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Ferrioxamine-Mediated Iron(III) Utilization by *Salmonella enterica*

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Utilization of ferrioxamines as sole sources of iron distinguishes *Salmonella enterica* serotypes Typhimurium and Enteritidis from a number of related species, including *Escherichia coli*. Ferrioxamine supplements have therefore been used in preenrichment and selection media to increase the bacterial growth rate while selectivity is maintained. We characterized the determinants involved in utilization of ferrioxamines B, E, and G by *S. enterica* serotype Typhimurium by performing siderophore cross-feeding bioassays. Transport of all three ferric siderophores across the outer membrane was dependent on the FoxA receptor encoded by the Fur-repressible *foxA* gene. However, only the transport of ferrioxamine G was dependent on the energy-transducing protein TonB, since growth stimulation of a *tonB* strain by ferrioxamines B and E was observed, albeit at lower efficiencies than in the parental strain. Transport across the inner membrane was dependent on the periplasmic binding protein-dependent ABC transporter complex comprising FhuBCD, as has been reported for other hydroxamate siderophores of enteric bacteria. The distribution of the *foxA* gene in the genus *Salmonella*, as indicated by DNA hybridization studies and correlated with the ability to utilize ferrioxamine E, was restricted to subspecies I, II, and IIIb, and this gene was absent from subspecies IIIa, IV, VI, and VII (formerly subspecies IV) and *Salmonella bongori* (formerly subspecies V). *S. enterica* serotype Typhimurium mutants with either a transposon insertion or a defined nonpolar frameshift (+2) mutation in the *foxA* gene were not able to utilize any of the three ferrioxamines tested. A strain carrying the nonpolar *foxA* mutation exhibited a significantly reduced ability to colonize rabbit ileal loops compared to the *foxA*$^+$ parent. In addition, a *foxA* mutant was markedly attenuated in mice inoculated by either the intragastric or intravenous route. Mice inoculated with the *foxA* mutant were protected against subsequent challenge by the *foxA*$^+$ parent strain.

The genus *Salmonella* shared an ancestor with *Escherichia coli* some 100 million to 160 million years ago (31) and has since become a pathogen in a wide range of warm- and cold-blooded vertebrate hosts. DNA has been either lost by deletion or introduced by bacteriophage- or plasmid-mediated horizontal transfer in both of these taxa since the divergence. The genetic material that is present in *Salmonella enterica* serotypes but absent from *E. coli* includes the *Salmonella* virulence plasmid, *Salmonella* pathogenicity island 1 (SPI1), SPI2, and smaller pathogenicity islets, such as the *lpf* operon and *sifA* (14). Other differences between the taxa involve genes whose products have a role in iron acquisition. For example, *E. coli* expresses the ferric dicitrate transport mechanism encoded by the *fecABCD* genes, but *Salmonella* spp. lack a comparable system (46). On the other hand, *S. enterica* expresses two outer membrane proteins, FepA and IroN, that mediate uptake of the catechol siderophore enterobactin, while *E. coli* possesses only the *fepA* gene (4). Moreover, many *Salmonella* strains are able to acquire iron complexed with ferrioxamines B, E, and G (22), hydroxamate siderophores that they do not themselves synthesize. In contrast, *E. coli* strains cannot efficiently utilize ferrioxamines (15).

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Since ferrioxamines are potent growth factors for the common *S. enterica* serotypes but not for a number of closely related bacteria, they have been used as supplements in standard enrichment and selection procedures to increase the speed and sensitivity of detection of members of the genus *Salmonella* in a number of food products (19, 32, 38). For example, standard procedures used for detection of small numbers of organisms in eggs require preenrichment of mixtures of yolk and albumin at room temperature for 1 to 2 days. In contrast, preenrichment of artificially infected albumin in buffered peptone water supplemented with ferrioxamine E allowed detection of contaminating *S. enterica* serotype Typhimurium within 6 h (19, 36). Similarly, two to five cells of *S. enterica* serotype Enteritidis per 25 g of egg albumin were detected within 24 h when selective preenrichment in buffered peptone water containing ferrioxamine E was used (3).

Little is known about the mechanism of uptake of iron(III)-ferrioxamine complexes by *Salmonella* spp. Since the ferrioxamines have molecular weights of approximately 600, which is greater than the theoretical limit for passive diffusion through porins (27), it is likely that, like uptake of other siderophores of enteric bacteria, ferrioxamine uptake requires binding to a specific receptor protein, followed by TonB- and ExbBD-dependent transport across the outer membrane. Transport of siderophores through the inner membrane normally depends on a periplasmic binding protein-dependent ABC transporter complex. It is possible that ferrioxamine uptake by *Salmonella* spp. involves the FhuBCD inner membrane permease complex

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
S. enterica serotype Typhimurium strains		
ATCC 14028	Wild type	ATCC[a]
AR1258	ATCC 14028, *entB*	44
RK804	AR1258, *tonB*	18
RK809	AR1258, *foxA*::pMAP	This study
enb-7	LT2, class II *ent* mutant	33
WR1024	enb-7, *fhuB*::MudJ	Laboratory stock
SL1344	*hisG46*	17
SL1344/nr	SL1344, Nal^r	This study
BK102	SL1344/nr, *foxA*	This study
TML	Wild type	12
TML/nr	TML, Nal^r	This study
RK102	TML/nr, *foxA*	This study
E. coli SM10λ*pir*	F^- *thi-1 thr-1 leuB6 supE44 tonA21 lacY1 recA*::RP4-2-tc::Mu Km^r	R. Haigh
Plasmids		
pUC18	ColE1, *bla*	Laboratory stock
pRA17	pUC18 carrying the 287-bp *foxA* fragment	This study
pRA5	pUC18 carrying the *foxA* 5′ region	This study
pBluescriptSK	ColE1, *bla*	Laboratory stock
pRA19	pBluescriptSK carrying the mutated 287-bp *foxA* fragment	This study
pRDH10	*sacRB cat ori*R6K *mobRP4*, Tet^r	R. Haigh
pRA21	pRDH10 carrying the 287-bp *foxA* fragment	This study
pIRS618	pSUKS1 carrying the serovar Typhimurium *tonB* gene	45
pMAP	*bla ori*R6K *mobRP4*	44

[a] ATCC, American Type Culture Collection.

required for utilization of other hydroxamate siderophores (21). However, none of this has been demonstrated experimentally, nor has the ability of *S. enterica* serotypes other than serotypes Typhimurium and Enteritidis to utilize ferrioxamines as iron sources been determined. In this paper we describe experiments aimed at characterizing the mechanism of ferrioxamine transport by *S. enterica* serotype Typhimurium and at assessing the distribution of the ability to utilize ferrioxamine in the genus *Salmonella*. The results of a preliminary investigation of the role of the ferrioxamine receptor FoxA in experimental salmonellosis are also presented.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. In addition, two previously characterized *Salmonella* strain collections were used for a hybridization analysis, the SARB collection consisting of 72 serotypes of subspecies I (7) and a collection consisting of representative strains of the other species and subspecies of the genus, including *Salmonella bongori* (35). Strains were cultured aerobically at 37°C as required. The ferrous iron chelator 2,2′-bipyridyl (Sigma) was added to media at the concentrations indicated below to impose iron limitation. Growth in liquid media was quantified by measuring the optical density at 620 nm. Bacteriophage P22 HT105/1 *int*$^-$ (39) was used for generalized transduction of markers between smooth strains of *S. enterica*. Transductants were routinely purified and made bacteriophage free by streaking them onto nonselective green indicator plates as described previously (23). All *foxA*$^+$-*foxA* isogenic pairs of strains had equivalent growth characteristics in nutrient broth (NB) (Oxoid no. 2), as judged by the length of the lag phase, the maximal growth rate, and the climax cell density during the stationary phase.

Siderophore assays. The abilities of bacterial strains to use siderophores as sources of iron were determined by performing cross-feeding tests. Vogel-Bonner medium supplemented with 2,2′-bipyridyl was used to test enterobactin-deficient strains (18). Enterobactin-producing strains were tested in bioassays by using egg white medium (EWM) consisting of nutrient agar base (25.6 g of NB per liter, 6 g of yeast extract per liter, 18 g of $Na_2HPO_4 \cdot 12H_2O$ per liter, 6 g of KH_2PO_4 per liter, 44 g of Oxoid agar no. 1 per liter) to which fresh sterile egg white was added. The egg white was passed repeatedly through a sterile 1.2-mm-gauge syringe needle in order to mix it before use; 9 ml of the resulting preparation was added to 11 ml of nutrient agar base, which was sufficient for a single assay plate. Ferrioxamines B and E were gifts from H. P. Schnebli, Novartis Pharma Ltd., Basel, Switzerland; ferrioxamine G was prepared and purified as previously described (37); coprogen was a gift from G. Winkelmann, University of Tübingen, Tübingen, Germany; and enterobactin was prepared and purified as described by Young (48).

Recombinant DNA techniques. Standard methods were used for isolation of chromosomal DNA, restriction endonuclease analysis, ligation, and transformation of plasmid DNA (24). Southern transfer by capillary action was performed as previously described (24). Labeling of DNA probes, hybridization, and immunological detection were performed by using Gene Images nonradioactive labeling and detection kits from Amersham. Hybridization was performed at 65°C in 5× SSC (1× SSC contains 8.77 g of NaCl per liter and 4.41 g of sodium citrate per liter, pH 7.0) containing 0.1% (wt/vol) sodium dodecyl sulfate, 5% (wt/vol) dextran sulfate, and 0.5% (wt/vol) blocking agent (supplied by the manufacturer). Filters were subsequently washed at high stringency with 0.1× SSC containing 0.1% sodium dodecyl sulfate. The plasmid DNA used for sequencing was isolated by using ion-exchange columns obtained from Qiagen.

Preparation of a *foxA* probe and attempts to clone the *Salmonella foxA* gene. The *foxA* gene of *Yersinia enterocolitica*, which encodes a 77-kDa outer membrane protein involved in the transport of ferrioxamine B, has been cloned and characterized (1). We previously identified part of an open reading frame (GenBank accession no. U62282) in the genome of *S. enterica* serotype Typhimurium whose deduced amino acid sequence exhibits 45% identity with the amino acid sequence of the FoxA protein of *Y. enterocolitica* (44). Using this sequence, we designed two primers, FX1 (5′ AGGCGGATCCATCGGCGGC 3′) and FX2 (5′ ACGGGATCCAGATCACCGTCC 3′) (incorporating *Bam*HI sites by including mismatching bases), which generated an approximately 300-bp PCR product that was subsequently cloned into the *Bam*HI site of pUC18 to give a recombinant plasmid designated pRA17. Sequencing confirmed that the insert was a 287-bp subgenic fragment corresponding to base pairs 55 to 342 of the *foxA* coding sequence described previously (44).

Three plasmid libraries and four cosmid libraries of *S. enterica* (serotype Typhimurium, serotype Paratyphi, or serotype Typhi) chromosomal DNA were screened, either by hybridization with the subgenic *foxA* probe or by hybridization in conjunction with complementation in a strain unable to utilize ferrioxamine E as a sole source of iron. No recombinant plasmids or cosmids that contained the entire *foxA* gene were found in these libraries. However, one positively hybridizing clone (designated pRA5) from a pUC18 library of partially *Sau*3A-digested *S. enterica* serotype Typhimurium chromosomal DNA contained part of the *foxA* gene and the 5′ flanking sequence; thus, this clone allowed us to determine the regulatory region (GenBank accession no. AF060876).

Construction of defined nonpolar *foxA* mutants. PCR primers FXM1 and FXM2 were designed by using the previously determined *foxA* sequence (44) to incorporate *Bgl*II restriction sites, as shown in Fig. 1B; these two primers were used along with primers P1L and P2L (which hybridized with sequences internal to the pUC18 vector sequence of pRA17), respectively, to amplify the *foxA* subgenic fragment borne by plasmid pRA17 as two separate PCR products that were approximately 190 and 230 bp long. These products were recovered, digested with *Bgl*II, ligated together, and cloned into pBluescriptSK at the *Xba*I-

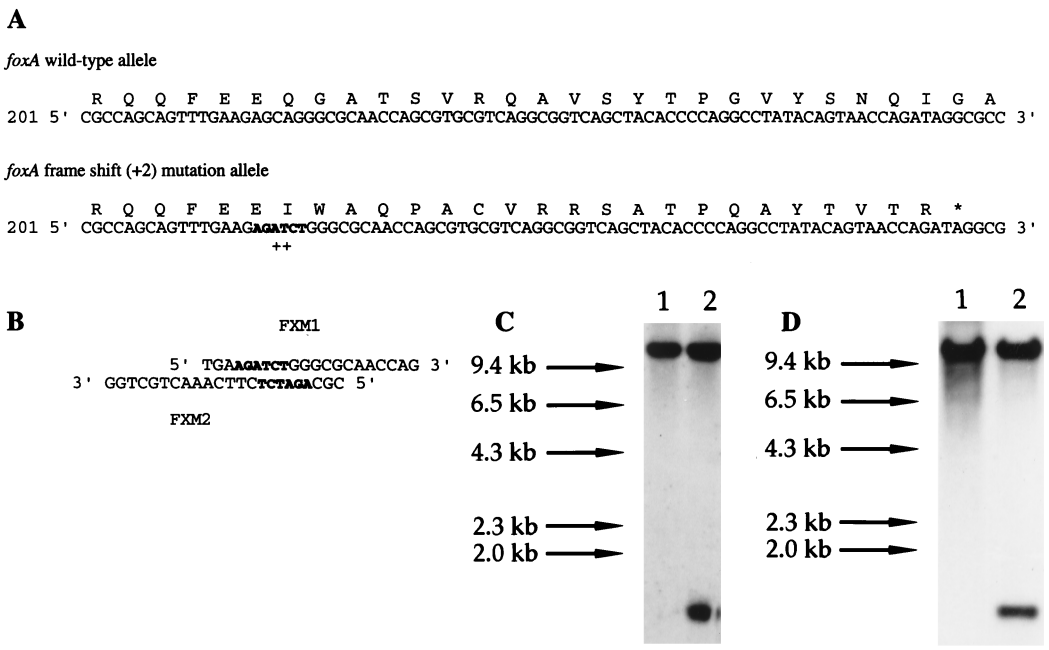

FIG. 1. Construction of a frameshift mutation in the *foxA* gene of *S. enterica* serotype Typhimurium. (A) Comparison of the nucleotide sequences of the wild-type and mutated *foxA* alleles, as confirmed by sequence analysis. The mutation consists of a 2-bp (AT) insertion (indicated by plus signs) and a *Bgl*II restriction enzyme recognition sequence (indicated by boldface type). The mutation was introduced into a cloned subgenic *foxA* fragment as described in the text by using primers FXM1 and FXM2, which have mismatching base pairs that include the *Bgl*II recognition site (B). (C and D) The mutations were confirmed by Southern hybridization analysis in which we used a subgenic *foxA* fragment as the probe. (C) Analysis of *Bgl*II-digested chromosomal DNA of parental strain TML (lane 1) and the *foxA* derivative strain RK102 (lane 2). (D) Analysis of parental strain SL1344 (lane 1) and the *foxA* derivative strain BK102 (lane 2).

*Sst*I sites to generate recombinant plasmid pRA19. Sequence analysis of the insert in pRA19 confirmed that a *Bgl*II cleavage site and a 2-bp insertion were introduced between base pairs 218 and 219 of the wild-type open reading frame (Fig. 1A). The +2 frameshift resulted in a termination codon 21 bases downstream of the insertion site.

To introduce the +2 frameshift mutation into the bacterial chromosome by allelic exchange, the insert was recloned into the suicide plasmid vector pRDH10 at *Bam*HI sites to generate plasmid pRA21. Plasmid pRDH10 contains *ori*R6K and so requires the protein product of λ*pir* for replication (20); derivative plasmid pRA21 was therefore maintained in *E. coli* SM10λ*pir* and introduced by conjugation into strains SL1344/nr and TML/nr (spontaneous nalidixic acid-resistant derivatives of *S. enterica* serotype Typhimurium strains SL1344 and TML, respectively), which were plated onto nutrient agar containing nalidixic acid (50 μg/ml) and chloramphenicol (20 μg/ml). Since the recipients lacked λ*pir*, growth on this medium directly selected for merodiploids in which the plasmid (encoding chloramphenicol resistance) had integrated into the host chromosome by a single recombination event. To select for loss of the integrated suicide vector by a second homologous recombination event, merodiploid strains were grown in medium containing tryptone (10 g/liter), yeast extract (5 g/liter), and 5% (wt/vol) sucrose to the mid-log phase, and dilutions of the cultures were plated onto tryptone-yeast extract-sucrose agar to enrich for bacteria that had lost the *sacRB* gene of the vector plasmid pRDH10 (6, 40). Bacteria growing on this medium were tested for sensitivity to tetracycline (10 μg/ml) as an indicator of loss of the vector. They were then screened for the presence of a new *Bgl*II recognition site at base pairs 217 to 222 by PCR amplification of chromosomal DNA with primers FX1 and FX2 and subsequent digestion of the product with *Bgl*II. Resolved merodiploid derivatives of SL1344/nr and TML/nr carrying the +2 frameshift *foxA* mutation were designated BK102 and RK102, respectively. The presence of the mutation was confirmed by performing a Southern blot analysis of *Bgl*II-digested chromosomal DNA with a *foxA*-specific probe (Fig. 1C and D). A *Bgl*II >10-kb fragment in the DNA of each parental strain hybridized with the probe, while two fragments (1.5 and 10 kb) hybridized in the *Bgl*II-digested DNA of both putative *foxA* mutants.

Rabbit ileal loop anastomosis test. Competitive colonization experiments to determine the effects of the *foxA* mutation were performed by using a modification of the rabbit ileal loop test with 2- to 3-kg specific-pathogen-free rabbits, as previously described (47). Each test loop (5 cm with 2-cm spacers) was inoculated by injecting 1 ml of a mixture (approximately 1:1) of RK102 (*foxA*) and the *foxA*$^+$ parent strain TML (total inoculum, 10^7 or 10^8 CFU in phosphate-buffered saline). To rule out the possibility that the nalidixic acid resistance mutation in RK102 had any effect, a control experiment involving similar mixtures of TML and TML/nr was also performed. The negative and positive controls consisted of 1 ml of sterile phosphate-buffered saline and 1 mg of cholera toxin in 1 ml of phosphate-buffered saline, respectively. Terminal anesthesia was administered 18 h after infection, and the loops were surgically removed for analysis. All test loops contained a small amount (0.5 to 1 ml) of purulent exudate. The positive control loops (containing cholera toxin) contained 15 to 20 ml of accumulated fluid. No accumulated fluid or purulent exudate was present in the negative control loops. The proportion of nalidixic acid-resistant bacteria (TML/nr or RK102) in each test loop was determined by plating serial dilutions of lumen contents and homogenized loop tissue onto unsupplemented MacConkey agar (Oxoid) and onto MacConkey agar containing nalidixic acid (50 μg/ml). The ratios of nalidixic acid-sensitive bacteria to nalidixic acid-resistant bacteria recovered were expressed as $\log_{10}$ values for statistical analysis. The Student *t* test was used to determine whether the ratio for the bacteria recovered was significantly different from the ratio for the inoculum.

Mouse typhoid model. Female BALB/c mice that were 6 to 8 weeks old and were housed under specific-pathogen-free conditions were used to determine the 50% lethal doses (LD_{50}s) of bacterial strains by intragastric and intravenous inoculation. Bacteria grown overnight in NB without shaking at 37°C were harvested by centrifugation and resuspended in sterile phosphate-buffered saline. Serial 10-fold dilutions containing 10^3 to 10^9 CFU were prepared and used for intragastric inoculation into groups of five mice, and serial 10-fold dilutions containing 10 to 10^4 CFU were prepared and used for intravenous inoculation into other groups of five mice. Mortality was recorded 28 days after infection, and LD_{50}s were calculated as described previously (34).

RESULTS

Role of TonB and FhuB in ferrioxamine-mediated iron uptake by *Salmonella* strains. Siderophore cross-feeding tests on Vogel-Bonner medium supplemented with 2,2′-bipyridyl were used to assess the ability of the *tonB* strain RK804 to utilize the catechol siderophore enterobactin and the hydroxamate siderophores coprogen and ferrioxamines B, E, and G (Table 2). No growth stimulation of RK804 was observed around discs loaded with coprogen or enterobactin, confirming that functional TonB protein is required for high-affinity transport of these siderophores. Similarly, no growth stimulation of RK804 was observed around a disc loaded with ferrioxamine G. In contrast, however, there was significant growth stimulation of RK804 by ferrioxamines B and E, although the growth zones

TABLE 2. Siderophore cross-feeding of various serotype Typhimurium strains[a]

Medium	Concn of 2,2′-bipyridyl (μM)	Strain	Relevant genotype	Results with the following siderophores:				
				Ferrioxamine B	Ferrioxamine E	Ferrioxamine G	Coprogen	Enterobactin
Vogel-Bonner[b]	200	AR1258	*entB*	28	29	26	28	30
		RK804	*entB tonB*	13	14	5	5	5
		RK804(pIRS618)	*entB tonB*$^+$	26	25	23	28	30
		WR1024	*ent fhuB*	5	5	5	5	30
		RK809	*entB foxA*	8	5	5	28	30
EWM[c]	400	SL1344/nr	Wild type	+	+	+	+	ND
		BK102	*foxA*	(+)	−	−	+	ND
		TML/nr	Wild type	+	+	+	+	ND
		RK102	*foxA*	(+)	−	−	+	ND

[a] Approximately 10^6 cells of each strain tested were spread over the surface of Vogel-Bonner or EWM agar. Sterile filter paper discs (diameter, 5 mm), each impregnated with 1 μg of a siderophore, were placed on the agar surfaces.

[b] For the Vogel-Bonner medium bioassays, the sizes (in millimeters) of growth zones around siderophore discs were recorded after 18 h of incubation at 37°C; a value of 5 mm indicates that there was no growth stimulation.

[c] In the EWM bioassays, growth was too diffuse to measure accurately as discrete zones, and so the results were recorded as follows: +, obvious growth; (+), limited growth around the disc; and −, no observable growth after 3 to 4 h of incubation at 37°C. ND, growth stimulation not determined.

were somewhat smaller than those of the *tonB*$^+$ parent strain AR1258. The growth of a derivative of strain RK804 harboring the recombinant plasmid pIRS618 (*tonB*$^+$) was stimulated by all of the siderophores tested, and the growth zones were comparable in diameter to those of strain AR1258 (Table 2) but were less dense, which probably reflected a lower growth rate of the plasmid-containing strain.

To test whether a mutation in the *fhuACDB* operon abolished ferrioxamine utilization, strain WR1024 (*fhuB*::MudJ) was tested for growth stimulation in a cross-feeding test on Vogel-Bonner medium supplemented with 2,2′-bipyridyl (Table 2). No growth stimulation was observed around discs loaded with the hydroxamate siderophore coprogen or ferrioxamine B, E, or G, while growth stimulation comparable to that of the parental strain was observed, as expected, around discs loaded with the catechol compound enterobactin.

Distribution of a *Y. enterocolitica foxA* homolog within the genus *Salmonella*. The requirement for FhuBCD and possibly TonB during uptake of iron complexed with ferrioxamines suggests that a receptor(s) for ferrioxamine binding is involved. We previously identified part of an open reading frame in the *S. enterica* serotype Typhimurium genome whose deduced amino acid sequence exhibited 45% identity with the amino acid sequence of the FoxA protein of *Y. enterocolitica* (44), an outer membrane protein involved in the transport of ferrioxamine B (1). Using the *Salmonella* sequence, we designed primers to generate a 287-bp PCR product (cloned in plasmid pRA17) corresponding to an intragenic fragment of the *S. enterica* serotype Typhimurium *foxA* gene. To determine how widespread the *foxA* homolog is among *Salmonella* isolates, chromosomal DNAs of representative strains were digested with *Eco*RI and analyzed by Southern blotting by using the insert fragment of plasmid pRA17 as the probe. All serotypes of *S. enterica* subspecies I, II, and IIIb were positive in this assay (Fig. 2B), while the DNA of strains belonging to subspecies IIIa, IV, VI, and VII and *S. bongori* did not hybridize with the *foxA*-specific probe. To test whether the distribution of *foxA* as determined by hybridization correlated with the ability to utilize ferrioxamines, growth stimulation of representative strains was assessed by performing bioassays on EWM. In each case, only isolates that were positive in hybridization experiments (isolates belonging to serotypes of subspecies I, II, and IIIb) were also positive in bioassays in which ferrioxamine E was the sole source of iron (Fig. 2A).

Genetic analysis of the *Salmonella foxA* locus. To confirm that the *Salmonella* DNA sequence homologous to *Y. enterocolitica foxA* plays a role in ferrioxamine uptake, a derivative of the enterobactin-deficient strain AR1258 (45) containing a pMAP insertion in the *foxA* homolog was constructed as previously described (44). This strain was designated RK809. The effect of the *foxA*::pMAP mutation was assessed by performing standard Vogel-Bonner medium diffusion plate bioassays. As noted above, growth of the *foxA*$^+$ parent strain AR1258 was stimulated by coprogen, enterobactin, and ferrioxamines B, E, and G. Stimulation of the growth of the *foxA*::pMAP mutant RK809 by ferrioxamine B, however, was limited, and this strain did not grow at all in the presence of ferrioxamines E and G (Table 2), although it responded normally to coprogen and enterobactin. Growth assays in tryptone soy broth essentially confirmed the data obtained in the plate bioassays. Ferrioxamines B, E, and G supported growth of strain AR1258 (*foxA*$^+$) in liquid culture (Fig. 3A) but not growth of the *foxA* mutant RK809 (Fig. 3B). These data suggest that the *Salmonella* FoxA protein acts as a receptor for all three ferrioxamines tested.

An alternative possibility is that the inability of RK809 to use ferrioxamines is due to polar effects of the pMAP insertion on downstream genes. To eliminate this possibility, we attempted to clone the entire *foxA* gene in order to test its ability to complement the insertion mutation but were not successful. Instead, we constructed a defined, nonpolar +2 frameshift mutation in the *foxA* gene as described above and tested the effect by performing diffusion plate bioassays on EWM supplemented with 2,2′-bipyridyl (Table 2). The phenotype of mutant strains BK102 and RK102 with regard to ferrioxamine uptake was essentially the same as that observed for strain RK809 carrying the potentially polar *foxA* mutation, confirming that FoxA acts as a receptor for all three ferrioxamines tested.

Sequence analysis of the *Salmonella foxA* locus. For reasons that we cannot explain, it was not possible to clone the entire *Salmonella foxA* gene, and thus the nucleotide sequence of the gene remains incomplete. However, hybridization screening of libraries (in which an intragenic *foxA* fragment was used as the probe) did yield clones containing part of *foxA* that enabled us to add to the previously reported sequence (44). In particular, plasmid pRA5 contained a 2,227-bp insert encoding part of *foxA* and a previously unidentified open reading frame approximately 100 bp upstream of *foxA*. Sequence analysis of this insert fragment indicated that the ATG start codon of the *Salmonella foxA* gene is preceded by a putative ribosome binding site (AATAAA at positions 1098 to 1103) and a putative Fur box (GGTAATAATTCTTATTTAC at positions 1078 to

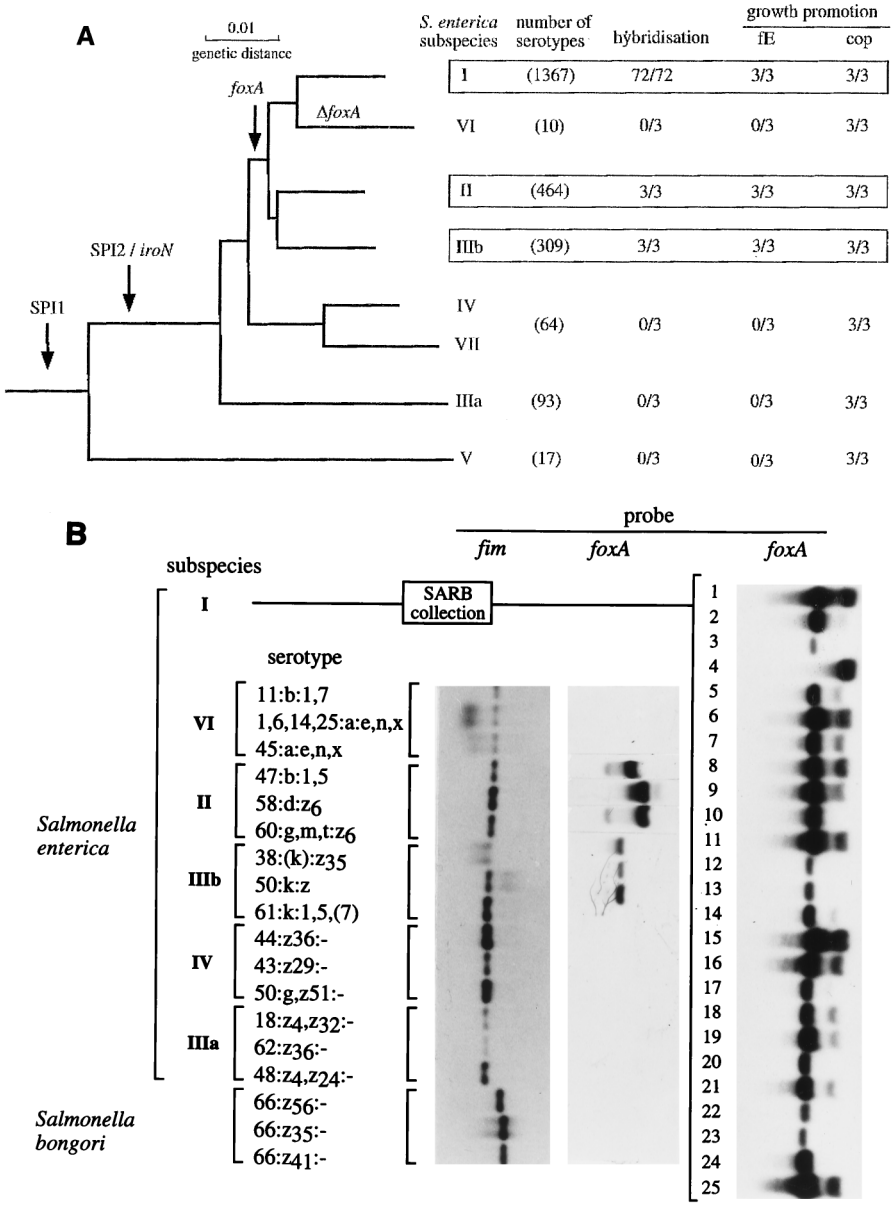

FIG. 2. Distribution of the *foxA* gene and the ability to utilize ferrioxamine E as a sole source of iron among subspecies of the genus *Salmonella*. (A) Neighbor-joining dendrogram showing the relatedness of the various subspecies, based on variation in the combined coding sequences of five housekeeping genes of *Salmonella* spp. (8). The arrows indicate probable introduction by horizontal transfer of SPI1 and SPI2 and of the *foxA* gene; Δ*foxA* indicates possible deletion of the *foxA* gene in the subspecies VI lineage. Note that Reeves et al. (35) did not differentiate *S. enterica* subspecies IV into subspecies IV and VII; therefore, the three serotypes tested were random examples of what was originally designated subspecies IV. The hybridization data are the number of strains that exhibited positive hybridization with the *foxA*-specific probe/total number of strains tested. The growth promotion data are the number of strains whose growth was promoted by ferrioxamine E (fE) or coprogen (cop) as a sole source of iron/total number of strains tested. (B) Southern blot analyses of chromosomal DNA of representative strains of each subspecies probed with a *foxA*-specific DNA fragment. For the SARB collection, only the first 25 of the 72 strains listed by Boyd et al. (7) were used, but all other isolates gave similar positive results with the *foxA* probe. For the representative serotypes of other subspecies, control hybridizations were performed by using a probe derived from the *Salmonella fim* operon (1) in order to validate negative *foxA* hybridizations.

1097) that is identical at 12 of 19 bases to the consensus sequence (9, 13, 41). The *Salmonella foxA* coding sequence determined so far encodes a deduced amino acid sequence containing 374 residues that exhibits 40.7% identity with the amino acid sequence of the *Y. enterocolitica* FoxA protein and significant similarity to the amino acid sequences of a number of other TonB-dependent outer membrane receptor proteins. It was predicted that a signal peptide identified by using the SignalP program (26) would be cleaved between residues 30 and 31. TonB box I and TonB box III were identified at residues 6 to 14 and 111 to 140, respectively, of the mature protein on the basis of a comparison with other previously identified TonB box sequences (1). A comparison with other proteins suggested that TonB box II is probably encoded by part of *foxA* not present in pRA5.

Effect of *foxA* on the colonization of rabbit ileal loops. Competitive colonization in the rabbit ileal loop anastomosis test (43, 47) was used to determine the potential role of the *Salmonella* FoxA protein in virulence. Approximately 1:1 mixtures of pairs of strains were inoculated into test intestinal loops, and

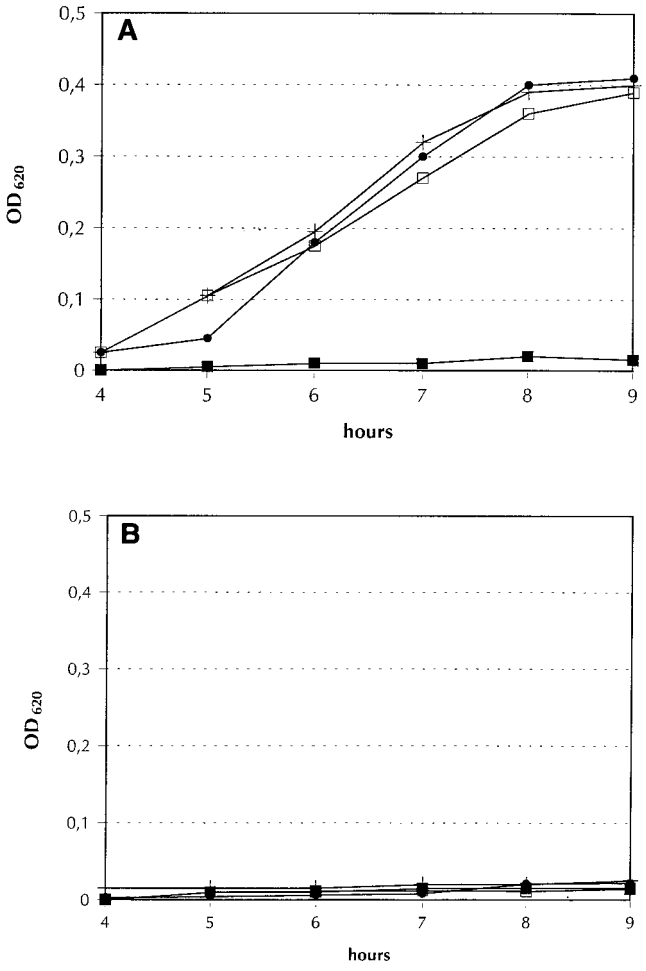

FIG. 3. Growth of *S. enterica* serotype Typhimurium strain AR1258 (*entB*) (A) and its *foxA* derivative RK809 (B) in tryptone soy broth that was supplemented with 200 μM 2,2′-bipyridyl and ferrioxamine B (●), E (+), or G (□) (at a concentration of 1 μg/ml) or not supplemented (■). Optical densities at 620 nm (OD_{620}) were determined at intervals during incubation with aeration by shaking at 37°C. The data are from a representative experiment; we performed at least five experiments in which similar results were obtained.

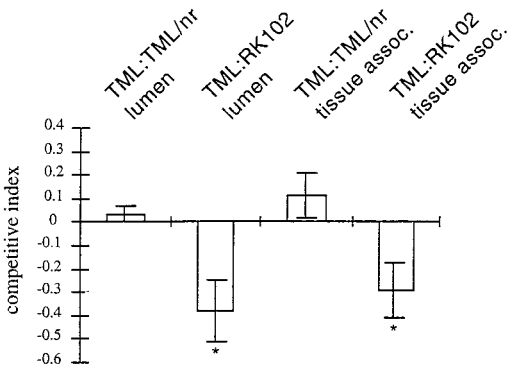

FIG. 4. Comparison of colonization of rabbit ileal loops by *S. enterica* serovar Typhimurium strain TML with colonization of rabbit ileal loops by derivative strains TML/nr and RK102 (*foxA*). We performed competitive colonization experiments involving wild-type strain *S. enterica* TML and either the nalidixic acid-resistant derivative strain TML/nr or the nalidixic acid-resistant *foxA* mutant strain RK102 by using a total of 10 ileal loops in two rabbits. Approximately 5-cm-long loops were inoculated with 1:1 mixtures containing 10^7 or 10^8 cells. After 28 h the proportion of nalidixic acid-resistant coliform bacteria present in the lumen and associated with the gut tissue was determined. The proportion of nalidixic acid-resistant coliform bacteria recovered was expressed as a $\log_{10}$ value (competitive index) and was subjected to a statistical analysis in which the Student *t* test was used. The asterisks indicate competitive indices significantly different ($P > 0.01$) from the input ratio ($\log_{10} 1 = 0$).

a significant variation from equivalence among bacterial mixtures recovered from the lumen contents or associated with the lumen walls was considered an indication that there were differences in the colonizing abilities of the two strains. There was no significant difference in the recovery of strains TML and TML/nr, which ruled out the possibility that the mutation causing nalidixic acid resistance had any effect in the latter strain. However, recovery of the *foxA* mutant RK102, a derivative of TML/nr, was significantly less than recovery of strain TML both in the lumen contents and associated with intestinal loop tissue (Fig. 4), strongly suggesting that FoxA plays a role in intestinal colonization by *Salmonella* strains. Addition of 1 mg of ferrioxamine E per ml to mixed inocula did not have a significant effect on the competitive indices observed (data not shown).

Mouse virulence of *foxA*$^+$ and *foxA* strains. To determine if a mutation in *foxA* affects the ability of *S. enterica* serotype Typhimurium to cause lethal infections in mice, LD_{50}s were determined after intragastric inoculation. The LD_{50} of strain SL1344/nr was calculated to be 10^5 CFU, which was approximately equal to the LD_{50} reported previously for the nalidixic acid-sensitive parent strain SL1344 (45). In contrast, the LD_{50} of the *foxA* mutant strain BK102 (a derivative of SL1344/nr) was $>10^9$ CFU, and no deaths occurred up to 28 days postinoculation at any inoculum dose.

Five mice from each group intragastrically inoculated with 10^3, 10^4, or 10^5 CFU and four mice from each group inoculated with 10^6, 10^7, 10^8, or 10^9 CFU of strain BK102 were challenged with a single intragastric dose containing approximately 5×10^8 CFU of the fully virulent strain SL1344/nr in 0.2 ml of phosphate-buffered saline on day 28 after the initial inoculation. The challenge dose, which was greater than the LD_{50}, would have resulted in the death of naive mice within 8 days. The number of deaths in each group was recorded 10 days after the challenge (Table 3). There were few or no survivors in the groups originally inoculated with 10^3, 10^4, or 10^5 CFU of strain BK102, indicating that there was little or no protection. However, there was significant protection in groups of mice originally inoculated with $>10^5$ CFU of strain BK102.

LD_{50}s were also determined following intravenous inoculation of mice; consistent with previous reports (45), the LD_{50} of the parent strain SL1344/nr was <10 CFU, but none of the

TABLE 3. Protection of mice previously inoculated with strain BK102 against subsequent challenge with *S. enterica* serotype Typhimurium strain SL1344/nr

BK102 inoculum (CFU)[a]	No. of survivors/no. of mice tested[b]
10^9	3/4
10^8	3/4
10^7	4/4
10^6	3/4
10^5	0/5
10^4	2/5
10^3	0/5

[a] The BK102 inoculum was administered 28 days prior to challenge with strain SL1344/nr.

[b] Number of mice surviving 10 days after inoculation with 5×10^8 CFU of SL1344/nr/number of mice tested.

mice inoculated with 10^4 CFU of the *foxA* strain died, indicating that the LD_{50} of BK102 was $>10^4$ CFU.

DISCUSSION

The *foxA* gene encoding the *Salmonella* ferrioxamine receptor was first identified as a cloned subgenic fragment having a deduced amino acid sequence that was 45% identical to the amino acid sequence of the specific outer membrane ferrioxamine receptor of *Y. enterocolitica* (44). Phenotypic analysis of *foxA* mutants carrying either a pMAP insertion (potentially polar) mutation or a defined nonpolar frameshift mutation demonstrated that the FoxA protein is required for utilization of all three ferrioxamine molecules tested. Moreover, using the subgenic *foxA* fragment as a probe, we showed that the *foxA* gene is not ubiquitous among *Salmonella* serotypes but is limited to subspecies I, II, and IIIb. The presence of *foxA* correlated with growth stimulation by ferrioxamine E in bioassays; strains that were negative as determined by hybridization were also negative in bioassays performed with ferrioxamine E. Although this observation may suggest that inclusion of ferrioxamines as medium supplements would not be useful in all cases, it is important to note that the three subspecies that express *foxA* account for >99% of the clinical isolates and 2,140 of 2,324 of the serotypes described so far. We therefore recommend that ferrioxamine E should be included in preenrichment and selection protocols for rapid identification of the vast majority of clinically significant serotypes of the genus *Salmonella* in food and feed.

If it is assumed that subspecies I, II, and IIIb contain a greater diversity of serotypes because they have adapted more successfully to the pathogenic lifestyle, then it is possible that *foxA* (or a closely linked determinant) may be selectively advantageous for *Salmonella* pathogenicity. It has been suggested that limited phylogenetic distribution is characteristic of introduction of a genetic determinant into a genome by horizontal gene transfer (28). Thus, ancestral *Salmonella* serotypes apparently became more successful by acquiring genetic determinants, such as SPI1 and SPI2, by bacteriophage- or plasmid-mediated transfer from other organisms. SPI1 is present in virtually all *Salmonella* serotypes but not in *E. coli* and so was probably acquired shortly after the taxa diverged from their common ancestor. SPI1 encodes determinants required for invasion of epithelial cells (10, 25), a basic requirement for *Salmonella* pathogenesis. Similarly, since SPI2 is present in all serotypes of *S. enterica* but not in *S. bongori*, it probably entered the *S. enterica* lineage shortly after *S. enterica* and *S. bongori* split from their common ancestor (29). Acquisition of SPI2, which is required for systemic infection (16, 30, 42), resulted in a more successful lineage, as judged by adaptive radiation into a greater range of hosts, including both cold- and warm-blooded animals, and a greater diversity of serotypes. It is tempting to speculate that introduction of *foxA*, which probably occurred after the split leading to subspecies I, II, IIIb, and VI on the one hand and subspecies IV and VII on the other, may have contributed to the further radiation of serotypes belonging to subspecies I, II, and IIIb. Fortuitous loss of the *foxA* gene by deletion, as in subspecies VI, could lead to underrepresentation of serotypes of a subspecies if the selective advantage is lost concomitantly.

As is typical for siderophores of enteric bacteria, utilization of ferrioxamine G by *Salmonella* strains is completely dependent on the energy-transducing inner membrane protein TonB for transport across the outer membrane following interaction with FoxA. In contrast, and very unusually, utilization of ferrioxamines B and E occurs in the absence of functional TonB protein, albeit at lower efficiency. One of the few other examples of TonB-independent iron supply in *Salmonella* strains is the iron supply mediated by the enterobactin precursor 2,3-dihydroxybenzoic acid (18); in this case it is believed that the small size of the molecule compared to other siderophores allows it to diffuse freely through the outer membrane porins. The molecular sizes of ferrioxamines B, E, and G, however, are not significantly different from one another, and so the mechanism by which ferrioxamines B and E traverse the outer membrane in the absence of TonB is not clear. This mechanism appears to depend on the FoxA receptor, however, since no leaky phenotype was observed in any strain containing a mutation in the *foxA* gene; this essentially eliminates the possibility that passive diffusion through porins occurs. Transport of ferrioxamines B, E, and G across the inner membrane involves the periplasmic binding protein-dependent permease comprising the FhuBCD proteins, which is the same route as the route for other hydroxamate siderophores in *E. coli* and *Salmonella* strains (21).

It is not clear why *Salmonella* strains express a receptor for siderophores that they do not synthesize. We assume that ferrioxamines are not generally available in the vertebrate hosts, except possibly in the intestinal lumen, where they may be secreted by members of the normal gut flora. However, although some members of the family *Enterobacteriaceae* do indeed synthesize ferrioxamines as their principal siderophores (37), there is no direct evidence that ferrioxamines are secreted by gut commensal species. Nevertheless, the role of FoxA was investigated in the context of experimental infections by *S. enterica* serotype Typhimurium in rabbit ileal loops and in the murine typhoid model. The former model has been reported to be a good model for enteric infection, which allows measurement of intestinal colonization and fluid secretion, while the latter model is widely used as a model for typhoid fever. In each case, *foxA*$^+$ and defined *foxA* derivatives of strains known to respond well in either model were used (TML in rabbits [12] and SL1344 in mice [17]). We observed that there was a small but significant decrease in the ability of the *foxA* mutant of TML to compete with the wild-type strain during colonization of rabbit ileal loops. Addition of ferrioxamine E with the inoculum did not affect the competitive index. This may suggest that sufficient ferrioxamine for maximal growth was already present in the loops, perhaps due to secretion by commensal bacterial species. However, it was not possible to demonstrate the presence of ferrioxamines in bioassays with appropriate indicator strains (data not shown). A more likely possibility is that the selective advantage of FoxA is not due simply to its function as a receptor for the transport of ferrioxamines as sources of iron. This possibility is strongly supported by the data obtained from the mouse typhoid model, in which a disease that occurs predominantly in the tissues comprising the reticuloendothelial system would not be expected to be abrogated by a mutation in genes involved in the early colonization stages of infection. Attenuation of the *foxA* mutants following both oral inoculation and intravenous inoculation was surprising and cannot be explained simply on the basis of the inability of these mutants to utilize ferrioxamines as sources of iron. The increases in LD_{50}s (>10,000-fold) were in fact considerably greater than the increases in LD_{50}s observed for mutations in the *lpf* and *inv* genes (10- to 50-fold with oral inoculation and no effect with parenteral inoculation), both of which have effects during the early stages of infection, including invasion of the intestinal mucosa (5, 11). Furthermore, a strain lacking a functional TonB protein was mildly attenuated in experimental infections of mice (there was an approximately fivefold increase in the LD_{50}), but only with oral inoculation,

and bacteria were recovered in significantly smaller numbers from the Peyer's patches, the normal focus of *S. enterica* serotype Typhimurium infections during the early stages of infection in the murine host (45). Although the reason for the significantly reduced virulence of *foxA* mutants is obscure, a number of possibilities may be considered. First, since the entire *foxA* gene could not be cloned and thus it was not possible to complement the frameshift mutation in virulence assays, indirect effects of the mutation (i.e., effects other than loss of the *foxA* function) cannot be eliminated (for example, expression of a truncated form of FoxA, which may compromise the integrity of the outer membrane). Perhaps more likely is the possibility that FoxA has an essential role in addition to its role in the uptake of ferrioxamines, just as, for example, OmpC has been implicated in bacterial association and internalization by macrophages (25a). The expression of *foxA* is derepressed under iron-limiting conditions (44); it is therefore probable that in the interstitial medium and blood serum where *Salmonella* cells initially interact with macrophages, the presence of transferrin results in high levels of FoxA in the outer membrane. Defining the precise role of FoxA in these circumstances requires further analysis; experiments to determine the mechanism of attenuation in *foxA* mutants and the ability of these mutants to protect against challenge with a fully virulent *foxA*$^+$ strain are in progress in our laboratories.

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