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Iron Uptake in *Plesiomonas shigelloides*: Cloning of the Genes for the Heme-Iron Uptake System

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The iron uptake systems of *Plesiomonas shigelloides* strains were determined. Siderophore production was not detected by chemical or biological assays, and the strains tested were unable to use enterobactin, aerobactin, or vibriobactin for growth in low-iron media. Both hemin and hemoglobin supported full growth of the bacteria in media lacking other iron sources, but neither transferrin nor lactoferrin served as a source of iron. Hemolysin was detected, and the production of hemolysin was iron repressible. DNA sequences encoding hemolysin production and DNA sequences encoding the ability to use heme or hemoglobin as a sole source of iron were cloned from *P. shigelloides* and expressed in *Escherichia coli*. The abilities to use heme and hemoglobin as iron sources were closely linked, and the cloned sequences encoded the ability to transport the porphyrin, as well as iron, into the cells.

Plesiomonas shigelloides, a gram-negative rod belonging to the *Vibrionaceae* family, is associated with outbreaks of diarrheal disease and food poisoning in humans (25). Recent reports have indicated that this organism is responsible for both sporadic and epidemic illnesses, including an acute secretory gastroenteritis (18), a more invasive form which resembles shigellosis (20), and a choleralike illness (30).

Although *P. shigelloides* is known to cause disease in humans, the mechanisms of its pathogenesis are unknown. Studies by Abbott et al. (1) and Herrington et al. (13) on the relative pathogenicity of *P. shigelloides* have indicated that the organism is not a highly virulent pathogen. In an intraperitoneally inoculated mouse model, the mean 50% lethal dose of 16 strains was 3×10^8 (1), and although human volunteers could be colonized with 1 strain, no illness was detected (13).

P. shigelloides has been screened for a variety of potential virulence factors. The organism has been reported to produce both choleralike (10) and heat-stable (19) toxins, although in other studies these toxins were not detected (1, 13). These results may represent differences in strains and/or methodologies. Neither invasion of tissue culture cells nor the presence of hemagglutinins or other cell surface adhesins has been observed (1, 25). Most strains possess a large (>120-MDa) plasmid, which one report suggests may facilitate invasion (13).

Iron acquisition systems have been shown to be involved in the virulence of a variety of bacterial pathogens (23). However, little is known about iron transport systems in *Plesiomonas* spp. Siderophores have not been found in this organism (1), suggesting that other iron transport systems may be important. The utilization of host iron sources such as transferrin or hemoglobin may provide an alternative method of iron acquisition. Iron transport systems associated with these host proteins have been found in human pathogens such as *Haemophilus influenzae* (12, 29), *Neisseria* spp. (2), and *Vibrio cholerae* (28).

Acquisition of iron from heme or hemoglobin may be facilitated by the production of hemolysins or cytotoxins which lyse host cells and release intracellular iron complexes. The production of hemolysin and the utilization of heme have been shown to contribute to the virulence of certain *Escherichia coli* strains (31, 32). Interestingly, the synthesis of several hemolysins has been found to be iron regulated; maximal production occurs under conditions of iron limitation (16, 28). Although one strain of *P. shigelloides* was reported to be hemolytic (9), the organisms are generally described as nonhemolytic (6). In the present study, several strains of *P. shigelloides* were analyzed to determine mechanisms of iron acquisition and the possible expression of hemolysins.

MATERIALS AND METHODS

Bacterial strains. *P. shigelloides* strains were provided by Steven E. Gardner, Veterans Administration Medical Center, Los Angeles, Calif. *E. coli* AN344 and RK4745 were provided by Robert Kadner, University of Virginia. *E. coli* 1017 is an Ent$^-$ Tn*5* insertion mutant of HB101 constructed for this study. Characteristics of bacterial strains and plasmids are indicated in Table 1. Strains were maintained at −80°C in L broth with 20% glycerol.

Media and reagents. Strains were grown on L agar or in L broth at 37°C. The iron chelator EDDA [ethylenediamine-di(*o*-hydroxyphenyl acetic acid)] (Sigma, St. Louis, Mo.) was deferrated by the method of Rogers (24) and added to L broth or L agar to induce iron limitation. T medium without added iron (27) was used to assay siderophore production. Chrome azurol S agar (26) was also used to detect siderophore production. Blood agar for the detection of hemolysin consisted of 1% washed human erythrocytes added to soft L agar.

Siderophores were prepared as described previously (11, 22). Transferrin, lactoferrin, hemin, and hemoglobin were obtained from Sigma and used as described previously (15). 5-Aminolevulinic acid was also obtained from Sigma.

Siderophore assays. Low-iron, T medium culture supernatants were screened for siderophore production by the chrome azurol S assay (26), the Arnow assay for catechols (3), and the ferric perchlorate (4) and Czaky (8) assays for hydroxamates. A bioassay was also used as follows to detect the secretion or utilization of siderophores. L agar with EDDA at 200 μg/ml was seeded with 10^4 indicator organisms

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Characteristic(s)
Strains	
P. shigelloides 3	Clinical isolate
P. shigelloides 9	Type strain
P. shigelloides 11	Clinical isolate
E. coli RK4745	RK4353 *hemA*, prototroph
E. coli AN344	*leu pro hemA*
E. coli 1017	HB101 *ent*::Tn*5*
Plasmid	
pJB8	Cosmid cloning vector, Amp^r
pAT153	Cloning vector, Amp^r Tet^r
pHPS1	30-kb fragment of *P. shigelloides* DNA encoding heme utilization cloned into pJB8
pHPS5	5.2-kb *Bam*HI fragment of pHPS1 subcloned into pAT153

per ml and allowed to solidify. Aliquots (10 μl) of fully grown bacterial cultures or dilutions of purified siderophores were spotted on plate surfaces to test for siderophore production and utilization. Plates were incubated at 37°C for 18 h and checked for zones of stimulation of indicator organisms by producer strains or siderophores. No growth of indicator organisms was detected in the absence of iron or usable siderophores.

Hemolysin assays. Cultures were grown in 5 ml of L broth or L broth with EDDA to a density of 10^9/ml. The cells were removed by centrifugation, and 200 μl of supernatant was added to 800 μl of the hemolysin assay mixture as described by Mercurio and Manning (21). The assay mixture contained 0.25% washed human erythrocytes, 0.02 M KH_2PO_4, 0.06 M Na_2HPO_4 (pH 7.0), and 0.12 M NaCl. The reaction mixtures were incubated at 37°C for 60 min and centrifuged to remove all unlysed erythrocytes. The A_{515} of the supernatant was measured to determine erythrocyte lysis. A hemolysin assay mixture incubated with L broth or L broth with EDDA was used as the blank.

For the detection of hemolytic colonies, cells were grown on L agar with or without EDDA for 18 h, overlaid with 4 ml of blood agar, and reincubated until zones of hemolysis appeared. To test for cell-associated hemolysin, we used the contact hemolysis assay of Clerc et al. (7).

Host iron compound utilization assays. Strains were grown to an A_{650} of 0.7, diluted, inoculated into 20 ml of L agar with EDDA (250 μg/ml) at 10^4 bacteria per ml, and poured into plates. Wells were punched in the agar and filled with 50 μl of the iron compound to be tested. Plates were incubated at 37°C and examined at 24 h for zones of growth.

Cloning. Chromosomal DNA from strain 9 was partially digested with *Sau*3A and ligated into *Bam*HI-digested pJB8. The DNA was packaged with Packagene (Promega) and plated on *E. coli* 1017. Colonies were screened on L agar plus carbenicillin (250 μg/ml), EDDA (75 μg/ml), and hemin (10 μM) for recombinants able to use hemin as the sole iron source. The library was also screened for hemolytic clones by the blood agar overlay method. *E. coli* 1017 containing cosmid clone pHSP1 was grown in L broth plus carbenicillin in the presence of EDDA and hemin to maintain expression of the plasmid. Plasmid DNA was isolated by the method of Kado and Liu (14).

Restriction fragments of pHPS1 were subcloned by random ligation into the multicopy plasmid vector pAT153 and transformation into *E. coli* 1017. Transformants were selected on L agar plus carbenicillin and streaked onto L agar plus carbenicillin (250 μg/ml), EDDA (50 μg/ml), and hemin (10 μM). After 48 h, colonies were inoculated into L broth plus EDDA (50 μg/ml) and hemin (10 μM) and allowed to grow for 24 h. Only transformants which allowed the growth of *E. coli* 1017 both in L broth and on L agar plus EDDA and hemin were analyzed further.

TABLE 2. Utilization of host iron sources by *P. shigelloides*

Iron source[a]	Growth[b] of strain:		
	3	9	11
Enterobactin (10 μM)	−	−	−
Aerobactin (10 μM)	−	−	−
Vibriobactin (10 μM)	−	−	−
Transferrin (30 mM)	−	−	−
Lactoferrin (30 mM)	−	−	−
Hemin (8 μM)	4+	4+	4+
Hemoglobin (10 μM)	4+	4+	4+
$FeCl_3$ (10 μM)	1+	1+	1+

[a] Transferrin and lactoferrin were tested as iron-saturated (100%) compounds.

[b] Diameter of the zone of growth around the well containing the iron compound in L agar with EDDA: −, no growth; 1+, ≤12 mm; 4+, >30 mm.

Effect of cloned genes on the growth of *hemA* mutants. Strains AN344 and RK4745 were transformed with subclone pHPS5. Transformants and parental strains were grown overnight in L broth plus 5-aminolevulinic acid (24 μM). Carbenicillin (250 μg/ml) was added to all cultures containing the plasmid. The cells were washed once in saline to remove any traces of 5-aminolevulinic acid and diluted 1/500 into L broth, L broth plus hemin (10 μM), or L broth plus 5-aminolevulinic acid (24 μM). Growth was monitored over a 26-h period.

RESULTS

Screening for siderophore production and utilization. Three strains of *P. shigelloides* were screened for iron acquisition systems. Siderophore production was assessed by both chemical and biological assays. As reported by Abbott et al. (1), no siderophores were detected by the chrome azurol S assay, a universal siderophore detection assay. The Arnow (3) and Czaky (8) tests for catechols and hydroxamates, respectively, were also negative. A sensitive bioassay in which the *P. shigelloides* strains were tested for their ability to stimulate the growth of homologous or heterologous strains in low-iron media was also used, but no stimulation of growth was detected. Some bacterial species which fail to make their own siderophores can use compounds secreted by other microbes. However, *P. shigelloides* did not utilize enterobactin, aerobactin, or vibriobactin in the bioassay (Table 2).

Utilization of host iron sources. Since the assays did not reveal the presence of siderophore-mediated iron transport systems, other methods of iron acquisition were investigated. Because these bacteria are found in mammalian hosts, it is likely that they are able to use one or more host iron compounds as a source of the essential element. The ability to utilize transferrin, lactoferrin, hemoglobin, or heme as a sole source of iron was tested (Table 2). Both hemin and hemoglobin supported growth of the bacteria in an iron-depleted medium, but transferrin and lactoferrin did not serve as iron sources. Hemin and hemoglobin appeared to be equally good iron sources (Fig. 1), and maximum growth of

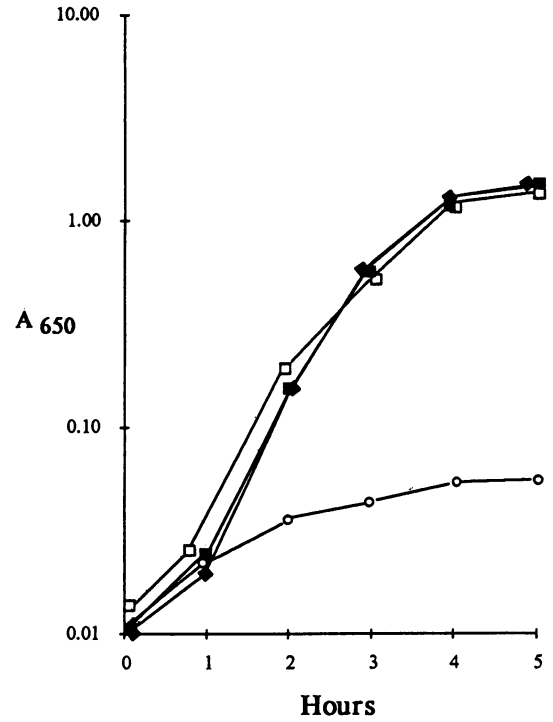

FIG. 1. Growth of *P. shigelloides* with hemin or hemoglobin as the sole source of iron. Cells were grown in L broth (□), L broth with EDDA (50 μg/ml) (○), or L broth with EDDA and supplemented with hemin (10 μM) (◆) or hemoglobin (2.5 μM) (■). Growth was monitored by measuring the A_{650}.

the bacteria was supported by approximately 1 μM hemoglobin or 5 μM hemin under the conditions tested (data not shown).

Iron-regulated synthesis of hemolysin. Because *P. shigelloides* used hemin and hemoglobin, the production of hemolysins which could release these compounds from host cells was determined. No zones of hemolysis were noted when the strains were streaked on blood agar containing either human or sheep erythrocytes, although Ferguson and Henderson (9) had detected hemolysis by one strain of *P. shigelloides* grown on veal infusion blood agar. Hemolytic activity was detected in broth cultures, however. Both cells and supernatants were positive, as determined by contact and liquid hemolysis assays, respectively. The amount of hemolysin produced and the influence of iron on its production were measured by the liquid hemolysis assay (Table 3). Synthesis of the hemolysin was repressed by iron, and maximum production of the hemolysin was detected in cells which had been grown under iron-restrictive conditions (Table 3).

TABLE 3. Hemolysin production by *P. shigelloides*

Strain	Hemolysin (A_{515})[a] in:		Growth (A_{650})[b] in:		Hemolysin (U)[c] in:	
	L	L-EDDA	L	L-EDDA	L	L-EDDA
3	0.000	0.061	1.65	0.227	0	26.9
9	0.029	0.221	2.16	0.135	1.3	163.7
11	0.000	0.088	1.92	0.177	0	49.7

[a] Supernatants from overnight liquid cultures with or without the iron chelator EDDA (20 μg/ml) were assayed for hemolysin as described in Materials and Methods.
[b] A_{650} of the culture at the time of the hemolysin assay.
[c] Hemolysin units are defined as (A_{515} of hemolysin assay/A_{650} of culture) × 100.

Although hemolysis was not seen when colonies were grown on the surface of blood agar plates, hemolytic colonies could be detected by an overlay method. Colonies grown on L agar were overlaid with soft agar containing 1% erythrocytes and reincubated for 2 to 6 h, at which time zones of hemolysis were detected. In agreement with the results of the liquid hemolysis assay, iron starvation induced by the addition of the iron chelator EDDA to the agar resulted in larger zones of hemolysis (data not shown).

Cloning of the hemolysin and hemin utilization genes of *P. shigelloides*. A library of *P. shigelloides* DNA in the cosmid vector pJB8 was screened in *E. coli* 1017. This strain is an Ent$^-$ derivative of HB101 which fails to grow in low-iron medium and lacks the ability to use hemin or hemoglobin as a source of iron. Recombinants were screened for growth on L agar with EDDA and hemin or hemoglobin and by a blood agar overlay assay for the production of hemolysin.

Several clones which produced zones of hemolysis in the blood agar overlay assay were obtained. These were analyzed further for hemolysin production in the liquid and contact hemolysis assays. These clones were positive for iron-regulated contact hemolysin but, in contrast to *P. shigelloides*, cell-free culture supernatants did not contain hemolytic activity. This result suggests that the hemolysin is secreted by *P. shigelloides* but that the cloned sequences do not encode all the components necessary for secretion in *E. coli*. These clones were negative for heme or hemoglobin utilization (data not shown).

Additional plasmids which permitted the growth of *E. coli* 1017 in low-iron medium supplemented with hemin were isolated. These clones also grew in the presence of hemoglobin but failed to grow in low-iron medium in the absence of heme sources. None of these recombinants encoded hemolysin production. One of the plasmids, pHPS1, was chosen for further study. Analysis of digests with the restriction enzymes *Bam*HI, *Sal*I, and *Eco*RI indicated that pHPS1 contained an approximately 30-kb insert cloned into pJB8. Subcloning of restriction fragments of the cosmid indicated that a single 5.2-kb *Bam*HI fragment allowed *E. coli* 1017 to utilize hemin as a sole iron source (Fig. 2); the parental strain lacking the plasmid did not grow under these conditions. This plasmid, pHPS5, also supported growth on hemoglobin (Fig. 2), suggesting that if separate genes are required for the two iron sources, they are closely linked.

Hemin transport by *E. coli hemA* mutants. Although the cloned *P. shigelloides* fragments allowed cells to use heme as an iron source, it was not clear whether the iron was being removed from the heme at the surface of the cells or whether the intact porphyrin was being transported into the cells. To address this problem, we transformed mutants which have a requirement for the porphyrin with plasmid pHPS5 and assayed them for growth. These *hemA* mutants fail to synthesize heme and require the precursor 5-aminolevulinic acid for aerobic growth. Their heme requirement normally cannot be met by hemin, since they are unable to transport that compound. Two *E. coli hemA* mutants, AN344 and RK4745 (Table 1), transformed with the plasmid were assayed for growth in medium containing 5-aminolevulinic acid or hemin (Fig. 3). Both parental strains grew poorly in L broth or L broth plus hemin but grew normally when the medium was supplemented with 5-aminolevulinic acid. The presence of the plasmid, however, allowed the cells to grow in L broth containing hemin. These data indicate that pHPS5

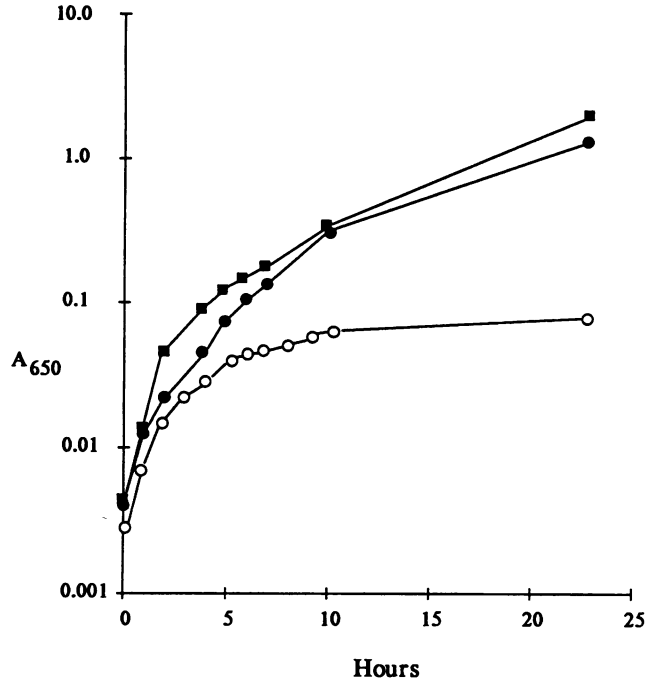

FIG. 2. Effect of cloned sequences on the growth of *E. coli* 1017 in medium with hemin or hemoglobin as the sole source of iron. *E. coli* 1017/pHPS5 was grown in L broth with EDDA (50 μg/ml) (○) or L broth with EDDA and supplemented with hemin (10 μM) (●) or hemoglobin (2.5 μM) (■). Growth was monitored by measuring the A_{650}.

encodes one or more genes which allow cells to use heme as an iron source and that the intact heme moiety is transported into the cells.

DISCUSSION

Assays of iron transport systems did not reveal the presence of siderophore-mediated iron transport systems in *P. shigelloides*, in agreement with the findings of Abbott et al. (1). The strains tested not only failed to synthesize the iron-chelating compounds but also failed to use siderophores synthesized by other enteric bacteria. However, *P. shigelloides* was able to use either hemin or hemoglobin as a source of iron. It is likely that these iron complexes serve as iron sources when these bacteria are within a human host.

Genes encoding the ability to use hemin or hemoglobin as an iron source could be cloned and expressed in *E. coli*, permitting observations of the influence of particular genes or mutations on this system and facilitating a more detailed study of this system. Transformation of *hemA* mutants of *E. coli* provided evidence that the porphyrin as well as the iron is transported into the cells. These mutants are unable to synthesize heme but do not grow with exogenous heme because they lack the ability to transport the molecule. Acquisition of the *P. shigelloides* genes permitted growth of the mutants in the presence of hemin or hemoglobin, indicating that the Fe-porphyrin complex is being transported into the cell rather than that the iron is being removed from the molecule extracellularly.

Acquisition of iron from hemin or hemoglobin may be facilitated by the expression of hemolysin. Some clinical

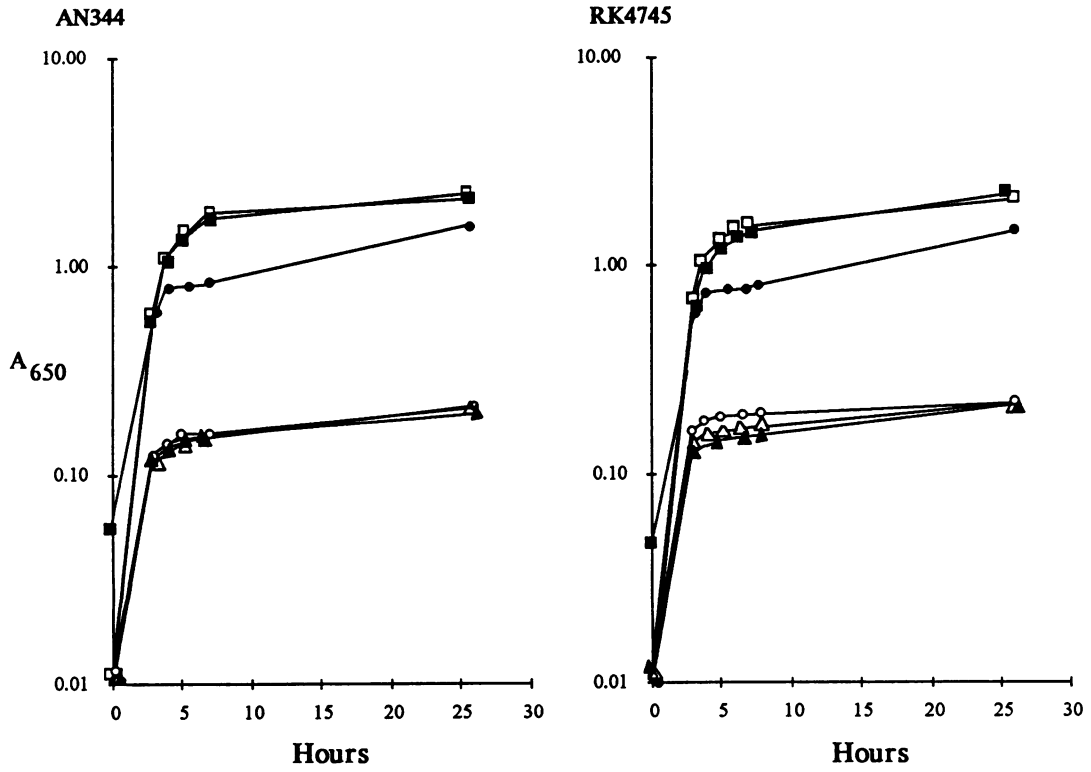

FIG. 3. Effect of cloned sequences on the growth of *hemA* mutants of *E. coli*. The parental strains (open symbols) and transformants carrying pHPS5 (solid symbols) were grown in L broth (△ and ▲) or L broth supplemented with 10 μM hemin (○ and ●) or 24 μM 5-aminolevulinic acid (□ and ■).

isolates of *E. coli* produce hemolysins and can utilize the iron present in heme or hemoglobin released from cells by hemolysis (17, 31). Waalwijk et al. (31) have shown that hemolysin production enhances the virulence of nephropathogenic *E. coli* by increasing the level of available iron in the host via the lysis of erythrocytes and the subsequent release of hemoglobin. Similarly, the virulence of extraintestinal *E. coli* infections, as measured in the rat peritonitis model, is enhanced by the production of hemolysin (32). The cytotoxic hemolysins of *V. cholerae* probably effect the release of intracellular heme from damaged epithelial cells in the intestine or hemoglobin from erythrocytes in a more invasive infection, to be utilized directly as iron sources (28). Since both localized and more invasive forms of disease are associated with *P. shigelloides* infections, the hemolysin detected in *P. shigelloides* could function in a similar manner.

In some bacteria, such as *E. coli* (16) and *V. cholerae* (28), hemolysin production is known to be influenced by iron concentration. Analysis of the synthesis of the *P. shigelloides* hemolysin indicated that it was also derepressed under conditions of iron starvation. This result suggests the presence of a *fur*-like regulatory system (5) which could influence the expression of iron transport system genes as well as other genes. Gardner et al. (10) reported that the production of both heat-stable and heat-labile choleralike toxins in *P. shigelloides* was detected only under iron-limited conditions. It is not known whether these toxins and the hemolysin are part of the same regulon.

Hemolysin production or activity in *P. shigelloides* is also influenced by other environmental conditions. No hemolytic activity was noted when colonies were grown on the surface of agar plates. Growth of the cells in liquid or in a soft agar overlay, however, produced detectable hemolysis. This result suggests that conditions such as oxygen tension or viscosity may influence expression.

Sequences encoding the hemolysin were cloned and expressed in *E. coli*. Iron regulation of hemolysin expression was maintained in *E. coli*, although the hemolysin was not secreted into the culture medium as it was in *P. shigelloides*. Genes for hemolysin production do not appear to be linked to those for heme iron utilization; none of the hemolysin-positive cosmid clones encoded heme utilization, nor did any of the heme utilization clones express hemolysin activity.

The presence of an iron-regulated hemolysin and a transport system for the iron found in heme or hemoglobin may represent an important pathway for iron acquisition within a mammalian host. The cloning and characterization of the genes and their products will permit a detailed characterization of the heme transport system and its role in bacterial survival in vivo.

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