JOURNAL OF BACTERIOLOGY, Sept. 1983, p. 949–955
0021-9193/83/090949-07$02.00/0
Copyright © 1983, American Society for Microbiology
Vol. 155, No. 3

Expression of Hydroxamate and Phenolate Siderophores by *Shigella flexneri*

SHELLEY M. PAYNE,* DAVID W. NIESEL, SUE S. PEIXOTTO, AND KATHLEEN M. LAWLOR

Department of Microbiology, The University of Texas at Austin, Austin, Texas 78712-1095

Received 5 May 1983/Accepted 13 June 1983

Shigella flexneri strains were assayed for the ability to synthesize and utilize phenolate and hydroxamate siderophores. The hydroxamate aerobactin was synthesized by all isolates tested, whereas phenolates were only rarely produced. Expression of aerobactin was accompanied by production of a single iron-regulated outer membrane protein (M_r = 74,000). This protein was not produced by a mutant defective in aerobactin utilization and may serve as the aerobactin receptor. Phenolate (enterobactin)-producing strains synthesized three additional outer membrane proteins (M_r = 74,000, 81,000, and 83,000) in response to iron starvation. These proteins are the same apparent size as those produced by *Escherichia coli* K-12 strains. Ent sequences are apparently present in strains which do not synthesize this compound. Although normally silent, *ent* genes can be activated in Ent$^-$ strains to produce Ent$^+$ variants. These laboratory variants are phenotypically indistinguishable from clinical Ent$^+$ isolates.

Many bacteria derepress high-affinity iron transport systems when starved for iron. These systems consist of an iron chelator, or siderophore, and specific membrane receptors for transport of the ferrisiderophore complex (16, 17). Laboratory strains of *Shigella flexneri* synthesize a single siderophore, aerobactin (19). Aerobactin is a secondary hydroxamate compound first isolated from low-iron culture supernatants of *Aerobacter aerogenes* (10) and more recently from *Escherichia coli*(ColV) strains (26). In addition to aerobactin, the phenolate siderophore enterobactin is synthesized by *A. aerogenes* (18) and *E. coli* (7). Phenolate siderophores are produced by most enteric bacteria including *Salmonella typhimurium* (21, 27), *Shigella sonnei* (20), and *Klebsiella pneumoniae* (20) but not *S. flexneri* (19). Because *S. flexneri* possesses extensive genetic homology with these species (6), it appeared unlikely that a phenolate-mediated iron transport system would be entirely absent. In this study, a large number of clinical and laboratory variants of *S. flexneri* were assayed for the ability to synthesize and transport phenolate siderophores.

MATERIALS AND METHODS

Chemicals. Tris was obtained as Trizma base (Sigma Chemical Co.) and ethylenediamine-di-(*o*-hydroxyphenylacetic acid) (EDDA) (Sigma) was deferrated by the method of Rogers (23) for use as an iron chelator. Chemicals for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad Laboratories. All other chemicals were reagent grade.

Bacterial strains and media. Bacterial strains and their sources are listed in Table 1. All other *Shigella* isolates were provided by Gloria Pierce, Texas Department of Health. Stocks were maintained frozen at −70°C in tryptic soy broth (Difco Laboratories) with 20% glycerol. Luria broth (L broth) was used for growth of *Shigella* strains. The iron chelator EDDA was added where indicated to induce iron starvation. Tris-buffered medium (T medium) (24) without added iron was used as described previously (19) to determine siderophore production.

Isolation of mutants. An overnight culture of SA100 in L broth was diluted 1:100 into fresh L broth and grown to mid-log phase. A 0.1-ml volume of the culture was added to 5 ml of minimal salts-0.1 ml of diethyl sulfate (Sigma). After 30 min at 37°C, the mutagenized cells were diluted 1:10 into L broth and allowed to grow at 37°C until turbid. The culture was enriched for iron transport mutants by two cycles of nalidixic acid selection as follows. The culture was diluted 1:50 into L broth containing 100 μg of EDDA per ml to inhibit growth of the mutants. After 2 h of incubation at 37°C, 20 μg of nalidixic acid was added per ml, and the culture was incubated for an additional 6 h at 37°C. The culture was filtered, and the cells were suspended in L broth. After overnight incubation at 37°C, the culture was diluted and plated on L agar. Individual colonies were picked onto L agar and L agar containing 12.5 μg of EDDA per ml to identify clones unable to grow in the presence of the iron chelator.

Siderophore bioassay. The bioassay described previously (19) was used to determine production and utilization of siderophores. EDDA was added to L agar and allowed to stand for 24 h before use. The agar was remelted, 10^3 indicator bacteria were added per ml, and the agar was poured into sterile petri plates. Strains to be tested for siderophore production were

streaked or spotted on the surface of the agar. Purified siderophores were tested by placing 10 μl of a 1 to 10 μM solution on a Sensi-Disc (BBL Microbiology Systems) on the surface of the agar. Zones of growth of the test strain around the producer strain or purified siderophore were measured after 24 h of incubation at 37°C. The concentration of EDDA was adjusted to suppress growth of the indicator strain. Hydroxamate synthesis or transport mutants were inhibited by 12.5 μg of EDDA per ml, wild-type aerobactin-producing strains were inhibited by 250 to 500 μg/ml, and 1 mg/ml was required to suppress growth of phenolate-producing strains.

Outer membrane preparation and SDS-PAGE. *S. flexneri* strains were grown in L broth with or without EDDA and harvested in mid-log phase. Iron transport mutants were allowed to grow to mid-log phase before addition of EDDA and were incubated an additional 2 h in the presence of the chelator. Cells were harvested by centrifugation, and the pellets were suspended in 10 mM sodium phosphate buffer (pH 7) with 5 mM $MgSO_4$. Total cell envelopes were prepared by the method of Inouye and Guthrie (13), using sonic disruption in the presence of 10 μg of DNase per ml. Outer membranes were prepared by the method of Filip et al. (9), using 0.5% Sarkosyl to solubilize the inner membrane (20 min; room temperature). The insoluble outer membrane was separated from the inner membrane by ultracentrifugation (38,000 × *g*; 30 min), and the pellet was washed twice with water to remove contaminating inner membrane proteins.

Isolated membrane fractions were solubilized in Laemmli solubilization buffer (14), and the proteins were separated by the discontinuous SDS-PAGE system of Ames (1). Slab gels (0.7 mm) consisted of a 5% stacking gel and a 10% separating gel. Gels were run at 25 mA until the tracking dye was 1 to 2 cm from the bottom of the gel. They were then stained with brilliant blue R (Coomassie blue), destained, and dried. Protein standards included in each gel were obtained from Pharmacia Fine Chemicals.

RESULTS

S. flexneri strains were screened for the ability to synthesize hydroxamate and phenolate siderophores. One hundred clinical isolates were grown in low-iron T medium, and the supernatants were assayed for siderophores. The ferric perchlorate assay of Atkin et al. (3) was used as an initial screen for hydroxamate siderophores. Formation of a purple complex with maximum absorbance at 485 nm at pH 1 was considered indicative of hydroxamate synthesis (Hyd^+). All 100 strains synthesized a compound of this type under conditions of iron limitation. In each case, synthesis was suppressed by addition of 5 μM iron to the medium.

Because the hydroxamate synthesized by *S. flexneri* strains tested previously had been identified as aerobactin (19), the siderophore was isolated from strains of three different serotypes (1-61, serotype 1a; SA100, serotype 2a; and 3733b, serotype 5) as described previously (19) and compared with aerobactin by thin-layer chromatography. In each case, the isolated siderophore was indistinguishable from aerobactin.

Low-iron culture supernatants of each strain were also assayed for phenolate compounds by the Arnow test, a colorimetric assay for dihydroxybenzoic acid-containing compounds (2). For 93 of the 100 strains, no evidence for phenolate production was obtained by this method. A bioassay was also employed to detect possible phenolate siderophores. Enterobactin synthesis mutants of *E. coli* or *S. typhimurium* were seeded in medium containing the iron chelator EDDA to inhibit growth. Low-iron culture supernatants or growing cultures of *S. flexneri* were placed on the surface of the agar, and the lawn was observed for growth around the *Shigella* strain or culture supernatant (Table 2). *S. flexneri* supernatants stimulated growth of *S. flexneri* owing to the presence of aerobactin. However, no enhancement of growth either of the *E. coli* or *S. typhimurium* strain was noted. The EDDA inhibition was overcome by iron, enterobactin, or supernatants of known enterobactin producers grown in the T medium.

Six of the strains were tested for their ability to utilize and synthesize enterobactin. When used as the indicator in the bioassay, these strains showed little or no response to enterobactin, whereas their growth was stimulated by aerobactin (Table 2). This suggests that neither synthesis nor transport of enterobactin is normally expressed in *S. flexneri*.

Of the 100 clinical isolates tested, 7 differed from the remaining strains in that phenolate compounds were detected in culture supernatants by the Arnow assay (2). The phenolate compound, like the hydroxamate aerobactin, was not synthesized when iron was present in

TABLE 1. Bacterial strains used

Strain	Description	Source
S. flexneri		
SA100	Serotype 2a	TDH[a]
SA301	SA100 Ent^+ Thi^-	This study
SA201	SA100 Hds^-[b]	This study
SA255	SA100 Hds^- Hdu^-[c]	This study
1-61	Serotype 1a	TDH
3733b	Serotype 5	TDH
SF100	Serotype 4b, Ent^+	TDH
340	Serotype 6, Ent^+	TDH
1-56	Serotype 6, Ent^+	TDH
S. typhimurium		
LT2		B. N. Ames
TA2442	LT2, Ent^-	B. N. Ames
E. coli		
ATCC 33475	K-12, Ent^-	

[a] TDH, Texas Department of Health.
[b] Hds, Hydroxamate (aerobactin) synthesis.
[c] Hdu, Hydroxamate (aerobactin) utilization.

TABLE 2. Production and utilization of siderophores by *S. flexneri*

Indicator[a]	Zone of stimulation[b]					
	S. flexneri[c]		Aerobactin	Enterobactin	*S. typhimurium*	Fe[d]
	Ent⁻	Ent⁺			LT2	
S. flexneri Ent⁻	+	+	+	+/−	+/−	+
E. coli ATCC 33475	−	+	−	+	+	+
S. typhimurium TA2442	−	+	−	+	+	+
S. flexneri SA301	+	+	+	+	+	+
S. flexneri 1-56	+	+	+	+	+	+

[a] 10^3 bacteria per ml in L agar containing 250 to 1,000 μg of EDDA per ml.

[b] +, >12-mm zone of stimulation; +/−, ≦12-mm zone of stimulation, no zone with some strains; −, no zone of stimulation.

[c] Ent⁻ (6 strains) and Ent⁺ (7 strains) by Arnow assay.

[d] 5 μl of 10 mM ferrous sulfate.

the growth medium. Therefore, this system, when expressed, is under normal derepressive control by iron. This phenolate was isolated from the culture medium by the procedure of Rogers (23) and compared with enterobactin by thin-layer chromatography. The *Shigella* phenolate migrated with the same R_f as enterobactin, displayed blue fluorescence under UV illumination, and formed a dark purple complex when sprayed with iron. This phenolate was tested in the bioassay and was as biologically active as enterobactin in stimulating growth of *E. coli* and *S. typhimurium* in iron-limiting medium (Table 2). In contrast to the majority of *S. flexneri* strains, the phenolate-producing strains (Ent⁺, e.g., 1-56, Table 2) were found to utilize exogenous enterobactin for growth in low-iron medium.

The Ent⁺ and Ent⁻ strains were compared for growth in low-iron medium. Growth of Ent⁺ Hyd⁺ strains SA301 and 1-56 in L broth was unaffected by EDDA levels up to 1 mg/ml. In contrast, growth of Ent⁻ Hyd⁺ strains SA100 and 3733b was inhibited by as little as 50 μg of EDDA per ml. Thus, the hydroxamate alone is sufficient to allow growth in the low-iron T medium but does not allow the cells to compete effectively with an iron chelator such as EDDA.

Although the Ent⁺ clinical isolates were of different serotypes, they were found to be of the same auxotype. All were found to require thiamine for growth. A requirement for thiamine was not found for any of the Ent⁻ strains.

Since Ent⁺, but not Ent⁻, cells were able to grow in the presence of high concentrations of EDDA, EDDA agar was used to select Ent⁺ variants of a wild-type Ent⁻ *S. flexneri* strain. Strain SA100 was mutagenized with diethyl sulfate and grown in L broth containing 500 μg of EDDA per ml to enrich for Ent⁺ variants. Individual colonies were isolated by plating on agar containing 500 μg of EDDA per ml. A variant obtained by this procedure, SA301, was grown in T medium and was found to synthesize both aerobactin and enterobactin. This laboratory variant, like the clinical Ent⁺ isolates, required thiamine for growth. To determine the relationship between the *thi* and *ent* mutations, a mutagenized culture of SA100 was subjected to penicillin enrichment for thiamine auxotrophs. Two classes of thiamine mutants were obtained. One class was indistinguishable from SA301, and mutants of this type were Ent⁺ in addition to Thi⁻. Additional mutants were found to require thiamine but remained Ent⁻. These mutants complemented the SA301 *thi* mutation and presumably represented mutations at a different locus. Attempts were made to revert the Ent⁺ Thi⁻ phenotype to the parental Ent⁻ Thi⁺ phenotype. No revertants were obtained when selecting for ability to grow in the absence of exogenous thiamine, even when the cells were mutagenized with diethyl sulfate before selection. Thus, the reversion rate is less than 10^{-9}.

As noted with the clinical Ent⁺ isolates, the laboratory variants, such as SA301, were found to utilize exogenous enterobactin for growth in low-iron medium (Table 2). Differences in the ability of Ent⁺ and Ent⁻ strains to respond to enterobactin suggested that the Ent⁻ strains lacked the receptors for this phenolate or were unable to remove iron from ferrienterobactin. To determine whether the enterobactin receptor(s) was expressed, strains were grown in medium containing EDDA to induce iron starvation, and the outer membranes were isolated. The outer membrane proteins were separated by SDS-PAGE and compared with outer membrane proteins of cells grown in the same medium with sufficient iron (Fig. 1). Two clinical Ent⁻ strains, SA100 and 3733b; two clinical Ent⁺ strains, SF100 and 1-56; and SA301, the Ent⁺ variant of SA100, were used in these experiments. A single outer membrane protein of M_r = 74,000 (74K protein), was induced by iron starvation of SA100 and 3733b. An iron-regulated

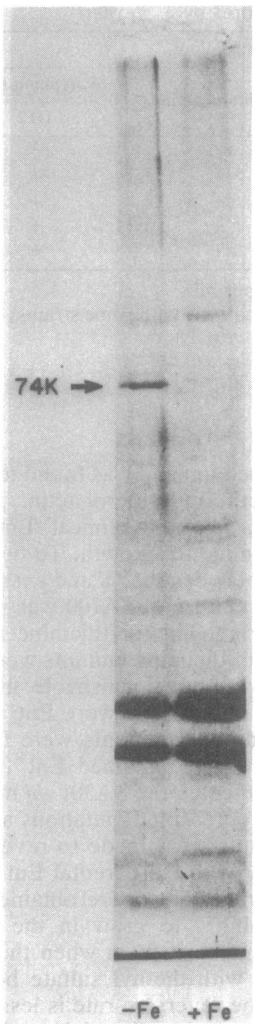

FIG. 1. SDS-PAGE of outer membrane proteins of *S. flexneri* SA100. Cells were grown in L broth with iron (+Fe) or in L broth with 250 μg of EDDA per ml (−Fe).

protein of this size has been found in the outer membrane of *E. coli* strains that express the aerobactin transport system and is the receptor for both aerobactin and cloacin, a bacteriocin synthesized by *Enterobacter cloacae* (4, 11, 25). Several proteins appeared to be synthesized in greater quantities in the cells grown in the presence of iron, but only the 74K protein was inducible by low iron levels. Absent from SA100 and 3733b are iron-regulated outer membrane proteins in the 81,000- to 83,000-molecular weight range (81K and 83K proteins) such as those seen in *E. coli* K-12 strains (5, 12, 15, 17). The 81K Fep protein serves as the enterobactin receptor in *E. coli* (12, 15, 22).

The Ent^+ isolates also expressed the 74K outer membrane protein during growth in iron-limiting medium. However, at least three additional iron-regulated outer membrane proteins were seen in these strains (Fig. 2). Proteins of the same apparent size as the *E. coli* 81K and 83K proteins were present, although the two proteins in *E. coli* and SA301 outer membranes did not resolve in this gel system. An additional protein band migrating near the 74K protein was also noted in these strains. Quantities of all these proteins were greatly reduced when the cells were grown in the presence of iron.

The presence of the 74K protein in aerobactin-utilizing strains, as well as its association with aerobactin utilization in *E. coli*(ColV) strains, suggested that it may be the receptor for aerobactin in *S. flexneri*. To determine the function of this protein, aerobactin synthesis and transport mutants of SA100 were isolated. Mutagenized cultures were subjected to two cycles of nalidixic acid selection to enrich for cells unable to grow in medium containing EDDA. Survivors were screened for the inability to grow on L agar containing small amounts of EDDA (12.5 μg/ml). Suspected mutants were assayed for the ability to synthesize and utilize aerobactin by both chemical and biological assays. Two classes of mutants were isolated by this procedure (Table 3). One class (e.g., SA201) was unable to synthesize aerobactin (*hds*). These mutants did not produce detectable siderophore by the ferric perchlorate assay and failed to stimulate growth of *S. flexneri* strains in the bioassay. Growth of these mutants in low-iron medium was restored by the addition of exogenous aerobactin. The second class neither synthesized nor utilized aerobactin. These mutants failed to grow in low-iron medium, even in the presence of exogenous aerobactin. Iron and the fungal hydroxamate siderophore ferrichrome both stimulated growth of this type of mutant (Table 3, SA255). Thus, this mutation is specific for the utilization of aerobactin (*hdu*). The ability to utilize ferrichrome suggests the presence of a receptor analogous to the 78K *tonA* protein of *E. coli* (17). However, no iron-regulated protein of this size was observed in these outer membrane preparations.

Since SA255 failed to utilize exogenous aerobactin, it appeared likely that the aerobactin receptor was affected by this mutation. Outer membranes of this mutant were prepared from cells grown in the presence or absence of EDDA and compared with the wild type (SA100) by SDS-PAGE (Fig. 3). The 74K outer membrane protein induced by iron starvation of the wild-type cells was not detected in iron-starved cells of the mutant. This suggests that this protein is the receptor for aerobactin or is required for some step in the utilization of iron from ferri-

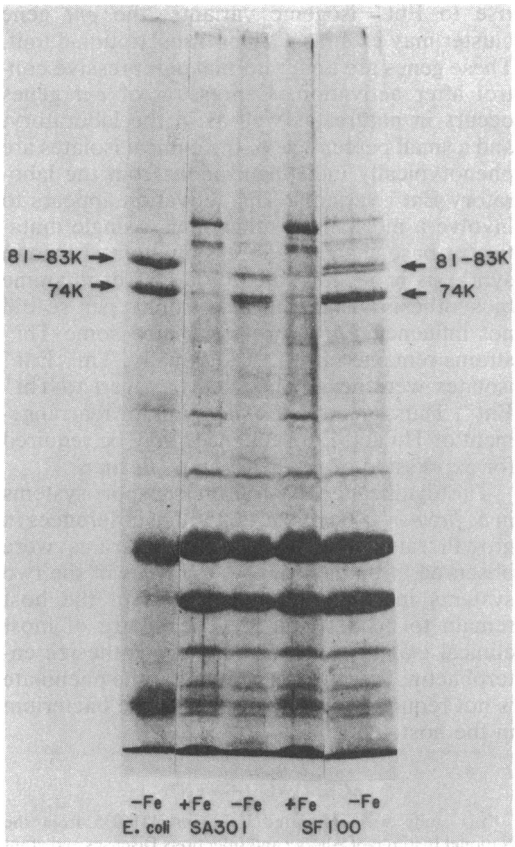

FIG. 2. SDS-PAGE of outer membrane proteins of a laboratory Ent$^+$ variant (SA301) and a clinical Ent$^+$ isolate (SF100). Cells were grown in L broth (+Fe) or in L broth with 1 mg of EDDA per ml (−Fe). Outer membrane proteins of iron-starved cells of *E. coli* are shown at the left for comparison.

aerobactin. The 74K protein was expressed in the synthesis mutant although at slightly lower levels than in the wild type.

The *S. flexneri* strains were also tested for their sensitivity to several bacteriocins which bind to siderophore receptors or iron-regulated proteins. Bacteriocins tested included colicin B, which binds to the 81K enterobactin receptor; colicin Ia, which recognizes the 74K *cir* protein; and cloacin, which binds to the 74K aerobactin receptor (4, 5, 11, 15, 17, 22, 25). All strains used in this study were found to be resistant to colicin B and Ia. SA100 was slightly sensitive to cloacin, whereas the transport mutant SA255 was resistant. However, the wild-type *S. flexneri* was at least 1,000-fold less sensitive to cloacin than *E. coli*(ColV) strains.

DISCUSSION

Phenolate siderophores such as enterobactin have been detected in a number of gram-negative enteric bacteria. *E. coli, S. sonnei*, and *S. typhimurium* each synthesize this compound, and related siderophores have been isolated from *Klebsiella* spp. and *Vibrio* spp. Compounds of this type are only rarely found in *S. flexneri*, however, and this species apparently relies on a hydroxamate compound, aerobactin, for acquisition of iron.

Occasional clinical isolates and laboratory variants of *S. flexneri* have been isolated which express the enterobactin system. Synthesis of enterobactin in these strains occurs in the presence of a functional aerobactin system. Syntheses of aerobactin and enterobactin have a number of common features in *S. flexneri*. Both systems are repressed by iron and each permits the growth of the bacteria in low-iron medium. Those strains synthesizing enterobactin, however, appear to be at a distinct advantage in the laboratory. Ent$^+$ strains grow faster than Ent$^-$ strains and are better able to compete with chelators for iron. The significance of these systems in acquisition of iron in vivo, however, remains to be determined.

Each transport system is associated with the expression of specific outer membrane proteins. A single 74K outer membrane protein is found in iron-starved cells of strains which synthesize aerobactin but not enterobactin. This protein is not detected in the outer membranes of mutants which are unable to utilize exogenous aerobactin for growth in low-iron medium and probably serves as the receptor for ferriaerobactin. A 74K outer membrane protein has been postulated to be the receptor for aerobactin in *E. coli*(ColV) strains (25), but it remains to be determined whether the proteins are homologous in the two species. Three additional iron-regulated outer membrane proteins are found in laboratory and clinical isolates which synthesize enterobactin. These have approximate molecular weights, by SDS-PAGE, of 74,000, 81,000, and 83,000 and are analogous to the iron-regulated proteins associated with enterobactin synthesis in *E. coli* K-12 strains (17). Only strains which express these outer membrane proteins have been found to utilize ferrienterobactin for growth in low-iron

TABLE 3. Synthesis and utilization of aerobactin by iron transport mutants of *S. flexneri*

Indicator	Zone of stimulation[a]			
	Aerobactin	SA100	SA201	SA255
SA100[b]	+	+	−	−
SA201	+	+	−	−
SA255	−	−	−	−

[a] +, >12-mm zone of stimulation by siderophore or growing culture; −, no zone of stimulation.

[b] 10^3 bacteria per ml in L agar containing 250 μg of EDDA per ml.

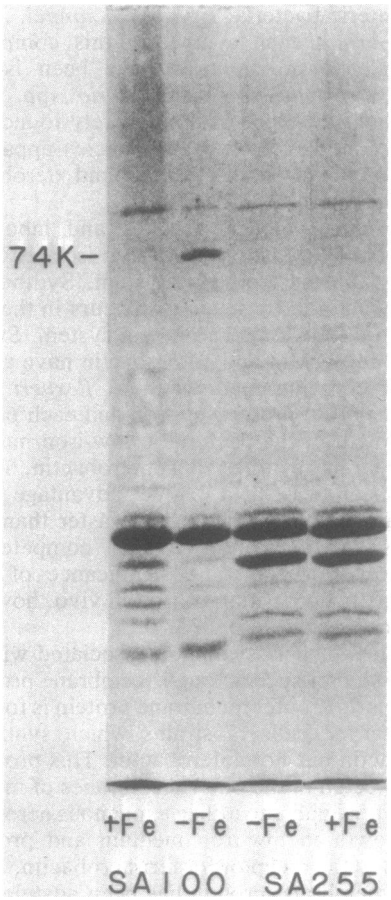

FIG. 3. SDS-PAGE of outer membrane proteins of wild-type SA100 and hydroxamate utilization mutant SA255. Cells were grown in L broth with iron (+Fe) or in L broth with EDDA (−Fe).

medium. One of the proteins is likely to be the enterobactin receptor in *S. flexneri*. Although the iron-regulated outer membrane proteins of *S. flexneri* are the same apparent molecular weight as those of *E. coli*, their presence does not appear to confer sensitivity to bacteriocins as is found in *E. coli*. It is possible that the bacteriocins recognize and bind to these outer membrane proteins in *S. flexneri* but fail to kill the cells.

It is apparent that *S. flexneri* has the genetic information to encode a phenolate-mediated transport system. An explanation for the failure of most *S. flexneri* strains to synthesize or utilize enterobactin is not clear. These strains resemble regulatory mutants in that both synthesis and transport genes are affected. If this is a regulatory mutation, it is distinct from *E. coli fur* (8) mutations because aerobactin synthesis and transport are unaffected in these strains. Since Ent^- laboratory strains of *S. flexneri* can give rise to Ent^+ isogenic variants, the *ent* gene cluster may exist as a silent transcriptional unit. These genes are under normal derepressive control after activation. Expression of *ent* genes occurs in nature, as well as in the laboratory, and a small percentage of the clinical isolates are phenotypically indistinguishable from the laboratory Ent^+ variants. The activation appears to involve a mechanism other than a single mutation or base change. Expression of enterobactin synthesis is accompanied by a loss of thiamine biosynthesis. Thiamine auxotrophy per se did not influence *Ent* expression since some Thi^- strains remained Ent^-. Additionally, Thi^- Ent^+ isolates were never observed to revert to Thi^+ Ent^-. Thus, an insertion, deletion, or rearrangement of Thi and Ent sequences may be required for expression of *ent* genes in *S. flexneri*.

The significance of two iron transport systems in *S. flexneri* is not clear. Although differences in growth rates of Ent^+ and Ent^- strains were observed in the laboratory, the roles of the two systems in the environment and in the host remain to be determined. The failure of most clinical isolates of *S. flexneri* to synthesize enterobactin, however, suggests that the phenolate is not required for the survival of the bacterium in the host.

ACKNOWLEDGMENTS

This study was supported by grant AI16935 from the National Institute of Allergy and Infectious Diseases and grant F-941 from the Robert A. Welch Foundation.

We thank Gloria Pierce, Texas Department of Health, for providing bacterial strains.

LITERATURE CITED

1. **Ames, G. F.-L.** 1974. Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. J. Biol. Chem. **249**:634–644.
2. **Arnow, L. E.** 1937. Colorimetric determination of the components of 3,4-dihydroxyphenylalanine–tyrosine mixtures. J. Biol. Chem. **228**:531–537.
3. **Atkin, C. L., J. B. Neilands, and H. J. Phaff.** 1970. Rhodotorulic acid from species of *Leucosporidium, Rhodosporidium, Rhodotorula, Sporidiobolus*, and *Sporobolomyces*, and a new alanine-containing ferrichrome from *Cryptococcus melibiosum*. J. Bacteriol. **103**:722–733.
4. **Bindereif, A., V. Braun, and K. Hantke.** 1982. The cloacin receptor of ColV-bearing *Escherichia coli* is part of the Fe^{3+}-aerobactin transport system. J. Bacteriol. **150**:1472–1475.
5. **Braun, V., R. E. Hancock, K. Hantke, and A. Hartmen.** 1976. Functional organization of the outer membrane of *Escherichia coli*: phage and colicin receptors as components of iron uptake systems. J. Supramol. Struct. **5**:37–58.
6. **Brenner, D. J., G. R. Fanning, G. V. Miklos, and A. G. Steigerwalt.** 1973. Polynucleotide sequence relatedness among *Shigella* species. Int. J. Syst. Bacteriol. **23**:1–7.
7. **Brot, N., J. Goodwin, and H. Fales.** 1966. *In vivo* and *in vitro* formation of 2,3-dihydroxybenzoylserine by *Escherichia coli* K-12. Biochem. Biophys. Res. Commun. **25**:454–461.
8. **Ernst, J. F., R. L. Bennett, and L. I. Rothfield.** 1978. Constitutive expression of the iron-enterochelin and fer-

richrome uptake systems in a mutant strain of *Salmonella typhimurium*. J. Bacteriol. **135**:928–934.
9. **Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart.** 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. **115**:717–722.
10. **Gibson, F., and D. I. Magrath.** 1969. The isolation and characterization of a hydroxamic acid (aerobactin) formed by *Aerobacter aerogenes* 62-1. Biochim. Biophys. Acta **192**:175–184.
11. **Grewal, K. K., P. J. Warner, and P. H. Williams.** 1982. An inducible membrane protein involved in aerobactin-mediated iron transport by ColV strains of *Escherichia coli*. FEBS Lett. **140**:27–30.
12. **Hollifield, W. C., Jr., and J. B. Neilands.** 1978. Ferric enterobactin transport system in *Escherichia coli* K12. Extraction, assay, and specificity of the outer membrane receptor. Biochemistry **17**:1922–1928.
13. **Inouye, M., and J. P. Guthrie.** 1969. A mutant which changes a membrane protein of *E. coli*. Proc. Natl. Acad. Sci. U.S.A. **64**:957–961.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of phage T4. Nature (London) **227**:680–685.
15. **McIntosh, M. A., S. S. Chenault, and C. F. Earhart.** 1979. Genetic and physiological studies on the relationship between colicin B resistance and ferrienterochelin uptake in *Escherichia coli* K-12. J. Bacteriol. **137**:653–657.
16. **Neilands, J. B.** 1981. Microbial iron compounds. Annu. Rev. Biochem. **50**:715–731.
17. **Neilands, J. B.** 1982. Microbial envelope proteins related to iron. Annu. Rev. Microbiol. **36**:285–309.
18. **O'Brien, I. G., and F. Gibson.** 1970. The structure of enterochelin and related 2,3-dihydroxy-N-benzoylserine conjugates from *Escherichia coli*. Biochim. Biophys. Acta **215**:393–402.
19. **Payne, S. M.** 1980. Synthesis and utilization of siderophores by *Shigella flexneri*. J. Bacteriol. **143**:1420–1424.
20. **Perry, R. D., and C. L. San Clemente.** 1979. Siderophore synthesis in *Klebsiella pneumoniae* and *Shigella sonnei* during iron deficiency. J. Bacteriol. **140**:1129–1132.
21. **Pollack, J. R., and J. B. Neilands.** 1970. Enterobactin, an iron transport compound from *Salmonella typhimurium*. Biochem. Biophys. Res. Commun. **38**:989–992.
22. **Pugsley, A. P., and P. Reeves.** 1977. The role of colicin receptors in the uptake of ferrienterochelin by *Escherichia coli* K-12. Biochem. Biophys. Res. Commun. **74**:903–911.
23. **Rogers, H. J.** 1973. Iron-binding catechols and virulence in *Escherichia coli*. Infect. Immun. **7**:445–456.
24. **Simon, E. H., and I. Tessman.** 1963. Thymidine-requiring mutants of phage T4. Proc. Natl. Acad. Sci. U.S.A. **56**:526–532.
25. **Van Tiel-Mengveld, G. J., B. Oudega, O. Kempers, and F. K. de Graaf.** 1981. The possible involvement of the cloacin DF13 receptor protein in the hydroxamate mediated uptake of iron by *Enterobacter cloacae* and *Escherichia coli* (ColV). FEMS Lett. **12**:373–380.
26. **Warner, P. J., P. H. Williams, A. Bindereif, and J. B. Neilands.** 1981. ColV plasmid-specified aerobactin synthesis by invasive strains of *Escherichia coli*. Infect. Immun. **33**:540–545.
27. **Wilkins, T. D., and C. E. Lankford.** 1970. Production by *Salmonella typhimurium* of 2,3-dihydroxybenzoylserine and its stimulation of growth in human serum. J. Infect. Dis. **121**:129–136.