Two Genes Encoding Serine Protease Homologues in *Serratia marcescens* and Characterization of Their Products in *Escherichia coli*¹

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A serine protease (SSP) of *Serratia marcescens* is one of the extracellular enzymes secreted from this Gram-negative bacterium. SSP is produced as a large precursor and converted to a mature protein by cleavages removing an NH₂-terminal signal sequence and a COOH-terminal pro-region. This COOH-terminal pro-region is integrated into the outer membrane and has a functional role for the export of the mature protein across the outer membrane. Southern hybridization analysis with a DNA fragment encoding the COOH-terminal pro-region as the probe showed a wide distribution of nucleotide sequences encoding SSP exporter-like proteins among *Serratia* species. Moreover, *S. marcescens* IFO 3046, from which the *ssp* gene had been cloned, was found to contain two *ssp* homologues (*ssp-h₁* and *ssp-h₂*). They were cloned and their nucleotide sequences were determined. The two *ssp* homologues were found to exist in tandem on the genome and their amino acid sequences showed 81% identity to each other. Both of them showed 55% identity in amino acid sequence to preproSSP. In addition, both showed end-to-end similarity to the 100 kDa serotype-specific antigen (Ssal) of *Pasteurella haemolytica*. *Escherichia coli* JM105 containing *ssp-h₁* gene produced a 53 kDa protein corresponding to the NH₂-terminal portion and a 49 kDa protein corresponding to the COOH-terminal portion, both of which were rigidly integrated in the outer membrane. Consistent with the significant similarity of the COOH-terminal portions of the homologues to that of SSP, they showed the ability to translocate the mature SSP part across the outer membrane into the medium. Furthermore, the NH₂-terminal portion of the homologue was not translocated into the outer membrane without its COOH-terminal part. All of these data show that the SSP homologues are outer membrane proteins that are translocated into the outer membrane with the aid of the translocator function of their COOH-terminal part.

Key words: chimeric protein, outer membrane protein, pro-sequence, protein secretion, *Serratia* serine protease.

Gram-negative bacteria have two distinct membrane systems, the outer membrane and the inner cytoplasmic membrane, spaced by the periplasmic compartment. Proteins from Gram-negative bacteria are therefore required to traverse two cell membranes if they are to be secreted extracellularly (1). We have studied the specific excretion of a serine protease (SSP) of *Serratia marcescens* through the outer membrane of *Escherichia coli* and elucidated its secretion system, including the folding mechanism of the mature protease domain (2-6). SSP is synthesized as a large precursor (preproSSP) in which the domain that will become the mature enzyme is flanked by a typical secretion signal sequence at the NH₂-terminus and a large pro-domain at the COOH-terminus. Although preproSSP is likely to employ the Sec-machinery to traverse the cytoplasmic membrane, it does not require any separately encoded accessory factors to translocate through the outer membrane. The COOH-terminal pro-domain of preproSSP is integrated into the outer membrane and is essential for transport through this membrane. The protease part is then released from the cell by an autoproteolytic cleavage, leaving the pro-domain embedded in the outer membrane. The specific excretion of SSP was also observed in *S. marcescens* (7). IgA proteases of *Neisseria* (8) and *Haemophilus* (9) are also excreted in the medium in the same way. In the case of SSP, the mature part is folded into the stable and active conformation with the aid of a guide peptide, named a junction region, corresponding to the NH₂-terminal portion of the COOH-terminal pro-domain (6). In addition to SSP and the IgA proteases, several proteins which are probably secreted in the same

1 Nucleotide sequence accession number. The nucleotide sequence encoding *ssp-h₁* and *ssp-h₂* has been registered in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number D78380.
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way have recently been discovered in Gram-negative bacteria of different species, such as vacuolating cytotoxin (VacA) of Helicobacter pylori (10, 11), temperature-sensitive haemagglutinin (Tsh) of avian pathogenic E. coli (12), an IgA protease-like protein (HApA) involved in in vitro attachment and entry of Haemophilus influenzae (13), the major extracellular protein (SepA) of Shigella flexneri (14), and EPEC-secreted protein C (EspC) of enteropathogenic E. coli (EPEC) (15). The growing number of extracellular proteins in this family in different species of Gram-negative bacteria suggest that the secretion pathway of this family is generally distributed among Gram-negative bacteria.

During our study on secretion of SSP, we found that S. marcescens contained two additional nucleotide sequences homologous to part of the ssp gene encoding the COOH-terminal pro-domain. For further investigation of the secretion system of SSP, we cloned and sequenced the two homologous genes. Despite significant similarity in the amino acid sequences of the homologues to SSP, neither showed any protease activity when expressed in E. coli. Both homologue proteins were translocated into the outer membrane with the aid of their COOH-terminal domain. Because of homology of the SSP homologues with several outer membrane proteins in other species, they are supposedly membrane proteins that are localized in the outer membrane in the same manner as SSP. In this paper, we describe the nucleotide sequences of SSP homologues and characterization of their products in E. coli. The role of the COOH-terminal part of the homologues as a translocator into the outer membrane, as determined by using chimeric proteins between SSP and the homologues, is also described.

MATERIALS AND METHODS

Bacterial Strains and Plasmids—E. coli JM105 [Δ(lac pro) thi rpsL endA sbcB15 hsdR4 F' traD36 proAB lacIq lacZAM15] (16), purchased from Amersham Japan (Tokyo), was used as the host for both phage M13 propagation and cloning and expression of the ssp-homologs. E. coli CJ236 [Δ(lac pro) thi rpsL endA sbcB15 hsdR4 F' traD36 proAB lacIq lacZAM15] (17), purchased from Takara Shuzo (Kyoto), to produce uracil-containing single-stranded DNA for site-directed mutagenesis. Plasmid pSP11tac contained the ssp gene under the control of the tac promoter on pBR322 (3).

Recombinant DNA Techniques—Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, and Klenow fragment of DNA polymerase I were purchased from Takara Shuzo. General techniques for DNA manipulation in E. coli were as described by Maniatis et al. (18). Site-directed mutagenesis was performed by the method of Kunkel et al. (17) using the Mutan-K site-directed mutagenesis system purchased from Takara Shuzo. Nucleotide sequences were determined by the dideoxy chain termination procedure (19) using an Autocycle Sequencer kit (Pharmacia) and Automated Fluorescence DNA sequence (DSQ 1, Shimadzu).

Southern Hybridization Analysis—Chromosomal DNAs from Serratia and related species were prepared according to the reported method (20). BamHI-digested DNA fragments were transferred from agarose gel to a positively charged nylon membrane (Hybond N*, Amersham Japan), as described by Southern (21). DNA fragments for hybridization probes were labeled with [α-32P]dCTP (110 TBEq/mm, Amersham Japan) and a BcaBEST labeling kit purchased from Takara Shuzo.

Cloning of the ssp-Homologues and Construction of Expression Plasmids—Among the three positive signals (14, 2.8, and 1.5 kb) detected by Southern hybridization with a 1.4 kb BglII-StuI fragment (encoding Ser104Phe of SSP) as a probe, the 1.4 kb signal was supposed to represent the ssp gene itself. We therefore cloned 1.5 and 2.8 kb BamHI-fragments into pUC19 by the standard DNA-probing method. We first isolated the 1.5 kb BamHI fragment by the colony hybridization method with the same probe, constructing pHA1 (see Fig. 2). We next searched for a restriction enzyme that produced a larger fragment giving positive hybridization signals with a 0.4 kb BamHI-PvuII fragment (probe 1, see Fig. 2) in the cloned 1.5 kb BamHI fragment. Sppl was found to produce two signals of 2.7 and 6.0 kb. The 2.7 and 6.0 kb Sppl fragments were cloned into pUC19, resulting in pHA2 and pHB1, respectively. The 2.7 kb Sppl fragment was found to include almost all of the originally cloned 1.5 kb BamHI fragment. The 6.0 kb Sppl fragment was found to include the full length of another ssp-homologue. We also cloned a 2.6 kb BamHI-PstI fragment into pUC19 using a 0.8 kb Sppl-BamHI fragment as a hybridization probe (probe 2, see Fig. 2), resulting in pHA3. This BamHI-PstI fragment contained a 3'-portion of one ssp homologue named ssp-h1 and a 5'-portion of the other homologue named ssp-h2, which indicated that both homologues were located in tandem.

For expression of the ssp homologues in E. coli JM105, the tac promoter in pUC18 was used. A 4.1 kb EcoRV-Sppl fragment covering the whole ssp-h1 gene was excised from pHB1 and cloned into pUC19 digested with Hinfl and Sppl, resulting in pSPH1/18 (see Fig. 2). A 4.1 kb Knpl-HindIII fragment of pSPH1/18 was cloned into the multi-linker of pUC19, resulting in pSPH1/19. A 2.0 kb PstI-HindIII fragment of pPHA2 was cloned into pHA3 digested with PstI and HindIII, resulting in pSPH2/19 (see Fig. 2). A 4.6 kb Knpl-HindIII fragment of pSPH2/19 was cloned into the multi-linker of pUC18, resulting in pSPH2/18.

Construction of Plasmids for Expression of Chimera Protein—A Nhel recognition sequence (GCTAGC) was introduced at four different sites within the SSP- and SSP-h2-coding regions by site-directed mutagenesis or PCR using a synthetic oligonucleotide (Table I) to construct pSP11tac1N, pSP11tac2N, pSPH2-1N, and pSPH2-2N. The 1.4 kb BamHI and HindIII fragment of pSPH2-2N was substituted for the 1.9 kb BamHI and HindIII fragment of pSPH1, constructing pSPH1-2N. A Mlu1 recognition sequence (ACCGGT) was similarly introduced at two different sites within the SSP- and SSP-h2-encoding regions to construct pSP11tac1M and pSPH2-1M, respectively.

Plasmid pSPH0222 was constructed by ligating a 4.6 kb Nhel-StuI fragment of pSP11tac1N and a 3.0 kb Nhel-HindIII fragment of pSPH2-1N after the HindIII site had been filled-in by Klenow fragment. Plasmid pSP0022 was constructed by ligating a 5.6 kb MluI-StuI fragment of pSP11tac1M and a 2.0 kb MluI-HindIII fragment of pSPH2-1M after the HindIII site had been treated with Klenow fragment. Plasmid pSP0002 was constructed by ligating a 5.4 kb Nhel-StuI fragment of pSP11tac2N and a 2.2 kb Nhel-HindIII fragment of pSPH2-2N after the
TABLE 1. Oligonucleotides used for this study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Position</th>
<th>Oligonucleotide*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSP111lacN</td>
<td>Upstream from the PstI site of pSP11lac</td>
<td>5'-TGGGATCTTGATAAGGGCGCATCAGGAAGATGA-3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'-GGTAAATTTTGCAGAGTCG-3'</td>
</tr>
<tr>
<td>pSP112acN</td>
<td>Ser^{<em>}(TCT)Leu^{</em>}(CTT)</td>
<td>Site directed mutagenesis</td>
</tr>
<tr>
<td>pSP111lacM</td>
<td>Thr^{*}(ACC)</td>
<td>Site directed mutagenesis</td>
</tr>
<tr>
<td>pSH2-1N</td>
<td>Gin^{*}(CAG)</td>
<td>Site directed mutagenesis</td>
</tr>
<tr>
<td>pSH2-2N</td>
<td>Ser^{<em>}(AGC)Val^{</em>}(GTG)</td>
<td>Site directed mutagenesis</td>
</tr>
<tr>
<td>pSH2-1M</td>
<td>Phe^{*}(TTC)</td>
<td>Site directed mutagenesis</td>
</tr>
<tr>
<td>pSH2-675Term</td>
<td>Ser^{*}(AGC)</td>
<td>Site directed mutagenesis</td>
</tr>
</tbody>
</table>

*Underlined nucleotides were those used to introduce restriction enzyme recognition sites or change the codon.

HindIII site had been treated with Klenow fragment.

Plasmid pSH1100 was constructed by ligating a 5.2 kb NheI–SphI fragment of pSH1-2N and a 1.2 kb NheI–SphI fragment of pSP111lac2N.

Plasmid pSH2-675Term was constructed by ligating a 0.9 kb PstI–HindIII fragment of pSP12 product and a 5.0 kb PstI–HindIII fragment of pSH2. Plasmid pSPH02Term was constructed by ligating the 4.6 kb NheI–SalI fragment of pSP111lac1N and the 1.9 kb NheI–HindIII fragment of pSH2-675Term after the HindIII site had been filled-in by Klenow fragment.

Preparation of Extracellular, Periplasmic, Cytoplasmic, and Insoluble Fractions—An overnight culture of E. coli transformants (100 μl) was inoculated in 10 ml of L-broth (22) containing 50 μg/ml ampicillin in a culture tube. After aerobic cultivation at 37°C for 4 h, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM to induce the lac or tac promoter and the incubation was continued for an additional 2 h. If induction by IPTG was not necessary, the incubation was continued for 8 h. The E. coli cells were harvested by centrifugation and the supernatant was used as an extracellular fraction. The precipitated cells were suspended in 5 ml of 10 mM Tris-HCl buffer (pH 8.5) containing 20% sucrose and exposed to cold osmotic shock (23) to obtain a periplasmic fraction. The cells were then disrupted with a sonicator (Branson sonifier cell disruptor 200) and centrifuged at 12,000 × g for 20 min to obtain cytoplasmic (supernatant) and insoluble (pellet) fractions. Proteins in the extracellular fraction were precipitated with 0.4 M trichloroacetic acid, washed with ethanol/diethyl ether (1:1), dried and suspended in 62.5 mM Tris-HCl buffer, pH 8.8, containing 2% SDS and 1% 3-mercaptopropanol. Each sample (extracellular fraction of 200 μl of culture, periplasmic fraction of 200 μl of culture, cytoplasmic fraction of 100 μl of culture, and insoluble fraction of 200 μl of culture) was analyzed by SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (24) and by immunoblot hybridization (see below). The gels were stained with Coo massie Brilliant Blue (CBB).

NH₂-Terminal Amino Acid Sequencing—The proteins separated by SDS-polyacrylamide gel electrophoresis were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilion Transfer, 0.45-μm pore size; Millipore). The membrane was stained with 0.1% amide black in 40% methanol/10% acetic acid and destained with 10% isopropanol/10% acetic acid. The protein band to be examined was cut out and washed with HPLC-grade water (Wako Pure Chemicals). Amino acid sequences were determined using an Applied Biosystems (ABI) model 447A pulsed liquid-phase sequencer equipped with model 120A on-line phenylthiohydantoin amino acid analyzer. All reagents for sequence analysis were purchased from ABI.

Membrane Fractionation—The inner and outer membranes of E. coli cells were prepared by sucrose density gradient centrifugation according to the method of Miura and Mizushima (25). An overnight culture (1.5 ml) of E. coli cells harboring pSH1/19 was inoculated in 150 ml of L-broth containing 50 μg/ml of ampicillin. After 12 h cultivation at 37°C with shaking, the cells were centrifuged at 6,000 × g for 5 min at 4°C and washed with buffer A containing 50 mM Tris-HCl (pH 7.5), 250 mM sucrose, and 1 mM dithiothreitol. The cells were then suspended in buffer A (5 ml/g of wet cells) and disrupted with a sonicator. Cell debris was removed by low-speed centrifugation at 2,500 × g for 10 min at 4°C, and the membrane fraction was collected by high-speed centrifugation of the supernatant at 155,000 × g for 2 h at 4°C. The precipitate was suspended in 1 ml of 10 mM Tris-HCl buffer (pH 7.5) and fractionated by 20–60% (w/w) sucrose density gradient centrifugation at 70,000 × g for 12 h at 4°C. Each fraction was analyzed by SDS–polyacrylamide gel electrophoresis.

Trypsin Treatments—E. coli cells grown as described above were washed with 10 mM Tris-HCl buffer (pH 8.0) containing 0.85% NaCl, and resuspended in the same buffer. Half of the suspension was used as the intact cell suspension and the other half was subjected to the cold osmotic shock described above. Trypsin (Sigma Chemical) was added to the intact and osmotically shocked cell suspension at a final concentration of 1 mg/ml, and the mixtures were incubated at 37°C for 30 min. The digestion was stopped by adding Nα-p-tosyl-L-lysine-chloromethyl ketone (TLCK; Sigma Chemical), and the mixture was placed on ice for 15 min. The cells were washed twice with the same buffer containing TLCK, suspended in 10 mM Tris-HCl buffer (pH 7.5) and disrupted with a sonicator. The insoluble fraction of 300 μl of culture was analyzed by SDS–polyacrylamide gel electrophoresis.

Detection of Protease Activity—E. coli cells harboring expression plasmids were inoculated on an L-broth agar plate containing 1% agar, 1% skim milk, and 50 μg/ml ampicillin. If the induction of lac or tac promoter was required, 1 mM IPTG was previously added to the plate. After overnight incubation at 37°C, turbid halo formation around the colonies was examined.

Immunological Analysis—The antibodies specific to preproSSP (anti-preproSSP antibody) (4) were used for immunological detection of SSP and its COOH-terminal pro-domain by the method of Burnett (26) with anti-rabbit (goat) antibodies conjugated with peroxidase (Bio-Rad) as a label.
secondary antibodies. PVDF membranes were used for Western blotting.

**RESULTS**

**Distribution of Nucleotide Sequences Homologous with the Region Encoding the COOH-Terminal Pro-Domain of PreproSSP among Serratia spp.**—PreproSSP (Met to 1048Phe) consists of a typical NH2-terminal signal peptide (Met to 27Ala), the mature protease domain (28Ala to 645Asp), and a large COOH-terminal pro-region (646Ser to 1048Phe) (see Fig. 3). The large COOH-terminal pro-region contains a junction region (646Ser to 718Gly) that serves as a guide peptide for folding of the mature part in the medium (6). In translocation of SSP across the outer membrane of E. coli, the COOH-terminal pro-domain may form a pore through which the mature and junction parts are excreted into the medium, playing a critical role in the excretion of SSP. In order to search for proteins that are excreted in the same manner in Serratia and related species, we analyzed the genomes of these bacteria by Southern hybridization by using a 32P-labeled 1.4 kb BglIII-StuI fragment encoding almost the entire part of the COOH-terminal pro-domain (677Ser to 1048Phe) of preproSSP. Several bands showed hybridization in the BamHI-digested genomes of S. ficaria, S. plymuthica, S. rubidaea, and S. odorifera (Fig. 1), suggesting the presence of proteins that would be secreted in the same manner as SSP in a wide range of species of Serratia. No hybridized band, however, was detected in S. fonticola (data not shown). Three hybridized bands were detected in the BamHI-digested genome of S. marcescens IFO 3046 from which the ssp gene had been cloned (Fig. 1). Among the three hybridized bands, the 14 kb signal was thought to represent the ssp gene on the basis of the restriction map of the fragment covering ssp (2). It was thus expected that this S. marcescens strain contained two ssp-homologues. On the other hand, no ssp-homologue was detected in the genomes of related bacteria, such as Enterobacter cloacae, Citrobacter freundii, Klebsiella pne-

**Fig. 1. Distribution of homologues of the COOH-terminal pro-domain of preproSSP among Serratia species.** Using a 32P-labeled 1.4 kb BglIII-StuI fragment encoding the COOH-terminal pro-domain of preproSSP (677Ser to 1048Phe) as a probe, BamHI-digested genomes of Serratia spp. were analyzed by Southern hybridization. Serratia spp. tested were S. ficaria IAM 13540 (lane 1); S. plymuthica IAM 13543 (lane 2); S. rubidaea IAM 13545 (lane 3); S. odorifera IAM 13542 (lane 4); and S. marcescens IFO 3046 (lane 5).
logues. Moreover, the serotype-specific antigen (Ssa1) of Pasteurella haemolytica (29, 30), which was identified as a 100 kDa outer membrane protein, showed significant homology (25% identity) to preproSSP and the SSP-homologues over the entire sequences. The alignment is also shown in Fig. 3.

Expression of ssp-h1 in E. coli—We expressed the homologue genes and determined the localization of the products in E. coli to see whether they were secreted in the same manner as SSP. E. coli JM105 cells containing pSPh1/19, which carried ssp-h1 in the orientation opposite to that of the lac promoter, were used for characterization of the products (Fig. 4A). A weak cross-reaction between SSP-h1 (especially its N-terminal portion) and the anti-preproSSP antibody hampered detection of products derived from ssp-h1 by immunoblotting (data not shown). Without the antibody, we could discriminate the products from the proteins derived from the E. coli host. In E. coli harboring pSPh1/19, two proteins which were estimated to be 53 and 49 kDa were detected in the insoluble fraction. NH₂-Terminal amino acid sequencing of the 53 and 49 kDa proteins gave sequences of 45YIENGK and 562SIGTLN, respectively, indicating that the 53 kDa protein corresponded to the NH₂-terminal portion with the signal peptide removed and the 49 kDa protein corresponded to the COOH-terminal portion (see Fig. 6). Because pSPh1/19 carried ssp-h1 in the orientation opposite to that of the lac promoter, the promoter sequence of ssp-h1 was functional in E. coli. In E. coli containing pSPh1/18, which carried ssp-h1 in the same orientation as that of the lac promoter, 53 and 49 kDa proteins were detected, just as for pSPh1/19, regardless of the induction of the lac promoter on addition of IPTG (data not shown).

The above data showed that the 53 kDa protein corresponding to the mature part of SSP was present in the insoluble fraction, but not in the extracellular fraction. For determination of cellular localization of the 53 and 49 kDa proteins, membrane fractionation by sucrose density gradient centrifugation and trypsin treatment of cells containing ssp-h1 were carried out. Besides major outer membrane proteins such as OmpF and OmpA, both proteins derived from SSP-h1 were detected in the outer membrane fraction on the fractionation (Fig. 4B). In addition, the 53 kDa protein was not digested by trypsin even when the cells were subjected to a cold osmotic shock to allow trypsin to penetrate into the periplasm (Fig. 4C). A 39 kDa protein that appeared on treatment of the intact cells with trypsin was found to be derived from the 49 kDa protein because its NH₂-terminal amino acid sequence was determined to be 67NGTSFA. This 39 kDa protein was also detected on the osmotically shocked cells with trypsin treatment. These results suggest that the 53 and 49 kDa proteins are rigidly integrated in the outer membrane. The cleavage site (67.Arg-671Asn) of the 49 kDa protein by trypsin is probably located on the cell surface.

The 53 kDa product was thought to contain about 80% of the corresponding mature SSP enzyme, including the catalytic triad of serine proteases, on the basis of its
Fig. 3. Alignment of amino acid sequences of SSP, SSP-h1, SSP-h2, and Seal. The serine, histidine, and aspartic acid residues which presumably compose the catalytic triad of serine proteases and the four conserved cysteine residues are indicated. The domain structure of preproSSP is illustrated. Among the four proteins, 3 or 4 identical amino acid residues in the alignment are indicated by black boxes. Dashes indicate gaps introduced for alignment.
Expression of ssp-h1 in E. coli and localization of its products in the outer membrane. (A) The E. coli cells containing pSPh1/19 and pUC19, as a control, were fractionated and analyzed by SDS-polyacrylamide gel electrophoresis. Proteins were stained with CBB. E, P, C, and I denote extracellular, periplasmic, cytoplasmic, and insoluble fractions, respectively. The protein bands of interest are indicated by arrows. Molecular mass markers are; 94 kDa (phosphorylase b), 67 kDa (bovine serum albumin), 43 kDa (ovalbumin), and 30 kDa (carbonic anhydrase). (B) SDS-polyacrylamide gel electrophoresis of membrane preparations of E. coli JM105 carrying pSPh1/19 after fractionation by sucrose density gradient centrifugation. The 53 kDa protein which corresponded to the NH2-terminal part was detected in the outer membrane fraction, along with the 49 kDa protein which corresponded to the COOH-terminal part. (C) Trypsin treatment of the E. coli cells carrying pSPh1/19. Trypsin-treated and non-treated E. coli cells containing pSPh1/19 were sonicated and their insoluble fractions were analyzed. The insoluble fractions prepared from the intact cells without the trypsin treatment (lane 1), and the insoluble fractions prepared from the intact cells treated with trypsin (lane 2), from the osmotically shocked cells without the trypsin treatment (lane 3), and from the osmotically shocked cells treated with trypsin (lane 4). The protein bands of interest are indicated by arrows. An about 26 kDa protein that appears on treatment of the osmotically shocked cells with trypsin (lane 4) is presumably derived from OmpA (34).

NH2-terminus and the size. However, no halo was formed around the colonies of the E. coli containing pSPh1/19, indicative of the absence of protease activity. We also compared the protease activities of the membrane fractions prepared by sonication from E. coli harboring pSPh1/19 and pUC19. Notwithstanding the accumulation of a considerable amount of the 53 kDa product in the outer membrane, no difference between them was observed (data not shown). We therefore concluded that the 53 kDa product showed little or no protease activity. The same was also true for the product from the ssp-h2 gene (see below).

Expression of ssp-h2 in E. coli—We next expressed ssp-h2 in E. coli by using pSPh2/18 and pSPh2/19. Plasmid pSPh2/18 contained ssp-h2 under the control of the lac promoter in pUC18. Plasmid pSPh2/19 contained it in the orientation opposite to that of the lac promoter. In E. coli harboring pSPh2/19, no protein derived from ssp-h2 gene was detected, suggesting the absence of a promoter sequence functional in E. coli in the region upstream from ssp-h2 (data not shown). In E. coli harboring pSPh2/18, however, a large amount of a 108 kDa protein was detected in the insoluble fraction when IPTG was added (Fig. 5). The NH2-terminal amino acid sequence of this protein was determined to be 'MTTTMG, indicating that this protein is a precursor of SSP-h2. Similar accumulation of the precursor with the NH2-terminal signal sequence was also detected in the insoluble fraction when the ssp gene under the tac promoter was introduced into E. coli. This preproSSP molecule is a dead-end product present in the periplasmic space in an insoluble form like an inclusion body (5). We therefore assume that 108 kDa precursor of SSP-h2 might be present in the periplasmic space in an insoluble form with its signal sequence in the cytoplasmic membrane as in the case of preproSSP. However, it is also possible that 108 kDa precursor is accumulated in the cytoplasm as an inclusion body. Large amounts of proteins of various sizes were leaked in the extracellular fraction in this culture, probably because lysis of cells took place due to the excessive accumulation of the precursor protein.

The accumulation of the SSP-h2 precursor led us to assume that the NH2-terminal signal sequence of SSP-h2 functioned less efficiently than those of SSP-h1 and SSP in E. coli. As mentioned above, the NH2-terminal region of SSP-h2 was unusually hydrophilic. We therefore substituted the NH2-terminal region ('Met to 'Ala) of SSP-h2 with the signal peptide ('Met to 'Ala) of SSP and produced the chimeric protein in E. coli. Upon induction with IPTG, pSPh0222 (Fig. 6) containing the chimeric gene under the control of the tac promoter directed the synthesis of 53 and 49 kDa proteins in large amounts in the insoluble fraction, in addition to the 108 kDa precursor (Fig. 5). NH2-Terminal amino acid sequencing of the 53 and 49 kDa proteins gave the sequences 'GDPASW and 'SIGTLN, respec.
serine protease homologues in S. marcescens

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The junction region of the homologues has the ability to translocate mature SSP through the outer membrane. The junction region [646Ser to 716Gly] of preproSSP has a role in folding the mature part into the active and stable conformation in the medium (6). Experiments to trim the junction region showed that deletion of at least 24 amino acids [677Val to 682Glu] had no effect on the role of the mature part (6). Since the deletion in the SSP homologues was 41 amino acids, we tested for the ability of the short junction region of the homologues to exert the same role as the junction region of preproSSP. For this purpose, we substituted almost all of the COOH-terminal domain of preproSSP (644Ala to 1044Phe) with its signal peptide buried in the inner membrane and the remainder in the periplasm was also seen in the insoluble fraction (5). In the insoluble fraction of E. coli harboring pSPh0002, the precursor protein and two proteins (38 and 36 kDa) were also seen, although the signals of the 38 and 36 kDa proteins were slightly weak because of the weak reactivity of the antibody with the SSP homologues. It is most probable that the mature part is liberated from the long peptide in the same way as for native SSP, leaving a COOH-terminal domain in the outer membrane. The 38 and 36 kDa proteins are considered to be processed at the same sites as for native SSP, because the processing sites [702Glu to 707Glu] and [716Gly to 717Phe] are present in the chimeric protein and the calculated molecular mass of the COOH-terminal protein of SSP is slightly larger than that of the corresponding portion of the homologues. All of these observations clearly show that the COOH-terminal region of the homologues has the ability to translocate mature SSP through the outer membrane.

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Fig. 6. Schematic representation of the wild type and several chimeras between SSP and its homologues used in this study. The signal peptide, the mature part, the junction region, and the COOH-terminal protein of preproSSP are indicated as black, striped, white, and stippled boxes, respectively. The precursors of SSP-h1 and SSP-h2 are divided on the basis of the homology with preproSSP. The deletion of 41 amino acid residues in the junction region of homologues is indicated by broken lines. The processed products detected in E. coli cells are also illustrated. Although the NH2-terminal amino acid sequences of the processed products were determined, their COOH-terminal amino acid residues were not analyzed.

Fig. 7. Secretion of mature SSP by using the COOH-terminal part of the homologues. E. coli cells containing each of the expression plasmids were fractionated and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblot hybridization. The antibody specific to preproSSP (anti-preproSSP antibody) was used for immunological detection of SSP and the COOH-terminal pro-domain. E, P, C, and I denote extracellular, periplasmic, cytoplasmic, and insoluble fractions, respectively. Molecular mass markers are the same as those in Fig. 4. PreproSSP, mature SSP, and processed COOH-terminal products (C-1 and C-2) are indicated. The processed COOH-terminal products of the homologue weakly cross-reacted with the anti-preproSSP antibody. In E. coli harboring pSP0002, mature SSP was secreted into the medium, just as in E. coli harboring pSP1113 carrying the wild type ssp gene.

Fig. 8. No secretion of the NH2-terminal part of SSP-h1 by using the COOH-terminal pro-domain of preproSSP. E. coli cells containing each of the expression plasmids were fractionated and analyzed by SDS-polyacrylamide gel electrophoresis. Proteins were stained with CBB. E, P, C, and I denote extracellular, periplasmic, cytoplasmic, and insoluble fractions, respectively. Molecular mass markers are the same as those in Fig. 4. The protein bands of interest are indicated by arrows. The homologues, 41 amino acids shorter than that of preproSSP, had no or very low activity as a guide peptide for folding the mature SSP part.
Involvement of the COOH-Terminal Region of the Homologue in Translocation of the NH₂-Terminal Part into the Outer Membrane—According to the above observations, the NH₂-terminal part of the homologues appeared to remain in the outer membrane. We next examined whether the NH₂-terminal part of the homologues was translocated into the outer membrane without the COOH-terminal region. For this purpose, we introduced a termination codon (TGA) after 666Ala in the sequence of the chimera protein encoded on pSPH0222, constructing pSPH02Term (Fig. 6). When E. coli harboring pSPH02Term was first cultured in L-broth containing 10 mM glucose for the purpose of completely preventing the expression and then transferred to fresh L-broth containing IPTG for induction of the tac promoter, a 66 kDa protein was produced in the insoluble fraction. In order to determine the localization of this 66 kDa protein, we carried out trypsin-treatment experiments (Fig. 9). The 66 kDa protein was not digested when trypsin was added to the intact cells (Fig. 9, lane 6). However, when trypsin penetrated into the periplasm in osmotically shocked cells, the protein was digested completely (Fig. 9, lane 8). On the other hand, the 53 kDa protein in E. coli containing pSPH0222 was not digested under the same conditions (Fig. 9, lane 4). These results suggest that the 66 kDa protein was not buried in the outer membrane. We speculate that it is in the periplasmic space in an insoluble form like an inclusion body. From these results, it is evident that the COOH-terminal region of the homologues plays a functional role in translocating the NH₂-terminal part into the outer membrane.

DISCUSSION

In this study, we cloned two ssp-homologues, designated ssp-h1 and ssp-h2, from S. marcescens IFO 3046 from which the ssp gene had been cloned. They are located in tandem on the genome, which suggests that one of them was generated by gene duplication during evolution. Since ssp-h1 was expressed from its own promoter in E. coli (see Fig. 4), it may also be expressed in S. marcescens. On the other hand, no promoter sequence is apparent in the region upstream from ssp-h2 and SSP-h2 appeared not to be produced without the lac promoter in E. coli. We therefore speculate that ssp-h2 may be a silent gene in S. marcescens, or transcribed to a small extent by read-through from the ssp-h1 promoter. The inverted repeat downstream of ssp-h1 seems to be less efficient as a ρ-independent transcriptional terminator because, in pSPH2/18, transcription of the ssp-h2 gene from the lac promoter upstream of this inverted repeat occurs (see Fig. 5). Although we attempted to detect the SSP-homologue proteins in the culture of S. marcescens by the immunological method with the anti-preproSSP antibody, we failed to detect the homologues. This is probably due to the weak cross-reactivity of the antibody with the homologues and to a low level of production of the homologues in S. marcescens.

Despite the significant similarity of the homologues to SSP which is excreted into the medium, they were integrated in the outer membrane so rigidly that trypsin-treatment

![Fig. 9. Trypsin treatment of the E. coli cells carrying pSPH0222 and pSPH02Term. Trypsin-treated and non-treated E. coli cells containing pSPH0222 (lanes 1–4) and pSPH02Term (lanes 5–8) were sonicated and their insoluble fractions were analyzed by SDS-polyacrylamide gel electrophoresis. Proteins were stained with CBB. The insoluble fractions prepared from the intact cells without the trypsin treatment (lanes 1 and 5), from the intact cells treated with trypsin (lanes 2 and 6), from the osmotically shocked cells without the trypsin treatment (lanes 3 and 7) and from the osmotically shocked cells treated with trypsin (lanes 4 and 8) are shown. The protein bands of interest are indicated by arrows.](image-url)
even with the cold osmotically shocked cells did not degrade them (see Fig. 4). The COOH-terminal portion of the homologues, which was shown to be essential for the export into the outer membrane (see Fig. 9), was functionally the same as that of SSP, because it was capable of exporting mature SSP into the medium across the outer membrane (see Fig. 7). By analogy with the secretion of SSP (5), we speculate that the large precursor of homologues is transported through the inner membrane via a Sec-dependent pathway with the cleavage of the signal peptide, and the NH2-terminal part is then brought to the outer membrane by the assistance of the COOH-terminal region rigidly integrated in the outer membrane. After integration of the long polypeptide into the outer membrane, or during its integration, it is cleaved by an E. coli protease. Since the NH2-terminal part of the homologues, corresponding to mature SSP, still remained in the outer membrane even when the COOH-terminal pro-domain of preproSSP was attached to it (see Fig. 8), we suppose that the NH2-terminal part of the homologues is destroyed to remain in the outer membrane. Comparison of the hydrophobicity of the mature parts of preproSSP and the corresponding region ("STyr-676Ala for SSP-h1 and 46Gly-674Ala for SSP-h2) of the homologues according to the method of Kyte and Doolittle (31) revealed the presence of a region in the higher hydrophobicity near the COOH-terminals of the region of homologues (data not shown). This region of high hydrophobicity may cause the homologue proteins to remain in the outer membrane.

It is not determined whether the SSP homologues are integrated in the outer membrane in the processed form in the original strain, S. marcescens. Concerning the localization and processing of the homologues in S. marcescens, other proteins that are outer surface membrane proteins in different genera provide a hint. They are the 120 kDa surface antigen (rOmpB) in R. rickettsii (27, 28), whose COOH-terminal portion shows 19% identity in amino acid sequence to that of the homologues, and the 100 kDa serotype-specific antigen (Ssa1) of P. haemolytica (29, 30), which shows significant homology (25% identity) to preproSSP and the SSP homologues over the entire sequence. The precursor of rOmpB is composed of an NH2-terminal signal peptide, a mature part, and a COOH-terminal pro-domain. The mature part (120 kDa) and the COOH-terminal domain (32 kDa) of rOmpB are accumulated in the outer membrane. The precursor of 100 kDa adhesion protein (AIDA-I) of enteropathogenic E. coli also has the same construction (32, 33). We therefore suppose that the SSP homologues are also localized and processed in the outer membrane in S. marcescens, as observed in E. coli. Although the physiological role of the homologues is still unclear, they may serve as outer membrane proteins expressing antigenicity via the trypsin-sensitive region in the COOH-terminal part, like Ssa1 in P. haemolytica.

REFERENCES

Serine Protease Homologues in S. marcescens