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Genetics and Regulation of Heme Iron Transport in *Shigella dysenteriae* and Detection of an Analogous System in *Escherichia coli* O157:H7

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***Shigella* species can use heme as the sole source of iron. In this work, the heme utilization locus of *Shigella dysenteriae* was cloned and characterized. A cosmid bank of *S. dysenteriae* serotype 1 DNA was constructed in an *Escherichia coli* siderophore synthesis mutant incapable of heme transport. A recombinant clone, pSHU12, carrying the heme utilization system of *S. dysenteriae* was isolated by screening on iron-poor medium supplemented with hemin. Transposon insertional mutagenesis and subcloning identified the region of DNA in pSHU12 responsible for the phenotype of heme utilization. Minicell analysis indicated that a 70-kDa protein encoded by this region was sufficient to allow heme utilization in *E. coli*. Synthesis of this protein, designated Shu (*Shigella* heme uptake), was induced by iron limitation. The 70-kDa protein is located in the outer membrane and binds heme, suggesting it is the *S. dysenteriae* heme receptor. Heme iron uptake was found to be TonB dependent in *E. coli*. Transformation of an *E. coli hemA* mutant with the heme utilization subclone, pSHU262, showed that heme could serve as a source of porphyrin as well as iron, indicating that the entire heme molecule is transported into the bacterial cell. DNA sequences homologous to *shu* were detected in strains of *S. dysenteriae* serotype 1 and *E. coli* O157:H7.**

The *Shigella* species have the ability to thrive in a variety of different habitats. They can grow extra- or intracellularly in the host as well as in the environment (8). This ability to occupy multiple niches implies that these bacteria can respond to changes in the environment and adapt to a variety of conditions, including changes in temperature, osmolarity, oxygen content, and concentrations of important nutrients (8).

One nutrient which is essential for all pathogenic bacteria is iron. Although iron is abundant in nature, its availability is restricted because of its poor solubility in aerobic environments (33). In mammalian hosts, most iron is sequestered. The majority of iron is found in erythrocytes as heme in the protein hemoglobin (3, 4). Host cells also contain ferritin, a storage form of iron. High-affinity iron-binding proteins, such as transferrin and lactoferrin, complex any extracellular iron (25, 28). Thus, the intracellular and extracellular pools of free iron are extremely small.

Enteric pathogens have several mechanisms to acquire iron. One method is the production of siderophores (5). Under conditions of iron starvation, these low-molecular-weight iron chelators are synthesized and secreted from the cell. Gram-negative bacteria transport ferric siderophores into the cell using specific outer membrane protein receptors, periplasmic proteins, and inner membrane protein complexes (26).

Alternatively, pathogenic bacteria may obtain iron directly from host iron sources. Pathogenic *Neisseria* spp. use specific receptors to obtain iron from human transferrin and lactoferrin (2). Heme proteins also provide an iron source for some pathogens. *Haemophilus influenzae* has been shown to acquire iron from hemoglobin, hemoglobin bound to haptoglobin, and heme-hemopexin (29), and mesophilic *Aeromonas* species grow well with hemoglobin, alone or complexed to haptoglobin, as the only iron source (22).

The free heme molecule itself may be a potential source of iron for pathogens. *Vibrio cholerae* (10), *Plesiomonas shigelloides* (6), and *Yersinia enterocolitica* (36) have been shown to possess heme transport systems. A number of other bacterial species, including *Aeromonas* species (22) and *Vibrio anguillarum* (23), are able to grow in iron-deficient media with heme as the only iron source.

Shigella spp. produce one or more siderophores (28). However, mutants unable to make siderophores are able to invade and multiply intracellularly, indicating the ability to acquire iron by other methods (16). Assays for utilization of potential iron sources found in the host indicate that the *Shigella* species are able to grow with heme as a sole iron source and that the ability to use heme as an iron source is siderophore independent (16). This investigation was undertaken to characterize the *Shigella dysenteriae* heme utilization system.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and their characteristics and sources are listed in Table 1. Representative *Escherichia coli* O157:H7 and *E. coli* O26:H11 strains were provided by James Kaper (University of Maryland). All other clinical isolates of *Shigella* spp. and *E. coli* were obtained through the Texas Department of Health (Austin, Tex.). Plasmids are also listed in Table 1.

Media. Bacterial strains were grown at 37°C in L broth or L agar. To restrict iron availability, the iron chelator ethylenediamine di(*o*-hydroxyphenylacetic acid) (EDDA) was deferrated (31) and added to liquid and solid media. The EDDA concentrations were 100 to 200 μg/ml for the screening of heme utilization clones in *E. coli* 1017 and 1 mg/ml for the assay of heme transport in clinical *E. coli* strains. When they were added to media, the concentrations of hemin and ferrous sulfate were 5 μg/ml and 20 μM, respectively. Antibiotics and standard concentrations were as follows: carbenicillin, 250 μg/ml; chloramphenicol (Cm), 30 μg/ml; kanamycin, 50 μg/ml; streptomycin, 200 μg/ml; and tetracycline (Tc), 12.5 μg/ml. For strains containing pAT153-based vectors, the concentration of Tc was decreased to 6.25 μg/ml. EDDA, $FeSO_4$, hemin, and antibiotics were purchased from Sigma Chemical Company (St. Louis, Mo.). Crystal violet was purchased from Allied Chemical Corporation (New York, N.Y.).

Cloning. Chromosomal DNA was isolated by a modification of the method of Marmur (20) and partially digested with *Sau*3AI. Fragments in the 17- to 35-kb size range were eluted from gels by the method of Maniatis et al. (18) and cloned into the *Bam*HI site of cosmid vector pLAFR3. The recombinant cosmid clones were packaged with the Packagene (Promega Corp., Madison, Wis.) bacteriophage lambda packaging system, which was then used to infect *E. coli* 1017.

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TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
Shigella dysenteriae		
O-4576	Serotype 1 clinical isolate	TDH[a]
SD-125	Serotype 1 clinical isolate	B. A. D. Stocker
Escherichia coli		
1017	HB101*ent*::Tn*5*	S. M. Payne
RV	Δ*lacx74 thi*	I. Molineux
MM51	*recA* mutant of RV	This study
P678-54	Minicell strain	1
RK4321	*entA*	R. Kadner
RK4338	Δ*tonB* mutant of RK4321	R. Kadner
RK1065	*hemA*	R. Kadner
NK5012	*supE* host for amplifying phage stocks	N. Kleckner
BE3-1511	Serotype O157:H7	TDH
Plasmids		
pACYC184	Cloning vector, Tc^r Cm^r	18
pAT153	Cloning vector, Am^r Tc^r	18
pLAFR3	Cosmid cloning vector, Tc^r	34
pSU19	Cloning vector, Cm^r	21
pSHU12	27-kb fragment of O-4576 DNA cloned into pLAFR3	This study
pSHU18	19-kb *Eco*RI fragment of pSHU12 cloned into pACYC184	This study
pSHU9	9-kb *Eco*RI-*Pst*I fragment of pSHU18 cloned into pAT153	This study
pSHU37	3.7-kb *Kpn*I fragment of pSHU9 cloned into pSU19	This study
pSHU262	2.6-kb *Eco*RV fragment of pSHU9 cloned into pACYC184	This study
pSHU912	pSHU9::mini-Tn*10*	This study

[a] TDH, Texas Department of Health.

Colonies were isolated on L agar containing Tc and then screened for growth on L agar containing EDDA and hemin. Restriction enzymes, calf intestinal phosphatase, and T4 DNA ligase were purchased from New England Biolabs, Inc. (Beverly, Mass.), and used according to the supplier's directions.

Transposon insertional mutagenesis. Heme utilization clones were mutagenized with a Cm^r λ mini-Tn*10* construct, derivative 105 (13), which was amplified in NK5012. Mutagenesis was performed in MM51 by the method of Kleckner et al. (13). Cm^r Tc^r colonies were pooled, and plasmid DNA was isolated by alkaline lysis (32). *E. coli* 1017 was transformed (18) with the pooled plasmids and screened for heme utilization on L agar containing Cm, Tc, EDDA, and hemin.

Crystal violet sensitivity. L agar plates were seeded with 100 μl of an overnight culture. A sterile 6-mm-diameter disk spotted with 10 μl of a 1-mg/ml crystal violet solution was applied to the center of the agar. The plates were incubated overnight and observed for growth inhibition.

^{35}S-minicell analysis. Volumes of L broth (300 ml each) containing EDDA, hemin, and the appropriate antibiotics were inoculated 1:50 with overnight cultures of *E. coli* P678-54 transformed with the indicated plasmids. After an overnight incubation, the minicells were purified on two sucrose step gradients by the method of Meagher et al. (24). Minicells were labeled with 50 μCi of ^{35}S Trans-label (ICN Biomedicals, Costa Mesa, Calif.) per ml. Labeling was performed in the presence of hemin and EDDA for heme utilization clones and mini-Tn*10* derivatives.

Cell fractionation. For the cellular localization of proteins, cultures were grown overnight in iron-restricted (L plus EDDA) or iron-replete (L plus $FeSO_4$) medium. The cultures were centrifuged at 8,000 × *g*, resuspended in sodium phosphate buffer (10 mM $NaPO_4$, 5 mM $MgSO_4$ [pH 7.0]), and frozen at −80°C. The cells were thawed and lysed by passage through a French press (Aminco, Urbana, Ill.). Total membranes were prepared by the method of Inouye and Guthrie (12) and treated with 0.5% *N*-laurylsarcosine (CIBA-GEIGY Corporation, Ardsley, N.Y.) to selectively solubilize the inner membrane (7). Inner and outer membrane fractions were then separated by centrifugation at 100,000 × *g*, and the outer membrane pellet was resuspended in Laemmli solubilization buffer (15). For hemin affinity chromatography, spheroplasts from mid-log, iron-starved cultures were lysed by sonication and total membranes were isolated. Membrane fractions were then separated by isopycnic centrifugation, and the outer membranes were isolated by the method of Osborn and Munson (27). The final pellets were resuspended in 50 mM Tris (pH 8.0)–1 M NaCl. The protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, Calif.).

Hemin affinity chromatography. Outer membranes (150 μg of protein) were preincubated with or without hemin (40 to 400 nmol) in a volume of 100 μl with gentle mixing for 1 h at 37°C. Preincubated membranes were then added to 50 μl of packed hemin-agarose (235 nmol of hemin; Sigma Chemical Company) which had been equilibrated in 50 mM Tris (pH 8.0)–1 M NaCl and incubated with mixing for 1 h at 37°C. After binding, solubilization buffer (9) was added to give a 250-μl total volume; final solubilization buffer concentrations were 10 mM Tris (pH 7.8), 150 mM NaCl, 10 mM EDTA, 1% (vol/vol) Triton X-100, 0.2% (wt/vol) sodium deoxycholate, and 0.1% (wt/vol) sodium dodecyl sulfate (SDS). Samples were solubilized for 90 min at 37°C with mixing. Nonadherent proteins were removed by centrifugation at 750 × *g* for 5 min, and the supernatants were discarded. Resin beds were then washed three times with a high-salt solubilization solution (50 mM Tris, pH 8.0; 1 M NaCl; 10 mM EDTA; 0.75% Triton X-100; 0.15% sodium deoxycholate; 0.075% SDS) and once with a final wash of 50 mM Tris (pH 8.0)–1 M NaCl. The wash volumes were 1 ml, and the samples were centrifuged between washes as described above. Heme-binding proteins were eluted from the resin by adding 100 μl of Laemmli solubilization buffer (15), boiling for 5 min, chilling on ice for 1 min, and centrifuging as described above.

SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were separated on SDS–12% polyacrylamide gels and stained with 0.1% Coomassie brilliant blue (Bio-Rad Laboratories). Protein standards were obtained from Pharmacia Biotech, Inc. (Piscataway, N.J.). ^{35}S-labeled proteins were visualized by autoradiography with Kodak X-Omat XAR film (Eastman Kodak Company, Rochester, N.Y.).

TonB bioassays. Cultures of RK4321 and RK4338 with and without heme utilization plasmids were grown to log phase and used to inoculate pour plates of L agar plus EDDA. The inoculum for RK4321 was 0.5×10^4 to 1×10^4 CFU/ml, and that for RK4338 was 1×10^5 to 5×10^5 CFU/ml. After solidification, each plate was spotted with 5 μl of 80 μM hemin and 5 μl of 200 μM ferrichrome. A sterile disk containing 20 μl of 10 mM $FeSO_4$ was also applied to each plate. The zones of growth around the $FeSO_4$, ferrichrome, and hemin were measured after 18 to 24 h of incubation at 37°C.

Growth of *hemA* mutant. *E. coli* RK1065 was transformed with the heme utilization plasmid pSHU262 and maintained on L agar plus Cm plus 24 μM aminolevulenic acid (ALA). Individual colonies were grown in L broth plus 48 μM ALA plus Cm and then were streaked onto L agar plus Cm with or without 24 μM ALA and with or without hemin. Growth and colony size were determined after 18 to 24 h of incubation.

Southern hybridization. Southern hybridizations to chromosomal or plasmid DNA were performed as described by Maniatis et al. (18) with GeneScreen membranes (NEN Research Products, Boston, Mass.). Naturally occurring plasmids of *S. dysenteriae* O-4576 were isolated by the alkaline lysis method of Sambrook et al. (32). After phenol-chloroform extraction and isopropanol precipitation, the DNA was resuspended in Tris-CDTA (*trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid) (50 mM Tris, pH 8; 1.5 mM CDTA) and LiCl (5 M final concentration) by the method of Marko et al. (19) and precipitated twice with ethanol. The 0.8-kb *Kpn*I-*Cla*I fragment of pSHU9 was isolated and labeled with [^{32}P]dCTP (14) with the GIBCO BRL Nick Translation System (Gaithersburg, Md.). Hybridization washes were performed under low-stringency conditions (1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.5% SDS at 65°C).

RESULTS

Cloning of the heme utilization locus from *S. dysenteriae*. The heme utilization gene from *S. dysenteriae* was cloned on the basis of its ability to confer the use of hemin as an iron source upon a laboratory strain of *E. coli*. To achieve this, a cosmid library of *S. dysenteriae* chromosomal DNA was constructed in *E. coli* 1017, a siderophore synthesis mutant of a strain incapable of hemin transport. Colonies were screened on iron-restricted medium in the presence or absence of hemin; of 4,000 clones screened in this manner, 14 displayed the desired phenotype of healthy growth on iron-restricted medium supplemented with hemin. Two of these clones were able to thrive also on iron-poor medium without hemin because of complementation of the siderophore synthesis mutation (data not shown) and were not characterized further.

Restriction endonuclease digestion of the 12 putative heme utilization clones showed them to contain overlapping DNA

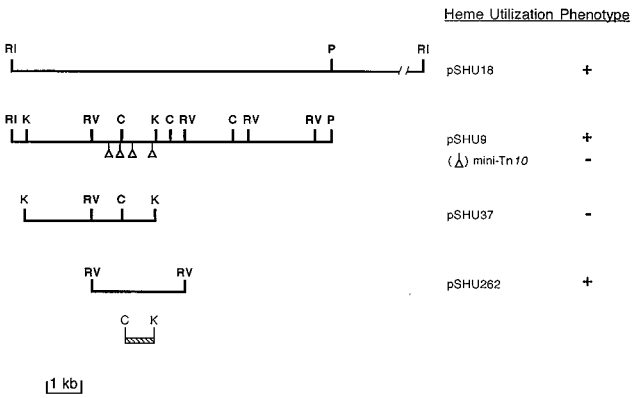

FIG. 1. Partial restriction enzyme map and mini-Tn*10* insertional analysis of heme utilization subclones. Open triangles indicate transposon insertion sites which abolish heme iron uptake. The 800-bp *Cla*I-*Kpn*I probe is shown as a hatched box. The restriction sites shown are *Cla*I (C), *Eco*RI (RI), *Eco*RV (RV), *Kpn*I (K), and *Pst*I (P).

fragments (data not shown). One clone, containing a 27-kb insert and designated pSHU12 (Table 1), was selected for further study. The insert was subcloned to obtain the smallest DNA fragment conferring growth on heme (Fig. 1).

To rule out the possibility that permeability changes in the cell envelope were permitting nonspecific diffusion of hemin into the cell, *E. coli* 1017 alone or transformed with heme utilization plasmid pSHU12, pSHU18, or pSHU9 was tested for crystal violet sensitivity. No significant differences between the clones and the original strain, 1017, were observed; all strains were observed growing within 2 to 4 mm of the disk.

Insertional mutagenesis of pSHU9. pSHU9 was mutagenized with a mini-Tn*10* derivative (13). Mutants that had lost the ability to grow with hemin as the iron source were isolated, and the insertion sites were mapped (Fig. 1). These insertions, four of which are depicted in Fig. 1, were clustered in a 1.2-kb region which spanned a *Cla*I site located within a 3.7-kb *Kpn*I fragment. The 3.7-kb *Kpn*I fragment was subcloned to create pSHU37 (Fig. 1). However, pSHU37 did not allow growth with hemin as the only iron source in *E. coli* 1017, indicating that sequences located outside this *Kpn*I fragment are required for heme transport. An overlapping 2.6-kb *Eco*RV fragment was isolated and cloned into the *Eco*RV site of pACYC184. This subclone, designated pSHU262, did yield the phenotype of heme utilization (Fig. 1).

Identification of proteins encoded by the heme utilization plasmid. The proteins encoded by pSHU9, by its heme utilization-negative derivatives pSHU37 and pSHU912 (pSHU9:: mini-Tn*10*), and by the minimal heme utilization clone pSHU 262 were identified by minicell analysis. pSHU9 encodes at least two proteins, a 70-kDa protein and a 36-kDa protein (Fig. 2, lanes 2 and 6). The mini-Tn*10* insertion mutant pSHU912, which had lost the ability to grow with hemin as the sole iron source, no longer produced the 70-kDa protein but did produce the 36-kDa protein (Fig. 2, lane 3). Upon longer exposure of the autoradiogram, a 50-kDa band, which may represent a truncated form of the 70-kDa protein, could be seen (Fig. 2, middle arrow). The *Eco*RV subclone, pSHU262, which does allow the transport of hemin, encodes only the 70-kDa protein (Fig. 2, lane 8). This indicates that the 70-kDa protein designated Shu (*Shigella* heme uptake) is essential and sufficient to yield the phenotype of heme utilization. The *Kpn*I subclone, pSHU37, which contained part of the presumed heme utilization region but did not restore the phenotype, encoded only the 36-kDa protein (Fig. 2, lane 5).

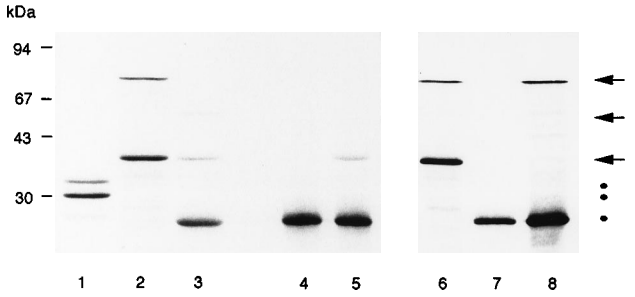

FIG. 2. Minicell analysis of proteins encoded by pSHU9 and derivatives. The autoradiogram shows proteins produced by P678-54 transformed with pAT153 (lane 1), pSHU9 (lanes 2 and 6), pSHU912 (lane 3), pSU19 (lane 4), pSHU37 (lane 5), pACYC184 (lane 7), and pSHU262 (lane 8). Dots indicate vector- or transposon-encoded proteins. The numbers to the left of the figure denote the sizes of the protein standards. The positions of the 70-, 50-, and 36-kDa proteins are designated by arrows on the right side of the figure.

Cellular localization of the 70-kDa protein and demonstration of iron regulation. To determine the cellular location of the 70-kDa protein and whether it showed the iron-stress-inducible phenotype common to many iron uptake systems, *E. coli* containing either the vector pAT153 or the cloned heme utilization system pSHU9 was grown in both iron-replete medium and iron-poor medium. The 70-kDa protein was found in the outer membrane compartment of the cell (Fig. 3). Expression of the 70-kDa outer membrane protein was induced in iron-poor media (Fig. 3, lane 3). To verify that the 70-kDa protein observed in minicells (Fig. 2, lanes 2, 6, and 8) and the 70-kDa iron-regulated outer membrane protein noted here were the same protein, an additional protein gel was run in which aliquots of both outer membrane proteins from pSHU9 grown in iron-restricted medium and the [^{35}S]methionine proteins encoded by pSHU9 were loaded into the same well. Results showed that these two proteins comigrated (data not shown).

The 70-kDa protein is a heme-binding protein. If this 70-kDa iron-regulated protein serves as the outer membrane receptor for heme transport, it should be a specific heme-binding protein. The ability of the 70-kDa protein to bind heme was assessed by hemin-agarose batch affinity chromatography. Outer membranes from *E. coli* P678-54 carrying either a heme utilization plasmid, pSHU9, or the vector pAT153 were isolated and combined with hemin-agarose resin as described in Materials and Methods. The primary hemin-binding protein in

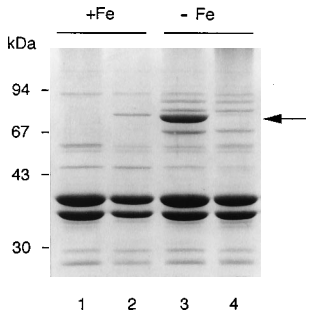

FIG. 3. SDS-PAGE analysis of outer membrane proteins from P678-54 transformed with pAT153 or pSHU9. Outer membranes were isolated from cells grown in L plus $FeSO_4$ (lanes 1 and 2) and L plus EDDA (lanes 3 and 4). Lanes 1 and 4 contain protein preparations from P678-54/pAT153. Lanes 2 and 3 contain protein preparations from P678-54/pSHU9. The 70-kDa protein is designated by the arrow to the right of the figure. The proteins were stained with Coomassie brilliant blue. The sizes of the protein standards are shown on the left.

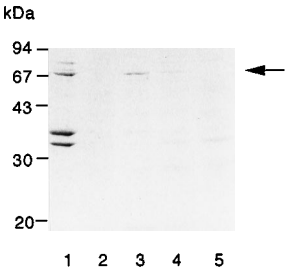

FIG. 4. Binding of the 70-kDa protein to hemin-agarose. Outer membranes were isolated from bacteria grown in low-iron conditions and were added to hemin-agarose resin. Heme-binding proteins were eluted as described in Materials and Methods, separated by SDS-PAGE, and stained with Coomassie blue. Lane 1 contains total outer membrane from P678-54/pSHU9. Lanes 2 to 5 contain proteins eluted from hemin-agarose resin. Lane 2, P678-54/pAT153; lane 3, P678-54/pSHU9; lane 4, P678-54/pSHU9 preincubated with hemin; lane 5, P678-54/pAT153 preincubated with hemin. The 70-kDa protein is indicated by an arrow at the right side of the figure. The sizes of the protein standards are marked on the left side.

outer membranes from cells carrying pSHU9 (Fig. 4, lane 3) comigrated with the 70-kDa Shu protein (Fig. 4, lane 1). No protein of this size was detected in eluants from the same strain carrying the vector alone (Fig. 4, lane 2). Specific binding of the protein to heme was confirmed by competition experiments in which the membranes were preincubated with a range of hemin concentrations. Binding of the 70-kDa protein was drastically reduced by preincubation with hemin; binding was inversely proportional to the amount of free hemin added (Fig. 4, lane 4, and data not shown). Even at the highest concentration of added hemin, however, some binding of the 70-kDa protein to the resin was observed. This incomplete competition with exogenous hemin is consistent with the observation of Lee (17) in studies of a heme-binding protein from *H. influenzae* type b. Because of solubility constraints, the highest concentration of free hemin used in these assays was only 1.7-fold higher than the concentration of hemin in the hemin-agarose resin.

Heme utilization reconstituted in *E. coli* K-12 is TonB dependent. In a number of systems, the *E. coli* TonB protein has been found to be essential for transport of iron complexes across the outer membrane. To ascertain if TonB was necessary for the transport of heme iron by the *S. dysenteriae* Shu protein, an isogenic pair of *E. coli* strains, RK4321 (*entA*) and RK4338 (*entA* Δ*tonB*), was transformed with pSHU262. Because of the defect in enterobactin synthesis, neither strain is capable of growth on iron-restricted medium. RK4321/pSHU262, the TonB$^+$ strain harboring the heme utilization plasmid, was able to grow on iron-limited medium supplemented with hemin (Table 2). In contrast, the *tonB* mutant strain, RK4338, carrying the same heme utilization clone showed no growth on this medium (Table 2).

TABLE 2. Effect of *tonB* mutation on heme utilization

Strain	Zone of growth (mm)[a]		
	Hemin	Ferrichrome	$FeSO_4$
RK4321 (*entA*)	0	34	30
RK4321/pSHU262 (*entA shu*$^+$)	20	35	35
RK4338 (*entA* Δ*tonB*)	0	0	30
RK4338/pSHU262 (*entA* Δ*tonB shu*$^+$)	0	0	24

[a] Bacteria were seeded in low-iron agar medium. Growth of the lawn around spots of potential iron sources was measured after an overnight incubation.

The transport of ferrichrome into the *E. coli* cell is known to be dependent on the TonB protein. The TonB$^+$ strain, in the presence or absence of pSHU262, was able to grow on the same iron-chelated medium supplemented with ferrichrome, whereas the *tonB* mutant, with or without pSHU262, was unable to grow, thus confirming the TonB phenotype of the strains (Table 2). As expected, all strains grew in this iron-poor medium in the presence of excess $FeSO_4$ (Table 2). These results indicated that the heme utilization system of *S. dysenteriae*, when reconstituted in *E. coli*, requires a functioning TonB protein.

The entire heme moiety is transported into the bacterial cell. The iron requirements of *S. dysenteriae* could be satisfied either by extracellular removal of iron from heme followed by transport of the iron into the bacterium or by transport of the entire heme moiety into the cell. To distinguish between these mechanisms, the ability of pSHU262 to permit hemin to satisfy the porphyrin requirement of an *E. coli hemA* mutant was determined. RK1065 (*hemA*) is unable to synthesize heme because of a defect in production of δ-ALA, an intermediate in heme biosynthesis. This strain showed no growth aerobically (no isolated colonies) on L agar unless ALA was added (colony diameter, 2 mm). No growth of RK1065 was observed on L plus hemin, presumably because this strain cannot transport the compound. RK1065 transformed with pSHU262 still failed to grow on unsupplemented medium, but it grew on either medium supplemented with hemin (colony diameter, 1 mm) or ALA (colony diameter, 2 mm). This indicated that the heme utilization clone enabled the transport of the intact heme molecule into the *hemA* mutant cell, bypassing the defective heme biosynthesis pathway.

Detection of homologous DNA sequences. An 800-bp internal *Kpn*I-*Cla*I fragment (Fig. 1) from the gene encoding the 70-kDa protein was used to probe *Kpn*I-digested chromosomal DNA from strains of *S. dysenteriae* serotypes 1 to 4 and *E. coli* serotypes O157:H7 and O26:H11. Chromosomal DNA from *S. dysenteriae* O-4576 was used as a positive control and showed the 3.7-kb *Kpn*I fragment from which the probe was derived (Fig. 5). Hybridization of the probe to DNA of other serotype 1 strains of *S. dysenteriae* (Fig. 5) but not to DNA of *S. dysenteriae* serotypes 2, 3, or 4 (Table 3) was observed. This internal fragment was also used to probe *Kpn*I-digested DNA from the naturally occurring plasmids of *S. dysenteriae* O-4576, the strain from which *shu* was cloned. No hybridization with plasmid DNA was detected, verifying that the heme utilization gene was located on the chromosome (data not shown).

The production of a hemolytic uremic syndrome by some *E. coli* strains suggested that these strains might have a similar heme transport system. Nine strains of *E. coli* O157:H7 were assayed for heme transport and for the presence of DNA homologous to the *shu* gene. All isolates tested were able to grow with heme as a sole iron source (Table 3). Additionally, DNA from each of the isolates, one of which is shown in Fig. 5, hybridized to the *Kpn*I-*Cla*I probe, while no homology was seen with DNA from a laboratory *E. coli* strain, HB101 (data not shown). This suggests that a heme transport system related to the one in *S. dysenteriae* exists in *E. coli* O157:H7. The size of the *Kpn*I fragment detected by hybridization was approximately 20 kb in all of the O157:H7 strains, compared with 3.7 kb in *S. dysenteriae* serotype 1. An enterohemorrhagic *E. coli* of a different serotype, O26:H11, tested negative for both the utilization of heme iron and the presence of a DNA sequence homologous to the heme transport gene of *S. dysenteriae* (Table 3).

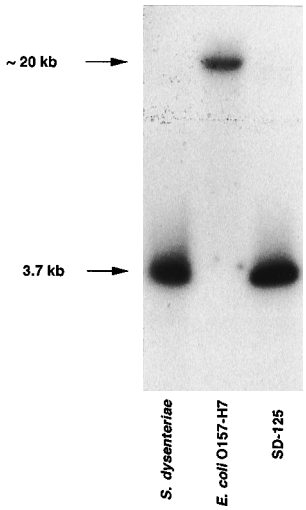

FIG. 5. Detection of DNA homologous to the *S. dysenteriae shu* gene in *E. coli* O157:H7 and *S. dysenteriae* serotype 1. Chromosomal DNA was digested with *Kpn*I and separated by agarose gel electrophoresis. Southern blots were prepared and probed with the 800-bp *Cla*I-*Kpn*I fragment shown in Fig. 1. The lanes contain DNA from *S. dysenteriae* O-4576, the source of the cloned heme utilization gene; *E. coli* O157:H7 BE3-1511; and *S. dysenteriae* SD-125 serotype 1.

DISCUSSION

The adaptation of bacteria to their environment is well illustrated in the variety of mechanisms employed for acquisition of the essential element iron. These range from high-affinity siderophore-mediated systems which solubilize and transport iron in aerobic environments to expression of receptors for binding of host iron carrier proteins. The largest potential pool of iron within a mammalian host is the heme molecule. Thus, it is not surprising that pathogens which encounter heme in their host have transport systems to take advantage of this abundant iron source.

The *Shigella* species, which produce a bloody diarrhea, are able to use heme as their sole iron source. In order to determine the mechanism of heme utilization, a DNA fragment conferring this phenotype was cloned in *E. coli*. Subcloning and transposon insertional mutagenesis accompanied by minicell analysis of the encoded proteins demonstrated a correlation between heme utilization and expression of a 70-kDa protein. Mutations which disrupt expression of this protein abolish the microbe's ability to use heme as an iron source, indicating that this protein is essential for heme utilization. Transformation of *E. coli* with a heme utilization plasmid which encodes only the 70-kDa protein allows heme transport, which confirms that this protein is sufficient for the phenotype of heme utilization.

TABLE 3. Homology of *S. dysenteriae* heme transport gene and DNA of other heme-utilizing bacteria

Organism	No. of strains	No. of strains using heme[a]	No. of strains showing homology[b]
S. dysenteriae			
Serotype 1	3	3	3
Serotype 2	1	0	0
Serotype 3	1	1	0
Serotype 4	1	1	0
E. coli O157:H7	9	9	9
E. coli O26:H11	1	0	0

[a] Determined by growth in L-EDDA-hemin agar.
[b] DNA homology by Southern blot with probe shown in Fig. 1.

The 70-kDa protein was observed in outer membrane fractions isolated by either Sarkosyl extraction or sucrose density gradient centrifugation. This protein has the ability to bind heme and could be affinity purified on hemin-agarose. Binding to hemin-agarose was specific; the binding was inhibited by the addition of free hemin. The location of the protein in the outer membrane and its ability to bind heme indicate that it could serve as the receptor for heme in this heme uptake system. As has been noted for many well-characterized receptors for bacterial iron transport systems, the expression of the Shu protein is regulated by iron. Maximum production of the heme receptor occurs when iron levels are limiting.

The mechanism of transport in *S. dysenteriae* appears to involve uptake of the entire heme molecule into the bacterial cell. The presence of the heme utilization plasmid, pSHU262, permitted an *E. coli hemA* mutant to use hemin as a source of porphyrin. This DNA, which encodes only the Shu protein, appears to allow the transport of the entire heme moiety, providing both an iron source and a porphyrin source for *E. coli.*

The heme receptor of *S. dysenteriae* appears to be dependent on the TonB protein for the transport of hemin in *E. coli*. It may be that the *S. dysenteriae* TonB analog will be required for heme transport in that organism. Comparison with other TonB-dependent iron transport systems suggests that additional proteins may be required for transport of heme into the cell, but additional genes required for heme utilization have not been identified in this system. A gene encoding a 36-kDa protein of unknown function and cellular location is linked to *shu*. It has not been determined whether this protein plays a role in heme transport in *S. dysenteriae*, but its presence is unnecessary to reconstitute the phenotype of heme utilization in *E. coli.*

Heme transport proteins with molecular masses ranging from 70 to 80 kDa have been identified in *V. cholerae*, *P. shigelloides*, and *Y. enterocolitica*. The *V. cholerae* HutA heme receptor is 77 kDa (10), while the HemR protein of *Y. enterocolitica* is 78 kDa (36). A 77-kDa protein has been observed by minicell analysis of a cosmid clone containing the heme utilization system of *P. shigelloides* (35). Each of these heme uptake proteins has been shown to exist in the outer membrane of the respective bacterium, and expression of the proteins is regulated by the concentration of iron in the environment. Transport of the intact heme molecule is another instance of similarity among these heme transport systems. The HutA heme receptor (10), the HemR protein (36), and the cloned sequences containing the heme-iron uptake system of *P. shigelloides* (6) have been shown to allow the transport of hemin as a porphyrin source. To utilize hemin as an iron source, the heme receptor of *Y. enterocolitica*, HemR, requires the presence of the *Y. enterocolitica* Ton B protein (36). In contrast, the HutA heme receptor of *V. cholerae* is able to transport hemin normally in an *E. coli tonB* mutant (10). This may not represent a significant difference between HutA and the other transport proteins, as there is some evidence that HutA requires a *V. cholerae* gene which may serve the same function as *E. coli tonB* (11). Thus, these heme utilization proteins and the Shu protein of *S. dysenteriae* may belong to a family of iron-regulated, outer membrane protein receptors.

Because the *Shigella* species and *E. coli* are closely related genetically, the possible presence of this heme utilization system in selected strains of *E. coli* was determined. A homologous DNA sequence was detected in *E. coli* O157:H7 strains which cause hemorrhagic colitis that, as is the case with *S.*

dysenteriae 1 (8), may be followed by a sometimes fatal hemolytic uremic syndrome (30). This sequela is associated with damage to the microcapillaries of the kidneys, which results in the release of significant amounts of blood. Therefore, sources of heme iron are provided for these pathogens as the disease progresses.

Surprisingly, although the gene encoding the heme receptor protein of *S. dysenteriae* was present in all serotype 1 strains tested, it was not detected by hybridization in serotype 2, 3, or 4. The presence of homologous sequences in two different genera but not in other serotypes of the species from which the gene was cloned suggests the possibility of horizontal gene transmission. Since the *S. dysenteriae* heme utilization system is located on the chromosome, gene transfer is not likely to be the result of simple plasmid exchange as is seen in some cases of antibiotic resistance. It may be that this heme utilization system is carried on some type of moveable DNA element, such as a transposon.

This heme transport system may be important for the acquisition of iron by *Shigella* species in vivo. It has already been shown that the production of siderophores is not essential for invasion and intracellular multiplication of *Shigella flexneri* (16). It may be that the *Shigella* species obtain iron for intracellular multiplication from the small pools of free heme inside host cells and extracellularly from blood or heme in the gut. To determine this, mutants of *S. dysenteriae* unable to transport hemin or produce siderophores will be tested for their capacity to invade and multiply in either tissue culture cells or animal models.

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REFERENCES

1. **Adler, H. I., W. D. Fisher, A. Cohen, and A. A. Hardigree.** 1967. Miniature *Escherichia coli* cells deficient in DNA. Proc. Natl. Acad. Sci. USA **57:**321–326.
2. **Blanton, K. J., G. D. Biswas, J. Tsai, J. Adams, D. W. Dyer, S. M. Davis, G. G. Koch, P. K. Sen, and P. F. Sparling.** 1990. Genetic evidence that *Neisseria gonorrhoeae* produces specific receptors for transferrin and lactoferrin. J. Bacteriol. **172:**5225–5235.
3. **Bottomley, S. S., and U. Muller-Eberhard.** 1988. Pathophysiology of heme synthesis. Semin. Hematol. **25:**282–302.
4. **Burman, D.** 1974. Iron metabolism in infancy and childhood, p. 543–562. *In* A. Jacobs and M. Worwood (ed.), Iron in biochemistry and medicine. Academic Press, London.
5. **Crosa, J. H.** 1989. Genetics and molecular biology of siderophore-mediated iron transport in bacteria. Microbiol. Rev. **53:**517–530.
6. **Daskaleros, P. A., J. A. Stoebner, and S. M. Payne.** 1991. Iron uptake in *Plesiomonas shigelloides*: cloning of the genes for the heme-iron uptake system. Infect. Immun. **59:**2706–2711.
7. **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.
8. **Hale, T. L.** 1991. Genetic basis of virulence in *Shigella* species. Microbiol. Rev. **55:**206–224.
9. **Hansen, E. J., C. F. Frisch, and K. H. Johnston.** 1981. Detection of antibody-accessible proteins on the cell surface of *Haemophilus influenzae* type b. Infect. Immun. **33:**950–953.
10. **Henderson, D. P., and S. M. Payne.** 1993. Cloning and characterization of the *Vibrio cholerae* genes encoding the utilization of iron from haemin and haemoglobin. Mol. Microbiol. **7:**461–469.
11. **Henderson, D. P., and S. M. Payne.** 1994. Characterization of the *Vibrio cholerae* outer membrane heme transport protein HutA: sequence of the gene, regulation of expression, and homology to the family of TonB-dependent proteins. J. Bacteriol. **176:**3269–3277.
12. **Inouye, M., and J. P. Guthrie.** 1969. A mutation which changes a membrane protein of *E. coli*. Proc. Natl. Acad. Sci. USA **64:**957–961.
13. **Kleckner, N., J. Bender, and S. Gottesman.** 1991. Uses of transposons with emphasis on Tn*10*. Methods Enzymol. **204:**138–181.
14. **Koch, J., S. Kolvraa, and L. Bolund.** 1986. An improved method for labeling of DNA probes by nick translation. Nucleic Acids Res. **14:**7132.
15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
16. **Lawlor, K. M., P. A. Daskaleros, R. E. Robinson, and S. M. Payne.** 1987. Virulence of iron transport mutants of *Shigella flexneri* and utilization of host iron compounds. Infect. Immun. **55:**594–599.
17. **Lee, B. C.** 1992. Isolation of an outer membrane hemin-binding protein of *Haemophilus influenzae* type b. Infect. Immun. **60:**810–816.
18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
19. **Marko, M. A., R. Chipperfield, and H. C. Birnboim.** 1982. A procedure for the large-scale isolation of highly purified plasmid DNA using alkaline extraction and binding to glass powder. Anal. Biochem. **121:**382–387.
20. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3:**208–218.
21. **Martinez, E., B. Bartolomé, and F. de la Cruz.** 1988. pACYC184-derived cloning vectors containing the multiple cloning site and *lacZ* α reporter gene of pUC8/9 and pUC18/19 plasmids. Gene **68:**159–162.
22. **Massad, F., J. E. L. Arcenaux, and B. R. Byers.** 1991. Acquisition of iron from host sources by mesophilic *Aeromonas* species. J. Gen. Microbiol. **137:**237–241.
23. **Mazoy, R., and M. L. Lemos.** 1991. Iron-binding proteins and heme compounds as iron sources for *Vibrio anguillarum*. Curr. Microbiol. **23:**221–226.
24. **Meagher, R. B., R. C. Tait, M. Betlach, and H. W. Boyer.** 1977. Protein expression in *E. coli* minicells by recombinant plasmids. Cell **10:**521–536.
25. **Morgan, E. H.** 1974. Transferrin and transferrin iron, p. 29–71. *In* A. Jacobs and M. Worwood (ed.), Iron in biochemistry and medicine. Academic Press, London.
26. **Neilands, J. B.** 1982. Microbial envelope proteins related to iron. Annu. Rev. Microbiol. **36:**285–309.
27. **Osborn, M. J., and R. Munson.** 1974. Separation of the inner (cytoplasmic) and outer membranes of gram-negative bacteria. Methods Enzymol. **31A:** 642–653.
28. **Payne, S. M.** 1988. Iron and virulence in the family Enterobacteriaceae. Crit. Rev. Microbiol. **16:**81–111.
29. **Pidcock, D. A., J. A. Wooten, B. L. Daley, and T. L. Stull.** 1988. Iron acquisition by *Haemophilus influenzae*. Infect. Immun. **56:**721–725.
30. **Riley, L. W.** 1987. The epidemiological, clinical, and microbiologic features of hemorrhagic colitis. Annu. Rev. Microbiol. **41:**383–407.
31. **Rogers, H. J.** 1973. Iron-binding catechols and virulence in *Escherichia coli*. Infect. Immun. **7:**445–456.
32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
33. **Spiro, T. G., and P. Saltman.** 1974. Inorganic chemistry, p. 1–28. *In* A. Jacobs and M. Worwood (ed.), Iron in biochemistry and medicine. Academic Press, London.
34. **Staskawicz, B., D. Dahlbeck, N. Keen, and C. Napoli.** 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. **169:**5789–5794.
35. **Stoebner, J., and S. M. Payne.** Unpublished data.
36. **Stojiljkovic, I., and K. Hantke.** 1992. Hemin uptake system of *Yersinia enterocolitica*: similarities with other TonB-dependent systems in gram-negative bacteria. EMBO J. **11:**4359–4367.