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Virulence of Iron Transport Mutants of *Shigella flexneri* and Utilization of Host Iron Compounds

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Mutants of *Shigella flexneri* defective in aerobactin-mediated iron transport were assayed for virulence in several model systems. A Tn*5* insertion mutant was invasive in HeLa cells, lethal in the chicken embryo, and produced keratoconjunctivitis in the guinea pig, indicating little or no loss of ability to invade and multiply intracellularly. Although the mutant failed to grow in low-iron medium in vitro, growth equivalent to that of the wild type was observed in HeLa cell lysates. Thus, there appears to be sufficient available iron inside the HeLa cell to allow growth in the absence of siderophore synthesis. Possible host iron sources were tested, and both the mutant and wild type utilized hemin or hematin as a sole source of iron. Only the wild-type, aerobactin-producing strain could remove iron from transferrin or lactoferrin. Two deletion mutants were also assayed for virulence and were found to be avirulent for the chicken embryo. These deletions encompass flanking sequences as well as the aerobactin genes; therefore, adjacent genes may be required for virulence.

The ability to compete for iron within the host is one of the factors which influences bacterial pathogenesis (24, 32). The concentration of free iron in the vertebrate host is limiting, and bacteria must have some mechanism for acquiring iron from host iron-binding compounds such as transferrin, lactoferrin, or heme. Pathogens such as *Haemophilus influenzae* (14) and some *Neisseria* species (20) can utilize iron from transferrin. In the case of *Neisseria meningitidis* the iron appears to be removed from transferrin at the cell surface (1). *Neisseria* species can also obtain iron from lactoferrin, although survival and invasiveness do not correlate with the ability to utilize this iron source (19). Heme compounds can serve as iron sources for a variety of bacteria. *H. influenzae* (8), *Neisseria* species (20), and *Yersinia pestis* (26), for example, can grow with hemin as the sole source of iron.

The acquisition of iron in vivo may require the synthesis of bacterial compounds which can effect the removal of iron from host sources. *Listeria monocytogenes* appears to remove iron from transferrin by production of a soluble reductant which can dissociate the iron-transferrin complex (9). More commonly, the bacteria synthesize low-molecular-weight iron-binding compounds, siderophores, and their associated outer membrane protein receptors. Many *Escherichia coli* and *Salmonella typhimurium* strains synthesize the phenolate siderophore enterobactin and its coordinate 81,000-dalton outer membrane receptor (21). A second predominant chemical class of siderophores is the hydroxamates, one of which is aerobactin (21). Siderophores have extremely high affinities for iron and are able to remove iron from transferrin (16).

The relationship between siderophore production and virulence is not absolute. The relative importance of siderophores appears to depend on the pathogen and the nature of the infection it produces. Williams and colleague (33, 34) studied the association between siderophore synthesis and the ability to cause disease by certain *E. coli* ColV strains. These studies showed that production of the hydroxamate siderophore aerobactin is associated with increased virulence in an animal model and conferred an advantage over synthesis of the phenolate, enterobactin. However, other pathogens such as *Vibrio cholerae* (31), *Y. pestis* (26), and *Salmonella typhimurium* (4) have been shown to retain virulence for the mouse in the absence of a siderophore-mediated iron transport system. Unlike *E. coli* ColV strains which are septicemic, *V. cholerae* is noninvasive, and *Y. pestis* and *S. typhimurium* are intracellular pathogens. Thus, siderophore synthesis may be more important for highly invasive, extracellular pathogens.

In this study, the role of siderophores in virulence of *Shigella flexneri* was investigated. The *Shigella* species are invasive pathogens which multiply within epithelial cells and produce dysentery (10, 11). These pathogens have been shown to produce one or more siderophores in vitro in response to iron stress (17, 22, 27), but there does not appear to be a correlation between the ability to produce disease by *Shigella* species and the type of siderophore synthesized. Most *S. flexneri* and *Shigella boydii* strains utilize only the aerobactin iron transport system (17, 22, 25), while many *Shigella sonnei* strains and *Shigella dysenteriae* strains utilize only the enterobactin system (17, 27). Some strains produce both types of siderophores (17, 25, 27).

Studies by Griffiths et al. (12) suggested that aerobactin synthesis was a virulence factor for *S. flexneri* since the genes were mapped to the xylose-mannitol region of the chromosome, which is known to encode a virulence factor. To define the role that the aerobactin iron transport system plays in *Shigella* virulence, we constructed a mutant of an *S. flexneri* 2a strain by transposon mutagenesis. This mutant was tested for its ability to invade cells and multiply intracellularly in several model systems, and its ability to utilize various iron sources was determined.

MATERIALS AND METHODS

Strains and media. Bacterial strains, plasmids, and their sources are listed in Table 1. Bacterial stocks were maintained at −70°C in Luria broth (18) with 20% glycerol. Luria agar (L agar) was used for routine growth of organisms. Screening for aerobactin mutants and the reversion assay were performed with L agar with added ethylenediamine-

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TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant phenotype[a]	Source or reference
Shigella flexneri		
SA100	Serotype 2a; Crb$^+$ Iuc$^+$ Iut$^+$	25
SA101	SA100 Crb$^-$	25
SA201	SA100 Iuc$^-$ Iut$^+$	25
SA255	SA100 Iuc$^-$ Iut$^-$	25
SA240	SA100 Iuc$^-$ Kan^r	This study
SA244	SA240(pKLS711); Iuc$^+$ Kan^r Cb^r Tet^r	This study
Shigella boydii 1392	Iuc$^+$ Iut$^+$	17
Escherichia coli		
C600(pRK340)	Tn*5* Kan^r Tet^r	R. Meyer
KLP711	RM1058(pKLS711); Iuc$^+$ Cb^r Tet^r	This study
Plasmids		
pKLS711	*S. flexneri* aerobactin genes cloned into pBR322; Iuc$^+$ Cb^r Tc^r	This study
pKLS711::Tn*5*	Iuc$^-$ Cb^r Tc^r Km^r	This study
pRK340	ts, Km^r (Tn*5*) Tc^r	R. Meyer

[a] Crb, Congo red binding; Iuc, aerobactin synthesis; Iut, ferri-aerobactin transport; ts, temperature sensitive.

di(*o*-hydroxyphenol acetic acid) (EDDA; 12.5 μg/ml; Sigma Chemical Co., St. Louis, Mo.). Low-iron Tris-buffered medium without added iron (T medium) was used to assay siderophore production (22), and Congo red agar (CR agar) (23) was used to screen for the presence of the large virulence plasmid of *S. flexneri*. Bochner medium (5) for isolation of tetracycline-sensitive clones was modified for use with *Shigella* by decreasing the amount of chlorotetracycline hydrochloride (Sigma) to 10 mg/liter and fusaric acid (Sigma) to 1.2 mg/liter.

Siderophore assays. Hydroxamate siderophores in supernatants of overnight cultures grown in T medium were detected by the ferric perchlorate assay (3). Phenolates were detected by the colorimetric assay of Arnow (2). Synthesis of the hydroxamate siderophore aerobactin was also detected by a bioassay in which *S. boydii* which utilizes aerobactin under iron stress, was seeded at a concentration of 10^4 CFU/ml into a lawn of L agar containing EDDA at a concentration of 100 μg/ml. The surface of the plate was then spotted or streaked with strains being tested for synthesis of the siderophore, and the ability to stimulate growth of *S. boydii* was determined after overnight incubation at 37°C.

Construction of mutants. *S. flexneri* deletion mutants SA201 and SA255 were described previously (25). The biosynthesis mutant *S. flexneri* SA240 was constructed by transposon mutagenesis. The temperature-sensitive transposon (Tn*5*) vector, pRK340, was transferred by conjugation to *E. coli* KLP711, which carries the cloned *S. flexneri* aerobactin genes. Cells were grown at 42°C in the presence of kanamycin (Kan) and carbenicillin (Cb) to enrich for cells in which transposition has occurred. Plasmids were isolated from the culture by the method of Kado and Liu (15). Calcium chloride-treated *E. coli* RM1058 cells were transformed with this plasmid pool as described by Maniatis (18). Cb^r Kan^r transformants were selected, and the plasmids were analyzed by agarose gel electrophoresis. Transformants which appeared to contain pKLS711 with a Tn*5* insertion were tested for inactivation of aerobactin biosynthesis genes by bioassay as described above. The appropriate pKLS711::Tn*5* which failed to stimulate growth of the indicator strain in the bioassay was isolated and digested with restriction enzymes (New England Biolabs, Inc., Beverly, Mass.) to determine the exact location of the insertion. *S. flexneri* SA100 was transformed with the mutant plasmid. The transformed cells were grown in nonselective medium for several rounds of replication to allow recombination to occur between the homologous regions on the plasmid and chromosome. These cells were then plated on modified Bochner medium containing kanamycin. Bochner medium selects for tetracycline sensitivity, i.e., loss of the Tc^r vector, and kanamycin selects for those cells retaining the transposon, presumably in the chromosomal aerobactin genes. The clones which grew on the Bochner-kanamycin medium were then screened on CR agar containing kanamycin and on L agar containing tetracycline. The *S. flexneri* isolates which produced red colonies on CR agar (Crb$^+$), demonstrating the presence of the large virulence plasmid, and were Tet^s were tested by bioassay for lack of aerobactin biosynthesis. The isolate designated SA240 is Crb$^+$ Iuc$^-$ Kan^r Tet^s.

Competent cells of SA240 were transformed with a plasmid preparation of pKLS711, and an isolate was selected which now had the ability to synthesize aerobactin while retaining the Crb$^+$ phenotype. This strain, SA244, was used as a control in virulence testing.

HeLa cell methodology. The ability to invade Hela cells was determined by the method of Hale and Formal (13), and intracellular growth was assayed by the method of Sansonetti et al. (29). Briefly, the bacteria were centrifuged onto the monolayer and allowed to adhere for 30 min before washing with Earle balanced salt solution. Cells were overlaid with fresh medium containing antibiotics to kill extracellular bacteria. The infected monolayers were incubated at 37°C in a humidified 5% CO_2 atmosphere. Plates were Giemsa stained to determine the percentage of infected cells. Duplicate plates were washed five times with Earle balanced salt solution and then treated with trypsin to remove the cells. The cells were counted, lysed with 0.5% sodium deoxycholate to release intracellular bacteria, and plated on L agar. The number of bacteria per cell was calculated as described by Sansonetti et al. (29).

Chicken embryo methodology. White Leghorn fertilized eggs (Ideal Poultry Breeding Farms) were incubated at 37°C in a Petersime incubator for 11 days. Viability was determined by candling. Overnight broth cultures of the organisms to be tested were diluted in minimal salts. A small hole was made in the eggshell over the allantoic cavity, and 0.1 ml of the appropriate dilution was injected into the allantoic fluid with a tuberculin syringe. A minimum of five embryos was used per dose. The inoculated chicken embryos were incubated at 37°C and candled at various times to determine deaths. The 50% lethal dose (LD_{50}) was calculated by the method of Reed and Muench (28).

The bacteria were reisolated from the allantoic fluid by aspiration with a tuberculin syringe, and the fluid was plated on both L agar and CR agar. The livers were removed from dead embryos and were minced in sterile saline with a sterile scalpel. These samples were also plated on L agar and CR agar.

In vivo growth of SA100 and SA240 was determined in the allantoic fluid of 11-day-old chicken embryos. Approximately 10^4 organisms were inoculated into the allantoic fluid, and 0.1 ml of allantoic fluid was removed from each of four embryos at 2, 4, and 8 h. Mean CFU were determined on L

agar for SA100 and on L-kanamycin agar for SA240. Growth was plotted to obtain generation times.

Guinea pig methodology. Strains were tested for the ability to cause keratoconjunctivitis as described by Serény (30). Albino Hartley strain guinea pigs, approximately 300 g each, were obtained from Murphy Breeding Lab, Plainfield, Ind. The guinea pigs were placed in separate cages upon arrival, and eye cultures were streaked on MacConkey agar for possible enteric contaminants. Only guinea pigs whose eyes proved negative for contaminants were used. Two guinea pigs were used for testing each strain. Overnight broth cultures of *S. flexneri* were centrifuged and washed with sterile saline and resuspended to a final concentration 10^{10} organisms per ml. A 10-μl drop was deposited in the conjunctival sac of the right eye of the guinea pig, and the eyelid was held gently shut for approximately 1 min to allow for adherence of the organism. The actual inoculum size was determined by plating serial dilutions of the resuspended culture. The left eye was inoculated with sterile saline as a control. The guinea pigs were checked at 18 h, and a positive response was determined by corneal opacity and evidence of conjunctivitis. All guinea pigs were observed for at least 3 weeks.

HeLa cell lysate growth. Approximately 4×10^7 HeLa cells were pelleted by centrifugation, washed three times with low-iron T medium, and lysed by sonication. The lysate was centrifuged to remove unbroken cells and debris and then diluted in T medium to a final concentration of 10% (vol/vol) based on an estimate of the volume of packed cells before sonication. The lysate or T medium alone was inoculated with 1×10^6 to 5×10^6 bacteria per ml from an overnight T medium culture. The mutant, SA240, grew in T medium on first passage from L broth (1:100 dilution) but failed to grow on subsequent passages. At various time points samples were withdrawn, serially diluted, and plated on L agar.

Utilization of iron-binding compounds. Bacteria (2×10^4 CFU/ml) were added to liquified L agar containing EDDA and poured into sterile petri dishes. EDDA was added to a final concentration of 250 μg/ml for the wild-type strain and 125 μg/ml for the aerobactin mutants. Wells, 5 mm in diameter, were made in the solidified agar and filled with filter-sterilized solutions of the compounds to be tested. Iron-saturated solutions of ovotransferrin, lactoferrin, and transferrin were prepared by mixing 30 μM solutions of each protein in 10 mM $NaHCO_3$ with 1 mM ferric ammonium sulfate. Excess iron was removed by dialysis against a 100 mM sodium citrate solution. The saturated and unsaturated compounds were mixed to obtain a final iron saturation of 33 or 75% and equilibrated for at least 2 h at 4°C. For hemin and hematin, concentrations ranging from 50 μM to 8 mM were used. After overnight incubation at 37°C, the plates were examined for growth around the wells. Protoporphyrin IX, hemin, hematin, ferritin, transferrin, conalbumin, lactoferrin, and hemoglobin were all obtained from Sigma.

RESULTS

Construction and characterization of iron transport mutants. A mutant defective in aerobactin biosynthesis, SA201, and one defective in both biosynthesis and transport, SA255, were isolated and described previously (25). These were shown to be deletion mutants by hybridization of an aerobactin gene probe to chromosomal DNA restriction fragments (17). The cloning and mapping of the wild-type genes revealed the presence of a copy of an *IS1*-like sequence, which may be responsible for these deletions, directly upstream of the aerobactin biosynthesis genes (C. L. Marolda, M. A. Valvano, K. M. Lawlor, S. M. Payne, and J. H. Crosa, submitted for publication). Since these deletions were relatively large, sequences adjacent to the aerobactin gene cluster may also have been deleted, making it difficult to assess the effect of the aerobactin mutation on virulence. Therefore, isogenic strains differing only in aerobactin biosynthesis were constructed by transposon mutagenesis to eliminate the problems of large deletions in spontaneous mutants and multiple mutations which may occur when using chemical mutagens. The strains were constructed by mutagenizing the cloned *S. flexneri* aerobactin genes with Tn*5* and replacing the wild-type chromosomal genes with the mutated plasmid sequences by marker exchange.

TABLE 2. Invasion and multiplication of *S. flexneri* in HeLa cells

Time after infection (h)	% of cells infected with:			Bacteria/cell[a]		
	SA100	SA240	SA101	SA100	SA240	SA101
1	21	21	0	2	2	0
2	22	19	0	12	8	0
4	27	23	0	47	65	0

[a] From reference 29.

As has been reported for the ColV aerobactin system (6, 7), transposon insertion in an aerobactin biosynthesis gene exerted a polar effect on expression of the aerobactin receptor. The receptor protein was not detected by polyacrylamide gel electrophoresis of outer membrane proteins of SA240 as compared with the wild-type strain (data not shown). However, bioassays indicated that SA240 is still able to utilize aerobactin, indicating that the 77-kilodalton receptor protein is present. It is not likely that SA240 transports aerobactin by an alternative pathway since mutants which fail to synthesize the receptor, e.g. SA251, are unable to utilize aerobactin (25).

The reversion rate for the mutation was determined before its virulence was tested. An overnight culture of SA240 was washed twice in sterile saline and resuspended in 1/10th of the original volume. Concentrations between 10^4 and 10^9 organisms were spread on L-EDDA (12.5 μg/ml) agar in the absence of kanamycin. Although the parent strain grew well under these conditions, no colonies were observed on plates of SA240. Thus, the reversion rate is less than 10^{-9}.

Virulence assays. The aerobactin mutant was tested for its ability to invade HeLa cells, one of the assays used to assess virulence of *Shigella* species (13). SA240 was invasive, and the percentage of infected cells was similar for the mutant and its parent. In contrast, strain SA101, a Crb$^-$ mutant containing a deletion in the 220-kilobase virulence plasmid, was unable to invade the HeLa cells. The ability of the aerobactin mutant to multiply intracellularly was also determined (Table 2). The inability to synthesize aerobactin had no apparent effect on intracellular multiplication.

The iron transport mutants were also screened for virulence in the chicken embryo model (Table 3). Allantoic inoculation of wild-type *S. flexneri* was previously shown to cause lethality in the chicken embryo, whereas LD_{50} values for noninvasive isolates were found to be 10^5 to 10^6 times higher (23, 24). The LD_{50} for wild-type *S. flexneri* SA100 at 24 h was 2.3×10^2, while SA101 failed to kill the embryos at doses of 10^6, the highest dose tested. The deletion mutants, SA201 and SA255, were avirulent in this system. The Tn*5* insertion mutant, SA240, was lethal for chicken embryos,

TABLE 3. *S. flexneri* virulence assays

Strain	Chicken embryo LD_{50}		Guinea pig Serény test[a]
	24 h	40 h	
SA100	2.3×10^2	7×10^0	+
SA101	$>1 \times 10^6$	$>1 \times 10^6$	−
SA201	$>1 \times 10^7$	$>1 \times 10^7$	ND
SA255	$>1 \times 10^6$	$>1 \times 10^6$	ND
SA240	5.0×10^3	3.8×10^2	+
SA244	8.6×10^2	2×10^0	+

[a] ND, Not determined; +, production of keratoconjunctivitis; −, no detectable change in the eye.

although the LD_{50} value for the mutant was 10^1 to 10^2 times higher than that for the wild type. To check for the possibility of reversion to the wild type, we isolated organisms from livers of dead embryos and plated them on a nonselective medium, L agar. Isolates were then screened for the presence of the transposon (Kan^r), as well as the ability to synthesize aerobactin as indicated by growth on L agar with EDDA (12.5 μg/ml). The organisms isolated from the embryos were uniformly Kan^r and failed to grow on low-iron medium.

In the chicken embryo model, the bacteria grow in the allantoic fluid before invasion of the embryo. An increase in the LD_{50} could be due to either a reduced growth rate in the allantoic fluid or a loss of invasiveness. To measure these parameters, we cultured both blood and allantoic fluid at various times after inoculation of 10^4 CFU of either SA100 or SA240 per embryo. Both strains were invasive, and bacteria were detected within the blood between 8 and 16 h after inoculation (data not shown). However, growth of SA240 in the allantoic fluid was slower, with a doubling time of 45 min as compared with 32 min for SA100. It is likely that the slower growth of SA240 in the allantoic fluid results in a higher LD_{50} in this model.

To further test the possible role for the aerobactin genes in invasion and virulence, we used a more stringent assay, the Serény test in guinea pigs (30) (Table 3). After the inoculation of 10^8 organisms into the conjunctival sac, both the wildtype and the aerobactin mutant produced keratoconjuctivitis within 18 h. There was no significant difference in the extent of inflammation, but inflammation was noted

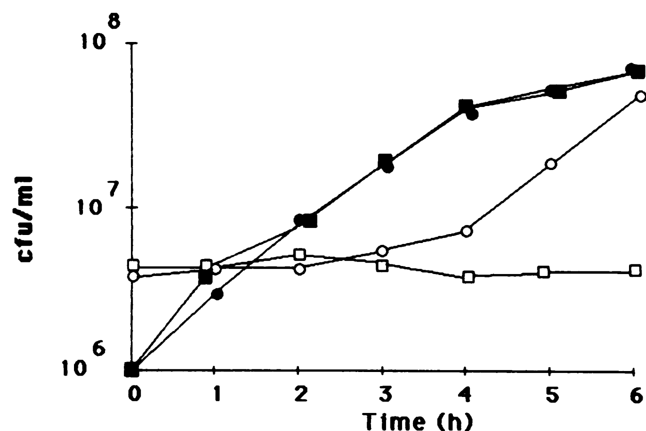

FIG. 1. Growth of strains SA100 (Iuc⁺) and SA240 (Iuc⁻) in HeLa cell lysates or T medium. Symbols: ●, SA100, HeLa lysate; ■, SA240, HeLa lysate; ○, SA100, T medium; □, SA240; T medium.

earlier in the eyes infected with the mutant. By comparison, the Crb⁻ mutant was avirulent, causing no detectable change in the inoculated eye.

Organisms were recovered from infected eyes and plated on nonselective medium to check for reversion of the mutant. As previously shown in the chicken embryo studies no revertants were isolated.

Growth of *S. flexneri* in HeLa cell lysates. The fact that aerobactin mutants of *S. flexneri* grew poorly in low-iron environments in vitro, yet retained virulence, suggested that iron must be available to the bacteria within the host. Since *Shigella* cells multiply intracellularly, the availability of iron to wild-type and Iuc⁻ *S. flexneri* in cell lysates was determined (Fig. 1). The lysates were prepared by washing HeLa cells with low-iron T medium salts and briefly sonicating a suspension of the cells in T medium (1:10, vol/vol). The bacteria were starved for iron by overnight growth in low-iron medium before inoculation into the lysate. Growth of the organisms in the lysates was compared with their growth in T medium alone. The wild-type *S. flexneri* and the iron transport mutant grew equally well in the lysate, and no lag occurred before the onset of growth. In T medium without cell lysate, SA240 failed to grow. The wild-type strain was able to grow in T medium but exhibited a lag of at least 2 h. These data indicate that sufficient iron is available in the cytosol of the HeLa cells to support growth of *Shigella* cells in the absence of a high-affinity iron transport system.

Utilization of iron-binding proteins and hemin by *S. flexneri*. The ability of *S. flexneri* to utilize iron from several host iron sources was assessed to determine potential iron sources for the bacteria in vivo. SA100 and SA240 were seeded into agar containing an inhibitory concentration of the iron-binding compound EDDA and observed for growth around wells containing hemin, hematin, or various iron-binding proteins (Table 4).

SA100 was able to utilize iron from transferrin, lactoferrin,

TABLE 4. Utilization of host iron sources by *S. flexneri*

Compound (% iron saturation)	Stimulation of growth in bioassay of strain[a]:	
	SA100	SA240
Lactoferrin		
0	−	−
33	+	−
75	+ +	−
100	+ +	−
Transferrin		
0	−	−
33	+	−
75	+ +	−
100	+ +	−
Ovotransferrin		
0	−	−
33	+	−
75	+ +	−
100	+ +	−
Ferritin	−	−
Hemoglobin	−	−
Hemin	+ +	+ +
Hematin	+ +	+ +
Protoporphyrin IX (iron-free)	−	−

[a] −, No growth around well; +, zone of growth ≤10 mm; + +, zone of growth >10 mm.

and ovotransferrin, but not ferritin or hemoglobin. The siderophore mutant, SA240, however, failed to utilize any of these iron-binding proteins. This indicates that utilization of iron from those proteins by strain SA100 was due to the ability of these organisms to produce aerobactin, which in turn was able to remove the iron from the iron-binding proteins and provide the cells with sufficient iron for growth.

Both the wild type and the aerobactin mutant were able to utilize hemin and hematin, indicating that the cells were able to take up the iron from those two compounds. No growth was observed around wells containing protoporphyrin IX, the iron-free precursor of heme. The ability of *S. flexneri* to utilize heme as an iron source may account for the growth of the Iuc$^-$ bacteria in the lysate and their growth and virulence in the guinea pig.

DISCUSSION

Siderophore-mediated iron transport systems have been implicated in bacterial virulence in several systems. *E. coli* ColV strains carrying the aerobactin genes were lethal for intraperitoneally inoculated mice, whereas siderophore mutants of these strains were avirulent in this model (34). Production of aerobactin appeared to be more important in vivo than production of enterobactin, and the presence of the aerobactin genes alleviated iron starvation induced by the host iron-binding protein transferrin. Konopka et al. (16) have reported that aerobactin has the ability to obtain iron from transferrin in serum, and ColV$^+$ *E. coli* grew more rapidly than ColV$^-$ strains in immunoglobulin-free blood (34).

Siderophores appear to be less important in virulence of other pathogens. *Salmonella typhimurium* lethality for mice was shown to be independent of siderophore production (4). Enterobactin synthesis mutants were equally as virulent as the parental strain. Similarly, *V. cholerae* infections of mice were not influenced by the ability of the strain to synthesize or transport the siderophore vibriobactin (31). No differences were detected in multiplication of *V. cholerae* in the mouse intestine or in the extent of diarrhea produced by siderophore mutants as compared with the parental strain. It is likely that the role of siderophores in virulence is highly dependent on the nature of the bacterial infection. Those bacteria which produce bacteremias may require siderophores such as aerobactin to compete successfully with host serum iron-binding proteins. Bacteria such as salmonellae which can multiply within cells may not require the high-affinity siderophore systems or may utilize alternative transport systems to acquire iron. *V. cholerae* and other noninvasive pathogens may find sufficient iron available on host mucosal surfaces. Although not required for virulence, the siderophores synthesized by these pathogens may be important in maintaining these organisms in the environment or between hosts.

The *Shigella* species are invasive organisms but do not usually cause disseminating disease. After ingestion, the organism penetrates and multiplies within the epithelial cells of the large intestine, causing localized destruction and eliciting an intense inflammatory reaction (10, 11). Shigellae rarely penetrate beyond the lamina propria. All the wild-type *Shigella* isolates that we have screened thus far have been found to synthesize and transport siderophores, but no single siderophore is associated with virulent strains (17, 25). Strains of *S. dysenteriae*, which cause the most severe form of dysentary among the *Shigella* species, may produce and utilize only the siderophore enterobactin. Hybridization studies with aerobactin biosynthesis genes have revealed the complete absence of aerobactin genes in these *S. dysenteriae* strains (17). Some *S. sonnei* strains synthesize only enterobactin, although the aerobactin genes are present (17). In contrast, aerobactin, but not enterobactin, is synthesized by many *S. flexneri* and *S. boydii* strains (17, 22, 25). Thus, unlike the ColV system, aerobactin does not appear to confer any advantage over enterobactin synthesis among the *Shigella* species.

Mutants of *S. flexneri* defective in aerobactin synthesis were constructed to determine the role of the siderophore in virulence of the organism. Mutants which contained deletions in the aerobactin region were avirulent for chicken embryos. However, the deletions in these mutants are relatively large and may cover genes adjacent to the aerobactin genes. Therefore, additional mutants were constructed by transposon mutagenesis to allow comparison of strains differing only in aerobactin synthesis. These *iuc*::Tn*5* mutants failed to grow in low-iron media in vitro, but retained the ability to invade and multiply within HeLa cells and produce keratoconjunctivitis in the guinea pig. Some increase in LD_{50} was noted when the mutant was inoculated allantoically into chicken embryos, and this appeared to be due to slower multiplication of the mutant in the allantoic fluid. This indicates that the mutant may not grow as well in extracellular environments as it does intracellularly. The difference in virulence between the transposon mutant and the deletion mutants suggests the presence of other virulence determinants in this region.

The ability of the aerobactin mutant to multiply intracellularly and produce a positive Serény test (30) indicates that sufficient iron is available intracellularly to preclude the necessity for siderophore-mediated iron transport. A potential source of iron in the cell is heme, and both the wild type and mutant were able to grow with hemin or hematin as a sole source of iron. This indicates that the hemin transport system is independent of the siderophore system. Other iron sources such as transferrin and lactoferrin were only utilized by the aerobactin-producing strain; thus, aerobactin is required to remove the iron from these proteins and makes it available to the bacterium.

The model systems used in this study are limited in that they do not mimic the conditions found in the extracellular environment of the normal human intestine. Thus, while aerobactin synthesis is not essential for either invasion or intracellular multiplication of *S. flexneri*, it may provide a selective advantage. Colonization in the intestine may be enhanced by the ability to compete with lactoferrin for iron, and survival of the bacteria in the environment between hosts may be influenced by synthesis of siderophores.

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