

Identification and Characterization of the Hemophore-Dependent Heme Acquisition System of *Yersinia pestis*

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Received 10 May 2001/Returned for modification 6 July 2001/Accepted 10 August 2001

Yersinia pestis possesses a heme-protein acquisition system (Hmu) that allows it to utilize heme and heme-protein complexes as the sole sources of iron. Analysis of the *Y. pestis* CO92 genomic sequence revealed a second heme-protein acquisition gene cluster that shares homology with the hemophore-dependent heme acquisition system (Has system) of *Serratia marcescens*. This locus consisted of the *hasR_{yp}* receptor gene, the *hasA_{yp}* hemophore gene, and genes encoding components of the HasA_{yp} dedicated ABC transporter factor (*hasDE_{yp}*), as well as a *tonB* homologue (*hasB_{yp}*). By using a reconstituted secretion system in *Escherichia coli*, we showed that HasA_{yp} is a secreted heme-binding protein and that expression of HasA_{yp} is iron regulated in *E. coli*. The use of a transcriptional reporter fusion showed that the *hasRADEB* promoter is Fur regulated and has increased activity at 37°C. Hemoglobin utilization via the Has_{yp} system was studied with both *E. coli* and *Y. pestis*, for which *has* and *has hmu* mutant strains were used. No contribution of the Has system to heme utilization was observed in either *E. coli* or *Y. pestis* under the conditions we tested. Previously it was shown that a deletion of the Hmu system had no effect on the virulence of *Y. pestis* in a mouse model of bubonic plague. An Hmu⁻ Has⁻ double mutant also retained full virulence in this model of infection. This report constitutes the first attempt to investigate the contribution of the hemophore-dependent heme acquisition system in bacterial pathogenicity.

Iron plays a central role in the metabolism of most bacteria, in which the Fe²⁺/Fe³⁺ redox pair and its wide range of electron transfer capacity are well suited to many enzymatic reactions. Despite iron abundance in nature, bacteria face an acute iron supply problem due to the extremely low concentration of soluble Fe³⁺ at physiological pH under aerobic conditions. In vertebrate hosts, iron deficiency is further increased by a set of iron-withholding defenses leading to the sequestration of most intracellular iron by ferritin or hemoproteins such as hemoglobin, whereas extracellular iron is complexed by transferrin, lactoferrin, hemopexin, and haptoglobin (5, 42).

To overcome this problem, gram-negative bacteria have evolved numerous energy-dependent iron-regulated systems to acquire iron from iron- or heme-containing compounds by three distinct acquisition strategies. These include (i) the release of small molecules with a high affinity for iron called “siderophores,” (ii) the direct binding of iron-containing protein compounds to outer membrane receptors, or (iii) the extracellular secretion of heme-binding proteins called “hemophores” that subsequently transport and deliver heme to their cognate outer membrane receptor. The coexistence of more than one of the above iron-scavenging systems is relatively common in bacteria (for review, see reference 42). Iron acquisition has been shown to be critical for the survival of pathogenic bacteria during the course of infection. In some instances, the first two strategies described above have been

directly related to bacterial virulence (2, 14, 27). However, it is still not known to what extent the more recently characterized hemophore-dependent heme acquisition system contributes to the virulence of the bacteria for which it has been described so far: *Serratia marcescens* (22), *Pseudomonas aeruginosa* (24, 25), and *Pseudomonas fluorescens* (29).

Yersinia pestis, the bacterial agent of bubonic and pneumonic plague, possesses several iron acquisition systems that may be important for survival in its various iron-deficient niches. Inorganic iron can be acquired via an iron and manganese uptake system (termed Yfe) or through the secretion of a siderophore called “yersiniabactin” (6, 15, 30a). *Y. pestis* can also use hemin and a wide variety of host heme-containing compounds through a heme transport system (termed “Hmu”) (17) composed of at least five components: HmuR, -S, -T, -U, and -V. Under iron-depleted conditions, HmuR, the outer membrane receptor, and HmuTUV, a putative ABC-dependent transport system, are required for the use of hemin, hemin-albumin, and myoglobin, while HmuR is the only Hmu component needed to use hemoglobin and heme-hemopexin as iron sources. HmuS (HemS in *Yersinia enterocolitica*) may participate in removal of iron from hemin; however, conclusive demonstration of its role has been elusive (38, 39, 41, 42). Studies performed with mice showed that although both the iron/manganese and the yersiniabactin transport systems are important for *Y. pestis*, a deletion of the Hmu system has no effect on virulence in mice by a subcutaneous route of infection (2, 41)

During the course of the Hmu study, an *hmu* deletion mutant still grew under iron-depleted conditions in the presence of high concentrations of hemoglobin, leading to the hypothesis that an Hmu-independent uptake system may be involved

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genetic markers or features	Source or reference
Strain^a		
<i>Y. pestis</i>		
KIM5(pCD1Ap)+	Wild type, Amp ^r	13
KIM6+	Lcr ⁻ (pCDI ⁻)	30
KIM6	Lcr ⁻ Pgm ⁻ (Ybt ⁻ Δ <i>pgm</i>)	30
KIM6-2030	Lcr ⁻ Pgm ⁻ Fur ⁻ (<i>fur::kan</i>), Km ^r	36
KIM6-2061.1	Lcr ⁻ Pgm ⁻ HmuR ⁻	This study
KIM6-2085	Lcr ⁻ Pgm ⁻ HmuR ⁻ Has ⁻	This study
KIM6-2045.1	Lcr ⁻ Psn ⁻	10
KIM6-2081.1+	Lcr ⁻ Hmu ⁻ Has ⁻	This study
KIM5-2045.7	Lcr ⁺ (pCD1Ap) Amp ^r Psn ⁻	This study
KIM5-2081.1+	Lcr ⁺ (pCD1Ap) Amp ^r Hmu ⁻ Has ⁻	This study
6/69	Wild type	3
6/69c	Lcr ⁻ (pYV ⁻)	This study
6/69c(<i>hasA</i>)	Lcr ⁻ HasA ⁻	This study
6/69c (pPla ⁻)	Lcr ⁻ pPla ⁻	This study
6/69 (pPla ⁻)	pPla ⁻	This study
6/69 (HPI ⁻)	Pgm ⁻ (Δ <i>pgm</i>)	7
<i>E. coli</i>		
C600	F ⁻ <i>thr leu fhuA lacY thi supE</i>	Laboratory collection
TG1	<i>supE thi Δlac-proAB F traD36 proAB lacI^qZΔM15</i>	Laboratory collection
MC4100	F ⁻ <i>araD139 ΔlacU169 relA1 rpsL150 thi mot flb5301 deoC7 ptsF25 rbsR</i>	Laboratory collection
MC4100 <i>fur::cat</i>	<i>fur</i>	Laboratory collection
MC4100 <i>hemA</i>	<i>hemA</i>	R. Kadner
Plasmids		
pACYC184	Medium copy no., Tet ^r Cm ^r	9
pSyc150	HasDE _{sm} Cm ^r	22
pEU730	Single-copy-no. reporter vector, promoterless <i>lacZ</i> , Sp ^r	11
pCD1Ap	<i>bla</i> cassette inserted into <i>yadA</i> of pCD1 from <i>Y. pestis</i> KIM5, Amp ^r	13
pLG338	Low copy number, Tet ^r Km ^r	40
pR10K	HasR _{xm} Km ^r	12
pBGS18+	Km ^r	35
pHas98	HasRADEB _{yp} 8.828-kb <i>Sau3A Y. pestis</i> KIM6 fragment inserted at <i>Bam</i> HI site	This study
pHas98ΔBg12	HasB _{yp} Deletion from <i>hasR</i> through <i>hasE</i> , 5.375-kb <i>Bgl</i> II deletion of pHas98	This study
pACYCHas	HasRADEB _{yp} Cm ^r , pHas98 8.5-kb <i>Eco</i> RI- <i>Stu</i> I into pACYC184 <i>Eco</i> RI- <i>Sca</i> I	This study
pHasRA	HasRA _{yp} ⁺ <i>D'</i> Amp ^r , pHas98 4.3-kb <i>Hind</i> III- <i>Asp</i> 718 into pUC19 <i>Hind</i> III- <i>Kpn</i> I	This study
PHasRA2	HasRA _{yp} ⁺ <i>D'</i> , Km ^r , pHasRA <i>Eco</i> RI- <i>Hind</i> III insert into pBGS18	This study
pHasA	HasRA _{yp} ⁺ Amp ^r , PCR fragment into <i>Eco</i> RI- <i>Bam</i> HI sites of pUC18	This study
pEUHas	Single-copy-no. <i>hasR::lacZ</i> reporter, 383-bp <i>hasR</i> promoter ligated into pEU730, Sp ^r	This study

^a For *Y. pestis* KIM strains, + designates strains with an intact *pgm* locus.

in hemoglobin utilization (41). One possible candidate was identified in the *Y. pestis* CO92 genomic sequence released by the Sanger Centre as a sequence sharing homology with the hemophore-dependent heme acquisition system (Has system) of *S. marcescens* (22), *P. aeruginosa* (24, 25), and *P. fluorescens* (29). HasA hemophores are small extracellular proteins secreted by an ABC-dependent pathway, a process involving their carboxy-terminal secretion signal and its recognition by a tricomponent membrane ABC transporter. HasA is a heme-binding protein that delivers heme to a dedicated outer membrane receptor, HasR. Interaction between HasA and HasR induces a 100-fold increase in the capacity of the system to bind heme compounds and broadens the spectrum of potential heme sources for HasR (22, 24, 42).

In this study, we have begun characterizing the Has system of *Y. pestis* and investigated its biological function. We showed that HasA_{yp} is a secreted heme-binding protein. Secretion, hemoglobin utilization, and induction conditions of the Has_{yp} system were studied with both *Y. pestis* and *Escherichia coli*. The effect of the deletion of the *Y. pestis* Has system in conjunction with a deletion of the Hmu system on the virulence of *Y. pestis* was investigated with a mouse model of infection. This

report constitutes the first attempt to investigate the contribution of the hemophore-dependent heme acquisition system to bacterial pathogenicity.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were cultivated in Luria broth (LB) (28). *Y. pestis* was grown in LB or on tryptose blood agar base (TBA). For growth under iron-restricted conditions, *Y. pestis* cells were grown in the defined medium PMH or PMH2 (13, 37). PMH2 is a modified version of PMH that contains 50 μM PIPES (piperazine-*N,N'*-bis[2-ethanesulfonic acid]) instead of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid). This change reduces medium acidification due to bacterial growth. PMH and PMH2 were deferrated with Chelex 100 resin (Bio-Rad) prior to filter sterilization as previously described (37). Alternatively, to reduce iron available to *E. coli* or *Y. pestis*, cells were grown in LB medium containing 0.2 mM 2,2'-dipyridyl (LBD). Hemin, hemin-agarose, and bovine hemoglobin were obtained from Sigma Chemical Co. Bovine hemoglobin agar plates were prepared as described in reference 12.

Molecular biological procedures. Standard procedures were used for cloning and analysis of DNA, PCR, electroporation, and transformation. Enzymes used to manipulate DNA were from New England Biolabs. Oligonucleotides were from Gibco BRL or Integrated DNA Technologies, Inc. Some DNA sequencing was performed by Genome Express S.A. In some instances, sequencing reactions were performed via the dideoxynucleotide chain termination method (33) by using ³⁵S-dATP (Amersham/USB), Sequenase version 2.0 (Amersham/USB),

and 7-deaza-dGTP (Boehringer Mannheim Biochemicals). Samples were electrophoresed through a 6% polyacrylamide gel containing 8.3 M urea (Sigma) cast in Tris-borate-EDTA buffer (32). Dried gels were exposed at room temperature to Kodak Biomax MR film.

Sequence analysis. Amino acid sequence comparisons were performed with the Clustal package program (16). Homology searches were performed with Blast 2.0 at <http://www.ncbi.nlm.nih.gov/BLAST/unfinishedgenome.html>. DNA sequence manipulations were done with DNASTrider 1.3 (26) or DNAMAN Version 4.21 (Lynnon Biosoft).

Construction of recombinant plasmids. pHasA was created as follows. The *hasA_{yp}* gene was amplified by PCR with oligonucleotides YpHasArbs-5 (5'-CCCGAATTCTTAATAATTAAGGACATTATCATGAGTA-3') and YhasAend-3 (5'-CCCAAGCTTAGCCCTAGCGGTGGTATCAATGACTCA GCC-3') as primers and with *Y. pestis* 6/69 genomic DNA as a template. The amplified DNA was digested with *Eco*RI and *Hind*III and ligated into the same sites of pUC18. For the construction of pEUHas, in which the *lacZ* reporter gene was placed under the control of the *hasR* promoter region, a 383-bp region upstream of the putative initiating methionine residue for HasR was amplified from pHas98 by using PCR primers Hasp1 (5'-GATCTTTTAACTTAATAG ACTG-3') and Hasp2 (5'-TTATGAACTTCATCCTTAAC-3'). The products were blunt-end ligated into the *Pme*I site of pEU730 (11), and the resulting plasmid was designated pEUHas. All constructs were checked by PCR with specific and vector-based primers. Then each construct was sequenced.

Construction of *has* mutations in *Y. pestis* strains. The Sanger Centre database of *Y. pestis* CO92 DNA was searched for *has*-related sequences by using the *hasRADE* sequence from *S. marcescens*. Based on the *Y. pestis* sequence, PCR primers HasYP1 (5'-CCGATTACAGCATCTCT-3') and HasYP2 (5'-GCGC ATCAGACATATAACC-3') were designed and used to produce a probe for screening a *Y. pestis* KIM6+ genomic DNA library. The resultant clone, pHas98, contains an approximately 8.8-kb fragment inserted into pLG338. An ~5.4-kb *Bgl*II fragment was removed from pHas98 to create pHas98Δ*Bgl*II. This deletion removes all of *hasA* and *hasD* as well as portions of *hasR* and *hasE*. A 2.5-kb *Stu*I-*Hpa*I fragment was cloned from pHas98Δ*Bgl*II into the *Sma*I site of pKNG101 (18) to yield pKNGhas. The mutated *has* alleles were introduced into *Y. pestis* KIM6 strains by allelic exchange as previously described (2). The genotype of all strains was confirmed by PCR with HasYP1 and HasYP2 primers, which are missing from the mutants, as well as HasD1 (5'-GATGCGCCATAC AGAGCC-3') and HasD2 (5'-GGCATTGTGCCATCAGG-3') primers, which flank the deleted region.

Protein analysis. *E. coli* strains harboring various plasmids, *S. marcescens* SM365, and *Y. pestis* strains were grown at 28°C (*Y. pestis*) or 37°C (*Y. pestis*, *S. marcescens*, and *E. coli*) in LB, LBD, or PMH2. Cells from overnight or exponential cultures were centrifuged for 10 min at 5,000 × *g* at 4°C. The supernatants were concentrated by precipitation with either 10% trichloroacetic acid (inactive supernatants that do not retain HasA heme-binding activity) or 80% ammonium sulfate (active supernatants that retain HasA heme-binding activity). Inactive concentrated cell extracts were prepared by boiling whole cells in sodium dodecyl sulfate (SDS) buffer. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by either Coomassie blue staining or Western blot analysis. Anti-HasA_{yp} antibodies were prepared against HasA_{yp} protein purified on hemin-agarose, separated on SDS-polyacrylamide gel, and electroeluted. The protein was mixed with Titer Max adjuvant (Interbiotech) and injected into a rabbit to raise antibodies. The rabbit serum was used in Western blot analysis at a dilution of 1/2,000. Nondenaturing PAGE was performed as described in reference 25.

For analysis of in vitro-synthesized products, ~5 μg of purified, circular, plasmid DNA was added to an *E. coli* S30 extract system (Promega Corp.) and supplemented with ³⁵S-labeled amino acids (DuPont NEN Research Products). After a 30-min incubation at 37°C, the products were acetone precipitated, resuspended in sample buffer, and electrophoresed on a 12% polyacrylamide gel containing SDS. The gels were dried and exposed overnight to Kodak BioMax MR film.

β-Galactosidase assays. Lysates of *Y. pestis* strains carrying pEUHas were prepared from cells exponentially growing in PMH2 in the presence or absence of iron through two transfers for a total of approximately 6 generations. The β-galactosidase activity, with ONPG (*o*-nitrophenyl-β-D-galactopyranoside) used as a substrate, was measured spectrophotometrically and expressed in Miller units (28).

HasA_{yp} purification heme affinity chromatography. Five hundred milliliters of *E. coli* C600(pSyc150, pHasA) culture grown overnight at 37°C was centrifuged for 10 min at 5,000 × *g* at 4°C. The supernatant was concentrated by 80% ammonium sulfate precipitation. The protein pellet was resuspended and extensively dialyzed against 10 mM Tris-HCl (pH 7.5) and then subjected to heme

affinity chromatography as described in reference 25. According to the manufacturer's specifications, there is a 10- to 100-fold excess of putatively available heme compared to the estimated number of HasA molecules used in the hemin-agarose procedure. Inactive and active supernatants (for biological tests) were prepared as described in reference 25.

Role of Has in hemoglobin utilization. Growth stimulation of an *E. coli hemA* mutant producing HasR_{sm} by exogenously supplied HasA hemophores was tested as follows. The HasR_{sm}-producing strain was mixed with 3 ml of top agar and poured onto LBD plates supplemented with 10⁻⁶ M hemoglobin. Five-millimeter-diameter wells were cut in the agar and filled with 50 μl of hemin-agarose-purified HasA_{yp} or HasA_{sm} protein. Growth around the wells was recorded after overnight incubation at 37°C.

For *Y. pestis* strains, cells were grown at 28°C in the deferrated, defined medium, PHM2, in the absence of iron through two transfers for a total of approximately 6 generations. Third-transfer cultures were inoculated to an optical density at 620 nm (OD₆₂₀) of 0.1 into PMH2 containing 50 μM ethylenediamine-*N,N'*-diacetic acid (EDDA) and either 0, 1.25, 2.5, 5, or 10 μM hemoglobin, assuming a molecular weight of 64,640 for hemoglobin. Growth of cells was monitored at regular intervals with a Genesys5 spectrophotometer (Spectronics Instruments, Inc.).

Dot blot HasA-binding assay. The interaction between HasA_{sm} or HasA_{yp} with HasR_{sm} was examined as follows: Cultures of MC4100 *fur::cat* carrying pBGS18+ or pR10K were grown exponentially in LB medium at 37°C and harvested when they reached an OD₆₀₀ of 1. Cell pellets were suspended in Tris-buffered saline (TBS) buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl) to an OD₆₀₀ of 0.5. Aliquots of 50 ml were applied to nitrocellulose filters. The filters were dried for 10 min at room temperature and saturated by incubation for 1 h at 37°C in BS blocking solution (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% skim milk, 0.001% antifoam, 0.01% NaN₃). The filters were then incubated for 30 min at 30°C in 1 ml of 50 mM Tris-HCl (pH 7.5) containing serial dilutions of either HasA_{yp} or HasA_{sm} or containing no HasA, washed three times with BS, and then incubated for 1 h at 37°C with polyclonal anti-HasA_{sm} or anti-HasA_{yp} antibodies diluted 1:2,000 in blocking solution. After three washes in BS, the filters were incubated with an alkaline phosphatase-conjugated goat anti-rabbit serum diluted 1:7,000 in BS. After three washes in BS, antibody binding was revealed as in other immunodetection tests (23).

Virulence testing. *Y. pestis* strains lacking the low-calcium-response virulence plasmid are avirulent by any route of infection. A derivative of this plasmid, containing an ampicillin resistance gene inserted into the pseudogene *yadA*/*'yadA* (pCD1Ap) (13), was electroporated into KIM6+, KIM6-2045.1, and KIM6-2081+, generating KIM5(pCD1Ap)+, KIM5-2045.7, and KIM5-2081.1+. All strains were constructed and tested for virulence in a BL3 facility. Bacteria were grown at 26°C in PMH2 containing 50 μM hemin and ampicillin (100 μg/ml) through two transfers for a total of approximately 7 generations. Cells were harvested at an OD₆₂₀ of between 0.5 and 0.7, pelleted, and resuspended in mouse isotonic phosphate-buffered saline (PBS; 149 mM NaCl, 16 mM Na₂HPO₄, 4 mM NaH₂PO₄ [pH 7.0]). Five- to 7-week-old female NIH/Swiss Webster mice were injected subcutaneously with 0.1 ml of 10-fold serial dilutions of the bacterial suspensions. Groups of four mice were used for each bacterial dose. The number of cells injected was determined by plating serial dilutions on TBA plates containing ampicillin. Mice were monitored daily for 2 weeks. Fifty percent lethal doses (LD₅₀s) were calculated by the method of Reed and Muench (31).

RESULTS

Identification of the *Y. pestis* Has system. Analysis of the *Y. pestis* CO92 (biotype *orientalis*) complete genomic sequence released by the Sanger Centre (http://www.sanger.ac.uk/Projects/Y_pestis) revealed the existence of a locus sharing homology with the hemophore-dependent heme acquisition system (Has, for heme acquisition system) of *S. marcescens*, *P. aeruginosa*, and *P. fluorescens*. The *Y. pestis has* operon contains five open reading frames (ORFs) (Fig. 1) called "*hasR_{yp}*," "*hasA_{yp}*," "*hasD_{yp}*," "*hasE_{yp}*," and "*hasB_{yp}*" according to the nomenclature used for the homologous genes of the *S. marcescens has* operon (12). The predicted sizes, protein locations, and putative functions, as well as percent identities of the deduced amino acid sequences with those found in protein databases, are shown in Table 2. A comparison of the CO92 *has* se-

GATAATGATAATCATTATC *E. coli* consensus
 ||| |||||
 GATTATGATAATCATTATC *Y. pestis* sequence
FUR binding-site

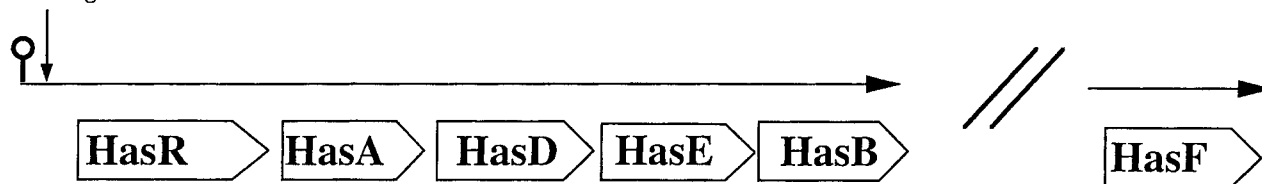


FIG. 1. Genetic organization of the *Y. pestis* Has system. Genetic organization of the 8.3-kb *Y. pestis* Has_{yp} system region identified in this study. The partial sequence comparison of the Fur binding site is shown above the diagram. The stem-loop structure located 40 bp upstream of the Fur binding site is indicated.

quences with those from *Y. pestis* KIM10+ (biotype *mediaevalis*) (http://maggie.genome.wisc.edu/cgi-bin/Authenticate.cgi/uwgp_blast.html), shows that the deduced amino acid sequences of these five ORFs are nearly identical between these two biotypes (data not shown). The deduced amino acid sequence of HasR_{yp}, the putative outer membrane receptor of the system, displayed a high level of identity with HasR_{sm}, except for a short N-terminal extension missing in HasR_{yp} (Fig. 2B). A well-conserved Fur binding site sharing 94% identity with the *E. coli* consensus sequence (GATAATGATAATCA TTATC) was identified 340 bp upstream of the *hasR*_{yp} initiation codon, suggesting that *hasR*_{yp} genes may be iron regulated. Further upstream of *hasR*_{yp}, a gene coding for a protein 90% identical to the *E. coli* argino-succinate lyase ArgH was identified. The intergenic region between *argH* and *hasR* contains a stem-loop structure, suggesting that *argH* is not part of the *hasRADEB* putative operon. While this stem-loop structure may act as a transcription terminator, its localization close to the Fur binding site suggests that this structure may play a role in the Has system regulation. Finally, the deduced amino acid sequence of a protein 50.6% identical to HasF_{sm}, the third component of the HasA transporter, was found to be located outside the main *hasR*_{yp} putative operon (Fig. 1). A single ORF was found in *hasF*_{yp} locus, located 281 kb away from *hasB*. Thus, HasF_{yp} does not seem to be part of an operon and does not contain any Fur binding site located upstream of the coding sequence.

HasA_{yp} is a secreted heme-binding protein. The homology between HasA_{sm} and HasA_{yp} (33.8% [Table 2]) suggested that, like HasA_{sm}, HasA_{yp} may be a secreted protein, although the differences between HasA_{yp} and other HasA hemophores (Fig. 2A), particularly in the C-terminal region corresponding to the secretion signal, raised some questions about the capacity of HasA_{yp} to be secreted. Previous studies showed that HasA_{sm} is secreted in *E. coli* by a hybrid transporter composed of two *S. marcescens* inner membrane proteins, HasD_{sm} and HasE_{sm}, and the *E. coli* outer membrane protein TolC, which complements HasF_{sm} function (22). We tested whether this transporter also secretes HasA_{yp}. *E. coli* MC4100, carrying pSYC150, which expresses *hasD*_{sm} and *hasE*_{sm}, was cotransformed with pHasA_{yp}, which contains *hasA*_{yp} cloned with its own ribosome binding site under *Plac* promoter control (Table 1). Figure 3 shows that HasA_{yp} was as efficiently secreted by the Has hybrid transporter as HasA_{sm}.

To study the biological function of HasA_{yp}, the culture su-

pernatant of strain *E. coli* MC4100(pSyc150, pHasA_{yp}) grown in LB medium was precipitated with 80% ammonium sulfate, solubilized, and subjected to affinity chromatography on heme-agarose. Unbound and bound proteins were analyzed by SDS-PAGE and Coomassie blue staining. A 19-kDa protein corresponding to HasA_{yp} was retained on heme-agarose, showing that HasA_{yp} is a heme-binding protein (Fig. 4A). To test whether HasA_{yp} produced in *E. coli* was able to acquire heme from hemoglobin, the samples were loaded in a non-denaturing polyacrylamide gel in which hemoproteins are known to migrate without dissociating from heme. In these gels, heme causes a significant shift in the migration of the bound polypeptide. As observed in previous studies (21, 25), the electrophoretic mobility of both HasA_{sm} and HasA_{yp} was increased in the presence of heme or hemoglobin (Fig. 4B), thus demonstrating that HasA_{yp} can acquire heme from hemoglobin.

Search for HasA_{yp} in *Y. pestis* supernatants. In order to investigate HasA_{yp} production and secretion in *Y. pestis*, the culture supernatants and cell pellets of strains 6/69 and KIM6+ grown under iron-rich or iron-depleted conditions were harvested and probed with anti-HasA_{yp} antibodies. Iron deprivation of *Y. pestis* was successfully obtained, since the iron-regulated high-molecular-weight proteins (8) were present in cell extracts (data not shown). Only a very faint band cross-reacting with the HasA antibodies was detected at ca. 15 kDa, in the cell pellet of iron-replete and iron-starved bacteria (data not shown). This band was still detected in the supernatant of *Y. pestis* 6/69c (*has* mutant), in which the chromosomal *hasA*_{yp} gene was replaced by a truncated copy by reverse ge-

TABLE 2. Properties and homologies of proteins of the *Y. pestis* Has system

Protein	Mass (kDa)	Location or putative function ^a	% Identity with system:		
			Has _{sm}	Has _{pa}	Has _{pf}
HasR	93.4/89.6 ^b	OM, HasA receptor for uptake	48.0	38.0	44.0
HasA	22.2	Secreted hemophore	33.8	28.7	20.3
HasD	65.0	IM, ATP-binding protein, HasA export	55.0	56.1	56.9
HasE	48.9	IM, ABC permease, HasA export	44.2	43.0	39.1
HasB	29.3	IM TonB-like protein	25.3	NA ^c	NA
HasF	50.6	OM, HasA export	69.6	13.4	27.8

^a OM, outer membrane; IM, inner membrane.

^b Unprocessed/processed.

^c NA, not applicable.

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HasApf      1 MTISVSYEAALGAYSVSDYLTDWALGFNTAGHGS-----SNIGGFSNGS--LSGDQYST
HasApa      1 MSISISYSTTYSQNLVADYLAQNSAYFGDYNHRPGQVVDGSNIGGFNPGP--FDGSQYAL
HasAsm      1 MAFSVNYDSSEFCYSIHDYLGQWASTFGDYNHTNGN-VTDANSGGFYGG--LSGSQYAL
HasAyyp     1 MSTLITQYNSNYADYSISSYLREWANNFGDIDQAP---AETKDRGSESGSSTLFSGQYAL
Con.        1 Mt sv Y t ygaysv dYL dWa Fgdv h          ntGgF gs sGSQYal

HasApf      53 HGANNSDYAFIADSNTSNGLIVVFNPALPASHNENHYLWGLDNLVQLGTGLGG--GNGSD
HasApa      59 K-STASDAAFIAGG----DLRYTLFS-----NPSHTLWGLKLDSTALGDTLTG--GASSG
HasAsm      58 SSTANQVTAFAIAGG----NLTYTLFN-----EPAHTLWGLDLSLFGDGLSG--GDTSP
HasAyyp     58 GSSHNSPECMIAEG----DLRYSEFMP-----QHTFHGQIDRIQFGKDLATNAGGPSA
Con.        61 s      afiA g      LhYt          HtlwGnlDsv G L g G S

HasApf      111 -ETLDAFKVAENGLDLSAAQCAG-----RVGNDVQNVIIYSLMCGNTSALETVNNILDD
HasApa      106 GVALDSQEVSFNGLDSPAICG-----RDG-TVHKVVYGLMSGDSSALQGQTDALKA
HasAsm      106 -MSHQVPPVSEGGINLSSLQACG-----HDG-VVHQVVYGLMSGDTGALETAINGILDD
HasAyyp     106 GKHLKIKIDTINELDLSGEFDSGKSMTEHQG-DVHKSVRGLMKGNPDPVLEVMKAKGIN
Con.        121 f ld evsF L Ls g G      r G vh vvygLM G t al l all

HasApf      164 FG--LSTANTFDEISAGLAAHAT--ATTTDVALVGVQDVAQDWALAA
HasApa      159 VDPSSLINSTFDQIAAAGVAHATPAAAAAEVGVGVQELPHDLALAA
HasAsm      158 YG--LSVNSTFDQIAAATA-----VGVQHADSPELLAA
HasAyyp     165 VDTAFKDLASIQYPSDGYMSDAP-----MVDTVGVVDC-HDMLLAA
Con.        181 ls stfdqi ag          v VGVqd d LAA

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B

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HasRyyp      1 -----MDNIIRDKKTIIISV
HasRsm      1 -----MFTHKGTTPAGRLATVRAALAMMLTQPAVALAAQAEASSAQAAQKFNIAA
HasRpa      1 MKHRGWSAVRGGK-KCAQLALCIGLVLLCTAALPLHAQDCADSASQQTALRRVRLDIPA
HasRpf      1 -----MSALTTKGRNTCSRLALAHHLGLFASACQ-VQAAEAPVGESQPVTLT----FDIAA
Con.        1          i g a la av l a          a          I a

HasRyyp      15 MKHTTCTII-----FALVITQGYSGQLAAN-----
HasRsm      55 QPLQSAMLRFAEQAGMVFDFEVKLDGMAAALNGSMSVEQGLRRLIGCNPVAFRLQPQG
HasRpa      60 QPLNRAILRFARQAGMVFDFDSQRFAGLGSAAVHGEYLLADGLSQMLQGSPEVRFSGKD
HasRpf      51 QPLGLAMLTFAEQAGLQVLFDSARLQGLTSTAALKGRYSAQEGLRVGSAPVEFRFTGDR
Con.        61 qpl aiL faeqagmqv fd k gm al g v Gl llaa pv fr

HasRyyp      38 ---TAETPTNNND---MALDKLNVEGKGAHDSDWVYDEPRSVSEITREQIDNRPARHAA
HasRsm      115 QIVLSRLPTANGDGGALALDSLTVLGCAGNNANDWVYDEPRSVSVISREQIDNRPARHAA
HasRpa      120 Q--LSLIRVSQDD--LVQMSPSVTS---AARPDWVYQTPESVSVIGREQIDNRPPRHAA
HasRpf      111 Q--VILTRVSADHDGALTGTGTTITGNTNGKPSDWYDTPRSVSVISREQIDKRPPRHAA
Con.        121 q ls n d m l v g ah DWvYd PrSVsvItREQId rP RHAA

HasRyyp      92 DILEQTFGVYSSVSQDPLGSLNIRGIQDYGRVNMNIDGMRQNFMSGHGQRNGSMYIDP
HasRsm      175 DILEQTFGAYSSVSQDPLLSVNIIRGIQDYGRVNMNIDGMRQNFQKSGHGQRNGTMYIDS
HasRpa      173 DILETFGVYSSVSQDPLSVNIIRGIQDYGRVNMNIDGMRQNFQSGHQQRNGTMYIDP
HasRpf      169 DILETAGVYTVNQRDPGLSVNIRGIQDYGRVNMNIDGMRQNFVNVNGHQQRNGTMLIDP
Con.        181 DiLE T GvYssVsQqDPgLSvNIIRGiQDYGRVNMniDGMRQNFq sGH QRNGtmyiDp

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FIG. 2. Clustal W amino acid sequence alignments of HasA and the N-terminal region. For both panels, identical amino acids are shown in black boxes, while similar amino acids are shown in gray boxes. The consensus line (Con.) shows identical (uppercase) and similar (lowercase) amino acids. (A) Amino acid sequence comparison of the N-terminal regions of the *Y. pestis* and *S. marcescens* HasR hemophore-dependent receptors HasR_{yyp} and HasR_{sm}, respectively. (B) Amino acid sequence comparison of HasA hemophores identified in *Y. pestis*, *S. marcescens*, *P. aeruginosa*, and *P. fluorescens* (HasA_{yyp}, HasA_{sm}, HasA_{pa}, and HasA_{pf}, respectively).

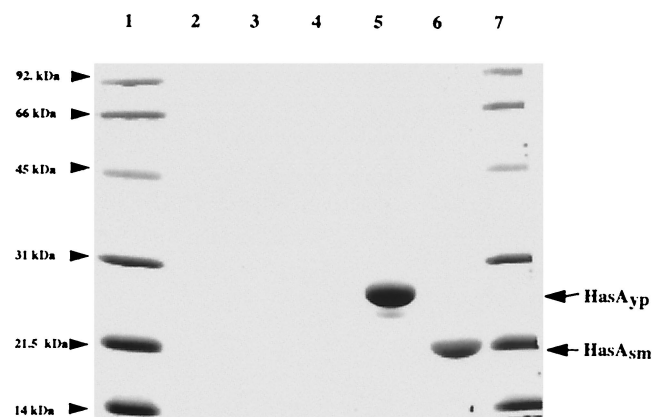


FIG. 3. HasA_{yp} is secreted through the *S. marcescens* Has system. Samples were prepared as described in Materials and Methods and Coomassie blue stained after SDS-PAGE analysis. The equivalent of 1 ml of supernatants from cultures at an OD₆₀₀ of 0.5 was loaded in each lane. Lanes: 1, molecular size markers; 2, MC4100 *hemA*; 3, MC4100 *hemA*(pSyc150); 4, MC4100 *hemA*(pHasA_{yp}); 5, MC4100 *hemA*(pSyc150, pHasA_{yp}); 6, MC4100 *hemA*(pSyc150, pHasA_{sm}); 7, molecular size markers.

netics, suggesting that it corresponds to some cross-reacting material. The effect of temperature on *hasA_{yp}* expression was evaluated by growing the cells at 28 or 37°C. Neither temperature allowed the visualization of HasA_{yp}. The influence of the presence or absence of the *Y. pestis* plasmids pYV and pPla and of the *pgm* locus (containing the high-pathogenicity island [HPI]) was also investigated by using the isogenic strains *Y. pestis* 6/69, 6/69_c, 6/69_c (pPla⁻), 6/69(pPla⁻), and 6/69(HPI⁻), but none of the mutants produced HasA_{yp}. Similar negative results were obtained with *Y. pestis* KIM6+ (data not shown).

The reasons for the absence of HasA detection in *Y. pestis* may be that (i) the induction conditions used were not appropriate, (ii) HasA is unstable or rapidly degraded, (iii) its secretion rate is too low to be detected, or (iv) the Has system is not functional. Some of these hypotheses were investigated.

In *Y. pestis*, the *hasRADEB* promoter is Fur regulated and presents increased activity at 37°C. To investigate transcription from the *hasRADEB* promoter region, we cloned the putative promoter region of *hasRADEB* upstream of *lacZ* in the reporter plasmid pEU730 (11). This construct, termed pEUHas, was electroporated into *Y. pestis* strains KIM6+, KIM6, and KIM6-2030. The cells were grown in the presence or absence of iron and assayed for β-galactosidase activity. The results indicated that expression of *lacZ* from this promoter fragment was iron regulated, showing an approximately three- to fivefold induction when the cells were grown in the absence of iron (Table 3). Transcription of *hasR::lacZ* appears to be regulated by Fur, because the levels of β-galactosidase activity were constitutively high in the *fur* mutant, KIM6-2030 (pEUHas). We also observed a minor but reproducible difference in the β-galactosidase values obtained with KIM6 (pEUHas)+ and KIM6(pEUHas), which were slightly higher in the KIM6+ strain, suggesting that a component of the 102-kb *pgm* locus may participate in the expression of *hasRADEB*. Finally, β-galactosidase activity was six- to sevenfold higher in KIM6(pEUHas)+ cells grown at 37°C than in those grown at 26°C.

Expression of HasA_{yp} is iron regulated in *E. coli*. To study expression from the *hasRADEB_{yp}* promoter region in *E. coli*, we monitored HasA_{yp} secretion in *E. coli* MC4100(pSyc150) carrying pHasRA2 containing *hasR_{yp}* and *hasA_{yp}* cloned with their own putative promoter region. As revealed by Coomassie blue staining or immunodetection with anti-HasA_{yp} antibodies, HasA_{yp} was secreted into the supernatant of *E. coli* MC4100 (pSyc150, pHasRA2) grown in iron-depleted but not iron-rich media (Fig. 5). These results indicate that in *E. coli*, *hasA_{yp}* (and possibly *hasR_{yp}*) is expressed from its own promoter and that expression is iron regulated.

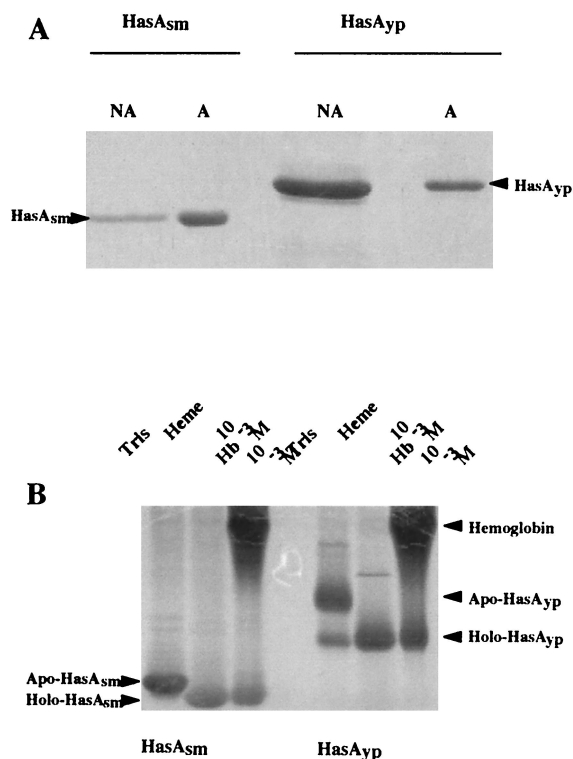


FIG. 4. HasA_{yp} is a heme-binding protein. (A) Isolation of HasA_{yp} by heme affinity chromatography. Coomassie blue staining is shown. Concentrated supernatants were incubated with hemin-agarose as described in Materials and Methods. (Left) HasA_{sm}, NA, *E. coli* MC4100 (pSyc150, pHasA_{sm}) concentrated supernatant not adsorbed (flow-through material in excess) on hemin-agarose; A, *E. coli* MC4100 (pSyc150, pHasA_{sm}) concentrated supernatant adsorbed on hemin-agarose. (Right) HasA_{yp}, NA, *E. coli* MC4100(pSyc150, pHasA_{yp}) concentrated supernatant not adsorbed on hemin-agarose; A, *E. coli* MC4100(pSyc150, pHasA_{yp}) concentrated supernatant adsorbed on hemin-agarose. The presence of HasA_{sm} and HasA_{yp} in NA lanes indicates that the capacity of the hemin-agarose columns was saturated by the supernatant concentrates used here. (B) Heme acquisition and formation of holoHasA_{sm} and holoHasA_{yp} after incubation of HasA_{sm} or HasA_{yp} with heme or hemoglobin. Four microliters (1 μg/ml) of HasA_{sm} prepared from ammonium sulfate-concentrated supernatants of *E. coli* MC4100(pSyc150, pHasA_{sm}) and 2 μl of HasA_{yp} (2 μg/ml) prepared from ammonium sulfate-concentrated supernatant of *E. coli* MC4100(pSyc150, pHasA_{yp}) were incubated with equal volumes of heme or hemoglobin (10⁻³ M) for 45 min at room temperature. The mixtures were loaded onto a 15% polyacrylamide gel and electrophoresed in the absence of SDS. The gel was stained with Coomassie blue. Protein bands complexed with heme (holoproteins) have a faster migration rate than uncomplexed protein (apoproteins).

TABLE 3. β -Galactosidase activities of *Y. pestis* strains containing *lacZ* reporters

Strain ^a	β -Galactosidase activity (mean \pm SE Miller units) ^a		-Fe/+Fe ratio
	+Fe	-Fe	
KIM6(pEU730)+	118 \pm 14	203 \pm 71	1.7
KIM6(pEUHas)+	8,264 \pm 1,532	39,738 \pm 6,182	4.8
KIM6(pEU730)	227 \pm 49	285 \pm 26	1.3
KIM6(pEUHas)	5,822 \pm 1,208	19,023 \pm 6,597	3.3
KIM6-2030(pEU730)	200 \pm 46	446 \pm 62	2.2
KIM6-2030(pEUHas)	22,152 \pm 4,492	28,674 \pm 5,235	1.3

^a Bacterial cells were grown in deferrated PMH2 at 37°C and harvested during mid-log-phase growth. Whole-cell lysates were assayed for β -galactosidase activity as described in reference 28.

Study of the functionality of the *Y. pestis* Has secretion system in *E. coli*. To test the functionality of the *Y. pestis* HasA_{yp} transport system, the entire *hasRADEB* locus was cloned into pLG338 or pACYC184 to produce plasmids pHas98 and pACYCHas (Table 1). pHas98 and pACYCHas were intro-

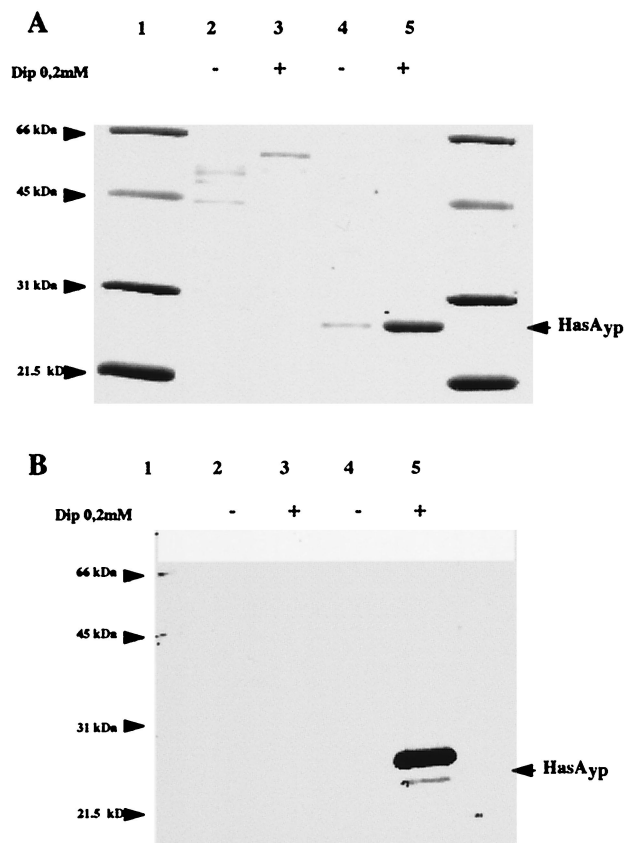


FIG. 5. Iron regulation of HasA_{yp} secretion in *E. coli*. Supernatant samples were prepared as described in Materials and Methods and subjected to SDS-PAGE analysis followed either by Coomassie blue staining (A [equivalent of 10 ml of supernatant from cultures at OD₆₀₀ of 0.5 loaded in each lane]) or immunodetection with anti-HasA_{yp} antibodies (B [equivalent of 1 ml of culture at OD₆₀₀ of 0.5 loaded in each lane]). (A and B) Lanes: 1, molecular size markers; 2, MC4100 (pHasRA2) in LB medium; 3, MC4100(pHasRA2) in LB medium supplemented with 0.2 mM 2,2'-dipyridyl; 4, MC4100(pSyc150, pHasRA2) in LB medium; 5, MC4100(pSyc150, pHasRA2) in LB medium supplemented with 0.2 mM 2,2'-dipyridyl.

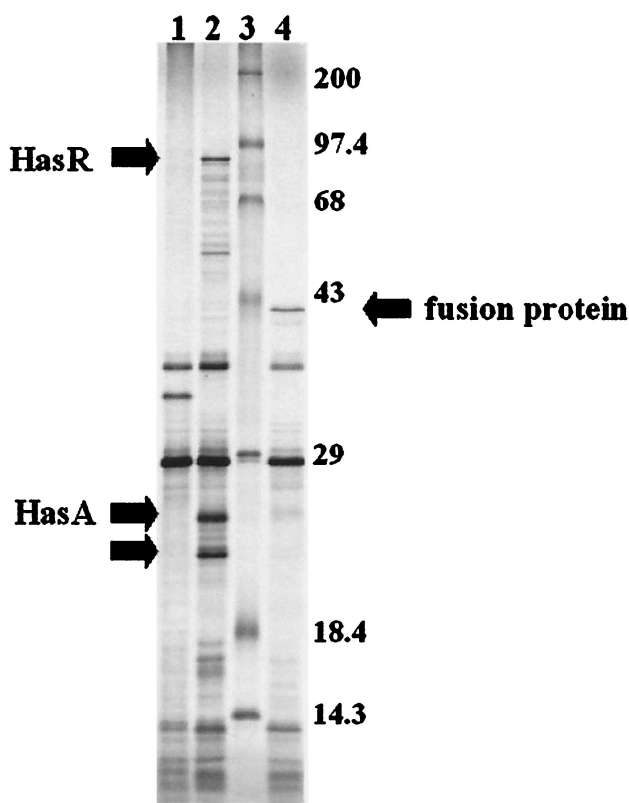


FIG. 6. Autoradiogram of plasmid-encoded proteins labeled with ³⁵S-labeled amino acids by in vitro transcription and translation. The sizes of molecular mass standards (lane 3) are shown on the right side of the figure. The locations of HasR, HasA, and a HasA degradation product (lane 2) are indicated by the arrows on the left side of the figure. The arrow on the right points to a fusion protein (lane 4) generated by a deletion within the *has* operon. The plasmids used for the in vitro transcription and translation reactions were pLG338 vector (lane 1), pHas98 (lane 2), and pHas98ΔBgI2 (lane 4).

duced into *E. coli* MC4100, and the recombinant strains were grown in iron-rich (LB) or iron-depleted (LBD) medium and monitored for HasA_{yp} secretion. No secreted HasA_{yp} could be detected in the iron-depleted or iron-rich medium (data not shown).

In vitro transcription or translation experiments with pHas98 showed Has-specific bands at ca. 87, 25, and 22 kDa (see arrows in Fig. 6, lane 2). The same bands were observed when a clone containing only *hasRAD'* (pHasRA) was used in the transcription/translation system (data not shown), but are absent from reactions containing the vector plasmid, pLG338 (Fig. 6, lane 1). The 87-kDa band likely corresponds to HasR_{yp}, while the 25- and 22-kDa proteins probably represent HasA_{yp} and a degradation product generated from HasA_{yp}. None of these bands was present in reactions containing pHas98ΔBgII (Fig. 6, lane 4). Instead, a new protein of ca. 41 kDa is seen, which is similar in size to the ca. 43-kDa fusion product predicted to occur as a result of the deletion. No bands corresponding to HasD, HasE, and HasB were convincingly observed. All three proteins contain at least one cysteine as well as several methionine residues and thus should have been labeled in the in vitro reactions. These results indicate that at least HasR_{yp} and HasA_{yp} are expressed from pHas98 and that

the HasA_{yp} secretion system encoded by pHas98 or pACYCHas may be expressed but not functional when reconstituted in *E. coli*. The biological function of the *Y. pestis* Has system was further investigated as described below.

Study of the *Y. pestis* HasA-dependent heme acquisition system in *E. coli*. The biological function of HasA_{sm} is the delivery of heme scavenged from various heme sources to the outer membrane receptor HasR_{sm}. In *S. marcescens*, HasA_{sm} is required for iron acquisition from heme and hemoglobin and was shown to interact with its outer membrane receptor, HasR_{sm}, both in vivo and in vitro (10). To test whether HasA_{yp} could substitute for the biological function of HasA_{sm}, we performed an in vivo growth stimulation test with hemoglobin plates (see Materials and Methods). In this test, a lawn composed of HasR_{sm}-expressing cells [MC4100 *hemA*(pR10K)] is plated on LB agar containing a low concentration of hemoglobin, and a solution of HasA_{sm} or HasA_{yp} is placed in a well in the agar. A productive interaction between HasA_{sm} and HasR_{sm} leads to bacterial growth around the HasA-containing well. As expected, HasA_{sm} promoted bacterial growth, but this promotion effect was not observed around the well containing native HasA_{yp} (data not shown). Consistently, in vitro dot blot binding experiments did not show any interaction between HasA_{yp} and HasR_{sm} (data not shown). These results suggest that HasA_{yp} cannot substitute for the biological function of HasA_{sm}, probably due to its lack of functional interaction with HasR_{sm}.

The expression of hasA_{yp} and hasR_{yp} from plasmids pHasRA and pHasRA2, carrying hasR_{yp} and hasA_{yp}, raised the possibility that HasR_{yp} could reach the outer membrane and is able to interact with hemophores available at the bacterial cell surface. To test this, we performed either in vivo growth stimulation tests with hemoglobin or in vitro dot blot binding experiments with cells potentially expressing HasR_{yp} [MC4100 *hemA*(pHasRA)]. We did not see any growth stimulation or dot blot interaction with either HasA_{yp} or HasA_{sm} (data not shown). These results suggest that if HasR_{yp} is correctly expressed in pHasRA and interacts with *Y. pestis* or *S. marcescens* HasA hemophores, this interaction does not lead to heme uptake under the conditions tested with *E. coli*.

We then investigated whether such a functional interaction could be detected with the whole *Y. pestis* Has system reconstituted in *E. coli*. To determine if the *Y. pestis* heme acquisition system allows cells to use hemoglobin as a source of iron, we introduced pHas98 or pACYCHas (encoding *hasRADEB*) and pHasRA (encoding *hasRA*) into an *E. coli hemA* mutant strain (Table 1). This mutant grows aerobically if supplemented with δ -aminolevulinic acid, but not with exogenously supplied hemoglobin. Growth was tested on iron-rich or iron-depleted agar plates in the presence of hemoglobin at concentrations ranging from 10^{-4} to 10^{-7} M. No growth was observed under any of the conditions tested, while a control strain carrying the Has_{sm} system grew at these various hemoglobin concentrations (data not shown). These results indicate that the *Y. pestis* Has-dependent heme acquisition system is not functional in *E. coli* and are consistent with the lack of HasA_{yp} secretion from pHas98- and pACYCHas-harboring cells.

Growth studies of *has* and *has hmu* mutant strains of *Y. pestis*. In *Y. pestis*, the Hmu system is the primary system that enables the bacteria to use heme and heme-containing compounds as a source of iron (17, 41). However, *hmu* mutants can

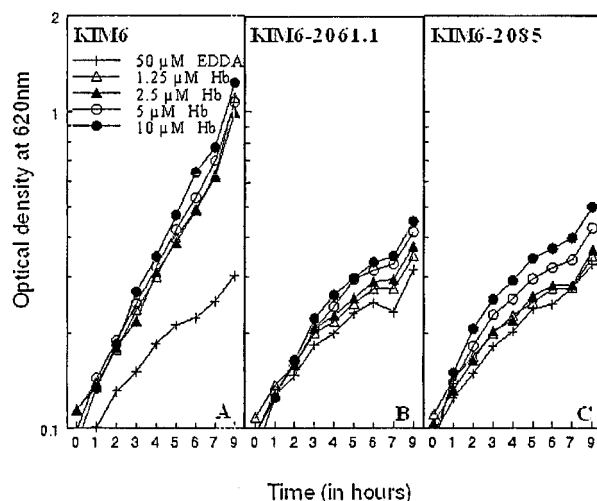


FIG. 7. Growth of *Y. pestis* KIM6 (*has⁺ hmu⁺*), KIM6-2061.1 (*has⁺ hmu* mutant), and KIM6-2085 (*has hmu* double mutant) cells at 28°C in deferrated PMH2 with hemoglobin as the sole iron source. Iron-depleted cells were transferred to PMH2 containing 50 μ M EDDA alone (+) or with hemoglobin at concentrations of 1.25 (Δ), 2.5 (\blacktriangle), 5 (\circ), or 10 (\bullet) μ M. These graphs depict one of two separate experiments with similar results.

still grow in the presence of high concentrations of hemoglobin, suggesting that there may be a second, lower-affinity system that the bacteria can use to obtain iron from hemoglobin. To determine if the Has system is responsible for this activity in *hmu* mutant strains, we examined the growth of KIM6 (*Ybt⁻ Has⁺ Hmu⁺*), KIM6-2061.1 (*Ybt⁻ Has⁺ Hmu⁻*), and KIM6-2085 (*Ybt⁻ Has⁻ Hmu⁻*) at 28°C in a defined medium containing 50 μ M EDDA and different concentrations of hemoglobin (Fig. 7). Similar results were obtained with cells grown at 37°C. KIM6 readily grew in this medium with as little as 1.25 μ M hemoglobin (Fig. 7A). However, the growth of KIM6-2061.1 (Fig. 7B) and KIM6-2085 (Fig. 7C) was not significantly stimulated by any tested concentration of hemoglobin. Furthermore, the growth of the *hmu* mutant was not significantly different from that of the *hmu has* mutant strain with any of the tested concentrations of hemoglobin. These results suggest that the Has system does not allow the bacteria to use hemoglobin as an iron source under the conditions tested in this study.

Virulence testing of *Y. pestis has* mutants. Previously, we had shown that a *Y. pestis* strain (KIM5-2060.21+) with a mutation in the hemin utilization system (*Hmu*) was fully virulent in mice by a subcutaneous route of infection (41). To determine if the *Y. pestis* Has system contributes to virulence in mammals, we constructed a *has hmu* double mutant, KIM6-2081.1+. The virulence plasmid with an ampicillin gene cassette inserted into the *yadA'*/*yadA* pseudogene (pCD1Ap) was electroporated into KIM6-2081.1+, generating KIM5-2081.1+. *Y. pestis* strains lacking the virulence plasmid are avirulent by any route of infection. Groups of four mice were infected subcutaneously with 10-fold dilutions of bacterial cells grown at 26°C. The LD₅₀s are listed in Table 4. The *has hmu* mutant was fully virulent by this route of infection, with an LD₅₀ identical to that of the wild-type strain [KIM5(pCD1Ap)+]. In contrast, a strain bearing a mutation in the receptor (*Psn*) for the sid-

TABLE 4. Virulence testing of *Y. pestis* derivatives in mice infected subcutaneously

Strain	Relevant characteristics	LD ₅₀ (CFU)
KIM5(pCDIAp)+	Psn ⁺ Hmu ⁺ Has ⁺ Lcr ⁺	<4.6
KIM5-2081.1+	Psn ⁺ Hmu ⁻ Has ⁻ Lcr ⁺	<4.2
KIM5-2045.7	Psn ⁻ Hmu ⁺ Has ⁺ Lcr ⁺	>5.4 × 10 ⁶

erophore yersiniabactin is completely avirulent. This result suggests that the heme uptake systems Hmu and Has do not play a role in the virulence of *Y. pestis* for mice, at least by a subcutaneous route of infection.

DISCUSSION

As a flea-mammal parasite, *Y. pestis* experiences a wide range of environmental conditions in which the ability to scavenge iron and heme contributes to its colonization potential and virulence. Accordingly, many inorganic iron or heme uptake systems in *Y. pestis* were characterized (Perry et al., submitted). The heme acquisition systems identified in *Y. enterocolitica* (HemRSTUV) and *Y. pestis* (HmuRSTUV) (17, 38, 39, 41) involve an outer membrane TonB-dependent receptor and a periplasmic binding protein transport apparatus. The availability of *Y. pestis* genome sequences allowed the identification of a potential second category of heme uptake system relying on type I secretion of an extracellular heme-binding protein hemophore called "HasA" (24, 41; Perry et al., submitted). Here we characterized the Has system of *Y. pestis* and studied its function in *E. coli* and *Y. pestis*.

The comparison of the four Has systems identified so far in *S. marcescens*, *P. aeruginosa*, *P. fluorescens*, and *Y. pestis* showed that the genetic organization of the *Y. pestis* Has system is closely related to the homologous system of *S. marcescens*. It encodes the outer membrane receptor (HasR) and the two inner membrane components (HasD and HasE) of the ABC transporter of the extracellular hemophore HasA. As in the *S. marcescens* *has* operon, a gene showing similarities in its deduced amino acid sequence to *tonB*, *hasB*, is located downstream of *hasE*. HasB was shown to be a TonB-like protein specifically dedicated to the HasA-dependent heme acquisition system (29a). A similar function may be associated with the *Y. pestis* protein HasB. A *hasB* equivalent was not found associated with the two other Has systems identified so far in *P. aeruginosa* and *P. fluorescens*.

Comparisons between *Y. pestis* HasA_{yp} and previously characterized hemophores revealed that they display relatively low levels of identity (33, 28, and 20% identity with HasA_{sm}, HasA_{pa}, and HasA_{pf}, respectively). Amino acid sequence comparisons showed that HasA_{yp} contains a 15-amino-acid insertion near the C terminus compared to HasA_{sm} (Fig. 2). This insertion was also found in *P. aeruginosa* and *P. fluorescens* HasA hemophores.

We showed here, by using a reconstituted secretion system in *E. coli*, that HasA_{yp} exhibits secretion and heme-binding properties very similar to those of HasA_{sm}. However, in vivo and in vitro experiments indicated that HasA_{yp} does not interact with HasR_{sm}. This lack of interaction between heterologous pairs of hemophores and the HasR receptor was previously investigated in the case of HasA_{pa} and HasR_{sm}, and it

was shown that the ability of HasA_{pf} to interact with HasR_{sm} depends on the removal of the C-terminal insertion. The additional stretch of 15 amino acids inserted at the C terminus of HasA_{pf} and HasA_{pa} was proposed to be responsible for the observed inhibition of heme delivery of HasA_{pf} or HasA_{pa} to HasR_{sm} (1, 24). While it may also be the case for HasA_{yp} (M. S. Rossi and J. M. Ghigo, unpublished results), these data further support the hypothesis that HasR-HasA couples constitute bipartite receptor entities that closely interact. These interactions may well be highly species specific, preventing eventual detrimental cross talk between hemophore-dependent systems expressed by two different bacterial species. Further characterization of functional HasR receptors may help clarify this point.

We showed that HasA_{yp} expressed from pHasA_{yp} can be secreted through a heterologous transporter in complementation experiments with the *S. marcescens* Has transporter. However, the introduction in *E. coli* of the whole *Y. pestis* *has* region led neither to HasA secretion nor to the reconstitution of a functional heme acquisition system. In particular, secretion studies did not provide evidence that the *Y. pestis* transporter components HasD_{yp} and HasE_{yp} are functional in *E. coli*. However, the lack of in vivo function in *E. coli* cannot be explained only by a HasA_{yp} secretion defect, since a heme-deficient strain expressing HasR_{yp} and HasA_{yp} was unable to acquire heme, even at a high hemoglobin concentration at which *hasR*-alone-dependent heme acquisition should be observed. This suggests either that HasR is not properly expressed at the cell surface or, less trivially, that other Has-specific components are missing in these heterologous experiments. These components may be required either for the proper function of the receptor or for further heme internalization.

Previous studies demonstrated that Has systems are iron regulated, in good agreement with the identification of Fur binding sites (or "Fur boxes") in these systems (21, 25, 29). Fur boxes are found in the promoter regions of iron-regulated genes and constitute fixation sites for the DNA-binding repressor Fur, which tightly regulates (represses) these genes when associated with ferrous iron as a corepressor. In *Y. pestis*, such a potential Fur binding site, albeit unusually distant upstream of the start codon for *hasR*, was also identified. Moreover, the use of a reporter plasmid where the *hasRADEB* putative promoter region was placed upstream of a promoterless *lacZ* gene showed that the *Y. pestis* *has* operon is active and iron regulated through a Fur-dependent control mechanism. We therefore tested and probed the supernatants of various *Y. pestis* 6/69 and KIM6 derivatives with anti-HasA_{yp} antibodies by using cells grown in iron-chelated synthetic and rich media at 28 and 37°C. None of these conditions allowed the detection of an extracellular HasA_{yp}. Although we provided evidence that the *has* operon is at least partially expressed in *E. coli* and that the *has* promoter is active and iron inducible when introduced in *Y. pestis*, we cannot formally rule out the possibility that the observed lack of function comes from the absence of expression of some of the components of the *Y. pestis* Has system. Such situations in which genes potentially involved in *Yersinia* environmental fitness are nonfunctional in *Y. pestis* have been reported (34). However, neither a frameshift mutation nor an obvious deletion was seen in the genes of the *hasRADEB* *Y. pestis* putative operon.

Previous studies suggested that, in addition to the Hmu system, there may be a second, lower-affinity system that *Y. pestis* can use to obtain iron from hemoglobin. However, the growth study presented here suggests that the Has system does not allow the bacteria to use hemoglobin as an iron source. The results obtained with the $\Delta hmuP'RSTUV$ mutant differ from those of earlier studies in which a graded response to increasing concentrations of hemoglobin was observed (41). Previous experiments used *Y. pestis* strains that produced the siderophore yersiniabactin (Ybt). Thus, the previous results with the $\Delta hmuP'RSTUV$ mutant were probably due to Ybt-mediated acquisition of iron from either hemoglobin degradation products or the EDDA-Fe complex, and not to the existence of a functional low-affinity heme transport system.

The recognition that an intact functional yersiniabactin system is a prerequisite for mouse virulence in the early stages of infection emphasized the fact that iron acquisition is critical for the progression of *Y. pestis* infections (30a). Our study, with a *has hmu* double mutant, constitutes the first investigation of the contribution of a hemophore-dependent system to bacterial pathogenicity. Our results with subcutaneous infection in mice suggest that the Has heme uptake system, as shown for the Hmu system, does not play a role in *Y. pestis* virulence in the mouse model of bubonic plague. Further experiments will be required to determine whether other routes of infection or infection in other animal hosts rely on Hmu and/or Has heme acquisition systems.

Taken together, our results suggest that a plausible reason for the absence of a HasA-related phenotype in *Y. pestis* may be that induction of the Has system was not appropriate in our experimental model and under the conditions used. While our data indicate that iron starvation participates in the regulation of the Has system, other factors may have to be considered. Comparison between HasR homologues revealed that HasR_{yp} lacks an N-terminal extension present in HasR_{sm} and other hemophore-dependent receptors (Fig. 2A) (data not shown). In *E. coli*, the amino terminus of the FecA outer membrane receptor is involved in regulating the Fec iron-dicitrate acquisition system (4, 19). Thus, regulation of the Has_{yp} system may be different from those of other Has systems and could require other factors. We noted the presence of strong stem-loop structures in the *hasRADEB* promoter region. Moreover, we showed that this promoter displays an increased *hasRADEB* promoter activity at 37°C compared to 26°C. This suggests that expression of the Has system may be temperature responsive, as shown to be the case for several bacterial virulence genes (20).

This study suggests that two distinct heme acquisition systems may coexist in *Y. pestis* and in other *Yersinia* species, supporting the hypothesis that hemophore-dependent heme acquisition systems are widespread in members of the family *Enterobacteriaceae*. While some of these systems have now been characterized at the structural and molecular level, their role in environmental survival and growth of gram-negative bacteria still awaits conclusive experimental demonstration.

ACKNOWLEDGMENTS

M.-S. Rossi was supported by the Conselho Nacional de Pesquisa (CNPq) grant no. 201031/97-3, Brazil. Work on this study by R.D.P. and J.D.F. was supported by Public Health Service grant AI-25098.

J.-M.G., S.L., and E.C. were supported by the Institut Pasteur, Paris, France.

We are grateful to Jan Thompson and Steve Zink for initial work on the Has system in *Y. pestis* KIM6+ and to Cécile Wandersman for helpful discussions and critical reading of the manuscript.

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Editor: J. T. Barbieri