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A New Type of Hemophore-Dependent Heme Acquisition System of *Serratia marcescens* Reconstituted in *Escherichia coli*

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The utilization by *Serratia marcescens* of heme bound to hemoglobin requires HasA, an extracellular heme-binding protein. This unique heme acquisition system was studied in an *Escherichia coli hemA* mutant that was a heme auxotroph. We identified a 92-kDa iron-regulated *S. marcescens* outer membrane protein, HasR, which alone enabled the *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 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.

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, whereas *hemA* mutants of other gram-negative bacteria, such as *Yersinia enterocolitica* and *Vibrio cholerae* (7, 27), grow aerobically in the presence of heme, suggesting 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 iron 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 polypeptide (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.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* C600 (F$^-$ *thr leu fhuA lacY thi supE*) and TG1 [*supE thi* Δ(*lac-proAB*) F′ *traD36 proAB lacI*^{q}*ZΔM15*] were from our laboratory collection. QC1782, which carries a Δ*fur*::*kan* insertion, and QC2517, which carries a Δ(*fur*::*cat*) insertion, were from D. Touati. Strain H5073 carries a *tonB* mutation and a Tn*10* insertion in *trp* genes and was a gift from K. Hantke. The Δ*hemA*::*kan* mutation present in strain H500 is described in reference 17. QC1782, QC2517, H5073, and H500 were used as allele donors in P1 transductions. *S. marcescens* SM365 was obtained from V. Braun. D2FR (*aro2*) and D2FR3 (*aro2 tonB*) were gifts from V. Braun and are described in reference 4. POP3 *hemA* (*araD139* Δ*lacU169 rpsL relA thi hemA*) was a gift from R. Kadner. Plasmids pUC18, pBGS18, pBGS19, pTZ18R, PAM238, pSYC34, pSYC134, and pSYC150 are described in reference 13. pSYCAC1 was created by subcloning the 7.7-kb *Hin*dIII-*Bam*HI insert of pSYC4 into pACYC184 which had been digested with *Hin*dIII and *Bam*HI; it carries the *hasA*, *hasD*, and *hasE* genes. Plasmids pSYC100, pX14, pSYCSK, pKSM4, pR10K, pR10PAM, and pRDH-PAM are described in this work (Fig. 1). pQE30 was obtained from Qiagen. The *hemA*::*kan* deletion, the *tonB* mutation cotransducible with the *trp*::Tn*10* insertion, and the *fur*::*kan* and *fur*::*cat* deletions were introduced into C600 and POP3 *hemA* by P1 transduction with selection for kanamycin, tetracycline, and chloramphenicol resistance as described in reference 16. The HemA phenotype was routinely checked by testing growth with and without 5-aminolevulinic acid on plates of Luria broth (LB) with agar. The TonB phenotype was routinely tested by determining resistance to colicin Ia and Ib and by checking for inhibition of growth on iron-chelated medium (LB plus 0.2 mM 2,2′-dipyridyl [LBD]).

Media. All media and antibiotics were used as described in reference 16. LBD medium consisted of LB medium supplemented with 0.2 mM 2,2′-dipyridyl to reduce available iron. Bovine hemoglobin, bovine hemin, bovine *N*,*N*′-dimethyl hemoglobin, and bovine myoglobin were obtained from Sigma Chemical Company. Bovine hemoglobin, bovine *N*,*N*′-dimethyl hemoglobin, and bovine myoglobin agar plates were prepared as follows. A 10^{-3} M solution of the porphyrin source (the concentration was calculated on a monomer basis) in 100 mM NaCl was filter sterilized with 0.45-μm-pore-size Millipore filters. Agar plates were prepared by mixing various concentrations of these solutions at 42°C with agar containing antibiotics with and without 0.2 mM 2,2′-dipyridyl. The bovine hemin stock solution (10^{-2} M) was prepared as described in reference 14.

Extraction and manipulation of plasmids. Isolation of plasmids, cloning, restriction map analysis, and transformation were carried out as described in reference 22.

Isolation of pKSM4. An *S. marcescens* SM365 genomic library was constructed in pUC18 as described in reference 22. Chromosomal DNA was prepared as described in reference 13. The *S. marcescens* chromosomal DNA was partially digested with *Sph*I and *Hin*dIII (see Fig. 1) and ligated with pUC18 which had been linearized with *Sph*I and *Hin*dIII, and the ligation mixture was used to transform *E. coli* TG1. Two successive, partially overlapping probes were used to identify the DNA region upstream from *hasA*. One was the 900-bp insert from pSYC134, which allowed the isolation of a 750-bp *Sph*I-*Kpn*I fragment that was inserted into pSYCSK (see Fig. 1). The fragment inserted in pSYCSK was used to isolate a 5.5-kb *Kpn*I-*Sma*I insert of *S. marcescens* DNA in pBGS19+ that was carried by pKSM4 (see Fig. 1). The restriction map and DNA sequence of this fragment in pSYCSK were identical to part of that in pKSM4 which was kept for further study.

Southern blot analysis, colony blotting, and preparation of hybridization probes. Aliquots of total DNA from SM365 were digested with various restriction endonucleases and analyzed by Southern blotting with non-radioactively labeled probes. Probes were labeled with digoxigenin–11-dUTP (Boehringer Mannheim) by PCR. Hybridization and immunological detection of the probe were performed with CSPD (1,2-dioxetrane chemiluminescent enzyme substrate; Tropix) as the chemiluminescent substrate for alkaline phosphatase.

DNA sequence analysis. DNA sequences were determined by Genome Express SA (Grenoble, France) by stepwise oligonucleotide synthesis with an Applied Biosystems sequencer. Synthetic oligonucleotides were obtained by using an Applied Biosystems synthesizer.

Membrane preparation and protein analysis. POP3 *hemA*(pBGS19) and POP3 *hemA*(pR10K) were each grown in 20 ml of LB medium at 37°C to an optical density (OD) of 0.2, these cultures were divided into two subcultures of 10 ml each, one of which was supplemented with 0.2 mM dipyridyl, and both cultures were reincubated. Cells were harvested when they reached an OD at 600 nm (OD_{600}) of 1 and were centrifuged for 10 min at 5,000 × *g* and 4°C. Cells were washed once in 50 mM Tris-HCl, pH 7.5. Each pellet was resuspended in 500 μl of 50 mM Tris-HCl (pH 7.5) and sonicated, and the crude membrane pellets were collected by centrifugation for 1 h at 15,000 × *g* and 4°C in a microcentrifuge. The total-membrane preparations were fractionated into inner and outer membrane components by selective solubilization in 2% Triton X-100 as described previously (24, 25). Briefly, the inner membrane proteins were solubilized by incubation for 1 h at 4°C in 50 mM Tris-HCl (pH 7.5)–2% Triton X-100–1 mM $MgCl_2$ and were separated by centrifugation for 1 h at 15,000 × *g* and 4°C in a microcentrifuge. The pellets were subsequently solubilized by incubation for 1 h at 4°C in 50 mM Tris-HCl (pH 7.5)–2% Triton X-100–5 mM EDTA followed by centrifugation for 1 hour at 15,000 × *g* and 4°C in a microcentrifuge. Supernatants, which contained solubilized outer membrane proteins, were kept, and pellets, which contained mostly aggregates, were discarded (23). Proteins present in the inner and outer membrane fractions of the various cultures were analyzed by sodium dodecyl sulfate–7% polyacrylamide gel electrophoresis (SDS–7% PAGE) followed by immunodetection with anti-His-HasR antibodies and anti-TolC antibodies.

Agar plate growth assays. Growth of *S. marcescens* SM365 and *E. coli* carrying various plasmids was tested on agar plates supplemented with bovine hemoglobin, bovine hemin, bovine *N*,*N*′-dimethyl hemoglobin, or bovine myoglobin, with or without 0.2 mM 2,2′-dipyridyl, at 37°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.

Growth assays on agar plates supplemented with purified HasA. Stimulation of growth of the HasR-producing *E. coli hemA* mutant by exogenously supplied HasA was tested as follows. The HasR-producing strain was mixed with 3 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.

***his-hasR* gene fusion construction—expression and purification of His-HasR.** We fused a hexahistidine tag to the HasR protein, allowing its purification over nitrilotriacetic acid-agarose. A 2.9-kb DNA fragment carrying a *hasR* gene lacking its 5′ end was obtained by digestion of pR10PAM with *Eco*47III and *Hin*dIII and was inserted into pQE30 which had been digested with *Sma*I and *Hin*dIII. This led to an in-frame fusion between the six histidines of the vector and the HasR protein lacking its signal peptide and the N-terminal eight residues of the mature form. *E. coli* TG1 transformants were screened for the production of recombinant proteins containing the hexahistidine epitope by Western blotting with antibodies directed against the RGSHis4 motif (Qiagen). One positive clone carrying phis-hasRQE30 was used for purification of the protein fusion. *E. coli* TG1(phis-hasRQE30) was grown in 100 ml of LB medium in the presence of ampicillin to an OD_{600} of 0.6, at which time expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 3 h of induction, when the culture reached an OD_{600} of 3.5, the cells were harvested by centrifugation and the cell pellet was disrupted by sonication at 50 W with a Branson B12 sonicator. Inclusion bodies were prepared as described previously (21). The cell extracts were centrifuged at 6,000 × *g* for 1 h. The pellet, which contained the inclusion bodies, was resuspended in 6 ml of Triton buffer (2% [vol/vol] Triton X-100, 50 mM Tris-HCl [pH 8], 10 mM EDTA, 100 mM NaCl) and incubated at 37°C overnight with shaking. After centrifugation at 6,000 × *g* for 20 min, the pellet was resuspended in 6 ml of TEN (50 mM Tris-HCl [pH 8], 10 mM EDTA, 100 mM NaCl). After 2 h of incubation at 37°C with shaking, centrifugation at 6,000 × *g* for 20 min yielded a white pellet which was solubilized in 2 ml of TEN containing 8 M urea. This solution was centrifuged for 15 min in an Eppendorf microcentrifuge at 15,000 × *g*. The supernatant, which contained the solubilized His-HasR protein, was mixed with 2 ml of packed Ni-nitrilotriacetic acid-agarose (Qiagen), and the suspension was rotated for 2 h at 4°C. Agarose beads were collected by centrifugation and washed several times with buffer C (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris [pH 8]), and His-HasR was eluted with 100 mM EDTA in 2 ml of buffer C. The eluate was subjected to preparative SDS–7% PAGE, and proteins were transferred to a nitrocellulose membrane. Ponceau red staining of the membrane revealed one major protein band with an apparent molecular mass of 90 kDa. The band was cut out, frozen in liquid nitrogen, and then pulverized and resuspended in 1 ml of phosphate-buffered saline; this was mixed with Titer Max adjuvant (Interbiotech) and injected into a rabbit to raise antibodies.

Nucleotide sequence accession number. The nucleotide sequence of *hasR* has been assigned GenBank accession no. Y08983.

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 from *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 *Sma*I-*Kpn*I DNA fragment carrying the *hasA* upstream region on pKSM4 (Fig. 1). To study the complete *hasA* upstream region, the *Sma*I-*Kpn*I insert of pKSM4 was introduced into pX14 which had been linearized by *Ecl*136II and *Kpn*I. pX14 is a deletion derivative of pSYC34 which carries the 5′ end of *hasA* and shares a common *Kpn*I site with pKSM4 (Fig. 1). The resulting plasmid (pR10K [Fig. 1]) carries the 5′ end of *hasA* and the 5.5-kb DNA fragment located upstream from *hasA*. We tested

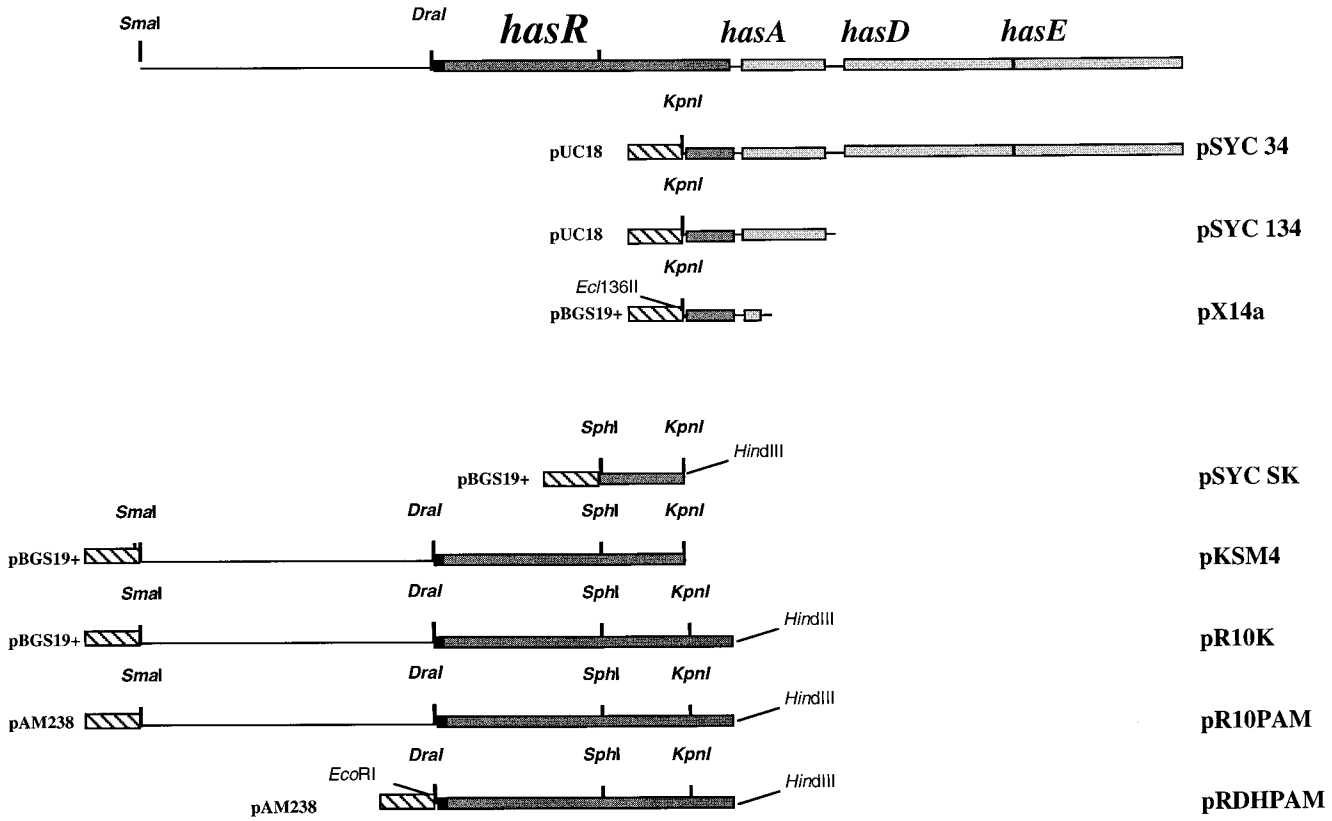

FIG. 1. Plasmid maps. pSYC134 carries the gene *hasA* alone. pSYC34 carries the gene *hasA* and the secretion factor genes *hasD* and *hasE*. Plasmids pX14a, pSYC100, pSYCSK, pKSM4, pR10K, pR10PAM, and pRDHPAM are described in Materials and Methods.

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^{-4} 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 *Hin*dIII 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 *Dra*I-*Hin*dIII 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

TABLE 1. Growth of various *E. coli hemA* mutants, carrying different plasmids, on agar plates containing different porphyrin sources[a]

Strain and relevant genotype	Lowest concn (M) required for growth with porphyrin source[b]:							
	Hb		He		MeHb		Myo	
	+ Dip	− Dip	+ Dip	− Dip	+ Dip	− Dip	+ Dip	− Dip
POP3 *hemA*	—[c]	—	—	—	—	—	—	—
POP3 *hemA*(pR10PAM)	10^{-4}	—	10^{-5}	—	10^{-4}	—	—	—
POP3 *hemA*(pR10PAM, pSYCAC1)	10^{-6}	—	10^{-5}	—	10^{-6}	—	—	—
C600Δ *hemA*::*kan fur*::*cat*(pR10PAM)	10^{-4}	10^{-4}	NT[d]	NT	NT	NT	NT	NT
C600Δ *hemA*::*kan fur*::*cat tonB trp*::Tn*10*(pR10PAM)	—	—	NT	NT	NT	NT	NT	NT
C600Δ *hemA*::*kan fur*::*cat*(pR10PAM, pSYCAC1)	10^{-6}	10^{-6}	NT	NT	NT	NT	NT	NT
C600Δ *hemA*::*kan fur*::*cat tonB trp*::Tn*10*(pR10PAM, pSYCAC1)	—	—	NT	NT	NT	NT	NT	NT

[a] 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.

[b] Plates were incubated for 15 h at 37°C. Abbreviations: Hb, hemoglobin; He, heme; MeHb, *N*,*N*′-dimethylhemoglobin; Myo, myoglobin; + Dip, with dipyridyl; − Dip, without dipyridyl.

[c] —, no growth after 15 h of incubation at 37°C.

[d] NT, not tested.

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 Δ*hemA* gene carrying the kanamycin resistance cassette from H500 to C600. Then, the *tonB* mutation was introduced into the resulting C600 Δ*hemA*::*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 Δ*hemA tonB*$^+$ and C600 Δ*hemA tonB* mutant strains. The strains were transformed with pR10PAM and tested for their ability to utilize hemoglobin as a porphyrin source. Strain C600 Δ*hemA fur*::*cat* harboring pR10PAM grew well on hemoglobin- and iron-rich medium, whereas strain C600 Δ*hemA tonB 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 pSYC150, 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 LBD 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^{-6} 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 LBD 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 Δ*hemA 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 Δ*hemA tonB trp*::Tn*10 fur*::*cat* carrying pR10PAM and pSYCAC1 did not grow on this

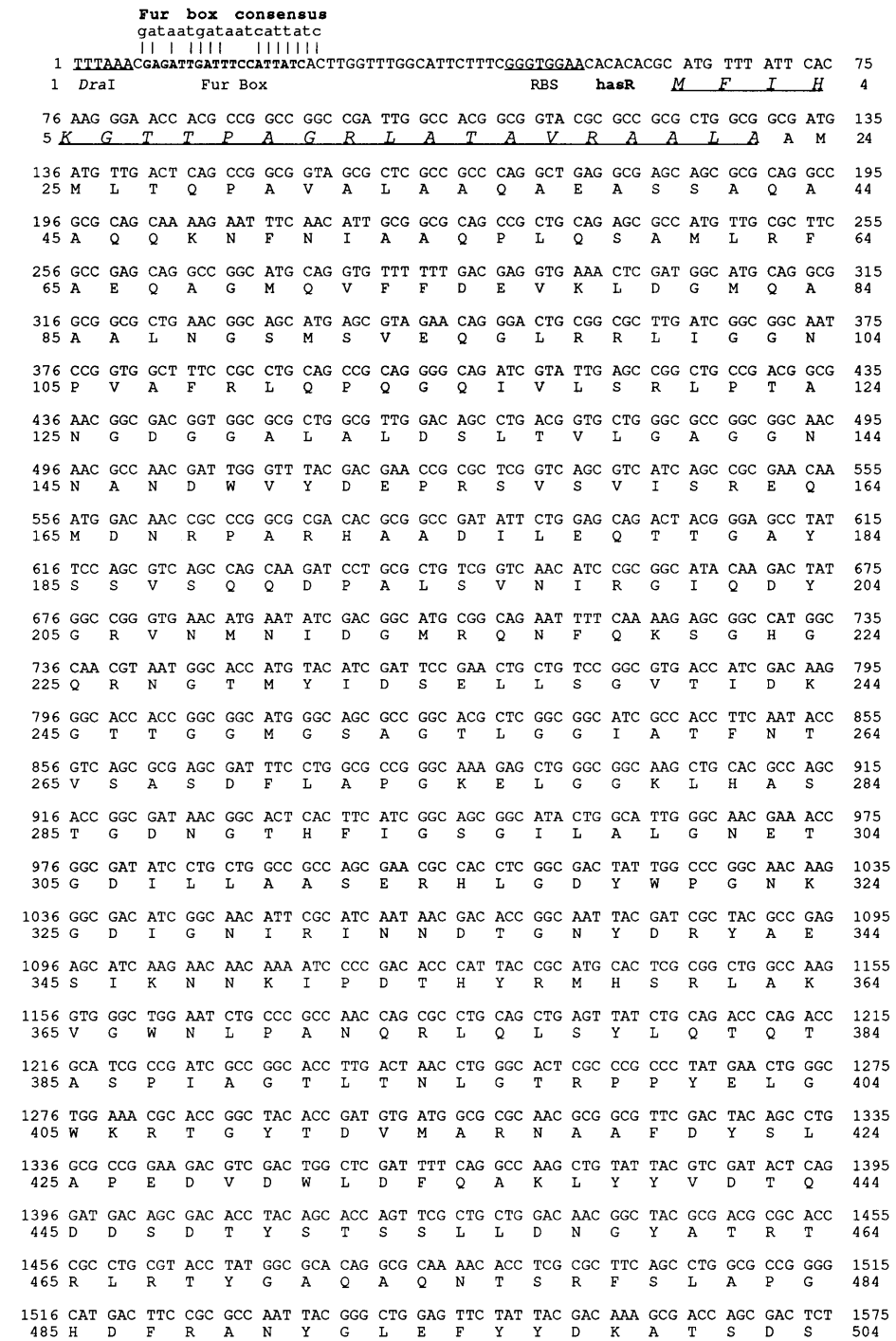

FIG. 2. Nucleotide sequence of the 2.9-kb *hasA* upstream region and the deduced amino acid sequence of HasR. The nucleotide sequence of the putative ribosome binding site is underlined. The amino acid sequence is shown in block letters below the nucleotide sequence. The amino acid sequence of the putative 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*.

medium even in the presence of high concentrations of hemoglobin.

Similarly, addition of the HasA preparation to wells of LB plates with 10^{-6} M hemoglobin containing a culture of strain C600 Δ*hemA tonB trp*::Tn*10 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

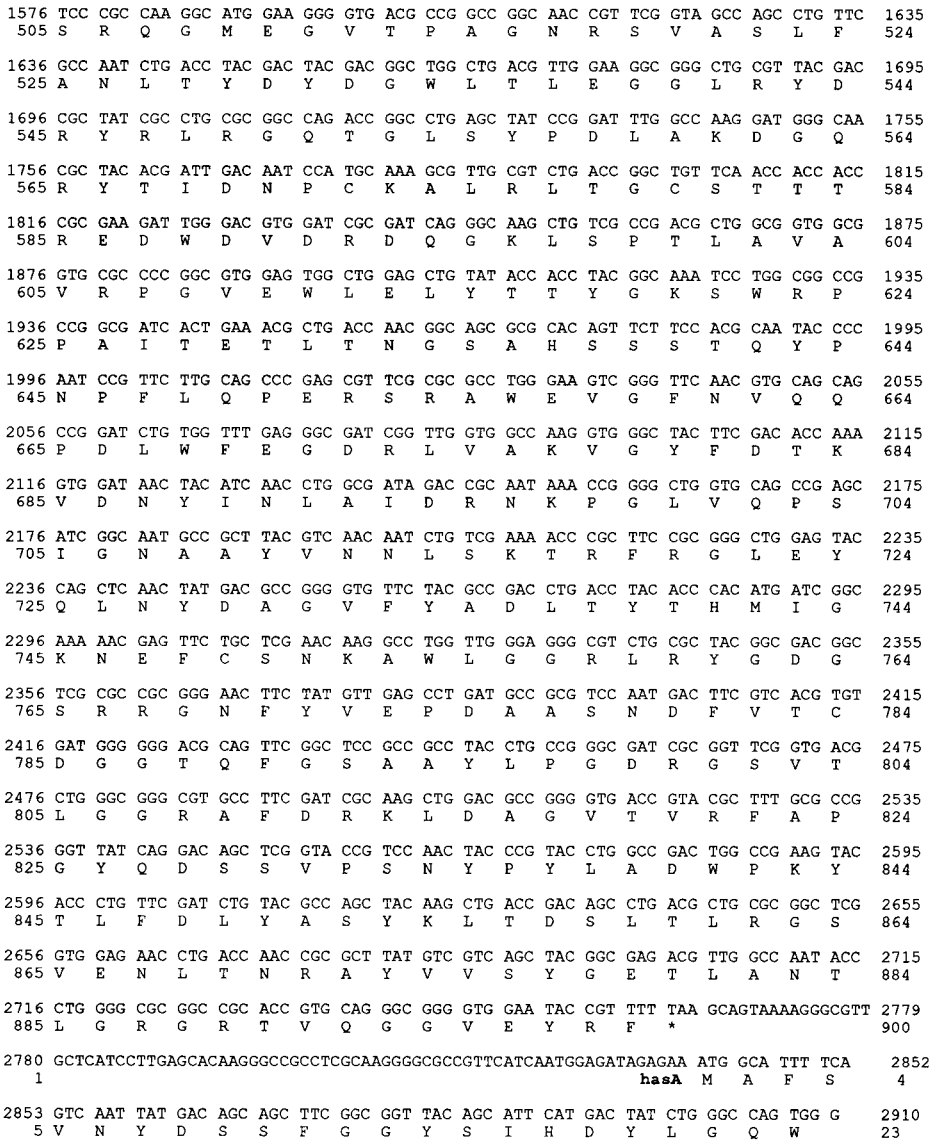

FIG. 2—*Continued.*

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*′-dimethyl hemoglobin (10^{-4} M) than did POP3 *hemA*(pR10PAM, pSYCAC1) (10^{-6} M). Myoglobin could not be used even at concentrations higher than 10^{-3} M. Therefore, *N*,*N*′-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*′-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

TABLE 2. Comparison of sequence of HasR with those of the TonB boxes of BtuB and HemR[a]

Protein	Sequence[b]	Position of D[c]
HasR	**D**SLT**V**VTAN	134
BtuB	**D**TLV**V**TANR	26
HemR	**D**TMV**V**TATG	44

[a] BtuB and HemR are TonB-dependent receptors of *E. coli* and *Y. enterocolitica*, respectively.
[b] Identical residues are shown in boldface type.
[c] Position in the unprocessed protein of the first amino acid shown.

on HxuA 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 hemophore. 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 HxuA-HxuC pair. HxuC, the outer membrane receptor, recognizes only free heme, not hemopexin. HxuA, the extracellular protein, is required for the recognition of hemopexin by HxuC. 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 HasA. Alternatively, HasA may have higher affinity for heme than that of globin for heme, and thus the equilibrium between

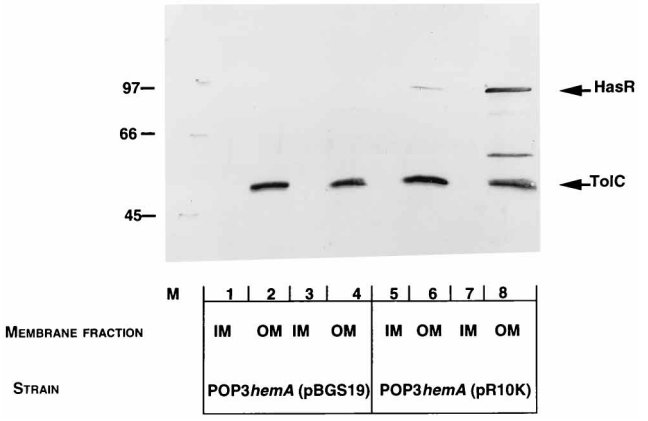

FIG. 3. Immunodetection with anti-TolC and anti-His-HasR antibodies of inner (IM) and outer (OM) membrane fractions of various strains grown under iron-rich or iron-depleted conditions. Inner and outer membrane fractions were prepared as described in Materials and Methods. Lanes 1 and 2, POP3 *hemA* (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-TolC (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.

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REFERENCES

1. **Bitter, W., J. D. Marugg, L. A. De Weger, J. Tommassen, and P. J. Weisbeck.** 1991. The ferric pseudobactin receptor PupA of *Pseudomonas putida* WCS 358: homology to TonB-dependent *Escherichia coli* receptors and specificity of the protein. Mol. Microbiol. **5:**647–655.
2. **Cope, L. D., R. Yogev, U. Muller-Eberhard, and E. J. Hansen.** 1995. A gene cluster involved in the utilization of both free heme and heme:hemopexin by *Haemophilus influenzae* type b. J. Bacteriol. **177:**2644–2653.
3. **Elkins, C.** 1995. Identification and purification of a conserved heme-regulated hemoglobin-binding outer membrane protein from *Haemophilus ducreyi*. Infect. Immun. **63:**1241–1245.
4. **Gaisser, S., and V. Braun.** 1991. The *tonB* gene of *Serratia marcescens*: sequence activity and partial complementation of *Escherichia coli tonB* mutants. Mol. Microbiol. **5:**2777–2787.
5. **Ghigo, J. M., and C. Wandersman.** 1994. A carboxyl-terminal four-amino acid motif is required for secretion of the metalloprotease PrtG through the *Erwinia chrysanthemi* protease secretion pathway. J. Biol. Chem. **269:**8979–8985.
6. **Gray-Owen, S. D., and A. B. Schryvers.** 1996. Bacterial transferrin and lactoferrin receptors. Trends Microbiol. **4:**185–191.
7. **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.
8. **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.

8a. **Izadi, N., Y. Henry, J. Haladjan, M. E. Goldberg, C. Wandersman, M. Delepierre, and A. Lecroisey.** Biochemistry, in press.

9. **Jarosik, G. P., J. D. Sanders, L. D. Cope, U. Muller-Eberhard, and E. J. Hansen.** 1994. A functional *tonB* gene is required for both utilization of heme and virulence expression by *Hemophilus influenzae* type b. Infect. Immun. **62:**2470–2477.
10. **Kadner, R. J.** 1990. Vitamin B12 transport in *Escherichia coli*: energy coupling between membranes. Mol. Microbiol. **12:**2027–2033.
11. **Killmann, H., R. Benz, and V. Braun.** 1993. Conversion of the FhuA transport protein into a diffusion channel through the outer membrane of *Escherichia coli*. EMBO J. **12:**3007–3016.
12. **Lee, B. C.** 1995. Quelling the red menace: haem capture by bacteria. Mol. Microbiol. **18:**383–390.
13. **Létoffé, S., J.-M. Ghigo, and C. Wandersman.** 1993. Identification of two components of the *Serratia marcescens* metalloprotease transporter: protease SM secretion in *Escherichia coli* is TolC dependent. J. Bacteriol. **175:**7321–7328.
14. **Létoffé, S., J.-M. Ghigo, and C. Wandersman.** 1994. Iron acquisition from heme and hemoglobin by *Serratia marcescens* extracellular protein. Proc. Natl. Acad. Sci. USA **91:**9876–9880.
15. **Létoffé, S., J.-M. Ghigo, and C. Wandersman.** 1994. Secretion of the *Serratia marcescens* HasA protein by an ABC transporter. J. Bacteriol. **176:**5372–5377.
16. **Miller, J. H.** 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
17. **Nakayashiki, T., K. Nishimura, R. Tanaka, and H. Inokuchi.** 1995. Partial inhibition of protein synthesis accelerates the synthesis of porphyrin in heme-deficient mutants of *Escherichia coli*. Mol. Gen. Genet. **249:**139–146.
18. **Nau, C. D., and J. Konisky.** 1989. Evolutionary relationship between the TonB-dependent outer membrane proteins: nucleotide and amino acid sequences of the *Escherichia coli* colicin I receptor gene. J. Bacteriol. **171:**1041–1047.
19. **Neilands, J. B.** 1981. Microbial iron compounds. Annu. Rev. Biochem. **50:**715–731.
20. **Nikaido, H., and M. H. J. Saier.** 1992. Transport proteins in bacteria: common themes in their design. Science **258:**936–942.
21. **Postle, K.** 1993. TonB protein and energy transduction between membranes. J. Bioenerg. Biomembr. **25:**591–601.
22. **Sambrook, J., E. F. Fritsch, and T. E. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
23. **Schmid, B., M. Kromer, and G. E. Schulz.** 1996. Expression of porin from *Rhodopseudomonas blastica* in *Escherichia coli* inclusion bodies and folding into exact native structure. FEBS Lett. **381:**111–114.
24. **Schnaitman, C. A.** 1971. Effect of ethylenediaminetetraacetic acid, Triton X-100, and lysozyme on the morphology and chemical composition of isolated cell walls of *Escherichia coli*. J. Bacteriol. **108:**553–563.
25. **Schnaitman, C. A.** 1971. Solubilization of the cytoplasmic membrane of *Escherichia coli* by Triton X-100. J. Bacteriol. **108:**545–552.
26. **Smith, M. L., J. Paul, P. I. Ohlsson, K. Hjortsberg, and K. G. Paul.** 1991. Heme-protein fission under nondenaturing conditions. Proc. Natl. Acad. Sci. USA **88:**882–886.
27. **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.
28. **Stojiljkovic, I., and K. Hantke.** 1994. Transport of haemin across the cytoplasmic membrane through a haemin-specific periplasmic binding protein-dependent transport system in *Yersinia enterocolitica*. Mol. Microbiol. **13:**719–732.
29. **Stojiljkovic, I., J. Larson, V. Hwa, S. Anic, and M. So.** 1996. HmbR outer membrane receptors of pathogenic *Neisseria* spp.: iron-regulated, hemoglobin-binding proteins with a high level of primary structure conservation. J. Bacteriol. **178:**4670–4678.
30. **Weinberg, E. D.** 1993. The iron-withholding defense system. ASM News **59:**559–562.