Serratia ATP-binding cassette protein exporter, Lip, recognizes a protein region upstream of C-terminus for specific secretion

Kenji Omori‡, Akiko Idei§, and Hiroyuki Akatsuka§

From the Discovery Research Laboratory, Tanabe Seiyaku Co., Ltd., 2-50, Kawagishi-2-chome, Toda, Saitama 335-8505, and the §Discovery Research Laboratory, Tanabe Seiyaku Co., Ltd., 16-89, Kashima-3-chome, Yodogawa-ku, Osaka 532-8505, Japan

‡To whom correspondence should be addressed. Tel. 81-48-433-8068; Fax 81-48-433-8159; E-mail: k-omori@tanabe.co.jp.

Running title: “Secretion determinant recognized by Lip exporter”

Key words: heme acquisition protein/secretion specificity/lipase/Serratia marcescens/ABC exporter/type I secretion

1 The abbreviations used are: ABC, ATP-binding cassette; MFP, membrane fusion protein; OMP, outer membrane protein; Has, heme-acquisition; PCR polymerase chain reaction.
ABSTRACT

*Serratia marcescens* ATP-binding cassette exporter, Lip system, secretes lipase (LipASM), metalloproteases, and cell surface layer protein homologue but not a heme-acquisition protein, HasA (HasASM). Secretion of HasASM is limited to HassM system. However, HasA proteins from *Pseudomonas fluorescens* (HasAPF) and *Pseudomonas aeruginosa* were exported through Lip and HassM systems. In order to investigate the specificity in Lip exporter-mediated secretion, secretion analysis was performed using chimeras containing HasAPF and HasASM sequences. The segment Val-Ala-Leu (designated R1 to R3 sites), which is present close to the C-terminus of HasAPF but not HasASM, was revealed to be involved in the substrate specificity of Lip exporter. Introduction of amino acid substitutions into the region R1 to R5 demonstrated that R1, R3, R4, and R5 sites require some specific amino acid residues for Lip-mediated secretion. The amino acid sequence of the region was conserved considerably among the proteins secreted by Lip exporter. On the contrary, the region was not related to HasA secretion through HassM system. Interestingly, a typical C-terminal motif, so far regarded as a secretion signal, was necessary for secretion through neither Lip nor HassM exporter. In LipASM secretion via Lip system, the typical C-terminal motif was not essential either, but the presence of a sequence similar to Val-Ala-Leu and its location from the C-terminus greatly affect the secretion level. Secretion analyses using hybrid exporters and competitors exhibited that the R1 to R5 region was recognized by ABC protein of Lip exporter, LipB, and that the mutations aborting Lip-mediated secretion in the region resulted in a loss of the affinity to LipB.
Thus, a determinant within the secretory protein for Lip-mediated secretion was fully defined.
INTRODUCTION

ATP-binding cassette (ABC) exportiner, which mediates translocation of proteins lacking an N-terminal signal sequence across the cell membranes, is known in Gram-negative bacteria. Secretion through the system is one-step and differs from that through the sec gene-mediated pathway. The system, termed type I, is categorized as an ABC transporter family (1, 2). The transport process requires ATP-hydrolysis as an energy source, and secretion through the system requires three specific components: an inner membrane protein (ABC protein), a membrane fusion protein (MFP), and an outer membrane protein (OMP).

One example to the present study is Serratia marcescens Lip system composed of LipB (ABC protein), LipC (MFP), and LipD (OMP) (3). The other example is S. marcescens HasSM system including HasD_{SM}, HasE_{SM}, and HasF_{SM} (4-6). HasF_{SM} is highly similar to Escherichia coli OMP, TolC (6), and TolC functions instead of HasF_{SM}. Lip system is involved in secretion of three unrelated proteins of S. marcescens, lipase (LipA_{SM}), metalloprotease (PrtA), and cell surface layer protein homologue (3, 7), while HasSM system dedicates to secretion of HasA_{SM} protein, which is an extracellular polypeptide of 19kDa exhibiting heme-binding activity and serving iron-acquisition (8, 9). Thus, two protein transport systems are present in S. marcescens, and they play a specific role for secretion of each protein in vivo. In regard to their secretion specificity, several reports were made using reconstituted exporters in the E. coli cells (10, 11, Omori et al., unpublished). Lip system can promote secretion of Erwinia chrysanthemi metalloprotease C (PrtC), Pseudomonas fluorescens lipase (LipA_{PF}) and alkaline protease (AprA_{PF}), and Pseudomonas aeruginosa alkaline protease (AprA_{PA}). HasSM system mediates secretion of LipA_{SM}. 

4
S. marcescens PrtA, and E. chrysanthemi PrtC. In contrast to the secretory proteins through Lip system, HasA<sub>SM</sub> can be secreted only by Has<sub>SM</sub> system. Lip system and E. chrysanthemi metalloprotease exporter Prt system (PrtD-PrtE-PrtF; 12) are unable to promote HasA<sub>SM</sub> secretion. The levels of sequence homology between each component of these transporters are considerable, and in fact, some components are exchangeable with those of other systems (10-12). Nevertheless, HasA<sub>SM</sub> secretion is specific and limited to Has<sub>SM</sub> exporter.

An analysis of hybrid exporters comprising components from two distinct ABC exporters revealed that one determinant for the secretion specificity of ABC exporter is ABC protein (10, 12). The secretion analysis of E. chrysanthemi metalloprotease PrtG through Prt system has demonstrated that a secretion signal is situated at C-terminus (13). The signal does not include any common primary sequence but contains a motif consisting of a negatively charged amino acid residues followed by several hydrophobic residues. Several reports described involvement of the C-terminal region in the secretion specificity (14-19). However, a sequence feature or protein region involved in the substrate specificity of ABC exporter has not been investigated in detail yet.

A small and unique secretory protein, HasA<sub>SM</sub>, is useful for the analysis of the substrate specificity. Three homologues of HasA have been identified from S. marcescens, P. aeruginosa (HasA<sub>PA</sub>), and P. fluorescens (HasA<sub>PF</sub>) (8, 20, 21). Two exporters, S. marcescens Has<sub>SM</sub> and P. fluorescens Has<sub>PF</sub> (HasD<sub>PF</sub>-HasE<sub>PF</sub>-HasF<sub>PF</sub>), have been reported to devote to secretion of HasA proteins, hitherto. Interestingly, Has<sub>SM</sub> exporter is able to secrete all three HasA proteins, whereas Has<sub>PF</sub> exporter secretes HasA<sub>PF</sub> and HasA<sub>PA</sub> but not HasA<sub>SM</sub> (21). Presently, Has<sub>SM</sub> is a sole exporter for HasA<sub>SM</sub>. The typical C-terminal motifs D-W-A-L-A-A, D-L-A-L-A-A, and E-L-L-A-A are found in HasA<sub>PF</sub>, HasA<sub>PA</sub>, and HasA<sub>SM</sub> sequences, respectively. The
motifs are analogous, and therefore, the secretion specificity of HasA<sub>SM</sub> cannot be elucidated simply by the motif.

Specific recognition by ABC exporter is an essential process for translocation of secretory proteins, and therefore, the specificity of HasA<sub>SM</sub> secretion is one of important subjects for understanding the secretion mechanism of ABC exporter. In this paper, we aim to describe the characteristics of the sequence recognized by ABC exporter using HasA<sub>SM</sub> and LipA<sub>SM</sub> mutants as a substrate and Lip system as an exporter. We reveal involvement of a protein region close to but not at the C-terminus in Lip exporter-mediated secretion and in recognition by ABC protein, LipB. Furthermore, we demonstrate that a typical C-terminal motif, which is considered as a secretion signal so far, is not essential for secretion. Our findings provide new important information for understanding the secretion mechanism and protein structure involved in the secretion specificity of ABC exporter.

**EXPERIMENTAL PROCEDURES**

*Strains and media*—E. coli K-12 DH5 was used as a host and LB medium was used as a rich medium (22). S. marcescens 413 that is a LipA<sub>SM</sub>-deficient strain (11) was used for a host of the secretion analysis of LipA<sub>SM</sub> mutants. Antibiotics were added at the following concentrations: ampicillin, 50 µg/ml; kanamycin, 50 µg/ml; and chloramphenicol, 20 µg/ml for E. coli, and ampicillin, 1000 µg/ml for S. marcescens 413.

*General methods*—DNA manipulations were carried out according to standard procedures (21). PCR was carried out using ExTaq purchased from Takara Shuzo (Kyoto, Japan). Nucleotide sequence was determined by dideoxy-chain-termination
method using a BigDye Terminator Cycle Sequencing Reaction kit (PE Applied Biosystems) and an automated DNA sequencer ABI PRISM™ 310. Transformation of the *S. marcescens* 413 cells was done as described previously (23).

**DNA constructs**—The HasA plasmids pUC/HasASM (HasASM), pUC/HasAPF (HasAPF), and pSYC1000 (HasAPA), and their chimeras (pMBF-HasA, pMBΔF-HasA, and pFBΔF-HasA) have been described previously (20, 21). HasA chimeras and their mutants were constructed based on pUC/HasASM by a conventional method using PCR-directed mutagenesis and synthetic oligonucleotide linkers. LipASM mutants were constructed from pLIPE121 (24), which is pUC19 carrying the lipASM gene, using PCR-directed mutagenesis and synthetic oligonucleotide linkers. Nucleotide sequences of the inserted DNAs of the resultant plasmids were confirmed by sequencing.

In order to produce hybrid exporters, the *hasADE*8000 genes (*hasADE* from *S. marcescens* 8000) were cloned from a *S. marcescens* 8000 genomic DNA library. The *hasDE*8000 genes were inserted into multiple cloning site of pMW218, to generate pMWHasDE. The plasmid pMW/HasE, carrying the *hasE*8000 gene was created by deleting the DNA fragment coding for the *hasD*8000 gene from pMWHasDE. The plasmids pACYC/LipB and pMW/LipCD carrying the *lipB* and *lipCD* genes from *S. marcescens* 8000 were created by deleting the *lipCD* and *lipB* fragments from pYBCD20 (7) and pMWBCD10 (25), respectively.

A FLAG-tagged HasA-VAL mutant encoded by pSTV/FLAG-HasA-VAL was generated as follows. A DNA fragment was amplified by PCR using an oligonucleotide, 5′-GGGAGCTCATTTTCACTTATGACGC-3′, the M4 primer 5′-GTTTTCCAGTCGAC-3′, and pMF-M-VAL as a template, digested by SacI and HindIII, and then inserted into the corresponding sites of pUC18 (22). After
digestion with EcoRI and SacI, the resultant plasmid was ligated with a synthetic linker (5’-AATTCGGATTACAAGGACGACGATGACAAGGAGCT-3’ plus 5’-CCTTGTCTACGTCGTCCTTGTAATCCG-3’), and then the SacI site was disrupted using T4 DNA polymerase. The EcoRI-HindIII fragment was inserted into the corresponding sites of pSTV28, a pACYC184 (22)-derived cloning vector (Takara Shuzo), generating pSTV/FLAG-HasA-VAL, which encodes HasA-VAL containing the sequence Met-Thr-Met-Asn-Ser-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys at the N terminus.

Analysis of protein secretion—The plasmids pLG575 (HlyBD/TolC; 14), pACYC/OI70 (HasDEF_{PF}; 21), pK150 (HasD{ESM}/TolC; 10), pRUW4 (PrtDEF; 26), pAGS8 (AprDEF_{PA}; 19), and pYBCD20 (LipBCD; 7), which encode ABC exporters in pACYC184, were used for the secretion analysis. The E. coli DH5 cells carrying plasmids encoding ABC exporter and HasA derivative were cultured in LB medium at 30°C for 28h with shaking vigorously. The proteins in the supernatants of the cultured media were concentrated by precipitation with trichloroacetic acid (TCA) at a final concentration of 10%. The proteins were subjected to SDS-PAGE. The recombinant S. marcescens 413 cells carrying the lipA_{SM} plasmids were cultured in the lipase medium (27) at 30°C for 28 h, and the proteins in the supernatants were directly loaded on the gel. The precast gel PAGEL (ATTO) was used for SDS-PAGE. The proteins in gels were stained by Coomassie brilliant blue G-250.

Secretion analysis with hybrid exporters—The HasA plasmid (pUC/HasA_{SM}, pMFM-VAL, or pMFM-VAA; ampicillin-resistant), the ABC protein plasmid (pACYC/LipB or pACYC/HasD; chloramphenicol-resistant), and the MFP-OMP or MFP plasmid (pMW/LipCD or pMW/HasE; kanamycin-resistant) were introduced into the E. coli cells. The resultant recombinant cells were cultured in LB medium at 30°C.
for 40h with shaking vigorously. After TCA-precipitation, the proteins in the supernatants of the cultured media were loaded on the gel as described above, and then electrophoretically transferred to an Immobilon P filter (Millipore). The blots were blocked by soaking in Block Ace (Dainippon Pharmaceutical, Japan) overnight at 4°C and incubated with an anti HasA<sub>SM</sub> antibody (a kind gift from Dr. Cecile Wandersman) at room temperature for two hours (diluted 1:5000 in PBS containing 0.1% Tween20). They were washed and incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G, and the bound antibody was detected with the enhanced chemiluminescence systems (Amersham).

**Secretion competition analysis**—The plasmid pSTV/FLAG-HasA-VAL coding for a FLAG-tagged HasA-VAL mutant as a secretion substrate and the plasmids encoding HasA mutant proteins as a competitor were introduced into the *E. coli* cells carrying pFBCD1 (*lipBCD* in the mini-F derivative pKPT1124 (28); Idei *et al.*, unpublished). The recombinants were cultured in LB medium at 30°C for 24 h with shaking vigorously. The proteins in the supernatants of the cultured media were subjected to SDS-PAGE and immunoblot analysis with anti-FLAG monoclonal antibody (Sigma) and anti-HasA<sub>SM</sub> antibody. In all experiments, protein analysis was carried out two or three times independently and similar results were obtained. In order to access cell lysis, β-galactosidase activity was measured using *o*-nitrophenyl-β-D-galactoside as a substrate according to the method described in ref 29. The levels of the extracellular β-galactosidase activity of the cells were <2% compared with those of cell lysates, indicating no significant cell lysis in all experiments.
RESULTS

_Secretion of the HasAPF protein through Lip exporter_—It has been reported that HasASM secretion is confined to HasASM exporter, whereas HasAPF secretion has not been examined except for HasPF and HasASM exporters. HasAPF secretion was investigated using the recombinant _E. coli_ cells expressing several ABC exporters. The proteins in the cultured supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1A). Interestingly, Lip exporter, which is unable to mediate HasASM secretion (11), secreted HasAPF at a high level as well as HasASM (TolC was utilized as OMP in the _E. coli_ cells), compared with a genuine exporter for HasAPF, HasPF. Prt and AprPA (exporter for AprPA) also promoted HasAPF secretion at low and very low levels, respectively. _E. coli_ haemolysin exporter, Hly, did not let the cells secrete HasAPF at a detectable level. Thus, the secretion capability of Lip exporter for a protein categorized as HasA was demonstrated. Secretion of three HasA homologues, HasASM, HasAPA, and HasAPF, were tested (Fig. 1B). HasASM exporter allowed secretion of all three HasA proteins as described previously (20, 21), whereas Lip exporter secreted both HasAPF and HasAPA but not HasASM. It is intriguing that secretion of HasAPF and HasAPA was achieved by an unrelated exporter, Lip system.

_Involvement of the HasAPF C-terminal segment in secretion through Lip exporter_—HasAPF and HasAPA proteins were suspected to contain a sequence necessary for Lip-mediated secretion but needless for secretion through HasASM exporter. The most notable difference in the amino acid sequences among three HasA homologues is the presence of segments, composed of 12 and 14 amino acid residues, close to but not at the C-termini of HasAPF and HasAPA, respectively (Fig. 2A). Involvement of the
segment in the Lip-mediated secretion was tested using HasA chimeras containing the HasA<sub>SM</sub> and HasA<sub>PF</sub> sequences (Fig. 2B). Secretion of all HasA chimeras through HasA<sub>SM</sub> exporter has been already confirmed (21). As shown in Fig. 2C, Lip system secreted HasA<sub>PF</sub> and a chimera carrying the HasA<sub>PF</sub> C-terminus (pMBF-HasA) but not HasA<sub>SM</sub> nor chimeras containing the HasA<sub>PF</sub> C-terminus without the segment of 12 amino acid residues (pMBΔF-HasA and pFBΔF-HasA). HasA chimera including the 12 amino acid segment in HasA<sub>SM</sub> sequence (pMFM-HasA; Fig. 3A) was secreted by both HasA<sub>SM</sub> and Lip (Fig. 3B). Thus, it was concluded that the HasA<sub>PF</sub> C-terminal segment is involved in secretion through Lip exporter but not required for HasA<sub>SM</sub>-mediated secretion.

Analysis of the amino acid sequence related to secretion by Lip exporter—The C-terminal segments of HasA<sub>PF</sub> and HasA<sub>PA</sub> contain a couple of common features: the sequence Ala-His-Ala-Thr, a Thr/Ala cluster and negatively charged residue (Asp or Glu) followed by three hydrophobic amino acid residues, in this order (Fig. 2A). In order to investigate a minimum segment and amino acid residues necessary for secretion via Lip exporter, secretion of HasA<sub>SM</sub> mutants containing derivatives of the HasA<sub>PF</sub> segment in the C-terminal region (Fig. 3A) was examined in the <i>E. coli</i> cells carrying Lip and HasA<sub>SM</sub> exporters (Fig. 3B). These alterations did not affect HasA<sub>SM</sub>-promoted secretion, indicating that all HasA mutants are functional for secretion.

Ala-substitutions at<sup>182</sup>His, <sup>184</sup>Thr, and <sup>189</sup>Asp of the C-terminal segment (pMFM-182A, pMFM-184A, and pMFM-189A, respectively) and deletion of a Thr cluster and its flanking region (pMFM-ADVAL, pMFM-AVAL, and pMFM-VAL) did not alter the levels of Lip-mediated secretion. The levels are the same as that of HasA chimera encoded by pMFM-HasA. However, other deletion mutants (pMFM-AL, pMFM-ATDVL, and pMFM-Δ(190-191)) and a mutant with Gly-replacements at <sup>190</sup>Val
and $^{191}$Ala (pMF-(190-191)G) reduced secretion through Lip exporter to undetectable levels. Finally, the minimum segment $^{190}$Val-$^{191}$Ala-$^{192}$Leu (carried by HasA$_{SM}$-VAL) was demonstrated to be closely associated with secretion promoted by Lip exporter.

**Roles of amino acid residues in the C-terminal segment in secretion via Lip system**—Roles of amino acid residues, $^{190}$Val, $^{191}$Ala, and $^{192}$Leu (designated R1, R2, and R3 sites, respectively), in Lip exporter-mediated secretion were examined by introducing the amino acid substitutions. Secretion of HasA mutants that are HasA$_{SM}$ containing a segment, Ala-Val-Ala-Leu or Val-Ala-Leu, with amino acid substitutions Val, Leu, Ile, Phe, Met, Ala, Gly, Arg, His, Glu, Gln, Thr, and Pro at R1, R2, and R3 sites (Fig. 4AB), were tested in the he E. coli cells carrying exporters. Has$_{SM}$ exporter secreted these HasA mutants but at various levels, indicating that mutations introduced did not cause a severe conformational change leading to abolishment of secretion.

Secretion of these HasA mutants through Lip exporter provided us several interesting observations. First, importance of R1 residue for secretion was exhibited (Fig. 4A). Replacement with Ile did not alter the secretion level, whereas other substitutions including hydrophobic residues, Leu, Phe, and Met, reduced it to undetectable levels. These observations implied that the presence of a hydrophobic residue at R1 is necessary but not sufficient for Lip-promoted secretion. Val and Ile at R1 are likely to play a specific role for secretion. Lip exporter secreted HasA mutants with replacements at R2 but except for Pro (Fig. 4A), indicating that Ala residue at R2 is not essential. Deletion of the R2 residue (pMF-ATDVL), however, disturbed Lip-mediated secretion (Fig. 3B), suggesting that the residue functions as a spacer to allocate the hydrophobic R1 at a proper position necessary for the secretion. R3 residue was replaceable with Ile (Fig. 4B). Decrease in the secretion levels (but at a detectable level) was observed by replacement with Val at R3. Thr-substitution
allowed secretion of the mutants at a low level. HasA mutants containing other residues at R3 were not secreted via Lip system at a detectable level.

Thus, the (Val/Ile)-X-(Leu/Ile/Val) sequence upstream of the C-terminus was demonstrated to be essential for secretion through Lip exporter. Because the sequence was unrelated to HasSM-mediated secretion, the region R1 to R3 was suggested to be involved in the recognition or specific transport process by Lip exporter.

Comparison of the C-terminal sequences of proteins secreted by Lip exporter—Fig. 5 shows the presence of a consensus similar to (Val/Ile)-X-(Leu/Ile/Val) in the R1 to R3 region of Lip-secreted proteins. A following sequence of the consensus also contained several conserved amino acid residues Val/Ile/Thr, Gly, and Val/Gln at R4, R5, and R6, respectively (Fig. 5). Further HasA mutants with substitutions at R4, R5, and R6 were generated to access a function of each residue in secretion via Lip and HasSM exporters (Fig. 4B).

In regard to R4 mutants, secretion of HasA mutants with replacements at R4 was achieved by HasSM exporter at various levels. Lip exporter secreted HasA mutant with Ile at an equal level to that with Val. HasA mutants with Met and Thr were secreted via Lip exporter at a reduced level. Replacements with other hydrophobic amino acid residues such as Leu and Phe, reduced the secretion to undetectable levels. Amino acid residues Val/Ile/Thr/Met at R4 allowing Lip-mediated secretion agreed with those found in the sequences of the proteins secreted by Lip (Fig. 5). The conserved amino acid residue Gly at R5 was essential for secretion through Lip and HasSM exporters. Immunoblot analysis with anti-HasASM antibody demonstrated production of HasA mutant proteins at equal levels in the cell extracts of E. coli without ABC exporter (data not shown). The amino acid residue at R6 was replaceable with several other amino acid residues although the secretion levels were various. Gln and Val conserved at R6
were dispensable to secretion.

In conclusion, the amino acid residues at R1, R3, R4, and R5 were strongly related to Lip-mediated HasA secretion. The residue Gly at the R5 site also played an important role in high-level secretion through HasSM.

**ABC protein LipB is responsible for Lip-mediated secretion of HasA mutants**—Hybrid exporter analysis using HasSM and Lip exporter components revealed that a hybrid, LipB-HasE SM-TolC, was functional for secretion of HasA SM-VAL (Fig. 6). However, neither HasA SM nor HasA SM-VAA (a HasA SM-VAL variant with Ala-substitution at R3) was secreted by Lip and hybrid exporters. HasD SM-LipC-LipD has been already known inactive for secretion (11), and an exporter lacking ABC protein did not direct secretion. Thus, the sequence Val-Ala-Leu was essential for secretion through exporters including the ABC protein LipB, confirming that LipB is a determinant of the substrate specificity of the exporter. In other words, LipB might recognize the region R1 to R3 of the secretion protein.

**C-terminal secretion signal is not essential for secretion via ABC exporters**—Proteins secreted by Lip exporter also contain a conserved motif consisting of a negatively charged amino acid residue followed by several hydrophobic residues at the C-terminus (Fig. 5). Involvement of the motif in secretion was investigated using HasA SM-VAL mutants (Fig. 7A). Interestingly, Ala-substitutions of 184Glu or/and 181Asp allowed secretion through Lip and HasSM systems although dual Ala-replacement of HasA SM-VAL (pMFM-A1A2) reduced the secretion level via Lip exporter (Fig. 7B). Furthermore, each C-terminal hydrophobic amino acid residue in the motif was replaceable with a negatively charged residue, Glu (Fig. 7AB). In Prt exporter, which secretes metalloproteases and lipases but not HasA SM as well as Lip exporter, the Val-Ala-Leu segment but not the C-terminal motif participated in secretion (Fig. 7C).
These findings indicated that the C-terminal motif so far considered as a secretion signal, is not essential for secretion through ABC exporter. Although the motif may have a role for something other than secretion, the importance of the region R1 to R5 in secretion via ABC exporters except for HasSM was generally recognized.

Involvement of the R1 to R5 region and C-terminal motif in Lip-mediated LipA<sub>SM</sub> secretion in vivo—Involvement of the region R1 to R5 in secretion through Lip exporter in vivo was tested. <i>S. marcescens</i> LipA<sub>SM</sub> mutants with Ala-substitutions at R1 to R6 (amino acid residues 596 to 601) were created and introduced into the <i>S. marcescens</i> 413 cells deficient in LipA<sub>SM</sub> (Fig. 8AB). The secretion level of LipA<sub>SM</sub> mutant with Ala at R3 was greatly reduced. Ala-substitution at R1 or R4 decreased the secretion level of the mutant to some extent compared with that of the wild-type LipA<sub>SM</sub> (pLIPE121). Replacement of Gly at R5 with Ala slightly affected LipA<sub>SM</sub> secretion in <i>S. marcescens</i>, although the Gly residue was essential for HasA mutant secretion through both Lip and Has<sub>SM</sub> exporters in <i>E. coli</i>. Further mutations were introduced into the LipA<sub>SM</sub> C-terminal sequence (Fig. 8C). As shown in Fig. 8D, Ala-substitution of the negatively charged residue<sup>609</sup>Asp (pLIPA-609A) and introduction of a charged or a bulky amino acid residue at the C-terminus did not change the secretion level except for Lys-replacement at the C-terminus (pLIPA-K1). In the same way as demonstrated in secretion of HasA mutants, a typical C-terminal motif was not essential for LipA<sub>SM</sub> secretion via Lip system in <i>S. marcescens</i>, either.

Influence of location of the region R1 to R5 from the C-terminus on secretion was examined (Fig. 8C). As shown in Fig. 8D, LipA<sub>SM</sub> mutants, having Gly (R5) 13 to 17 amino acid residue upstream from the C-terminus, were secreted but at various levels, whereas the secretion levels were low on mutants containing Gly (R5) 11-12 and 19 amino acid residue upstream from the C-terminus. All LipA<sub>SM</sub> mutants expressed
in the *E. coli* cells without exporter exhibited intracellular lipase activity, indicating production of lipase proteins (data not shown). Position of the R1 to R5 region, but not a specific sequence of the C-terminal tail, was demonstrated to be critical for Lip-mediated secretion based on the similarity of the C-terminal tails of proteins secreted by Lip system and the results from the secretion analysis of LipA<sub>SM</sub> mutants.

*Function of the R1 to R5 region in Lip-mediated secretion*—Secretion competition analysis was performed to know whether inability of Lip exporter to secrete HasA<sub>SM</sub> and secretion-deficient HasA-VAL mutants is due to a lack of their affinity to ABC protein or a defect in the secretion process. FLAG-tagged HasA-VAL (pSTV/FLAG-HasA-VAL) was secreted in the *E. coli* cells carrying a mini-F derived low-copy-number (one-two copies per cell) Lip exporter plasmid, pFBCD1. HasA<sub>SM</sub> (pUC/HasA<sub>SM</sub>), HasA-VAA (pMFM-VAA), and HasA-R5A (pMFM-R5A), which are not secreted through Lip exporter, and HasA-VAL (pMFM-VAL), a substrate of Lip system, were employed as a competitor. As shown in Fig. 9, a FLAG-tagged HasA-VAL protein of 22 kDa was secreted through Lip system as well as HasA-VAL without a tag (21 kDa). Immunoblot analysis demonstrated reduction of the secretion levels of the 22 kDa FLAG-tagged HasA-VAL when a competitor, HasA-VAL of 21 kDa, was co-expressed and secreted, indicating secretion competition between HasA-VAL proteins. Interestingly, the presence of pUC18, pUC/HasA<sub>SM</sub>, pMFM-VAA, and pMFM-R5A did not affect the secretion levels of FLAG-tagged HasA-VAL. Under conditions that secretion competition between HasA-VAL proteins was observed, neither HasA<sub>SM</sub> nor other HasA-VAL mutants exhibited secretion competition with HasA-VAL and disturbed the secretion.

**DISCUSSION**
Lip system exports several types of secretory proteins, whereas HasA\textsubscript{SM} is a poor secretion substrate of the system. One possible explanation for this is a lack of a protein region necessary for Lip-mediated secretion in the HasA\textsubscript{SM} sequence. Interestingly, a C-terminal motif, so far regarded as a secretion signal, was not involved in secretion through Lip and Has\textsubscript{SM} exporters. The same was the case with LipA\textsubscript{SM} secretion via Lip system \textit{in vivo}. Secretion analysis using HasA chimeras and their mutants revealed a protein region including a new motif, (Val/Ile)-X-(Leu/Ile/Val)-(Val/Ile/Met/Thr)-Gly (designated the region R1 to R5) close to but not at the C-terminus. The motif was involved in secretion through Lip or Prt system, whereas the motif was not necessary for Has\textsubscript{SM}-mediated secretion. Similar sequences corresponding to the region R1 to R5 were found in Lip-secreted proteins, including LipA\textsubscript{SM}. A component within Lip exporter, determining the secretion specificity, was LipB, ABC protein of Lip system, coinciding with our previous observation (11). No competition for Lip-mediated HasA-VAL secretion with competitors HasA\textsubscript{SM} and HasA-VAL derivatives (HasA-VAA and HasA-R5A) was observed, that is, proteins retaining an R1 to R5 motif inactive for Lip-mediated secretion, did not cause a blockade of secretion. These findings indicate that proteins including the inactive R1 to R5 motif do not have high affinity to ABC protein, which leads to an impediment to the secretion process. The protein region carrying an active R1 to R5 motif is recognized by ABC protein with higher affinity than that containing inactive one. Secretion competition between LipA\textsubscript{SM} and PrtA in \textit{S. marcescens}, which is previously reported in ref 25, is explained by competition in the process of recognizing the R1 to R5 regions of these proteins by Lip system. Thus, a new determinant within secretory proteins, which enables secretion through Lip system, in
other words, which Lip system recognizes, was revealed. Secretion profile of LipA<sub>SM</sub> mutants with Ala-replacements differed from that of HasA<sub>SM</sub> mutants, for example, a conserved Gly residue at R5 was demonstrated necessary for HasA mutant secretion via Has<sub>SM</sub> and Lip exporters but not essential for LipA<sub>SM</sub> secretion via Lip system. These observations indicate that a motif in the region is not rigorous and that amino acid residues in the region R1 to R5 possibly affect secretion through Lip exporter each other. In addition, LipA<sub>SM</sub> mutants containing Gly (R5) at positions -14 to -17 from the C-terminus were secreted through Lip exporter, whereas those with a longer or shorter C-terminal tail were a poor substrate. Any specific feature was not found in the sequence between R5 and the C-terminus, and the alteration of the C-terminal tail sequence by insertion or deletion of one or two amino acid residues did not change the secretion level severely, indicating that location of the region R1 to R5 from the C-terminus is another important factor.

One report (13) supports this idea. In <i>E. chrysanthemi</i> PrtG, the C-terminal 15 amino acid sequence

Val-Asn-Ile-Val-Gly-Ala-Ala-Leu-Gln-Pro-Ser-Asp-Val-Ile-Val-COOH, which includes the region R1 to R5 (underlined) at position -11 from the C-terminus, has been shown to be a minimum functional part for Prt-mediated secretion. Furthermore, PrtG mutant lacking the C-terminal 14 amino acid residues but exhibiting a newly created typical C-terminal motif, was secreted, and therefore, a conserved C-terminal motif was regarded as a secretion signal. The deletion, however, located the sequence Leu-His-Leu-Ser-Gly, which is similar to the region R1 to R5, at position –9. Their previous findings might imply the importance of the region R1 to R5 at a proper position in secretion.

What is a function of the conserved C-terminal motif? At least, the motif is not
necessary for secretion through Lip, Prt, and Has\textsubscript{SM} exporters. Considering that the motif is conserved among proteins secreted through ABC exporters including highly conserved repeat toxin family exporters, the motif may be necessary for protein-folding after secretion through ABC exporters. However, its real function has not been defined yet.

In order to clarify recognition mechanism by Lip system, the analysis of structural features laying the region R1 to R5 is necessary. Although 3D-structure of Lip\textsubscript{ASM} is unknown presently, crystallographic analyses of Apr\textsubscript{PA} and PrtA, both of which are secreted by Lip system and possess the sequence homologous to the region R1 to R5, show very similar C-terminal structure (30, 31). The conserved C-terminal motif and a protein region corresponding to the region R1 to R5 are $\beta$-sheet structured. On the contrary, conformation of the Has\textsubscript{ASM} C-terminus produced in \textit{E. coli}, which is the last 15 residues containing the region R4 to R5, has been studied and revealed to be highly flexible and unstructured (32), suggesting that the C-terminal structure of Has\textsubscript{ASM}-VAL having the region R1 to R5 is unstructured. Probably, C-terminal tails of secretory proteins are unstructured during a process of secretion, and therefore, no rigid conformation seems to be necessary for secretion through Lip system although the region R1 to R5 plays an important role for the secretion.

Our findings reported here opened a new avenue for investigating a secretion mechanism of ABC exporter. Besides the crystallographic analysis of Lip\textsubscript{ASM} mutants or HasA chimeras, studies on a protein region of the ABC protein LipB, which is involved in the specific recognition by Lip system, are intriguing. Although 3D-structure of the \textit{E. coli} outermembrane protein TolC, which demonstrates that a tunnel connected to the external environment is constituted with trimeric TolC protein, has been reported recently (33, 34). However, 3D-structure of ABC protein and
mechanism of secretion is still unknown. Chimeric ABC proteins containing the LipB and HasD_{SM} sequences or mutants of ABC proteins will be helpful and informative for the exploration of the secretion specificity of Lip and Has_{SM} exporters. Through these approaches, a mechanism of the substrate recognition by ABC exporter will be totally understood.

Acknowledgements—We gratefully acknowledge Dr. C. Wandersman for her kind advice and generous gifts of the plasmids encoding *S. marcescens* and *P. aeruginosa* HasA proteins and *S. marcescens* Has_{SM} and *E. chrysanthemi* Prt exporters, and the anti-HasA antibody. The *P. aeruginosa* aprDEF_{PA} genes were kindly provided by Dr. M. Murgier. We thank Dr. I. B. Holland for his kind gift of the plasmid pLG575.

REFERENCES

1. Binet, R., Létoffé, S., Ghigo, J. -M., Delepelaire, P. and Wandersman, C. (1997) *Gene* 192, 7-11
2. Young, J. and Holland, I. B. (1999) *Biochim. Biophys. Acta.* 1461, 177-200
3. Akatsuka, H., Kawai, E., Omori, K. and Shiba, T. (1995) *J. Bacteriol.* 177, 6381-6389
4. Létoffé, S., Ghigo, J. -M. and Wandersman, C. (1993) *J. Bacteriol.* 175, 7321-7328
5. Binet, R. and Wandersman, C. (1996) *Mol. Microbiol.* 22, 265-273
6. Wandersman, C. and Delepelaire, P. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 4776-4780
7. Kawai, E., Akatsuka, H., Idei, A., Shibatani, T. and Omori, K. (1998) Mol. Microbiol. 27, 941-952
8. Létoffè, S., Ghigo, J.-M. and Wandersman, C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9876-9880
9. Létoffé, S., Ghigo, J.-M. and Wandersman, C. (1994) J. Bacteriol. 176, 5372-5377
10. Binet, R. and Wandersman, C. (1995) EMBO J. 14, 2298-2306
11. Akatsuka, H., Binet, R., Kawai, E., Wandersman, C. and Omori, K. (1997) J. Bacteriol. 179, 4754-4760
12. Létoffé, S., Delepelaire, P. and Wandersman, C. (1990) EMBO J. 9, 1375-1382
13. Ghigo, J.-M. and Wandersman, C. (1994) J. Biol. Chem. 269, 8979-8985
14. Mackman, N., Nicaud, J.-M., Gray, L. and Holland, I. B. (1986) Curr. Top Microbiol. Immunol. 125, 159-181
15. Kenny, B., Haigh, R. and Holland, I. B. (1991) Mol. Microbiol. 5, 2557-2568
16. Kenny, B., Taylor, S. and Holland, I. B. (1992) Mol. Microbiol. 6, 1477-1489
17. Zhang, F., Greig, D. I., and Ling, V. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 4211-4215
18. Jarchau, T., Chakraborty, T., Garcia, F. and Goebel, W. (1994) Mol. Gen. Genet. 245, 53-60
19. Duong, F., Lazdunski, A. and Murgier, M. (1996) Mol. Microbiol. 21, 459-470
20. Létoffé, S., Redeker, V. and Wandersman, C. (1998) Mol. Microbiol. 28, 1223-1234
21. Idei, A., Kawai, E., Akatsuka, H. and Omori, K. (1999) J. Bacteriol. 181, 7545-7551
22. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

23. Sakurai, N. and Komatsubara, S. (1996) *Lett. Appl. Microbiol.* **23**, 23-26

24. Akatsuka, H., Kawai, E., Omori, K., Komatsubara, S., Shibatani, T. and Tosa, T. (1994) *J. Bacteriol.* **176**, 1949-1956

25. Akatsuka, H., Kawai, E., Omori, K., Komatsubara, S. and Shibatani, T. (1996) *J. Ferment. Bioeng.* **81**, 115-120

26. Wandersman, C., Delepelaire, P., Létoffé, S. and Schwartz, M. (1987) *J. Bacteriol.* **169**, 5046-5053

27. Matsumae, H., Furui, M. and Shibatani, T. (1993) *J. Ferment. Bioeng.* **75**, 93-98

28. Takagi, T., Imai, Y., Sugiura, M., and Kisumi, M. (1985) *J. Biotechnol.* **3**, 59-71

29. Miller, J. H. (1992) *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

30. Baumann, U. (1994) *J. Mol. Biol.* **242**, 244-251

31. Baumann, U., Wu, S., Flaherty, K. M. and McKay, D. B. (1993) *EMBO J.* **12**, 3357-3364

32. Izadi-Pruneyre, N., Wolff, N., Redeker, V., Wandersman, C., Delepierre, M. and Lecroisey, A. (1999). *Eur. J. Biochem.* **261**, 562-568

33. Koronakis, V., Li, J., Koronakis, E., and Stauffer, K. (1997) *Mol. Microbiol.* **23**, 617-626

34. Koronakis, V., Sharff, A., Koronakis, E., Luisi, B., and Hughes, C. (2000) *Nature* **405**, 914-919
**Figure legend**

**FIG. 1.** *Secretion of HasA proteins via several ABC exporters.* *A,* *E. coli* DH5 cells carrying pUC/HasA<sub>PF</sub> and ABC exporter plasmids were cultured at 30°C for 40 h. The polypeptides in the supernatants of the cultured media (1.5 OD equivalents) were subjected to 12.5% SDS-PAGE, and stained by Coomassie brilliant blue G-250. Position of HasA<sub>PF</sub> is shown by an *arrowhead.* Lane 1, molecular mass markers; lane 2, pACYC184 (a vector); lane 3, pLG575 (HlyBD/TolC); lane 4, pACYC/OI70 (HasDE<sub>PF</sub>); lane 5, pK150 (HasDE<sub>SM</sub>/TolC); lane 6, pRUW4 (PrtDEF); lane 7, pAGS8 (AprDE<sub>PA</sub>); lane 8, pYBCD20 (LipBCD). *B,* *E. coli* DH5 cells harboring HasA plasmid (pUC/HasA<sub>SM</sub>, pUC/HasA<sub>PF</sub>, or pSYC1000) and ABC exporter plasmid (pACYC184, pK150, or pYBCD20) were cultured at 30°C for 40 h. The polypeptides in the media were analyzed as described above. The positions of molecular mass markers are shown on the left. Positions of HasA proteins are shown by *arrowheads* on the right. The exporters used are indicated above. Lane 1, pUC/HasA<sub>SM</sub>; lane 2, pUC/HasA<sub>PF</sub>; lane 3, pSYC1000.

**FIG. 2.** *Secretion of HasA chimeras composed of the HasA<sub>SM</sub> and HasA<sub>PF</sub> sequences via Lip and Has<sub>SM</sub> exporters.* *A,* Comparison of the predicted amino acid sequences among HasA proteins. The entire amino acid sequences are presented in one-letter code. To maximize homology, gaps (*dashes*) were introduced into the sequences. Identical amino acid residues are shown in an outline typeface with black background. The C-terminal segments present in HasA<sub>PF</sub> and HasA<sub>PA</sub> are boxed. *B,* HasA chimeras are schematically illustrated. The closed and open boxes represent amino acid sequences of HasA<sub>SM</sub> and HasA<sub>PF</sub>, respectively. The HasA<sub>PF</sub> C-terminal
segment (amino acid residues 181-192) is indicated by a crosshatched box. Amino acid residue numbers of the HasA_{PF} sequence are printed in *italics*. The *Bgl*II site is shown. C, The plasmids encoding HasA_{SM}, HasA_{PF}, and HasA chimeras were introduced into *E. coli* DH5 cells carrying the plasmids pACYC184 and pYBCD20. Secretion of these HasA chimeras via the Has_{SM} exporter has been confirmed (21). The polypeptides in the supernatant of the media cultured at 30°C for 40 h (1.5 OD equivalents) were subjected to 12.5% SDS-PAGE and then stained with Coomassie brilliant blue G-250. The positions of molecular mass markers are shown on the *left*. Positions of HasA proteins are shown by *arrowheads*. The exporters used are indicated *above*.

Lane 1, pUC/HasA_{PF}; lane 2, pUC/HasA_{SM}; lane 3, pMBF-HasA; lane 4, pMBΔF-HasA; lane 5, pFBΔF-HasA.

**FIG. 3.** *Construction of HasA_{SM} mutants and their secretion through Lip and Has_{SM} exporters.* A, The HasA_{PF} C-terminal segment of 12 amino acid residues and its derivatives were inserted into the HasA_{SM} sequence (between amino acid residues 174 and 175). The inserted sequences are presented in *one-letter* designations. Gaps (*dashes*) indicate the deletions, and amino acid replacements are shown in an *outline* typeface with *black* background. The *numbers* indicated below the sequences correspond to amino acid residue numbers of the HasA_{PF} C-terminal segment. The C-terminal segments of HasA_{PF} and HasA_{PA} are shown at the *bottom*. B, The HasA mutant plasmids were introduced into *E. coli* DH5 cells carrying pACYC184, pK150, or pYBCD20. The polypeptides in the supernatants of the media cultured at 30°C for 28 h were subjected to 12.5% SDS-PAGE. The protein amounts equivalent for optical densities of 3, 1.5, and 1.5 were loaded for none, Has_{SM} and Lip, respectively. The gels were stained by Coomassie brilliant blue G-250. The positions of molecular...
mass markers are shown on the left. The exporters used are indicated above each gel. Lane 1 pUC/HasA<sub>SM</sub>; lane 2, pMFM-HasA; lane 3, pMFM-182A; lane 4, pMFM-184A; lane 5, pMFM-189A; lane 6, pMFM-Δ(190-191); lane 7, pMFM-(190-191)G; lane 8, pMFM-ADVAL, lane 9, pMFM-AVAL; lane 10, pMFM-VAL; lane 11, pMFM-AL; lane 12, pMFM-ATDVL.

**FIG. 4.** Secretion of HasA<sub>SM</sub> mutants with amino acid substitutions at R1 to R6. Substitutions at R1 and R2 (A) were introduced into the HasA<sub>SM</sub>-AVAL encoded by pMFM-AVAL, and substitutions at R3, R4, R5, and R6 (B) were introduced into HasA<sub>SM</sub>-VAL encoded by pMFM-VAL (consult Fig. 3A). The primary structures of these HasA mutants are schematically illustrated above. Bars present HasA<sub>SM</sub> sequence, and the amino acid sequences of the inserted segments are boxed. The HasA<sub>SM</sub> sequences surrounding the segments are shown in one-letter designations with an outline typeface. The HasA mutant plasmids were introduced into *E. coli* DH5 cells carrying pACYC184, pK150, or pYBCD20. The polypeptides in the supernatants of the media cultured at 30°C for 28 h (1.5 OD equivalents) were subjected to 15% SDS-PAGE, and then stained by Coomassie brilliant blue G-250. Exporters used are shown in the left of each gel. Amino acid substitutions are indicated in one-letter designations above each lane. No extracellular HasA mutant proteins were detected in the cultured media of *E. coli* cells carrying pACYC184 (data not shown).

**FIG. 5.** Comparison of the C-terminal amino acid sequences of proteins secreted through Lip exporter. The amino acid sequences at the C-termini of HasA<sub>SM</sub> and the proteins secreted through Lip exporter are shown in one-letter designations. Gaps (dashes) are introduced into the sequences to maximize homology.
The C-terminal hydrophobic amino acid residues and negatively charged residues are shown in an *outline* typeface with *black* background, and the negatively charged amino acid residues are *boxed*. The amino acid residues of the region R1 to R6, valine, alanine, leucine, valine, glycine, valine, and glutamine at the positions 175, 176, 177, 178, 179, and 180 of the HasASM-VAL sequence, are *boxed*.

**Fig. 6.** *Secretion of HasASM mutants through hybrid exporters.* The plasmids encoding HasA plasmids (HasASM, HasASM-VAL, and HasASM-VAA; consult Fig. 4B) were introduced into *E. coli* DH5 cells carrying pACYC184 (None) plus pMW/LipCD, pACYC/LipB plus pMW/LipCD, pACYC/HasDASM plus pMW/HasEASM, and pACYC/HasDASM plus pMW/HasEASM. The polypeptides in the supernatants of the media cultured at 30°C for 40 h (1.5 OD equivalents) were subjected to 15% SDS-PAGE. Proteins were subjected to immunoblot analysis using anti-HasASM antibody. Lane 1, pUC/HasASM; lane 2; pMFM-VAL; lane 3, pMFM-VAA. Combinations of hybrid exporter are shown above each gel.

**Fig. 7.** *Secretion analysis of HasA mutants carrying amino acid substitutions at the C-terminus.* A, Substitutions introduced into the C-terminal sequence of HasASM-VAL encoded by pMFM-VAL (consult Fig. 4A) are shown at the top in *one-letter* designations with amino acid residue numbers. The conserved C-terminal motif is shown in an *outline* typeface with *black* background, and negatively charged amino acid residue is *boxed*. The inserted segment is *boxed*. An asterisk presents a termination codon. The amino acid residues substituted are printed in an *outline* typeface with *black* background. B, The HasA mutant plasmids were introduced into *E. coli* DH5 cells carrying pACYC184, pK150, or pYBCD20. The
polypeptides in the supernatants of the media cultured at 30°C for 28 h were
concentrated (1.5 OD equivalents), subjected to 15% SDS-PAGE, and then strained by
Coomassie brilliant blue G-250. The positions of molecular mass markers are shown
on the left. The exporters used are indicated above each gel. Lane 1, pUC18; lane 2,
pMFM-VAL; lane 3, pMFM-A1; lane 4, pMFM-A2; lane 5, pMFM-A1A2; lane 6,
pMFM-E4; lane 7, pMFM-E3; lane 8, pMFM-E2; lane 9, pMFM-E1; lane 10,
pMFM-A1E4; lane 11, pMFM-A1E3; lane 12, pMFM-A1E2; lane 13, pMFM-A1E1;
lane 14, pMFM-A2E4; lane 15, pMFM-A2E3; lane 16, pMFM-A2E2; lane 17,
pMFM-A2E1. C, The HasA plasmids (pUC/HasASM, pMFM-VAL, pMFM-A1,
pMFM-E1) were introduced into E. coli DH5 cells carrying pACYC184, pK150,
pYBCD20, or pRUW8. The polypeptides in the supernatants of the media cultured at
30°C for 28 h (1.5 OD equivalents) were analyzed as described above. The exporters
used are indicated above each gel. Lane 1, pUC/HasASM; lane 2, pMFM-VAL; lane 3,
pMFM-A1; lane 4, pMFM-E1.

**FIG. 8.** Secretion analysis of LipASM mutants in *S. marcescens.* A,
Ala-substitutions were introduced at the positions R1 to R6 of LipASM. The
C-terminal sequence of LipASM is shown at the top in *one-letter* designations with
amino acid residue numbers. A C-terminal motif is shown in an *outline* typeface with
*black* background, and a negatively charged amino acid residue is boxed. The amino
acid residues at the positions R1 to R6 were *boxed.* The amino acid residues
substituted are printed in an *outline* typeface with *black* background. B, Secretion of
LipASM mutants from *S. marcescens* 413 cells. The polypeptides in the supernatants
of the lipase media cultured at 30°C for 28 h (0.24 OD equivalents) were subjected to
12.5% SDS-PAGE and strained by Coomassie brilliant blue G-250. The positions of
molecular mass markers are shown on the left. The positions of LipASM (L), PrtA (P), and flagellin (F) are indicated with an arrowhead on the right. Lane 1, pUC19; lane 2, pLIPE121; lane 3, pL IPA-596 A; lane 4, pLIPA-597 A; lane 5, pLIPA-598 A; lane 6, pLIPA-599 A; lane 7, pLIPA-600 A; lane 8, pLIPA-601 A. C, LipASM mutants carrying Ala-substitutions and deletions in the C-terminal sequence are schematically illustrated as described above. To maximize homology, gaps (dashes) were introduced into the sequences. Spans between Gly at R5 and the negatively charged Asp in the C-terminal motif, and distances of the R5 Gly from the C-terminus are indicated on the right. D, Secretion of the LipASM carrying mutations at C-terminus from S. marcescens 413 cells. The polypeptides in the supernatants of the lipase media cultured at 30°C for 28 h (0.24 OD equivalents) were subjected to 12.5% SDS-PAGE as described above. The positions of molecular mass markers are shown on the left. The positions of LipASM (L), PrtA (P), and flagellin (F) are indicated with an arrowhead on the right. Lane 1, pUC19; lane 2, pLIPE121; lane 3, pLIPA-609 A; lane 4, pLIPA-S4; lane 5, pLIPA-S5; lane 6, pLIPE121E; lane 7, pLIPA-S10; lane 8, pLIPA-S12; lane 9, pUC19; lane 10, pLIPE121; lane 11, pLIPA-PA2; lane 12, pLIPA-PA1; lane 13, pLIPA-M1; lane 14, pLIPA-M2; lane 15, pLIPA-M3; lane 16, pLIPA-F1, lane 17, pLIPA-K1; lane 18, pLIPA-E1.

**FIG. 9.** **Secretion competition analysis of HasA-VAL through Lip exporter in E. coli.** Secretion of FLAG-tagged Has-VAL was tested in the E. coli cells carrying Lip exporter (pFBCD1 for lanes 1 to 5; pYBCD20 for lane 6) and competitor plasmids. The polypeptides in the supernatants of the cultured media at 30°C for 24 h (2 OD equivalents for lanes 1 to 5; 1.5 OD equivalents for lane 6) were subjected to 15% SDS-PAGE and stained by Coomassie brilliant blue G-250 (upper). Proteins were
also analyzed with anti-FLAG (middle) and anti-HasA\textsubscript{SM} antibodies. The positions of molecular mass markers are shown on the left. The positions of FLAG-HasA-VAL and HasA-VAL proteins are indicated with an arrowhead on the right. Lane 1, pUC18; lane 2, pUC/HasA\textsubscript{SM}; lane 3, pMFM-VAL; lane 4, pMFM-VAA; lane 5, pMFM-R5A; lane 6, pUC/HasA-VAL.
Fig. 1AB. Omori et al. 2001
Fig. 2A Omori et al. 2001
Fig. 2BC Omori et al. 2001
Fig. 3A Omori et al. 2001

S. marcescens HasA

P. fluorescens HasA 181 AHAT--ATTDDVAL 192
P. aeruginosa HasA 178 AHATPAAAAAEVG 191
Fig. 3B Omori et al. 2001
Fig. 4 Omori et al. 2001
|                 | -/LipC/LipD | LipB/LipC/LipD | LipB/HasE/TolC | HasD/HasE/TolC |
|----------------|-------------|----------------|---------------|---------------|
| 1              |             |                |               |               |
| 2              |             |                |               |               |
| 3              |             |                |               |               |

(kDa)

Fig. 6 Omori et al. 2001
A

-ATA VAL VGVQHADSPELLAA*

plasmid

pMFM-VAL -ATA VAL VGVQHADSPELLAA*
pMFM-A1 -ATA VAL VGVQHADSPELLAA*
pMFM-A2 -ATA VAL VGQHAASPELLAA*
pMFM-A1A2 -ATA VAL VGQHAASPELLAA*
pMFM-E4 -ATA VAL VGVQHADSPELLAA*
pMFM-E3 -ATA VAL VGVQHADSPELLAA*
pMFM-E2 -ATA VAL VGVQHADSPELLAA*
pMFM-E1 -ATA VAL VGVQHADSPELLAA*
pMFM-A1E4 -ATA VAL VGVQHADSPELLAA*
pMFM-A1E3 -ATA VAL VGVQHADSPELLAA*
pMFM-A1E2 -ATA VAL VGVQHADSPELLAA*
pMFM-A1E1 -ATA VAL VGVQHADSPELLAA*
pMFM-A2E4 -ATA VAL VGQHAASPELLAA*
pMFM-A2E3 -ATA VAL VGQHAASPELLAA*
pMFM-A2E2 -ATA VAL VGQHAASPELLAA*
pMFM-A2E1 -ATA VAL VGQHAASPELLAA*

Fig. 7A Omori et al. 2001
Fig. 7B Omori et al. 2001
|    | None | Has$_{SM}$ | Lip | Prt |
|----|------|-----------|-----|-----|
| 1  |      |           |     |     |
| 2  |      |           |     |     |
| 3  |      |           |     |     |
| 4  |      |           |     |     |

Fig. 7C Omori et al. 2001
### A

| Plasmid     | Sequence                        |
|-------------|---------------------------------|
| pLIPE121    | -NDLVLAFGHSQVTLLGVSQSLDHFPDQVLA* |
| pLIPA-596A  | -NDLVLAFGHSQVTLLGVSQSLDHFPDQVLA* |
| pLIPA-597A  | -NDLVLAFGHSQVTLLGVSQSLDHFPDQVLA* |
| pLIPA-598A  | -NDLVLAFGHSQVTLLGVSQSLDHFPDQVLA* |
| pLIPA-599A  | -NDLVLAFGHSQVTLLGVSQSLDHFPDQVLA* |
| pLIPA-600A  | -NDLVLAFGHSQVTLLGVSQSLDHFPDQVLA* |
| pLIPA-601A  | -NDLVLAFGHSQVTLLGVSQSLDHFPDQVLA* |

### B

Fig. 8AB Omori et al. 2001
C

| plasmid       | LipA<sub>SM</sub> sequence | span | position of R5 Gly |
|---------------|-----------------------------|------|-------------------|
| pLIPE121      | -NDDLVLAFGHSQVTLIGVSL-DH-FNPDQVVLA* | 8    | -15               |
| pLIPE-609A    | -NDDLVLAFGHSQVTLIGVSL-DH-FNPAOQVVLA* | 8    | -15               |
| pLIPE-S4      | -NDDLVLAFGHSQVTLIGVSL-D---DQVVLA*   | 4    | -11               |
| pLIPE-S5      | -NDDLVLAFGHSQVTLIGVSL-DH------DQVVLA* | 5    | -12               |
| pLIPE-S10     | -NDDLVLAFGHSQVTLIGVSLAADD--DQVVLA*  | 10   | -17               |
| pLIPE-S12     | -NDDLVLAFGHSQVTLIGVSLAAADHPNPDQVVLA* | 12   | -19               |
| pLIPE-PA2     | -NDDLVLAFGHSQVTLIGVSLAAD--DQVVLA*  | 8    | -17               |
| pLIPE-PA1     | -NDDLVLAFGHSQVTLIGVSLAAD--DQVVLA*  | 8    | -16               |
| pLIPE-M1      | -NDDLVLAFGHSQVTLIGVSL-DH-FNPDQVVL*  | 8    | -14               |
| pLIPE-M2      | -NDDLVLAFGHSQVTLIGVSL-DH-FNPDQVV*   | 8    | -13               |
| pLIPE-M3      | -NDDLVLAFGHSQVTLIGVSL-DH-FNPDQV*    | 8    | -12               |
| pLIPE-F1      | -NDDLVLAFGHSQVTLIGVSL-DH-FNPDQVLF*  | 8    | -16               |
| pLIPE-K1      | -NDDLVLAFGHSQVTLIGVSL-DH-FNPDQVLK*  | 8    | -15               |
| pLIPE-E1      | -NDDLVLAFGHSQVTLIGVSL-DH-FNPDQVLAE* | 8    | -16               |

D

(kDa)

1  2  3  4  5  6  7  8  9  10  11  12  13  14  15  16  17  18

L  P  F
Serratia ATP-binding cassette protein exporter, Lip, recognizes a protein region upstream of C-terminus for specific secretion
Kenji Omori, Akiko Idei and Hiroyuki Akatsuka

J. Biol. Chem. published online May 2, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M101410200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts