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Secretion of Nuclease across the Outer Membrane of *Serratia marcescens* and Its Energy Requirements

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Extracellular secretion of *Serratia marcescens* nuclease occurs as a two-step process via a periplasmic intermediate. Unlike other extracellular proteins secreted by gram-negative bacteria by the general secretory pathway, nuclease accumulates in the periplasm in its active form for an unusually long time before its export into the growth medium. The energy requirements for extracellular secretion of nuclease from the periplasm were investigated. Our results suggest that the second step of secretion across the outer membrane is dependent upon the external pH; acidic pH effectively but reversibly blocks extracellular secretion. However, electrochemical proton gradient, and possibly ATP hydrolysis, are not required for this step. We suggest that nuclease uses a novel mechanism for the second step of secretion in *S. marcescens*.

Extracellular secretion of N-terminal signal sequence-bearing proteins by gram-negative bacteria often occurs by the general secretory pathway (GSP) (36). It is a two-step process involving a periplasmic intermediate en route to the extracellular milieu. ATP (or GTP) and an energized membrane are required for translocation across bacterial, mitochondrial, and chloroplast membranes (11, 14, 45). Some aspects of this process for which an energy requirement has been implicated are (i) maintenance of proteins competent for translocation, (ii) binding to the membrane receptor of the translocation machinery, (iii) the driving force for protein movement, and (iv) unidirectional translocation (12, 14, 32, 42, 45). The available data indicate that the requirement for the energization of the membrane is independent of the requirement for ATP. Often, distinct energy requirements for translocation reflect mechanistically different translocators involved in the process (10, 16, 40, 43).

In bacteria, the initiation of translocation absolutely requires ATP hydrolysis by the SecA ATPase, whereas the ΔP, the proton motive force across the cytoplasmic membrane, acts in part during the latter stage of preprotein translocation (14, 42). SecD and SecF, components of the translocation machinery which are known to act at a later stage of translocation in *Escherichia coli* (6, 29), play a role in ΔP-dependent translocation step but not the ATP-dependent step (2).

Current genetic and biochemical analysis of conserved components of the GSP has revealed at least one inner membrane protein which contains a consensus ATP binding motif and several inner membrane proteins specifically involved in the second step of secretion across the outer membrane (36, 41). It has been suggested that the function of these inner membrane proteins is to couple the energy of ATP hydrolysis and/or ΔP across the inner membrane to the translocation of proteins across the outer membrane (1, 21, 39). Supporting this hypothesis is the observation that the second step of aerolysin secretion across the outer membrane of *Aeromonas salmonicida*, which is known to use the *exe* terminal branch of the GSP (18), is blocked by dissipation of ΔP and low pH of the growth medium (52).

Proteins do not cross the inner membrane in a fully folded conformation. However, the periplasm provides an oxidizing environment which favors formation of disulfide bonds and also contains molecules that facilitate the folding and subunit assembly of nascent proteins (5, 19, 24, 28). It has been shown that only disulfide-bonded and fully assembled proteins, rather than an unfolded polypeptide chain, are translocated across the outer membrane of gram-negative bacteria (7, 17, 19, 33, 35, 53).

The slow secretion by *Serratia marcescens* of its extracellular nuclease provides a good opportunity for experimentally dissecting the energy requirements of the first and second steps of secretion of this protein. Nuclease is synthesized with an N-terminal signal sequence to initiate secretion across the cytoplasmic membrane into the periplasm. This step is blocked by the pretranslational addition of CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) or sodium azide, suggesting the requirement for the electrochemical proton gradient and the involvement of SecA in this process. Once nuclease crosses the inner membrane, it remains in the periplasm as active, processed mature nuclease for an unusually long time and is slowly released across the outer membrane, without cell lysis, over a period of up to 1 to 2 h (47).

In this report, we demonstrate the dependence of nuclease secretion from the periplasm upon the external pH, similar to that of aerolysin secretion by *A. salmonicida* (52). More importantly, our results suggest that the electrochemical proton gradient across the inner membrane and probably ATP hydrolysis are not coupled to nuclease secretion across the outer membrane. These results support our hypothesis (9, 46, 47) that extracellular secretion of nuclease by *S. marcescens* uses a novel mechanism which is different from the known terminal steps of the GSP.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Strain SM6 is a wild-type *S. marcescens* strain. W1050 (obtained from U. Winkler) is a derivative of SM6 (F′ *pro lac*) isolated as a nuclease overproducer after chemical mutagenesis (51). It carries a pleiotropic mutation that causes overproduction of other extracellular enzymatic activities as well. Nuclease overproduction has been shown to result from increased transcription of the *nucA* gene (8) in this strain.

Cells were grown routinely at 30°C. The medium used for pulse-labeling with

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[^{35}S]methionine was the MOPS (morpholinepropanesulfonic acid) minimal medium of Neidhardt (31) at pH 7.4 supplemented with 0.7% glycerol and 20 μg of each amino acid except methionine per ml (MOPS-Gly-AA). The medium used for other experiments was MOPS-Gly-CAA, which is MOPS minimal medium at pH 7.4 supplemented with 0.7% glycerol and 0.5% Casamino Acids. For pH experiments, MOPS minimal medium was adjusted with KOH to pH 7.4, 6.6, 5.2, or 4.6. Experimental cultures were inoculated from fresh overnight cultures which had been washed with fresh medium to remove nuclease and other factors from the inoculum. Chloramphenicol was used at 200 μg/ml, a concentration which we have previously shown blocks further protein synthesis (46).

Nuclease analysis. For cellular localization of nuclease, a culture was grown at 30°C in MOPS-Gly-AA from a single colony, and the saturated overnight culture was washed once with fresh medium before inoculation. The culture was then diluted 1,000-fold in the same medium and grown further. At an A_{600} of 2.8, the cells were centrifuged to remove extracellular nuclease, washed once, and resuspended in sonication buffer. Whole cells were sonicated as described below and designated the whole-cell fraction. The periplasmic fraction was prepared by the method of Randall and Hardy (38). The remaining shocked cells were washed once and resuspended in sonication buffer and then sonicated to release cell bound nuclease (cytoplasmic fraction). To half of the sonicated shocked cells, Triton X-100 was added to 0.1%; this mixture was placed on ice for 10 min to release membrane-bound nuclease (cytoplasmic plus membrane fraction). Equivalent sample volumes were measured for nuclease activity by the microtiter dish assay (4). Nuclease activities are presented as the dilution factor required to show no loss of fluorescence. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting, and immunoprecipitation of pulse-labeled proteins were all performed as described previously (46, 47).

Steady-state secretion experiments. The effect of external pH was tested by transferring cells into fresh medium at each different pH. The cells were taken in the late exponential phase of growth at an A_{600} of 2.5 (*S. marcescens* routinely grows to an A_{600} of ≈8), washed once, and resuspended at each pH in fresh medium containing chloramphenicol to block de novo synthesis of protein. The cells were then incubated further at 30°C. Samples were taken at appropriate time points, and cells were separated from the supernatant by centrifugation. The supernatant was saved on ice as the extracellular fraction. The remaining cell pellets were washed, resuspended, and sonicated on ice to release intracellular nuclease as described previously (46). To confirm that most of the intracellular nuclease reflects the periplasmic intermediate during export, the periplasmic fraction was prepared by the method of Randall and Hardy (38) after blocking protein synthesis. The cell pellet remaining after removal of the periplasm was washed, sonicated, and treated with Triton X-100 at 0.1% to release cytoplasmic membrane-bound protein without affecting nuclease activity. The level of nuclease in each sample was measured by the microtiter dish assay and/or visualized by immunoblotting.

RESULTS

Cellular localization of nuclease. We have previously demonstrated that extracellular secretion of nuclease by *S. marcescens* occurs in a growth phase-dependent manner (46). The enzyme produced during exponential growth accumulated in the cell as active nuclease, and this intracellular pool was secreted into the growth medium during the transition from the exponential to the stationary phase of growth (A_{600}, >2.5). The intracellular nuclease is fully active. Since disulfide bond formation is essential for nuclease activity (3) and occurs in the periplasm and not the cytoplasm (5), the cell-bound enzyme found in exponentially growing cells is presumed to have been exported to the cell envelope (3, 46).

To test whether the active intracellular nuclease recovered from a sonicated cell extract represents periplasmic or membrane-associated active nuclease, a culture of W1050 in the late exponential phase at an A_{600} of 3 was fractionated. The nuclease activity in each fraction was measured by the microtiter dish assay (whole-cell fraction, 81; periplasmic fraction, 81; cytoplasmic fraction, 3; cytoplasmic plus membrane fraction, 3), and protein was visualized by immunoblotting. The results show that nuclease activity and the protein levels in sonicated whole cells (intracellular fraction) are similar to those of the periplasmic fraction, indicating that the majority of intracellular nuclease activity indeed represents the periplasmic pool of nuclease during the export (Fig. 1).

Delayed export of nuclease extracellularly from the periplasm. Growth phase-dependent secretion of nuclease can be observed for the wild-type strain SM6 as well as the nuclease-overexpressing strain W1050 (8, 46). However, our previous experiments demonstrating that nuclease is secreted via a periplasmic intermediate were done only with the latter strain. Is the periplasmic intermediate in fact the normal route for nuclease secretion, or is it an aberrant event arising from the increased expression by strain W1050? Unfortunately, we are unable to directly detect immunoprecipitated pulse-labeled nuclease from strain SM6 due to its low level of expression; therefore, we here demonstrate the delayed secretion of nuclease from this strain by other methods.

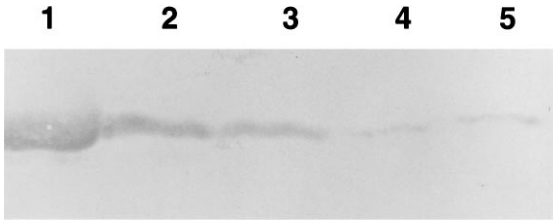

FIG. 1. Cellular localization of nuclease in *S. marcescens*. Samples described in Materials and Methods were analyzed by SDS-PAGE and immunoblotted with nuclease antiserum. For the preparation of the cytoplasmic plus membrane fraction, shocked cells were treated with SDS loading buffer at 70°C. Lanes: 1, purified nuclease; 2, whole-cell fraction; 3, periplasmic fraction; 4, cytoplasmic fraction; 5, cytoplasmic plus membrane fraction.

Cells grown to the late exponential phase at an A_{600} of 2.8 were centrifuged, washed to remove extracellular nuclease, and resuspended in medium with 200 μg of chloramphenicol per ml to block new protein synthesis. Extracellular and periplasmic nuclease activity was then measured at each time point displayed in Fig. 2. The results show that nuclease is slowly secreted from the periplasm into the growth medium and requires about 30 min to achieve secretion of nearly 70%

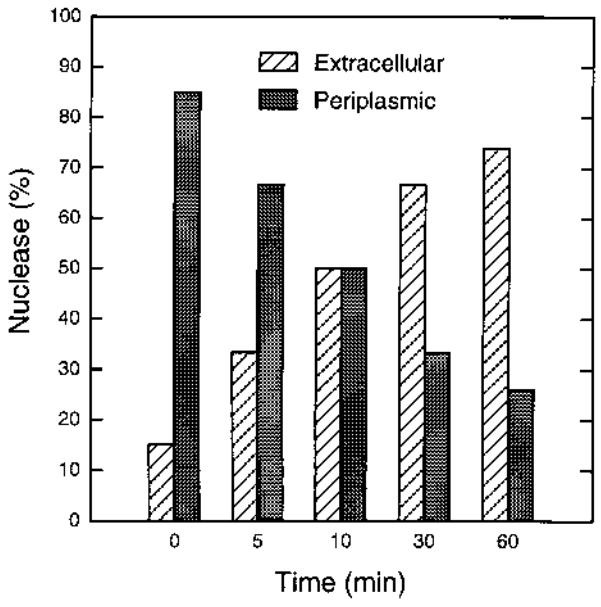

FIG. 2. Extracellular release of nuclease from the periplasm in *S. marcescens*. Strain SM6 was grown at 30°C in MOPS-Gly-CAA as described in Materials and Methods. At a cell density equivalent to an A_{600} of 2.8, cells were centrifuged and washed to remove extracellular nuclease and resuspended in medium containing 200 μg of chloramphenicol per ml and further incubated at 30°C. Samples were taken at designated times, and the nuclease activities in the growth medium (extracellular) and periplasm were measured and are displayed as percentages of the total.

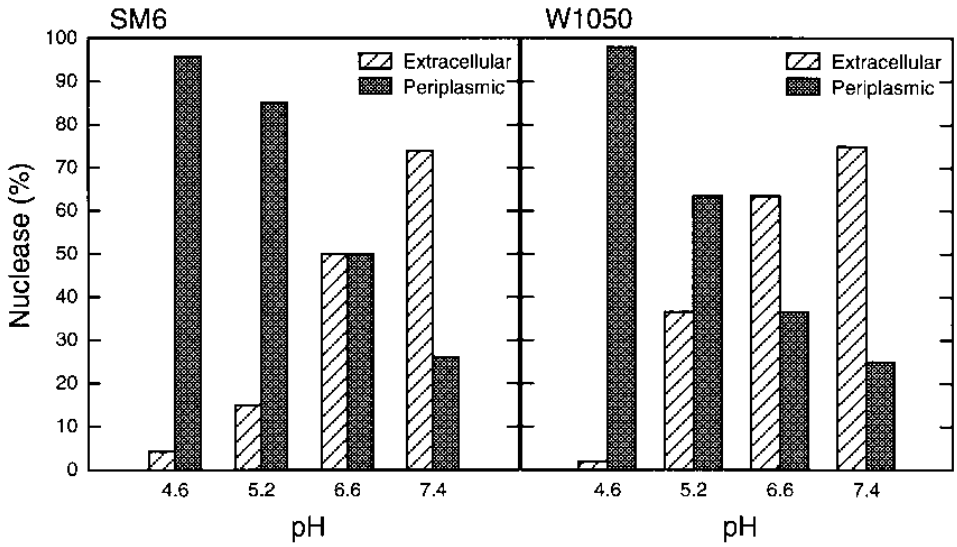

FIG. 3. pH-dependent secretion of nuclease. Strains SM6 and W1050 were grown as described in the legend to Fig. 2. At a cell density of A_{600} of 2.8, cultures were washed once and further incubated at 30°C in the same medium containing 200 μg of chloramphenicol per ml at the different pHs indicated. After an additional incubation of 30 min for SM6 and 10 min for W1050, the nuclease activities of the extracellular and periplasmic fractions were measured.

of total nuclease activity (Fig. 2). Strain W1050 treated under identical conditions secreted a similar percentage of nuclease more rapidly, within 10 min (data not shown). The level of periplasmic activity decreased commensurately with the increase in extracellular activity. These data support our previous findings that nuclease is secreted in a two-step process, the second step of extracellular secretion being relatively slow. Nuclease found in the periplasm is on the normal route for secretion and is not aberrantly shunted there as a result of overexpression in strain W1050 used in previous experiments (46).

The second step of secretion depends upon the pH of the medium. The energy requirement for protein secretion across the outer membrane of gram-negative bacteria has been studied in only a few cases. Alpha-hemolysin of *E. coli* uses a signal sequence-independent pathway, requiring two inner membrane proteins, HlyB and HlyD, and an outer membrane protein, TolC, to cross both membranes in one step. Koronakis et al. (25) have shown that this one-step process requires ΔP, the proton motive force across the cytoplasmic membrane, but only at an early stage. Late stages of secretion do not require total proton motive force nor either of its components, membrane potential or proton gradient. In contrast, the toxin aerolysin of *A. salmonicida*, which is secreted in a two-step process by the GSP system, requires total proton motive force for both steps of secretion (52). However, each of these two different systems shows a marked pH dependence of secretion; extracellular secretion decreases as the pH of the medium is reduced (25, 52).

Taking advantage of the fact that enzymatically active nuclease accumulates in the periplasm before its delayed secretion to the culture supernatant, we were able to probe the energetic requirements for nuclease secretion as well. We have previously shown that the first step of translocation to the periplasm requires proton motive force; nuclease precursor accumulates in the presence of CCCP (46).

To test whether extracellular nuclease secretion is also dependent upon the pH of the medium, secretion across the outer membrane was analyzed at different pHs. Both SM6 and W1050 grown in MOPS-Gly-CAA medium at pH 7.4 to an A_{600} of 2.8 were washed once and transferred to fresh medium containing chloramphenicol at the different pHs indicated in Fig. 3. After additional incubation for 30 min for SM6 or 10 min for W1050 at 30°C, the nuclease activities in the extracellular and periplasmic fractions were measured. Figure 3 shows that in both strains, the efficiency of extracellular secretion decreases as the pH of the medium decreases. The total nuclease activity remained constant regardless of the pH (data not shown). For SM6, nuclease was distributed equally in both fractions at pH 6.6, whereas for W1050, a lower pH was required. At pH 4.6, extracellular secretion of nuclease is almost completely blocked in both strains and release of nuclease is not seen even after 1 h at pH 4.6 (data not shown).

The low pH secretion block is reversible. The effect of low pH should be reversible if it is due to electrochemical perturbations but not if low pH inactivates the secretory system, such as by damaging one or more of its components. To test whether the effect of low pH is reversible as in the case of aerolysin, W1050 cells treated at pH 4.6 for 30 min were washed and resuspended in pH 7.4 medium for an additional 10 min of incubation at 30°C. Extracellular and periplasmic nuclease activities were measured in samples at each pH (Fig. 4). These results show that secretion resumed rapidly, within the 10-min incubation period, after normal pH was restored. The total nuclease activities were similar at both pHs, so the low pH effect is not due to inactivation of the extracellular nuclease.

This was further confirmed by immunoblotting to visualize protein rather than activity. A culture of W1050 treated at pH 4.6 in medium containing chloramphenicol as described above was incubated at 30°C for 10- and 30-min time periods. Protein samples from the extracellular and periplasmic fractions were prepared, and nuclease was visualized by SDS-PAGE and immunoblotting. A control experiment was performed similarly but in pH 7.4 medium. As shown in Fig. 5, no nuclease protein was secreted after 30 min of treatment at pH 4.6. However, nuclease from cells kept in pH 7.4 medium was mostly extracellular within 10 min. Reversibility was again confirmed by returning the cells to pH 7.4 medium. Nuclease which had

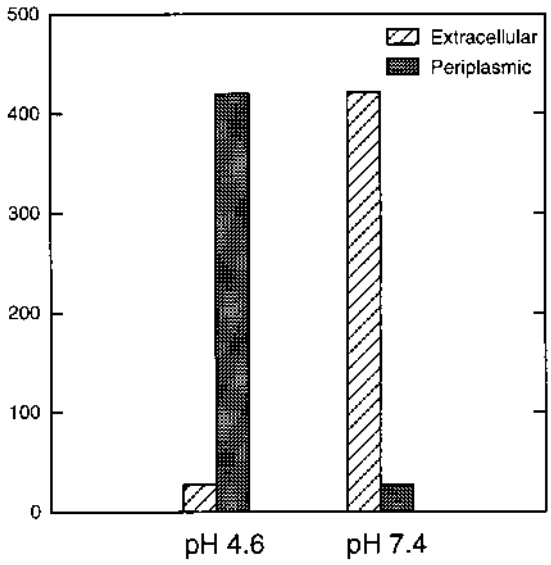

FIG. 4. Reversibility of low-pH secretion block. Strain W1050 was treated as described in the legend to Fig. 2. Thirty minutes after incubation at pH 4.6, a sample was taken. The remaining cells were washed once, resuspended in medium at pH 7.4 containing 200 μg of chloramphenicol per ml, and further incubated at 30°C for 10 min. Nuclease activities in the extracellular and periplasmic fractions of samples from each pH were determined.

remained in the periplasm was now found to be extracellular (Fig. 5, lanes AW).

Proton motive force is not required for nuclease translocation across the outer membrane. Uncouplers such as CCCP, which dissipate the proton motive force across the inner membrane, are known to block protein translocation across the inner membrane as well as signal sequence processing. This is also true for nuclease, for which the addition of 60 μM CCCP 30 s before pulse-labeling blocks the processing of nuclease precursor (46). CCCP has also been shown to disrupt translocation of aerolysin across the outer membrane (52). To test the effect of CCCP on the second step of nuclease secretion, 60 μM CCCP was added after 1 min of pulse-labeling and chased with excess unlabeled methionine and chloramphenicol for the times indicated in Fig. 6. A control was treated identically without CCCP but with the same amount of ethanol used in the CCCP stock solution. Figure 6 shows that the posttranslational presence of CCCP did not inhibit mature nuclease protein from being secreted across the outer membrane into the growth medium. Cell lysis was carefully monitored by coimmunoprecipitation with maltose-binding protein antiserum. The absence of maltose-binding protein and a second periplasmic marker protein (nonnuclease protein) (46, 47) in the extracellular fraction demonstrates that cell lysis was not occurring. Interestingly, in the presence of CCCP, the rate of the second step of secretion appeared to increase. We do not know the reason for this. These data indicate that the second step of nuclease secretion in *S. marcescens* does not require a proton motive force across the inner membrane, and likely not ATP, because when the same concentration of CCCP (60 μM) was added 1 min before pulse-labeling, it completely blocked protein labeling, suggesting that the cellular ATP levels had been fully depleted (data not shown).

Nuclease secretion at alkaline pH. Eaves and Jeffries (13) first suggested that the production of nuclease is growth phase dependent by showing that nuclease accumulates extracellularly during the stationary phase of *S. marcescens* growth. They suggested that extracellular nuclease production was correlated with the rapidly increasing pH of the medium, which in turn is associated with the late log phase. Our data in this paper suggest that extracellular secretion of nuclease is dependent upon the pH of the growth medium and decreases as the pH of the growth medium decreases. To test whether alkaline pH favors the extracellular secretion step, pulse-chase experiments were performed in growth media at different pHs. Cultures grown in pH 7.4 medium were pulse-labeled, washed, and then chased in medium with a pH of 7.4, 8.0, or 8.8. However, there was no significant difference in the rate of nuclease secretion under these conditions (data not shown).

DISCUSSION

Extracellular secretion of nuclease occurs in a two-step process in which nuclease first is rapidly secreted across the cytoplasmic membrane to the periplasm and then, in a second step, crosses the outer membrane slowly (46). In this work, we investigated the energy requirement for secretion of nuclease across the outer membrane of *S. marcescens*. Our results show that this second step is dependent upon the pH of the growth medium; low pH effectively but reversibly blocked periplasmic nuclease from leaving the cells. However, the secretion block by low external pH is independent of proton motive force across the cytoplasmic membrane and the hypothetical proton gradient across the outer membrane (52) since CCCP did not inhibit the second step of secretion across the outer membrane. When CCCP was added 1 min before the pulse-labeling in the same concentration as that added after the pulse-label-

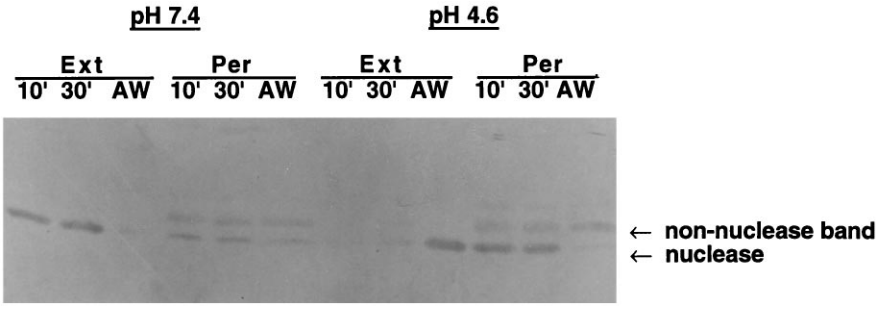

FIG. 5. Immunoblotting of nuclease secreted at pH 7.4 and 4.6. Strain W1050 was grown as described in the legend to Fig. 3. Samples were taken after 10 and 30 min of incubation (lanes 10′ and 30′) from each pH. After 30 min of incubation, the cells were washed again with fresh medium and resuspended in pH 7.4 medium. The cells were further incubated for 10 min after the wash (lanes AW). Samples were fractionated into the extracellular and the periplasmic fractions, and nuclease was visualized by SDS-PAGE and immunoblotting.

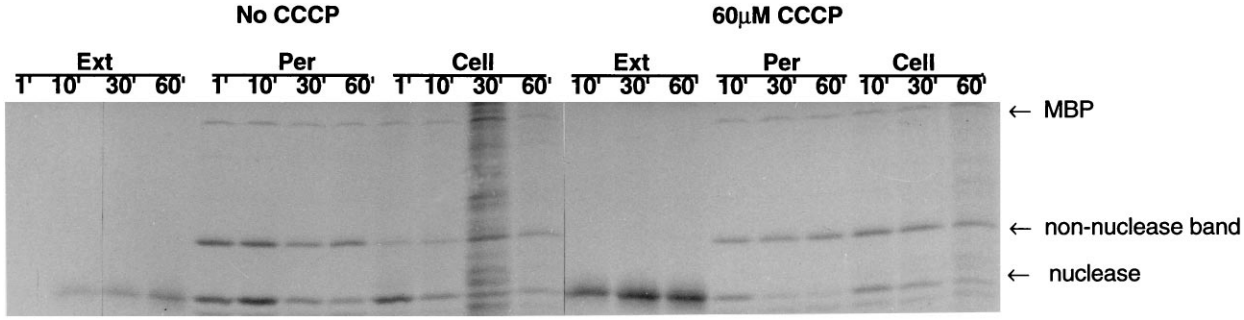

FIG. 6. Effect of CCCP on extracellular secretion. Strain W1050 was grown as described in the legend to Fig. 2 except in MOPS-Gly-AA medium plus 1% maltose. At a cell density of A_{600} of 1.8, cells were pulse-labeled for 1 min with 100 μCi of [^{35}S]methionine per ml. At the end of the pulse period, 1 mg of unlabeled methionine per ml was added. The sample was removed (lanes 1′), and the remaining culture was split. For one sample, 60 μM CCCP was added, and for the other, an ethanol blank was added as a control without CCCP. The chase continued for 10′, 30′, and 60′ (lanes 10′, 30′, and 60′, respectively). Cells were fractionated into supernatant, periplasmic, and remaining cellular fractions and immunoprecipitated as described in Materials and Methods. The immunoprecipitated proteins were separated on SDS–12% PAGE, and the bands were visualized by autoradiography.

ing, it completely blocked protein synthesis, suggesting that it fully depleted cellular ATP. This suggests that nuclease does not require proton motive force, and possibly not ATP, for this step. In light of the fact that the energy sources required for the translocation step differ from the known systems (16, 40, 43), we are left with the thought that nuclease uses a novel mechanism which may be specific for nuclease.

We have previously shown that two-step secretion of *S. marcescens* nuclease is different from the GSP in many ways. Efficient secretion occurs when nuclease is produced only from its native promoter and not from a heterologous promoter such as *lac* (9). The expression of a nuclease amino-terminal fragment, when overexpressed from high-copy-number plasmid, competes specifically and only with nuclease at the cytoplasmic membrane. This competition does not occur in response to overexpression of another secreted protein such as β-lactamase or maltose-binding protein (9). These data suggest that there is a limiting factor(s) essential for nuclease secretion. We are attempting to identify this component(s).

Extracellular secretion of nuclease is also growth phase dependent, occurring at the onset of stationary phase, as are many other extracellular proteins secreted via the GSP (1, 27). However, unlike those proteins which show no saturation of their secretion system during exponential growth even upon overproduction (1, 37), nuclease accumulates in the periplasm for an unusually long time during the exponential growth phase before its release into the growth medium. Our data showing that nuclease does not require proton motive force to cross the outer membrane further supports the hypothesis that nuclease may use a different mechanism than the GSP or a previously unknown variation of the terminal branch of the GSP for its extracellular secretion. As noted by Pugsley (36), no GSP has been reported to date in *S. marcescens.*

The mechanism by which low pH blocks the second step of secretion remains an open question. It could dissipate proton motive force by reducing the proton gradient. However, we have already discarded the necessity of proton motive force by demonstrating that CCCP disruption of proton motive force does not block extracellular secretion. Another possibility is that it may directly affect the translocation of nuclease via changes (i) on some component of the outer membrane such as phospholipids or polysaccharide, altering the physical properties of the outer membrane, (ii) on the net charge of some component of the secretion machinery or even nuclease itself, or (iii) on the nature of some interaction between nuclease and its secretion components. However, these changes must be transient since we have shown that the effect of pH is readily reversible. It is interesting to note that secretion is very rapid upon return of the cells to neutral pH. This may suggest that the slow, essential step occurring in the periplasm can continue to occur at low pH, but a different step, not normally rate limiting, is blocked at low pH. Alternately the low-pH-to-high-pH treatment may somehow trigger more rapid secretion.

The only transmembrane potential known to exist across the bacterial outer membrane is the Donnan potential, which has been suggested to cause a ΔpH across the outer membrane (44) that may regulate the function of a voltage-gated channel; however, nuclease is presumed to be too large (26 kDa) to cross the outer membrane through any porin. Lipids are also reported to be involved in protein secretion in *E. coli* (48); however, the role that lipids play in protein secretion is through their interaction with proteinaceous membrane components of the translocation machinery (26), and protein translocation across the *E. coli* cytoplasmic membrane occurs by an entirely proteinaceous transport mechanism (23). There is a report that lipopolysaccharide is involved in the export process by playing a role in the correct insertion of the outer membrane protein component of translocation machinery rather than by export itself (50).

The diverse mechanisms of extracellular protein secretion used by gram-negative bacteria all involve at least one outer membrane protein (36, 41, 49), even those rare self-secreting extracellular proteins which carry a domain later found in the outer membrane (30, 34). Two lines of evidence suggest that nuclease is not a self-secreting protein. First, when nuclease is produced in *E. coli*, it is primarily periplasmic (3). Second, even in *S. marcescens*, expression of nuclease does not guarantee its secretion into the growth medium; when nuclease is produced from the *lac* promoter, it is found only in the periplasm (9). Nuclease must require some host-encoded factor(s) for efficient secretion. We have described a candidate for one such component which does not have any homology with known secretion machinery elsewhere (22).

Some proteins appear not to require any cellular energy sources for translocation. These proteins are thought to be thermodynamically driven by conformational change either upon binding to or interaction with the translocation machinery (15, 20). Like that of nuclease, the secretion and assembly of P pili, which are assisted by specific periplasmic chaperones and occur via a periplasmic intermediate, have been shown to occur independently of cellular energy. However, the kinetics of assembly and secretion is a very fast process, occurring in

less than 5 min, and is not dependent upon the external pH (20). The unusually long half-life of the periplasmic nuclease species makes it tempting to speculate that essential steps occur in the periplasm as a prerequisite for extracellular secretion. We hope to elucidate these soon.

ACKNOWLEDGMENTS

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