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Cloning and Characterization of the *Pseudomonas fluorescens* ATP-Binding Cassette Exporter, HasDEF, for the Heme Acquisition Protein HasA

AKIKO IDEI,[1] ERI KAWAI,[1] HIROYUKI AKATSUKA,[1] AND KENJI OMORI[2]*

Discovery Research Laboratory, Tanabe Seiyaku Co., Ltd., Yodogawa-ku, Osaka 532-8505,[1] and Toda, Saitama 335-8505,[2] Japan

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Two ATP-binding cassette (ABC) exporters are present in *Pseudomonas fluorescens* no. 33; one is the recently reported AprDEF system and the other is HasDEF, which exports a heme acquisition protein, HasA. The *hasDEF* genes were cloned by DNA hybridization with a DNA probe coding for the LipB protein, one of the components of the *Serratia marcescens* ABC exporter Lip system. *P. fluorescens* HasA showed sequence identity of 40 to 49% with HasA proteins from *Pseudomonas aeruginosa* and *Serratia marcescens*. The *P. fluorescens* Has exporter secreted HasA proteins from *P. fluorescens* and *P. aeruginosa* but not *S. marcescens* HasA in *Escherichia coli*, whereas the Has exporter from *S. marcescens* allowed secretion of all three HasA proteins. The *P. fluorescens* HasDEF system also promoted the secretion of the lipase and alkaline protease of *P. fluorescens*. Hybrid exporter analysis demonstrated that the HasD proteins, which are ABC proteins, are involved in the discrimination of export substrates. Chimeric HasA proteins containing both *P. fluorescens* and *S. marcescens* sequences were produced and tested for secretion through the Has exporters. The C-terminal region of HasA was shown to be involved in the secretion specificity of the *P. fluorescens* Has exporter.

Many transport systems are involved in protein translocation across the cell membranes in gram-negative bacteria. One is the ATP-binding cassette (ABC) exporters, which are termed type I (6, 34). This system mediates a one-step secretion of proteins. The secretion differs from that through the *sec* gene-mediated pathway in that the secretory proteins lack an N-terminal signal sequence. The secretion system is composed of three specific components. The first component is situated in the inner membrane and belongs to the ABC transporters (17, 27). The second component is a member of the membrane fusion protein (MFP) family (8) and is a transport accessory protein that may associate with both the inner and outer membranes. The third component is an outer membrane protein (OMP).

ABC exporters translocate various polypeptides. One example is the *Serratia marcescens* Has_{SM} system (23) composed of $HasD_{SM}$ (ABC protein), $HasE_{SM}$ (MFP), and $HasF_{SM}$ (OMP) that promote secretion of the heme-binding protein HasA ($HasA_{SM}$) (25). *S. marcescens* also possesses another ABC exporter, the Lip system (LipB-LipC-LipD) (2), which mediates secretion of three unrelated proteins: the lipase $LipA_{SM}$ (1), a metalloprotease, and the cell surface layer protein homologue SlaA. The structural gene of the secretory protein is usually linked to the genes encoding its specific ABC exporter. In *S. marcescens*, the genes for $HasA_{SM}$, $HasD_{SM}$, and $HasE_{SM}$ are encoded in the *has* operon (23) but the gene coding for $HasF_{SM}$ is located in another locus (5). Three components of the Lip system are encoded in the *lipBCD* operon, and the gene for SlaA is situated upstream of the operon (20).

Many proteins secreted through the ABC exporters possess a common C-terminal secretory signal. Some of them can be secreted by heterologous ABC exporters. For examples, the *Erwinia chrysanthemi* metalloprotease PrtC can be secreted via the Has_{SM} and Lip systems (3, 4), the *Pseudomonas aeruginosa* Apr_{PA} system ($AprD_{PA}$-$AprE_{PA}$-$AprF_{PA}$) (14) mediates secretion of the *Pseudomonas fluorescens* lipase $LipA_{PF}$ (9), and the *E. chrysanthemi* Prt system (PrtD-PrtE-PrtF) (22) promotes secretion of the *P. aeruginosa* alkaline protease $AprA_{PA}$ (10). However, efficient secretion of the proteins with the C-terminal signal through heterologous ABC exporters is not always possible. $HasA_{SM}$ cannot be secreted via the Prt and Lip exporters (3, 4). Likewise, $LipA_{SM}$ is secreted neither by the reconstituted Has_{SM} system in *Escherichia coli* cells nor by the native Has_{SM} system in *S. marcescens* (3). Analysis of hybrid exporters comprising components from ABC exporters revealed that one determinant of substrate specificity is the ABC protein (3, 4). Novel ABC exporters or secretory proteins showing unique secretion specificities would be useful tools for the analysis of the mechanism of substrate selectivity.

Among the secretory proteins exported through ABC exporters, HasA shows a unique secretion profile. Two HasA proteins have been identified from *S. marcescens* and *P. aeruginosa* (24, 26). Secretion of HasA is specific, and the Has_{SM} system is the only one known. The *P. aeruginosa* Has exporter has been predicted but not identified yet. Since *P. fluorescens* belongs to the same rRNA homology group as *P. aeruginosa* (29), the presence of the Has system was expected in *P. fluorescens*. In this paper, we describe a Has system including a HasA protein and an ABC exporter from *P. fluorescens*. Specificity of HasA secretion through the Has exporter of this bacterium was studied by using chimeric HasA proteins.

MATERIALS AND METHODS

Strains, plasmids, and media. *E. coli* K-12 DH5 (31) and *P. fluorescens* no. 33 (21) were used. The plasmids pSYC1000 (26), pUC/PFLipA13 (19), and pAK42 (19) encoding the *P. aeruginosa* HasA ($HasA_{PA}$), *P. fluorescens* LipA ($LipA_{PF}$), and *P. fluorescens* AprA ($AprA_{PF}$) proteins, respectively, were used for secretion analysis. The $aprDEF_{PF}$ plasmid pACYC/AK60 was described previously (19). Luria-Bertani medium (31) was used for *E. coli* and *P. fluorescens* cells. Antibi-

* Corresponding author. Mailing address: Discovery Research Laboratory, Tanabe Seiyaku Co., Ltd., 2-50, Kawagishi-2-chome, Toda, Saitama 335-8505, Japan. Phone: (81-48) 433-8069. Fax: (81-48) 433-8159. E-mail: k-omori@tanabe.co.jp.

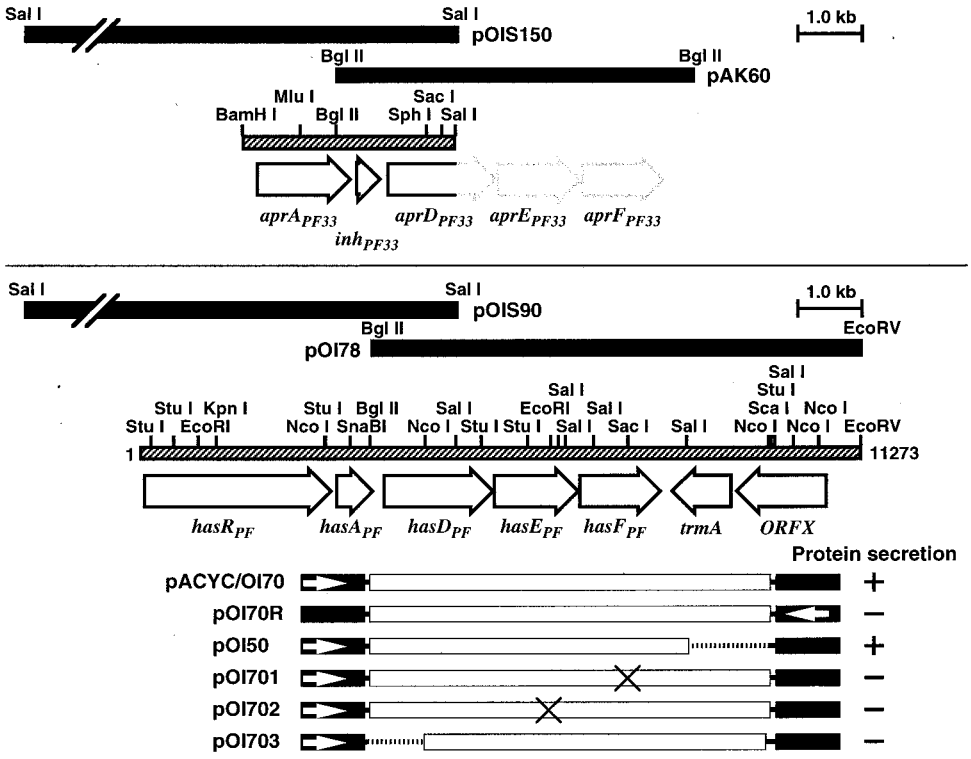

FIG. 1. Physical maps of inserted DNA. The black bars and hatched bars represent the chromosomal DNA inserts and the regions for which the nucleotide sequences were determined, respectively. The plasmids pOIS150 and pOIS90 are shown in the upper and lower panels, respectively. The open arrows indicate ORFs. The plasmids encoding the $HasDEF_{PF}$ exporter mutants are shown below. The open and solid boxes represent the inserted DNA and the pACYC184 DNA, respectively. The open arrows show the *tet* promoter of pACYC184. The crosses and dotted lines indicate the positions of the mutations introduced and deletions, respectively. The ability to secrete $HasA_{PF}$ (+) is shown on the right.

otics were added at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; and chloramphenicol, 20 μg/ml.

General methods. DNA manipulations and hybridization analysis were carried out according to standard procedures (31). PCR was carried out through 30 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 30 s with ExTaq purchased from Takara Shuzo (Kyoto, Japan). After being subcloned into pUC18 (31), pUC19 (31), pHSG299 (33), and pBluescript II SK(+) (31), the nucleotide sequence was determined with an automated DNA sequencer model 373A and a dideoxy chain termination procedure with fluorescence-labeled primers according to a protocol of the manufacturer (Perkin-Elmer Applied Biosystems). Nucleotide and amino acid sequence data were analyzed with the computer program GENETYX (Software Development, Tokyo, Japan).

Southern blot analysis. The *P. fluorescens* chromosomal DNA was digested with one or a combination of the following restriction enzymes: *Eco*RI, *Hin*dIII, *Bam*HI, *Sal*I, and *Eco*RV. From each digested DNA, 10 μg was separated on a 0.7% agarose gel and then transferred onto a nylon membrane. The 0.9-kb *Pst*I fragment of the *S. marcescens lipB* gene was prepared from pMWBCD10 (2). The blot was hybridized with the ^{32}P-labeled DNA fragment at 55°C for 16 h and washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate (SDS) at room temperature, followed by a 5-min wash in 2× SSC–0.5% SDS at 45°C (low-stringency conditions). The filter was exposed to X-ray film at −70°C for 2 days. To confirm that the DNA fragment was related to the $aprD_{PF}$ gene, the 1.7-kb *Bgl*II-*Sac*I fragment encoding the $AprD_{PF}$ N-terminal half was prepared from pAK42 (19). The blot was hybridized with the ^{32}P-labeled probe at 65°C for 16 h and washed twice in 0.1× SSC–0.5% SDS at room temperature, followed by two 10-min washes in 0.1× SSC–0.5% SDS at 65°C (high-stringency conditions), and then exposed to X-ray film at −70°C overnight.

Cloning of the ABC exporter genes from *P. fluorescens*. A genomic DNA library was constructed in *E. coli* with ligation of 1 μg of *Sal*I-digested pUC19 and 10 μg of 9- to 23-kb *Sal*I-digested *P. fluorescens* chromosomal DNA. The clones were isolated by colony hybridization with the above-described ^{32}P-labeled *lipB* fragment under low-stringency conditions. To isolate all of the *P. fluorescens* ABC exporter genes, a genomic DNA library was constructed in *E. coli* with ligation of 1 μg of *Bam*HI-*Eco*RV-digested pACYC184 (31) and 10 μg of *Bgl*II-*Eco*RV-digested *P. fluorescens* chromosomal DNA (6.4 to 9.7 kb). pOI78 was obtained from the library as the clone that hybridized with the ^{32}P-labeled *Sal*I-*Bgl*II (1.4-kb) fragment from pOIS90.

Plasmid construction. The $HasDEF_{PF}$ plasmids pACYC/OI70 and pOI70R carrying the 6.4-kb *Bgl*II-*Stu*I $hasDEF_{PF}$ fragment (blunt ended) were constructed in the *Eco*RV site of pACYC184 in the same and opposite orientations as the *tet* gene, respectively (Fig. 1). Deletion of the 1.4-kb *Sal*I-*Stu*I fragment from pACYC/OI70 produced pOI50. pOI701 and pOI702 were generated from pACYC/OI70 by destroying the *Sac*I and *Eco*RI sites in the $hasF_{PF}$ and $hasE_{PF}$ genes, respectively. The $hasEF_{PF}$ plasmid pOI703 was produced by ligating the 5.4-kb *Nco*I fragment (blunt ended) with the *Eco*RV-digested pACYC184. Construction of the $hasD_{PF}$ plasmid pACYC/$HasD_{PF}$ was done by introducing the *Hin*dIII and blunt-ended *Eco*RI fragments of pACYC/OI70 into the *Hin*dIII-*Eco*RV-digested pACYC184. The 6.5-kb *Hin*dIII-*Sca*I $hasDEF_{PF}$ fragment of pACYC/OI70 was ligated with the *Hin*dIII-*Hin*cII-digested pMW219 (36), and then the 2.1-kb *Hin*dIII-*Not*I fragment was removed, resulting in the $hasEF_{PF}$ plasmid pMW/$HasEF_{PF}$. The plasmids pACYC/$HasD_{SM}$ and pMW/$HasE_{SM}$ containing the $hasD_{SM}$ and $hasE_{SM}$ genes in pACYC184 and pMW218 (36), respectively, were produced from pMW/HasDE7 carrying the $hasDE_{SM}$ genes of *S. marcescens* 8000 (unpublished data).

Construction of HasA plasmids. Inserting the 4.1-kb *Eco*RI-*Sal*I fragment of pOIS90 into the corresponding sites of pUC18 produced the $hasA_{PF}$ plasmid pUC/$HasA_{PF}$. The 0.6-kb DNA fragment containing the $hasA_{SM}$ gene was amplified by PCR with a primer set (5′-GGGAATTCTAATTCATCAATGGAGA TAGAGAAATG-3′ and 5′-GGGGATCCGGCGGGCAAACGGCCGCGATC AGG-3′) and pSYC134 (25) as a template. After digestion with *Eco*RI and *Bam*HI, the fragment was inserted into the corresponding sites of pUC18, generating pUC/$HasA_{SM}$. The plasmids encoding chimeras between $HasA_{SM}$ and $HasA_{PF}$ were produced by creating the *Xho*I sites at Leu-Glu (amino acid residues 153 to 154 in $HasA_{PF}$ and amino acid residues 147 to 148 in $HasA_{SM}$) by PCR with pUC/$HasA_{SM}$ and pUC/$HasA_{PF}$ as templates (Fig. 2). The fragments encoding the N-terminal regions of $HasA_{SM}$ and $HasA_{PF}$ were generated with the primer sets RV primer (5′-CAGGAAACAGCTATGAC-3′) plus 5′-C CGGATCCCTCGAGCGCGCCGGTATCGCCGGACATCAGG-3′) and 5′-G CGAATTCGAGGTTAAGTGATGACTATTTCTG-3′ plus 5′-CCGGATCCC TCGAGTGCCGAGGTGTTGCCTTGCATCAG-3′, respectively. The *Eco*RI-*Bam*HI-digested PCR products (ca. 0.45 kb) were introduced into the corresponding sites of pUC18, resulting in pMX and pFX encoding the $HasA_{SM}$ and $HasA_{PF}$ N-terminal regions with the *Xho*I site, respectively. The DNA fragments coding for the $HasA_{SM}$ and $HasA_{PF}$ C-terminal regions were obtained by the primer sets 5′-TTCCTCGAGACCGCGCTGAACGGCATCCTCGACG ACTA-3′ plus 5′-GTTTTCCCAGTCACGAC-3′ and 5′-GGGAATTCCTCGA

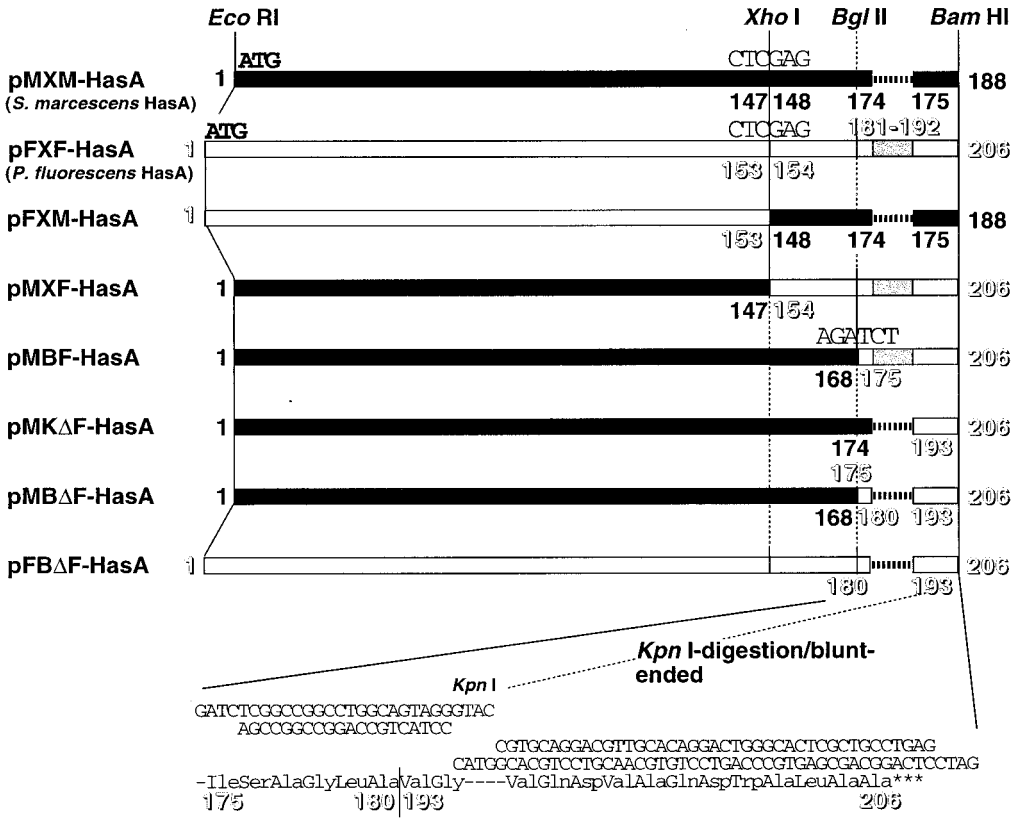

FIG. 2. Construction of the HasA chimera plasmids encoding HasA chimeras between $HasA_{SM}$ and $HasA_{PF}$. The construction of the plasmids is illustrated schematically. The solid and open boxes represent amino acid sequences of $HasA_{SM}$ and $HasA_{PF}$, respectively. The $HasA_{PF}$ C-terminal inserts are shaded. The amino acid residue numbers of the $HasA_{PF}$ sequence are shown in outline. The *Xho*I, *Bgl*II, and *Kpn*I sites introduced are shown. The synthetic oligonucleotides encoding the C-terminal sequences of $HasA_{PF}$ (amino acid residues 175 to 180 and 193 to 206), which are introduced in pMKΔF-HasA, pMBΔF-HasA, and pFBΔF-HasA, are shown, with a deduced amino acid sequence below. The dashed lines indicate gaps to maximize homology at the C terminus.

GACCGTGCTCAACAACCTGCTGGACG-3′ plus 5′-CCGGATCCTCAGGC AGCGAGTGCCCAGTCCTG-3′, respectively. The amplified fragments were cloned into pMX and pFX by using the *Xho*I and *Bam*HI sites, producing four HasA plasmids, pMXM-HasA, pMXF-HasA, pFXM-HasA, and pFXF-HasA (Fig. 2).

The DNA fragment encoding $HasA_{SM}$ (amino acid residues 1 to 168) was produced by PCR with the RV primer, the primer 5′-GGAGATCTGATCGA AGGTGGAGTTGACGC-3′, and the template DNA pUC/$HasA_{SM}$. The amplified fragment (0.5 kb) was digested by *Eco*RI and *Bgl*II and then introduced into the corresponding sites of pFXF-HasA, resulting in pMBF-HasA. The synthetic oligonucleotides 5′-CGTGCAGGACGTTGCACAGGACTGGGCAC TCGCTGCCTGAG-3′ and 5′-GATCCTCAGGCAGCGAGTGCCCAGTCCT GTGCAACGTCCTGCACGGTAC-3′, encoding the $HasA_{PF}$ C terminus (amino acid residues 195 to 206) were inserted into the *Kpn*I and *Bam*HI sites of pUC18, generating pUC/PFlinker12. The DNA encoding $HasA_{SM}$ (amino acid residues 1 to 175) was produced by PCR with the RV primer and the primer 5′-CCGGTACCCCACCGCCGTCGCCGCCGCCAC-3′ and the template DNA pUC/$HasA_{SM}$. The PCR product was digested with *Eco*RI and *Kpn*I and introduced into the corresponding sites of pUC/PFlinker12. The *Kpn*I site of the resultant plasmid was destroyed to produce pUC/MKΔF-HasA.

The pMBΔF-HasA plasmid was created as follows: the 0.95-kb *Sca*I-*Kpn*I fragment of pUC/PFlinker12 was ligated with the 2.2-kb *Sca*I-*Bgl*II fragment of pMBF-HasA and the *Bgl*II-*Kpn*I linker 5′-GATCTCGGCCGGCCTGGCAGT AGGGTAC-3′ plus 5′-CCTACTGCCAGGCCGGCCGA-3′. The resultant ampicillin-resistant plasmid was digested with *Kpn*I and then blunt ended to produce pMBΔF-HasA. The plasmid encoding $HasA_{PF}$ lacking the C-terminal insert (pFBΔF-HasA) was produced by inserting the 0.5-kb *Eco*RI-*Bgl*II fragment of pFXF-HasA into the corresponding sites of pMBΔF-HasA.

The nucleotide sequences of all of these *hasA* genes were confirmed by sequencing. These *hasA* genes were expressed under control of the *lacZ* promoter of pUC18.

Analysis of protein secretion. The *E. coli* cells carrying the plasmids coding for the exporter and secretory proteins were cultured in Luria-Bertani medium at 30°C for 40 h with vigorous shaking. The polypeptides in the supernatants were precipitated with trichloroacetic acid at a final concentration of 10% and were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Protein analysis was carried out independently three times, and similar results were confirmed. β-Galactosidase activity was measured as described by Miller (28) to assess cell lysis. The levels of extracellular β-galactosidase activity were <0.5% of that of whole cells, indicating no significant cell lysis.

SDS-PAGE and immunoblot analysis. The precast gel PAGEL (12.5%) (ATTO, Tokyo, Japan) was used for SDS-PAGE. The proteins in gels were stained by Coomassie brilliant blue G-250 or electrophoretically transferred to an Immobilon P filter (Millipore) for immunodetection. Peptide corresponding to amino acid residues 41 to 60 of $HasA_{PF}$ was synthesized, and the antiserum was obtained by injecting rabbits with the peptide in Freund's complete adjuvant (15). The antiserum to $LipA_{SM}$ (1) was used for detection of $LipA_{PF}$. The antibody against $HasA_{SM}$ was a generous gift of Cécile Wandersman. The antiserum raised against *P. fluorescens* AprA was kindly provided by Tamotsu Hoshino. The blots were blocked by soaking them in Block Ace (Dainippon Pharmaceutical, Osaka, Japan) overnight at 4°C and incubated with antiserum at room temperature for 2 h (diluted 1:1,000 to 1:4,000 in phosphate-buffered saline containing 0.1% Tween 20). They were washed and incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G, and the bound antibody was detected with the enhanced chemiluminescence system (Amersham).

Nucleotide sequence accession number. The *has$RADEF_{PF}$* sequence has been submitted to the GenBank, EMBL, and DDBJ databases with accession no. AB023289.

RESULTS

Cloning of the ABC exporter homologue genes from *P. fluorescens*. The ABC proteins of the Prt, Lip, Has_{SM}, and Apr_{PA} systems have 54 to 60% identity. Therefore, Southern analysis of the isolated *P. fluorescens* chromosomal DNA was performed with the ^{32}P-labeled *lipB* fragment encoding the N-terminal portion well conserved among ABC proteins under low-stringency conditions. Two positive signals were consis-

tently detected, irrespective of the enzyme used (data not shown). Hybridization analysis revealed that one of the signals was found to come from the $aprD_{PF}$ DNA fragment. To identify the origin of the other signal, a genomic DNA library of *P. fluorescens* was constructed. Two clones, pOIS90 and pOIS150, which contained *Sal*I fragments of 9 and 15 kb, respectively, with different restriction patterns, were isolated by colony hybridization with the *lipB* probe under low-stringency conditions (Fig. 1).

Nucleotide sequence analysis revealed that pOIS150 and pOIS90 encode a part of the apr_{PF} operon (19) and the N-terminal-to-central region of a novel ABC protein, respectively. The entire complex of novel ABC exporter genes was isolated as the 7.8-kb *Bgl*II-*Eco*RV fragment cloned into pACYC184 (pOI78 [Fig. 1]). Southern hybridization revealed that the cloned fragments came from the *P. fluorescens* chromosomal DNA without rearrangement (data not shown).

Nucleotide sequence analysis of the new ABC exporter genes. The inserted DNA fragment of pOI78 contained five open reading frames (ORFs) predicted to encode proteins of 580 (M_r, 61,803), 443 (M_r, 48,491), 439 (M_r, 48,658), 363 (M_r, 41,240), and 431 (M_r, 44,894) amino acid residues. The first three ORFs are contiguous, and the last two ORFs are located on the opposite strand (Fig. 1). A putative ribosome binding site (32) is present upstream of each ORF. The ORF1 product possessed features of ABC proteins, which are ATP-binding and ABC motifs, and was 67, 60, 62, 59, and 60% identical to $HasD_{SM}$, $AprD_{PF}$, $AprD_{PA}$, PrtD, and LipB proteins, respectively. The ORF2 product showed 50 to 52% identity to the MFPs of the exporters. The ORF3 product, a putative OMP, was 57, 58, 52, 54, 22, and 26% identical to $AprF_{PF}$, $AprF_{PA}$, PrtF, LipD, TolC (35), and $HasF_{SM}$, respectively.

Upstream of the exporter genes, two ORFs encoding proteins of 206 (M_r, 21,243) and 916 (M_r, 101,186) amino acid residues were found (Fig. 1). The product of the former ORF ($hasA_{PF}$) was a homologue of the heme-binding proteins, showing 41 and 40% identity with $HasA_{SM}$ and $HasA_{PA}$, respectively. The latter (the $hasR_{PF}$ gene product) was 43% identical to *S. marcescens* $HasR_{SM}$ (12), which is an OMP capable of binding HasA. On the basis of this gene organization and the secretion function described below, the exporter genes downstream of the $hasRA_{PF}$ genes were designated $hasD_{PF}$, $hasE_{PF}$, and $hasF_{PF}$, respectively. No obvious promoter sequence (16) was found upstream of or within the $hasRADEF_{PF}$ complex. Thus, *P. fluorescens* appears to possess a heme acquisition system and at least two kinds of ABC exporter, $AprDEF_{PF}$ and $HasDEF_{PF}$.

The $hasRADEF_{PF}$ genes were not followed by a typical rho-independent terminator (30), and the downstream ORF was in the opposite orientation. The product of this ORF was 48% identical to tRNA (uracil-5-)-methyltransferases (EC 2.1.1.35) from *E. coli* (13), and the ORF was designated *trmA*. ORFX, predicted to encode a protein that was 52% identical to hypothetical proteins reported in *E. coli* (7), was found upstream of the *trmA* gene in the same orientation as *trmA*.

Protein secretion through the *P. fluorescens* ABC exporter. The abilities of Apr_{PF} and Has_{PF} to secrete protein were examined in recombinant *E. coli* (Fig. 3). First, we investigated secretion of $HasA_{PF}$. $HasA_{PF}$ secretion was promoted by the Has_{PF} exporter, but secretion of $HasA_{PF}$ through Apr_{PF} encoded by pACYC/AK60 (19) was not detectable even on immunoblot analysis with the antiserum against $HasA_{PF}$ (data not shown). The Has_{PF} exporter plasmids lacking one of the three components (pOI701, pOI702, or pOI703) were unable to secrete $HasA_{PF}$ (Fig. 1). Since pOI701 deficient in the gene

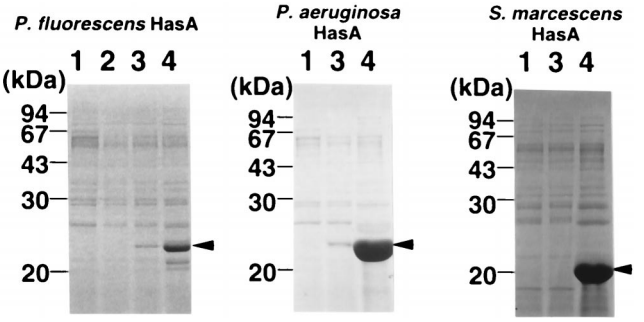

FIG. 3. HasA secretion through the Has exporters. The plasmids $pUC/HasA_{PF}$ (left), pSYC1000 (middle), and $pUC/HasA_{SM}$ (right) encoding the HasA proteins from *P. fluorescens*, *P. aeruginosa*, and *S. marcescens*, respectively, were introduced into the *E. coli* cells carrying the Apr_{PF} (pACYC/AK60), Has_{PF} (pACYC/OI70), and Has_{SM} (pK150) exporters. The polypeptides in the supernatants of the cultured media (1.5 optical density equivalents) were concentrated and then subjected to SDS-PAGE (12.5% acrylamide). The gels were stained with Coomassie brilliant blue G-250. The arrowheads indicate the positions of the HasA proteins, and the positions of molecular mass markers are shown on the left of each gel. Lanes 1, pACYC184; lane 2, pACYC/AK60; lanes 3, pACYC/OI70; lanes 4, pK150.

for the OMP $HasF_{PF}$ did not allow $HasA_{PF}$ secretion in *E. coli* DH5 (carrying the *tolC* gene), $HasF_{PF}$ function cannot be replaced by the *E. coli* TolC. pOI70R did not direct the *E. coli* cells to secrete $HasA_{PF}$, showing that expression of $hasDEF_{PF}$ is driven by an exogenous promoter, P_{tet} in pACYC184. Interestingly, the Has_{PF} exporter also secreted $HasA_{PA}$ but not $HasA_{SM}$. On the other hand, the Has_{SM} system encoded by pK150 (4) secreted $HasA_{PF}$, $HasA_{PA}$, and $HasA_{SM}$ (Fig. 3). The levels of HasA secretion through the Has_{PF} exporter were low compared with those through the Has_{SM} exporter.

In addition to HasA secretion, the Has_{PF} exporter also promoted secretion of the *P. fluorescens* lipase $LipA_{PF}$ and alkaline protease $AprA_{PF}$ in *E. coli* cultured at 30°C (Fig. 4). Under these conditions, the Apr_{PF} exporter, which is temperature sensitive (19), failed to secrete these proteins. Interestingly, $LipA_{PF}$ was secreted through the Has_{SM} exporter although the exporter did not promote secretion of $LipA_{SM}$, which is 66% identical to $LipA_{PF}$.

Secretion analysis of the hybrid Has exporters. To investigate the functional overlap of two Has exporters, Has_{PF} and Has_{SM}, secretion analysis was carried out with hybrid exporters having components from each system (Fig. 5). The $hasD_{SM}$ or $hasD_{PF}$ plasmid and the $hasE_{SM}$ or $hasEF_{PF}$ plasmid were introduced into the *E. coli* cells carrying the plasmids encoding $LipA_{PF}$, $HasA_{PF}$, and $HasA_{SM}$. Only exporters composed of components from the same system ($HasD_{SM}$-$HasE_{SM}$-TolC or $HasD_{PF}$-$HasE_{PF}$-$HasF_{PF}$) were functional for $LipA_{PF}$ and $HasA_{PF}$ secretion (Fig. 5A and B, lanes 3 and 6). The hybrid exporter $HasD_{SM}$-$HasE_{PF}$-$HasF_{PF}$ allowed $HasA_{SM}$ secretion at a very low level (3%) compared with that through $HasD_{SM}$-$HasE_{SM}$-TolC (Fig. 5C, lanes 3 and 4); all other hybrid systems were nonfunctional. The finding that $HasE_{PF}$ and $HasF_{PF}$ operated as an MFP and an OMP for $HasA_{SM}$ secretion, respectively, albeit very inefficiently, suggested that $HasD_{PF}$ plays a key role for substrate specificity in the Has_{PF} system.

Chimeric analysis of HasA secretion through the Has exporter. The most notable difference among the HasA sequences in the three species is the apparent deletion, in $HasA_{SM}$, of a segment close to the C terminus corresponding to amino acid residues 181 to 192 of $HasA_{PF}$ (Fig. 2). To investigate whether the substrate specificity of the Has_{PF} exporters on HasA secretion depends on this sequence diversity,

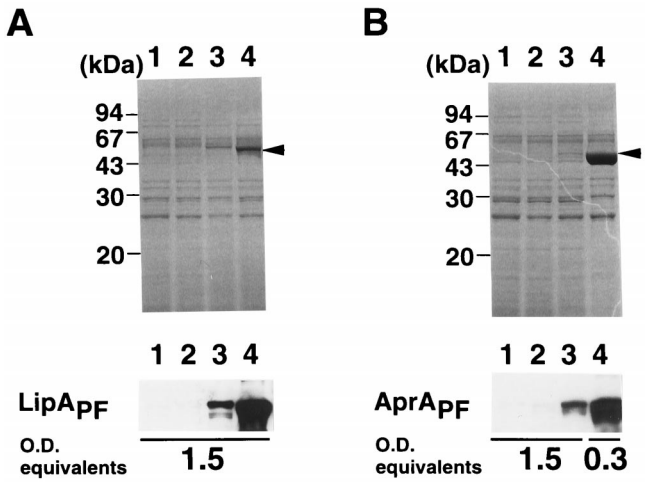

FIG. 4. Secretion of LipA$_{PF}$ (A) and AprA$_{PF}$ (B) through the Has exporters. *E. coli* cells carrying LipA$_{PF}$ and AprA$_{PF}$ plasmids (pUC/PFLipA13 and pAK42, respectively) with the various exporters were cultured and tested for protein secretion as described in the legend to Fig. 3. The proteins were visualized by Coomassie brilliant blue G-250 (upper gels) and analyzed by immunoblotting with antisera against LipA$_{SM}$ and AprA$_{PF}$ (lower gels). The arrowheads indicate the positions of the LipA$_{PF}$ and AprA$_{PF}$ proteins, and the positions of molecular mass markers are shown on the left of each gel. The positions of molecular mass markers are shown on the left of the upper gels. Optical density (O.D.) equivalents are indicated below. Lanes 1, pACYC184; lanes 2, pACYC/AK60; lanes 3, pACYC/OI70; lanes 4, pK150.

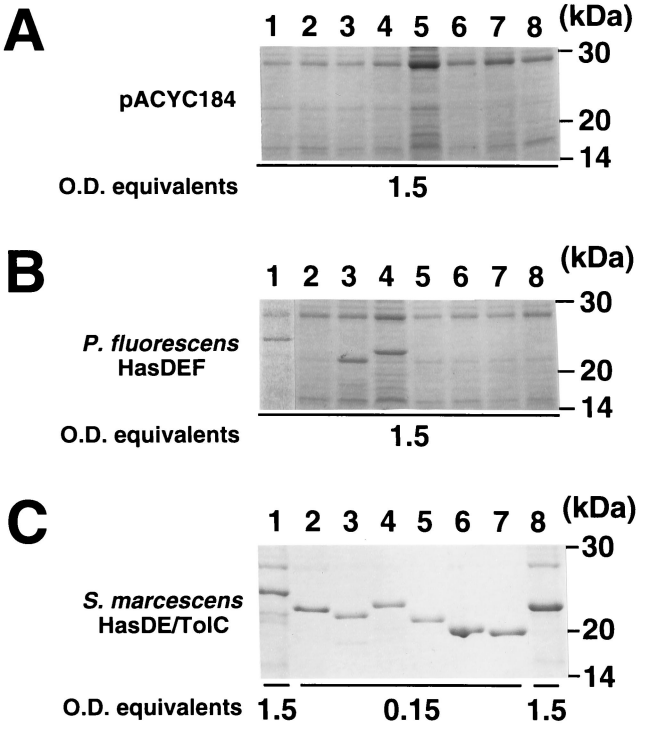

FIG. 6. Secretion of chimeras between HasA$_{SM}$ and HasA$_{PF}$. Eight *hasA* plasmids encoding HasA$_{SM}$ and HasA$_{PF}$ and six HasA chimeras were introduced into the *E. coli* cells carrying pACYC184 (A), pK150 (B), and pACYC/OI70 (C). The cells were cultured and tested for protein secretion as described in the legend to Fig. 3. The gels were stained with Coomassie brilliant blue G-250. Optical density (O.D.) equivalents are indicated below each gel. Lanes 1, pFXF-HasA; lanes 2, pFXM-HasA; lanes 3, pMXF-HasA; lanes 4, pMBF-HasA; lanes 5, pMBΔF-HasA; lanes 6, pMKΔF-HasA; lanes 7, pMXM-HasA; lanes 8, pFBΔF-HasA.

we constructed chimeras containing the HasA$_{SM}$ and HasA$_{PF}$ sequences and examined their secretion by Has exporters, Has$_{PF}$ and Has$_{SM}$ (Fig. 2) (see Materials and Methods). First, C-terminal HasA chimeras (pFXM-HasA and pMXF-HasA) were created and then tested for secretion from the *E. coli* cells carrying the Has exporters (Fig. 6). The Has$_{SM}$ system allowed

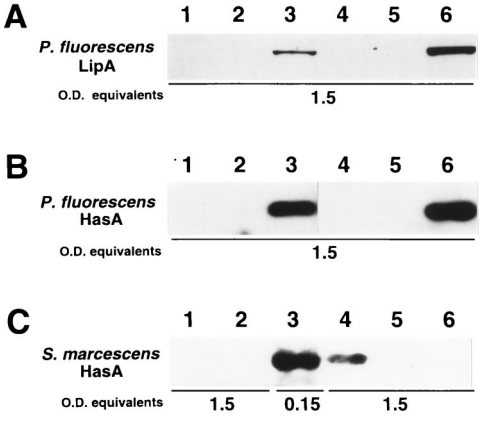

FIG. 5. HasA and LipA secretion by hybrid Has exporters. The plasmids pACYC/HasD$_{PF}$ and pACYC/HasD$_{SM}$ were used as the plasmids encoding the ABC proteins HasD$_{PF}$ and HasD$_{SM8000}$, respectively. MFP and OMP were provided by pMW/HasEF$_{PF}$ (*hasEF$_{PF}$*) and pMW/HasE$_{SM}$. These plasmids were introduced into the *E. coli* cells harboring the LipA$_{PF}$ plasmid pUC/PFLipA13 (A), the HasA$_{PF}$ plasmid pUC/HasA$_{PF}$ (B), and the HasA$_{SM}$ plasmid pUC/HasA$_{SM}$ (C). The cells were cultured and tested for protein secretion as described in the legend to Fig. 3. The gels were analyzed by immunoblotting with antisera against LipA$_{SM}$, HasA$_{PF}$, and HasA$_{SM}$. Secretory proteins are indicated on the left and optical density (O.D.) equivalents are shown below. Lanes 1, pACYC184 plus pMW/HasE$_{SM}$; lanes 2, pACYC184 plus pMW/HasEF$_{PF}$; lanes 3, pACYC/HasD$_{SM}$ plus pMW/HasE$_{SM}$; lanes 4, pACYC/HasD$_{SM}$ plus pMW/HasEF$_{PF}$; lanes 5, pACYC/HasD$_{PF}$ plus pMW/HasE$_{SM}$; lanes 6, pACYC/HasD$_{PF}$ plus pMW/HasEF$_{PF}$.

secretion of all these chimeras (Fig. 6C). The Has$_{PF}$ exporter secreted HasA$_{PF}$ and a chimera produced by pMXF-HasA (Fig. 6B, lanes 1 and 3) but failed to secrete HasA$_{SM}$ and a chimera from pFXM-HasA (Fig. 6B, lanes 2 and 7), indicating that the C-terminal region of HasA$_{PF}$ is involved in HasA$_{PF}$ secretion via the Has$_{PF}$ exporter. To define the sequence related to the substrate specificity, we created further C-terminal chimeras (Fig. 2). A pMBF-HasA-encoded chimera that is HasA$_{SM}$ containing the HasA$_{PF}$ C-terminal sequence of 32 amino acid residues (amino acid residues 175 to 206) was secreted by both Has exporters (Fig. 6B and C, lanes 4). Chimeras encoded in pMKΔF-HasA and pMBΔF-HasA, which carry a C terminus of HasA$_{PF}$ but lack the HasA$_{PF}$ insert (Ala181 to Leu192), were exported by Has$_{SM}$ (Fig. 6C, lanes 5 and 6) but not by Has$_{PF}$ (Fig. 6B, lanes 5 and 6). Deletion of the insert from HasA$_{PF}$ (pFBΔF-HasA) did not affect secretion of the chimera through the Has$_{SM}$ system (Fig. 6C, lane 8), whereas the Has$_{PF}$ exporter aborted secretion (Fig. 6B, lane 8). These findings showed that the inserted segment close to the HasA$_{PF}$ C terminus may play an important role in HasA$_{PF}$ recognition by the Has$_{PF}$ exporter.

DISCUSSION

The *P. fluorescens* genes encoding an unidentified ABC exporter were isolated together with the secretory protein gene. The secretory protein HasA$_{PF}$, which is similar to HasA$_{SM}$ and also lacks an N-terminal signal peptide, was shown to possess

heme-binding activity, a typical feature of HasA (unpublished data). The gene organization of the *hasRADEF*$_{PF}$ operon showed a typical structure of the genes coding for the ABC exporter and its secretory protein. The Has$_{PF}$ exporter secreted HasA$_{PF}$ and also mediated secretion of LipA$_{PF}$ and AprA$_{PF}$ more efficiently than Apr$_{PF}$ did in the recombinant *E. coli* system. The presence of two distinct ABC exporters, Apr$_{PF}$ and Has$_{PF}$, in *P. fluorescens* demonstrated that *S. marcescens* is not unique in possessing two ABC exporters, the Has and Lip systems (2, 23).

Several differences between the Has$_{PF}$ and Has$_{SM}$ systems were observed. The OMP HasF$_{PF}$ was essential for secretion, and its function could not be replaced with TolC, in spite of HasF$_{SM}$ being replaceable with TolC. The *hasDEF*$_{PF}$ operon coded for all three secretory components, whereas the Has$_{SM}$ system is encoded by the *hasDE*$_{SM}$ operon and the unlinked *hasF*$_{SM}$ gene. Of interest was the substrate specificity of the Has$_{PF}$ exporter. This system promoted secretion of HasA$_{PF}$ and HasA$_{PA}$ but not HasA$_{SM}$, whereas the Has$_{SM}$ exporter allowed secretion of all HasA proteins tested. Hybrid exporter analysis indicated that an ABC protein (HasD$_{PF}$) was responsible for the substrate specificity, as in other ABC exporters (3, 4).

HasA proteins were 40 to 49% identical to each other, but several insertions and deletions were observed. The secretion signal of the proteins secreted by the ABC exporter is known to be situated at the C terminus (11), often containing a motif consisting of negatively charged amino acid residues followed by several hydrophobic residues. The sequences D-W-A-L-A-A, D-L-A-L-A-A, and E-L-L-A-A are identified in the C-termini of HasA$_{PF}$, HasA$_{PA}$, and HasA$_{SM}$, respectively (26). These motifs are expected to be essential but not sufficient for secretion. In some cases, the signal was located in the last 15 to 30 amino acids but at a low secretion level (10, 11).

We postulated that the inserts close to the C terminus, which are found in HasA$_{PF}$ and HasA$_{PA}$ but not HasA$_{SM}$ and contain several conserved amino acid residues, may be involved in the specific secretion via the Has$_{PF}$ exporter. Interestingly, a HasA$_{PF}$ inserted segment (Ala181 to Leu192) close to the C terminus was shown to be responsible for the substrate specificity on HasA secretion via the Has$_{PF}$ exporter. Lack of the insert caused failure of secretion. Very recently, conformation of the C terminus of HasA$_{SM}$, which is the last 15 residues, containing the motif essential for secretion, has been studied by ^{1}H nuclear magnetic resonance (18). This peptide was shown to be highly flexible and unstructured. It is of interest that the C-terminal insert, which we showed to be responsible for the specificity of secretion, is situated just upstream of the last 15 amino acid residues. Since Has$_{SM}$ allows the secretion of all three HasA proteins and HasA chimeras, further secretion analysis of the HasA chimeras and mutants with amino acid substitutions through Has$_{PF}$ and other ABC exporters may be of interest.

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