JOURNAL OF BACTERIOLOGY, Aug. 2000, p. 4401–4405 Vol. 182, No. 16
0021-9193/00/$04.00+0

Functional Characterization of the $HasA_{PF}$ Hemophore and Its Truncated and Chimeric Variants: Determination of a Region Involved in Binding to the Hemophore Receptor

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Received 2 March 2000/Accepted 29 May 2000

Hemophores are secreted by several gram-negative bacteria (*Serratia marcescens*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Yersinia pestis*) and form a family of homologous proteins. Unlike the *S. marcescens* hemophore ($HasA_{SM}$), the *P. fluorescens* hemophore $HasA_{PF}$ has an additional region of 12 residues located immediately upstream from the C-terminal secretion signal. We show that $HasA_{PF}$ undergoes a C-terminal cleavage which removes the last 21 residues when secreted from *P. fluorescens* and that only the processed form is able to deliver heme to the *S. marcescens* outer membrane hemophore-specific receptor, $HasR_{SM}$. Functional analysis of variants including those with an internal deletion of the extra C-terminal domain show that the secretion signal does not inhibit the biological activity, whereas the 12-amino-acid region located upstream does. This extra domain may inhibit the interaction of the hemophore with $HasR_{SM}$. To localize the hemophore regions involved in binding to HasR, chimeric $HasA_{PF}$-$HasA_{SM}$ proteins were tested for biological activity. We show that residues 153 to 180 of $HasA_{PF}$ are necessary for its interaction with the receptor.

Bacteria have diverse high-affinity heme uptake systems for the various heme sources that they might encounter (3, 15). One such system is dependent on hemophores which bind heme with high affinity and fulfill a function similar to that of siderophores (2): they are secreted into the extracellular medium, where they scavenge free or protein-bound heme and then deliver it to a specific cell surface receptor. The *Serratia marcescens* hemophore-dependent heme acquisition system has been reconstituted in *Escherichia coli* (5). Exogenously added *S. marcescens* hemophore ($HasA_{SM}$) increases the efficiency of heme uptake via the specific receptor ($HasR_{SM}$) and also makes available new heme sources (such as hemopexin and myoglobin) which are not recognized by HasR alone (S. Létoffé et al., unpublished results). The *S. marcescens* hemophore is a monomer which binds heme with a stoichiometry of 1 and an affinity lower than 10^{-9} M (8). The crystal structure of holoprotein has been solved and found to consist of a single module with two residues in interaction with heme (1). Both heme-free and heme-loaded hemophores bind to HasR with similar apparent affinities (10^{-10} M), indicating direct protein-protein interactions (10).

HasA-type hemophores are found in *S. marcescens*, *Pseudomonas aeruginosa* (11), *Pseudomonas fluorescens* (7), and *Yersinia pestis* (J. M. Ghigo, personal communication) and form a family of homologous proteins which do not share extensive similarity with any other known proteins. They are secreted by ABC transporters in a Sec-independent process (14). Like most proteins using this pathway, they do not have an N-terminal signal sequence but rather have an uncleavable C-terminal secretion sequence consisting of at least the last 15 residues. This extreme C-terminal signal contains one or two negatively charged residues followed by a hydrophobic stretch (6). It is unstructured and highly accessible to the solvent (16).

P. aeruginosa, *P. fluorescens*, and *S. marcescens* hemophores are secreted from *E. coli* by their reconstituted ABC transporters. However, they have apparent molecular weights higher than that of the proteins secreted by their natural hosts. Mass spectrometry has shown a single cleavage of the hemophore secreted from *S. marcescens* which removes the last 12 residues (9) and multiple cleavages of that from *P. aeruginosa* which remove 15 to 21 residues (11). C-terminal cleavage is not required for secretion and presumably occurs in the extracellular medium, a result of the activity of extracellular proteases produced by *S. marcescens* and *P. aeruginosa* but not by *E. coli*. Both uncleaved and cleaved $HasA_{SM}$ and *P. aeruginosa* HasA ($HasA_{PA}$) bind heme and can acquire heme from hemoglobin. Both forms of $HasA_{SM}$ can deliver heme to the *S. marcescens* outer membrane receptor $HasR_{SM}$ in *E. coli*, allowing heme uptake. In a similar test performed with Has_{PA}, we found that the recombinant form of $HasA_{PA}$ (uncleaved) cannot deliver heme to $HasR_{SM}$ whereas the cleaved form can. $HasA_{PA}$ has an additional region of 14 residues close to the C terminus not found in $HasA_{SM}$ but which is removed in the processed form (11). We suggested that this additional domain close to the C terminus could inhibit heme delivery by preventing a direct interaction between $HasR_{SM}$ and $HasA_{PA}$. However, the occurrence of multiple cleavages of $HasA_{PA}$ in *P. aeruginosa* and the difficulty of separating the different processed forms complicate the study of the interaction between $HasA_{PA}$ and $HasR_{SM}$.

Here, we found that $HasA_{PF}$ has properties very similar to those of $HasA_{PA}$: it is cleaved when secreted from *P. fluorescens*; both the processed and unprocessed forms bind heme, whereas only the processed form can deliver heme to $HasR_{SM}$. However, unlike $HasA_{PA}$, it undergoes a single cleavage in *P. fluorescens*. This prompted us to construct variants of $HasA_{PF}$

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and $HasA_{PF}$-$HasA_{SM}$ chimeras and to study their biological activity and binding to $HasR_{SM}$ to localize hemophore domains involved in binding to $HasR_{SM}$.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* C600 (F^- *thr leu fhuA lacY thi supE*) and *E. coli* POP 3 *hemA* (*araD139* Δ*lacU169 rpsL relA thi hemA*) were from the laboratory collection. *P. fluorescens* 33 was a gift from Haruo Kumura. pR10K carries $hasR_{SM}$ on pBGS18 (5). Plasmid pSYC134 is pUC18 carrying $hasA_{SM}$, and pSYC150 is pACYC184 carrying *hasD* and *hasE* (11). pFXF-HasA carries $hasA_{PF}$, pFXM-HasA encodes the $HasA_{PF}$1-153-$Cter_{SM}$148-188, polypeptide, and pFBΔF-HasA encodes the $HasA_{PF}$Δ181-192 polypeptide; they are derived from pUC18 and described in reference 7.

Plasmid constructions. pUC/$HasA_{PF}$1-180-$Cter_{SM}$148-188 was created as follows. A DNA fragment encoding amino acid residues 1 to 180 of $HasA_{PF}$ was produced by inserting the phosphorylated synthetic oligonucleotide linkers 5′-GATCTCTGCTGGGTTGGCCTGCA-3′ and 5′-GGCCAACCCAGCAGA-3′ between the *Bgl*II and *Pst*I sites of pUC18 encoding $HasA_{PF}$, resulting in pUC/$HasA_{PF}$-PstI. The DNA fragment coding for amino acid residues 148 to 188 of $HasA_{SM}$ was generated by PCR with the oligonucleotide 5′-GGGCTGCAG AGACCGCGCTGAACGGCATC-3′, the universal primer M4 5′-GTTTTCCC AGTCACGAC-3′, and pUC/$HasA_{SM}$ as a template DNA. The amplified fragment was digested with *Pst*I and *Bam*HI and then introduced into the corresponding sites of pUC18. The *Pst*I-*Bam*HI fragment of ca. 0.1 kb was blunt ended and inserted between the *Pst*I and *Hin*dIII (blunt-ended) sites of pUC/$HasA_{PF}$-PstI. The resultant plasmid was digested with *Pst*I, treated with T4 DNA polymerase, and then ligated to destroy the *Pst*I site, generating pUC/$HasA_{PF}$1-180-$Cter_{SM}$148-188.

The plasmid encoding a chimeric HasA protein consisting of residues 1 to 180 of $HasA_{PF}$ and residues 174 to 188 of $HasA_{SM}$ was created by inserting oligonucleotides. The PCR product of ca. 0.1 kb amplified using the oligonucleotide 5′-GGGCATGCCGTGGGCGTGCAGCACGCC-3′, the universal primer M4, and a *Sph*I-disrupted pUC/$HasA_{SM}$ DNA was inserted between the *Sph*I and *Hin*dIII sites of pUC/$HasA_{PF}$. This plasmid was digested with *Bgl*II and *Sph*I and blunt ended with T4 polymerase, and the oligonucleotides 5′-CGGCCAGGCC GGCCGA-3′ and 5′-GATCTCGGCCGGCCTGGCCGCATG-3′ were inserted, generating a plasmid encoding Has_{PF}1-180-$Cter_{SM}$174-188. DNA sequencing of the hybrid genes showed that the genetic manipulations did not introduce any change in the hybrid protein amino acid sequences. DNA manipulations were carried out according to standard procedures (13).

Media. All media and antibiotics were as described by Miller (12). LBD contained 0.2 mM 2,2′-dipyridyl, to reduce iron available to *E. coli*. LBD* contained 0.4 mM 2,2′-dipyridyl to chelate iron in *P. fluorescens* cultures. Hemin, hemin-agarose, and bovine hemoglobin was obtained from Sigma Chemical Co. Bovine hemoglobin agar plates were prepared as described in reference 5.

Protein analysis. The $HasA_{PF}$ protein secreted from *E. coli* was prepared from the supernatant of an overnight culture (grown at 37°C in LBD) of C600 harboring plasmids encoding $HasA_{PF}$ (pFXF-HasA) and Has_{SM} transporter (pSYC150). The $HasA_{PF}$ protein secreted from *P. fluorescens* was prepared from the supernatant of *P. fluorescens* grown in LBD* at 23°C for 96 h. The supernatants were concentrated either by precipitation with 10% trichloroacetic acid (TCA) (inactive supernatants) or by ammonium sulfate precipitation: 60% for $HasA_{SM}$ and 80% for $HasA_{PF}$ (active supernatants).

Proteins in *P. fluorescens* active supernatant were purified by hemin-agarose affinity chromatography as described previously. The bound protein (as optical density [OD] equivalents) was eluted with 200 μl of the sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or 200 μl of 6 M guanidine-HCl. The guanidine-eluted sample was incubated for 3 h at room temperature on a wheel and then placed on an empty disposable column with mesh to recover the eluted proteins. The elute was diluted with 200 μl of 0.2 M Tris-HCl (pH 7.5) and incubated overnight at 4°C on a wheel. After dialysis against 10 mM Tris-HCl (pH 7.5), aliquots were subjected to SDS-PAGE and stained with Coomassie blue to evaluate protein concentration and purity, and other aliquots were used for further experiments.

Amino acid sequence analysis. N-terminal amino acid sequences were determined using the G1005A protein sequencing system (Hewlett-Packard).

Mass spectrometry. The spectra of positive ions were recorded in linear mode with a MALDI-TOF (matrix-assisted laser desorption–ionization time-of-flight) mass spectrometer (Voyager Elite; Perceptive Biosystems) using a saturated solution of sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) in 30% acetonitrile as matrix. External calibration was performed with apomyoglobin and trypsinogen using the protonated ions of the monomer with average *m/z* ratios of 16,952.6 and 23,983, respectively.

Electrophoresis and immunological techniques. Proteins were analyzed by SDS-PAGE followed either by Coomassie blue staining or Western blot analysis. Coimmunoprecipitations were performed as described previously (10). Anti-HasA and anti-HasR rabbit polyclonal sera, both at a 1/5,000 dilution, were used for immunodetection.

***E. coli* growth assays on LBD hemoglobin agar plates supplemented with purified HasA.** Growth stimulation of POP 3 *hemA*(pR10K) by exogenously supplied HasA was tested as follows. Cells of the HasR-producing strain were mixed with 3 ml of top agar and poured onto LBD plates supplemented with 10^{-6} M hemoglobin (Hb-LBD). Five-millimeter-diameter wells were cut in the agar and filled with 50 μl of serial dilutions of various HasA preparations. Growth around the wells was recorded after overnight incubation at 37°C (5).

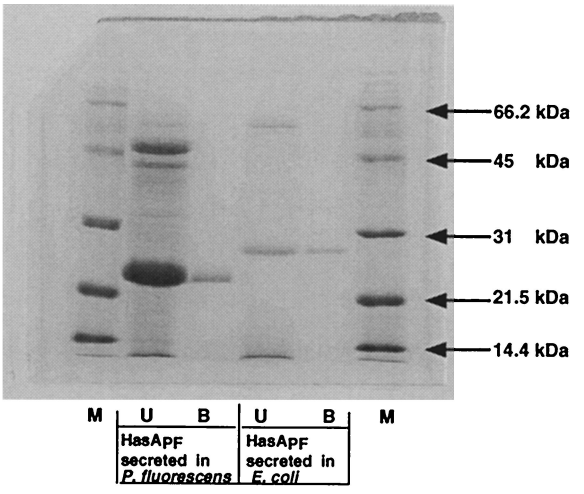

FIG. 1. Hemin-agarose chromatography of $HasA_{PF}$ secreted from *P. fluorescens* and *E. coli*. Lane 1 (M), molecular weight markers. Lane 2 (U [unbound]) was loaded with 5 OD equivalents of concentrated *P. fluorescens* PF33 supernatant not bound to hemin-agarose. Lane 3 (B [bound]) was loaded with hemin-bound material eluted by boiling in SDS-sample buffer corresponding to 3 OD equivalents of ammonium sulfate-concentrated supernatant. Lane 4 was loaded with 3 OD equivalents of concentrated supernatant of *E. coli* strain C600 (pSYC150, pFXF-HasA) not bound to hemin-agarose. Lane 5 was loaded with hemin-bound material eluted by boiling in SDS-sample buffer corresponding to 1.5 OD equivalents of ammonium sulfate-concentrated supernatant. Samples were subjected to SDS-PAGE (15% gel) followed by Coomassie brilliant blue G-250 staining.

RESULTS AND DISCUSSION

Characterization of the $HasA_{PF}$ proteins secreted by *P. fluorescens* and by *E. coli* expressing the Has_{SM} transporter. The supernatants were concentrated by TCA precipitation, resolved by SDS-PAGE, and probed with anti-$HasA_{SM}$ antibodies. One protein band was detected in each sample. The form secreted by *E. coli* exhibited a molecular weight higher than that of the form secreted from *P. fluorescens* (data not shown). To determine the origin of the difference in molecular weight, both forms (that secreted by *P. fluorescens* and that secreted by *E. coli* expressing the Has_{SM} transporter) were concentrated and purified by hemin-agarose chromatography. Both forms bound heme (Fig. 1). The recombinant form had an apparent molecular mass 3 kDa higher (Fig. 1). N-terminal amino acid sequencing of the proteins purified on hemin-agarose showed sequences (TISVSEAA) identical to the deduced amino acid sequence of $HasA_{PF}$ (amino acid residues 2 to 10). Both lacked only the first methionine at the N terminus. When analyzed by mass spectrometry, purified $HasA_{PF}$ from the *P. fluorescens* culture gave one major peak showing a molecular weight of 18,987.0. The value agreed well with the calculated molecular weight of the monoprotonated $HasA_{PF}$ composed of amino acid residues 2 to 185 (18,985.1). Thus, $HasA_{PF}$ from the *P. fluorescens* culture lacked also the 21 C-terminal residues (Fig. 2B).

Comparison of the biological activity of the recombinant and processed $HasA_{PF}$. Growth stimulation of POP3 *hemA* (pR10K) by addition of serial dilutions of concentrated $HasA_{PF}$ preparations was tested on Hb-LBD. The unprocessed form

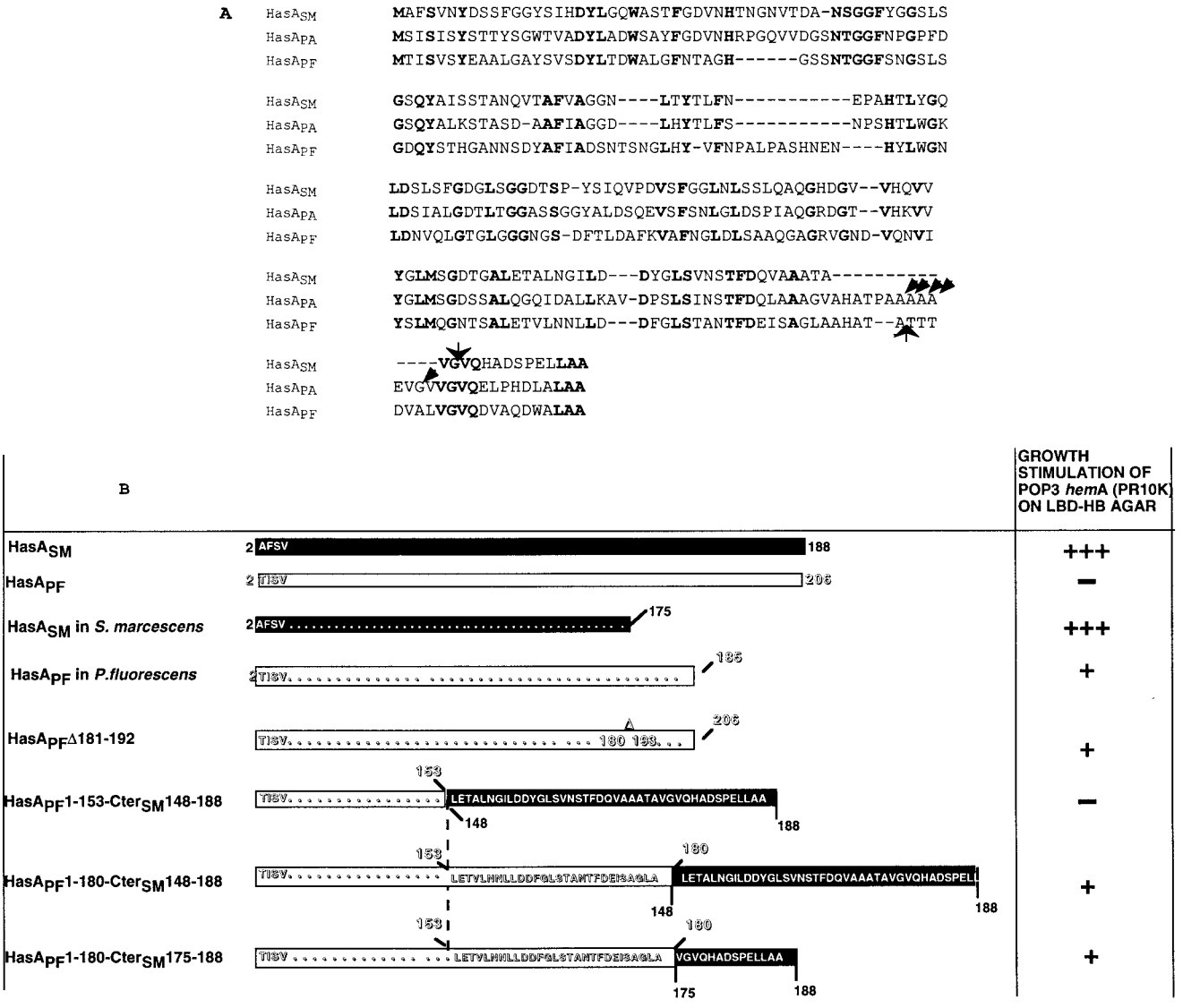

FIG. 2. Chimeric HasA polypeptides. (A) Alignment of the amino acid sequences of *S. marcescens*, *P. aeruginosa*, and *P. fluorescens* HasA proteins. The residues are numbered starting from the N-terminal methionine. However, mature $HasA_{SM}$, $HasA_{PA}$, and $HasA_{PF}$ polypeptides lack the N-terminal methionine. The C-terminal proteolytic cleavage sites in *S. marcescens*, *P. aeruginosa*, and *P. fluorescens* are indicated by arrows. Bold characters indicate identical residues in the three polypeptides. (B) Schematic representation of $HasA_{PF}$ variants and $HasA_{PF}$-$HasA_{SM}$ hybrid proteins. The solid and open boxes represent amino acid sequences of $HasA_{SM}$ and $HasA_{PF}$, respectively. The deletion of the region between residues 180 and 193 of $HasA_{PF}$ (AHATATTTDVAL) is indicated by a Δ above the corresponding box. The C-terminal amino acid sequences of the chimera are shown. The ability of each polypeptide to stimulate growth of the $HasR_{SM}$-producing strain is shown on the right. The wells contained 2 to 10 μg of the $HasA_{PF}$ wild-type and variant proteins. Similar growth stimulation was obtained with 2 to 10 ng of $HasA_{SM}$. Growth around the wells was recorded after overnight incubation at 37°C. +, bacterial growth ring of 2 mm; +++, bacterial growth ring of 5 mm; —, no growth.

did not show any growth stimulation, whereas the processed form had significant activity (Fig. 2B). This suggests that the 21 C-terminal residues interfere with the heterologous complementation. The 14 C-terminal residues of this region can promote (alone or fused to passenger proteins) efficient secretion via the Has_{SM} transporter and therefore constitute the secretion signal (7). This 14-amino-acid secretion signal is well conserved in the three hemophores $HasA_{PF}$, $HasA_{SM}$, and $HasA_{PA}$ (Fig. 2A). In contrast, the 12 amino acids in $HasA_{PF}$ (from residues 181 to 192) located just upstream from this secretion signal are absent from $HasA_{SM}$ (Fig. 2A). To determine whether the presence of this nonhomologous region was inhibiting the heterologous complementation, we tested a $hasA_{PF}$ variant (encoded by pFBΔF-HasA) consisting of an internally deleted protein in which the N-terminal 180 amino acids are fused directly to the C-terminal 14 amino acids ($HasA_{PF}$Δ181-192 [Fig. 2B]) (7). This construct lacks the extra 12-residue domain and is therefore similar to the active $HasA_{SM}$ produced by *E. coli*.

Biological activity of $HasA_{PF}$Δ181-192. The $HasA_{PF}$Δ181-192 variant was secreted by the Has_{SM} transporter (Fig. 3). Added exogenously, this protein showed the same growth stimulation of POP3 *hemA*(pR10K) as did the naturally processed protein on Hb-LBD plates (Fig. 2B). Hence, the deletion of the 12 amino acids restored biological activity in the heterologous complementation test, indicating that it is not the secretion signal per se which blocks the hemophore function.

As both the naturally processed form (at residue 185) and

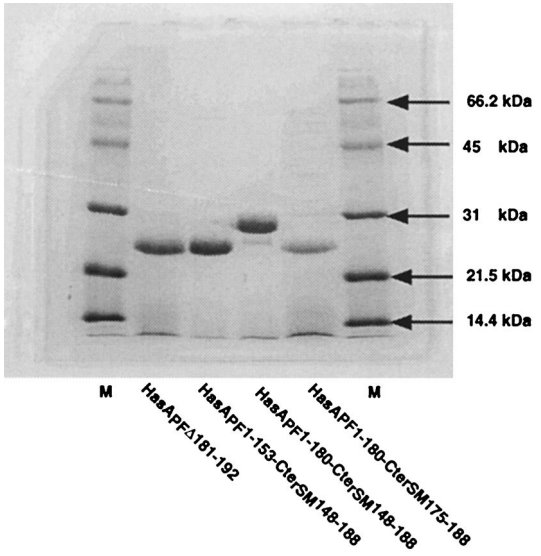

FIG. 3. Secretion of $HasA_{PF}$ chimeras and variants. C600 carrying pSYC150 and the various pUC derivatives encoding the $HasA_{PF}$ polypeptides were grown overnight. The proteins in the culture supernatants were concentrated by TCA precipitation and subjected to SDS-PAGE analysis. The gels were stained with Coomassie brilliant blue G-250. Each lane was loaded with 3 OD equivalents of concentrated supernatant.

the internally deleted variant lacking residues 181 to 192 are active, the first 180 amino acids of $HasA_{PF}$ bear all of the determinants required for heme binding and delivery to the receptor $HasR_{SM}$. To map more precisely the domain required for heme delivery to $HasR_{SM}$, we constructed chimeric $HasA_{PF}$ having either the 153 or 180 N-terminal amino acids of $HasA_{PF}$ fused to the 41-amino-acid C-terminal secretion signal of $HasA_{SM}$.

Biological activity of chimeric $HasA_{PF}$-$HasA_{SM}$ proteins. $HasA_{PF}$1-180-$Cter_{SM}$148-188 and $HasA_{PF}$1-153-$Cter_{SM}$148-188 were both efficiently secreted by *E. coli* expressing the Has_{SM} transporter (Fig. 3). Both bound heme (data not shown). Only $HasA_{PF}$1-180-$Cter_{SM}$148-188 had activity in the heterologous complementation test (Fig. 2B). The absence of biological activity of the shorter $HasA_{PF}$ hybrid could be due to its failure to interact with the receptor. As direct interactions between $HasR_{SM}$ and $HasA_{Sm}$ have been previously demonstrated by coimmunoprecipitation (10), we used this technique to determine the binding capacity of the two chimeras. Both chimeras were immunoprecipitated with a monoclonal antibody directed against the C-terminal 50 amino acids of $HasA_{SM}$. HasR was detected only in the immunoprecipitate of the longer fusion (Fig. 4). This suggests that the $HasA_{PF}$ residues 153 to 180 are required for the interaction between $HasA_{PF}$ and $HasR_{SM}$. The $Cter_{SM}$148-188 region shares 50% identity with the corresponding region of $HasA_{PF}$ (Fig. 2A). However, the inactivity of $HasA_{PF}$1-153-$Cter_{SM}$148-188 indicated that the fusion did not lead to the reconstitution of a functional binding domain. To confirm that the $Cter_{SM}$148-188 domain does not contain the elements necessary for binding, we tested the activity of a chimera with a shorter $Cter_{SM}$ of 15 amino acids.

$HasA_{PF}$1-180-$Cter_{SM}$174-188 was efficiently secreted from *E. coli* by the Has_{SM} transporter (Fig. 3) and was active for heterologous complementation (Fig. 2B). $HasR_{SM}$ was coimmunoprecipitated with this last chimera (Fig. 4).

As $HasA_{SM}$ residues 174 to 188 can be deleted without changing the properties of HasA, the activity of $HasA_{PF}$1-180-$Cter_{SM}$174-188 is clearly not dependent on the added $HasA_{SM}$ domain. Thus, residues 153 to 180 of $HasA_{PF}$ are necessary for its biological activity and in particular for its binding to the receptor. $HasA_{PF}$ shares 41% identity with $HasA_{SM}$. This degree of similarity all along the protein sequence suggests that the two proteins have similar secondary and tertiary structures (4). The last 14 residues of the $HasA_{SM}$ secretion signal are not seen in the three-dimensional structure, and nuclear magnetic resonance spectroscopy indicates that they are poorly structured (9). The region located just upstream from the secretion signal forms two α helices (residues 144 to 155 and 167 to 174) on the side of the globular HasA molecule opposite the heme pocket (1). Similarly, the corresponding region of $HasA_{PF}$, between residues 144 and 180, is also likely to be on the

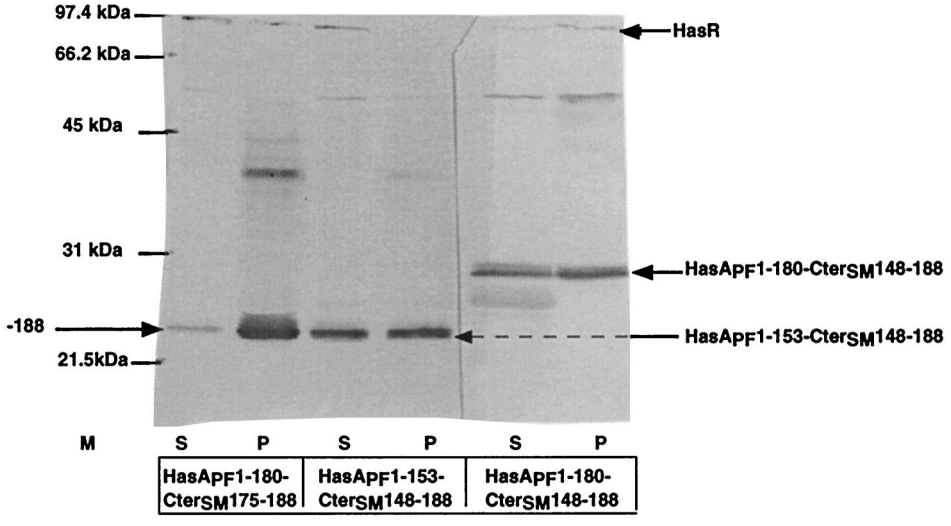

FIG. 4. Coimmunoprecipitation of HasA and HasR with a mouse monoclonal anti-Cter-HasA antibody. Western blots were probed with rabbit anti-HasA and anti-HasR antisera, both at a 1/5,000 dilution. Approximately 40 OD culture equivalents of solubilized whole membrane proteins were incubated with 10 μg of various $HasA_{PF}$ polypeptides as indicated at the bottom. S, supernatant of the immunoprecipitate (4 OD culture equivalents); P, immunoprecipitated proteins (20 OD culture equivalents).

opposite face of the protein relative to the heme pocket. Possibly, recognition of the heterologous receptor by $HasA_{PF}$ does not involve the heme binding site but rather uses a C-terminal domain of the molecule located upstream from the secretion signal.

We have identified a 12-amino-acid sequence (181 to 192) in $HasA_{PF}$ located immediately upstream from the 14 C-terminal secretion signal which is absent from $HasA_{SM}$ and which interferes with heterologous complementation (heme delivery via $HasA_{PF}$ to $HasR_{SM}$). The small size of the inhibitory region suggests that it inhibits by masking the functionally important neighboring domain (amino acids 150 to 180).

The physiological significance of the C-terminal cleavage that all three studied hemophores undergo is unclear. The unprocessed and in vitro processed forms of $HasA_{SM}$ appear to have the same biological activity when exogenously added to *S. marcescens hemA* or *hasA* mutants or to *E. coli hemA* mutants expressing $HasR_{SM}$ (data not shown). Thus, the inhibitory effect is revealed only in the heterologous systems. Cleavage of the HasA C termini when secreted from their natural hosts might simply be a consequence of their high accessibility to the extracellular proteases also produced by these species.

ACKNOWLEDGMENTS

We are grateful to Philippe Delepelaire, Jean-Marc Ghigo, Laurent Debarbieux, Pascal Arnoux, and Mirjam Czjzek for helpful discussions.

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