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Identification of Two Components of the *Serratia marcescens* Metalloprotease Transporter: Protease SM Secretion in *Escherichia coli* Is TolC Dependent

SYLVIE LÉTOFFÉ, JEAN-MARC GHIGO, AND CÉCILE WANDERSMAN*

Unité de Génétique Moléculaire, Institut Pasteur (Centre National de la Recherche Scientifique URA 1149), 25 rue du Dr. Roux, 75724 Paris Cedex 15, France

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The *Serratia marcescens* metalloprotease (protease SM) belongs to a family of proteins secreted from gram-negative bacteria by a signal peptide-independent pathway which requires a specific transporter consisting of three proteins: two in the inner membrane and one in the outer membrane. The $prtD_{SM}$ and $prtE_{SM}$ genes encoding the two *S. marcescens* inner membrane components were cloned and expressed in *Escherichia coli*. Their nucleotide sequence revealed high overall homology with the two analogous inner membrane components of the *Erwinia chrysanthemi* protease secretion apparatus and lower, but still significant, homology with the two analogous inner membrane components of the *E. coli* hemolysin transporter. When expressed in *E. coli*, these two proteins, $PrtD_{SM}$ and $PrtE_{SM}$, allowed the secretion of protease SM only in the presence of TolC protein, the outer membrane component of the hemolysin transporter.

In gram-negative bacteria, a large number of proteins are secreted by a signal peptide-independent pathway (37). These proteins lack the typical amino-terminal signal peptide (10). They cross both membranes without a periplasmic intermediate. Most of them have a C-terminal targeting sequence and a tandem series of glycine-rich repeated hexapeptides located close to their C termini (11, 26, 41). Secretion is achieved by a specific secretion apparatus consisting of three proteins located in the cell envelope: two in the inner membrane and one in the outer membrane (6, 37). Comparing different secretion systems, these three proteins share sequence homology and one of the inner membrane components is a member of the ABC protein family able to bind ATP and involved in various transport processes in prokaryotes and eukaryotes (18). Proteins using this secretion pathway belong to distinct families, the toxin family and the protease family.

The toxin family is a group of related proteins found in many gram-negative pathogens, having the ability to lyse erythrocytes and/or nucleated cells. It includes hemolysins of *Escherichia coli*, *Proteus* spp., and *Morganella* spp.; the *Bordetella pertussis* adenylate cyclase; and *Pasteurella haemolytica* and *Actinobacillus* leukotoxins (14, 15, 17, 21, 27, 36). The secreted polypeptides as well as their specific secretion systems are highly homologous. In addition, functional complementation has been demonstrated in several cases (28, 36).

The protease family comprises the four extracellular metalloproteases produced by *Erwinia chrysanthemi* (12, 13, 37), the alkaline protease secreted by *Pseudomonas aeruginosa* (16), and the *Serratia marcescens* extracellular metalloprotease (protease SM) (24). All of these proteases are highly homologous (60% identity). Besides their amino acid sequence identity, they share several characteristics. They are synthesized as inactive precursors having an N-terminal extension named propeptide, the cleavage of which is not required for secretion (4), and they are inhibited by a group of small intracellular inhibitors produced by the same species (22). Also in this family there is efficient complementation between the various secretion systems. Moreover, these proteases can be secreted by the hemolysin translocator with significant albeit low efficiency (5, 16).

E. coli colicin V represents a special case in this secretion pathway. Like the other proteins, it is devoid of a typical N-terminal signal peptide. Its secretion apparatus is homologous to the toxin and protease systems. The *E. coli* hemolysin and *E. chrysanthemi* protease secretion systems can promote colicin V secretion. However, its targeting sequence is close to the N terminus and it lacks the glycine-rich repeats (9).

Usually the genes involved in the synthesis and secretion of these polypeptides are clustered, located either on the chromosome or on a plasmid. This is the case for the *E. chrysanthemi* proteolytic determinant, in which $prtD_{EC}$, which encodes the ABC component, $prtE_{EC}$, which encodes the second inner membrane component, and $prtF_{EC}$, which encodes the outer membrane component, are adjacent and contiguous with the protease structural genes (13, 39). Similar genetic organization is found for the proteolytic determinant of *P. aeruginosa* (16) and for the adenylcyclase determinant of *B. pertussis* (14). However, slightly different genetic organization is found for the α-hemolysin determinant (27) and the colicin V determinant (9), in which only the two inner membrane specific secretion proteins (HlyB and HlyD in the first system and CvaA and CvaB in the second) are encoded by genes contiguous to those encoding the secreted polypeptides HlyA and CvaC, respectively. The third outer membrane component, TolC protein, is common to the two systems and is encoded by an unlinked chromosomal gene (38).

In the case of the protease SM, its structural gene was cloned and expressed in *E. coli* (32). The protease was produced but not secreted in the heterologous system unless the *E. chrysanthemi* protease secretion apparatus was coexpressed in *E. coli* (24).

This functional heterologous secretion system allowed the study of the features of protease SM secretion; in particular, we showed the existence of a C-terminal targeting sequence, indicating that protease SM secretion is analogous to the *E. chrysanthemi* protease secretion pathway (24). The lack of protease SM secretion in *E. coli* could be explained by the

* Corresponding author. Electronic mail address: cwander@pasteur.fr.

absence of its own secretion genes on the recombinant cosmid carrying the prt_{SM} gene. This simplest hypothesis could be easily tested by introducing an *S. marcescens* DNA library into an *E. coli* strain expressing the prt_{SM} gene. In this way, a proteolytic clone secreting protease SM was found and is characterized in this work.

MATERIALS AND METHODS

Strains, plasmids, phage, and media. *E. coli* C600 (F^- *thr leu fhuA lacY rpsL thi supE*), AR 1062 (F^- *thr leu ara azi fhuA lacY tsx minB gal rpsL xyl mtl thi hsdR*), and TG1 [Δ(*lac pro*) *thi rpsL supE endA sbcB hsdR* F′*traD36 proAB lacI*^{q} *Z* ΔM15] are from our laboratory collection. The parental *tolC*::Tn*10* strain GC7459 is described in reference 30. Strain C600 *tolC*::Tn*10* was constructed by phage P1 transduction of the *tolC*::Tn*10* insertion from GC7459 into the C600 recipient strain. *S. marcescens* 365 was obtained from V. Braun. The M13 phage derivative K07 was a gift from J. Vieira. Phage P1vir was used for general transduction. Plasmids pAM238 and pAM239 (kindly provided by J. P. Boucher), pBR322 (2), pEMBL18 and pEMBL19 (7), pBGS18 and pBGS19 (35), and pACYC184 (3) were used as vectors. Plasmid pSM4Δ21 carrying the *prtSM* and inh_{SM} genes is described in reference 24. Plasmid pRUW4 carrying *prtD*, *prtE*, and *prtF* genes is described in reference 39. Plasmid pRUW500 carrying *prtB* is described in reference 23, and plasmid pRUW9 is described in reference 39.

A 2-kb *Hin*dIII-*Eco*RI DNA fragment from pSM4Δ21 was recloned in the vector pAM239. The resulting plasmid was pSYC1.

The *prtB* gene from pRUW500 was recloned in pAM238 to produce plasmid pRUW538. All media have been described previously, and antibiotics were used as described previously (29).

Extraction and manipulation of plasmids and in vitro cloning. Isolation of plasmids, transformation of *E. coli*, restriction endonuclease mapping, ligation with T4 DNA ligase, agarose gel electrophoresis of DNA, and purification of DNA fragments were done as described in reference 33.

DNA sequence determination. Unidirectional deletions were generated with the exonuclease III/S1 system as described in reference 33. Deletions were made in one direction starting from pUC18 carrying the *Kpn*I-*Bam*HI 7-kb DNA insert of pSYC4 and in the other direction from pUC19 carrying the same insert in the opposite orientation. The various deleted DNA fragments were recloned in pBGS18 and pBGS19. Single-stranded DNA from these phagemids carrying the various DNA inserts was obtained by infection with the M13 phage derivative K07. DNA sequences were determined by the dideoxy nucleotide method of Sanger et al., using [α-^{35}S]dATP and T7 DNA polymerase (1). DNA sequencing was done for the DNA fragments common to pSYC8 and pSYC9, which are the smaller DNA fragments containing plasmids remaining proteolytic when introduced into C600(pSYC1) (see Fig. 2). Nonoverlapping regions were sequenced with oligonucleotide primers. DNA sequences were analyzed according to the method of Lipman and Pearson (25). Amino acid sequence comparison was performed with the Clustal package program (19).

Construction of a genomic library. The following steps were performed as described in reference 33: isolation of total DNA, partial digestion with *Sau*3A, size fractionation on an agarose gel, extraction from low-melting-point agarose of DNA fragments ranging from 7 to 20 kb, and ligation to pBR322 digested with *Bam*HI and dephosphorylated.

Isolation and radiolabeling of minicells. Minicells were isolated from *E. coli* AR 1062 containing various recombinant plasmids and proteins were labelled with [^{35}S]methionine by methods described previously (8). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and labelled products were identified by direct autoradiography of the dried gel with Kodak X-Omat ARS film.

Protein analysis. *S. marcescens* and *E. coli* were grown at 37°C in ML medium (29). Cells were harvested during the exponential growth phase (optical density at 600 nm [OD_{600}] = 1) or stationary phase (OD_{600} = 4) and centrifuged for 10 min at 5,000 × *g* at 4°C. The supernatants were concentrated by precipitation with 10% trichloroacetic acid as described previously (39). The cell pellets were solubilized in SDS sample buffer to yield a preparation of total cellular proteins. Proteins were analyzed by SDS-PAGE and Coomassie blue staining or immunodetection. Immunodetection was carried out as described previously (24). Anti-protease SM antibodies were used at a 1/500 dilution. Anti-protease B antibodies were used at a 1/5,000 dilution.

RESULTS

Cloning of the *S. marcescens* genes encoding the specific secretion functions. A genomic library of *S. marcescens* was constructed in pBR322. The chromosomal DNA was prepared as described in Materials and Methods. It was partially digested with restriction endonuclease *Sau*3A and size fractionated in an agarose gel. DNA fragments ranging from 7 to 20 kb were recovered and ligated with *Bam*HI-linearized and dephosphorylated pBR322 and transformed into *E. coli* C600 carrying the protease SM gene and the inhibitor gene on a compatible plasmid, pSYC1. Recombinant clones were screened on skim milk agar. Three proteolytic colonies were found. The corresponding recombinant plasmids were isolated and used to transform *E. coli* C600 or C600(pSYC1). Two recombinant plasmids, pSYC2 and pSYC3, led to proteolytic colonies in C600 as well as in C600(pSYC1). Immunodetection with anti-protease SM antibodies showed that neither the cell pellets nor the culture supernatants of C600(pSYC2) or C600(pSYC3) contained protease SM. Moreover, the culture supernatants of C600(pSYC1, pSYC2) and C600(pSYC1, pSYC3) did not contain protease SM, although cell pellets did (data not shown). These results indicate that plasmids pSYC2 and pSYC3 express neither protease SM nor specific proteins allowing its secretion. Besides the major protease SM, *S. marcescens* synthesizes several minor extracellular proteases (34). It is therefore possible that the extracellular proteolytic activity found in *E. coli* carrying these plasmids comes from one of the minor *S. marcescens* proteases.

The third recombinant plasmid, pSYC4, led to proteolytic activity only in C600(pSYC1), not in C600. Proteins present in the culture supernatants of *S. marcescens* and *E. coli* C600 carrying either pSYC1, pSYC4, or both plasmids together were separated by SDS-PAGE followed by immunodetection with antibodies directed against protease SM. Figure 1 shows that protease SM was present only in the supernatants of *S. marcescens* and the *E. coli* strain carrying both plasmids. Immunodetection performed on the corresponding cell pellets showed the presence of protease SM only in C600(pSYC1) and C600(pSYC1, SYC4), not in C600(pSYC4) (data not shown). These results indicate that pSYC4 does not express protease SM but allows the secretion of protease SM encoded by the second plasmid, pSYC1. Thus, plasmid pSYC4 might carry the *S. marcescens* specific secretion genes and was kept for further study.

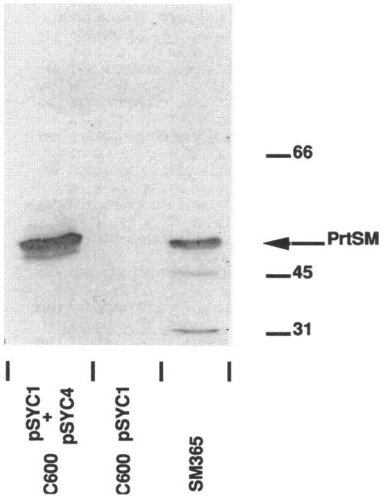

FIG. 1. Immunodetection of supernatants of various strains. Cultures of *E. coli* C600 cells carrying various plasmids and *S. marcescens* cells were grown in ML medium until they reached an OD_{600} of 1. The cells were harvested and the culture supernatants were concentrated 100-fold by precipitation with trichloroacetic acid as described in Materials and Methods. SDS-PAGE was followed by immunodetection with anti-Prt_{SM} antibodies. The SM365 lane was loaded with 2 OD equivalent units of concentrated supernatant sample, the C600 (pSYC1) lane was loaded with 5 OD equivalent units of concentrated supernatant sample, and the C600(pSYC1, pSYC4) lane was loaded with 4 OD equivalent units of concentrated supernatant sample. The positions of molecular mass standards (in kilodaltons) are indicated to the right. PrtSM, protease SM.

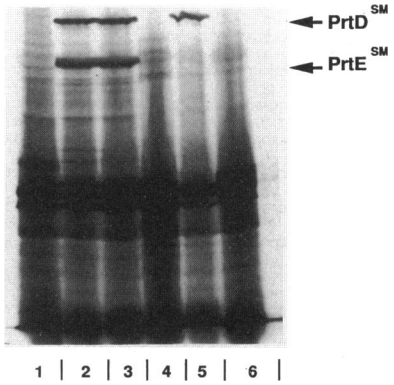

FIG. 3. Identification of plasmid-encoded proteins in a minicell system. Samples were run by SDS-PAGE (10% polyacrylamide). Lanes show products encoded by pBR322 (lane 1), pSYC4 (lanes 2 and 3), pUC19 (lane 4), pSYC5 (lane 5), and pSYC6 (lane 6). The positions of $PrtD_{SM}$ and $PrtE_{SM}$ are indicated.

Subcloning and identification of the plasmid-encoded proteins. pSYC4 carries an 8-kb DNA insert in pBR322. It was subjected to subcloning and to unidirectional deletions as described in Materials and Methods and in the legend to Fig. 2. The various subclones were tested for their ability to promote protease SM secretion. The initial positive plasmid as well as two subclones, each carrying approximately half of the insert, were analyzed in a minicell system to identify the encoded proteins (Fig. 3).

The minicell-producing strain, AR1062, was transformed with each of the plasmids, minicells were isolated and labelled

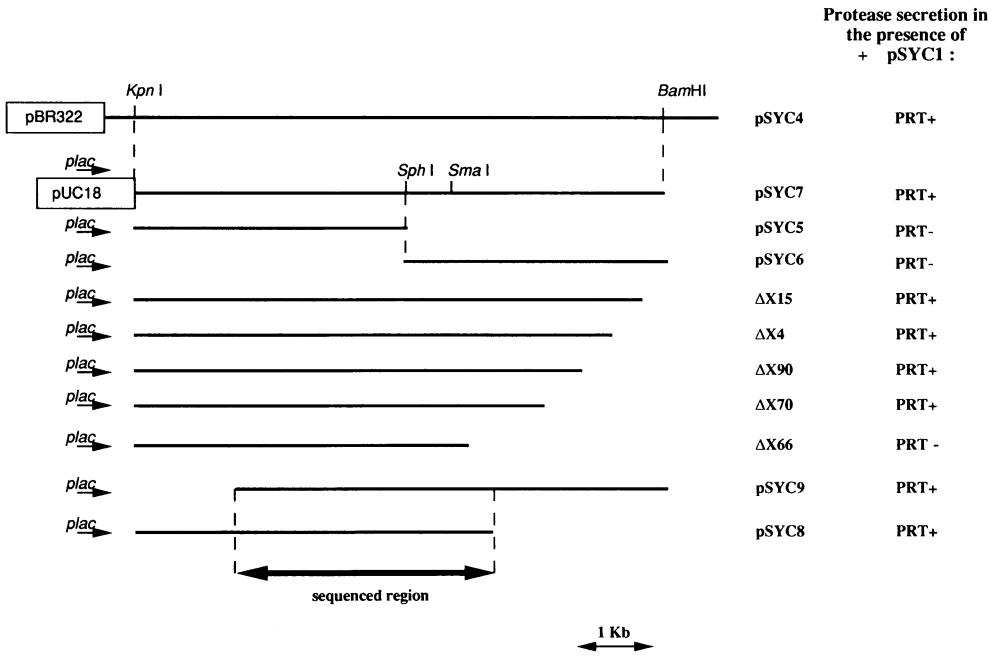

FIG. 2. Map of the different plasmids used in this study. pSYC4 contains the secretion factor genes $prtD_{SM}$ and $prtE_{SM}$. The arrows above pSYC4 indicate the direction of transcription of $prtD_{SM}$ and $prtE_{SM}$. Plasmids pSYC7 contains the *Kpn*I-*Bam*HI fragment of pSYC4 under *lac* promoter control. Plasmids pSYC5, pSYC6, pSYC8, pSYC9, ΔX15, ΔX4, ΔX90, ΔX70, and ΔX66 were created either by subcloning or unidirectional deletion. The sequenced region is indicated in boldface and corresponds to the overlapping region between pSYC8 and pSYC9. pSYC1 contains the protease SM determinant as well as the inh_{SM} gene. The proteolytic phenotype of the strain C600(pSYC1) carrying the different plasmids is indicated and was monitored by the presence of a halo (PRT +) on skim milk agar plates (see Materials and Methods).

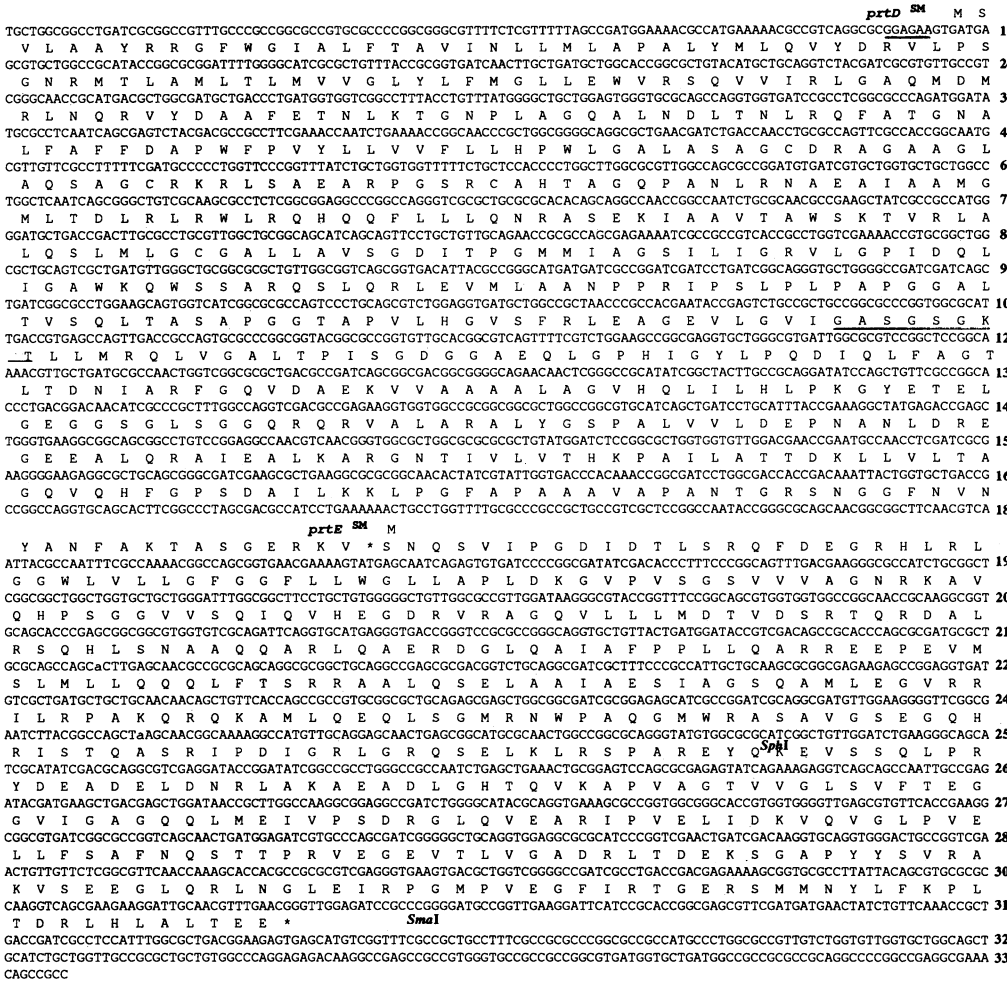

FIG. 4. Nucleotide sequence of the 3.368-kb DNA fragment together with the deduced amino acid sequences for $PrtD_{SM}$ and $PrtE_{SM}$. The nucleotide sequences of the putative ribosome binding sites are underlined. The amino acid sequence is shown in boldface letters above the sequence. The amino acid sequence of the putative ATP-binding site in $PrtD_{SM}$ is underlined. The first methionine codon of *prtE* overlaps the stop codon of the *prtD* gene (see text). The putative ribosome binding site is underlined at the beginning of $prtD_{SM}$.

with [^{35}S]methionine, and proteins were analyzed by SDS-PAGE and autoradiography. Figure 3 shows that pSYC4 codes for two detectable proteins with apparent molecular masses of 60 and 50 kDa, while pSYC5, which carries the left half of the fragment, encodes only the 60-kDa protein and pSYC6 expresses neither protein. In analogous systems, the secretion apparatuses consist of three proteins. However, only two proteins were identified in the minicell analysis, indicating either that these two proteins are sufficient to promote protease SM secretion in *E. coli* or that a third protein was necessary but not identified in the present study.

The *S. marcescens* protease secretion proteins are expected to be closely related to the *E. chrysanthemi* secretion proteins PrtD, PrtE, and PrtF because this apparatus can promote efficient secretion of protease SM. We therefore tested whether antisera directed against PrtD, PrtE, and PrtF recognized the proteins encoded by pSYC4. No cross-reaction was observed by immunodetection (data not shown).

DNA sequence of the 3.368-kb DNA fragment allowing specific protease SM secretion. The DNA sequence was determined for the smallest DNA fragment still able to allow the secretion of protease SM. This fragment was defined as the overlapping DNA fragment present in pSYC8 and pSYC9, both of which have one end deleted and lead to a proteolytic phenotype when introduced into C600(pSYC1). This overlapping sequenced region is indicated in Fig. 2 by a thick line.

The strategy used to determine the nucleotide sequence of the 3.368-kb DNA fragment present on pSYC8 is described in Materials and Methods. The nucleotide sequence is shown in Fig. 4. Analysis of the nucleotide sequence revealed the presence of two open reading frames encoded on the same DNA strand. The first gene, named $prtD_{SM}$, codes for a 576-residue protein (predicted molecular mass, 60.8 kDa). A putative ribosome binding site and methionine initiation codon are indicated in Fig. 4. A search for homology in a protein data bank showed that this protein has 58.5% identity with $PrtD_{EC}$ of *E. chrysanthemi* (Fig. 5A) and 24% identity with HlyB.

Like $PrtD_{EC}$ and HlyB, this protein, in its terminal half, has five or six highly hydrophobic domains which might correspond to transmembrane segments and a rather hydrophilic C-terminal half with a putative ATP-binding site indicated in Fig. 4.

FIG. 5. Amino acid sequence comparison of the *S. marcescens* and *E. chrysanthemi* inner membrane transporter components. (A) Comparison between $PrtD_{EC}$ and $PrtD_{SM}$. (B) Comparison between $PrtE_{EC}$ and $PrtE_{SM}$. Dashes indicate gaps introduced for alignment, stars indicate matches across all of the sequences, and points indicate conservative substitutions. The amino acid sequence of the conserved putative ATP-binding site in $PrtD_{SM}$ and $PrtD_{EC}$ is boxed.

The second gene, named $prtE_{SM}$, codes for a 437-residue polypeptide (predicted molecular mass, 47.8 kDa) which is probably initiated at an ATG codon overlapping the last stop codon of $prtD_{SM}$. The putative ribosome binding site consensus sequence is underlined in Fig. 4. A search for homology showed that the protein has 51.6% identity with $PrtE_{EC}$ (Fig. 5B) and 24% identity with HlyD. This protein might have one N-terminal hydrophobic domain. No signal sequences were found in these two proteins.

These two secretion proteins are homologous to the inner membrane components found in analogous secretion systems. However, the gene encoding the outer membrane component was not found, and no open reading frame exhibiting even a low degree of homology with *prtF* could be detected on the fragment. This result could either indicate that two specific components are sufficient to promote protease SM secretion or, more likely, that the third outer membrane component was provided by the *E. coli* host strain. The best candidate was the TolC protein, already known to be involved in the constitution of similar secretion systems, HlyB/HlyD/TolC (40) and CvaA/CvaB/TolC (9).

Protease SM secretion by the $PrtD_{SM}$, $PrtE_{SM}$ transporter is TolC dependent. *E. coli* C600 carrying a Tn*10* insertion in the *tolC* gene was transformed with pSYC1 and pSYC8. Unlike the parental $tolC^+$ strain carrying the same plasmids, the *tolC*::Tn*10* strain was not proteolytic. Protease SM was undetectable in concentrated supernatants of C600 *tolC*::Tn*10* (pSYC1, pSYC8) (Fig. 6), although the amount of intracellular protease detected by Western blot (immunoblot) analysis of the cell pellet was similar in $tolC^+$ and *tolC*::Tn*10* strains (data not shown). This result indicates that a third outer membrane component is also required for protease SM secretion, as is the case in other systems, and that the recombinant plasmids isolated do not carry this *Serratia* gene. The fact that a larger recombinant plasmid, pSYC7, was also unable to promote protease secretion in a *tolC* mutant background (data not shown) suggests that in *E. coli*, the TolC protein is able to substitute for the *S. marcescens* component and that this hybrid apparatus is functional.

The hybrid secretion apparatus can also promote *E. chrysanthemi* protease B secretion. In order to test whether the $PrtD_{SM}$, $PrtE_{SM}$, TolC apparatus can promote the secretion of heterologous proteins, C600(pSYC8) and C600 *tolC*::Tn*10* (pSYC8) were transformed with pRUW538, which carries the *prtB* gene of *E. chrysanthemi*. The proteolytic phenotype on skim milk plates was observed only with the $tolC^+$ strain. Proteins present in the supernatants were analyzed by SDS-PAGE followed by Coomassie blue staining. Figure 7 shows that the $PrtD_{SM}$, $PrtE_{SM}$, TolC apparatus was able to specifically secrete protease B as efficiently as the *E. chrysanthemi*

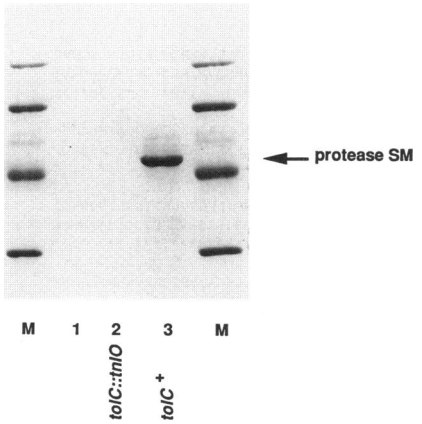

FIG. 6. Secretion of protease SM in *E. coli* as shown by Coomassie blue staining of culture supernatants. Cultures were grown in ML medium to an OD_{600} of 1. The supernatants were concentrated as described in Materials and Methods. Lanes: M, molecular mass markers (from the top): 92, 66, 45, and 31 kDa; 1, C600 *tolC*$^+$(pSYC1); 2, C600 *tolC*::Tn*10*(pSYC1, pSYC8); 3, C600 *tolC*$^+$ (pSYC1, pSYC8). Lanes 1 and 2 were loaded with 5 OD equivalent units of concentrated supernatant sample, and lane 3 was loaded with 2 OD equivalent units.

secretion apparatus ($PrtD_{EC}$, $PrtE_{EC}$, $PrtF_{EC}$) encoded by pRUW9. However, no secreted protease at all was found in the *tolC*::Tn*10* strain supernatant (data not shown).

Activity of other hybrid complexes. The TolC outer membrane component can interact with $PrtD_{SM}$ and $PrtE_{SM}$ to form an active transporter. We therefore tested whether TolC can also interact with $PrtD_{EC}$ and $PrtE_{EC}$ for secretion of

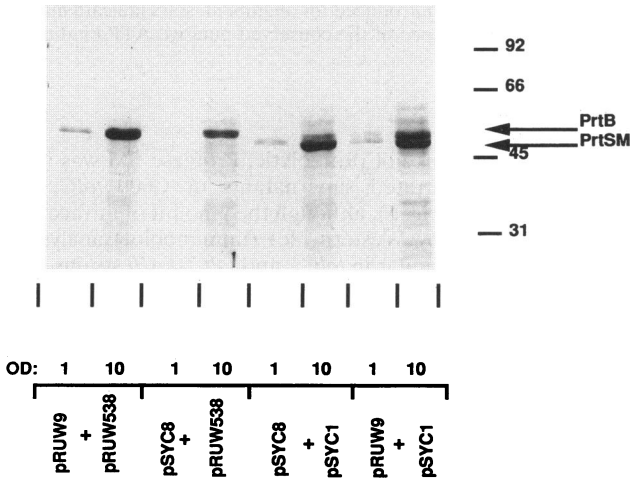

FIG. 7. Comparison of protease B (PrtB) and protease SM (PrtSM) secretion in *E. coli* through Coomassie blue staining of culture supernatants. Culture supernatants were prepared and concentrated as described in Materials and Methods. All plasmids were present in the C600 strain. From left to right, the two first lanes were loaded with 1 and 10 OD equivalent units of concentrated supernatant sample of C600(pRUW9, pRUW538), the next two were loaded with concentrated supernatant sample of C600(pSYC8, pRUW538), the next two were loaded with concentrated supernatant sample of C600(pSYC8, pSYC1), and the last two were loaded with concentrated supernatant sample of C600(pRUW9, pSYC1). The positions of protease B and protease SM are as indicated. The molecular mass standards (in kilodaltons) are indicated on the right.

protease B or protease SM. C600(pRUW34), which carries the $prtD_{EC}$ and $prtE_{EC}$ genes, was transformed with either pRUW538 or pSYC1, which express protease B and protease SM, respectively. The transformants were not proteolytic on skim milk plates, and the culture supernatants did not contain detectable amounts of either protease (data not shown), indicating that this hybrid secretion apparatus is not functional.

DISCUSSION

S. marcescens protease SM is secreted by a signal peptide-independent pathway (32). Like many proteins belonging to this pathway, it has a C-terminal targeting sequence (26). In *E. coli*, it can be secreted by the heterologous protease secretion apparatus $PrtD_{EC}$, $PrtE_{EC}$, $PrtF_{EC}$ of *E. chrysanthemi* (24).

In the present work, we have identified an 8-kb *S. marcescens* DNA fragment which allows the secretion of SM protease in *E. coli*. The DNA fragment does not carry the prt_{SM} gene, confirming our previous hypothesis that the genes encoding the secretion proteins are not closely linked to the protease structural gene (24). This DNA fragment carries two genes coding for the secretion proteins $PrtD_{SM}$ and $PrtE_{SM}$. $PrtD_{SM}$ is highly homologous to $PrtD_{EC}$, one of the inner membrane components of the *E. chrysanthemi* secretion system. Like $PrtD_{EC}$, it belongs to a family of ATP-binding membrane transporters identified in bacteria, yeasts, protozoans, and mammals. $PrtE_{SM}$ is highly homologous to $PrtE_{EC}$, the second inner membrane component of the *E. chrysanthemi* transporter. Such homology between the *S. marcescens* and *E. chrysanthemi* transporters was expected because protease SM secretion by the heterologous system is efficient (24). However, the heterologous system (from *E. chrysanthemi*) requires a third specific component, the $PrtF_{EC}$ protein coded for by a gene adjacent to $prtE_{EC}$ (23). No protein homologous to $PrtF_{EC}$ was encoded by the 8-kb DNA fragment required for protease SM secretion in *E. coli*. Considering the homology between the two inner membrane components of the *E. chrysanthemi* and *S. marcescens* transporters, it seemed likely that a third specific outer membrane component is part of this transporter but is coded for by a gene absent from the cloned DNA. In the *E. coli* clone, a host protein could substitute for this *S. marcescens* component. We showed that indeed the *E. coli* TolC protein is required with $PrtD_{SM}$ and $PrtE_{SM}$ to form an active transporter for the secretion of protease SM. It is therefore a hybrid secretion apparatus formed by one protein from *E. coli* and two proteins from *S. marcescens* which is functional.

The TolC protein is thus able to form several active transporters: the HlyB, HlyD, TolC transporter for α-hemolysin (38), the CvA, CvB, TolC transporter for colicin V (9), and the $PrtD_{SM}$, $PrtE_{SM}$, TolC transporter for protease SM. On the other hand, it cannot replace $PrtF_{EC}$ or AprF because the hybrid transporter $PrtD_{EC}$, $PrtE_{EC}$, TolC is unable to secrete protease B or protease SM and the hybrid transporter AprD, AprE, TolC is unable to transport the alkaline protease of *P. aeruginosa* (data not shown). Conversely, $PrtF_{EC}$ cannot replace TolC in the hybrid transporter HlyB, HlyD, $PrtF_{EC}$ to promote the α-hemolysin secretion (data not shown). These results indicate that the outer membrane component in these apparatuses is not just an aspecific pore allowing transport across the outer membrane. Because some hybrid transporters including TolC are functional while others are not, it is possible that the components allowing a functional interaction might share a common motif, a kind of TolC box absent in the nonpermissive components. To determine which membrane secretion protein is specific for the constitution of an active

transporter, new hybrids carrying a single component of each system—$PrtD_{EC}$ and $PrtE_{SM}$ or $PrtD_{SM}$ and $PrtE_{EC}$—will be constructed, combined with TolC protein, and tested for functionality.

In addition to the interaction between themselves, the secretion components must also recognize the substrate, the targeting sequence on the secreted polypeptide. It is not known whether this site interacts sequentially with each component. The hybrid transporter $PrtD_{SM}$, $PrtE_{SM}$, TolC is able to secrete proteases B and SM, while the hybrid transporter $PrtD_{EC}$, $PrtE_{EC}$, TolC is unable to do so. The substrates can therefore use the TolC protein if it is in contact with $PrtD_{SM}$ and $PrtE_{SM}$ but not if it is in contact with $PrtD_{EC}$ and $PrtE_{EC}$. This indicates that the specificity does not arise from a direct interaction between the signal and the outer membrane component and suggests that secretion is not via sequential interactions first with the inner membrane and then with outer membrane. It is more plausible that the substrate interacts specifically with one inner membrane component and is then driven through a specific channel crossing both membranes.

In these secretion systems, the genes encoding the secretion proteins are often adjacent and also are often contiguous to the structural genes of the secreted polypeptides. The *S. marcescens* protease SM secretion determinant does not follow this rule: the prt_{SM} gene is unlinked to the $prtD_{SM}$ and $prtE_{SM}$ genes, and the third unidentified gene is contiguous to none of these. The *S. marcescens* TolC analog will be cloned and identified in order to determine whether it has only one specific function, like $PrtF_{EC}$ and AprF, or several functions, like TolC, which, besides secretion, is involved in colicin E1 adsorption (31), vancomycin permeability (40), resistance to hydrophobic compounds (31), and chromosome segregation (20).

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