Salmonella typhi Iron Uptake Mutants Are Attenuated in Mice

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Iron starvation interferes drastically with the multiplication and virulence of Salmonella typhi mutants defective in enterochelin synthesis or enterochelin transport. Growth of these mutants is inhibited in the presence of human sera and unsaturated transferrin and is restored by fully saturated transferrin. The mutants exhibit decreased ability to grow in HeLa cell monolayers and are attenuated in mice. These findings are consistent with the S. typhi enterochelin system playing a role in the pathogenesis of typhoid fever.

Because iron is absolutely required for normal in vitro multiplication of Salmonella typhi, the organism has developed active mechanisms for iron uptake from the environment that include production of the high-affinity iron chelator enterochelin (8, 9). The importance of this chelator to S. typhi metabolism is underscored by the strict requirement for 2,3-dihydroxybenzoic acid in S. typhi araA mutants unable to synthesize enterochelin (7). The situation is less clear in infected hosts, but indirect evidence suggests that iron is also critical to S. typhi growth in vivo. All clinical isolates of S. typhi produce enterochelin (8, 9), patients with typhoid develop high titers of antibodies to outer membrane proteins (OMPs) potentially involved in enterochelin transport (2, 3, 9), patients with conditions leading to hyperferremia (malaria, sickle cell anemia, Oroya fever, and schistosomiasis) have an increased frequency of typhoid (6, 12, 16), and iron appears to be absolutely necessary for the establishment of S. typhi infections in mice (15, 17). However, the role of the enterochelin iron uptake system in the infection of mice with Salmonella typhimurium (a model for typhoid fever in humans) remains controversial, and establishment of S. typhimurium infections in this species is not clearly iron dependent (2, 4, 22). In order to examine the role of the enterochelin system in S. typhi infections more directly, we have generated mutants in both the enterochelin synthesis and enterochelin transport systems of S. typhi. Growth of these mutants in the presence of human sera and in unsaturated and saturated human transferrin has been evaluated, and their ability to invade HeLa cells and virulence in mice have been determined.

Strains, media, and chemicals. The following strains were used: a nalidixic acid-resistant (Nal') derivative of tryptophan-requiring S. typhi Ty2 isolated in our laboratory (2); Escherichia coli SM10 containing the suicide vector pRT733 provided to us by J. Mekalanos (13, 20); and enterochelin-deficient E. coli mutants AN90-60 (entD), AN192-60 (entB), and AN102 (lepA) (4). Luria-Bertani medium and nutrient broth (Difco Laboratories, Detroit, Mich.) and Tris-glucose minimal medium were prepared and used as described elsewhere (9). Where required, the following were added: 2,2'-dipyridyl (DPD; 100 μM/ml; Sigma Chemical Co., St. Louis, Mo.), alkaline phosphatase substrate indicator 5-bromo-4-chloro-3-indolylphosphate-p-toluidine salt (XPh; 40 μM/ml; Sigma), nalidixic acid (100 μg/ml; Sigma), and kanamycin (25 μg/ml; Sigma).

Isolation of mutants. TnphoA mutants were isolated by mating S. typhi Ty2 (Nal') with SM10(pRT733) as the TnphoA donor (13, 20); 106 kanamycin-resistant S. typhi transconjugants were obtained. From 400 blue colonies containing TnphoA fusions, 7 isolates potentially defective in their iron uptake system were identified by their inability to grow in iron-poor media. They were then purified; to rule out auxotrophies, their ability to grow in Tris-glucose minimal medium plus tryptophan was confirmed; and they were kept for further studies. Characterization of S. typhi mutants as enterochelin synthesis defective or enterochelin transport defective was done by cross-feeding, as previous work indicated that cross-feeding was more reliable than the Arnow reaction to detect enterochelin production in S. typhi (9).

Growth curves. Human sera were collected from five healthy individuals from an area in which typhoid is not endemic and heat inactivated at 56°C (1). Serum antibodies cross-reacting with S. typhi Ty2 Nal' were removed from pooled sera by absorption with live S. typhi Ty2 cells until agglutination was not detected; absorbed sera were stored at −20°C (1, 3). Human transferrin (40 μM; Sigma) was determined to be 13% iron saturated by a Hitachi 117 chemistry analyzer (Boehringer Mannheim, Indianapolis, Ind.); 100% saturated human transferrin was prepared (11), and its degree of saturation was confirmed as described above. Viable counts on Luria-Bertani agar plates were used to determine the number of bacteria under different growth conditions (9).

DNA hybridization. Southern hybridization of chromosomal DNA of S. typhi Ty2 and its mutants was performed with TnphoA DNA probes using the Genius 1 DNA labeling and detection kit (Boehringer Mannheim) (8, 9).

OMP analysis. Outer membranes were isolated and visualized by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described previously (3, 8, 9).

Invasion of HeLa cells. The ability of S. typhi Ty2 Nal' and its iron uptake mutants to invade and multiply inside monolayers of HeLa cells was determined as described elsewhere (14).

Virulence in the mouse mucin model. The virulence of S. typhi Ty2 Nal' and its iron uptake mutants was assayed in the mouse mucin model using female CFW mice weighing 22 to 25 g each (17). Animals were injected with 0.5 ml of bacterial suspensions intraperitoneally and observed twice daily for a period of 7 days (17), and the 50% lethal dose (LD50) was calculated by the Reed and Muench method (19).

Isolation and characterization of S. typhi iron uptake-deficient mutants. Seven TnphoA mutants were unable to grow...
TABLE 1. Cross-feeding of *S. typhi* TnphoA and *E. coli* iron uptake mutants by *E. coli* *fepA* and *ent* mutants and *S. typhi* Ty2 and JA055

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<th>Cross-fed mutant</th>
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<tr>
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<td><em>E. coli AN102</em></td>
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<td><em>S. typhi</em></td>
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<td>JA032</td>
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<td>JA138</td>
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* –, +, and ++, presence of 0, <50, approximately 50, and approximately 100 colonies in the zone of growth stimulation produced by the cross-feeding strain (9). With *E. coli* AN192-60 *entB* as the cross-feeding strain, no cross-fed colonies grew.

on iron-poor Tris-glucose medium supplemented with 100 μM DPD, suggesting a defect in the enterobactin-mediated iron-uptake system (9). Iron starvation increased the blue color of the colonies of these mutants in the presence of XP (13, 20). Three of these *S. typhi* mutants, JA032, JA055, and JA138, were used in further studies. Cross-feeding experiments in agar plates (Table 1) suggested that JA032 and JA138 were probably defective in the ability to synthesize enterochelin, while JA055 appeared to be defective in enterochelin transport and did not cross-feed *E. coli* *ent* mutants AN192-60 and AN90-60 and *S. typhi* JA032. Consistent with this interpretation, spent media of the *fepA* mutant *E. coli* AN102 corrected DPD-produced growth inhibition of *S. typhi* JA032 and JA138 but had no effect on *S. typhi* JA055 (data not shown). SDS-PAGE analysis of OMPs of wild-type and mutant bacteria grown in nutrient broth with DPD indicated the presence of three proteins (68, 78, and 83 kDa) in *S. typhi* Ty2 and in *S. typhi* JA032 and JA138 (data not shown). These OMPs have been previously characterized by us as induced by iron depletion (3, 8, 9). In contrast, growth of *S. typhi* JA055 in iron-poor media resulted in induction of only the 78-kDa iron depletion-induced protein (data not shown) (9). These results provide additional evidence for believing that *S. typhi* JA032 and JA138 are enterochelin synthesis mutants and that *S. typhi* JA055 is an enterochelin transport mutant. DNA hybridization studies with chromosomal DNA of wild-type *S. typhi* Ty2 and its iron uptake-deficient derivatives digested first with the restriction enzyme HindIII and later with BglII or NcoI and probed with *XhoI-EcoRI* (1.3-kb) and *HindIII* (3.4-kb) DNA fragments of TnphoA, respectively, indicated that mutants JA032 and JA138 have only one insertion of the transposon (20). Mutant JA055 appears to have two insertions. In *S. typhi* JA032 and JA055, a mouse monoclonal antibody against *E. coli* alkaline phosphatase permitted the detection of iron starvation-induced fusion products in immunoblots (9a).

**Growth of *S. typhi* Ty2 and its iron uptake-deficient derivatives in human sera and human transferrin.** Growth of *S. typhi* mutants JA032, JA138, and JA055 was severely inhibited in 50% heat-inactivated, *S. typhi*-absorbed normal human serum, a concentration that permitted growth of wild-type *S. typhi* Ty2 (Fig. 1A). Similar growth inhibition was observed when mutants were grown in nutrient broth supplemented with 40 μM 13% iron-saturated human transferrin (Fig. 1B), approximately the concentration of transferrin found in human serum. However, when the transferrin was 100% iron saturated, growth of *S. typhi* iron uptake-deficient mutants was restored to a rate similar to that of *S. typhi* Ty2 (data not shown). Neither the wild-type *S. typhi* nor the mutants grew in the presence of 75% heat-inactivated, *S. typhi*-absorbed normal human serum (data not shown).

**Growth of *S. typhi* Ty2 and its iron uptake-deficient derivatives in monolayers of HeLa cells.** Continuous microscopic observation of HeLa cell monolayers after simultaneous infection with different *S. typhi* strains indicated that lysis of the monolayer by *S. typhi* iron uptake-deficient mutants JA032, JA055, and JA138 was significantly slower than lysis induced by *S. typhi* Ty2 (Fig. 2) (14). Bacterial viable counts of six wells in six different experiments indicated that *S. typhi* mutants JA055 and JA138 multiplied intracellularly in the monolayers to only 37 and 49%, respectively, of numbers obtained with wild-type *S. typhi* Ty2. Unexplainedly, we could not obtain reliable viable counts with *S. typhi* JA032.

**FIG. 1.** Growth of nalidixic acid-resistant *S. typhi* Ty2 (●) and its iron uptake-deficient derivatives JA032 (○), JA055 (△), and JA138 (□) in 50% normal human sera (A), nutrient broth with 40 μM 13% saturated human transferrin (B), and nutrient broth with 40 μM 100% saturated human transferrin (C).
Virulence of S. typhi Ty2 and its iron uptake-deficient derivatives in the mouse mucin model. Figure 3 shows the mortality of mice inoculated with different doses of wild-type and mutant S. typhi strains. The LD₅₀ values of S. typhi Ty2, its enterochelin-deficient derivative JA032, and its enterochelin transport-deficient derivative JA055 were 8.5 × 10⁶, 3.5 × 10⁶, and 6.1 × 10⁵ CFU, respectively.

Two classes of iron uptake mutants of S. typhi were generated by TnphoA mutagenesis. One class (JA032 and JA138), cross-fed by enterochelin-producing strains of E. coli and S. typhi, was defective in the synthesis of enterochelin. The second class (JA055) could not be cross-fed by enterochelin but was able to support the growth of E. coli and S. typhi enterochelin synthesis mutants, suggesting a deficient transport of this chelator (9). Analysis of OMPs of these mutants by SDS-PAGE confirmed this interpretation, as JA055 lacked expression of two of the three iron starvation-induced proteins, including the 83-kDa protein previously identified as the FepA-like protein of S. typhi (9). The expression of all three of these iron starvation-induced proteins was conserved in mutants JA032 and JA138 (9).

Iron starvation substantially restricts the growth of S. typhi mutants defective in either synthesis or transport of enterochelin, as manifested by the reduced viable counts in the presence of human sera, in media supplemented with unsaturated transferrin, and in epithelial cell monolayers of HeLa cells. That this decreased growth is the result of iron starvation is also suggested by the normal multiplication of these mutants in Tris-glucose medium without DPD, in L broth, and in the presence of 100% saturated transferrin and by the ability of iron starvation to upregulate alkaline phosphatase expression of these mutants (9, 20). Moreover, the degree of inhibition of growth of wild-type S. typhi Nat⁺ produced by human sera and unsaturated human transferrin is similar to that produced by the iron chelator DPD (9). The inhibition of wild-type S. typhi growth by iron limitation and its growth inhibition in 50% heat-inactivated human sera may be explained by the production of only small amounts of enterochelin, as previously demonstrated (18). The limited ability of wild-type S. typhi to cross-feed putative S. typhi ent and E. coli ent mutants (Table 1) confirms this. The cross-feeding assay results (9), changes in the profile of OMPs (9), the fact that TnphoA is inserted only once in two mutants (13, 20), the ability of iron starvation to upregulate the expression of alkaline phosphatase in the mutants, and restoration of the rate of growth of these mutants to that of the S. typhi Ty2 wild-type strain by iron-rich media and 100% saturated transferrin also suggest that the observed inhibitory effect on growth is due to iron starvation and not due to mutagenesis of other uncharacterized virulence genes or to polarity affecting the expression of other genes secondary to insertion of TnphoA (13, 20). This appears to be the case even for mutant JA055, which may have an added TnphoA insertion outside the enterochelin genes.

These results, and our previous findings that the enterochelin system is present in all clinical isolates of S. typhi (8) and appears to be induced in patients with typhoid fever (2, 9), suggest that S. typhi iron transport mediated by enterochelin plays a critical role in the ability of S. typhi to multiply in the human host in the extra- and intracellular niches of the human host, where little, if any, free iron is available (2, 6, 10, 16). The postulated critical role of enterochelin-mediated iron transport in S. typhi pathogenicity is fully consistent with the decreased mortality and marked increase in LD₅₀ for mice infected with S. typhi iron transport mutants and could also explain the increased frequency of infections with S. typhi in individuals with clinical syndromes associated with increases of saturated transferrin above the physiological levels (6, 10), as enterochelin preferentially acquires iron from transferrin (5).

The very low numbers of S. typhi cells in the bloodstream (2, 16), the increased frequency of typhoid in hyperferremic states (6, 16), the low levels of enterochelin produced by S. typhi (18) (Table 1), and the fact that high temperature appears to downregulate both the synthesis of enterochelin in Salmonella cells (21) and plasma iron concentrations in humans (6) seem to suggest that iron-withholding mechanisms of the human host restrict S. typhi growth in an efficient and successful manner. The differences in iron uptake requirements of S. typhi and S. typhimurium may have a role in the inability of the former species to cause disease in mice and other animals (2, 4, 15, 18, 22).

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REFERENCES


