

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 (Ssa1) 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 auto-proteolytic 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

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Abbreviation: SSP, *Serratia* serine protease.

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 [$\Delta(lac\ pro)\ thi\ rpsL\ endA\ sbcB15\ hsdR4\ F'\ traD36\ proAB\ lacI^q\ lacZ\ \Delta M15$] (16), purchased from Amersham Japan (Tokyo), was used as the host for both phage M13 propagation and cloning and expression of the *ssp*-homologues. *E. coli* CJ236 [*dut-1 ung-1 thi-1 relA1/pCJ105*] (17) was 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 Sequencing Kit (Pharmacia) and Automated Fluorescence DNA sequencer (DSQ 1, Shimadzu).

Southern Hybridization Analysis—Chromosomal DNAs from *Serratia* and related species were prepared according to the reported method (20). *Bam*HI-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/mmol, Amersham Japan) and a *Bca*BEST labeling kit purchased from Takara Shuzo.

Cloning of the *ssp*-Homologues and Construction of Expression Plasmids—Among the three positive signals (1.4, 2.8, and 1.5 kb) detected by Southern hybridization with a 1.4 kb *Bgl*III-*Stu*I fragment (encoding ⁶⁷⁵Ser to ¹⁰⁴⁵Phe 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 *Bam*HI-fragments into pUC19 by the standard DNA-probing method. We first isolated the 1.5 kb *Bam*HI 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 *Bam*HI-*Pvu*II fragment (probe 1, see Fig. 2) in the cloned 1.5 kb *Bam*HI fragment. *Sph*I was found to produce two signals of 2.7 and 6.0 kb. The 2.7 and 6.0 kb *Sph*I fragments were cloned into pUC19, resulting in pHA2 and pHB1, respectively. The 2.7 kb *Sph*I fragment was found to include almost all of the originally cloned 1.5 kb *Bam*HI fragment. The 6.0 kb *Sph*I fragment was found to include the full length of another *ssp*-homologue. We also cloned a 2.6 kb *Bam*HI-*Pst*I fragment into pUC19 using a 0.8 kb *Sph*I-*Bam*HI fragment as a hybridization probe (probe 2, see Fig. 2), resulting in pHA3. This *Bam*HI-*Pst*I 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 *lac* promoter in pUC18 was used. A 4.1 kb *Eco*RV-*Sph*I fragment covering the whole *ssp-h1* gene was excised from pHB1 and cloned into pUC18 digested with *Hinc*II and *Sph*I, resulting in pSPh1/18 (see Fig. 2). A 4.1 kb *Kpn*I-*Hind*III fragment of pSPh1/18 was cloned into the multilinker of pUC19, resulting in pSPh1/19. A 2.0 kb *Pst*I-*Hind*III fragment of pHA2 was cloned into pHA3 digested with *Pst*I and *Hind*III, resulting in pSPh2/19 (see Fig. 2). A 4.6 kb *Kpn*I-*Hind*III fragment of pSPh2/19 was cloned into the multilinker of pUC18, resulting in pSPh2/18.

Construction of Plasmids for Expression of Chimera Protein—A *Nhe*I 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 *Bam*HI and *Hind*III fragment of pSPh2-2N was substituted for the 1.9 kb *Bam*HI and *Hind*III fragment of pSPh1, constructing pSPh1-2N. A *Mlu*I recognition sequence (ACGCGT) 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 *Nhe*I-*Stu*I fragment of pSP11tac1N and a 3.0 kb *Nhe*I-*Hind*III fragment of pSPh2-1N after the *Hind*III site had been filled-in by Klenow fragment. Plasmid pSP0022 was constructed by ligating a 5.6 kb *Mlu*I-*Stu*I fragment of pSP11tac1M and a 2.0 kb *Mlu*I-*Hind*III fragment of pSPh2-1M after the *Hind*III site had been treated with Klenow fragment. Plasmid pSP0002 was constructed by ligating a 5.4 kb *Nhe*I-*Stu*I fragment of pSP11tac2N and a 2.2 kb *Nhe*I-*Hind*III fragment of pSPh2-2N after the

TABLE I. Oligonucleotides used for this study.

Plasmid	Position	Use	Oligonucleotide*
pSP11tac1N	Ser ²⁶ (TCT)Leu ²⁶ (CTT)	PCR	5'-TGGGATCCTGATAAGCGGCTAGCGAAGAATGAAT-3'
	Upstream from the <i>Pst</i> I site of pSP11tac	PCR	5'-GTTAATAGTTTGGCGAACGTTGTTG-3'
pSP11tac2N	Ser ⁶⁴ (AGC)Leu ⁶⁴ (CTG)	Site directed mutagenesis	5'-GCCAGTTTTGACTCGCTAGCCAGCACT-3'
pSP11tac1M	Thr ⁷⁷ (ACC)	Site directed mutagenesis	5'-ACAGGCGACGCGTCAACTG-3'
pSPh2-1N	Gln ⁴⁴ (CAG)	Site directed mutagenesis	5'-GGGTATGCGCTAGCGGCGCCG-3'
pSPh2-2N	Ser ⁷⁸ (AGC)Val ⁷⁸ (GTG)	Site directed mutagenesis	5'-ACGAGCTTCGCCTCGCTAGCGCAGACGCC-3'
pSPh2-1M	Phe ⁷¹ (TTC)	Site directed mutagenesis	5'-CGGCAGGCGACGCGTCAGCTG-3'
pSPh2-675Term	Ser ⁶⁷⁵ (AGC)	PCR	5'-CCCAAGCTTAGGCGAAGCTCGTGCCGT-3'
	Upstream from the <i>Pst</i> I site of pSPh2	PCR	5'-GGTGTCCGACTACGGCAA-3'

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

*Hind*III site had been treated with Klenow fragment.

Plasmid pSP1100 was constructed by ligating a 5.2 kb *Nhe*I-*Sph*I fragment of pSPh1-2N and a 1.2 kb *Nhe*I-*Sph*I fragment of pSP11tac2N.

Plasmid pSPh2-675Term was constructed by ligating a 0.9 kb *Pst*I-*Hind*III fragment of PCR product and a 5.0 kb *Pst*I-*Hind*III fragment of pSPh2. Plasmid pSPh02Term was constructed by ligating the 4.6 kb *Nhe*I-*Stu*I fragment of pSP11tac1N and the 1.9 kb *Nhe*I-*Hind*III fragment of pSPh2-675Term after the *Hind*III site had been filled-in by Klenow fragment.

Preparation of Extracellular, Periplasmic, Cytoplasmic, and Insoluble Fractions—An overnight culture of *E. coli* transformants (100 μ l) was inoculated in 10 ml of L-broth (22) containing 50 μ g/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 (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 \times *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 6.8, containing 2% SDS and 1% 2-mercaptoethanol. Each sample (extracellular fraction of 200 μ l of culture, periplasmic fraction of 200 μ l of culture, cytoplasmic fraction of 100 μ l of culture, and insoluble fraction of 200 μ l 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 Coomassie 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- μ m 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 pSP11/19 was inoculated in 150 ml of L-broth containing 50 μ g/ml of ampicillin. After 12 h cultivation at 37°C with shaking, the cells were centrifuged at 6,000 \times *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 \times *g* for 10 min at 4°C, and the membrane fraction was collected by high-speed centrifugation of the supernatant at 155,000 \times *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 \times *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 0.1 mg/ml, and the mixtures were incubated at 37°C for 30 min. The digestion was stopped by adding *N*- α -*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 μ l 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 μ g/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

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 (¹Met to ¹⁰⁴⁵Phe) consists of a typical NH₂-terminal signal peptide (¹Met to ²⁷Ala), the mature protease domain (²⁸Ala to ⁶⁴⁵Asp), and a large COOH-terminal pro-region (⁶⁴⁶Ser to ¹⁰⁴⁵Phe) (see Fig. 3). The large COOH-terminal pro-region contains a junction region (⁶⁴⁶Ser to ⁷¹⁶Gly) 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 *Bgl*III–*Stu*I fragment encoding almost the entire part of the COOH-terminal pro-domain (⁶⁷⁶Ser to ¹⁰⁴⁵Phe) of preproSSP. Several bands showed hybridization in the *Bam*HI-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 *Bam*HI-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 pneu-*

moniae, and *Erwinia carotovora* (data not shown).

Cloning and Sequencing of the *ssp*-Homologues—The above observation prompted us to clone the two *ssp* homologues from *S. marcescens*. We cloned the DNA sequences showing a positive signal on the Southern blot by the standard DNA-probing method, including colony hybridization. Restriction mapping (Fig. 2) and nucleotide sequencing of the cloned fragments revealed that the two *ssp*-homologues, designated *ssp-h1* and *ssp-h2*, were present in tandem on the genome. The nucleotide sequences of the cloned DNA fragments showed that *ssp-h1* and *ssp-h2* encoded open reading frames (ORFs) of 1,036 and 1,034 amino acid residues, respectively. In Fig. 2, parts of the nucleotide sequence are shown, and the entire nucleotide sequence has been registered in the databases. The calculated molecular mass of both SSP-h1 and SSP-h2 were 108 kDa. The start codon (ATG) of *ssp-h2* was 121 bp downstream from the termination codon (TAA) of *ssp-h1*. There are inverted repeat sequences which might function as a ρ -independent transcriptional terminator immediately after the termination codons of both homologue genes. Putative promoter sequences were found in the region upstream from the *ssp-h1*-encoding sequence. In the upstream region from *ssp-h2*, however, no such sequences were found.

Comparison of Amino Acid Sequences of the SSP Homologues with Those of Other Proteins—The amino acid sequences of SSP-h1 and SSP-h2 deduced from their nucleotide sequences are aligned in Fig. 3. SSP-h1 and SSP-h2 showed 81% identity in amino acid sequence to each other. The similarity was not uniformly distributed along the two sequences. The COOH-terminal 531 amino acid residues of both homologues exhibited 99.8% identity (only one amino acid residue was different and only five bases were different even in the nucleotide sequences), although the remaining part of about 500 amino acid residues exhibited 62% identity.

Both homologues showed 55% identity in amino acid sequence to preproSSP; the mature protein region exhibited 49% identity and the COOH-terminal pro-domains exhibited 67% identity. The serine, histidine, and aspartic acid residues presumably composing the catalytic triad of serine proteases and four cysteine residues in SSP were all conserved in both homologues. However, as described below, *E. coli* JM105 containing each of these genes on expression plasmids formed no halo on a skim milk-containing agar plate, indicative of the absence of protease activity. Comparison of the amino acid sequences among preproSSP and these homologues revealed no similarity in the regions corresponding to the NH₂-terminal signal peptides. In a comparison of the consensus signal peptides, the NH₂-terminal region of SSP-h2 was extraordinarily hydrophilic, and was later shown to be less functional as a signal peptide than the corresponding regions of SSP-h1 and SSP (see below). In addition, it is noteworthy that a deletion of 41 amino acid residues, corresponding to the junction region which is required for the folding of the mature protease in the case of SSP, was found in the sequences of both homologues.

A computer-aided homology search revealed that the COOH-terminal pro-domain of the 120 kDa protein (rOmpB) of *Rickettsia rickettsii* (27, 28) showed 19% identity in amino acid sequence to that of the SSP homo-

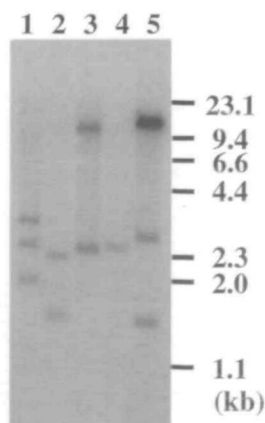


Fig. 1. Distribution of homologues of the COOH-terminal pro-domain of preproSSP among *Serratia* species. Using a ³²P-labeled 1.4 kb *Bgl*III–*Stu*I fragment encoding the COOH-terminal pro-domain of preproSSP (⁶⁷⁶Ser to ¹⁰⁴⁵Phe) as a probe, *Bam*HI-digested genomes of *Serratia* spp. were analyzed by 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 ⁶⁷¹NGTSFA. 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 (⁶⁷⁰Arg-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

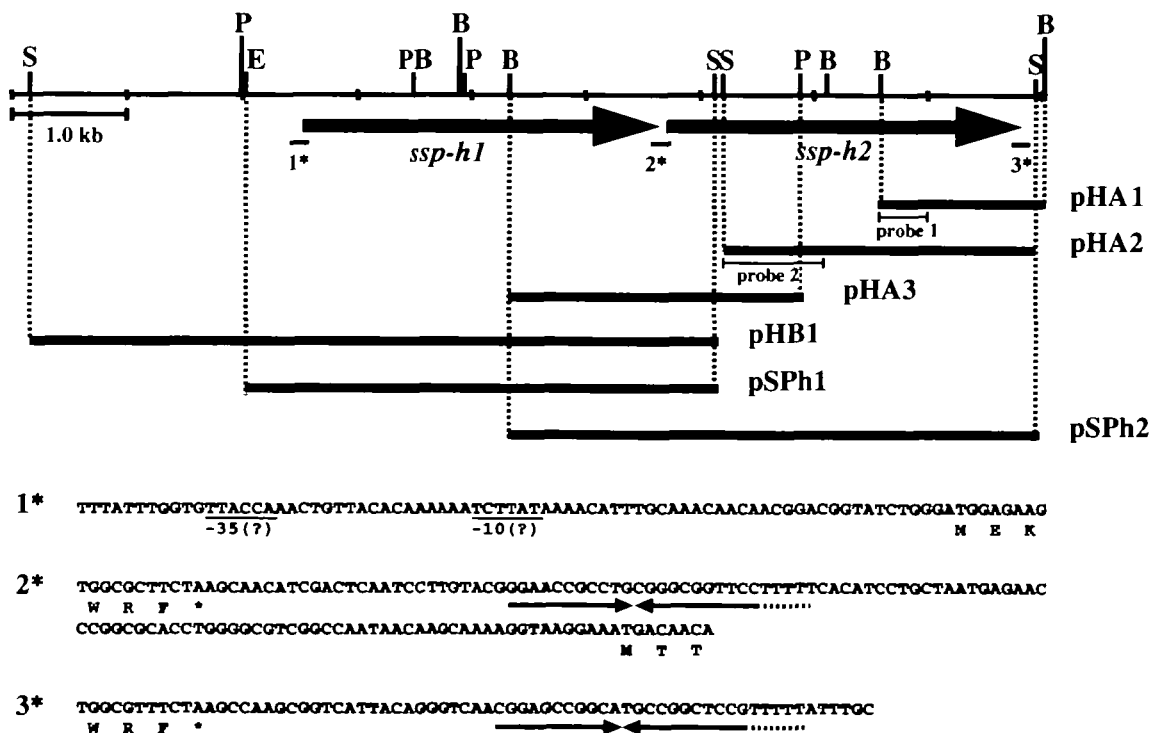


Fig. 2. Restriction map of the DNA fragment containing *ssp-h1* and *ssp-h2*. S, P, E, and B denote *Sph*I, *Pst*I, *Eco*RV, and *Bam*HI, respectively. Two *ssp*-homologues, designated *ssp-h1* and *ssp-h2*, are present in tandem on the genome of *S. marcescens*. The arrows indicate the direction and extent of the coding regions. Below the restriction map, a DNA fragment on each plasmid is shown. The DNA

fragments used as hybridization probes are also shown. The nucleotide sequences upstream and downstream of the coding regions are exhibited. Upstream of the initiation codon of *ssp-h1*, promoter-like sequences (-35 and -10) are present. An inverted repeat sequence downstream of the coding region is shown by opposing arrows.

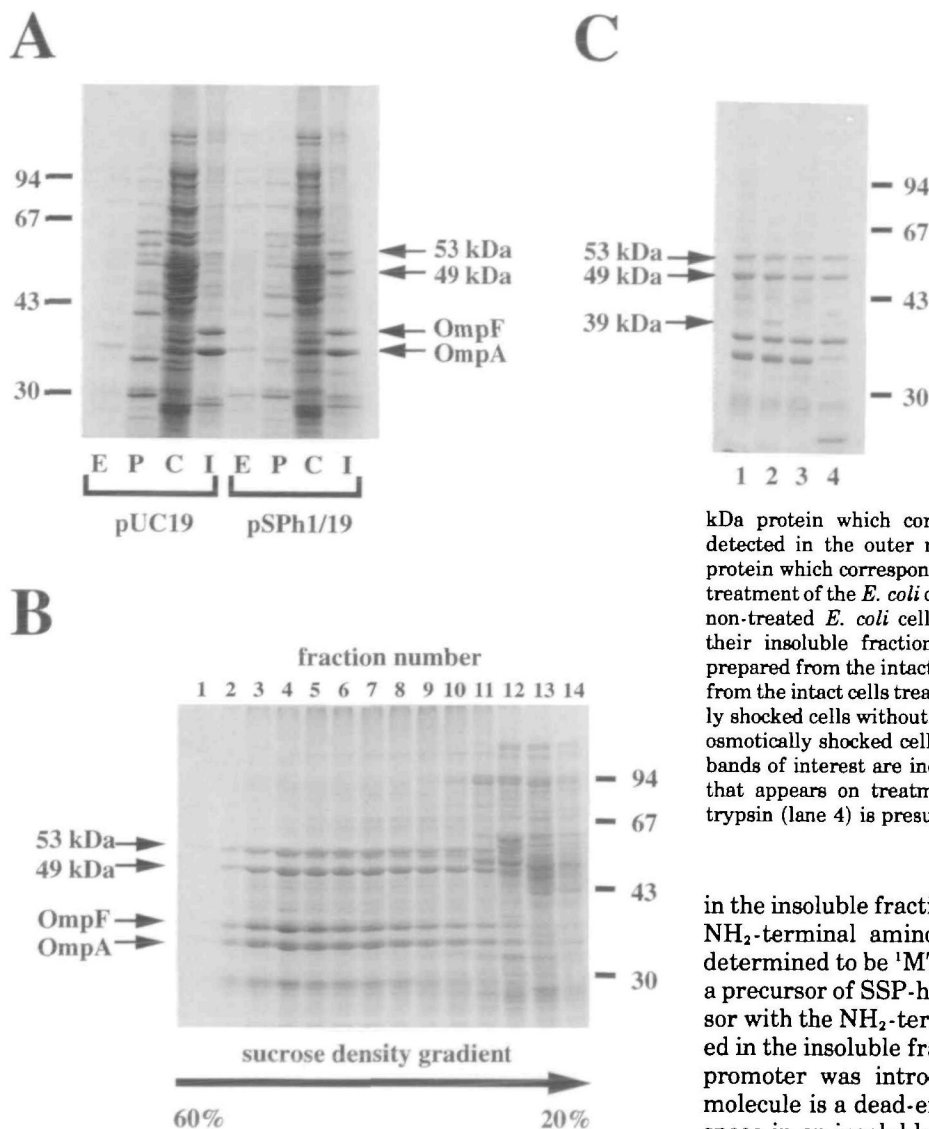


Fig. 4. Expression of *ssp-h1* in *E. coli* and localization of its products in the outer membrane. (A) The *E. coli* cells containing pSPH1/19 and pUC19, as a control, were fractionated and analyzed by SDS-polyacrylamide gel electrophoresis. Proteins were stained with CBB. E, P, C, and, I denote extracellular, 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), 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).

in the insoluble fraction when IPTG was added (Fig. 5). The NH₂-terminal amino acid sequence of this protein was determined to be 'MTTMTMG, 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 (1Met to 35Ala) of SSP-h2 with the signal peptide (1Met to 27Ala) 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 ⁴⁵GDPASW and ⁶⁰SIGTLN, respec-

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

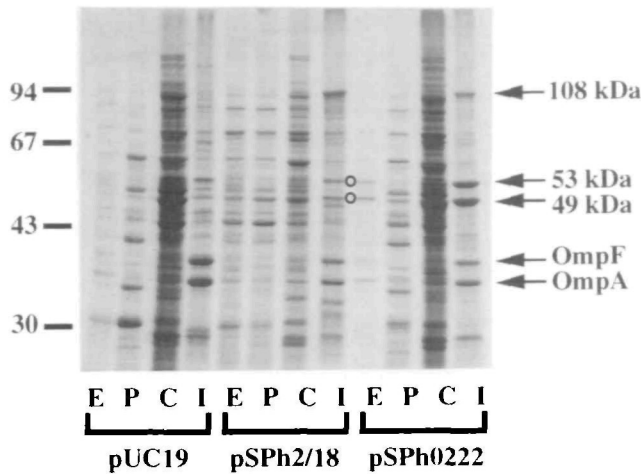


Fig. 5. Expression of *ssp-h2* in *E. coli*. The *E. coli* cells containing pUC19, pSPh2/18, and pSPh0222 were fractionated and analyzed by SDS-polyacrylamide gel electrophoresis. Proteins were stained with CBB. E, P, C, and, I denote extracellular, periplasmic, cytoplasmic, and insoluble fractions, respectively. The protein bands of interest are indicated by arrows. The 53 and 49 kDa proteins of the insoluble fraction prepared from *E. coli* harboring pSPh2/18 are marked by open circles. Molecular mass markers are the same as those in Fig. 4.

tively. Both the 53 and 49 kDa proteins were supposedly localized in the outer membrane because the processing pattern was the same as for SSP-h1. The 108 kDa precursor was digested completely by trypsin when the cells were subjected to a cold osmotic shock to allow trypsin to penetrate into the periplasm, while the 53 and 49 kDa proteins were not digested, as was observed for those derived from SSP-h1 (Fig. 9). This suggested that the 108 kDa precursor was in the periplasmic space in an insoluble form like an inclusion body, just as in the case of preproSSP.

Comparison of the electrophoretic pattern of the insoluble fraction prepared from *E. coli* harboring pSPh2/18 with that of *E. coli* harboring pSPh0222 revealed that small amounts of the 53 and 49 kDa proteins were also produced by *E. coli* harboring pSPh2/18 (see Fig. 5). It is most likely that the 53 and 49 kDa proteins detected in the insoluble fraction of *E. coli* harboring pSPh2/18 result from the same processing as in the case of the chimeric protein, although the NH₂-terminal amino acid sequences of these products were not determined. All of these data suggest that both SSP-h1 and SSP-h2 are translocated into the outer membrane and processed into two in the same manner. As in the case of *E. coli* harboring pSPh1/19, no halo was formed around the *E. coli* colonies harboring pSPh0222 on a skim milk-containing agar plate.

Secretion of Mature SSP by Using the COOH-Terminal Portion of the Homologues—The COOH-terminal regions of both homologues were identical in amino acid sequence and showed 67% identity to that of preproSSP, although they had a deletion of 41 amino acid residues in the sequence corresponding to the junction region of preproSSP. To investigate whether the COOH-terminal region of the homologues had the ability to excrete mature SSP into the medium, we substituted almost all of the COOH-terminal region (⁷²⁸Arg to ¹⁰⁴⁵Phe) of preproSSP for the corre-

sponding region (⁷¹⁶Arg to ¹⁰³⁴Phe) of SSP-h2, constructing pSPh0002 (Fig. 6). *E. coli* containing pSPh0002 was fractionated and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting with the anti-preproSSP antibody (Fig. 7). In *E. coli* harboring pSPh0002, mature SSP of 66 kDa was excreted into the medium, as observed for *E. coli* harboring pSP11tac carrying the wild-type *ssp* gene (Fig. 7). A turbid halo was formed around the colony of *E. coli* harboring pSPh0002 on a skim milk-agar plate, indicating that active SSP was produced and secreted from the cells. In the secretion of native SSP, the liberation of the mature part from the long peptide by its own protease activity leaves two major proteins, C-1 (⁷⁰²Ala to ¹⁰⁴⁵Phe, 40 kDa) and C-2 (⁷¹⁷Phe to ¹⁰⁴⁵Phe, 38 kDa), derived from the COOH-terminal pro-domain in the outer membrane (Fig. 7). The two proteins seen in the medium and cytoplasmic fraction presumably represent contamination during the cell-fractionation. PreproSSP 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 (⁷⁰¹Glu-⁷⁰²Ala and ⁷¹⁶Gly-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.

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 ⁷⁰⁰Glu) had no effect on the role in folding 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 (⁶⁴⁸Ala to ¹⁰⁴⁵Phe) of preproSSP for the ⁶⁷⁷Ala to ¹⁰³⁴Phe region of SSP-h2, constructing pSPh0022. In *E. coli* harboring pSPh0022, mature SSP was not detected in any fraction of the cells or in the medium, although a protein of about 106 kDa probably representing the precursor and a 41 kDa protein probably corresponding to a processed COOH-terminal pro-domain were detected in the insoluble fraction (Fig. 7). Expression of a mutant *ssp* gene encoding preproSSP with deletion of almost the whole junction region (⁶⁴⁷Leu to ⁷¹⁵Leu) resulted in the same situation; when the mutant gene was expressed, mature SSP was not detected in any fraction, whereas a processed COOH-terminal protein and preproSSP were found in the insoluble fraction (6). The absence of the mature part can be explained in terms of proteolysis by *E. coli* protease. These observations suggest that the junction region of the homo-

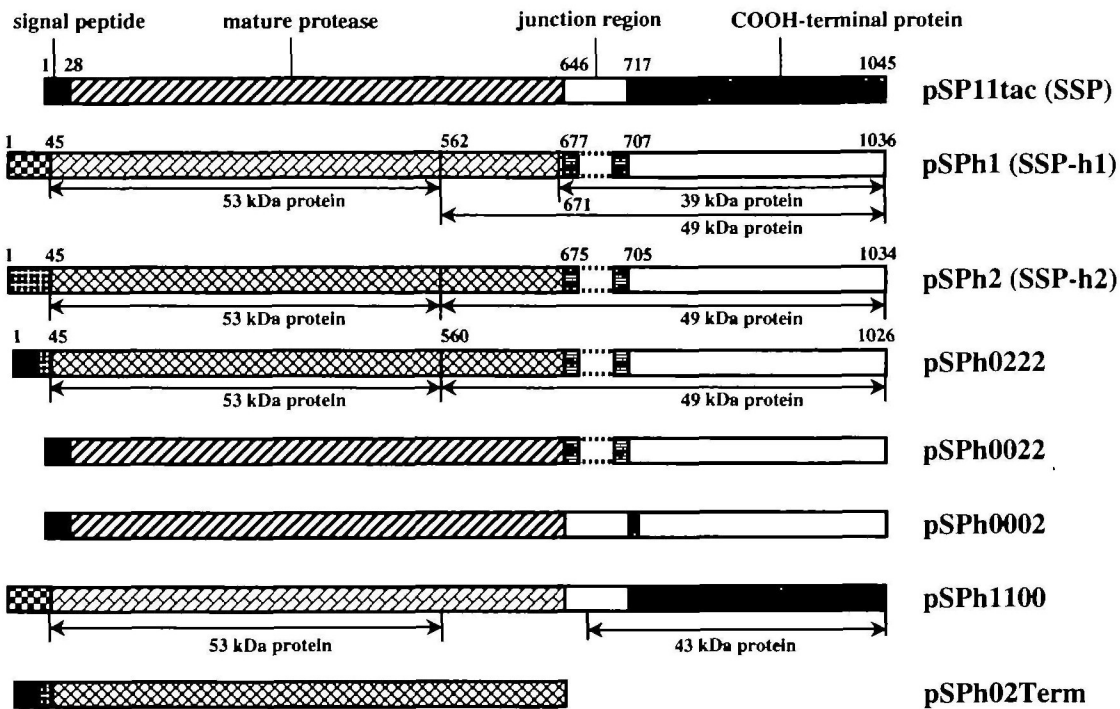


Fig. 6. Schematic representation of the wild type and several chimeras between SSP and its homologues used in this study. The signal peptide, the mature part, the junction region, and the COOH-terminal protein of preproSSP are indicated as black, striped, white, and stippled boxes, respectively. The 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.

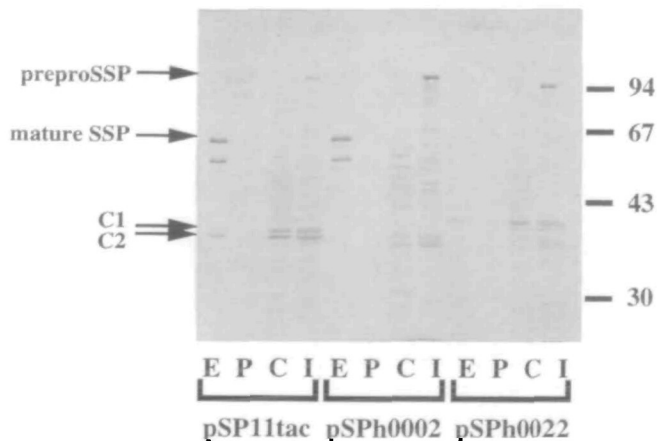


Fig. 7. Secretion of mature SSP by using the COOH-terminal part of the homologues. *E. coli* cells containing each of the expression plasmids were fractionated and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblot hybridization. The antibody specific to preproSSP (anti-preproSSP antibody) (4) 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 pSPH0002, mature SSP was secreted into the medium, just as in *E. coli* harboring pSP11tac carrying the wild type *ssp* gene.

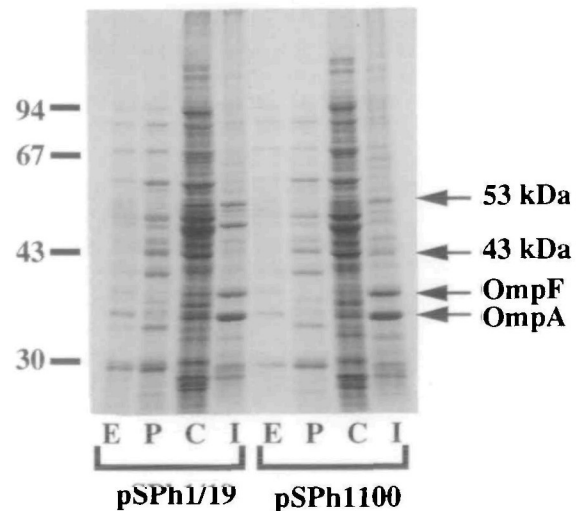


Fig. 8. No secretion of the NH₂-terminal part of SSP-h1 by using the COOH-terminal pro-domain of preproSSP. *E. coli* cells containing each of the expression plasmids were fractionated and analyzed by SDS-polyacrylamide gel electrophoresis. Proteins were stained with CBB. E, P, C, and I denote extracellular, 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.

logues, 41 amino acids shorter than that of preproSSP, had no or very low activity as a guide peptide for folding the mature SSP part.

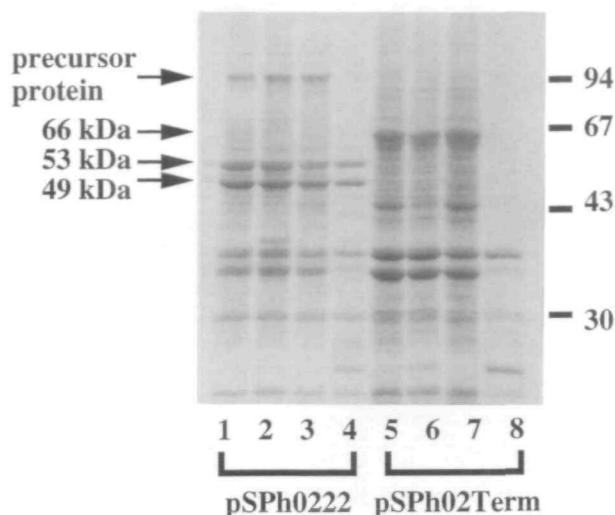


Fig. 9. Trypsin treatment of the *E. coli* cells carrying pSPh0222 and pSPh02Term. Trypsin-treated and non-treated *E. coli* cells containing pSPh0222 (lanes 1–4) and pSPh02Term (lanes 5–8) were sonicated and their insoluble fractions were analyzed by SDS-polyacrylamide gel electrophoresis. Proteins 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.

Substitution of the COOH-Terminal Domains of Homologues with That of PreproSSP—When the *ssp*-homologues were expressed in *E. coli*, the 53 kDa protein which corresponded to the mature SSP part was not excreted into the medium, but was found in the outer membrane. Furthermore, as described above, the junction region of 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 portion. We next examined whether the NH₂-terminal portion of the homologues was secreted into the medium when attached to the junction region and COOH-terminal protein of preproSSP. We substituted the COOH-terminal portion (⁶⁷⁹Ala to ¹⁰³⁶Phe) of SSP-h1 for the corresponding region (⁶⁴⁸Ala to ¹⁰⁴⁵Phe) of preproSSP, constructing pSPh1100 (Fig. 6).

In *E. coli* harboring pSPh1100, a 53 kDa protein presumably corresponding to the NH₂-terminal portion was detected in the insoluble fraction, just as in the case of *E. coli* containing pSPh1 (Fig. 8). Furthermore, careful examination of the electrophoretic pattern of the insoluble fraction prepared from *E. coli* harboring pSPh1100 revealed the presence of a 43 kDa protein (Fig. 8). This 43 kDa protein is assumed to be an OmpT protease-processed COOH-terminal protein, because the junction region of preproSSP contains sites cleaved by OmpT and because an OmpT-processed protein in *E. coli* harboring pSP121tac carrying an active site-mutated *ssp* gene was also 43 kDa in size (5). This result suggests that the NH₂-terminal portion of SSP-h1 would not be excreted into the medium even with the aid of the COOH-terminal pro-domain of preproSSP. The NH₂-terminal portion of SSP-h2 also appeared to remain in the outer membrane, but not to be secreted into the medium, even with the aid of the signal peptide and the

COOH-terminal pro-domain of preproSSP (data not shown). From these observations, the NH₂-terminal portion of homologues is thought to be destined to remain in the outer membrane.

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

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 destined to remain in the outer membrane. Comparison of the hydrophobicity of the mature parts of preproSSP and the corresponding region (⁴⁵Tyr-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-terminus 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*.

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