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Enterochelin (Enterobactin): Virulence Factor for *Salmonella typhimurium*

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The ability of *Salmonella typhimurium* to synthesize enterochelin (enterobactin; ENT) affects its capacity to grow both in vivo and in vitro. An ENT mutant (96-1), blocked in the conversion of chorismate to 2,3-dihydroxybenzoate, was derived from SR-11, a strain of high mouse virulence. This mutant was unchanged in the other characteristics tested: colonial, biochemical, antigenic, and cellular. In contrast to SR-11, growth of this mutant in complement-inactivated human serum was strongly inhibited. However, addition of 5 μM ENT to the cultures relieved their inhibition. Viable counts of bacteria injected into the mouse peritoneal cavity showed that without ENT, growth of 96-1 was inhibited markedly; with ENT, the apparent growth rate of 96-1 exceeded that of SR-11. The 50% lethal dose (LD_{50}) of 96-1 was 2 to 3 log units higher than that of SR-11. When ENT was injected, the ENT^- mutant exhibited an ENT-dose-related decrease in its LD_{50}. A single injection of 300 μg of ENT per mouse with the inoculum reduced the LD_{50} of 96-1 to that of the wild-type strain. These findings support the contention that ENT is a virulence factor for *S. typhimurium*.

All microorganisms with the possible exception of the lactic acid bacteria require iron. Although iron is one of the most prevalent of the earth's elements, in aerobic environments the amount of free iron available for assimilation by microorganisms is restricted due to the proclivity of ferric iron to form large, insoluble aggregates at neutral or alkaline pH (26). To acquire the necessary iron from these aggregates, aerobic microorganisms have evolved specialized iron-solubilizing and -transporting ligands, chelating agents, which have been termed siderophores (10, 14).

Siderophores can be divided into two general types, hydroxymate and phenolate (14). In vitro, *Enterobacter aerogenes* (15, 17), *Escherichia coli* (15, 17), *Salmonella typhimurium* (20), and possibly other enteric bacteria secrete a phenolate-type siderophore named enterochelin (ENT; 15) or enterobactin (20).

A pathogen does not have to contend with the insolubility of ferric iron, but must obtain its iron from its host. Although the amount of iron present in host fluids is more than adequate for microbial growth, the iron-binding proteins (transferrin and ferritin in the serum, lactoferrin in secretions) sequester essentially all the iron in these environments (3, 23, 28, 30). The most likely mechanism whereby a pathogen successfully competes with these iron-binding proteins is via siderophore production. Phenolate-type siderophores were demonstrated to compete successfully with purified transferrin for iron (22; R. J. Yancey and C. E. Lankford, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, D26, p. 55). Also, siderophores reverse the inhibitory effect of blood serum for *E. coli* (7, 22), *Mycobacterium tuberculosis* (6, 8, 11), and *S. typhimurium* (31; D. J. Purifoy and C. E. Lankford, Tex. Rep. Biol. Med. **23**:637, 1965; D. J. Purifoy, T. D. Wilkens, and C. E. Lankford, Bacteriol. Proc., p. 55, 1966; Yancey and Lankford, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, D26, p. 55).

While there is a preponderance of evidence that the availability of iron will enhance the apparent virulence of a pathogen for a host (3, 18, 28, 30), the importance of siderophores is unproven. Rogers (22) found that phenolate siderophore production influences *E. coli* infection. Two serologically different strains, varying in ability to produce siderophore in low-iron media, differed in their virulence for mice. Rogers concluded that siderophores were a virulence factor for *E. coli*. However, a comparison of two different serotypes, which might differ qualitatively or quantitatively in one or several virulence factors unrelated to siderophore production, raises questions about the validity of Rogers' conclusion.

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Jones et al. (5) found that the iron-chelating agents desferrioxamine and 2,3-dihydroxybenzoic acid (DBA) increase the iron availability to *S. typhimurium*, increasing the apparent virulence of the pathogen for mice. Iron overload, induced by injection of heated human blood, enhanced this virulence increase. Though statistically significant, the observed differences between siderophore-treated and untreated mice were small and lasted for a few days only.

To more properly assess the relationship between virulence and siderophore production, we derived ENT synthesis mutants of a strain of *S. typhimurium*. We found that production of ENT is a requirement for growth in blood serum and for high mouse virulence, and we conclude that ENT is a virulence factor for *S. typhimurium*.

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MATERIALS AND METHODS

Bacteria. For most of these studies, two strains of *S. typhimurium* were used. The wild-type strain was *S. typhimurium* SR-11, from the laboratory of L. Joe Berry of this department. This strain was a highly virulent, xylose-fermenting descendant of strain RIV (24). Strain 96-1 was an ENT$^-$ derivative of SR-11.

For bioassay of ENT, *S. typhimurium enb-7* was used (19). This ENT$^-$ strain and its parent, *S. typhimurium* LT-2, were provided by J. B. Neilands, University of California, Berkeley.

ENT was purified from cultures of *E. coli* AN102, an *fep*$^-$ derivative of *E. coli* K-12 (4) obtaind from F. Gibson, Australia National University, Canberra.

These cultures were maintained on brain heart infusion agar (BHIA) slants. Inocula for all experiments were from the second of two consecutive 12-h slant cultures. Cells were harvested from the slants, washed three times by centrifugation, and diluted in either phosphate buffer (pH 7.0) or physiological saline. Viable counts were made by spreading 0.1-ml volumes of the appropriate dilution onto nutient agar.

Culture media. For bioassay of ENT production and for selection of ENT mutants, the citrate-glucose-salts medium of Vogel and Bonner (27), medium E (ME), was used. The citrate in this medium inhibits growth of ENT$^-$ strains (19), presumably by sequestering all the iron. ENT$^-$ strains, therefore, are siderophore auxotrophs in this medium. The growth medium for ENT purification from culture supernatant fluids of *E. coli* AN102 was that of O'Brien et al. (16).

Serum and serum cultures. Human serum was obtained from four to eight healthy volunteers. The heat-labile complement components were inactivated by heating the serum at 56°C for 30 min.

Serum cultures were incubated at 37°C in a candle jar. Supplements and inocula were added in quantities of 1.0% or less of the final volume. Growth of the bacteria was monitored by periodic viable counts.

Isolation of ENT$^-$ mutants. Strain SR-11 was treated with ethyl methane sulfonate by the method of Meynell and Meynell (12). After segregation in ME, the cells were incubated in nitrogen-free ME supplemented with 0.1 μM ENT to exhaust metabolite stores. The cells then were diluted into complete ME (without ENT) and subjected to 300 U of penicillin G per ml at 37°C for 90 min. After the penicillin was washed from the cells, the bacteria were plated onto ME solidified with 1.0% Ionagar (Oxoid). The plates were incubated at 37°C for 43 h, and the colonies that developed were marked. The plates then were sprayed lightly with an aqueous suspension from an ethyl acetate extract of an ME culture filtrate of SR-11. The plates were incubated for an additional 48 h, and the new colonies were picked and transferred to BHIA. The suspected mutant clones were screened for the ability to grow with, but not without, the addition of ENT.

Chemicals. Chemicals were of analytical grade when possible. Ethyl acetate was redistilled before use. Authenic ENT was a gift from J. B. Neilands.

Preparation of purified ENT. ENT was isolated from supernatant fluids of late-log-phase cultures of *E. coli* AN102 by modification of the methods of Gibson and co-workers (9, 16). Twenty liters of culture supernatant fluid was passed through a column of diethylaminoethyl-cellulose at 4°C. The adsorbed siderophore was eluted with 3 M NH_4Cl containing 5 mM H_2SO_4 and 20% (vol/vol) ethanol. After evaporation of the ethanol, the eluant was adjusted to pH 2.0 and extracted three times with one-fifth volumes of ethyl acetate. The combined extracts were evaporated over 100 ml of 0.1 M KH_2PO_4 buffer (pH 7.0) containing 25 mM $FeSO_4$. This magenta-colored solution was washed three times with equal volumes of ethyl acetate, acidified, and again extracted with three equal volumes of ethyl acetate. The combined extracts were washed with Chelex 100 (deferrated)–0.1 M phosphate buffer (pH 7.2) and dried with sodium sulfate. After the purity and identity of the preparation was established by comparison with authentic ENT in two-dimensional thin-layer chromatographs (16), the concentration of ENT was determined from the absorbance at 315 nm (E = 9,390 cm^{-1} M^{-1} in ethyl acetate; 17).

Since ferric ENT is more stable than ENT, stock solutions of siderophore were stored as ferric ENT at −20°C. Aqueous deferri-ENT (referred to as ENT) solutions were prepared as needed in 0.1 M tris-(hydroxymethyl)-aminomethane-hydrochloride buffer (pH 6.5) containing 20% (vol/vol) ethanol (9).

Examination of culture supernatant fluids for ENT or related compounds. *S. typhimurium* SR-11 and its suspected ENT$^-$ mutants were grown to stationary phase in medium A (19) and in mannitol-salts minimal medium (31). Phenolate production was measured on 10-fold concentrates of the culture filtrates in three ways: (i) by the colorimetric method of Arnow (1); (ii) by thin-layer chromatography (16); and (iii) by bioassay with *S. typhimurium enb-7*.

S. typhimurium enb-7, when growing in ME, gave a dose response to ENT, 2,3-dihydroxybenzoylserine, DBA, and the hydroxamic acid schizokinen. The limit of ENT detection by this assay was approximately 5 nM, whereas a 50% maximal response was obtained at 100 nM ENT.

Mice. The mice, 6- to 8-week-old CFW females averaging 20 g, were obtained locally.

LD_{50} determinations. Fifty percent lethal dose (LD_{50}) determinations, on groups of six to eight mice per dose, were estimated by the method of Reed and Muench (21). Inoculum cells, diluted in physiological saline, were injected intraperitoneally in 0.1-ml aliquots.

Viable counts in the mouse peritoneal cavity. Both SR-11 and 96-1 (with or without ENT) were injected into the peritoneal cavity of groups of 18 to 20 mice. At intervals, two mice from each group were sacrificed with CO_2 gas. Five milliliters of sterile saline (0.85% NaCl) was injected into the peritoneal cavity, and the abdomen was massaged for 1 min. The wash fluid was recovered by opening the cavity and withdrawing the fluid with a Pasteur pipette. This material was diluted appropriately and plated onto nutrient agar. Recovery of bacterial cells from the peritoneal cavity by this technique was 90 to 101% at time zero. Gentle homogenization of the peritoneal wash solution with a tissue homogenizer (2 to 5 min) did not significantly influence these counts up to 12 h postinfection.

RESULTS

Characterization of mutants. Several suspected ENT mutants were found not to produce ENT or its hydrolysis products by the three assays listed in Materials and Methods (theoretical detection limit, 5 nM ENT). One of these mutants (96-1) was tested further to determine the approximate location of the lesion in ENT synthesis, its biochemical, colonial, cellular, and antigenic characteristics, its growth pattern in human serum, and its virulence for mice.

Lesion in ENT synthesis. Since 96-1 was not auxotrophic for aromatic amino acids or vitamins as determined by the screening procedure in ME, the biosynthetic block in ENT synthesis must be beyond chorismate, the branch-point intermediate (10). Addition of 10 μM DBA allowed maximal growth of this mutant in ME and conversion of DBA to ENT. Ethyl acetate extracts of stationary-phase culture filtrates, when assayed by thin-layer chromatography, showed that this strain was capable of converting DBA completely to ENT or to the ENT hydrolysis product, 2,3-dihydroxybenzoylserine. No accumulation of DBA was detected in medium A or in mannitol-salts medium. Therefore, strain 96-1 was similar to the class II *enb* mutants of *S. typhimurium* LT-2 (19) in that it was blocked in the synthesis of ENT between chorismate and DBA. Further characterization of the lesion in ENT synthesis was not attempted.

Other characteristics. Colonial characteristics, 34 biochemical test reactions, and the O- and H-antigenic determinants of 96-1 were identical to those of the wild type, SR-11. Both strains were xylose fermenting, with O-antigenic formulae 1,4,12. The 5 O-antigenic determinant, characteristic of most strains of *S. typhimurium*, was lacking in both strains. These results were corroborated by the Texas State Department of Health Resource Laboratories. Cellular characteristics, as determined by electron microscopy, were similar also. Both strains were flagellated and heavily pilated.

Growth in heat-inactivated serum. The growth patterns of SR-11 and 96-1 in complement-inactivated serum were compared by viable count (Fig. 1). From a large inoculum, the wild-type strain grew to maximal cell density after a short lag. Small inocula showed an initial growth period characteristic of inoculum cells grown on iron-rich BHIA, followed by a 5- to 6-h period of bacteriostasis. The bacteriostatic period ended with resumption of growth at 10 to 12 h post-inoculation. This inoculum-dependent pattern of growth in complement-inactivated serum is similar for all ENT-producing strains of *S. typhimurium* that we have tested.

After two or three divisions, however, 96-1 was markedly inhibited at both inoculum sizes. This pattern of growth is identical to that reported previously for the *enb* mutants obtained from J. B. Neilands (Yancey and Lankford, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, D26, p. 55). The addition of 5 μM ENT to serum

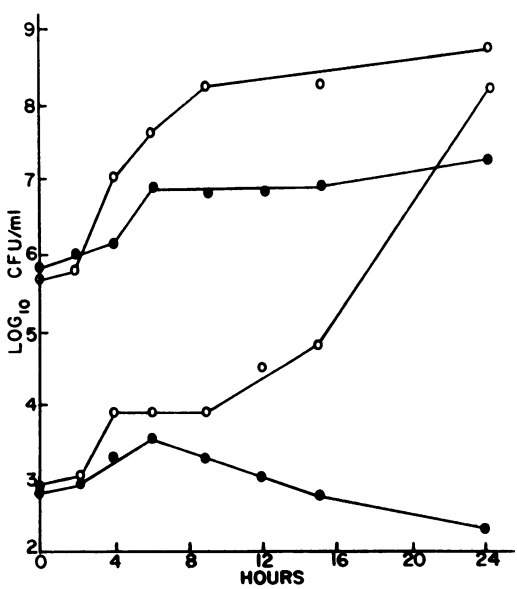

FIG. 1. *Growth of S. typhimurium SR-11 and 96-1 in complement-inactivated serum. Growth was monitored by viable plate count. Symbols:* (○) *S. typhimurium SR-11;* (●) *S. typhimurium 96-1. CFU, Colony-forming units.*

cultures of 96-1 permitted exponential growth similar to that of the wild type at large inoculum sizes (Fig. 2). The smallest amount of ENT required for the growth of 96-1 in human serum was 20 nM (D. Moore, unpublished data). The control culture exhibited extended bacteriostasis after the initial division period. The initial division period in serum was characteristic of all strains of *Salmonella* that we tested when the inoculum cells were taken from an iron-rich medium such as BHIA. This period was due to storage of iron by the inoculum cells while growing on BHIA and depletion of this stored iron during the first few division periods. After depletion of the iron, the cells entered bacteriostasis (Yancey and Lankford, Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, p. 89; Yancey, Moore, and Lankford, manuscript in preparation).

These experiments and experiments with other ENT$^-$ strains indicate that synthesis of ENT is necessary for the growth of *S. typhimurium* in human serum. The siderophore requirement also can be fulfilled by addition of iron to the serum adequate to saturate the serum transferrin (Fig. 3).

Peritoneal counts. With an intraperitoneal inoculum of approximately 10^5 cells per mouse, growth of 96-1 was inhibited in the peritoneal cavity over a 12-h period after infection (Fig. 4). The wild-type strain showed a period of growth followed by a small decrease in cell numbers. With injection of 300 μg of ENT per mouse with the inoculum cells, the increase in cell numbers of 96-1 surpassed that of the wild-type strain over this observation period. At a larger inocu-

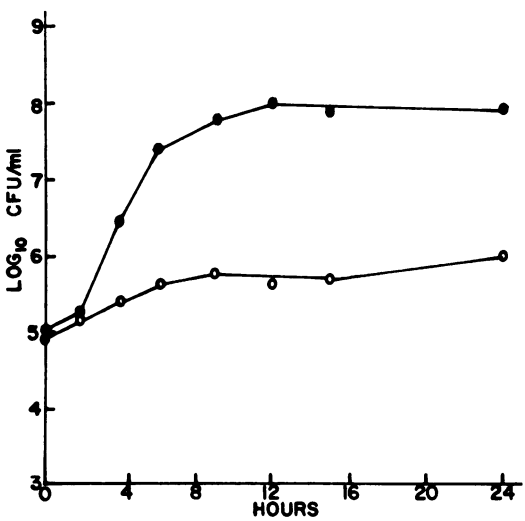

FIG. 2. *Effect of ENT on the growth of S. typhimurium 96-1 in human serum. Growth was monitored by viable plate count. Symbols:* (○) *Unsupplemented;* (●) *supplemented with 5 μM ENT. CFU, Colony-forming units.*

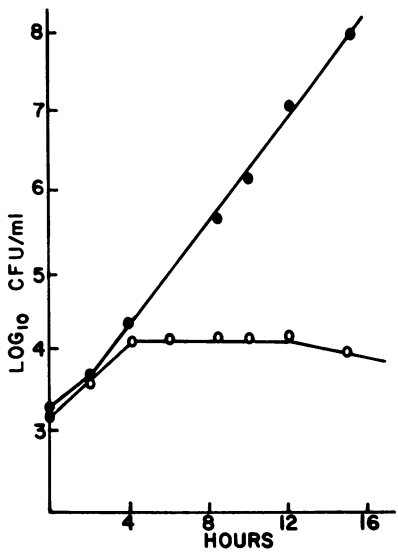

FIG. 3. *Effect of iron on growth of 96-1 in human serum. Growth was monitored by viable plate count. Symbols:* (○) *Unsupplemented;* (●) *supplemented with 53.7 μM iron (as $FeSO_4$; 124% saturation of transferrin). CFU, Colony-forming units.*

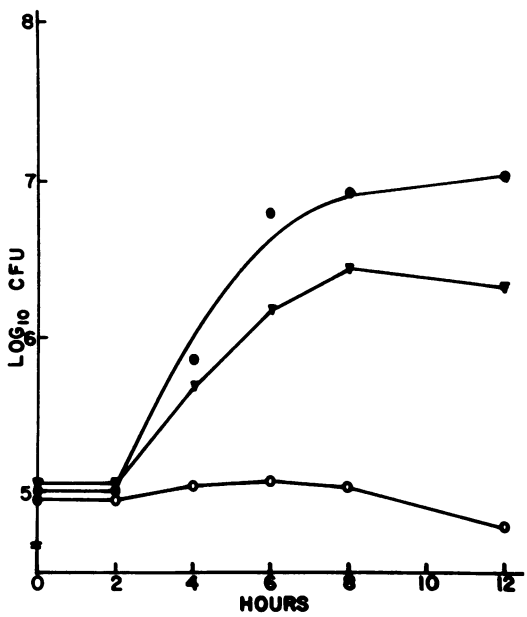

FIG. 4. *Effect of ENT on the growth of 96-1 in the mouse peritoneal cavity. Growth was monitored by viable plate count as described in the text. Each point represents the average count of two mice. Symbols:* (○) *96-1;* (●) *96-1 plus 300 μg of ENT per mouse;* (▼) *SR-11. CFU, Colony-forming units.*

lum size, the same pattern of growth occurred, although the difference between 96-1 ENT-supplemented and ENT-unsupplemented inocula was not as pronounced as with the smaller inoculum (Yancey, unpublished data). The kinetics of bacterial growth in the SR-11-infected mice was virtually identical to that in the 96-1-infected, ENT-supplemented animals.

Late in infection (>12 h), microscopic observations of concentrated wash suspensions stained with 0.25% safranin or observations by phase-contrast microscopy revealed that little phagocytosis of either strain occurred, although numerous phagocytes were present.

Relation of virulence for mice to ENT production. Preliminary experiments with *S. typhimurium* LT-2 revealed that, although this strain produced siderophore and grew in an inoculum-dependent fashion, the LD_{50} was rather high (LD_{50} at 5 days after intraperitoneal infection, 7×10^5 colony-forming units). The mean LD_{50} of the mutants of this strain was only slightly higher: 1.9×10^6 and 8.5×10^5 colony-forming units for *enb-7* and *enb-1*, respectively. We decided that to study the role of ENT in virulence more clearly, a more virulent wild-type strain should be used. Therefore, the virulence of *S. typhimurium* strain SR-11 and its ENT^- mutant 96-1 was compared in mice after intraperitoneal inoculation.

Virulence comparison (LD_{50} determinations) of strains SR-11 and 96-1. Table 1 demonstrates differences in the LD_{50} values of the two strains. Without ENT, the mean LD_{50} of 96-1 was more than 630 times higher than that of SR-11 at 5 days and for a period of up to 2 weeks postinfection. However, injection of 300 μg of ENT per 20-g mouse in one dose with the inoculum or in three 100-μg doses (on consecutive days starting at day zero) lowered the LD_{50} of 96-1 to that of the wild type. With one 100-μg dose of ENT injected with the inoculum, the virulence of 96-1 was markedly potentiated, but it remained approximately 12 times higher than the wild-type LD_{50}. Injection of ENT with the wild-type strain also reduced the 5-day LD_{50} of SR-11; however, this was simply a kinetic potentiation of virulence, since the LD_{50} at 14 days, with or without ENT, was approximately one to three cells. The LD_{50} of 96-1 without ENT at 14 days remained 500 to 1,000 times higher than that of the wild type (Fig. 5).

Figure 5 demonstrates differences in the LD_{50} of the strains with time to 14 days. Over this time period, the LD_{50} of 96-1 was 2 to 3.5 log units higher than that of SR-11. However, injection of a total of 300 μg of ENT per mouse in 100-μg doses lowered the LD_{50} to that of the wild type over the 5- to 7-day period. With a single 300-μg dose of ENT per mouse given with the inoculum, strain 96-1 exhibited wild-type kinetics of mouse killing over the 4- to 7-day period. With one 100-μg dose of ENT injected with the inoculum, the LD_{50} curve of 96-1 was midway between the lethality-kinetic curve of 96-1 without ENT and of 96-1 plus 300 μg of ENT.

TABLE 1. *Effect of enterochelin on virulence of S. typhimurium strains SR-11 and 96-1*

Strain	ENT injected[a] (μg)	Arithmetic mean of LD_{50} at 5 days	No. of tests	Ratio of LD_{50} doses[b]
SR-11	0	8.4×10^2 (1.3×10^3–4.1×10^2)[c]	3	1.0
	1×300	1.1×10^2	1	0.13
96-1	0	5.3×10^5 (7.5×10^5–2.2×10^5)	4	632.0
	1×100	1.0×10^4	1	12.0
	3×100[d]	1.3×10^3 (1.9×10^3–6.1×10^2)	2	1.5
	1×300	9.3×10^2	1	1.1

[a] ENT was injected intraperitoneally after mixing with the inoculum cells at day zero.

[b] Mean LD_{50} tested/mean LD_{50} of SR-11 (without ENT).

[c] Numbers in parentheses represent the range of LD_{50} in this number of tests.

[d] ENT was injected with the inoculum cells at day zero and again on days 1 and 2.

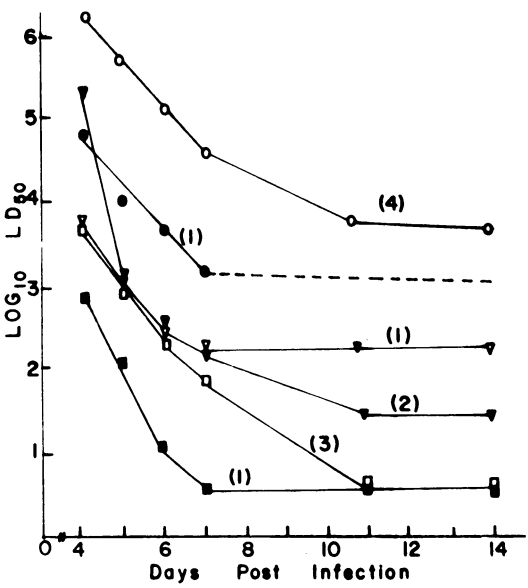

FIG. 5. *Influence of ENT on the time course LD_{50} comparison of SR-11 and 96-1. ENT was injected with the inoculum cells at day zero (and, in the case of 96-1 plus 3 × 100 μg of ENT, on days 1 and 2). Numbers in parentheses indicate the number of determinations. If there was more than one determination, these data represent the arithmetic mean of those determinations. Symbols:* (○) *96-1;* (●) *96-1 plus 100 μg of ENT per mouse;* (▽) *96-1 plus 300 μg of ENT per mouse;* (▼) *96-1 plus 3 × 100 μg of ENT per mouse;* (□) *SR-11;* (■) *SR-11 plus 300 μg of ENT per mouse.*

All *S. typhimurium* isolates recovered from dead or dying mice (30 isolates from 6 different mice) were of the original phenotype for ENT production.

Mice injected only with various doses of ENT (10 to 300 μg of ENT per mouse; 40 mice total) showed no ill effects due to the compound.

DISCUSSION

While there is a preponderance of evidence that the availability of iron affects the apparent virulence of a pathogen for experimental hosts (or, more accurately, the resistance of the host for the pathogen), the importance of siderophores as an infection determinant is unproven. Purifoy and Lankford (Texas Rep. Biol. Med. **23**:637, 1965) found that administration of desferal with *S. typhimurium* reduced its LD_{50} by three- to fivefold. Rogers (22), using two serologically dissimilar strains of *E. coli*, found that a catechol siderophore isolated from the more virulent strain abolished apparent bacteriostasis of the less virulent strain in vivo. However, the latter strain produced some catechol in vitro and could be induced to produce higher levels under conditions of iron deprivation. As suggested earlier, a comparison of two different serotypes, which may differ qualitatively or quantitatively in one or several virulence factors unrelated to siderophore production, raises questions concerning the validity of a direct conclusion that siderophores are virulence factors. To properly assess the relationship between virulence and siderophore production, an ENT^- mutant (96-1) of a virulent strain of *S. typhimurium* (SR-11) was derived. In vitro, this mutant appeared to be identical phenotypically to the wild type except for its inability to produce ENT.

In vivo experiments demonstrated that 96-1 was 500- to 700-fold less virulent than the wild type. Injections of deferri-ENT increased the virulence to that of the wild type in a dose-related fashion. Viable cell counts of the peritoneal fluid after injection of SR-11 and 96-1 correlated with the LD_{50} determinations. In the presence of ENT, the increase in cell numbers of 96-1 during the first 8 to 10 h postinfection mimicked the increase in numbers in the peritoneum. It is reasonable to assume that deferri-ENT can provide iron only by drawing it from some host iron store.

The initial increase of approximately 2 logs in numbers of viable bacterial cells in the mouse peritoneum might explain the 2- to 3-log difference in virulence (as determined by LD_{50}) of SR-11 and 96-1. This early rapid growth of the virulent wild type and of its ENT-potentiated mutant may be a major determining factor in the subsequent outcome of infection. The virulence-enhancing effect of ENT might be important only in this environment. Growth of the mutant after penetration beyond the peritoneal cavity, for example, in the blood or in the liver, spleen, and other organs, may not be strongly inhibited by iron-binding proteins. S. R. Richardson (M.S. thesis, University of Texas, Austin, 1967) and Wilkens et al. (T. D. Wilkens, S. R. Richardson, and C. E. Lankford, Bacteriol. Proc., 1968, p. 83) found that blood serum of mice was not inhibitory for *S. typhimurium* and other *Salmonella*, even when the serum of the mice was made hypoferremic (about 15% iron saturation of transferrin) by prior injection of endotoxin. Nevertheless, the LD_{50} dose of *S. typhimurium* could be reduced about 2 log units by simultaneous injection of relatively small doses (10 to 50 μg per mouse) of ferric iron. These results suggested that a major resistance-lowering effect of iron injection in mice may be an impairment of cellular defenses. Although strain 96-1 grew less well than SR-11, the ENT^- strain did not show bacteriostasis up to 8 h (end of observation period) after inoculation of approximately 500 cells into heat-inactivated serum of mice pretreated with lipopolysaccharide (Yancey, unpublished data). It will be important to determine whether ENT can alter the effectiveness of the reticuloendothelial system in clearing and destroying blood-borne pathogens.

The smallest amount of ENT that was stimulatory for 96-1 in vivo was 100 μg per 20-g mouse. Assuming that this entire amount of siderophore was present in the bloodstream of the mouse (approximately 3-ml blood volume; L. J. Berry, personal communication) in an unaltered state, this amount of ENT would represent a maximal blood concentration of 50 μM. This concentration is approximately 2,500 times that required for growth of the strain in serum (20 nM). However, since nothing is known about the distribution, clearance, and/or metabolism of ENT by the mammalian host, it is not possible to extrapolate directly from the in vitro experiments in order to determine a relevant concentration for the in vivo studies. Due to its small size (670 daltons), ENT probably was eliminated efficiently and rapidly in the urine, thus reducing the hypothesized maximal blood level.

After surveying randomly selected strains and serotypes of *E. coli* and *Klebsiella pneumoniae* for siderophore production and for virulence, Miles and Khimji (13) found no significant correlation between the parameters. From this lack of consistent correlation, they argued that siderophores should not be considered a virulence determinant. However, with three wild-type strains of *S. typhimurium*, we found a relationship between ENT production and the virulence

of the strains. Strain SR-11 produced approximately two and five times as much ENT in vitro as did the LT-2 and Fisher strains, respectively (data not presented). SR-11 was more virulent (5-day LD_{50}, 8×10^2) than LT-2 (5-day LD_{50}, 7×10^5), which was more virulent than strain Fisher (5-day LD_{50}, 3×10^6). Nevertheless, these data are not necessarily significant, since virulence is the result of several factors (25), and loss of any of these factors could affect partial or complete loss of virulence. The avirulent, randomly selected strains used by Miles and Khimji (13) may have lacked one or more other virulence factors while maintaining the ability to produce siderophore(s). This probably was the case with *S. typhimurium* LT-2, which produces ENT but is only moderately virulent for mice. The *enb* derivatives of this strain were only slightly less virulent. In the directed approach reported here, an ENT^- mutant, apparently impaired only in the ability to produce siderophore, was derived from a strain of high virulence. Loss of siderophore synthesis decisively reduced the virulence of the mutant.

Virulence factors have been described as "substances that are either directly toxic to the host . . . or antagonize the antibacterial mechanisms of the host" (13). Since ENT apparently antagonizes the iron-restricting mechanisms of the host, an antibacterial mechanism (6, 29), this compound should be considered a virulence factor for *S. typhimurium*.

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