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Differential Secretion of Isoforms of *Serratia marcescens* Extracellular Nuclease

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Extracellular secretion of the *Serratia marcescens* nuclease occurs in a two-step process: (i) rapidly to the periplasm via a signal sequence-dependent pathway and then (ii) slowly to the extracellular growth medium without cell lysis. There are two major isoforms of the nuclease in the culture supernatant of *S. marcescens*. We have isolated, purified, and determined the sequences of both isoforms. The first isoform, the mature nuclease (Sm2), is the result of signal sequence processing. The second isoform (Sm1) has three additional amino acids missing from the N terminus of the mature nuclease. Sm1 starts to appear extracellularly only during prolonged growth of a culture (16 to 48 h), probably because of cell lysis. However, pulse-chase experiments show that it is made early with Sm2 but is not secreted efficiently.

Serratia marcescens is an evolutionarily adept gram-negative enteric bacterium. It lives in diverse habitats, including soil, water, and on plants, and it also is an insect, fungal, and opportunistic human pathogen (14, 36, 41, 42). It is a prolific producer of extracellular proteins that allow exploitation of its habitats for nutrients (7, 11, 14, 24) and that are important for colonization and invasion (14, 24, 27).

Extracellular nuclease is one of the proteins found in the growth medium of *S. marcescens* (11). It has potent nucleolytic activity on nucleic acids without a strong preference for type, composition, or length and is equally active on both DNA and RNA (13, 29). Nuclease is synthesized as a higher-molecular-weight precursor protein containing a typical N-terminal signal sequence (3, 6, 37). Extracellular secretion of nuclease occurs in a two-step process, first to the periplasm and then to the growth medium of *S. marcescens* in a very slow (30 to 60 min) second step (37). The signal sequence is processed from nuclease upon translocation across the inner membrane to the periplasm in both *S. marcescens* and *Escherichia coli* (4, 37). However, efficient extracellular secretion into the medium only occurs from *S. marcescens*, suggesting that nuclease requires host-specific gene products to complete the extracellular secretion step (4, 37). One such gene has recently been identified (17a).

There are two major nuclease isoforms produced from the *nucA* gene by *S. marcescens* (12), and we have isolated and purified both isoforms from the culture supernatant of strain SM6. One is the mature nuclease (Sm2) formed after the signal sequence is processed, and the second is an isoform of nuclease (Sm1) which has had an additional three amino acids deleted from the N terminus. The Sm1 and Sm2 nomenclature is derived from the elution order of these isoforms after anion-exchange chromatography (12). With these purified isoforms, kinetic studies have shown that the enzymatic properties of both isoforms are virtually identical (13).

Here, we report that nuclease isoforms differ in secretion properties. Nuclease isoforms differentially appear in the culture supernatant, with Sm1 appearing only very late during the growth of a culture. Sm1, however, is made early along with Sm2, but it is not efficiently secreted. The appearance of Sm1 accompanies the release of cellular proteins, suggesting that nonspecific cell lysis is responsible for its release into the growth medium. Mutational studies of the amino acids differing between isoforms suggest that the N terminus of the mature nuclease plays a role in secretion.

MATERIALS AND METHODS

Strains and plasmids. *S. marcescens* W1050 (43, 44) is a nuclease-overexpressing strain isolated after chemical mutagenesis of wild-type strain SM6(F′ *prolac*). Nuclease overproduction results from derepressed transcription of the nuclease gene *nucA* (9). This is our standard strain for nuclease studies, so that experiments can be performed in the absence of SOS-inducing agents. Strain SMB104 is W1050Δ*nucA*, created by recombination with a plasmid carrying an internal deletion of *nucA* that removes virtually the entire *nucA* coding region (37). This strain makes neither isoform nor does it produce any cross-reacting material detected by nuclease antisera. Strains W1050 and SMB104 still produce extracellular proteases. Strain SMB1066 is a Tn*5*-induced protease-deficient derivative of SM6 (SM6Prt::Tn*5*-A). *E. coli* JM101 (39) was used for routine clonings and the propagation of plasmids.

The plasmid pAC9 (10) was used as a low-copy-number vector. It is a derivative of the p15A origin plasmid pACYC177 (8) and carries the *lacZ*α complementation region and polylinker of pUC9. pAC9Nuc2 contains *nucA* on a 2.1-kb *Eco*RI-*Pst*I fragment and expresses nuclease from the *nucA* promoter. pAC9Nuc4 carries the *nucA* gene on a 1.46-kb *Rsa*I fragment and has had a portion of to within about 50 nucleotides of the promoter's −35 region deleted, removing an upstream activator region (9). Nuclease expression is regulated normally but at a 10-fold lower level than that in pAC9Nuc2. Nuclease expressed from either plasmid is correctly localized in SMB104 (37). Plasmids were introduced into *E. coli* cells by transformation and were introduced into *S. marcescens* by electroporation.

Nuclease was purified from the culture supernatant of SMB1066 carrying the overexpression plasmid pUC19Nuc4O^c as previously described (25).

Media and growth conditions. Cells were routinely grown at 30°C for *S. marcescens* and at 37°C for *E. coli*. For plasmid maintenance, 25 μg of kanamycin per ml was used for *E. coli* and 100 μg of kanamycin per ml was used for *S. marcescens*. LB medium (10 g of yeast extract, 5 g of tryptone, and 10 g of NaCl per liter) was used for the rich medium. For the pulse labeling with [^{35}S]methionine, cells were grown in MOPS (3-*N*-morpholinopropanesulfonic acid)-Gly-AA, which is MOPS minimal medium (28) supplemented with 0.7% glycerol and 20 μg of each of the 19 amino acids except methionine per ml. In both LB medium and minimal medium, strains of *S. marcescens* grow to a density (A_{600}) of >6 to 8. At the culture densities used in this work (A_{600} of between 1 and 3), the cells continue to grow rapidly.

Mutagenesis. Site-directed mutagenesis of the N-terminal amino acids of the mature nuclease was accomplished by the method of Kunkel et al. (18). M13mp19Nuc2 containing the *Eco*RI-*Pst*I clone of *nucA* was used as the template DNA for the mutagenesis. Mutagenic primers were synthesized to generate the following mutations: (i) D1A (Asp at position 1 changed to Ala); (ii) D1K, and (iii) ΔD1-T2-L3 (or Δ1–3), a deletion of the first three residues such that

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                                        ↓Nuclease isoform (Sm1): pI 7.4
                                  ↓Mature nuclease (Sm2): pI 6.6
-7  -6  -5  -4  -3  -2  -1  1   2   3   4   5   6   7   8   9   10  11  12  13  14
TTC GCC GCG CAG GCG TCG GCC GAC ACG CTC GAA TCC ATC GAC AAC TGC GCG GTC GGC TGC CCG
Phe Ala Ala Gln Ala Ser Ala Asp Thr Leu Glu Ser Ile Asp Asn Cys Ala Val Gly Cys Pro
                Mutants
                D1A         Ala.....................................................
                D1K+L3V     Lys.....Val.............................................
                Δ1-3              Glu.....................................................
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FIG. 1. Amino acid sequence around the signal sequence processing site. The nuclease signal sequence is underlined. The Sm2 and Sm1 amino-terminal sequences and respective pI values determined from IEF gels are indicated. Mutants are shown below the wild-type sequence.

only Sm1 is produced. *E. coli* JM101 was transfected with in vitro-synthesized DNA, and a single plaque was isolated for the preparation of single-stranded DNA to verify the mutation by sequence determination by the method of Sanger et al. (34). The D1K change was never found; possibly, the change is detrimental to the cell. Out of 30 independent plaques from the D1K mutagenesis whose sequences were determined, 2 had the desired change at Lys-1 but also carried additional mutations, resulting in the change L3V as well. An *Eco*RI-*Pst*I fragment containing the mutated *nucA* gene was purified and subcloned into pAC9 to produce the pAC9Nuc2 derivative. A 1.5-kb *Sty*I-*Pst*I fragment containing *nucA* from the middle of the signal sequence was purified and ligated with the purified 3.2-kb *Sty*I-*Pst*I fragment from pAC9Nuc4 to produce pAC9Nuc4′, which carries an additional 0.2 kb downstream of the 3′ *Rsa*I of pAC9Nuc4.

SDS-PAGE and immunoblotting. Proteins were precipitated and separated by electrophoresis on a discontinuous 12% polyacrylamide gel (19) or with the Pharmacia Phast gel system and gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), isoelectric focusing (IEF), and PAGE (non-SDS) as stated in the text. Proteins in gels were electroblotted to Immobilon P membrane (Millipore). The membrane was probed with anti-nuclease serum, and alkaline phosphatase-conjugated goat anti-rabbit antibody was used as a second antibody. The signal was developed by the addition of *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt.

Pulse-labeling, cell fractionation, and immunoprecipitation. *S. marcescens* W1050 or SMB104 carrying an appropriate plasmid was grown in MOPS-Gly-AA supplemented with 0.2% maltose from a single colony, and the saturated overnight culture was washed once with fresh medium. Cultures were then diluted 10^{-3}-fold in the same medium and grown further. The culture was pulse-labeled for 1 min with 100 μCi of [^{35}S]methionine at the cell density stated in the text. At the end of the pulse period, unlabeled methionine was added to a concentration of 1 mg/ml, and the chase continued for the time designated. Aliquots were removed at appropriate times and placed into prechilled microcentrifuge tubes, and samples were kept cold afterwards. Cells were pelleted, and the supernatant was saved on ice as the extracellular fraction. After washing of the cell pellet with wash buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl), the periplasmic fraction was prepared by the osmotic shock method of Randall and Hardy (33). The cell pellet was washed again and resuspended in 50 mM Tris (pH 8.0)–1% SDS and incubated at 70°C for 15 min to release the cytoplasmic and membrane proteins as a cellular fraction, followed by a brief sonication to shear the chromosomal DNA. All fractions were adjusted to a final concentration of 150 mM NaCl–1% Triton X-100–0.5% sodium deoxycholate–0.1% SDS–50 mM Tris (pH 8.0), and anti-nuclease and anti-maltose binding protein (MBP [antiserum was purchased from New England Biolabs]) sera were added. After rotation of the tubes at room temperature for 30 min, 60 μg of protein A-conjugated Sepharose CL4B beads per ml was added to all of the tubes. After rotation of the tubes for at least another 2 h at room temperature, the beads were pelleted, washed twice with the wash buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl), resuspended in SDS-PAGE sample buffer, and then incubated at 70°C for 15 min. The immunoprecipitated proteins were separated by SDS-PAGE (12% polyacrylamide) (19), and the dried gel was exposed for autoradiography.

RESULTS

Differential appearance of nuclease isoforms in the culture medium of *S. marcescens*. Nuclease isoforms were isolated from the culture supernatant of protease-deficient SMB1066 carrying the nuclease overexpression plasmid pNuc4O^c (25). Two major nuclease isoforms were purified, their N-terminal residues were determined (C. Y. Yang, Baylor College of Medicine), and their apparent pIs were measured by IEF gels. These values of 7.4 for Sm1 and 6.6 for Sm2 coincide well with the predicted values of 7.0 and 6.7 (Fig. 1). One isoform is the mature nuclease (Sm2) after signal sequence processing, and the second isoform (Sm1) has had an additional three amino acids deleted from the N terminus of the mature nuclease. During the growth of the culture, we noticed a difference in the kinetics of the appearance of the isoforms in the culture medium. Nuclease prepared from the culture supernatant at different incubation times was analyzed by native PAGE (Fig. 2). When a sample was in the early stationary phase (10 h), only Sm2 (lower band) was detected in the supernatant (lane 1). Sm1 (upper band) started to appear when cultures were grown further and is clearly visible after 24 h of growth (lane 3). However, when cultures were incubated for an additional 24 h, Sm1 became the predominant form in the culture supernatant (lane 4).

The second isoform is not processed from the mature nuclease in growth medium. The gradual appearance of Sm1 in the culture supernatant led us to test if proteolytic cleavage of Sm2 in the growth medium is responsible for the production of Sm1. That would be possible since *S. marcescens* is a prolific producer of extracellular proteases (7, 27, 46). The purified mature nuclease was added to filter-sterilized stationary-phase culture supernatant of Nuc$^-$ *S. marcescens* SMB104, and this mixture was then incubated for 10 to 24 h. However, there was no processing of Sm2 to Sm1 during this incubation period (Fig. 2, lane 5).

To confirm that the proteolytic cleavage of Sm2 does not occur in the culture medium, a pulse-chase experiment was performed. *S. marcescens* W1050 was pulse-labeled at an A_{600} of 1.8 for 1 min with [^{35}S]methionine and chased for 1, 10, 24, and 48 h. Extracellular secretion of nuclease occurs slowly within 2 h during this stage of cell growth, as has been previously observed (37). Proteins from the culture supernatant and the remaining cell pellet were analyzed by immunoprecipitation, SDS-PAGE, and autoradiography. Note that on the SDS-PAGE gel, unlike on the previous native gel, Sm2 corresponds to the upper band. The results show that Sm1 started to appear in the culture supernatant by 10 h; however, by that time the periplasmic marker protein (PP) was also found extracellularly, indicating that nonspecific release of periplasmic proteins, perhaps by cell lysis, has occurred (Fig. 3). On the other hand, the amount of Sm2 dramatically decreased during a 10-h chase, suggesting that the protein has been degraded in the culture medium. By 24 and 48 h, Sm1 was the predominant species and was stable during this extended time period.

Taken together with the results in Fig. 2, this finding suggests that Sm2 in the extracellular media does not convert into Sm1; instead Sm1 appears gradually during prolonged incubation with concomitant release of cellular proteins. The loss of Sm2 does not parallel the appearance of Sm1. It would also seem true that the loss of Sm2 requires the presence of intact cells—perhaps cell surface proteases are involved or intracellular proteases are released by cell lysis at these later times. There is no obvious loss of Sm2 when cell supernatants are incubated for prolonged periods as shown in Fig. 1, lane 5.

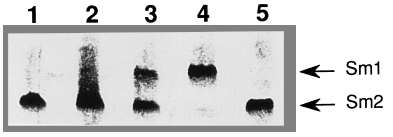

FIG. 2. Differential appearance of extracellular nuclease isoforms in *S. marcescens*. A culture of SMB1066(pUC19Nuc4O^c) (25) cells was diluted 200-fold into fresh LB medium with 1 mg of ampicillin per ml. Samples were taken at the times indicated, concentrated by precipitation, and fractionated by nondenaturing PAGE. By the first time point (10 h), the culture density (A_{600}, ~4) was in early stationary phase; at all subsequent time points, the culture was saturated (A_{600}, ~7). Lanes: 1, 10 h; 2, 16 h; 3, 24 h; 4, 48 h; 5, 10 h of incubation of the purified Sm2 in the cell culture supernatant of a Nuc$^-$ *S. marcescens* stationary-phase culture.

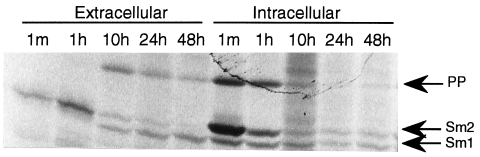

FIG. 3. Pulse-labeling of nuclease in *S. marcescens*. Saturated overnight cultures of W1050 in MOPS-Gly-AA (Materials and Methods) were washed once with fresh medium and then diluted 10^{-3}-fold and grown further. At an A_{600} of 1.8, cultures were pulse-labeled with 100 μCi of [^{35}S]methionine for 1 min and chased for the designated times with excess unlabeled methionine. At each time point, 0.25 ml of cells was taken and proteins from the culture supernatant and remaining cell pellet were immunoprecipitated, fractionated by SDS-PAGE (12% polyacrylamide), and visualized by autoradiography. Sm1 and Sm2 bands are marked by arrows, and the band PP is an unidentified periplasmic protein which we use as a cellular marker (37). It is weakly immunoprecipitated by our anti-nuclease serum but is unrelated to nuclease and is still produced in a *nucA*-deleted strain. m, minute.

Production of isoforms at different stages of *S. marcescens* growth. One thing noticeable in Fig. 3 is that at this culture density, the majority of nuclease produced during the 1-min pulse is Sm2. To investigate the production of each isoform during culture growth, W1050 cells were pulse-labeled at different culture densities. The results show that Sm2 is the predominant product during early exponential growth, but Sm1 appears as the culture reaches saturation until both forms are present at nearly equal levels (Fig. 4).

Sm1 is not efficiently secreted. The fact that Sm1 is synthesized much earlier than it appears extracellularly led us to investigate if differential secretion of isoforms is responsible for the differential appearance of nuclease in the culture medium. W1050 cultures were pulse-labeled at an A_{600} of 2.5 for 1 min and chased for the times indicated. The result in Fig. 5 shows that both isoforms were rapidly secreted into the periplasm in a 1-min pulse; however, only Sm2 is secreted efficiently to the growth medium. Sm1 was not secreted extracellularly during the 2-h chase, by which time all Sm2 in the periplasm had been completely secreted to the medium. Taken together with Fig. 3, our results indicate that the second isoform is made but not secreted efficiently. It is released into the culture medium late in growth concurrently with cellular proteins, indicating that a nonspecific mechanism such as cell lysis is responsible for its appearance in the culture medium.

Effects of mutations in the N terminus of mature nuclease on extracellular secretion. The existence of these two similar isoforms, having very different secretion properties, suggests an important role in the N-terminal residues of nuclease for extracellular secretion. One obvious difference between the two isoforms is the presence of the charged amino acid Asp at the +1 residue of Sm2. In *Pseudomonas aeruginosa*, N-terminal acidic residues in mature exotoxin A protein are known to be important for both efficient signal sequence processing and extracellular secretion of the protein from the periplasm (23).

To investigate the possible role of the N-terminal amino acids in extracellular secretion of nuclease, site-directed mutagenesis was used to replace amino acids in this region. Because this region is the signal processing site, we paid strict attention to introducing appropriate substitutions. To introduce a neutral residue, we replaced Asp with Ala, which is known to be compatible as a signal peptide processing site (20). For the introduction of a positive charge, we selected Lys, which not only is compatible at a processing site but also has a lower pK_a than Arg. However, this mutant was only recovered as the double mutant D1K+L3V. We also deleted all three amino acids from +1 to +3 to produce only Sm1 from the nuclease gene. Cells of strain SMB104 carrying plasmid pAC9Nuc4′, pAC9Nuc4′D1A, pAC9Nuc4′Δ1–3, or pAC9Nuc4′D1K+L3V were grown to the stationary phase in LB medium, and nuclease was monitored by Western blotting (immunoblotting) (data not shown). In all cases, extracellular nuclease was seen, but the D1K mutant made significantly lower levels of nuclease protein.

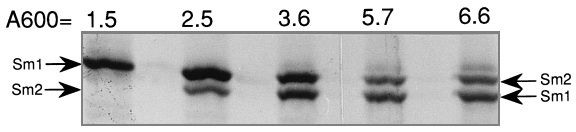

FIG. 4. Production of nuclease isoforms during different stages of cell growth. W1050 cultures were grown as described in the legend to Fig. 3. At the indicated cell density (A_{600}), cells were pulse-labeled with 100 μCi of [^{35}S]methionine per ml for 1 min, and nuclease protein from lysed cells was analyzed as described in the legend to Fig. 3.

To analyze kinetically the ability of *S. marcescens* to process the signal sequence of mutant nucleases and secrete them, we carried out a final pulse-chase experiment. Nuclease produced from a plasmid-carrying *S. marcescens* strain is not secreted as efficiently as nuclease produced from the chromosomal locus, most likely because of tight regulation of secretion and expression of nuclease (37). Nevertheless, pulse-chase experiments with the plasmid-carrying strain still allow the qualitative comparisons of secretion kinetics. SMB104 cells carrying each mutant grown to an A_{600} of 2.0 were pulse-labeled and chased for the indicated times. The results show that, without exception, there is no accumulation of precursor protein in any of the mutants, indicating that the processing of the signal sequence and the secretion across the cytoplasmic membrane were not impaired by the mutational changes (Fig. 6). Both nuclease isoforms are produced from the wild-type, pAC9Nuc4′, and only Sm2 is secreted extracellularly, as is nuclease from the chromosomal locus (Fig. 5 and 6). However, only Sm1 is produced from pAC9Nuc4′Δ1–3, and it is secreted efficiently into the growth medium, without lysis, and with kinetics similar to that of Sm2 from the wild type (Fig. 6). Sm2 and Sm1 from the D1A mutant were incompletely resolved (Fig. 6). However, it appears that only Sm2 is secreted with kinetics similar to that of wild-type nuclease, indicating that this form still has better secretion efficiency than Sm1. When nuclease was produced from the D1K+L3V mutant, both the extent of production and the rate of secretion were significantly lower without affecting the production of extracellular nuclease (Fig. 6). However, because of the altered mobility of this mutant protein, we did not determine whether the extracellular form was in fact Sm2 or Sm1. The results from the Δ1–3 and D1A mutants allow us to conclude that an acidic residue, that of Asp, in the N-terminal region is not critical for nuclease secretion, unlike in the case of exotoxin A (23).

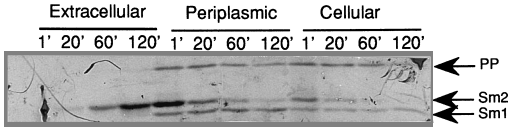

FIG. 5. Sm1 is not secreted efficiently. W1050 cultures were grown as described in the legend to Fig. 3, pulse-labeled at an A_{600} of 2.5 for 1 min with 100 μCi of [^{35}S]methionine per ml, and chased for 20, 60, and 120 min with excess unlabeled methionine. At each time point, 0.25 ml of cells was taken and fractionated into extracellular, periplasmic, and cellular fractions as described in Materials and Methods. All samples were analyzed as described in the legend to Fig. 3.

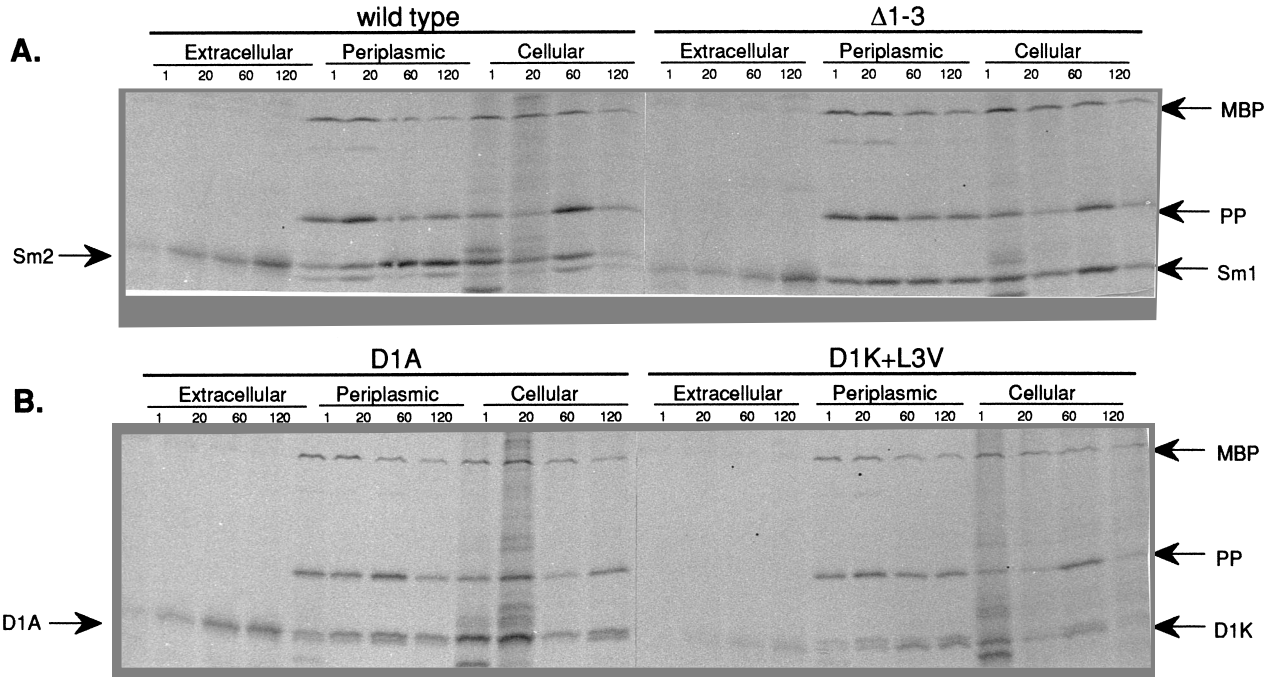

FIG. 6. Kinetic analysis of extracellular secretion of nuclease mutants. SMB104 cells carrying pAC9Nuc4′ and pAC9Nuc4′Δ1–3 (A) and pAC9Nuc4′D1A and pAC9Nuc4′D1K+L3V (B) were grown as described in the legend to Fig. 3, except that MOPS-Gly-AA was supplemented with 0.2% maltose. Cultures were pulse-labeled at an A_{600} of 2.0 for 1 min with 100 μCi of [^{35}S]methionine and chased for 20, 60, and 120 min with an excess amount of unlabeled methionine. At each time point, fractions were prepared and visualized by immunoprecipitation as in Fig. 5. Anti-MBP serum was added to precipitate the periplasmic MBP marker.

DISCUSSION

The general secretory pathway (GSP) has been known to provide the major route for the extracellular secretion of N-terminal signal sequence-bearing proteins produced by gram-negative bacteria (31). This is a two-step process in which the first step is similar to that used by periplasmic and outer membrane proteins, requiring all of the components of the Sec machinery for translocation across the cytoplasmic membrane into the periplasm. The second step of secretion across the outer membrane requires host-specific gene products, which are highly conserved in diverse gram-negative bacteria. Despite a wealth of information about the terminal branch genes of the GSP, the targeting signal and its interaction with and/or recognition by the secretion apparatus are virtually unknown.

Extracellular secretion of the signal sequence-bearing nuclease of *S. marcescens* also occurs in a two-step process via a periplasmic intermediate in *S. marcescens* (37). However, there are several unique features in this process which make it different from the GSP. The periplasmic intermediate of nuclease has an unusually long half-life: 1 to 2 h, depending upon growth conditions. Extracellular secretion of proteins secreted via GSP generally occurs very rapidly within 1 to 15 min (15–17, 23). In fact, the kinetics of secretion of some proteins are so fast that the periplasmic intermediates are not detected under normal growth conditions (15, 22).

In this report, we demonstrate that the Sm2 isoform of nuclease is processed by signal peptidase at the predicted cleavage site, is not converted to Sm1 extracellularly, and is efficiently secreted extracellularly, unlike Sm1. How Sm1 is produced in *S. marcescens* is not known. The two most likely scenarios are that Sm2 is processed by an unidentified periplasmic peptidase(s) in *S. marcescens* to Sm1 or that Sm1 is not derived from Sm2 but instead is a result of signal peptide processing at an alternate site. The recently published crystal structure of the *S. marcescens* nuclease reveals that this portion of the enzyme is readily accessible to external interactions (26) such as protease cleavage. However, this does not rule out alternative signal sequence processing. In fact, we have shown that proteolytic cleavage from Sm2 in the culture medium does not occur.

Our data in this report, showing that Sm2 is secreted better than Sm1, suggest that there is competition between the two nuclease isoforms for recognition by the secretory machinery. When Sm1 is produced alone, it can be secreted as efficiently as Sm2, suggesting that the deletion of three amino acids at the N terminus does not interfere with passage of the protein through the outer membrane. The defect of Sm1 on secretion in the presence of Sm2 is that it may have reduced affinity for the secretion machinery. An alternative explanation is that only protein processed at the normal signal processing site is competent to be secreted. If, as is likely, secretion to the periplasm is linked to the extracellular secretory step, then processing at a secondary site (generation of Sm1) could prevent the second step. Hence, the difference in secretion ability of Sm2 compared with Sm1 is not intrinsic to nuclease or its N terminus but instead is intrinsic to processing of the signal peptide. In this model, the efficient secretion of Δ1–3 occurs because with this mutant the normal nuclease signal peptide is processed, such as in the generation of Sm2.

To further address the role of these N-terminal residues in secretion, mutations were made in this region. In the case of exotoxin A of *P. aeruginosa*, which is known to use the *xcp* terminal branch of the GSP (1, 5, 38), N-terminal acidic residues of the mature exotoxin A protein are known to be important for both processing of signal peptide and extracellular secretion of the toxin (23). However, by changing the negatively charged Asp at the +1 residue of the mature nuclease to

a neutral Ala, we have shown that the negative charge per se is not responsible for the efficient secretion of Sm2.

Strains carrying the D1K+L3V mutant accumulated low but still detectable levels of protein with an apparent molecular weight lower than that of Sm1. There is no accumulation of precursor of this mutant (Fig. 6), suggesting that the position of the signal processing site may have shifted for this mutant or that the mutation dramatically alters the protein's mobility. Because of the low level of production and its aberrant processing, it was hard to conclude what effect this change had on extracellular secretion. This mutation was isolated with difficulty and always carried additional second site mutations, such as the L3V change, suggesting that the D1K change is detrimental to the cells. Exported proteins from bacteria generally do not carry a net positive charge (40) in this region, and the introduction of this positive charge in the first five amino acids of the mature protein may interfere with its secretion across the bacterial cytoplasmic membrane (2, 21, 32, 45). The N terminus of Sm2 has three negatively charged residues within the first seven amino acids, and therefore this change was not expected to interfere with processing and secretion across the inner membrane. However, the +1 Asp-to-Lys change may not be compatible as a signal peptide cleavage site in *S. marcescens*. Sequence comparisons of cleavage sites of many prokaryotic signal sequences have identified a conserved pattern of amino acids at the −1 (Ala, Gly, and Ser) and −3 (Ala, Gly, Ser, Val, and Ile) residue positions (30).

It is tempting to speculate that the second site mutation of +3 Leu to Val may provide a new cleavage site of a nuclease signal peptide in *S. marcescens*. If this is the case, the most likely candidate for the N terminus of this protein would be the +6 Ile of the wild-type Sm2, which would have Ser at the −1 position and Val at the −3 position (Fig. 1). However, this site does not provide the conserved feature of a helix-breaking residue at −4 to −6 (30, 35). This mutant nuclease is active and produces a halo on nuclease indicator medium, indicating that the first disulfide bond which is formed between +9 and +13 Cys is intact. However, to confirm this hypothesis, amino-terminal amino acid sequencing of the mutant would need to be performed.

Given that Sm2 is not efficiently secreted by the cell, why is it made? The fact that it is only released in a nonspecific manner concomitant with cell lysis under the normal growth conditions provides a clue as to its role in *S. marcescens*. It may reduce viscosity caused by chromosomal DNA released from lysed cells, aiding the spread of the remaining viable bacteria, or more importantly, it may provide nutrients by degrading and recycling the DNA, rendering this bacterium more adaptable in diverse environments. The data showing that more Sm1 is produced during the late stage of growth make this plausible.

Nevertheless, these findings not only add information about the differential production and secretion of nuclease isoforms but also support the hypothesis that there might be secretion factors involved in extracellular secretion of nuclease in *S. marcescens.*

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