Two-Step Secretion of the *Serratia marcescens* Extracellular Nuclease

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The extracellular nuclease of *Serratia marcescens* is one of a wide variety of enzymes secreted into the growth medium. Its appearance occurs late in the growth of a culture, and its gene, *nucA*, is transcriptionally regulated in a complex fashion by growth phase and other factors. Pulse-labeling studies reveal that extracellular secretion of nuclease occurs as a two-step process. In the first step, nuclease is rapidly translocated across the cytoplasmic membrane into the periplasm, where it accumulates as a mature active nuclease. A precursor protein, nuclease still carrying its signal sequence, was detected in the presence of carbonyl cyanide *m*-chlorophenylhydrazone or sodium azide, suggesting that this initial translocation and signal processing step involves an energy-dependent and Sec-dependent pathway in *S. marcescens*. The second step of secretion across the outer membrane is a slow process requiring between 30 to 120 min, depending on growth conditions.

Gram-negative bacteria use diverse mechanisms for the secretion of extracellular proteins (33, 44, 47, 54). Proteins with N-terminal signal sequences often utilize the general secretory pathway (GSP) (44). The GSP consists of a two-step process in which the first step is initiated by the signal sequence and its interaction with the Sec proteins to translocate the protein across the cytoplasmic membrane to the periplasm (48). The second step, which involves translocation across the outer membrane to the extracellular medium, requires an accessory secretion apparatus encoded by numerous genes. One of the best-characterized proteins using this system, the pullulanase of *Klebsiella oxytoca*, requires 14 gene products for the second secretion step (44). Components of this secretion pathway are well conserved among gram-negative bacteria, such as *Pseudomonas aeruginosa* (13, 53), *Xanthomonas campestris* (10, 22), *Aeromonas hydrophila* (25), *Erwinia chrysanthemi* (18, 32), and *E. carotovora* (46). However other extracellular proteins are known to utilize mechanisms which differ from that of the GSP, such as the type III secretion systems (47) and the ABC transporter systems (54) as well as some unique systems (38, 49).

Serratia marcescens secretes a variety of enzymes into the extracellular milieu, including a nuclease (3, 11), a phospholipase A (17), a lipase (19), two chitinases (27, 39), proteases (7, 40), and an iron chelator, HasA (30), and it has multiple mechanisms to secrete them. As such, it provides a good model with which to study different pathways of secretion. The extracellular HasA, the metalloprotease, and the lipase of *S. marcescens* utilize ABC transporter systems, a signal sequence-independent secretion pathway analogous to that used for secretion of α -hemolysin in *Escherichia coli* (1, 31, 51). The serine protease from *S. marcescens* is a self-secreting protein that utilizes an N-terminal signal sequence to cross the inner

membrane but mediates its own translocation across the outer membrane by using its carboxy-terminal domain (38). A hemolysin encoded by the *shlA* gene in *S. marcescens* carries an N-terminal signal sequence and requires a single gene product, ShlB, for its activation as well as its release from the cell surface (49). This two-gene system is quite different from the α -hemolysin system of *E. coli* (54). Synthesis and excretion of the phospholipase A require expression of the intact flagellum assembly system, suggesting that phospholipase A may expropriate the flagellum export apparatus for its secretion (16).

The nuclease and chitinases of *S. marcescens* have typical N-terminal signal sequences, but very little is known about the molecular mechanisms involved in their extracellular secretion. The production of these proteins is coordinately regulated in a growth phase-dependent manner; they appear in the medium as the cells begin to enter stationary phase. Both are also regulated by an SOS-like system (5). The extracellular nuclease (NucA) is the major focus of this study. This nuclease has been well characterized biochemically and structurally (15, 37), but little is known about its biosynthesis or export. Here we show that the secretion of this nuclease occurs in two steps. In the first step, nuclease is rapidly translocated across the cytoplasmic membrane to the periplasm via an energy-dependent and Sec-mediated pathway. The second step across the outer membrane occurs in a growth phase-dependent manner. The periplasmic-intermediate form of nuclease has a surprisingly long half-life.

MATERIALS AND METHODS

Bacterial strains and plasmids. SM6 is a wild-type *S. marcescens* strain. Strain W1050 (obtained from U. Winkler) is a derivative of SM6(F' *pro-lac*) isolated after chemical mutagenesis as a nuclease overproducer (55). This mutation is pleiotropic and causes overproduction of a number of extracellular enzymes, including lipase, chitinase, and a bacteriocin. Nuclease overproduction in this strain results from increased transcription of the nuclease gene (9). *S. marcescens* TT392(F' *pro-lac*) (52) is defective for nuclease activity and produces no immunologically detectable nuclease protein. *E. coli* JM101 was used for routine cloning and the propagation of plasmids. The *dut ung* mutant strain CJ236 (29) was used to prepare DNA for site-directed mutagenesis. Plasmids pAC9 and pAC18 are derived from the low-copy-number plasmid pACYC177 and carry the *lacA* cassette of pUC9 or pUC18. They confer kanamycin resistance.

Media and growth conditions. Cultures were grown at 30°C for *S. marcescens* and 37°C for *E. coli*. For plasmid maintenance, the growth medium was supplemented with ampicillin (100 μ g/ml) or kanamycin (25 μ g/ml) for *E. coli* and ampicillin (1 mg/ml) or kanamycin (100 μ g/ml) for *S. marcescens*. LB (10 g of yeast extract, 5 g of tryptone, and 10 g of NaCl per liter) was used as a rich

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medium. DNase test agar (Gibco or Difco) supplemented with 80 μ g of methyl green per liter was used for nuclease indicator plates. Medium used for pulse-labeling with [35 S]methionine was morpholinepropanesulfonic acid (MOPS) minimal medium (41) supplemented with 0.7% glycerol and all amino acids except methionine at 20 μ g/ml (final concentration) (MOPS-glycerol-AA). Medium used in growth experiments was MOPS minimal medium supplemented with glycerol and 0.5% Casamino Acids (MOPS-Glycerol-CAA). Maltose was added to a concentration of 0.2% when needed for induction of maltose-binding protein (MBP) expression. Isopropyl-1-thio- β -D-galactopyranoside (IPTG) at a concentration of 2 mM was added to cultures to induce the *lac* promoter. Experimental cultures were inoculated from overnight cultures which had been centrifuged and washed with fresh medium to remove nuclease or other factors from the spent medium. Optical density measurements were taken at A_{600} ; when necessary, cultures were diluted in medium such that final readings were in the range of 0.2 to 0.8. Cultures of *S. marcescens* reached an A_{600} of 8.0 in standard shake flasks and were in exponential growth until the A_{600} was 2.0 to 3.0.

Enzyme assays. Nuclease activity was measured by the microtiter dish assay (5) and is presented as the dilution factor required to give a loss of fluorescence equal to that of a negative control. Intracellular and extracellular nuclease activity during cell growth was measured by taking a sample at each time point of an experiment. The samples were centrifuged in a microcentrifuge, and the supernatants were saved on ice as the extracellular fraction. The cell pellets were washed once with 10 mM MgSO_4 , resuspended in sonication buffer (50 mM Tris-HCl [pH 8.0], 50 mM NaCl), and sonicated for three cycles of 20 s each on ice followed by 20 s of cooling to prepare the cellular fraction for enzyme measurements. β -Galactosidase activity was measured by the method of Miller (35) and expressed as the ΔA_{420} per minute. β -Lactamase activity was measured at room temperature by the addition of 10 μ l of each sample to 90 μ l of 50 mM potassium phosphate (pH 6.6) containing 0.75 mg of ampicillin per ml. The decrease in A_{240} was monitored by using the kinetics program on a Milton Roy Spectronic 3000 spectrophotometer. Activity is expressed as ΔA_{240} per minute.

Construction of signal peptide hybrids. Plasmid pAC9Nuc14 carries the nuclease gene expressed solely from its own promoter in the kanamycin-resistant low-copy-number plasmid pAC9. Two identical *Ava*I sites were introduced into the nuclease gene of pAC9Nuc14 by site-directed mutagenesis (29). The first is in the spacer between the ribosome binding site and the start codon made by changing four bases; the other is at +4 of the mature nuclease made by a single silent substitution. The double mutant pAC9Nuc14-2Ava expressed significantly less nuclease than its parent. Reconstruction and analysis of the single mutants revealed that only the first *Ava*I mutation resulted in reduced nuclease expression, presumably by altering the spacer region after the ribosome binding site, which reduced the efficiency of translational initiation. This was advantageous for these experiments in that nuclease expression was therefore reduced to below saturation levels for efficient secretion. All of the hybrid constructs carry this same mutation.

Deletion of the region between these two *Ava*I sites creates a unique single *Ava*I site and a gene lacking a start codon and signal peptide (pAC9Nuc14 Δ Ava). This Nuc⁻ derivative was used as a recipient for cloning foreign signal peptides. DNA fragments encoding the signal peptides of the periplasmic MBP and the outer membrane lambda receptor (LamB) were synthesized as overlapping oligonucleotides which were annealed, cleaved by *Ava*I, and cloned into pAC9Nuc14 Δ Ava. Nuc⁺ recombinants were picked from DNase test agar plates, and their DNA sequences were verified. These fusions precisely substituted the foreign signal peptide for that of nuclease except that the -1 residue of nuclease was retained in all cases to preserve the signal peptidase cleavage site.

The β -lactamase fusion plasmid pJBSNuc1-Bla was made by inserting a fragment (*Eco*RI-*Ava*I) carrying the *nucA* promoter and signal sequence up to the second *Ava*I site described above at the +4 residue of mature nuclease into the β -lactamase fusion vector pJBS633 (8), which carries only the mature portion of the β -lactamase gene. Recombinants which expressed β -lactamase were selected, and the junction was sequenced to verify that the fusion was correct.

Pulse-chase experiments. *S. marcescens* W1050 was grown from a single colony in MOPS-Glycerol-AA medium supplemented with 0.2% maltose. Overnight cultures were washed once with fresh medium before inoculation, diluted 1,000-fold in the same medium, and grown. At the appropriate cell density, cells were pulse-labeled for 1 min with 100 μ Ci of [35 S]methionine per ml at 30°C. Following the pulse period, unlabeled methionine was added to a concentration of 1 mg/ml, and the chase was continued at 30°C for various times. To study the effects of inhibitors, cells were pretreated with carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) or sodium azide at various concentrations for 30 s before the 1-min pulse. Cells were removed at appropriate times and placed into prechilled microcentrifuge tubes containing 0.1M potassium cyanide on ice to stop further processing.

Cellular fractionation. The extracellular fraction was obtained following removal of cells by centrifugation in a microcentrifuge, and the supernatant was saved on ice. After the cell pellet was washed with wash buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl), the periplasmic fraction was prepared by the spheroplast shock method of Randall and Hardy (45). The shocked pellet was washed again and resuspended in 50 mM Tris (pH 8.0) containing 1% sodium dodecyl sulfate (SDS) and incubated at 70°C for 15 min to release the cytoplasmic and membrane proteins collectively as the cellular fraction. This preparation was treated by a brief sonication to shear the chromosomal DNA and reduce viscos-

ity. Some of the MBP periplasmic marker protein appeared in this fraction (Fig. 4), suggesting that our periplasmic sample fractionation was not complete. We tested several other methods of preparing the periplasmic contents from *S. marcescens*, such as osmotic shock (42) and polymyxin B treatments (21); however, none significantly improved the efficiency of the periplasmic sample separation. In this study, the method of Randall and Hardy (45) was used in all experiments.

Immunoprecipitation. All fractions were adjusted to a final concentrations of 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris (pH 8.0). Sera (rabbit) prepared against purified nuclease (this laboratory) and MBP (New England Biolabs) were added to all tubes. After slow rotation of the tubes at room temperature for 30 min, protein A-conjugated Sepharose CL4B beads (60 μ g/ml) were added. After rotation of the tubes for 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-polyacrylamide gel electrophoresis (PAGE) sample buffer, and incubated at 70°C for 15 min. The immunoprecipitated proteins were separated on an SDS-12% polyacrylamide gel, and the dried gel was exposed for autoradiography. The intensity of the labeled protein bands was measured with a Tri Microscan gel analysis system.

RESULTS

Growth phase-dependent secretion of nuclease. Transcription of the *nucA* gene in *S. marcescens* is regulated in a growth phase-dependent manner, as was previously shown by monitoring a transcriptional *nucA-lacZ* fusion whose expression increased more than 10-fold during the transition from exponential to early stationary phase (9). To determine whether the secretion of nuclease is similarly regulated by growth phase, the level of intracellular and extracellular nuclease was measured during the growth of wild-type *S. marcescens* SM6 and the nuclease-overproducing strain W1050. When nuclease began to appear during exponential growth of strain W1050, 80 to 90% of the enzyme was found as active nuclease in the cells (Fig. 1A). When the culture reached an A_{600} of >2.5 to 3.0, the intracellular pool of nuclease began decreasing and nearly 75% of the nuclease appeared in the extracellular medium. In contrast, strain SM6 produced much less nuclease at all times (Fig. 1B), and only about 40% of total nuclease was found extracellularly. However, the percentage of extracellular nuclease showed a twofold increase during the same growth period (A_{600} >2.5 to 3.0). These data suggest that secretion of nuclease is regulated by growth phase in *S. marcescens*. Interestingly, strain W1050 not only overproduces nuclease but is more efficient at its secretion.

To demonstrate that the appearance of nuclease in the culture supernatant is not from nonspecific mechanisms such as cell lysis or leakage through the outer membrane, extracellular levels of cytoplasmic and periplasmic marker proteins, β -galactosidase and β -lactamase, respectively, were assayed from W1050(pUC18) in parallel with nuclease. At an A_{600} of 2.8, when the majority (75%) of nuclease was localized in the extracellular media, only 12% of β -galactosidase and 4.0% of β -lactamase were found extracellularly. Throughout the growth of the culture, the bulk of β -galactosidase and β -lactamase remained associated with the cells (Fig. 2); the supernatant fraction never showed more than 18% of the total activity of either enzyme. These results suggest that a specific export system is involved in the extracellular secretion of nuclease in *S. marcescens*.

Identification of the nuclease precursor. Comparison of the predicted amino acid sequence of the *nucA* translation product (3) and the N-terminal amino acid sequence determined for extracellular nuclease (6, 50) suggest that nuclease is synthesized as a precursor containing a typical N-terminal signal sequence of 21 amino acids. The presence of such a signal sequence indicates that the secretion of nuclease might be Sec dependent. To test this hypothesis, and to identify the precursor form of nuclease in vivo, cells were pulse-labeled in the presence of inhibitors which are known to block translocation

FIG. 1. Growth curves and activity of secreted and cell-bound nuclease. Saturated overnight cultures of *S. marcescens* SM6 and W1050 grown in MOPS-Glycerol-CAA were washed once with fresh medium, diluted 1,000-fold (at time zero), and grown further. Growth curves and nuclease assays were performed on fractionated cells as described in Materials and Methods. Note the change in scale between the two panels. The last four samples in panel A were at saturation for the assay.

of proteins across the inner membrane in *E. coli*. CCCP prevents protein export via energy uncoupling (2), whereas sodium azide inhibits the SecA ATPase (14, 43). *S. marcescens* W1050 was used for pulse-labeling experiments because SM6 produces very little nuclease (Fig. 1B) in the absence of SOS-inducing agents.

In the absence of CCCP, two nuclease isoforms were detected in the whole cell fraction (Fig. 3A, lanes 1 and 6), one of which ($M_r = 26,000$) is the mature form of nuclease and the

second of which is the major isoform that has an additional three amino acids deleted from the mature N terminus (12, 50). A band at a higher apparent molecular weight ($M_r = 29,000$), presumably corresponding to the nuclease precursor, appeared when cells were pulse-labeled in the presence of

FIG. 2. Nuclease secretion does not involve cell lysis. *S. marcescens* W1050 carrying plasmid pUC18 was grown in MOPS minimal medium with 0.5 mg of ampicillin per ml. The culture was sampled over time, and levels of enzymes were measured in the culture supernatant or in a sonicated cell extract (9). The percentage of each enzyme found extracellularly relative to the total is plotted against the cell density.

FIG. 3. Identification of the precursor form of nuclease. Cultures of W1050 were grown as described for Fig. 1 but in MOPS-Glycerol-AA medium. At an A_{600} of 1.0, cells were labeled for a 1-min pulse with 100 μ Ci of [35 S]methionine per ml. Samples which had been treated with CCCP or sodium azide had the inhibitor added 30 s before the label. After the pulse, unlabeled methionine was added to 1 mg/ml in the absence or presence of 200 μ g of chloramphenicol per ml. At the end of the indicated chase period, cells were separated from the supernatant, lysed, and immunoprecipitated with antinuclease serum. The immunoprecipitated proteins were separated by SDS-PAGE (12% gel) and visualized after autoradiography. (A) Cells were pretreated with CCCP at the indicated concentrations, and the chase was continued for 10 min. Lanes: 1, untreated; 2, 15 μ M CCCP; 3, 30 μ M CCCP; 4, 45 μ M CCCP; 5, 60 μ M CCCP; 6, untreated but chased in the presence of 200 μ g of chloramphenicol per ml. (B) Cells were pretreated with 5 mM sodium azide and chased as indicated. Samples in lanes 1 to 3 were not treated with sodium azide. Lane 1, no chase; lane 2, 10-min chase; lane 3, 30-min chase. Samples in lanes 4 to 6 were treated with 5 mM sodium azide. Lane 4, no chase; lane 5, 10-min chase; lane 6, 30-min chase. pNuclease, the precursor form of nuclease; mNuclease, the mature form; iNuclease, the isoform.

FIG. 4. Conversion of precursor to mature nuclease after CCCP removal. Pulse-chase experiments were performed as described for Fig. 3. After pretreatment with 60 μ M CCCP for 30 sec, W1050 cells were pulse-labeled for 1 min with [35 S]methionine. After a 10-min chase with excess unlabeled methionine, samples were washed twice with medium to remove the CCCP and resuspended in medium free of the uncoupler. Incubation continued as indicated. Lanes: 1, 10-min chase with CCCP; 2, 10 min after wash; 3, 30 min after wash; 4, 60-min chase with CCCP. Bands are labeled as in Fig. 2. The signal decreases in lanes 3 and 4 because a portion of nuclease was secreted extracellularly during this period and was not recovered.

CCCP (Fig. 3A). The ratio of precursor to processed nuclease increased as the CCCP concentration increased. Little labeling occurred after excess methionine was added for the chase, since lanes 1 and 6 had bands of similar intensities whether or not chloramphenicol was added just after the pulse to block further protein synthesis and incorporation of label.

A 30-s incubation with 5 mM sodium azide prior to pulse-labeling significantly delayed or halted processing of the precursor (Fig. 3B, lane 4 to 6). By 30 min, precursor was not fully processed to the mature form and in fact was significantly degraded instead. In samples not incubated with sodium azide (Fig. 3B, lane 1 to 3), only processed nuclease was observed at the end of a 1-min pulse.

A similar experiment was used to confirm the precursor-product relationship between the putative nuclease precursor and the mature nuclease. Cells were pulse-labeled for 1 min after a 30-s pretreatment with CCCP, chased for 10 min with excess unlabeled methionine and chloramphenicol, and then washed twice to remove the inhibitor. The precursor was processed and was no longer visible within 10 min after the CCCP was removed (Fig. 4, lane 2). However, the precursor band remained throughout a 60-min chase in the presence of inhibitor (Fig. 4, lane 4). These results support the conclusion that the nuclease signal sequence is cleaved in an energy-dependent and presumably SecA-dependent pathway.

Cellular localization of the signal peptide hybrids. The signal sequence is known to be important in initiating the translocation process, and it has also been shown to play a role in modulating folding of the precursor. Such secondary effects of the signal might play a role in extracellular secretion of nuclease. To test this, we made signal sequence substitutions which precisely substituted the nuclease signal with that from the periplasmic MBP or the outer membrane protein LamB. *S. marcescens* TT392(F' *pro-lac*) cells carrying the different plasmids were grown overnight in LB. Supernatants and the sonicated cell fractions were prepared, and nuclease levels measured from each (Table 1). Cell lysis, as monitored by the release of β -galactosidase, was always less than 5%. Immunoblots (not shown) were also prepared with samples from all fractions to confirm that nuclease activity levels corresponded to nuclease protein levels. Nuclease was secreted with similar efficiencies from all hybrids, as judged both by activity and immunologically detectable protein.

Nuclease is secreted in a two-step process. The intracellular nuclease that accumulates in exponentially growing *S. marcescens* cells should be localized primarily to the periplasm or membrane, because it is enzymatically active and its signal sequence has been processed (Fig. 3). Active nuclease contains two disulfide bonds (4, 6) which cannot form in the reducing

TABLE 1. Secretion of nuclease-signal hybrids^a

Plasmid	% Nuclease extracellular	% Cell lysis
pAC9	Undetectable	2.1
pAC9Nuc14-2Ava	64	1.2
pAC9Nuc14-MalE	57	2.0
pAC9Nuc14-LamB	75	1.8

^a *S. marcescens* TT392(F' *pro-lac*) carrying each plasmid was grown in LB medium with kanamycin at 30°C until cells reached stationary phase. Enzyme levels (nuclease or β -galactosidase to monitor lysis) were measured as described in Materials and Methods and expressed as a percentage of extracellular activity relative to total activity.

environment of the cytoplasm and would be lethal if it did. Therefore, any active nuclease must have been translocated out of the cytoplasm.

The pathway and the kinetics of extracellular secretion of nuclease were also investigated by pulse-chase experiments. An exponentially growing culture of strain W1050 at an early stage of growth ($A_{600} = 1.2$) was pulse-labeled with [35 S]methionine for 1 min and chased with excess unlabeled methionine for various times. At this stage of growth, more than 80% of total nuclease is in the cellular fraction (Fig. 1). Figure 5A demonstrates that nuclease is secreted during the 1-min pulse into the periplasm, where it accumulates as mature nuclease. Nuclease is then slowly secreted to the medium over a period of 1 to 2 h, and the pool of periplasmic nuclease decreases. These results agree well with those of the nuclease assay data (Fig. 1). Together, they strongly suggest that nuclease accumu-

FIG. 5. Two-step secretion of 35 S-labeled nuclease. Cultures of strain W1050 were grown as described for Fig. 1 except that MOPS-Glycerol-AA media was supplemented with 0.2% maltose to induce MBP synthesis. At the indicated cell densities, cells were pulse-labeled with 100 μ Ci of [35 S]methionine per ml for 1 min and chased for 20, 60, and 120 min with excess unlabeled methionine. At each time point, samples were taken and fractionated into extracellular, periplasmic, and cellular fractions as described in Materials and Methods. All samples were immunoprecipitated with antinuclease serum and anti-MBP antiserum concurrently, and proteins from the same culture volume were separated by SDS-PAGE (12% gel) and visualized after autoradiography. A representative experiment is shown. (A) Cells were pulse-labeled at an A_{600} of 1.2; (B) cells were pulse-labeled at an A_{600} of 2.3. Bands are labeled as in Fig. 2 except that non-nuclease protein is a periplasmic marker described in the text.

FIG. 6. Kinetic profile of nuclease secretion at different stages of cell growth. Pulse-chase and immunoprecipitation of nuclease from strain W1050 during early ($A_{600} = 0.8$ to 1.2 ; A) and late ($A_{600} = 2.3$ to 2.5 ; B) stages of growth were performed as described for Fig. 5. The intensities of the protein bands in each autoradiogram were quantitated by a Tri Microscan system. At each point, total nuclease was calculated as the sum of intensities of the nuclease band in the extracellular, periplasmic, and cytoplasmic fractions. The percentage of nuclease at each point was calculated by dividing the intensity of the band in each fraction by the sum of all nuclease bands. The percentage of MBP appearing in the extracellular fraction was less than 10% throughout the chase (data not shown). Each point shows the standard deviation of the mean for three independent experiments.

lates in the periplasm before its release into the growth medium.

In the same experiment, the periplasmic MBP was shown to remain in the periplasm (Fig. 5A). Thus, nuclease is not released from the periplasm by cell lysis. MBP is also found in the cytoplasmic fraction because procedures for releasing periplasmic proteins from *S. marcescens* are inefficient. Nuclease seen in the cellular fraction similarly is likely to represent periplasmic or membrane protein. The band labeled non-nuclease protein in Fig. 5A represents an *S. marcescens* periplasmic protein which cross-reacts with antinuclease serum (50) and is found in a *nucA* deletion strain of *S. marcescens* (data not shown). This protein band remained in the cellular and periplasmic fractions and serves to demonstrate that lysis did not occur.

Kinetic comparison of nuclease secretion at different cell densities. Figure 1A shows that nuclease secretion occurred in the late stages of growth of a culture and that nuclease accumulated transiently in the periplasm during exponential growth. We investigated the time course of nuclease secretion during the early and late stages of culture growth to determine if nuclease is secreted faster during the later stages of growth. Samples of strain W1050 were pulse-labeled at an A_{600} of 0.8 to 1.2 or at A_{600} of 2.3 to 2.5 and chased for 1, 20, 60, and 120 min. After cell fractionation and immunoprecipitation, nuclease was visualized after SDS-PAGE and autoradiography as in Fig. 5. The intensity of each band appearing in the autoradiogram was measured by densitometry, and quantitation of each experiment allowed a comparison of the kinetics of nuclease secretion at different culture densities. The first step of secretion from the cytoplasm into the periplasm occurs rapidly within the first minute and is not rate limiting. Figure 5 shows a representative experiment; a compilation of three independent experiments analyzed by densitometry is presented in Fig. 6.

When cells were pulse labeled at an A_{600} of 0.8 to 1.2, 55% of nuclease was found in the total intracellular fraction at 1 min

(Fig. 6A). When cells were pulse-labeled at an A_{600} of 2.3 to 2.5, only 30% of nuclease was in the cellular fraction (Fig. 6B). Only mature nuclease was detected in the cellular fraction, suggesting that the signal sequence was processed completely within the 1-min chase. This nuclease may be cytoplasmic membrane-bound or residual periplasmic nuclease contaminating the cellular fraction. The rate of the second step of secretion differs in cells at the two culture densities. When cells were pulse-labeled at an A_{600} of 0.8 to 1.2, about 70% of the nuclease was found in the medium by 60 min (Fig. 6A), and 50% was secreted by about 40 min. At an A_{600} of 2.3 to 2.5, only 25% of nuclease was extracellular by 60 min, and 50% secretion was reached at about 90 min (Fig. 6B). However, much more nuclease is made at this time point (Fig. 1). At both cell densities, the level of extracellular nuclease increased as the level of periplasmic nuclease decreased, reaching 70 to 80% extracellular nuclease by the end of the 2-h chase.

Nuclease regulation does not explain its ability to be secreted. An unusual feature of nuclease expression is that it is controlled transcriptionally both at the level of growth phase and also by the SOS system (5) as well as by at least two other systems (9, 26). It remains possible that nuclease secretion is only an apparent by-product of SOS regulation. For example, if a small fraction of cells were fully SOS induced and expressed a high level of nuclease, they likely would undergo cell lysis and release nuclease extracellularly. Hence, nuclease would appear to be secreted efficiently because it is made only in this small fraction of cells, whereas other markers would appear not to be secreted because the bulk of the cells which express those marker proteins do not undergo lysis. To investigate this further, we constructed a hybrid (pJBSNuc1-Bla) which expresses the mature periplasmic protein β -lactamase using the nuclease signal peptide. This hybrid protein is transcribed from the nuclease promoter and, like nuclease, is growth phase and SOS regulated. This plasmid was introduced into wild-type Nuc⁺ *S. marcescens* strain SM6, and the levels of extracellular nuclease made from the normal chromosomal

locus as well as β -lactamase made from the nuclease promoter-signal peptide fusion were measured after overnight growth of the culture. Nuclease was secreted efficiently, with 80% found extracellularly, whereas just under 10% of the total β -lactamase activity expressed from the nuclease- β -lactamase fusion was found extracellularly. Therefore, nuclease secretion is not a by-product of SOS regulation in these cells because both proteins were expressed coordinately from the same promoter and utilized the same signal peptide.

DISCUSSION

The pathways used by bacteria to release proteins in the extracellular milieu are complex and varied. Here we have analyzed the path of nuclease secretion by cellular fractionations and pulse-chase experiments and shown that it occurs in two steps. In the first step, nuclease is translocated across the inner membrane, and the signal sequence is rapidly processed. The mature nuclease accumulates in the periplasm. The second step of secretion, from the periplasm to the growth medium, occurs slowly over a period of 1 to 2 hours, and the pool of periplasmic nuclease concomitantly decreases. The presence of periplasmic nuclease during secretion is not due to aberrant compartmentalization of excess nuclease overproduced by strain W1050, since we could also recover nuclease from the periplasm of wild-type strain SM6 (Fig. 1B). The release of nuclease to the medium without the concurrent release of periplasmic β -lactamase and cytoplasmic β -galactosidase also demonstrates that an export system rather than cell lysis or nonspecific leakage is responsible for the secretion. Equally important, we demonstrated that secretion of normal nuclease is not an indirect effect due to its mode of regulation, by the SOS system, for example. When β -lactamase was expressed under identical conditions, it was not found extracellularly. We therefore conclude that information for extracellular targeting of the *S. marcescens* nuclease does not reside in the signal peptide and that specificity most likely resides in the mature protein itself, presumably interacting with secretory proteins.

Pulse-chase experiments in the presence of CCCP or sodium azide allowed detection of a precursor of nuclease inside the cells. This precursor was completely chased to mature nuclease upon removal of the energy poison. This result suggests that nuclease initially is synthesized as a precursor with a signal sequence and then is translocated across the inner membrane in an energy-dependent and Sec-dependent pathway. The conclusion that the N-terminal signal sequence functions only in export from the cytoplasm is supported by signal peptide substitution experiments showing that nuclease hybrids containing signal peptides from *E. coli* envelope proteins are secreted as efficiently as wild-type nuclease.

The profile of the expression and localization of nuclease in *S. marcescens* depends on the cell physiology. The total level of nuclease expression and the proportion of nuclease that remains cell bound are different under different growth conditions. However, conditions under which nuclease expression increases, such as growth in minimal medium, always promote more efficient secretion of nuclease (51a).

Nuclease is actually secreted faster during the early stages of growth than in the later stages. Nuclease remained in the periplasm for 30 to 60 min before it was released into the growth medium when cells were pulse-labeled during the exponential phase. When cells were pulsed during the early stationary phase, nuclease remained in the periplasm for 90 to 120 min before secretion. Yet the static picture seen in Fig. 1 suggests that most nuclease is in the periplasm during early stages of growth. These observations can be reconciled by

considering the dramatic increase in total nuclease expression during this period, resulting in a large accumulation of nuclease which could transiently overwhelm the secretory system at this later time point. The amount of cell-bound nuclease found at an A_{600} of 1.2 of Fig. 1 is in fact similar to the amount of extracellular nuclease found 90 min later.

Secretion functions do seem to be transiently saturable for nuclease in *S. marcescens*, as demonstrated both by the difference in behavior of SM6 and W1050 and by the saturation of secretion observed when nuclease is overexpressed from plasmids (50). Since the wild-type strain SM6 produces a low level of nuclease and secretes it inefficiently, whereas the pleiotropic mutant W1050 produces more nuclease (9) and secretes it more efficiently, it is reasonable to propose that components of the secretion machinery are coordinately overexpressed with the nuclease structural gene (*nucA*) in strain W1050. Additionally, the accumulation of nuclease in the periplasm during exponential growth may reflect the time required for some step in nuclease secretion.

The accumulation of nuclease in the periplasm before its release into the growth medium makes nuclease different from other extracellular proteins secreted via the GSP found in many gram-negative bacteria (44). These proteins do not normally accumulate in the periplasm prior to release. Unlike other gram-negative bacteria which are known to possess the GSP, no pleiotropic extracellular-export-deficient mutants have been isolated in *S. marcescens* (20, 44). A newly identified putative secretory protein for nuclease does not have any significant homology to other known secretory proteins in GSP, but clearly its existence supports the notion that a specific mechanism is involved in the extracellular secretion of nuclease (26).

Generally, the kinetics of extracellular protein secretion are relatively fast. It takes 1 to 15 min for the proteins to be secreted via the GSP, and secretion is sometimes too fast for detection of the periplasmic intermediates under normal growth conditions (34). Similar kinetics are observed for extracellular export of α -hemolysin, which is secreted in a signal sequence-independent, one-step process (28). The assembly and secretion of P pilin on the surface of the outer membrane of pyelonephritic *E. coli*, which are mediated by the action of a periplasmic chaperone, are completed in less than 5 min (24). Unlike these proteins, nuclease experiences a lag period between its synthesis and extracellular secretion, and the rate-limiting step for secretion is not its synthesis or export across the cytoplasmic membrane but rather some events occurring in the periplasm. This is similar to, albeit slower than, the time required for the extracellular secretion step of cholera toxin from *Vibrio cholerae* (21). Our data, in fact, suggest that during the late stages of growth, when extracellular secretion of nuclease appears, cells are preferentially secreting nuclease which was made earlier over newly synthesized nuclease which remains in the periplasm after pulse-labeling.

The unusually long half-life of periplasmic nuclease suggests that some steps may be occurring in that compartment before secretion into the medium. Such steps could involve (i) differential folding or dimerization, since nuclease is known to exist as a dimer (36), but the rate of dimer formation or its necessity for secretion are uncertain; (ii) intra- or intermolecular interaction with chaperone proteins (there may be periplasmic molecular chaperones in *S. marcescens* like those involved in the secretion and assembly of pilin [23, 24] that bind tightly to folded periplasmic pilin intermediates that are in, or close to, their final conformation and lead them to the outer membrane secretion machinery; a periplasmic nuclease associated with a chaperone or other factor may arrive at the site of transport

across the outer membrane and be held in the order of synthesis until they can be transported sequentially when the translocation machinery across the outer membrane is assembled, and in this way, the secretion machinery could distinguish nuclease made early from nuclease made later; or (iii) modification or assembly of the secretion machinery itself. However, these are all speculative suggestions in the case of nuclease.

In summary, the two-step secretion of nuclease is reminiscent of the secretion pathways of many extracellular proteins. But when nuclease secretion is examined in more detail, its route and the mechanism appear to be different from any described so far, especially with the highly conserved two-step mechanism of the GSP.

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