Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 9876–9880, October 1994
Microbiology

Iron acquisition from heme and hemoglobin by a *Serratia marcescens* extracellular protein

(ABC transporter/heme-binding protein)

S. LÉTOFFÉ, J. M. GHIGO, AND C. WANDERSMAN

Unité de Physiologie Cellulaire, Centre National de la Recherche Scientifique, Unité de Recherche Associée 1300, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France

Communicated by Jonathan Beckwith, June 6, 1994 (received for review March 3, 1994)

ABSTRACT **Several pathogenic bacteria are able to use heme and hemoproteins as iron sources independent of siderophore production by mechanisms involving outer membrane heme-binding proteins and heme transport systems. Here we show that *Serratia marcescens* has such a property and we identify an extracellular heme-binding protein, HasA (for *h*eme *a*cquisition *s*ystem), allowing the release of heme from hemoglobin. This protein is secreted by *S. marcescens* under conditions of iron depletion and is essential for heme acquisition.**

Free soluble iron is unavailable at the level required for growth of microorganisms (1). Most bacteria excrete low molecular weight iron chelators called siderophores, with very high affinity for iron—able, for example, to pick up iron from host carriers (2). Besides siderophores, many bacterial pathogens have other ways to acquire iron. They can acquire it from iron–transferrin complexes by a direct interaction with a specific outer membrane receptor (3). In the animal host, iron is also present in heme-containing compounds such as heme, hemopexin, and hemoglobin, the most important heme reservoir being intracellular hemoglobin. This potential iron source becomes available when erythrocytes are lysed by hemolysins produced by various pathogenic bacteria during invasion.

Outer membrane receptors for hemoproteins have been identified in some pathogens (4, 5), suggesting that the selective recognition of heme-containing compounds occurred at the cell surface. However, the involvement of extracellular proteins in heme uptake was not proposed.

In the present work we identify HasA (for *h*eme *a*cquisition *s*ystem), an extracellular protein secreted by *Serratia marcescens* under conditions of iron depletion. This unusual extracellular protein lacks an N-terminal signal peptide and is secreted from transformed *Escherichia coli* by a previously identified *S. marcescens* transporter that is highly homologous to the metalloprotease transporters found in *Erwinia chrysanthemi* and *Pseudomonas aeruginosa* and that promotes also in *E. coli* the secretion of the *S. marcescens* metalloprotease (6). It belongs to a well-characterized family of translocators called ABC transporters (7).

We show here that *S. marcescens* is able to use heme and hemoglobin as iron sources, whereas a *hasA* mutant cannot, and that anti-HasA antibodies specifically prevent this process. We show that HasA is a heme-binding protein involved in the release of heme from hemoglobin. This study demonstrates the role of an extracellular protein in heme acquisition.†

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* C600 (F⁻ *thr leu fhuA lacY rpsL supE*) and POP3 (*araD139* Δ*lac-169 rpsL relA thi*) are from our laboratory collection. pUC18, pBGS18, pSYC1, pSYC34, and pSYC134 are described in ref. 6.

Media. LB medium was described previously (8). LBD medium contained 0.4 mM 2.2′-dipyridyl, and LBD* medium contained 2 mM 2.2′-dipyridyl, to reduce available iron.

Extraction and Manipulation of Plasmids. Isolation of plasmids, cloning, restriction map analysis, transformation, and electroporation were carried out as described (9).

DNA Sequence Analysis. pSYC34, which carries *hasA*, $prtD^{SM}$, and $prtE^{SM}$, was subjected to unidirectional deletions generated by the exonuclease III/nuclease S1 system (9). pSYC134 DNA carrying only *hasA* was recloned in pBGS18 (see Fig. 2). DNA sequences were determined and analyzed as described (10, 11).

Protein Analysis. *E. coli* harboring various recombinant plasmids and *S. marcescens* SM365 and SM365 *hasA*::*kan* were grown at 37°C or 30°C in LBD or LB medium. Cells from overnight cultures were centrifuged for 10 min at 5000 × *g* at 4°C. The supernatants were concentrated either by precipitation with 10% trichloroacetic acid (inactive supernatants) or by ammonium sulfate precipitation (active supernatants) (12). Inactive concentrated cell extracts were prepared by boiling whole cells in SDS buffer (12). The amino acid sequence of the N terminus of HasA was determined as described (12).

Electrophoresis and Immunological Techniques. Proteins were analyzed by SDS/PAGE. Antibodies were raised in a rabbit against pure HasA protein and used in Western blot analysis (13) at a 1:2000 dilution. The other rabbit sera used have been described (14–16). For growth experiments, the sera were heated at 56°C for 10 min to inactivate complement. The sera were then centrifuged for 10 min at 10,000 × *g* to eliminate precipitates. IgG was partially purified from sera by two consecutive ammonium sulfate fractionations (45% and 40% saturation). Pellets were suspended in sterile phosphate-buffered saline (PBS) and dialyzed extensively against sterile PBS. The PBS volume added was half the initial serum volume. The IgG preparations were tested by immunodetection with the specific antigens.

Heme Affinity Chromatography. HasA protein produced by *S. marcescens* was prepared from 1 liter of an overnight culture of SM365 grown in iron-limiting conditions (LBD medium). The culture supernatant was precipitated with ammonium sulfate (80% saturation). HasA protein produced in *E. coli* was prepared from 1 liter of an overnight culture of C600(pSYC34) grown in LB medium (induction by iron-limiting conditions was not necessary because HasA synthesis and secretion were under P_{lac} promoter control). The culture supernatant was precipitated as described above. Five hundred microliters of the concentrated SM365 supernatant was mixed with 500 μl of heme agarose and 80 μl of concentrated C600(pSYC34) supernatant was mixed with 100 μl of heme agarose, as described (17). The heme-agarose contained 4 μmol of ligand per ml. The mixtures were

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

†The sequence reported in this paper has been deposited in the GenBank database (accession no. X81195).

incubated at 37°C for 1 hr and, then centrifuged for 3 min at 3000 × g. The supernatants were electrophoresed in an SDS/15% polyacrylamide gel. The pellets were washed seven times (17). The pellets were suspended in 100 μl of SDS sample buffer and boiled for 5 min. The affinity gel was pelleted at 3000 × g for 5 min and the eluted proteins were electrophoresed in an SDS/15% polyacrylamide gel.

Nondenaturing PAGE. HasA or albumin (10 μg) was incubated at room temperature for 30 min with various concentrations of heme or hemoglobin. The mixtures were loaded onto a 14% polyacrylamide gel and were electrophoresed in the absence of SDS. The protein bands complexed with heme took on a brown color. The color was fixed in 10% acetic acid. The gel was then stained with Coomassie blue to show the HasA, hemoglobin, and albumin bands.

Iron Supply Assays. Growth of *E. coli* carrying various plasmids and *S. marcescens* SM365 and SM365 *hasA*::*kan* was determined in liquid cultures at 30°C. 2.2′-Dipyridyl (2 mM) was added to LB medium to chelate free iron (LBD*). Since dipyridyl also chelates other essential metals, LBD* was supplemented with a mixture of magnesium, manganese, zinc, copper, and cobalt salts at 0.1 mM each. This medium did not support bacterial growth unless iron was added at >2 mM. This medium was used unsupplemented or containing iron (2 mM $FeSO_4$), or hemoglobin (20 mg/ml), or heme (10 mM). Overnight cultures of *S. marcescens* were grown in LBD to induce HasA secretion. Overnight cultures of *E. coli* POP3(pSYC34) and POP3(pSYC34, pSYC1) were grown in LB medium. POP3 was used in these experiments because it was more resistant to iron-chelating agent. pUC18 and pAM238 are the vectors corresponding to pSYC34 and pSYC1 without foreign DNA inserts. The various cultures were diluted in all the media to ≈10^5 bacteria per ml. The cell density was determined just after dilution by plating on LB agar and counting the colonies that formed. After 24 hr at 30°C, the bacteria were again assayed on LB agar. For the mixed-culture growth experiments, *S. marcescens* SM365 and the *hasA*::*kan* mutant were grown overnight separately in LBD. The two cultures were then diluted and mixed in all the media at ≈10^5 bacteria per ml. The cultures were then handled as described above. The cell density of the mutant was determined by plating the mixtures on LB agar containing kanamycin, which allows the growth of only the mutant *hasA*::*kan*.

For growth experiments in the presence of either rabbit immune sera or partially purified IgG, *S. marcescens* SM365 was grown overnight in LBD. The culture was diluted in a medium constituted by equal volumes of LBD* with metal salts as above and serum or PBS containing IgG. In these media, the 2.2′-dipyridyl concentration (1 mM) and the metal salts concentration (0.05 mM) were sufficient to completely stop growth in the absence of added iron. These media, named LBD* metals 1/2, were used either as is or supplemented with iron, heme, or hemoglobin at the same concentrations as above. The various SM365 cultures were then handled as above. After the growth period, antibodies present in the culture supernatants were tested by immunodetection with the specific antigens. They were active at the same dilution as before the growth period, indicating that they had not been extensively degraded.

Marker Exchange in the *hasA* Gene. The SM365 *hasA*::*kan* mutant was obtained by inserting a *Bam*HI adaptor at the *Dra* III site in *hasA* in pSYC34. A selective marker, the kanamycin Genblock (Pharmacia), was introduced into this unique new *Bam*HI site in pSYC34. This plasmid was shown to be unable to direct HasA synthesis or secretion in *E. coli*. The mutated plasmid was introduced into *S. marcescens* by electroporation with ampicillin and kanamycin selection. Cells in which a homologous recombination between the *hasA*::*kan* gene and the *S. marcescens* chromosome had occurred were selected by introducing a second plasmid, pUCcat, incompatible with the first one and carrying another antibiotic-resistance marker (18). The recombination frequency was enhanced by 15 sec of UV irradiation at 0.49 W/m^2. After 20 generations of growth with simultaneous selection for kanamycin and chloramphenicol resistance, ampicillin-sensitive colonies were obtained. One such *hasA*::*kan* mutant was cured of the pUCcat plasmid by growth without chloramphenicol and was kept for further experiments. Aliquots of total DNA from SM365 and SM365 *hasA*::*kan* were digested with various restriction endonucleases and analyzed by Southern gel transfer and hybridization (19). Two DNA probes were labeled with [α-^{32}P]dCTP (Amersham, 6000 Ci/mmol, 1 Ci = 37 GBq) by using a random priming kit. The probes used were the 1.3-kb kanamycin-resistance gene block and the 941-bp DNA insert of pSYC134. Analysis of restriction fragments which hybridized with the two probes indicated that SM365 contained only one copy of *hasA*, that SM365 *hasA*::*kan* contained only one kanamycin cassette insertion, and that the mutated *hasA*::*kan* gene had replaced in SM365 the wild-type *hasA* gene (data not shown).

RESULTS

An Extracellular Protein, HasA, Is Encoded by a Gene Contiguous to *prtD*^{SM} and *prtE*^{SM}, Whose Products Are Constituents of the Specific HasA Transporter. We previously cloned a *S. marcescens* 4.2-kb DNA fragment coding for two inner membrane proteins—PrtD^{SM} (a membrane ATPase) and PrtE^{SM}, both required for the secretion of the *S. marcescens* metalloprotease (PrtSM) in *E. coli* via a signal peptide-independent pathway (6, 16). In addition, this 4.2-kb insert encodes a third protein, HasA, of apparent molecular mass 19 kDa, which was secreted in large amounts (Fig. 1). The *hasA* structural gene is adjacent to *prtD*^{SM}, and HasA secretion requires the transporter encoded by *prtD*^{SM} and *prtE*^{SM} (Fig. 1 and ref. 22).

***hasA* Nucleotide Sequence.** The nucleotide sequence of *hasA* was determined (Fig. 2). The predicted HasA protein

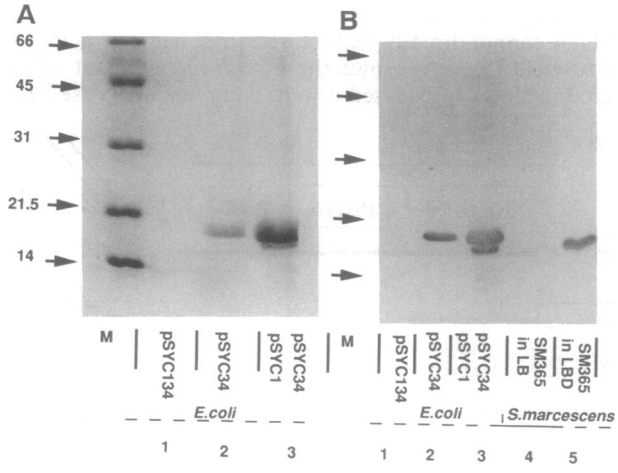

FIG. 1. Analysis of proteins secreted by *S. marcescens* and *E. coli* carrying pSYC34, pSYC134, or pSYC34 and pSYC1. *S. marcescens* strain SM365 was grown at 30°C in either LB (lane 4) or LBD (lane 5) medium. Strain C600 carrying pSYC134 (lanes 1), pSYC34 (lanes 2), or pSYC34 and pSYC1 (lanes 3) was grown at 37°C in LB medium. Inactive concentrated supernatants were prepared. Proteins were separated by SDS/15% PAGE and stained with Coomassie blue stained (*A*) or immunodetected with anti-HasA antibodies (*B*) after transfer to nitrocellulose membrane. The amounts loaded are expressed as OD equivalents of concentrated supernatants: in *A*, lanes 1–3 were loaded with 5, 0.5, and 2 OD equivalents; in *B*, lanes 1–3 were loaded with 1, 0.5, and 0.5 OD equivalents, lane 4, with 5 OD equivalents, and lane 5, SM365 with 3 OD equivalents.

has 188 aa with a calculated molecular mass of 19,271 Da. The *hasA* gene, preceded by a potential ribosome binding site, does not have a 5′ sequence resembling a signal sequence. Moreover, the amino-terminal 11 aa of purified HasA matched the N-terminal amino acid sequence deduced from the open reading frame (Fig. 2). This confirms that the protein found in the supernatant is indeed encoded by the *hasA* gene and lacks a signal peptide.

Both PrtSM and HasA lack a signal peptide and they use the same transporter in *E. coli*. However, these two proteins do not share any sequence homology. Extensive searching in protein sequence data bases did not reveal significant similarities of HasA with other proteins.

HasA Production in *S. marcescens*. To get clues about HasA function, we looked for HasA secretion in *S. marcescens*. Among various growth conditions tested, only iron-limiting medium led to HasA immunodetection in the extracellular medium (Fig. 1). The HasA polypeptide found in *S. marcescens* supernatants was 17 kDa by SDS/PAGE, compared with 19 kDa for the polypeptide secreted by *E. coli*. The size difference was shown to result from processing in the extracellular medium by proteolytic cleavage in the C terminus of HasA, catalyzed by *S. marcescens* proteases (Fig. 1 and ref. 22). Processing also took place in *E. coli* supernatants when the recombinant strain, in addition to pSYC34, carried plasmid pSYC1, encoding exoprotease PrtSM (Fig. 1). The iron regulation of HasA could indicate a role of HasA in iron uptake. To test this hypothesis, we constructed a *S. marcescens hasA* mutant with an insertional inactivation of the chromosomal *hasA* gene.

Use of Heme and Hemoglobin as Iron Sources by *S. marcescens* SM365 and SM365 *hasA*::*kan*. The *hasA* gene was disrupted as described in *Materials and Methods*. The *S. marcescens* wild-type strain was able to grow under iron-limiting conditions in the presence of hemoglobin or heme as sole iron source (with a doubling time of about 3 hr). In contrast, the *S. marcescens hasA*::*kan* mutant did not grow in the presence of heme or hemoglobin. This result strongly suggests that HasA is necessary for the utilization of heme and hemoglobin iron (Table 1).

HasA cannot directly extract iron from heme or hemoglobin to yield free iron, since the *E. coli* recombinant strains secreting HasA (processed or not) were unable to use heme or hemoglobin as iron source even though they could use free iron (Table 1). More likely, HasA may bind free heme or release heme from hemoglobin and deliver it to the cells; the lack of growth of HasA-producing *E. coli* may reflect the absence of heme transport proteins.

The inability of the SM365 *hasA*::*kan* mutant to use heme or hemoglobin could result from an indirect effect of the *hasA* disruption, since the *kan* insertion had a polar effect on the downstream genes $prtD^{SM}$ and $prtE^{SM}$ in *E. coli* and in *S. marcescens* as demonstrated by the loss of PrtSM secretion in an *E. coli* strain carrying pSYC34 *hasA*::*kan* and pSYC1, which encodes the PrtSM, and by the absence of HasA secretion in the *S. marcescens* mutant *hasA*::*kan* complemented by pSYC134 (data not shown). Thus, it cannot be excluded that this operon contains a gene required for heme uptake.

The *hasA*::*kan* Mutation Inactivates Other Functions Involved in Heme Uptake. Since HasA is an extracellular protein, it was possible to test whether the lack of HasA was the only impediment to heme and hemoglobin utilization by the SM365 *hasA*::*kan* mutant. Mixed cultures of SM365 and SM365 *hasA*::*kan* were carried out in low-iron medium with heme or hemoglobin as iron source. The *hasA*::*kan* mutant did not grow in the presence of the SM365 strain, which secretes HasA into the extracellular medium (Table 1). This indicates that the simple addition of HasA was not sufficient to restore heme utilization, suggesting that the *kan* insertion inactivates other genes required for this process. Consequently, this experiment did not allow us to come to a conclusion about a direct role of HasA in heme uptake. We therefore tested whether the neutralization of HasA activity in the extracellular medium had an effect on heme and hemoglobin utilization.

Effect of Polyclonal Antibodies Directed Against HasA on the Use of Heme and Hemoglobin as Iron Sources. An overnight culture of SM365 grown in LBD medium was diluted in medium containing complement-depleted polyclonal antiserum partially purified IgG suspended in PBS. These media did not support bacterial growth whether the serum or the IgG preparation was used, indicating that they do not contain free iron, heme, or hemoglobin at concentrations high enough to allow bacterial growth. When supplemented with iron, all these media supported bacterial growth, indicating that the

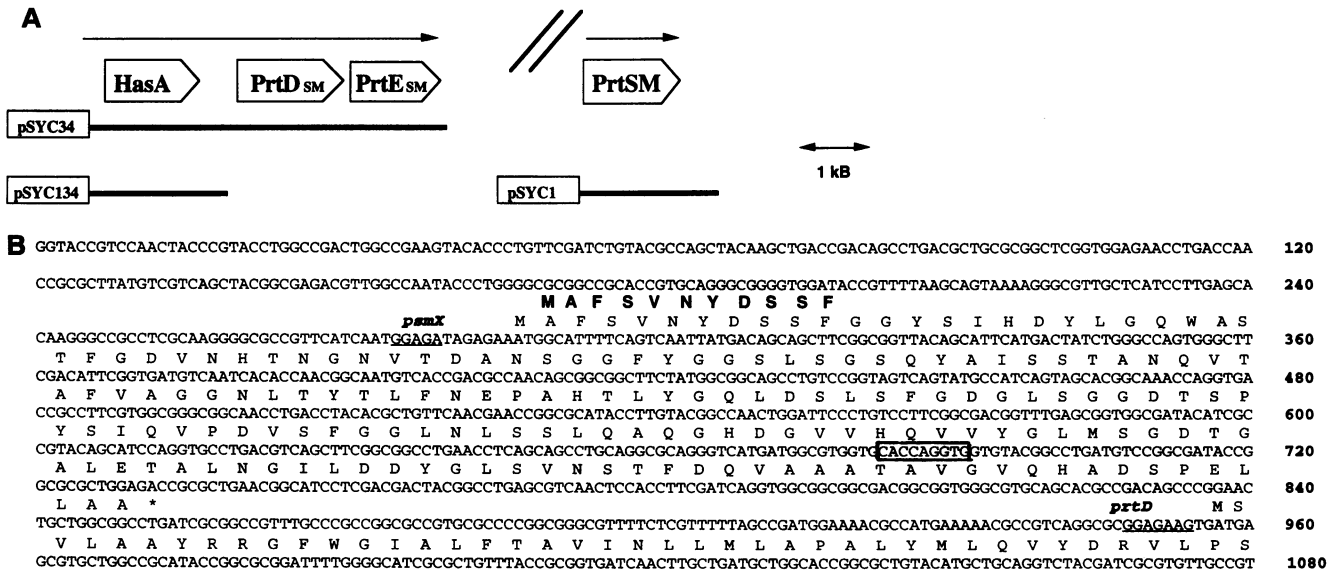

FIG. 2. (*A*) Restriction map of pSYC34 DNA insert of 4208 bp and location of the *hasA*, $prtD^{SM}$, and $pstE^{SM}$ genes. Arrow above pSYC34 indicates the direction of transcription. (*B*) Nucleotide and predicted amino acid sequences of *hasA*. The nucleotide sequence of the putative ribosome binding site is underlined. The *Dra* III site used to introduce the *kan* cassette is boxed. The N-terminal 10 aa of the purified HasA protein determined by the Edman technique (20) are shown above the deduced amino acid sequence.

Table 1. Growth of *S. marcescens* and *E. coli* carrying various plasmids in medium with heme or hemoglobin as iron source

Bacteria	No addition	+ iron	+ hemoglobin	+ heme
S. marcescens				
SM365 (wild type)	1	10^3	10^2	10^2
SM365 *hasA*::*kan*	1	10^3	1	1
E. coli				
POP3(pUC18)	1	10^3	1	1
POP3(pUC18, pAM238)	1	10^3	1	1
POP3(pSYC34)	1	10^3	1	1
POP3(pSYC34 + pSYC1)	1	10^3	1	1
Mixed cultures				
SM365 plus	NT	NT	NT	NT
SM365 *hasA*::*kan*	NT	NT	1	1

Overnight cultures grown in LBD medium were diluted in the various LBD* media as described in *Materials and Methods*. After 24 hr of growth, the bacteria were again assayed on LB agar. For the mixed cultures the bacteria were assayed on LB agar containing kanamycin. The values for the increase in colony-forming units (cfu)/ml correspond to the ratio between the cell density after 24 hr of growth and the initial cell density. Each experiment was reproduced five times and the average ratios are given. NT, not tested.

complement-depleted sera do not have bactericidal activity. Polyclonal serum directed against pure HasA as well as partially purified anti-HasA IgG inhibited specifically the growth of SM365 in the medium supplemented with heme or hemoglobin. The growth inhibition was not observed with all the other sera or IgG preparations tested containing polyclonal antibodies directed against *E. coli* or *E. chrysanthemi* proteins or directed against PrtSM, another extracellular protein secreted by *S. marcescens* SM365. The growth inhibition by the specific anti-HasA serum or IgG was not complete but was significant when compared with the growth observed in the presence of the other sera (Table 2). Thus, HasA is directly involved in the process of heme and hemoglobin utilization as iron sources. We therefore tested whether HasA protein could bind heme or hemoglobin *in vitro*.

HasA Is a Heme-Binding Protein. The retention of HasA on heme-agarose was tested by using a concentrated culture supernatant of *S. marcescens* grown under iron-starvation conditions. HasA was indeed retained by this affinity chromatography (Fig. 3) and was absent in the various wash fractions, indicating that it remained attached to heme-agarose even in the presence of detergent or high-salt buffers (data not shown). Similar results were obtained with concentrated culture supernatants of *E. coli* producing either the unprocessed or the processed form of HasA (Fig. 3). Serum albumin, which binds heme with low affinity, did not bind the resin in the high-ionic-strength buffer used in these experiments, showing that HasA retention was not due to nonspecific ionic interactions (data not shown). In addition, the interaction of HasA with heme-agarose was prevented by preincubation of the HasA preparation with heme or with hemoglobin. In contrast, no binding inhibition was observed after preincubation of HasA with protoporphyrin IX, the immediate precursor of heme (Fig. 3), or with lactoferrin, a non-heme iron-containing protein (data not shown).

These results demonstrate that HasA is a heme-binding protein. To test whether HasA could acquire heme from hemoglobin *in vitro*, we set up a nondenaturing polyacrylamide gel system in which one can see complexes between heme and the apoprotein. This system allowed protein separation without dissociating heme from the proteins. Bound heme produced a brown stain of the polypeptide bands in the gel (Fig. 4). Staining of HasA was observed after incubation with either heme or hemoglobin. Albumin, used as control in similar experiments, was stained after preincubation with heme at higher concentrations and not at all with hemoglobin. HasA is thus able to bind heme and to take it up from hemoglobin, and it is likely that this function makes HasA indispensable for bacterial utilization of heme and hemoglobin iron.

Table 2. Growth of SM365 wild-type strain in medium containing heme or hemoglobin as iron source in the presence of various polyclonal sera or partially purified IgG

Antibodies	No addition	+ iron	+ hemoglobin	+ heme
Anti-PhoA	1	10^4	10^4	2×10^3
Anti-PrtG	10	NT	5×10^4	3×10^3
Anti-PrtSM	3	NT	2×10^4	10^3
Anti-HasA	1	10^4	5×10^2	50

An overnight culture of SM365 grown in LBD medium was diluted in the various LBD* metals 1/2 media (see *Materials and Methods*) and handled as for Table 1. The following antibodies were tested: serum raised against *E. coli* alkaline phosphatase (PhoA), serum and partially purified IgG raised against *E. chrysantthemi* protease G (PrtG), serum raised against *S. marcescens* protease SM (PrtSM), and serum and partially purified IgG raised against *S. marcescens* protein HasA. Most of the experiments were reproduced three times and the average ratios are given. NT, not tested.

DISCUSSION

We have identified an iron-regulated extracellular protein, HasA, secreted by *S. marcescens*. The protein lacks a typical N-terminal signal peptide and is secreted by a *S. marcescens* ABC transporter. However, HasA does not share any sequence homology with other proteins secreted by this pathway or with any known protein.

S. marcescens can use heme and hemoglobin as iron sources whereas the *hasA* mutant cannot. This suggests that HasA is required for this function. However, since *hasA* disruption had a polar effect on downstream genes, the lack of utilization of heme or hemoglobin iron could be an indirect effect of *hasA* inactivation. To test this eventuality, the SM365 *has*::*kan* strain was provided with extracellular HasA by growing the

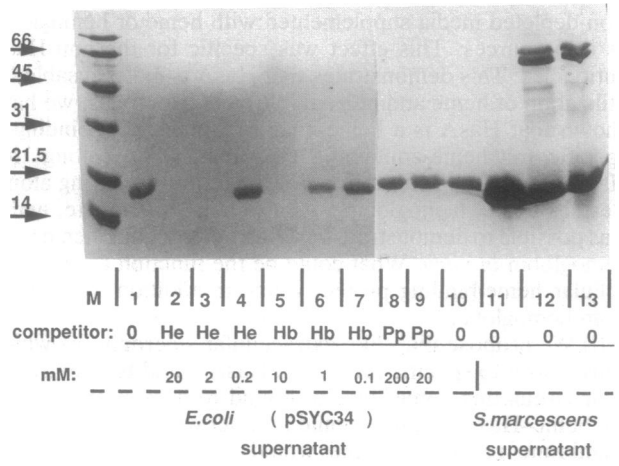

FIG. 3. Isolation of HasA by heme affinity chromatography and binding specificity as measured by competition experiments. Concentrated supernatant proteins were incubated with heme-agarose as described in *Materials and Methods*. Lane 1, *E. coli*(pSYC34) concentrated supernatant proteins retained on heme-agarose alone; lanes 2–4, in the presence of 20, 2, and 0.2 mM heme (He); lanes 5–7, in the presence of 10, 1, and 0.1 mM hemoglobin (Hb); lanes 8 and 9, in the presence of 200 and 20 mM protoporphyrin IX (Pp); lane 10, the same sample as in lane 1; lane 11, SM365 concentrated supernatant proteins retained on heme-agarose; lane 12, SM365 supernatant fraction not retained on heme-agarose; lane 13, proteins of the SM365 concentrated supernatant.

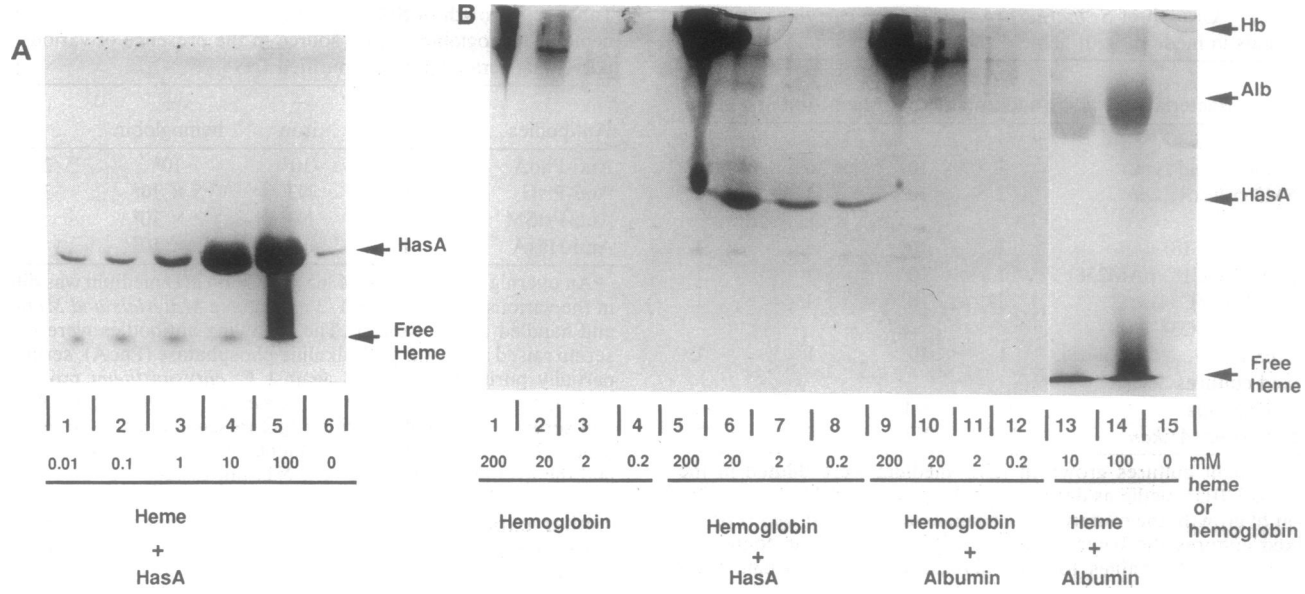

FIG. 4. Complex formation between heme and HasA or albumin. HasA or albumin (10 μg) was incubated at room temperature for 30 min with various concentrations (mM) of heme or hemoglobin. The mixtures were loaded onto a 14% polyacrylamide gel and electrophoresed in the absence of SDS. The protein bands complexed with heme took on a brown color after fixation in 10% acetic acid. Coomassie-stained HasA, hemoglobin (Hb), and albumin (Alb) bands are indicated by arrows.

mutant in the presence of the wild-type SM365 strain, which secretes HasA. The absence of growth recovery showed that the mutant lacks other functions required for utilization of heme or hemoglobin iron. Since the SM365 *hasA*::*kan* mutant was shown to carry a unique *kan* insertion in *hasA*, it indicated that the insertion exerted a polar effect on genes required for heme and hemoglobin utilization. Hence, *hasA* is localized in an operon involved in heme acquisition. In addition the *hasA* gene product was shown to be directly required for heme and hemoglobin acquisition by testing the HasA-neutralizing activity of the anti-HasA serum. The addition of anti-HasA serum or IgG to SM365 cultures grown in medium supplemented with heme or hemoglobin as iron sources significantly reduced bacterial growth. This effect was observed only in iron-depleted media supplemented with heme or hemoglobin as iron sources. This effect was specific for the anti-HasA antibodies. This demonstrates that HasA is indispensable for utilization of heme and hemoglobin iron. Moreover, we have shown that HasA is a heme-binding protein. The binding is specific for heme-containing compounds, suggesting that HasA recognizes neither iron nor the tetrapyrrole ring alone. Besides heme, hemoglobin was also able to compete, and it was possible to demonstrate that HasA can acquire heme from hemoglobin *in vitro*. What could be the function of an extracellular heme-binding protein which in addition traps heme from hemoglobin?

(*i*) We propose that, like extracellular siderophores which deliver iron to specific receptors, HasA could bind extracellular circulating heme, free or bound to hemoproteins, and the heme–HasA complex could interact with a specific outer membrane receptor. Heme would be delivered solely to bacteria which have such a receptor. We speculate that *S. marcescens* may possess a specific outer membrane receptor for the heme HasA complex.

(*ii*) Free heme, like iron, is restricted. In plasma, hemoglobin released from erythrocytes by hemolysis or tissue damage binds rapidly and quite irreversibly with haptoglobin and forms stable complexes which are very rapidly internalized by liver cells, where they are degraded (21). HasA could trap heme from hemoglobin before it forms a complex with haptoglobin. Although *S. marcescens* is only an opportunistic pathogen (20), it is likely that similar proteins are secreted by some virulent hemolytic bacteria, thereby dramatically aggravating the microbial infection.

We thank O. Raibaud, P. Delepelaire, R. D'Ari, D. Mattei, and K. Hinterberg for helpful discussions, R. D'Ari and A. Ullmann for critical reading of the manuscript, and M. Schwartz for constant interest in this work.

1. Weinberg, E. D. (1978) *Microbiol. Rev.* **42**, 45–60.
2. Neilands, J. B. (1981) *Annu. Rev. Biochem.* **50**, 715–731.
3. Williams, P. & Griffiths, E. (1992) *Med. Microbiol. Immunol.* **181**, 301–322.
4. Stojiljkovic, J. & Hantke, K. (1992) *EMBO J.* **11**, 4359–4367.
5. Henderson, D. P. & Payne, S. M. (1994) *J. Bacteriol.* **176**, 3269–3277.
6. Létoffé, S., Ghigo, J. M. & Wandersman, C. (1993) *J. Bacteriol.* **175**, 7321–7328.
7. Higgins, C. F. (1992) *Annu. Rev. Cell Biol.* **8**, 67–113.
8. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab. Press, Plainview, NY).
9. Sambrook, J., Fritsch, F. & Maniatis, T. E. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
10. Biggin, M. D., Gibson, T. J. & Hong, G. F. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 3963–3968.
11. Lipman, D. J. & Pearson, W. R. (1985) *Science* **227**, 1435–1441.
12. Delepelaire, P. & Wandersman, C. (1989) *J. Biol. Chem.* **264**, 9083–9089.
13. Wandersman, C., Delepelaire, P., Létoffé, S. & Schwartz, M. (1987) *J. Bacteriol.* **169**, 5046–5053.
14. Létoffé, S., Delepelaire, P. & Wandersman, C. (1989) *Mol. Microbiol.* **3**, 79–86.
15. Ghigo, J. M. & Wandersman, C. (1994) *J. Biol. Chem.* **269**, 1–7.
16. Létoffé, S., Delepelaire, P. & Wandersman, C. (1991) *J. Bacteriol.* **173**, 2160–2166.
17. Tsutsui, K. & Mueller, G. C. (1982) *Anal. Biochem.* **121**, 244–250.
18. Ruvhkun, G. B. & Ausubel, F. M. (1981) *Nature (London)* **289**, 85–88.
19. Southern, E. M. (1975) *J. Mol.* **98**, 503–517.
20. Grimont, F. & Grimont, P. A. D. (1992) in *The Prokaryotes*, eds. Balow, A., Trüper, H. G., Dworkin, M., Harder, W. & Schleifer, K. H. (Springer, Berlin), pp. 2822–2848.
21. Takami, M. (1993) *J. Biol. Chem.* **268**, 20335–20342.
22. Létoffé, S., Ghigo, J. M. & Wandersman, C. (1994) *J. Bacteriol.* **176**, in press.