units)[a]	
		+Fe	−Fe
AR1258	*entB*::*lacZ*	42 ± 2.5	260 ± 77
AIR17	*entB*::*lacZ feoB*	306 ± 7.5	334 ± 29
AIR17(pUH18)	*entB*::*lacZ feoB* (*E. coli feoB* gene on plasmid)	38 ± 7.5	226 ± 55

[a] Calculated as described by Miller (27); averages of at least three independent experiments ± standard error. +Fe, grown in NB + 40 μM $FeCl_3$; −Fe, grown in NB + 0.2 mM 2,2′-dipyridyl.

TABLE 3. Utilization of ferrioxamine B on NBD agar

Strain	Genotype	Growth zone (mm)[a]
CL1509	*aroA*	24
AJB29	*fhuB aroA*	0
AIR51	*tonB aroA*	0
AIR51(pIRS618)	*tonB aroA* (*tonB* on plasmid)	26

[a] Growth zone around filter disk containing 3 μg of ferrioxamine B (Fe-Desferal).

as a sole iron source (26), an *S. typhimurium tonB* mutant should be unable to take up and utilize iron from ferrioxamine B. To prevent scavenging and uptake of ferrioxamine-iron by the siderophore enterobactin, ferrioxamine-iron utilization was assayed in an *aroA* strain background, using *S. typhimurium* CL1509 (10). Because of its inability to produce a precursor common to both enterobactin and aromatic amino acids, the *aroA* mutant is unable to produce enterobactin. As a negative control, a *fhuB aroA* mutant (AJB29) was assayed for ferrioxamine B utilization (36). This mutant is defective in a cytoplasmic permease necessary for internalization of ferrioxamine B and ferrichrome and is therefore unable to grow on ferrioxamine B as a sole iron source (2, 24, 36). Strains were tested in an agar diffusion assay for growth on ferrioxamine B (Table 3). In contrast to the parent CL1509 (*aroA*), AIR51 (*aroA tonB*) and AJB29 (*aroA fhuB*) did not grow around a ferrioxamine B-soaked disk (Table 3). Growth of AIR51 could be restored by introduction of plasmid pIRS618, carrying an intact copy of *S. typhimurium tonB* (Table 3).

Growth of *feoB* and *tonB* mutants in vitro. We examined the effects of *feoB* and *tonB* mutations singly and in combination with other mutations affecting iron uptake on aerobic growth of *S. typhimurium* in broth. Under iron-rich conditions, no effect of these mutations on growth was observed (Table 4). Under iron-limited conditions, however, mutations either in *tonB* or in *entB*, which encodes an enzyme necessary for enterobactin biosynthesis, led to a growth defect in liquid media (Table 4).

We next examined the role of TonB and Feo during growth in normal mouse serum. The *entB* mutant was deficient for growth in serum, a finding which was consistent with an earlier report (6). The *tonB* mutant, like the *entB* mutant, was unable to grow during an 8-h incubation in mouse serum (Fig. 2). The *feoB* mutant was able to grow like the wild type, indicating that

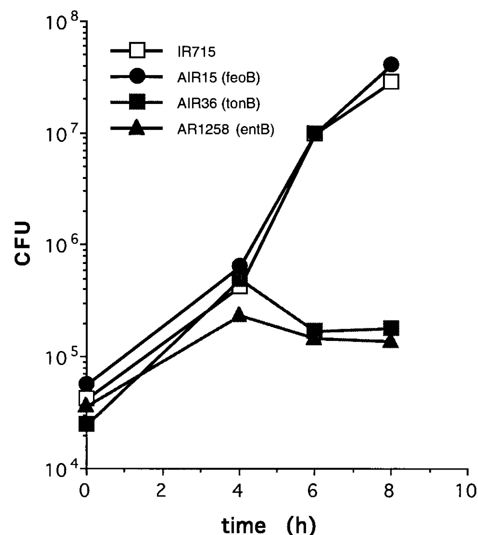

FIG. 2. Comparison of the abilities of *S. typhimurium* mutants to grow in pooled normal mouse serum. Sera from 15 mice were pooled for this experiment. Datum points represent averages of duplicate wells.

the Feo system is not required for growth in serum. Since we and others have found that *ent* mutants are unable to grow in serum but are fully virulent in BALB/c and several other inbred mouse strains (6), growth of *S. typhimurium* during mouse typhoid does not appear to occur in the bloodstream.

Intracellular growth. The ability to grow within epithelial cells has been shown to be required for virulence of *S. typhimurium* (25), and *S. typhimurium* must cross the epithelial barrier of the intestine to cause systemic infection. Bacterial multiplication in the liver and spleen of the mouse has also been shown to be intracellular (13, 28). We therefore assessed the abilities of the *tonB* and *feoB* mutants to grow in cultured epithelial cells. Interestingly, neither the single mutants nor the double mutant differed from the wild type in the ability to replicate within HEp-2 cells (Table 5). Since the *tonB* mutant is unable to grow in mouse serum (Fig. 2), it does not utilize iron compounds (heme and transferrin) present in the serum used to culture the HEp-2 cells. However, it is possible that the HEp-2 cells utilize heme and transferrin from the tissue culture medium and make it available to intracellular *S. typhimurium*. Similar results were obtained with HeLa cells and J774 cells (data not shown). Thus, the TonB and Feo uptake systems appear not to be required for intracellular growth of *S. typhimurium.*

Colonization of mouse intestine. After oral infection, *S. typhimurium* first passes through the stomach and the surviving bacteria colonize the intestine. To assess the contribution of TonB and Feo-mediated iron uptake in this early step of infection, recovery of mutant strains from the feces of orally

TABLE 4. Growth under iron-limiting and iron-replete conditions

Strain	Genotype	OD_{578} after 24 h in[a]:	
		+Fe	−Fe
IR715	Wild type	3.00 ± 0.158	0.748 ± 0.024
AR1258	*entB*	3.04 ± 0.390	0.220 ± 0.032
AIR15	*feoB*	3.14 ± 0.447	0.656 ± 0.044
AIR17	*feoB entB*	3.23 ± 0.257	0.270 ± 0.042
AIR36	*tonB*	3.09 ± 0.240	0.270 ± 0.006
AIR62	*feoB tonB*	2.67 ± 0.599	0.266 ± 0.022
AIR62(pIRS618)	*feoB tonB* (*tonB* on plasmid)	2.96 ± 0.649	0.477 ± 0.021
AIR62(pUH18)	*feoB tonB* (*feoB* on plasmid[b])	2.98 ± 0.370	0.206 ± 0.011

[a] Cultures were inoculated to a starting optical density at 578 nm (OD_{578}) of 0.01; 24-h values are the means of three experiments ± standard error. +Fe, NB + 40 μM $FeCl_3$; −Fe, NB + 0.2 mM 2,2-dipyridyl.
[b] *E. coli feoB.*

TABLE 5. Intracellular growth in HEp-2 cells

Strain	Genotype	Fold growth after 18 h[a]
IR715	Wild type	10.8 ± 1.5
AIR15	*feoB*	8.9 ± 1.6
AIR36	*tonB*	10.9 ± 1.9
AIR62	*tonB feoB*	11.7 ± 2.2

[a] Mean of three experiments ± standard error.

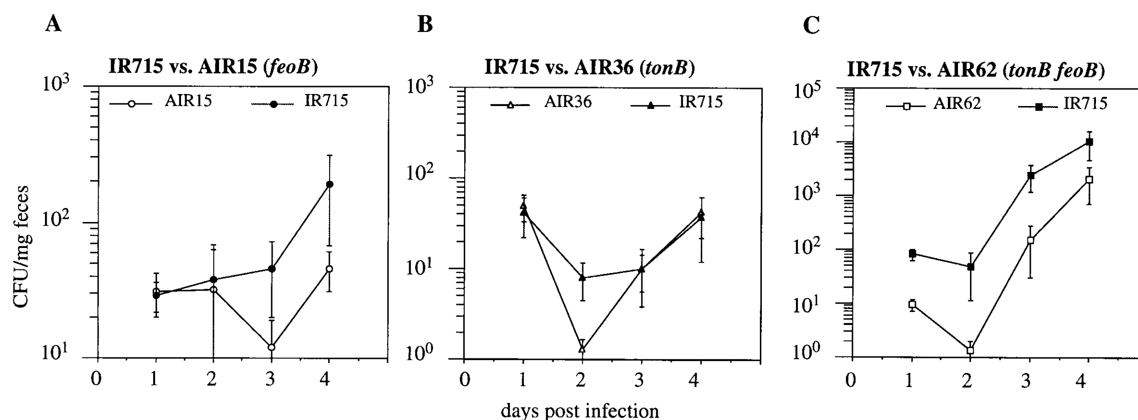

FIG. 3. Comparison of the abilities of *S. typhimurium* mutants AIR15 (*feoB*) (A), AIR36 (*tonB*) (B), and AIR62 (*tonB feoB*) (C) to compete with the parent strain (IR715) for colonization of the mouse intestine. On day 0, a 1:1 mixture of the parent strain and each mutant, containing approximately 10^7 bacteria, was administered i.g. to 10 mice, and bacterial numbers in the feces were quantified during 4 days postinfection. Each datum point represents the average of 10 mice ± standard error.

infected mice was compared with that of the wild type. To this end, mixtures (at a 1:1 ratio) of AIR36 (*tonB*) plus IR715 and AIR15 (*feoB*) plus IR715 were administered i.g. to groups of 10 mice. Between 1 and 4 days postinfection, bacterial counts in the feces of infected mice were determined. Although the numbers of *tonB* mutant and wild-type bacteria recovered between 1 and 4 days postinfection differed only on day 2 postinfection, the *feoB* mutant was recovered in lower numbers at days 3 and 4 (Fig. 3). Numbers of the *tonB feoB* double mutant in the feces were consistently lower than those of the wild type during all 4 days of infection assessed (Fig. 3). These data show that *feoB* mutants have a reduced ability to colonize the intestine and that this defect can be potentiated by a second mutation in *tonB*. The greatest difference in numbers of mutant and wild-type bacteria is observed at early time points, suggesting that the *tonB feoB* mutants may be cleared more efficiently than the parent strain.

Effects of mutations affecting iron uptake on mouse virulence. To determine whether mutations in *tonB* and *feoB* affect the ability of *S. typhimurium* to cause disease in the host, we examined the abilities of these mutants to cause lethal infection in the mouse. Previous studies on the role of iron uptake in *S. typhimurium* infection of the mouse have been performed by using the intravenous and i.p. routes of infection (6, 38). These routes of infection bypass the intestine, where the initial phase of infection takes place. To include the intestinal phase of infection in our study, we determined the LD_{50}s of mutants administered i.g. in addition to those after i.p. infection (Table 6). We confirmed the previous finding by Benjamin et al. that the enterobactin biosynthesis pathway is not required for mouse typhoid, since the LD_{50} of the *entB* mutant by both routes of infection was not higher than that for the parent strain (6). Similarly, a mutation in *feo* had no effect on LD_{50}s. When mutations in *entB* and *feoB* (AIR17) or *feoB* and *tonB* (AIR62) were combined, no additive effects on LD_{50} were observed by either route of infection. Only strains AIR36 and AIR62, carrying *tonB* mutations, exhibited increased LD_{50}s when given i.g.

The average times to death of mice infected i.g. at the doses above and below the LD_{50} were determined. If given at these doses, both AIR36 (*tonB*) and AIR62 (*tonB feoB*) required, on the average, 2 to 3 days longer to kill mice than the parent strain (Table 6). The LD_{50} of AIR62 could be complemented to the wild-type level by introducing an intact copy of *tonB* on pIRS618 (Table 6). All strains tested were as virulent as the wild type when administered i.p. These results indicate that the *tonB* mutant has a reduced ability to colonize a compartment encountered during i.g. infection but not during i.p. infection.

Recovery of the *tonB* mutant from different mouse organs. To determine at what point in infection the *tonB* mutants are affected, bacteria were enumerated in Peyer's patches, mesenteric lymph nodes, livers, and spleens of mice 5 days after i.g. infection (Table 7). To compensate for large differences between individual animals, each mouse was infected with a 1:1 mixture of *tonB* mutant and wild-type bacteria. This type of analysis allowed for a direct comparison of wild type and mutant in each animal.

Since the two strains carry different antibiotic resistance genes, it is possible to determine the numbers of each strain in the organs by plating on the appropriate antibiotics. Significant differences between the numbers of *tonB* and wild-type bacteria were found in the Peyer's patches ($P < 0.01$) and the mesenteric lymph nodes ($P < 0.025$) (Table 7). Numbers of the two strains recovered from the liver and spleen were not significantly different. These results show that the *tonB* mutant has a decreased ability to colonize Peyer's patches and the mesenteric lymph nodes but is able to reach the same numbers in the liver and spleen as the wild type. A further indication that TonB contributes to establishing infection in these organs was obtained during in vivo complementation of the *tonB feoB* mutant AIR62 with plasmid pIRS618 carrying *tonB*. At 4 days postinfection, the spleen of a mouse infected with AIR62(pIRS618)

TABLE 6. LD_{50}s of iron uptake mutants

Strain	Relevant genotype	i.g. infection		i.p. LD_{50}
		LD_{50}	Mean time to death (days)[a]	
IR715	Wild type	1.3×10^5	7.2 ± 0.98	<10
AR1258	*entB*	1.4×10^5	10 ± 4.4	<10
AIR15	*feoB*	1.5×10^5	8.3 ± 1.1	<10
AIR36	*tonB*	7.8×10^5	13.3 ± 5.9	<10
AIR17	*entB feoB*	2×10^5	8.0 ± 1.4	<10
AIR62	*tonB feoB*	1.1×10^6	11.8 ± 3.5	<10
AIR62(pIRS618)	*tonB feoB* (*tonB* on plasmid)	1.9×10^5	7.0 ± 0.0	ND[b]

[a] Mean ± standard error for mice infected at doses above and below the LD_{50}.
[b] ND, not determined.

TABLE 7. Bacterial load in organs of mice 5 days after mixed i.g. infection[a]

Organ	Mean CFU/organ ± SE[b]		Significance of difference between IR715 and AIR36[c] (probability)
	IR715 (wild type)	AIR36 (*tonB* mutant)	
Peyer's patches[d]	$6.1 \times 10^2 \pm 3.0 \times 10^2$	$1.2 \times 10^2 \pm 67$	Significantly different ($P < 0.01$)
Mesenteric lymph node	$3.4 \times 10^4 \pm 2.7 \times 10^4$	$8.5 \times 10^3 \pm 2.4 \times 10^3$	Significantly different ($P < 0.025$)
Liver	$1.2 \times 10^5 \pm 1.0 \times 10^5$	$8.8 \times 10^4 \pm 4.7 \times 10^4$	Not significantly different ($P > 0.05$)
Spleen	$7.5 \times 10^5 \pm 7.5 \times 10^5$	$3.2 \times 10^5 \pm 1.8 \times 10^5$	Not significantly different ($P > 0.05$)

[a] Mice were infected i.g. with a 1:1 mixture of IR715 and AIR36 containing 10^7 bacteria, and organs were collected after 5 days.
[b] Mean of between 9 and 16 mice.
[c] Significance of differences observed was determined by the Wilcoxon signed rank test.
[d] Three Peyer's patches proximal to the cecum were collected and pooled.

was examined to determine the stability of pIRS618 in vivo. We found that although pIRS618 could complement the defect in virulence of AIR62 to the wild-type level, less than 1% of bacteria isolated from the spleen still carried the plasmid. In contrast, 50% of the bacteria in the Peyer's patches and 10% of the bacteria in the mesenteric lymph nodes still carried plasmid pIRS618. Our virulence data show that *tonB* is not essential during i.p. infection (Table 6), and loss of pIRS618 in the liver and spleen may thus be the result of growth under conditions which do not select for presence of the *tonB* plasmid. The fact that pIRS618 was recovered in higher numbers from bacteria colonizing Peyer's patches and mesenteric lymph nodes, on the other hand, would be consistent with a role of *tonB* in iron acquisition in these compartments.

DISCUSSION

This study examined the role of iron(II) uptake, mediated by the Feo uptake system, and the role of iron(III) uptake, mediated by TonB, on the ability of *S. typhimurium* to grow in different organs of the mouse. Using allelic exchange, we constructed defined *S. typhimurium* mutants in *feoB* and *tonB*. Whereas the *feoB* mutant was capable of growing like the wild type in vitro, it was at a disadvantage compared with its parent strain during colonization of the mouse intestine (Fig. 3). These results indicate that *S. typhimurium* has access to an iron(II) source which is taken up by Feo in the mouse intestine. Similarly, a study of *E. coli feo* mutants showed that they, too, are impaired in the ability to colonize the mouse intestine (34). The introduction of a *tonB* mutation into the *S. typhimurium feoB* mutant resulted in further reduction of bacterial numbers recovered from the feces, suggesting that in the anaerobic environment of the gut, both ferrous and ferric iron are available. In serum and in epithelial cells, however, the growth of the *feo* mutant was unaffected (Table 5; Fig. 2). Furthermore, both i.g. and i.p. LD_{50}s were the same as those of the parent strain, showing that Feo-mediated iron(II) uptake is not required by *S. typhimurium* to cause systemic disease. The *S. typhimurium tonB feoB* mutant exhibited the same degree of attenuation as the *tonB* mutant and could be complemented to the i.g. LD_{50} of the wild type by introduction of a plasmid-encoded copy of *tonB*. These results show that even in the absence of TonB-mediated ferric iron uptake, ferrous iron uptake via Feo does not contribute to growth at extraintestinal sites.

Under iron-replete in vitro growth conditions, *S. typhimurium tonB* and *entB* mutants grew like the wild type but showed reduced growth under iron-limited conditions. This was expected, since enterobactin is the only siderophore produced by *S. typhimurium* 14028 and TonB is necessary for the uptake of this siderophore. In the mouse, the *S. typhimurium tonB* mutant was recovered in the same numbers from the feces as the wild type but required fivefold-higher inocula to cause lethal infection by the i.g. route. TonB-mediated uptake of enterobactin is necessary for growth in one of the compartments encountered during i.p. infection, the blood, as shown by the inability of *S. typhimurium entB* and *tonB* mutants to grow in mouse serum. However, growth in serum does not appear to be responsible for the observed attenuation of the *tonB* mutant, since it was fully virulent if the intestinal tract was circumvented by the i.p. route of infection (Table 6). This observation is also consistent with findings of Benjamin et al., who showed that biosynthesis of enterobactin is not required for mouse typhoid (5). Rather, our data suggest that TonB contributes to growth at a site not encountered by *S. typhimurium* when administered i.p., such as the intestine, Peyer's patches, or mesenteric lymph nodes. The ability of the *tonB* mutant to compete successfully in the intestine with the wild type for colonization indicates that the intestinal lumen is not the site where the *tonB* mutation affects growth (Fig. 3). This compartment appears to be in either the Peyer's patches or the mesenteric lymph nodes, as significantly ($P < 0.01$) lower numbers of the *tonB* mutant than the wild type were recovered from these organs during mixed infection. Furthermore, during in vivo complementation of the *tonB* and *tonB feoB* mutants with a plasmid carrying the *tonB* gene, the plasmid was recovered at a much higher rate from bacteria isolated from Peyer's patches or the mesenteric lymph node than from bacteria isolated from the liver and spleen. Gulig and Doyle assessed the division of *S. typhimurium* in vivo by measuring segregation of a temperature-sensitive plasmid in mice (17). They found that only 1% of the bacteria recovered from Peyer's patches and 0.1% of the bacteria recovered from the spleen still contained the plasmid 5 days after infection. The finding that during complementation of the *tonB* and *tonB feoB* mutants with a plasmid carrying the *tonB* gene, 50% of bacteria recovered from Peyer's patches still contained the plasmid suggests that there is selective pressure in vivo to maintain it. On the other hand, there seems to be no selective advantage for TonB-dependent growth in the spleen, since less than 1% of the bacteria recovered from this organ contained the *tonB* plasmid. The higher recovery of plasmid-containing bacteria from the Peyer's patches and the mesenteric lymph nodes is hence most likely the combined result of (i) selection for the plasmid-encoded *tonB* gene in the Peyer's patches and the mesenteric lymph nodes and (ii) increased loss of the plasmid in the liver and spleen by segregation of the plasmid, as a result of the high growth rate of *S. typhimurium* in these organs and lack of selection. Together, these data suggest that the attenuation of the *S. typhimurium tonB* mutant is caused by a defect in colonizing the Peyer's patches and mesenteric lymph nodes. This reduced colonization is unlikely to be solely the result of the inability of the *tonB* mutant to take up enterobactin, as *entB* mutants were found to be fully virulent by the i.g. route of infection. It is still unclear

whether TonB mediates iron uptake in an intracellular or an extracellular location in the Peyer's patches and the mesenteric lymph nodes.

During the course of a *S. typhimurium* infection, bacteria pass through a series of compartments, each of which contains different iron sources for bacterial growth. Growth at certain sites, e.g., the blood, is not required for *S. typhimurium* to cause mouse typhoid. Consequently, iron uptake systems, such as enterobactin, which allow iron uptake in these compartments are not virulence factors in this animal model. In contrast, growth in other compartments such as the liver, spleen, mesenteric lymph nodes, or Peyer's patches contributes to the development of disease. For mouse typhoid fever, the most important compartments are the liver and spleen, in which the bacteria are filtered out of the blood by resident macrophages (11, 14). These are the cells in which mouse-pathogenic *Salmonella* species are thought to grow (9). The resident macrophages of the liver and spleen are rich in iron, as they have the function of removing aged erythrocytes from the circulation. Furthermore, in response to infection, the amount of iron (stored as ferritin) in these resident macrophages is actually increased (37). Thus, the strategy used by *S. typhimurium* (and possibly by other intracellular pathogens as well) to obtain iron in the host may be to multiply within these sites of iron storage (3). Our own results (unpublished) show that *S. typhimurium entB* mutants are unable to utilize ferritin in vitro. However, since the growth conditions in vitro differ from the intracellular milieu, this result does not exclude the possibility that *S. typhimurium* expresses a system for utilization of ferritin in vivo. In either case, *S. typhimurium* apparently is able to obtain iron in this intracellular niche independently of Feo- and TonB-dependent iron uptake systems. These findings suggest that another, yet undescribed mechanism for iron acquisition may be involved in growth of *S. typhimurium* in the liver and spleen.

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