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Identification of *shuA*, the Gene Encoding the Heme Receptor of *Shigella dysenteriae*, and Analysis of Invasion and Intracellular Multiplication of a *shuA* Mutant

MELODY MILLS† AND SHELLEY M. PAYNE*

Department of Microbiology and Institute for Cellular and Molecular Biology, University of Texas, Austin, Texas 78712

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***shuA* encodes a 70-kDa outer membrane heme receptor in *Shigella dysenteriae*. Analysis of the *shuA* DNA sequence indicates that this gene encodes a protein with homology to TonB-dependent receptors of gram-negative bacteria. Transport of heme by the ShuA protein requires TonB and its accessory proteins ExbB and ExbD. The *shuA* DNA sequence contains a putative Fur box overlapping the −10 region of a potential *shuA* promoter, and the expression of *shuA* is repressed by exogenous iron or hemin in a Fur-dependent manner, although hemin repressed expression to a lesser extent than iron salts. Disruption of this open reading frame on the *S. dysenteriae* chromosome by marker exchange yielded a strain that failed to use heme as an iron source, indicating that *shuA* is essential for heme transport in *S. dysenteriae*. However, *shuA* is not essential for invasion or multiplication within cultured Henle cells; the *shuA* mutant invaded and produced normal plaques in confluent cell monolayers.**

The ability to bind and transport heme is a phenomenon commonly observed among pathogenic bacteria (5, 14, 15, 27). Because heme is the most abundant source of iron in mammals, it is not surprising that bacteria which infect these hosts would have a mechanism to transport this compound and use it as an iron source. Among gram-negative pathogens, several systems have been identified for obtaining iron through utilization of free heme or heme proteins (6, 13, 15, 17, 24, 28, 29). In *Shigella dysenteriae*, an intestinal pathogen that causes bloody diarrhea, heme binds to an outer membrane protein (ShuA) and subsequently is transported into the bacterial cell in a TonB-dependent manner (20). To further characterize this heme transport system, the sequence of the gene encoding ShuA was determined and analyzed, and a mutant defective in *shuA* was constructed and tested in cell culture assays. (For a list of strains and plasmids used in this study, see Table 1).

Features of the nucleotide sequence of *shuA*. The DNA sequence of the 2.6-kbp *Eco*RV fragment of pSHU262, the smallest clone conferring heme utilization upon a laboratory strain of *Escherichia coli* (20) (Fig. 1), was determined. Nested sets of deletions were made in a derivative of pSHU262 (pMTLSHU26) with the Erase-a-Base System (Promega Corporation, Madison, Wis.), used according to the manufacturer's guidelines. Plasmid DNA was sequenced with the Sequenase Version 2.0 DNA Sequencing Kit (U.S. Biochemicals, Cleveland, Ohio), used according to the manufacturer's standard protocol. A single long open reading frame, containing 1,980 bp and designated *shuA*, was present in pSHU262. To verify that this open reading frame encodes the heme receptor, we determined by restriction endonuclease mapping the site of a previously constructed mini-Tn*10* (Cm^r) transposon insertion (pSHU912) (20) and found that the insertion mapped within this open reading frame (Fig. 1 and data not shown). To determine the effect of a chromosomal *shuA* mutation, the insertion mutation of pSHU912 was transferred to the *S. dysenteriae* chromosome by marker exchange, as follows. The insert from pSHU912 was cloned into pWSc-1, a vector carrying the *sacB* gene encoding sucrose sensitivity, to produce pSc912. *S. dysenteriae* 0-4576-S1 was transformed with pSc912, and marker exchange mutants were obtained by selecting colonies that were Cm^r Suc^r, indicating retention of the transposon insertion but loss of the Suc^r plasmid. The chromosomal mutation was confirmed by Southern hybridization analysis of the chromosomal DNA of the putative mutant. Hybridization with the internal *Kpn*I-*Cla*I fragment of *shuA* (Fig. 1) indicated that the marker exchange mutant had an insertion within the chromosomal *shuA* gene, generating a 4.0-kb *Eco*RV fragment, and that no uninterrupted copies of the gene (2.6-kb *Eco*RV fragment) were present (data not shown). The mutant failed to express ShuA, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cell proteins, confirming that this open reading frame encodes ShuA. The mutant also failed to grow with heme as the sole iron source, suggesting there is no other heme transport receptor in *S. dysenteriae* (data not shown). It is the interruption of *shuA*, not a polar effect on downstream genes, that is responsible for the heme transport defect in the mutant, since minicell analysis of the proteins encoded by *shuA* plasmids showed that only the ShuA protein was affected by this insertion (20).

Analysis of the DNA sequence identified a possible promoter region that closely resembled the consensus promoter of *E. coli* (Fig. 2). The potential −10 region shared five of six bases with the *E. coli* consensus −10 region, while four of six bases were identical between the two −35 regions. The promoter is 328 bp upstream of the putative translation start site, yielding an unusually long 5′ untranslated region in the *shuA* gene. The heme receptor gene *hemR* of *Yersinia enterocolitica* has a similar long untranslated region (28). A small open reading frame of unknown function, designated *hemP*, is present upstream of *hemR* in *Y. enterocolitica* (28). While small open reading frames are present upstream of *shuA*, none has homology to *hemP* (data not shown).

* Corresponding author.
† Present address: Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814-4799.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
E. coli		
1017	HB101*ent*::Tn*5*	6
CC118	F$^-$ Δ(*ara-leu*)*7697 araD139* Δ*lacX74 galE galK* Δ*phoA20 thi rpsE rpoB argE*(Am) *recA1 appR1*	19
GM1	P90C/F′ *lac pro*	30
KP1037	GM1 *exbB*::Tn*10*	26
KP1143	GM1 *exbD*::Tn*phoA*	26
MFT-5	*fur*::Tn*5* Km^r	M. McIntosh
S. dysenteriae		
0-4576	Serotype 1 clinical isolate; Cm^r Sm^r Tc^r	16
0-4576-S1	Cm^s Sm^s Tc^s 0-4576	This study
MMH2	0-4576-S1 *shuA*::miniTn*10*	This study
S. flexneri SA100		23
S. boydii 0-1392		16
S. sonnei		
PB66		D. Winsor
0-1245		16
Plasmids		
pSHU262	2.6-kb *Eco*RV fragment of pSHU9 cloned into pACYC184	20
pSHU912	pSHU9::mini-Tn*10*	20
pSHU251	pSHU262::Tn*phoA*	This study
pSc912	~11-kb *Pst*I-*Sal*I fragment of pSHU912 cloned into pWSc-1	This study
pMTLSHU26	2.6-kb *Eco*RV fragment of pSHU262 cloned into the *Sma*I site of pMTL22	This study
pABN203	*E. coli fur* gene cloned into pBR322; Tc^r	7
pACYC184	Cloning vector; Tc^r Cm^r	18
pMTL22	Cloning vector; Cb^r	3
pWKS30	Cloning vector; Cb^r	32
pWSc-1	pWKS30 containing *sacB* gene; Cb^r Suc^s	E. Wyckoff

A potential Fur box overlaps the −10 region of the *shuA* gene (Fig. 2). This putative binding site for the transcriptional regulator Fur was found to have 84% identity (16 out of 19 bases) with the *E. coli* consensus Fur box and included the two highly conserved sequences AAT and ATT (9). Binding of Fur dimers at this site could block transcription when the corepressor, iron, is present (1).

Iron regulation of *shuA* expression is mediated by Fur in *E. coli*. The synthesis of ShuA is influenced by the concentration of iron in the medium (20). Identification of a potential Fur box within the promoter region suggested that the iron regulation of *shuA* is mediated by Fur. This was analyzed by constructing an alkaline phosphatase reporter gene fusion to *shuA*. pSHU262, the minimal heme utilization clone encoding only the 70-kDa ShuA protein, was mutagenized with Tn*phoA* (19), yielding pSHU251. This mutated plasmid lost the ability to confer heme transport to *E. coli* 1017, suggesting insertion within *shuA*. DNA sequencing of the Tn*phoA* insertion site confirmed that *phoA* was inserted after the codon for amino acid 318 in the *shuA* coding sequence (Fig. 1 and 2). Alkaline phosphatase activity in a strain carrying the *shuA-phoA* fusion was iron regulated; colonies of *E. coli* CC118 (Δ*phoA*) transformed with pSHU251 were dark blue when plated on low-iron medium [L agar with 250 μg of ethylenediamine-di(*o*-hydroxyphenyl-acetic acid) (EDDA) per ml] containing the phosphatase indicator 5-bromo-4-chloro-3-indolylphosphate (BCIP) and white when plated on high-iron medium (L agar with 20 μM $FeCl_3$) containing BCIP.

E. coli MFT-5, which is Fur$^-$, was transformed with pSHU251 to test whether regulation of *shuA* expression by iron is dependent on Fur. The endogenous phosphatase activity of MFT-5 was repressed under the conditions used for these iron regulation assays and thus did not interfere with the use of PhoA as the reporter (data not shown). The reporter gene fusion was also moved into MFT-5 complemented with pABN203 (7), a compatible plasmid carrying the wild-type *fur* gene. In the wild-type Fur$^+$ strain, a 13-fold reduction of activity was seen in iron-rich medium compared to the activity under iron-poor conditions (Table 2). In the *fur* mutant strain, iron regulation of expression was lost. The level of expression under high-iron conditions was the same as that seen under low-iron conditions (Table 2), indicating constitutive expression in the *fur* mutant. Complementation of the *fur* mutant strain with the *fur* gene on a plasmid restored iron regulation (Table 2).

The reporter gene fusion was placed in *S. dysenteriae* to investigate further the regulation of *shuA*. High-iron conditions caused a sevenfold reduction in activity compared to the activity under low-iron conditions (Table 2). In contrast to the *E. coli* 1017 constructs, which are unable to transport hemin, the *S. dysenteriae* strain has an intact chromosomal *shuA* locus. Therefore, the effect of hemin on expression of the plasmid-encoded *shuA-phoA* fusion could be determined in this strain. The observed alkaline phosphatase activity in iron-restricted medium containing hemin (10 μg/ml) as the sole iron source was 276.25 ± 16.47 U, representing a 1.7-fold reduction of activity compared to that under low-iron conditions (463.50 ± 21.8 U). Therefore, the addition of hemin to the growth medium repressed the expression of *shuA* but not to the extent observed with the addition of ferrous sulfate. The failure of heme to repress completely the expression of this gene suggests that the amount of iron removed intracellularly from the heme molecule was insufficient to permit Fur-regulated gene repression. Whether heme can directly regulate the expression of this gene and how that regulation might affect Fur regulation are not known.

Predicted amino acid sequence of ShuA. Translation of the *shuA* open reading frame (Fig. 2) indicated that the ShuA precursor consists of 660 amino acids, with a molecular mass of 72,533 Da and a pI of 4.87. The amino-terminal 28 amino acids resemble a standard signal sequence (25), supporting earlier data that ShuA was located in the outer membrane (20). Cleavage at the putative signal peptidase I site would yield a 69.5-kDa

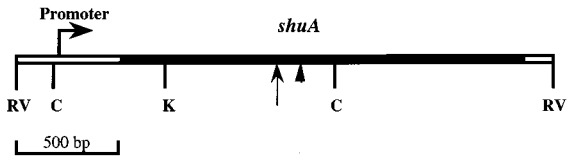

FIG. 1. Map of the 2.6-kb *Eco*RV fragment encompassing the *shuA* gene of *S. dysenteriae*. The position of the 1,980-bp open reading frame corresponding to *shuA* is shown as a dark box within the 2,662-bp *Eco*RV insert in pSHU262 (20). The location of the putative promoter region and the direction of transcription are indicated by the arrow bent at a right angle. The site of the Tn*phoA* insertion in pSHU251 is indicated by the arrowhead, while the site of the mini-Tn*10* transposon insertion in pSHU912 is marked by the straight arrow. The abbreviations used to indicate restriction endonuclease sites are as follows: RV, *Eco*RV; C, *Cla*I; and K, *Kpn*I.

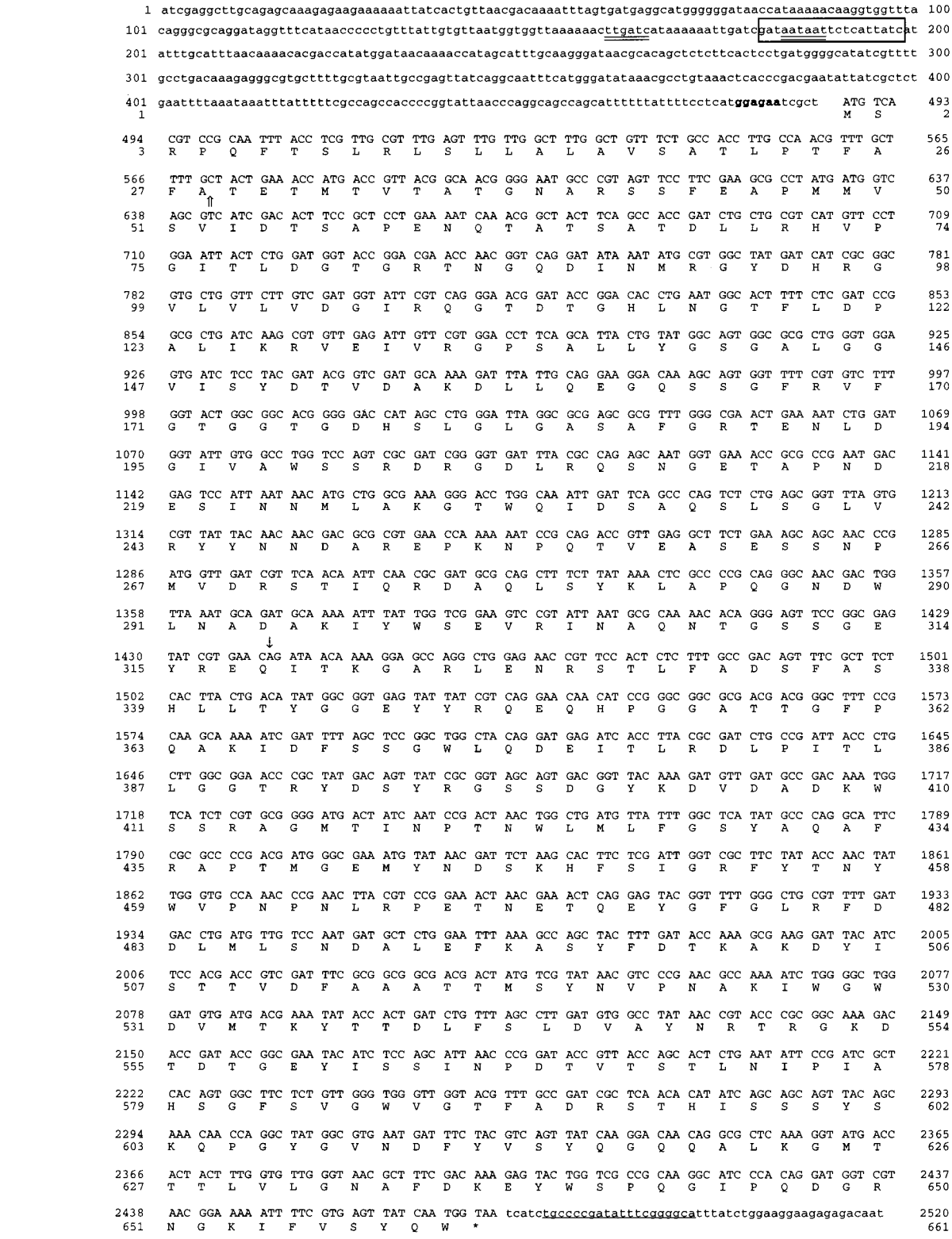

FIG. 2. Nucleotide sequence of *shuA* and predicted amino acid sequence of the ShuA protein of *S. dysenteriae*. The nucleotide sequence of *shuA* and the predicted protein sequence are shown. The sequences with homology to *E. coli* −35 and −10 promoter regions are indicated by double underlining, and the potential Fur box, which overlaps the −10 region, is boxed. The Shine-Dalgarno sequence is shown in boldface letters. The arrow between amino acids 28 and 29 marks the predicted signal peptidase cleavage site. The site of the Tn*phoA* insertion in pSHU251 is indicated by the arrow above the codon for amino acid 318. The termination codon is indicated by an asterisk, and a downstream region of dyad symmetry that is a probable transcription terminator site is underlined once.

TABLE 2. Regulation of *shuA* by iron and Fur

Strain transformed with pSHU251	Relevant phenotype	PhoA activity (U)[a]		Fold repression[b]
		+Fe	−Fe	
E. coli				
1017	Fur^+	11.95 ± 10.7	161.22 ± 21.4	13
MFT-5	Fur^-	117.44 ± 1.9	121.30 ± 3.6	1
MFT-5/pABN203	Fur^+	11.50 ± 1.6	136.40 ± 19.6	12
S. dysenteriae 0-4576-S1	Fur^+	65.90 ± 6.9	463.50 ± 21.8	7

[a] Strains transformed with pSHU251 were grown in L broth to mid-log phase and then divided into the same medium supplemented with 20 to 40 μM $FeSO_4$ (+Fe) or containing the iron chelator EDDA to restrict iron (−Fe). Concentrations of EDDA were 200 μg/ml (1017), 500 μg/ml (MFT-5), or 1,000 μg/ml (0-4576-S1). Supplemented cultures were incubated until the A_{600} was 0.6 to 0.8 and then assayed for alkaline phosphatase activity by the method of Brickman and Beckwith (2). Values shown are the means ± standard deviations for at least three independent experiments.

[b] Fold repression is expressed as the ratio of activity under low-iron conditions to that under high-iron conditions.

mature ShuA protein, in agreement with the size estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (70 kDa) (20).

Examination of the predicted amino acid sequence of the amino terminus of the mature ShuA protein revealed the presence of a well-conserved TonB box (ETMTVTA) (amino acids 30 to 36 in Fig. 2), including the two residues considered to be the most highly conserved in all TonB boxes, the T residue at position 2 and the V residue at position 5 (21). The presence of a TonB box correlated with the observation that heme transport by ShuA is TonB dependent (20) and suggested a requirement for the TonB accessory proteins ExbB and ExbD. To determine whether these proteins were required for heme transport, an isogenic set of *exb* mutant strains (GM1, KP1037, and KP1143) was transformed with a *shuA* plasmid, pSHU262, and tested for the ability to use hemin as an iron source. Cultures were streaked onto iron-rich medium (L agar), iron-restricted medium (L agar with 250 μg of EDDA per ml), or iron-restricted medium containing hemin (5 μg/ml) for the isolation of colonies. At least 12 isolated colonies were measured, and the average size was determined. As expected, all of the strains grew on L agar, which is relatively iron rich, and had equivalent colony diameters (1.2 mm at 18 h of incubation at 37°C). None of the strains grew on iron-restricted L agar, which served as the negative control. The wild-type strain transformed with the *shuA* plasmid grew on iron-restricted medium supplemented with hemin (1.2-mm colony diameter). However, neither the *exbB* mutant nor the *exbD* mutant containing the plasmid was able to grow on the iron-restricted medium containing hemin as the iron source. Therefore, uptake of heme by the ShuA protein resembles that in the typical TonB-dependent transport system in that TonB, ExbB, and ExbD are all required for transport.

Distribution of *shuA* in other enteric species and homology of ShuA to other heme transport proteins. Although the ability to use heme as an iron source is common to all the *Shigella* species, the *shuA* gene was not detected in the chromosome of *Shigella flexneri*, *Shigella boydii*, or *Shigella sonnei* (Fig. 3). A faint band could be seen in Southern hybridizations of *S. sonnei* chromosomal DNA to the *shuA* probe upon prolonged exposure when the stringency was reduced (1× SSC [0.15 M NaCl plus 0.015 M sodium citrate]) (data not shown), but no bands were detected in *S. flexneri* or *S. boydii* DNA under the same conditions, suggesting that systems other than that encoded by *shuA* are responsible for heme transport in these species. Therefore, among the *Shigella* species, *shuA* is restricted to *S. dysenteriae* type 1 and is absent even from the other serotypes of *S. dysenteriae* (20). However, homology between *shuA* and the genes of some strains of *E. coli*, including *E. coli* O157:H7, had been observed and was the basis for our recent report of the cloning and DNA sequence of the almost-identical *chuA* gene from *E. coli* O157:H7 (31).

ShuA is 99.5% identical to ChuA (31) and is similar to TonB-dependent heme transport proteins of other gram-negative pathogens. Proteins with significant homology to ShuA include HmuR (14) from *Y. pestis* (70% identity to ShuA), HemR (28) from *Y. enterocolitica* (68% identity), HxuC (4) from *Haemophilus influenzae* (31% identity), HasR (8) from *Serratia marcescens* (37% identity), and HutA (12) from *Vibrio cholerae* (27% identity). Thus, ShuA appears to be a member of a group of proteins that function to bind and transport heme for utilization by the bacterial cell.

Effect of *shuA* mutation on invasion and intracellular multiplication by *S. dysenteriae*. *Shigella* spp. have both heme transport and siderophore-mediated iron transport systems that could promote iron acquisition within the host. Our earlier observation that the *S. flexneri* siderophore, aerobactin, was not essential for intracellular multiplication within HeLa cells (15) suggested that the heme transport system, rather than siderophores, could provide the bacteria with iron in the intracellular environment. Therefore, the *shuA* mutant MMH2 was compared to the parent strain for invasion, intracellular multiplication, and ability to spread to adjacent cells (Table 3). Henle cell invasion (10) and plaque assays (22) were performed as described previously (15). The mutant and wild-type strains were equally invasive (Table 3). The bacteria in each infected cell were counted after 2 and 3 h of invasion to determine whether the mutant was multiplying intracellularly at the same rate as in the wild type, but no differences were noted (data not shown). Plaque assays, which require that the bacteria multiply intracellularly and spread to adjacent cells, were performed also. If the mutant did not grow as efficiently as the wild type in the intracellular environment, it would be expected that it would spread more slowly and produce smaller plaques, but the plaque sizes were the same (Table 3). These

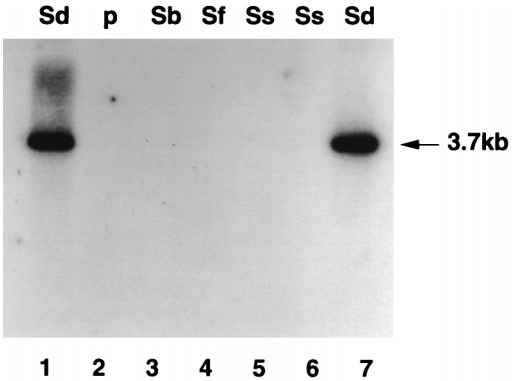

FIG. 3. Presence of *shuA* in the chromosome of *S. dysenteriae* but not other species of *Shigella*. Total or plasmid DNA was isolated, digested with *Kpn*I, separated by agarose gel electrophoresis, and transferred to nitrocellulose. The blot was hybridized under reduced-stringency conditions (1× SSC; 65°C) with the 858-bp *Kpn*I-*Cla*I internal fragment of *shuA* (Fig. 1). The *Kpn*I fragment that hybridizes is indicated by the arrow. Lanes 1 and 7, total DNA from *S. dysenteriae* 0-4576 (Sd), the strain from which *shuA* was cloned; lane 2, *S. dysenteriae* 0-4576 plasmid DNA (p); lane 3, *S. boydii* 0-1392 (Sb); lane 4, *S. flexneri* SA100 (Sf); lane 5, *S. sonnei* PB66 (Ss); lane 6, *S. sonnei* 1-1245 (Ss).

TABLE 3. Effect of *shuA* mutation on Henle cell invasion and plaque formation

S. dysenteriae strain	Relevant phenotype	Invasion (%)[a]	Plaque size (mm)[b]
0-4576-S1	ShuA$^+$	31	2.6 ± 0.8
MMH2	ShuA$^-$	35	2.7 ± 0.9

[a] Strains were grown in L broth plus the iron chelator EDDA to mid-log phase and used to infect subconfluent monolayers of Henle cells (10). After 2 h of infection, at least 300 Henle cells were counted, and all those containing three or more intracellular bacteria were considered invaded.

[b] Confluent Henle cell monolayers were infected with 10^2 bacteria per 35-mm-diameter plate and overlaid with medium containing agarose plus gentamicin. After 48 h, the monolayers were stained and the plaques were measured. Sizes are reported as the means ± standard deviations for at least 25 plaques.

data suggest that the heme transport and siderophore systems are functionally redundant and that the loss of one of these systems is compensated for by the presence of the other when the bacteria are growing within host cells. Alternatively, the cultured cells may not accurately reflect the intracellular environment in vivo, and it is possible that the cultured Henle cells contain abnormally high levels of iron from contaminating iron in the medium. This would allow the lower-affinity iron transport systems of *Shigella* to permit growth of the bacteria in cultured cells, masking the effects of loss of high-affinity iron transport systems. Support for the latter hypothesis was obtained by measuring expression of the iron-regulated aerobactin promoter and synthesis of the aerobactin receptor in *Shigella* growing within cultured cells (11). There was no derepression of the promoter, and synthesis of the outer membrane protein was decreased in the intracellular environment, indicating that the levels of iron in the Henle cells were sufficient to repress one high-affinity iron transport system.

These studies, along with our earlier observations on *Shigella* heme transport (20), indicate that *shuA* encodes a Fur-regulated, TonB- and Exb-dependent heme receptor. A gene almost identical to *S. dysenteriae shuA* is present in *E. coli* O157:H7 (31), but *shuA* is not present in heme-utilizing *Shigella* species other than *S. dysenteriae* type 1 (20, 31). Thus, there is evidence for additional heme transport systems in *Shigella* and other gram-negative pathogens. These systems allow the pathogen to bind free heme or heme proteins to an outer membrane receptor and transport the heme into the cell. In each case, synthesis of the receptor is regulated by the concentration of iron. Transport of heme across the outer membrane appears to require the participation of a TonB system, but the requirements for transport across the cytoplasmic membrane are less clear. Additional studies are needed to define the steps subsequent to heme binding.

The fact that heme transport systems are widely distributed among pathogenic bacteria suggests that these systems play a role in iron acquisition in the host. However, testing this in the currently available tissue culture assays does not provide a definitive result, and animal models are needed for a more complete assessment of the role of heme transport in *Shigella* pathogenesis.

Nucleotide sequence accession number. The sequence data for *shuA* has been deposited in GenBank under accession no. U64516.

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