Cloning and Expression in *Escherichia coli* of the *Serratia marcescens* Metalloprotease Gene: Secretion of the Protease from *E. coli* in the Presence of the *Erwinia chrysanthemi* Protease Secretion Functions

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The *Serratia marcescens* extracellular protease SM is secreted by a signal peptide-independent pathway. When the *prtSM* gene was cloned and expressed in *Escherichia coli*, the cells did not secrete protease SM. The lack of secretion could be very efficiently complemented by the *Erwinia chrysanthemi* protease B secretion apparatus constituted by the PrtD, PrtE, and PrtF proteins. As with protease B and α-hemolysin, the secretion signal was located within the last 80 amino acids of the protease. These results indicate that the mechanism of *S. marcescens* protease SM secretion is analogous to the mechanisms of protease B and hemolysin secretion.

Serratia marcescens secretes a 50-kDa metalloprotease (SM protease) in large amounts into the culture medium (4). The gene encoding the exoprotease has been cloned and expressed in *Escherichia coli*, and its nucleotide sequence has been reported (32). It was shown that the mature protein is preceded by a propeptide of 16 amino acids which does not have the characteristics of a signal peptide, indicating that the secretion pathway is independent of the cleavage of an N-terminal signal peptide. In this regard, the SM protease is similar to the *Erwinia chrysanthemi* proteases B and C (7) and to *E. coli* hemolysin (12), which contain a C-terminal secretion signal (8, 34) and have partial amino acid sequence homology in a repeated glycine-rich nonapeptide located close to the C terminus (7). The SM protease also contains the same repeats. Moreover, the SM and B proteases share 60% identity (7).

When the *E. chrysanthemi* genes involved in the synthesis and secretion of proteases B and C are expressed in *E. coli*, the proteases are made and secreted from this foreign host (45). This secretion requires three specific functions (27). On the other hand, when expressed in *E. coli*, the SM protease was not secreted but accumulated inside the cells as an inactive precursor of higher molecular mass (51 kDa), consistent with the existence of a 16-amino-acid propeptide (32). The absence of SM protease secretion in *E. coli* might indicate the absence of the genes required for secretion on the recombinant plasmid that carries the *prtSM* gene.

On the basis of the high degree of homology between the two proteases, we hypothesized that the SM protease could be secreted by the *E. chrysanthemi* protease secretion system; proceeding from this hypothesis, we have introduced an *S. marcescens* cosmid DNA library into an *E. coli* strain expressing the *E. chrysanthemi* protease secretion functions. Proteolytic *E. coli* recombinant clones were found and characterized in this work. We have shown that the SM protease is efficiently produced in *E. coli* and secreted only in the presence of the *E. chrysanthemi* secretion functions. The data reported here suggest that the molecular mechanisms for the secretion of SM protease and protease B are similar. It therefore follows that the SM protease is a

member of the family of proteins secreted from gram-negative bacteria without the intervention of an N-terminal signal peptide (37), but rather with a C-terminal secretion signal (35).

MATERIALS AND METHODS

Strains, plasmids, phage, and media. *E. coli* C600 (F⁻ *thr leu fhuA lacY rpsL thi supE*), PAP105 [Δ(lac-pro) F'*lacI*�q *lac*ZM15 *pro*⁺ Tn*10*], and BZB1019 (F⁻ *met hsdR rpsL gal*) are from the laboratory collection. *E. coli* K5 (*hsdR hsdM lac rpsL thi thr*) is described in reference 3. Plasmids pBR322 (2), pACYC 184 (5), pEMBL8⁺ and pEMBL9⁺ (10), and pBGS18⁺ and pBGS19⁺ (39) and cosmid pHC79 (22) were used as vectors. Plasmid pRUW4*inh1* encodes the *E. chrysanthemi* protease secretion functions PrtD, PrtE, and PrtF (27) and has an *inh* mutation that inactivates the protease inhibitor (45). Plasmid pLG575 expresses the *E. coli* hemolysin secretion functions HlyB and HlyD (28). Plasmid pHP45 Ω has an Ω interposon which carries the spectinomycin resistance gene (36). *S. marcescens* 365 (4) was obtained from V. Braun. The M13 phage derivative KO7 was a gift from J. Vieira. All media have been described previously (30), 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 previously (29).

DNA sequence determination. Single-stranded DNA from plasmid pBGS19⁺ carrying the *Hin*dIII-*Bam*HI DNA fragment shown in Fig. 1 was obtained by infection with M13 phage derivative KO7. The DNA sequence of a 200-bp fragment starting from the *Bam*HI site was determined by the Sanger dideoxy method using [α-³⁵S]dATP (1).

5'-Terminal deletion of the *prtSM* gene and construction of a hybrid gene between *lacZ* and *prtSM*. The *Hin*dIII-*Fsp*I 2.4-kb DNA fragment from pSM4-9 was recloned into *Hin*dIII-*Sma*I-linearized pSB118 vector, and the same 2.4-kb insert was cut out with endonucleases *Kpn*I-*Eco*RI and inserted in pBGS19⁺ linearized with *Kpn*I-*Eco*RI in order to place the 5' end of the 2.4-kb fragment close to the *Eco*RI site and to generate unidirectional 5' deletions of the *prtSM*
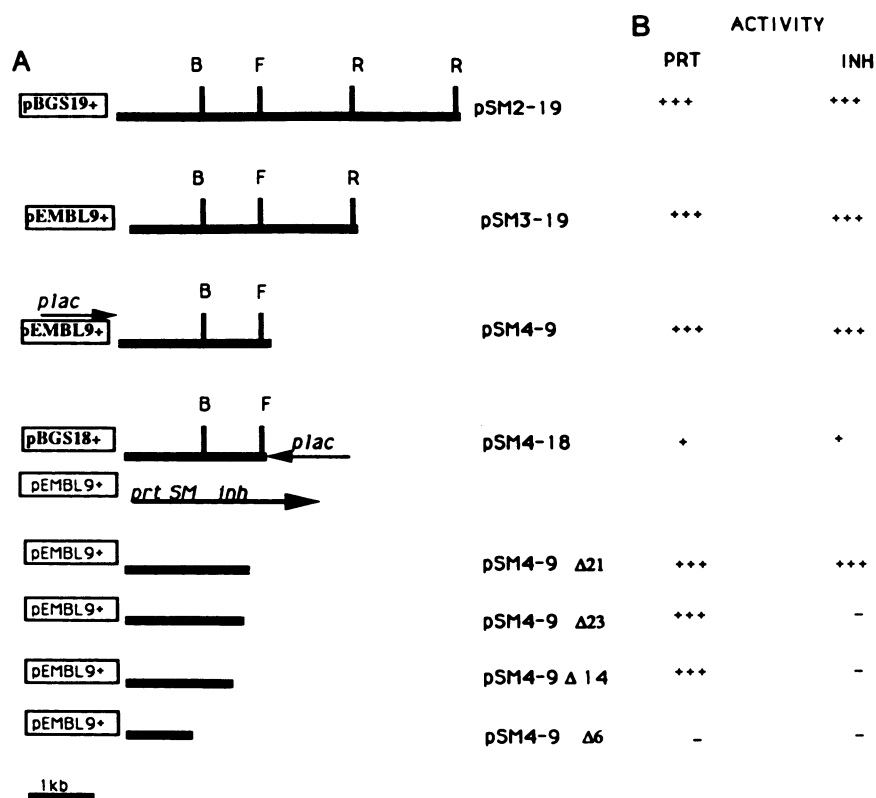
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FIG. 1. (A) Restriction endonuclease map of the plasmids used. Cleavage sites determined by restriction endonuclease analysis are indicated for the following restriction enzymes: B, *Bam*HI; F, *Fsp*I; R, *Eco*RI. Transcriptional orientations of the *prtSM* and *inh* genes are indicated by an arrow showing the positions of the genes. The vectors shown in boxes are described in Materials and Methods. The *lac* promoter (*plac*) transcriptional orientations on pSM4-9 and pSM4-18 are indicated by arrows. (B) Extracellular proteolytic (PRT) and protease inhibitor (INH) activities produced by cells carrying the various plasmids together with pRUW4*inh1*. Symbols: +++, maximum enzymatic activity; +, 1/30 of the maximum activity; −, no enzymatic activity.

gene starting from the *Eco*RI site, using the 3'→5' exonuclease activity of T4 DNA polymerase (6). Fifteen derivatives of pSM4-19 that carry deletions were digested with *Eco*RI and *Hin*dIII and cloned into pEMBL8 digested with the same enzyme. In-frame fusions between the first codons of the *lacZ* gene and the 5'-end-deleted *prtSM* gene which encode a LacZ-PrtSM hybrid protein are expected to occur for one-third of the deletions. Four of the fifteen deletions created were in frame with the *lacZ* gene and encode a hybrid protein.

Protein analysis. *S. marcescens* and *E. coli* were grown at 37°C in ML medium. Cells were centrifuged for 10 min at 5,000 × *g* at 4°C during the exponential growth phase (optical density at 600 nm [OD$_{600}$] = 1) or during the stationary growth phase (OD$_{600}$ = 4). The supernatants were concentrated by precipitation with 10% trichloroacetic as described previously (45). The cell pellets were solubilized in sodium dodecyl sulfate (SDS) sample buffer to yield a preparation of total cellular proteins. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (24) and Coomassie blue staining or immunodetection. Immunodetection with anti-SM protease antibodies used at a 1/500 dilution was carried out as described previously (26, 41).

Genomic library construction. The *S. marcescens* 365 chromosomal DNA library used in this work has been described elsewhere (3). The recombinant cosmids were present in *E. coli* K5 and were plated either directly on skimmed milk agar, with ampicillin resistance selection, or

after transformation with pRUW4, with ampicillin and chloramphenicol resistance selection.

Measurements of enzyme activities. Protease activity was measured by monitoring the hydrolysis of azocasein or by the cup plate technique (26, 43). The intracellular inhibitor activity was measured after disruption of the cells by sonication in a Branson Sonifier model B12 by the cup plate technique (26).

RESULTS

Cloning of the SM protease structural gene. A genomic DNA library of *S. marcescens* 365 was constructed in cosmid pHC79 and introduced into *E. coli* K5. A total of 2,000 transfectant clones were screened for their proteolytic phenotype on skimmed milk agar. No such colonies could be found. These *E. coli* recombinant clones were transformed with pRUW4*inh1*, which encodes the three secretion functions, PrtD, PrtE, and PrtF, necessary for protease B and C secretion and were screened again. In this case, several proteolytic colonies were found on skimmed milk agar. Cosmid pHC79SM1 carried by one proteolytic recombinant was isolated and used to transform *E. coli* BZB1019 or *E. coli* BZB1019(pRUW4*inh1*), with selection for ampicillin resistance in the first case and for ampicillin and chloramphenicol resistance in the second. Proteolytic colonies were obtained only when pHC79SM1 was introduced into the strain carrying pRUW4*inh1*, suggesting that the protease
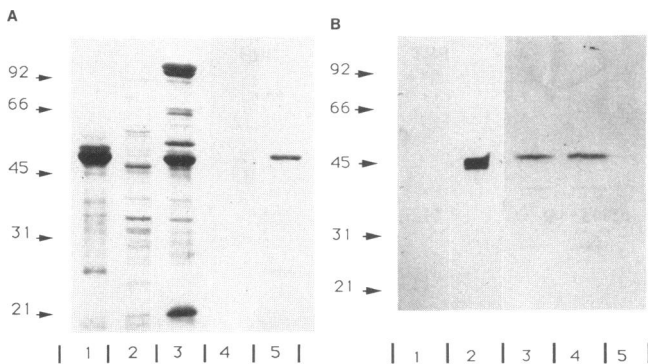
FIG. 2. Analysis of supernatants and cell pellets of *S. marcescens* 365 and of several *E. coli* C600 strains containing various recombinant plasmids. Cultures were grown in ML medium to an OD_{600} of 1; the cells were harvested, concentrated 20-fold in sample buffer, and boiled for 5 min, and 10-μl portions were subjected to SDS-PAGE analysis. Culture supernatants were concentrated 100-fold by precipitation with trichloroacetic acid as described in Materials and Methods, and 10-μl portions were subjected to SDS-PAGE analysis. (A) Coomassie blue staining after SDS-PAGE. Lanes: 1, supernatant of strain 365; 2, supernatant of strain C600(pHC79SM); 3, supernatant of C600(pHC79SM1, pRUW4*inh1*); 4, supernatant of C600(pSM4-9); 5, supernatant of C600(pSM4-9, pRUW4*inh1*; 6, supernatant of C600(pRUW4*inh1*). (B) Immunodetection with anti-SM protease antibodies. Lanes: 1, supernatant of C600(pSM4-9); 2, supernatant of C600(pRUW4*inh1*, pSM4-9); 3, pellet of C600 (pSM4-9); 4, pellet of C600(pSM4-9, pRUW4*inh1*); 5, supernatant of C600(pRUW4*inh1*).

encoded by the cosmid is secreted only in the presence of pRUW4.

To subclone the protease structural gene, cosmid pHC79SM1 DNA was digested with *Cla*I and ligated with *Acc*I-linearized pBGS19⁺. The ligation mixture was used to transform *E. coli* C600(pRUW4*inh1*), with selection for ampicillin and chloramphenicol resistance, and transformants were screened for proteolytic activity on skimmed milk agar. One proteolytic clone carried a plasmid (pSM2-19) with a 5.8-kb *Cla*I insert. The restriction map of pSM2-19 is shown in Fig. 1. Several subclones were constructed and tested for the ability to confer a proteolytic phenotype after transformation of C600(pRUW4*inh1*) (Fig. 1). This defines a 2.4-kb *Hin*dIII-*Fsp*I DNA fragment present on pSM4-9 within which the *prtSM* gene must lie.

Proteins present in culture supernatants of *S. marcescens* 365 and of *E. coli* C600(pRUW4*inh1*, pHC79SM1) and C600(pRUW4*inh1*, pSM4-9) were separated by SDS-PAGE and subjected to either Coomassie blue staining or immunodetection with antibodies raised against pure SM protease. Figure 2A shows one major protein band of approximately 50 kDa present in these supernatants. Immunodetection (Fig. 2B) indicates that this protein is recognized by antibodies directed against SM protease.

In strain C600(pSM4-9), no SM protease secretion occurred. Instead, an inactive intracellular form of higher molecular weight was recognized by the anti-SM protease antibodies (Fig. 2B), confirming the role of pRUW4 in SM protease secretion in *E. coli*.

Genetic organization of the 2.4-kb DNA fragment. The *Hin*dIII-*Fsp*I protease encoding DNA from pSM4-9 was recloned into pBGS18⁺ in reverse orientation relative to the *lacZ* promoter carried by these plasmids. The proteolytic activity in strain PAP105(pSM4-9, pRUW4*inh1*) was induc-

ible by isopropyl-β-D-thiogalactopyranoside (IPTG) and was 30 times higher than the activity present in PAP105(pSM4-18, pRUW4*inh1*), indicating that the *prtSM* gene is transcribed from the *Hin*dIII site toward the *Eco*RI site (Fig. 1).

The nucleotide sequence of a small 200-bp DNA fragment starting at the *Bam*HI site was determined; it was highly homologous to the published sequence of the *prtSM* gene (32) and allowed us to localize the 3' end of the *prtSM* gene 250 nucleotides downstream from the *Bam*HI site.

Identification of a protease inhibitor. The existence of a protease inhibitor in *E. chrysanthemi* whose structural gene is located close to the protease structural gene led us to test whether such an inhibitor was synthesized in *S. marcescens*. An inhibitor of SM protease was found in *S. marcescens* 365 and also in *E. coli* C600 carrying pHC79SM1, pSM4-9, or pSM4-18. The inhibitor is active against SM protease and the mixture of proteases B and C. It is a small (approximately 10-kDa), heat-stable protein located partially in the periplasmic space, very similar in its properties to the *E. chrysanthemi* protease inhibitor. However, an immunoblot with antibodies raised against pure *E. chrysanthemi* protease inhibitor showed no cross-reactivity between these two inhibitors (data not shown).

Comparison of the inhibitor activity in PAP105 carrying pSM4-9 or pSM4-18 showed that the inhibitor activity was inducible by IPTG in a strain carrying pSM4-9 but not pSM4-18 and that it was 20 times higher in the first strain, indicating that the *inh* gene is transcribed in the same direction as the *prtSM* gene (Fig. 1).

The polar Ω interposon was introduced at the *Bam*HI site in the *prtSM* gene. When introduced in C600(pRUW4*inh1*), this mutated plasmid pSM5-9 failed to produce proteolytic activity or inhibitor activity, showing that the interposon insertion in *prtSM* has a polar effect on *inh* expression and indicating that the two genes belong to the same operon.

Unidirectional deletions of 200 bp starting from the *Eco*RI site on pSM4-9 were obtained by using the 3'→5' exonuclease activity of T4 DNA polymerase. This deletion mapping (Fig. 1) indicated that the *inh* gene is located on a 0.4-kb DNA fragment contiguous to the *prtSM* gene.

Protease inhibitor function. Two deletions in pSM4-9 (Δ14 and Δ23; Fig. 1) which end in the *inh* gene led, after transformation of C600(pRUW4*inh1*), to a Prt⁺ Inh⁻ phenotype, indicating that the inhibitor is not required for the SM protease secretion, as was previously shown for *E. chrysanthemi* protease secretion (Fig. 1) (45). However, *E. coli* C600 carrying pSM4-9Δ14 or SM4-9Δ23 (Fig. 1) does not grow well and forms flat translucent colonies, a phenotype not observed with the parental pSM4-9 plasmid. Whereas intracellular proteolytic activity is never detected in *E. coli* strains carrying Inh⁺ Prt⁺ plasmids or in *S. marcescens*, intracellular activity is found in *E. coli* carrying (pSM4-9Δ14 or pSM4-9Δ23 and may be harmful to cells, which would explain the slow-growth phenotype. Figure 3 shows an immunoblot of the intracellular forms of SM protease found in cell extracts of C600(pSM4-9), and C600(pSM4-9Δ14), and C600(pSM4-9Δ23). In the presence of the inhibitor, only the precursor form of the protease could be detected inside the cells; in the absence of the inhibitor, a significant fraction (about 20 to 50%) of the protease had the molecular weight of the mature protease and the remainder had that of the zymogen, indicating that the inhibitor is necessary to protect the cells; against an intracellular zymogen activation.

Characterization of SM protease secretion in *E. coli* with the help of *E. chrysanthemi* PrtD, PrtE, and PrtF functions. Three secretion functions are required for *E. chrysanthemi* prote-
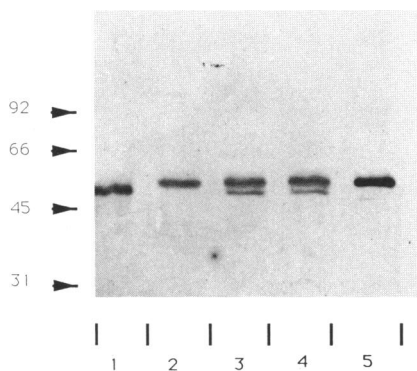
FIG. 3. Immunodetection of SM protease in cell pellets of various strains. Cell pellets (20-fold concentrated) were prepared as described for Fig. 2, and 1-μl portions were subjected to SDS-PAGE analysis followed by immunodetection with anti-SM protease antibodies. Lanes: 1, concentrated supernatant of C600(pSM4-9, pRUW4inhl) showing the mature SM protease band; 2, pellet of C600(pSM4-9Δ21); 3, pellet of C600(pSM4-9Δ14); 4, pellet of C600 (pSM4-9Δ29); 5, pellet of C600(pSM4-9, pRUW4inhl).

ase B secretion (27). To test whether all three are also required for SM protease secretion in *E. coli*, strain C600(pSM4-9) was transformed with pRUW4 *prtD1*, pRUW4 *prtE1*, or pRUW34, which carry nonpolar mutations in each of the genes encoding the secretion functions. Each mutation abolished the secretion of SM protease in *E. coli* without affecting the synthesis of the protease (Fig. 4).

E. chrysanthemi protease B is synthesized as an inactive higher-molecular-weight precursor (zymogen) that is secreted and activated in the external medium. When cation chelators such as EDTA are present in the culture medium, zymogen activation is inhibited (7).

In the presence of 200 μM EDTA in the culture medium, the protease SM zymogen is secreted very efficiently (Fig. 5). The small amount of zymogen found in the supernatant might be due either to EDTA toxicity or to a faster degradation of the zymogen than the mature form. In any case, the presence of the zymogen in the supernatant indicates that the cleavage of the propeptide is not essential for the secretion process.
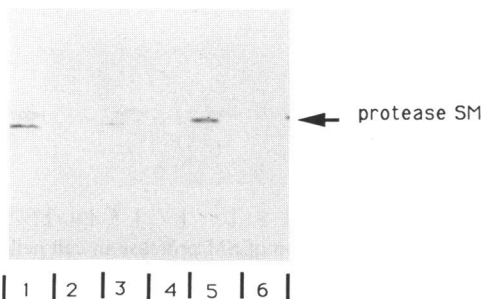

FIG. 4. Immunodetection of SM protease in cell pellets and supernatants of various strains. Cell pellets and supernatants were prepared as described for Fig. 2 and subjected to SDS-PAGE (10% acrylamide) and immunodetection with anti-SM protease antibodies. Lanes: 1, cell pellet of strain C600(pRUW4 *prtD1*, pSM4-9); 2, supernatant of the same strain; 3, cell pellet of strain C600(pRUW4 *prtE1*, pSM4-9); 4, supernatant of the same strain; 5, cell pellet of strain C600(pRUW34, pSM4-9); 6, supernatant of the same strain. Each lane was loaded with 10 μl of supernatant or 5 μl of pellet.
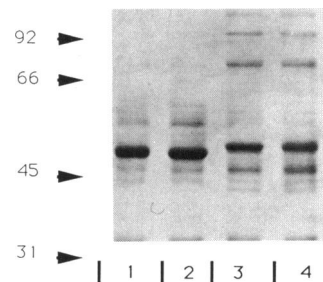

FIG. 5. Analysis of supernatants of strain C600(pSM4-9, pRUW4inhl) cultivated in the presence of various EDTA concentrations. Supernatants (100-fold concentrated) were prepared as described for Fig. 2, and 10-μl portions were subjected to SDS-PAGE analysis (7.5% acrylamide). Lanes: 1, no EDTA; 2, 100 μM EDTA; 3, 200 μM EDTA; 4, 400 μM EDTA.

The secretion signal of *E. chrysanthemi* protease B is located within the last 40 C-terminal amino acids (8). To test whether such a C-terminal secretion signal was present on SM protease, the DNA insert present in pSM4-9 was recloned in pBGS19⁺ and successive unidirectional deletions were made, starting from the *Eco*RI site at the 5′ end of the *prtSM* gene (Fig. 6). Fifteen such deletions were recloned in pEMBL8 under the *lac* promoter in the expectation that some would be in phase with the 5′ end of the *lacZ* gene. The corresponding plasmids (Fig. 6) were introduced into C600 and C600(pRUW4inhl). Supernatants of the different cultures were analyzed by SDS-PAGE and Coomassie blue staining. Truncated hybrid proteins were found to be encoded by four such plasmids (Fig. 7). They had apparent molecular masses of 42, 41, 28, and 8 kDa, respectively, and were actively secreted only in the presence of the three
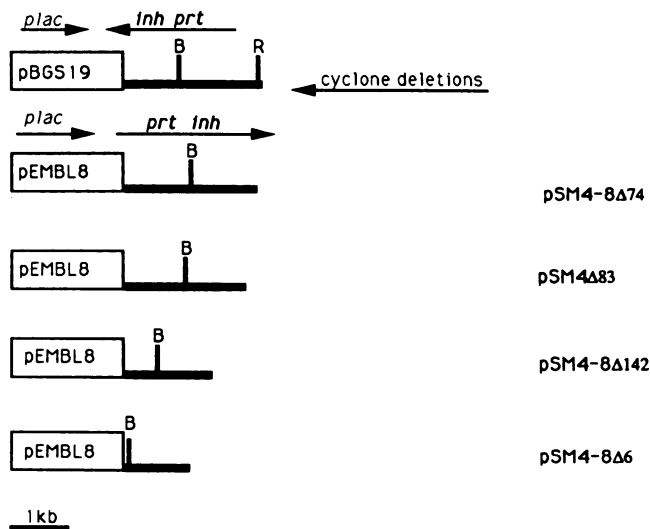

FIG. 6. Unidirectional deletions of the 5′ end of the *prtSM* gene. Plasmid pSM4-19 contains the *Fsp*I-*Eco*RI DNA fragment shown in Fig. 1 that carries the *inh* and *prtSM* genes. Transcriptional orientations of the genes are indicated by arrows above the lines. The arrow below the top line indicates the sense of the unidirectional deletions generated on pSM4-19, starting from the 5′ end of *prtSM*. The DNA fragments carrying truncated *prtSM* genes were recloned in pEMBL8 under control of the *lac* promoter (*plac*). Four such plasmids encoding LacZ-PrtSM hybrid proteins are indicated.

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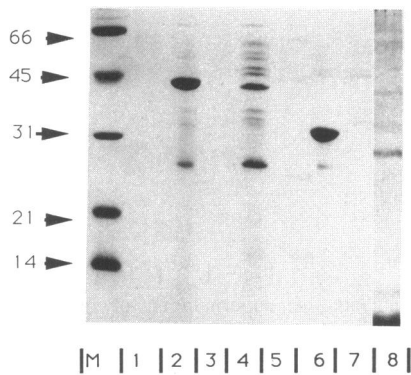14 ►

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FIG. 7. Analysis of supernatant of *E. coli* C600 containing various plasmids. Supernatants (100-fold concentrated) were prepared as described for Fig. 2, and 10-μl portions were subjected to SDS-PAGE analysis (15% acrylamide) followed by Coomassie blue staining. Lanes: 1, C600(pSM4-8Δ74); 2, C600(pRUW4*inh1*, pSM4-8Δ74); 3, C600(pSM4-8Δ83); 4, C600(pRUW4*inh1*, pSM4-8Δ83); 5, C600(pSM4-8Δ142); 6, C600(pRUW4*inh1*, pSM4-8Δ142); 7, C600 (pSM4-8Δ6); 8, C600(pRUW4*inh1*, pSM4-8Δ6).

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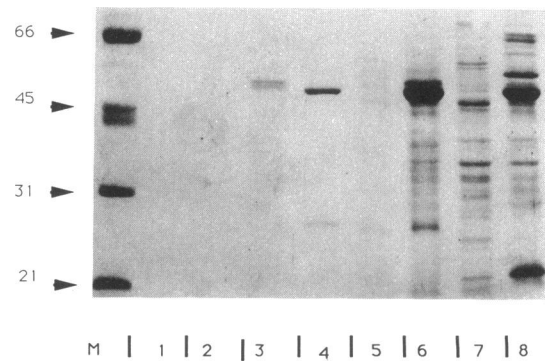
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FIG. 8. Analysis of supernatants of *S. marcescens* 365 and of *E. coli* C600 containing various plasmids. Concentrated supernatants were prepared as described for Fig. 2. Exponentially growing cultures were harvested at an OD_{600} of 1. Stationary cultures were harvested at an OD_{600} of 4 after overnight growth. For exponentially growing 365 and C600(pHC79SM1, pRUW4*inh1*), the supernatants were 750-fold concentrated and 20-μl portions were loaded. For stationary cultures of 365 and C600(pHC79SM1, pRUW4*inh1*), the supernatants were 150-fold concentrated and 10-μl portions were loaded. For exponentially growing C600(pSM4-9, pRUW4*inh1*) and C600(pSM4-9), the supernatants were 100-fold concentrated and 10-μl portions were loaded. For stationary cultures of C600(pSM4-9, pRUW4*inh1*) and C600(pSM4-9), the supernatants were 25-fold concentrated and 10-μl portions were loaded. Lanes: 1, C600(pSM4-9), exponential phase; 2, C600(pSM4-9), stationary phase; 3, C600(pSM4-9, pRUW4*inh1*), exponential phase; 4, C600(pSM4-9, pRUW4*inh1*), stationary phase; 5, C600(pHC79SM1, pRUW4*inh1*), exponential phase; 6, C600(pHC79SM1, pRUW4*inh1*), stationary phase; 7, 365, exponential phase; 8, 365, stationary phase.

specific secretion functions. The smallest hybrid gene constructed encoded an 8-kDa protein which was secreted, showing that the secretion signal is located in the C-terminal portion of the protein.

We have previously shown that the hemolysin secretion apparatus can provide for protease B secretion, although inefficiently (1% of the secretion observed with pRUW4-encoded secretion functions [8]). Such a residual secretion of SM protease was not observed in the presence of hemolysin secretion functions (data not shown).

Relationship between bacterial growth and production of SM protease. Like several extracellular enzymes produced by *S. marcescens*, SM protease is synthesized during the late logarithmic growth phase (38), whereas the *E. chrysanthemi* extracellular proteases are secreted during the exponential growth phase (43). Measurement of extracellular proteolytic activity showed that very low activity was measurable from the exponentially growing cultures ($OD_{600} = 1$) of *S. marcescens* 365 and *E. coli* C600(pHC79SM1, pRUW4*inh1*) (about 1% of the activity found in late stationary phase [$OD_{600} = 4$]). Supernatants and cell pellets were prepared from exponential and stationary cultures and analyzed by SDS-PAGE and Coomassie blue staining or immunodetection with anti-SM protease antibodies. In *S. marcescens* and in *E. coli* C600(pRUW4*inh1*, pHC79SM1), the SM protease was detected neither in supernatants nor in cell pellets during the exponential growth phase (Fig. 8 and 9), indicating that the protease does not accumulate inside the cell during the exponential growth phase prior to secretion. During the stationary phase, the protease was present in the cell pellets and in the supernatant, indicating the existence of an intracellular pool of SM protease. This result suggested that it was expression of the *prtSM* gene that was regulated by the growth phase rather than the secretion step itself. In strain C600(pRUW4*inh1*, pSM4-9), an intracellular pool of SM protease was present during the exponential growth phase and secretion had already occurred.

DISCUSSION

S. marcescens is known to produce large amounts of several extracellular enzymes (nuclease, hemolysin, lipase,

proteases, and chitinases) which might be involved in the pathogenesis of *S. marcescens* opportunistic infections (3). In this work, we have studied the secretion of one of these extracellular enzymes, the metalloprotease SM. Its structural gene has been isolated from a cosmid library of *S. marcescens* 365 chromosomal DNA and subcloned in several plasmids. The SM protease encoded by these cosmids and plasmids accumulated inside *E. coli* cells but was not secreted. The lack of secretion could be specifically and functionally complemented by the *E. chrysanthemi* protease secretion apparatus, which is constituted by three envelope proteins: PrtD, PrtE, and PrtF (27). This allowed the study

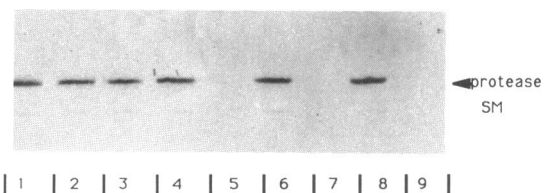◄protease
SM

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |9 |

FIG. 9. Immunodetection of SM protease in cell pellets of various strains during the exponential and stationary growth phases. Cells were harvested at an OD_{600} of 1 and of 4 as described for Fig. 8. Cell extracts were prepared as described for Fig. 2, and 10-μl portions were subjected to SDS-PAGE (10% acrylamide) followed by immunodetection with anti-SM protease antibodies. Lanes: 1, C600(pSM4-9), exponential phase; 2, C600(pSM4-9), stationary phase; 3, C600(pSM4-9, pRUW4*inh1*), exponential phase; 4, C600 (pSM4-9, pRUW4*inh1*), stationary phase; 5, C600(pHC79SM1, pRUW4*inh1*), exponential phase; 6, C600(pHC79SM1, pRUW4 *inh1*), stationary phase; 7, 365, exponential phase; 8, 365, stationary phase.

of SM protease secretion in *E. coli*. We showed that the SM protease is synthesized as an inactive higher-molecular-weight precursor which can be secreted, indicating that cleavage of the propeptide is not prerequisite for secretion. Adjacent to the *prtSM* gene was located a gene which encodes a protease inhibitor: a small periplasmic, heat-stable protein that is active on protease SM and also on proteases B and C. Hence, as in *E. chrysanthemi*, regulation of intracellular proteolytic activity is achieved by two mechanisms: the presence of inactive intracellular protease precursors and synthesis of a specific intracellular protease inhibitor. Whereas in *E. chrysanthemi* or in *E. coli* expressing proteases B and C, the inhibitor is useless for protection against intracellular proteolytic activity, in *E. coli* expressing the SM protease, the inhibitor is essential for bacterial cell viability. Its function is to prevent intracellular activation of the zymogen either by preventing the autoproteolytic cleavage of the zymogen or by inhibiting the proteolytic activity of undetectable amounts of mature protease present in the cells. Such a block in the conversion of the zymogen to the mature form achieved by a specific protease inhibitor is often found in eukaryotic proteolytic cascades (33, 34, 42).

A common feature of many bacterial exoenzymes, including those from *Serratia* spp., is growth-phase-dependent expression (4, 38). Little or no protein is synthesized in exponentially growing cells. When the cells reach stationary phase, enzyme production is turned on. The *E. coli* extracellular toxin microcin B17 and the SM protease behave in this manner (20), and it was shown that the growth phase regulation takes place at the transcriptional level (20).

The late expression is conserved in *E. coli* harboring cosmids pHC79SM1 and pRUW4 but is lost after the subcloning step in *E. coli* harboring pSM4-9 and pRUW4. This finding strongly indicates that the natural promoter is present and active on pHC79SM1 and can be regulated in *E. coli*.

The very efficient SM protease secretion which occurs in *E. coli* with the help of PrtD, PrtE, and PrtF proteins indicates the existence of analogies in the mechanisms of SM and B protease secretion. Hence, the SM protease belongs to the expanding family of proteins secreted in gram-negative bacteria in a signal peptide-independent fashion.

This family now comprises the α-hemolysin of *E. coli* (12), the hemolysinlike proteins from the genera *Proteus*, *Morganella* (23), and *Actinobacillus* (19), the leucotoxin of *Pasteurella haemolytica* (40), the cyclolysin of *Bordetella pertussis* (16), the *E. chrysanthemi* metalloproteases B and C (8), the *Pseudomonas aeruginosa* alkaline protease (18), the *Rhizobium leguminosarum* NodO protein (11), and the colicin V and microcin B17 secreted by *E. coli* (14, 15). The components of the secretion systems of this family of proteins (including microcin B17 and colicin V [14, 15]) show amino acid sequence similarities. In particular, one of the components in each system is an ATP-binding membrane protein that shows significant amino acid sequence homologies with the P glycoprotein or MDR protein, which in mammals is responsible for resistance to several unrelated drugs (17).

Not only sequence similarities but also cross-complementation exist between the different secretion systems. The *E. chrysanthemi* protease B secretion apparatus can promote the secretion of at least two proteases produced by other organisms: the *P. aeruginosa* alkaline protease (18) and, as shown in this work, the SM protease of *S. marcescens*. Similarly, the hemolysin secretion apparatus can promote the secretion of several other proteins: leucotoxin (40), protease B (8), and alkaline protease (18). It appears that the

different secretion mechanisms are analogous, can complement each other more or less efficiently, and are conserved and widespread among gram-negative bacteria.

The secretion signal recognized by the different secretion mechanisms was localized on some proteins; it consists of the last 27 amino acids of α-hemolysin (35) and the last 40 amino acids of protease B (8). In this work, we show a similar location for the SM protease secretion signal. In the case of colicin V, however, the secretion signal is located at the N terminus (15). Moreover, amino acid sequence comparison of the secretion signals does not reveal a conserved domain or even an amino acid consensus for the secretion signal. The only homologous sequence found in most of these proteins is a domain rich in glycine repeats located close to the C-terminal secretion domain, which is not required for the secretion of α-hemolysin, protease B, and protease SM (8, 40). It is unclear what the secretion apparatus recognizes on the proteins or how it can recognize different substrates. Hence, it appears important to find the functional signals on the proteins in order to gather more information on primary sequence and localization on the protein.

The locations of the specific secretion genes close to the structural gene for the secreted protein have been reported for several secretion systems, such as the *E. chrysanthemi* proteases B and C (45) and the *Klebsiella pneumoniae* pullulanase (9). Yet the secretion genes may have other locations, as is the case for the *E. coli* hemolysin determinant, for which the *tolC* gene and the *hlyB* and *hlyD* genes, all three involved in hemolysin secretion, are not linked (44), or for the *P. aeruginosa xcp* genes involved in elastase, toxin, and phospholipase secretion (13). Moreover, in other reported cases, such as *Pasteurella haemolytica* leucotoxin (40) and *B. pertussis* cyclolysin (16, 25), some secretion genes are located close to the secreted toxin structural gene but are not expressed efficiently in *E. coli*, most probably because of the lack of a positive regulator in *E. coli* (21, 31).

As we proposed above, *S. marcescens* must possess analogous protease secretion functions. The absence of secretion in *E. coli* harboring only the cosmid indicated either that the *S. marcescens* secretion genes are not clustered near the *prtSM* structural gene or that the secretion proteins are not expressed or are not functional in *E. coli*.

In conclusion, our use of functional cross-complementation to clone the *prtSM* gene was successful and allowed the study of the protease SM secretion in *E. coli*. In another study, the *S. marcescens* secretion functions will be characterized.

ACKNOWLEDGMENTS

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