Iron, an essential nutrient for living organisms, is mostly insoluble or tightly sequestered by high-affinity iron-binding proteins (30). In vertebrate hosts, most iron is intracellular, stored in ferritin or linked to the protoporphyrin ring as heme or heme-containing compounds such as hemoglobin. The small amounts of extracellular iron are bound to transferrin or lactoferrin (6). Bacteria have various mechanisms for scavenging iron, allowing survival in iron-poor environments. A general mechanism of bacterial iron acquisition involves siderophore-mediated ferric uptake systems: excretion of small inorganic iron chelators termed siderophores in response to low environmental iron concentrations followed by iron-siderophore complex assimilation via high-affinity specific transport (19). These systems involve iron-siderophore recognition by a specific outer membrane receptor, energy-consuming TonB-dependent translocation through the outer membrane (11, 21), and transport across the cytoplasmic membrane by a periplasmic binding protein-dependent transport mechanism (20). Heme iron utilization is widespread among bacterial pathogens. Various heme-containing compounds are used, such as free heme or heme bound to hemopexin, hemoglobin, the haptoglobin-hemoglobin complex, or albumin (12). The outer membranes of gram-negative bacteria are impermeable to heme, which is too large to diffuse through the porins. Thus, heme transport across the outer membrane requires interaction of the substrate with specific outer membrane receptors followed by energy-driven translocation, which in most cases is TonB dependent (12). Once in the periplasm, heme is imported across the cytoplasmic membrane by a specific periplasmic binding protein-dependent transport system. Heme is either incorporated into cytochrome apoproteins or degraded in the cytoplasm, providing an iron source. Heme-deficient mutants with enzymatic lesions in the heme biosynthesis pathway have been isolated in many organisms, including Escherichia coli. Such hemA mutants are blocked in an early step in this pathway and require 5-levulinic acid moieties to synthesize heme. E. coli hemA mutants cannot use exogenously supplied heme or heme-containing compounds as a porphyrin source. This concomitant secretion of HasA by the HasR-producing hemA mutant greatly facilitates the acquisition of heme from hemoglobin. This is the first report of a synergy between an outer membrane protein and an extracellular heme-binding protein, HasA, acting as a heme carrier, which we termed a hemophore. That E. coli does not have a system for transport of heme across the cell envelope. E. coli hemA mutants have been used for cloning and characterization of heme utilization systems from other bacteria by complementation of the E. coli hemA defect in heme biosynthesis in the presence of exogenously supplied heme or hemoproteins. This strategy has led to the identification of outer membrane heme and hemoprotein receptors which confer to E. coli hemA mutants the ability to use heme as a porphyrin and an iron source. This demonstrates the impermeability of the E. coli outer membrane to heme and the presence of heme transport across the cytoplasmic membrane in wild-type E. coli (8, 27–29).

In most systems that have been described, the outer membrane receptor directly recognizes either heme alone or the heme moiety of the holoprotein (3, 29). An alternative system, involving an extracellular heme-binding protein which catches heme and shuttles it back to a specific outer membrane receptor, has been reported in two species. In Haemophilus influenzae type b, the extracellular HxuA protein is required for acquisition of heme from the heme-hemopexin complex (2). In Serratia marcescens, a 19-kDa extracellular heme-binding protein, HasA (for heme acquisition system), is required for uptake of free and hemoglobin-bound heme and for heme utilization (14). HasA has no signal peptide but does have a secretion signal in the C-terminal 50 amino acids, with the extreme C-terminal motif (a negatively charged residue followed by several hydrophobic residues) being conserved among many proteins that use the ABC pathway (5). Its secretion depends on ABC protein-mediated exporters: two inner membrane proteins (an ATPase [the ABC protein] and a membrane fusion protein) and an outer membrane polyepitope (15). HasA secretion by an E. coli hemA mutant is not sufficient in itself to allow growth on heme or hemoglobin. Thus, HasA, like extracellular siderophores, may bind heme and deliver it to an outer membrane receptor specific for the heme-HasA complex.

In this report, we describe the identification and characterization of an iron-regulated S. marcescens outer membrane protein, HasR, which alone enables an E. coli hemA mutant to grow on heme or hemoglobin as a porphyrin source. The concomitant secretion of HasA by the HasR-producing hemA mutant reduces by 100-fold the minimum hemoglobin concentration required to satisfy the cell’s need for porphyrin.
The supernatants, which contained solubilized outer membrane proteins, and outer membrane components by selective solubilization in 2% Triton X-100 pellets were collected by centrifugation for 1 h at 15,000 g. Each pellet was resuspended in 10 ml of buffer C (8 M urea, 0.1 M NaH2PO4, 0.05 M Na2HPO4, 0.01 M Tris [pH 8]). The supernatant, which contained the solubilized proteins, was collected by centrifugation and washed several times with buffer C (8 M urea, 0.1 M NaH2PO4, 0.05 M Na2HPO4, 0.01 M Tris [pH 8]). The supernatant, which contained solubilized outer membrane proteins, was kept, and pellets, which contained mostly aggregates, were discarded.

**Materials and Methods**

**Bacterial strains and plasmids.** E. coli C600 (F' thy leu tet [lacY] supE) and TG1 [supE thi [lac-proAB] F' traD36 proAB lacP2D15] were from our laboratory collection. QC752, which carries a ΔfurB::kan insertion, and QC2517, which carries a ΔfurB::lacZ::kan insertion in the FurB gene, were a gift from K. Hantke. The ΔhemA::kan mutation present in strain H500 is described in reference 17. QC752, QC2517, HS073, and H500 were used as allele donors in F1 transductions. E. coli SM365 was obtained from V. Braun. D2FR (aro2 tonB) was gifted from V. Braun. and are described in reference 4. POP3 hemA (araD129 lacU169 proI relA thi hemA) was a gift from R. Kadner. Plasmids pUC18, pBG819, pTZ18R, pAM238, pSC34, pSYC34, and pSC54 were digested with restriction enzyme, as described in reference 13. pSC54 was created by subcloning the 7.7-kb HindIII-BamHI insert of pSC4 into pAC841, which had been digested with HindIII and BamHI; it carries the hasA, hemA, and hasC genes.

**Agar plate growth assays.** Growth of S. marcescens SM365 and E. coli carrying various plasmids was observed on agar plates supplemented with bovine hemoglobin, bovine hemin, bovine N,N'-dimethyl hemoglobin, or bovine myoglobin, with or without 30°C overnight (15 h). The assays were performed as follows. A colony (picked from an LB plate) of each strain to be tested was resuspended in 30 μl of liquid LB medium. This resuspension was used as a homogeneous inoculum source for streaking of the agar plates with a streaking needle. Each growth assay was repeated at least five times.

**Southern blot analysis, colony blotting, and preparation of hybridization probes.** Aliquots of total DNA from SM365 were digested with various restriction enzymes and were separated by centrifugation for 1 h at 15,000 g. HasA was tested as follows. The HasR-producing strain was mixed with 5 ml of top agar and poured onto LB plates supplemented with 0.2 mM 2,2'-dipyridyl and 10-6 M hemoglobin. Wells (5 mm in diameter) were cut in the agar, and each was filled with 50 μl of sterile HasA extract prepared from a C600(pSYC34) culture supernatant as described previously (14). Growth around the wells was recorded after overnight incubation at 37°C.

**RESULTS**

Cloning of the gene located upstream of hasA. Production of HasA protein by S. marcescens is iron regulated. However, there is another open reading frame and no conserved Fur box upstream of hasA. This suggests that hasA is not the first gene in the operon. To investigate whether this adjacent gene is involved in heme uptake, we isolated a 5.5-kb SmaI-KpnI DNA fragment carrying the hasA upstream region on pKSM4 (Fig. 1). To study the complete hasA upstream region, the Sm5-KpnI insert of plasmid pKSM4 was introduced into S. marcescens, which had been linearized by Ecl136I and KpnI. In the resulting derivative of pSYC34 which carries the 5' end of hasA and shares a common KpnI site with pKSM4 (Fig. 1). The resulting plasmid (pRI10K [Fig. 1]) carries the 5' end of hasA and the 5.5-kb DNA fragment located upstream from hasA. We tested
whether this region located upstream from hasA carries a gene(s) required for heme import across the outer membrane.

Complementation of E. coli hemA with pR10K allows utilization of hemoglobin as a porphyrin source. pR10K was introduced into E. coli POP3 hemA, a mutant which grows aerobically if supplemented with 5-aminolevulinic acid but not when provided with exogenously supplied hemoglobin. Growth was tested on solid iron-rich medium (LB) or under dipyridyl iron-depleted conditions (LBD) in the presence of hemoglobin at various concentrations from $10^{-2}$ to $10^{-8}$ M. Single POP3 hemA (pR10K) colonies grew in 15 h at 37°C only on LBD plates supplemented with hemoglobin at concentrations equal to or higher than $10^{-5}$ M. The pR10K insert was then transferred into a low-copy-number vector, pAM238 (Fig. 1). The resulting plasmid, pR10PAM, allowed the utilization of hemoglobin by E. coli POP3 hemA only on LBD plates supplemented with hemoglobin at concentrations equal to or higher than $10^{-4}$ M (Table 1). pR10PAM carries a 5.5-kb DNA insert. Fragments of this insert, carrying the same 3' end as pR10K from the HindIII site in the polylinker and having various 5' ends (various sites in the insert), were introduced into pAM238. POP3 hemA was transformed with these constructs and tested for the ability to use hemoglobin as a porphyrin source. The smallest plasmid, pRDHPAM, which allowed the iron-regulated utilization of hemoglobin, carried a 2.9-kb DraI-HindIII DNA fragment of the pR10K insert (Fig. 1).

We tested whether the iron regulation of hemoglobin utilization was also controlled by the Fur repressor, as is the case for many iron-regulated genes. A double mutant, POP3 fur::kan hemA, was constructed by P1 transduction of the fur::kan allele into POP3 hemA and was transformed with either pR10PAM or pRDHPAM. Both strains grew on LB and LBD plates supplemented with hemoglobin at concentrations equal to or higher than $10^{-4}$ M. Thus, iron repression is mediated by Fur and the 2.9-kb insert contains the determinants of this regulation. The DNA sequence of the 2.9-kb DNA insert was determined.

Nucleotide sequence analysis of the 2.9-kb DNA insert allowing iron-regulated utilization of hemoglobin. The nucleotide sequence of the 2.9-kb DNA insert is shown in Fig. 2. There is a well-conserved putative Fur box (19) 36-bp upstream from the beginning of an open reading frame coding for an 899-amino-acid protein with a predicted molecular mass of 98,220 Da. The putative ribosome binding site and methionine initiation codon are indicated in Fig. 2. The N terminus of the open reading frame appears to be a typical signal sequence, suggesting that the encoded product could be an envelope protein. It was named HasR.

The downstream 200 amino acids of hasR display about 30% identity with the regions encoding the C termini of many TonB-dependent outer membrane receptors, such as the H. influenzae heme-hemopexin receptor HxuC (2) (19% identity for the last 298 residues), the Y. enterocolitica hemin receptor HemR (27) (27% identity for the last 176 residues), and the E. coli vitamin B12 receptor BtuB (10) (22% identity for the last 66 residues).

The N-terminal part of HasR is 21.9% identical to the N-terminal 242 residues of Pup A (1), the ferric-pseudobactin M114 receptor of Pseudomonas putida. Most of the outer membrane receptors to which HasR exhibits similarity are...
TonB dependent and contain a conserved 8-amino-acid sequence close to the N terminus of the mature receptor. This motif is called the TonB box (18, 21). No such well-conserved peptide is found close to the N terminus of the mature HasR protein. However, HasR contains a putative TonB box at position 134, two residues of which are identical and two of which are homologous to the consensus sequence (Table 2). Since several TonB-dependent outer membrane receptors have poorly conserved TonB boxes, we therefore investigated whether the HasR-dependent heme utilization system was TonB dependent.

Role of E. coli TonB in hemoglobin utilization. To avoid hemA revertants, which would have a growth advantage in the tonB background, a C600 hemA deletion mutant was first constructed by P1 transduction of a ΔhemA gene carrying the kanamycin resistance cassette from H500 to C600. Then, the tonB mutation was introduced into the resulting C600 ΔhemA:kan strain by P1 transduction from H5073. As tonB mutants do not grow in iron-restricted medium, a fur mutation was introduced into these strains to allow constitutive expression of HasR without iron depletion. The fur:cat mutation was transferred by P1 transduction from QC2517 into the C600 ΔhemA tonB− and C600 ΔhemA tonB mutant strains. The strains were transformed with pR10PAM and tested for their ability to utilize hemoglobin as a porphyrin source. Strain C600 ΔhemA fur:cat carrying pR10PAM had lost its ability to utilize hemoglobin in iron-rich medium (Table 1). This result shows that TonB is required for HasR-dependent utilization of heme as a porphyrin source.

Identification of HasR. As a tool to identify HasR and determine its cellular localization, we raised anti-HasR antibodies by using a hexahistidine-tagged HasR protein as described in Materials and Methods. Crude membrane extracts from cells harboring either pR10PAM or only the vector, pBGS19, grown under iron-rich or iron-depleted conditions were prepared and separated into a fraction soluble in Triton X-100 alone and a fraction solubilized in Triton X-100 and EDTA. These fractions contained inner and outer membrane proteins. Proteins from each fraction were analyzed by SDS-PAGE followed by immunoblotting. The efficiency of the membrane partitioning was tested by estimating the amount of a known outer membrane protein, TolC, in each fraction. TolC was immunodetected in all cultures, but only in the outer membrane fractions. Anti-His-HasR antibodies labeled only the outer membrane fractions of cells carrying pR10PAM that had been grown under iron-deficient conditions (Fig. 3). Thus, HasR resides in the outer membrane and is produced only under iron-limiting conditions. We compared hemoglobin utilization of strains expressing hasR with or without concomitant HasA secretion to investigate the function of HasA in hemoglobin acquisition.

Reconstitution of HasA-dependent hemoglobin utilization as a porphyrin source. POP3 hemA(pR10PAM) was transformed with pSYCAC1 carrying the hasA, hasD, and hasE genes, which direct HasA synthesis and secretion in E. coli, or with pSYS150, which produces HasD and HasE but not HasA. Only the presence of both plasmids pR10PAM and pSYCAC1 allowed hemoglobin porphyrin utilization by E. coli POP3 hemA on LB plates supplemented with 10^{-6} M hemoglobin (Table 1). Therefore, cells need both the outer membrane receptor HasR and the extracellular protein HasA to be able to use exogenous hemoglobin at concentrations between 10^{-4} and 10^{-8} M, whereas only HasR is required for utilization of relatively high hemoglobin concentrations (100 times higher). Since HasA is secreted, its function in HasR-dependent heme acquisition is presumably extracellular. If that is the case, HasA added externally to a HasR-producing hemA strain should reduce the concentration of exogenous hemoglobin required for its growth.

Effect of addition of exogenous HasA on growth of HasR-producing strains. POP3 hemA(pR10PAM) and POP3 hemA-(pAM 238) were grown in iron-rich medium supplemented with 5-aminolevulinic acid to an OD_{600} of 1 and poured either on LB plates or on plates with that medium supplemented with 10^{-6} M hemoglobin. Fifty microliters of a HasA preparation or of buffer was added to each of all wells in the plates. After 16 h at 37°C, only POP3 hemA(pR10PAM) grew, and growth occurred only around the HasA-containing wells on plates supplemented with hemoglobin. This demonstrates that HasA can be supplied extracellularly to facilitate hemoglobin heme uptake and that this requires HasR. Since heme acquisition via HasR is TonB dependent, we tested whether the complete system comprising the hemophore HasA and the outer membrane component HasR was also TonB dependent.

Role of E. coli TonB in hemoglobin utilization by the complete HasA-HasR system. C600 ΔhemA fur:cat harboring pR10PAM and pSYCAC1 grew well on iron-rich medium supplemented with hemoglobin at a concentration of 10^{-6} M (Table 1). In contrast, strain C600 ΔhemA tonB trp::Tn10 fur:cat carrying pR10PAM and pSYCAC1 did not grow on this

### Table 1. Growth of various E. coli hemA mutants, carrying different plasmids, on agar plates containing different porphyrin sources

<table>
<thead>
<tr>
<th>Strain and relevant genotype</th>
<th>Hb</th>
<th>He</th>
<th>MeHb</th>
<th>Myo</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ Dip</td>
<td>− Dip</td>
<td>+ Dip</td>
<td>− Dip</td>
<td>+ Dip</td>
</tr>
<tr>
<td>POP3 hemA</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>POP3 hemA(pR10PAM)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>POP3 hemA(pR10PAM, pSYCAC1)</td>
<td>10^{-4}</td>
<td>10^{-5}</td>
<td>10^{-6}</td>
<td>NT</td>
</tr>
<tr>
<td>C600Δ hemA::kan fur:cat(pR10PAM)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>C600Δ hemA::kan fur:cat tonB trp::Tn10(pR10PAM)</td>
<td>10^{-6}</td>
<td>10^{-5}</td>
<td>10^{-6}</td>
<td>NT</td>
</tr>
<tr>
<td>C600Δ hemA::kan fur:cat(pR10PAM, pSYCAC1)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>C600Δ hemA::kan fur:cat tonB trp::Tn10(pR10PAM, pSYCAC1)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

* LB medium with or without 0.2 mM 2,2'-dipyridyl, which induces iron-regulated promoters, was used. Strains were streaked on various agar plates as described in Materials and Methods. Each experiment was repeated five times.

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**S. MARCESCENS HEMOPHORE-DEPENDENT HEME ACQUISITION**

Vol. 179, 1997

*E. coli* hemA mutants were constructed by P1 transduction of a *D. hemA*- and iron-rich medium, whereas strain C600 was transformed with pR10PAM and tested for their **TonB is required for HasR-dependent utilization of heme as a globin in iron-rich medium (Table 1)**. This result shows that cat carrying pR10PAM had lost its ability to utilize hemoglobin porphyrin utilization by the *E. coli* POP3 hemA on LB plates supplemented with 10^{-6} M hemoglobin (Table 1). Therefore, cells need both the outer membrane receptor HasR and the extracellular protein HasA to be able to use exogenous hemoglobin at concentrations between 10^{-4} and 10^{-8} M, whereas only HasR is required for utilization of relatively high hemoglobin concentrations (100 times higher). Since HasA is secreted, its function in HasR-dependent heme acquisition is presumably extracellular. If that is the case, HasA added externally to a HasR-producing hemA strain should reduce the concentration of exogenous hemoglobin required for its growth.

**Effect of addition of exogenous HasA on growth of HasR-producing strains.** POP3 hemA(pR10PAM) and POP3 hemA-(pAM 238) were grown in iron-rich medium supplemented with 5-aminolevulinic acid to an OD_{600} of 1 and poured either on LB plates or on plates with that medium supplemented with 10^{-6} M hemoglobin. Fifty microliters of a HasA preparation or of buffer was added to each of all wells in the plates. After 16 h at 37°C, only POP3 hemA(pR10PAM) grew, and growth occurred only around the HasA-containing wells on plates supplemented with hemoglobin. This demonstrates that HasA can be supplied extracellularly to facilitate hemoglobin heme uptake and that this requires HasR. Since heme acquisition via HasR is TonB dependent, we tested whether the complete system comprising the hemophore HasA and the outer membrane component HasR was also TonB dependent.

**Role of E. coli TonB in hemoglobin utilization by the complete HasA-HasR system.** C600 ΔhemA fur:cat harboring pR10PAM and pSYCAC1 grew well on iron-rich medium supplemented with hemoglobin at a concentration of 10^{-6} M (Table 1). In contrast, strain C600 ΔhemA tonB trp::Tn10 fur:cat carrying pR10PAM and pSYCAC1 did not grow on this
medium even in the presence of high concentrations of heme.

Similarly, addition of the HasA preparation to wells of LB plates with 10^{-6} M hemoglobin containing a culture of strain C600 ΔhemA tonB trp::Tn10 fur::cat carrying pR10PAM did not stimulate growth around the wells. This shows that HasR function is TonB dependent even when heme is delivered via HasA. We have previously shown that HasA binds free or hemoprotein-associated heme and that HasA is required by S. marcescens for iron heme utilization (14). To determine whether HasA facilitates heme acquisition from various heme sources, we compared the efficiencies of utilization of various

![FIG. 2. Nucleotide sequence of the 2.9-kb hasA upstream region and the deduced amino acid sequence of HasR. The nucleotide sequence of the putative ribosome binding site is underlined. The amino acid sequence is shown in block letters below the nucleotide sequence. The amino acid sequence of the putative N-terminal signal sequence is underlined and in italics. The putative Fur binding site sequence (Fur box) is indicated in boldface letters. A comparison of the hasR Fur box with the Fur box consensus sequence is shown above the sequence. The beginning of the hasA sequence is shown 3' of the hasR sequence. The asterisk indicates the stop codon of hasR.](http://jb.asm.org/)

on October 14, 2017 by guest
http://jb.asm.org/download
heme sources by the *E. coli* hemA mutant producing HasR alone or producing HasR and secreting HasA.

Utilization of heme and various hemoproteins by *E. coli* hemA strains producing HasR alone or producing HasR and secreting HasA. We did studies to determine the minimum concentrations of heme compounds required for the growth of single colonies on LBD plates within 15 h (Table 1). POP3 *hemA* (pR10PAM) and POP3 *hemA* (pR10PAM, pSYCAC1) needed similar free heme concentrations for growth. POP3 *hemA* (pR10PAM) required a 100 times higher concentration of N,N<sup>9</sup>-dimethyl hemoglobin (10<sup>-2</sup> M) than did POP3 *hemA* (pR10PAM, pSYCAC1) (10<sup>-2</sup> M). Myoglobin could not be used even at concentrations higher than 10<sup>-2</sup> M. Therefore, N,N<sup>9</sup>-dimethyl hemoglobin is a substrate for HasR, and HasA recognizes heme in this hemoprotein and decreases the minimum concentration allowing growth. Myoglobin was not recognized by HasR alone and was not used via HasA. Free heme is a substrate for HasR, but secretion of HasA did not significantly improve the utilization of free heme by *E. coli*.

**DISCUSSION**

We isolated a DNA fragment from *S. marcescens* corresponding to the 5′ end of the has operon which carries a gene, *hasR*, whose expression enables an *E. coli* heme auxotroph mutant to use exogenously supplied free heme and hemoglobin as porphyrin sources. Free heme, hemoglobin, and N,N<sup>9</sup>-dimethyl hemoglobin, but not myoglobin, were used by HasR-producing strains, showing that HasR differs from most other heme compound outer membrane receptors, which are more specific, in that it is able to transport either free heme or heme bound to hemoglobin or other hemoproteins (12). The *H. influenzae* HxuC outer membrane protein is another receptor which is also required for both heme and heme-hemopexin uptake, but heme uptake from hemopexin is totally dependent.
on HxA secretion to the extracellular medium (2). HasR enables the use of heme, hemoglobin, and a modified (methylated) hemoglobin, suggesting that HasR recognizes the heme moiety of the hemoproteins. Heme is buried more deeply in myoglobin than in the other hemoproteins tested, which may explain why it was not recognized.

HasR-dependent heme utilization was iron regulated in a fur+ strain. In a fur mutant, heme was used even in iron-rich medium. This suggests that HasR is iron regulated in a Fur-dependent manner. Not surprisingly, just upstream from the beginning of the hasR gene there is a well-conserved Fur box. The hasR product was identified and its cellular localization was determined by using antibodies raised against a histidine-tagged HasR protein. HasR is a 98-kDa iron-regulated outer membrane protein. It exhibits substantial similarity to other outer membrane receptors. However, HasR does not have a typical TonB box close to the N terminus of its processed form (18). Nevertheless, heme utilization in an E. coli hemA mutant expressing HasR required a functional TonB protein. A region similar to the TonB box with only two conserved residues is found at position 134 in the HasR protein. Introducing mutations into this putative TonB box would determine whether it is required for heme acquisition. Using isogenic tonB+ and tonB mutant strains of S. marcescens, which both secrete HasA under iron-depleted conditions, we observed that the tonB+ strain, but not the tonB mutant, grew at a high iron chelator concentration (0.2 mM dipyridyl) (data not shown). In the presence of exogenous hemoglobin, both strains grew on this medium, suggesting that hemoglobin can be used by S. marcescens as an iron source and thus be internalized even in the absence of a functional TonB protein (data not shown). This might be due to an unidentified S. marcescens TonB-like protein which in E. coli could be replaced by TonB. The poor conservation of the TonB box in HasR might reflect its dependence on this putative second energy-transducing protein. A heme utilization system in V. cholerae which may be dependent on a TonB analog has been described. Like HasR, the V. cholerae HutA receptor does not have a typical TonB box (8). The existence in H. influenzae of a TonB-independent outer membrane receptor which is functional for growth only when heme is in excess has been postulated. However, no such protein has been characterized, and it is not known whether it would depend on a TonB analog (9). In E. coli, utilization of free heme and hemoproteins via HasR also required relatively high exogenous concentrations (about 10^{-4} M) of these compounds and required only the production of HasR, whereas acquisition of iron from these compounds in S. marcescens required the presence of HasA in the extracellular medium (14). Since hasR and hasA are adjacent in an operon, we compared hemoglobin utilization by E. coli hemA strains producing HasR with or without concomitant HasA secretion. The strains producing only HasR required 100 times higher hemoglobin concentrations than the strains producing HasR and secreting HasA. Growth was stimulated at an exogenous hemoglobin concentration of 10^{-6} M by addition of a HasA preparation to HasR-producing cells, confirming that HasA functions from the outside in a soluble form. Thus, for efficient function, this system needs both proteins: HasR, the outer membrane protein, and HasA, the extracellular hemoprotein. This is the first report of an extracellular protein working in synergy with an outer membrane receptor.

HasR alone allowed the uptake of free heme and heme bound to hemoglobin or to N,N'-dimethyl hemoglobin but not myoglobin. The presence of HasA in the supernatants of HasR-producing strains (either added or concomitantly secreted) facilitated heme acquisition but did not broaden the range of the heme compounds utilized. The HasA-HasR pair thereby differs from the HxA-HxC pair. HxA, the outer membrane receptor, recognizes only free heme, not hemopexin. HxA, the extracellular protein, is required for the recognition of hemopexin by HxC. The extracellular protein increases the number of potential heme sources but does not change the minimum concentration required for growth (2). Surprisingly, HasA secretion by E. coli had no effect on the efficiency of free-heme uptake, whereas it was required for acquisition of iron heme in S. marcescens (14). However, this result is difficult to interpret because the overall free-heme uptake by E. coli is inefficient with or without HasA (10^{-5} M), whereas an S. marcescens heme auxotroph can use both heme and hemoglobin as porphyrin sources, at concentrations as low as 10^{-7} M (data not shown). Possibly, S. marcescens has another HasA-dependent outer membrane receptor for free heme or S. marcescens excretes factors which increase free-heme solubilization.

The mechanism by which HasA extracts heme from hemoproteins is unknown. HasA may interact with the apoprotein, changing its conformation such that the heme is transferred to HasR. Alternatively, HasA may have higher affinity for heme than that of globin for heme, and thus the equilibrium between

### Table 2. Comparison of sequence of HasR with those of the TonB boxes of BtuB and HemR

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
<th>Position of D</th>
</tr>
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<tbody>
<tr>
<td>HasR</td>
<td>DTLVVTAN</td>
<td>134</td>
</tr>
<tr>
<td>BtuB</td>
<td>DTLVVTANR</td>
<td>26</td>
</tr>
<tr>
<td>HemR</td>
<td>DTMVVTATG</td>
<td>44</td>
</tr>
</tbody>
</table>

* BtuB and HemR are TonB-dependent receptors of E. coli and Y. enterocolitica, respectively.

* Identical residues are shown in boldface type.

* Position in the unprocessed protein of the first amino acid shown.

FIG. 3. Immunodetection with anti-ToIC and anti-His-HasR antibodies of inner (IM) and outer (OM) membrane fractions of various strains grown under iron-rich or iron-depleted conditions. Inner and outer membrane fractions were prepared as described in Materials and Methods. Lanes 1 and 2, POP3 hemA (pBGS19) grown in iron-rich medium; lanes 3 and 4, POP3 hemA (pBGS19) grown in iron-depleted medium; lanes 5 and 6, POP3 hemA (pR10K) grown in iron-rich medium; lanes 7 and 8, POP3 hemA (pR10K) grown in iron-depleted medium. Inner membrane lanes (1, 3, 5, and 7) were loaded with 2 OD equivalent units of cell sample. Outer membrane lanes (2, 4, 6, and 8) were loaded with 1 OD equivalent unit of cellular sample. Both the anti-ToIC (diluted 1/2,000) and the anti-His-HasR (diluted 1/50,000) antibodies were used to probe the blots.
hemoglobin and globin may be shifted to globin by HasA, which binds the free heme (26).

How HasA lowers the concentration of heme required for HasR-dependent heme uptake is also unknown. It could be due to simply a better presentation of the heme moiety to the receptor, or it could be because of a direct interaction with HasR. Finally, heme complexes tightly with HasA but presumably must be transferred to HasR, internalized across the outer membrane, and released inside the periplasm in a TonB-dependent mechanism. Possibly, the interaction of HasA with HasR leads to a conformational change in HasA, allowing the transfer of heme from HasA, which has a very high affinity for heme (K_d, <10^{-8} M [8a]), to HasR, which does not bind heme with a very high affinity (as suggested by the inefficient uptake of heme promoted by HasR alone). We are presently investigating whether this is an energy-dependent step and, if so, what energy source is involved. The low affinity of HasR for heme may also facilitate the release of heme inside the cells. This model is also being studied.

ACKNOWLEDGMENTS

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REFERENCES