

Pseudomonas aeruginosa Alkaline Protease: Evidence for Secretion Genes and Study of Secretion Mechanism

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A 6.5-kb DNA fragment carrying the functions required for specific secretion of the extracellular alkaline protease produced by *Pseudomonas aeruginosa* was cloned. The whole 6.5-kb DNA fragment was transcribed in one direction and probably carried three genes involved in secretion. The expression in *trans* of these genes, together with the *apr* gene, in *Escherichia coli* allowed synthesis and secretion of the alkaline protease, which was extensively investigated by performing pulse-chase experiments under various conditions. We demonstrated the absence of a precursor form, as well as the independence of alkaline protease translocation from SecA. The absence of secretion genes impaired alkaline protease secretion; the protein then remained intracellular and was partially degraded.

The export and secretion of proteins are fundamental properties of living cells. Secretion can be considered a special case of protein export, in that the final destination is extracellular.

The mechanism of protein secretion in gram-negative bacteria, which involves the crossing of both the cytoplasmic and the outer membranes, is still poorly understood. Several specific and independent pathways appear to be used for the secretion of different proteins in various gram-negative bacteria (28). We studied the alkaline protease (APR) of *Pseudomonas aeruginosa* as an example of a protein secreted by gram-negative bacteria. *P. aeruginosa* produces various extracellular enzymes and toxins, some of which are important in the pathogenicity of *Pseudomonas* infections (20).

APR secretion is known to be different from secretion of the other extracellular proteins in *P. aeruginosa*. Pleiotropic mutations (*xcp*) have defined a general secretion pathway that mediates secretion of several proteins, including elastase, phospholipase, lipase, alkaline phosphatase, and exotoxin A (7, 32). In this system, several genes that are scattered on the *P. aeruginosa* chromosome are involved; none of the *xcp* mutations which have been studied affects APR secretion. These results support the hypothesis that APR has its own specific, independent secretion pathway (7, 17, 32).

We recently described cloning of the APR structural gene (*apr*) (12). We showed that the APR determinant spans approximately 8.8 kb of contiguous DNA which contains all of the information necessary for the synthesis and secretion of active protease when it is expressed in *Escherichia coli* (12). In this study, we found that the information contained in structural gene *apr* is not sufficient to translocate APR across both membranes of the cell envelope. The gene products involved in APR secretion were characterized by using deletion analysis and minicell expression. The conditions of APR synthesis and secretion were extensively investigated. We demonstrated the absence of a precursor form, as well as the independence of APR translocation from SecA. Taken together, our results suggest that the APR

secretion pathway is independent of the general export apparatus involved in targeting of proteins to the envelope in *E. coli*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are listed in Table 1.

E. coli was grown in LB broth and LB agar at 37°C with aeration. Antibiotics were used in selective media at the following concentrations: tetracycline, 20 µg/ml; and ampicillin, 50 µg/ml. To study the secretory phenotype, skim milk agar plates were used (15% skim milk [vol/vol] in tryptic soy agar [Difco]). Genes downstream from the *lac* or *tac* promoters were induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to liquid medium as well as to skim milk agar plates. M9 minimal medium was used for pulse-labeling experiments (22).

Preparation of cell lysates and supernatants. Cells of *E. coli* were harvested by centrifugation, and the culture supernatant was rapidly frozen. The cells were washed in 10 mM Tris hydrochloride (pH 8) and then sonicated on ice by using four 15-s pulses. After centrifugation at 10,000 × *g* for 10 min, the cell lysates were collected and frozen. When necessary, cell lysates and supernatants were concentrated by precipitation with 17% trichloroacetic acid overnight at 4°C. Samples were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

SDS-PAGE and immunoblotting. SDS-PAGE was performed by using the method of Lugtenberg et al. (21). The separating gels contained 11% acrylamide. Purified PAO1 APR was obtained from Nagase Biochemicals, Japan. Antiserum against this protease was prepared as previously described (7). Immunoblotting was done as described elsewhere (7).

Enzyme assays. β-Galactosidase activity was assayed by using the *o*-nitrophenyl-β-galactopyranoside assay described by Miller (23). APR activity was determined by the sensitive assay described by Howe and Iglewski (15), using Hide powder azure (Sigma Chemical Co., St. Louis, Mo.).

DNA procedures. Small-scale purification (culture vol-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype	Source or reference
<i>E. coli</i> strains		
TG1	<i>supE hsdΔ5 Δthi (lac-proAB)</i>	22
AR1062	<i>F⁻ thr leu ara azi fhuA lacY tsx minA gal rpsL xyl mtl thi hsdR</i>	4
MC4100	<i>F⁻ Δ(lac)U169 araD136 rela rpsL thi</i>	22
MM52	MC4100, <i>secA51</i>	25
Plasmids		
pACYC184	<i>Cm^r Tc^r oripP15A</i>	2
pUC19	<i>ColE1 Ap^r lacI φ80dlacZ</i>	33
pJF119	<i>lacI^a rrnB Ap^r (expression vector with tac promoter)</i>	8
pJUEK72	8.8-kb <i>KpnI-EcoRI</i> insert in pUC19	12
pJF2518	2.5-kb <i>EcoRI</i> insert in pJF119	This study ^a
pAGS7	7-kb <i>KpnI</i> insert in pACYC184	This study ^a
pUXE621	<i>KpnI-2-EcoRI-3</i> deletion of pJUEK72	This study ^a
pUXB34	<i>KpnI-2-BamHI-3</i> deletion of pJUEK72	This study ^a
pAGS70	pAGS7 with interposon Ω-Hg insertion into <i>BamHI</i>	This study ^a
pJUE25	2.5-kb <i>EcoRI</i> insert in pUC19	This study

^a See Fig. 1.

umes, 1 to 10 ml) of plasmid DNA from *E. coli* was performed by using a modification of the rapid boiling method (14). Large-scale purification was carried out by using the method of Birnboim and Doly (1), followed by cesium chloride gradient centrifugation (22). Restriction endonucleases, T4 DNA ligase, and bovine intestinal phosphatase were used as recommended by the manufacturer (Boehringer GmbH, Mannheim, Federal Republic of Germany). Plasmids were introduced into calcium chloride-treated *E. coli* cells (18). Gel electrophoresis in 0.6% agarose was performed by using Tris-borate-EDTA buffer (22).

Identification of plasmid-encoded proteins. Minicells were isolated from an overnight culture of *E. coli* AR1062 which had been transformed with the appropriate plasmid. These minicells were separated from nucleated cells by two cycles of glycerol gradient (10 to 30%) centrifugation. Purified minicells were suspended in M63-glucose medium containing IPTG, (final concentration, 2 mM) and incubated at 37°C. In control cultures, the IPTG was omitted. Labeling was carried out by using 50 μCi of L-[³⁵S]methionine per ml (1,200 Ci/mmol; Amersham) in the presence of 0.5% methionine assay medium (Difco). The minicells were sedimented, suspended in 30 μl of sample buffer, boiled for 5 min, and then subjected to SDS-PAGE.

Construction of recombinant plasmid pAGS7. Recombinant plasmid pAGS7 was generated in two steps. First, a 1.4-kb *PvuII-EcoRI* fragment from plasmid pJF119 was subcloned in pACYC184 (*ScaI* and *EcoRI* sites). This fragment carried the *tac* promoter and the multiple cloning sites of pJF119. Second, *KpnI-2-KpnI-3*, which was 7 kb long, was inserted into the *KpnI* site of the multiple cloning sites. In our construction (pAGS7), the orientation of the *KpnI* fragment was such that the *tac* promoter was not used; instead, the *Cm* promoter of pACYC184 functioned to promote expression of *apr* genes.

Pulse-chase experiments. All pulse-labeling experiments were performed at an optical density at 600 nm of 0.8 after growth in M9 minimal medium in the presence of 0.5% methionine assay medium (Difco). Under these conditions the bacteria were in early-log-phase growth at this optical density. Induction with 1 mM IPTG was always performed 10 min before preincubation at 25°C.

After preincubation for 20 min at 25°C, [³⁵S]methionine (200 μCi/ml) was added to small volumes of the cultures. An excess of unlabeled methionine was added after 30 s, and samples were removed at different times (see the figure legends). Each sample was immediately frozen in liquid nitrogen in the presence of chloramphenicol (100 μg/ml). Fractionation of supernatants and cellular extracts was carried out only where mentioned.

Solubilization and immunoprecipitation. The methods used for solubilization and immunoprecipitation were the methods described by Pagés and Bolla (27), with slight modifications. The samples were solubilized at 30°C twice (5 min each), and the preincubation time was 30 min instead of 15 min.

RESULTS

Expression of the *apr* gene in *E. coli*. Previously we have described the cloning of the *apr* gene (12). The smallest plasmid that allowed normal APR synthesis and secretion in *E. coli* carried an 8.8-kb DNA fragment (12) (plasmid pJUEK72) (Fig. 1). On this plasmid, the structural gene *apr* was located on a 2.5-kb fragment (*EcoRI-4-EcoRI-5*) (Fig. 1). There was approximately 6.2 kb between the *tac* promoter, which allowed the expression of all of the genes encoded by this DNA fragment, and the *apr* gene (12). It should be kept in mind that as the *Pseudomonas* promoters functioned very poorly in *E. coli*, we had to subclone every gene which we studied under the control of an expression promoter (12).

Secretion of APR from *E. coli* containing the cloned *apr* determinant was monitored by performing a Western blot analysis. Figure 2 shows a Western blot of whole-cell extracts and culture supernatants expressed from various plasmids in *E. coli* TG1, analyzed by SDS-PAGE, and probed with an anti-APR polyclonal antiserum. As described previously, pJUEK72 allowed synthesis and secretion into the culture medium by *E. coli* (Fig. 2, lane 1S). APR did not accumulate inside the cells to any great extent, at least within the limits of our detection technique (Fig. 2, lane 1C). Enzymatic assays of cell extracts and culture media confirmed these results (Table 2); the protease activity was detected only in the culture supernatants, and no cell-bound activity was detected (Table 2).

In contrast, *E. coli* carrying plasmid pJF2518 did not secrete APR into the supernatant; this was shown by the results of enzymatic assays (Table 2) and immunodetection experiments (Fig. 2, lane 3S). However, no intracellular accumulation of the protease was observed; only a faint band of protease was observed intracellularly in immunodetection experiments (Fig. 2, lane 3C). These assays were carried out under the same conditions that were used for the assays with plasmid pJUEK72 (the same culture turbidity and induction time with IPTG). This absence of intracellular accumulation of protease was later explained by a rapid degradation of cell-bound APR (see Fig. 6).

From the results of this experiment, we concluded that in *E. coli* the APR structural gene by itself (pJF2518) is not sufficient for APR to be secreted into the medium.

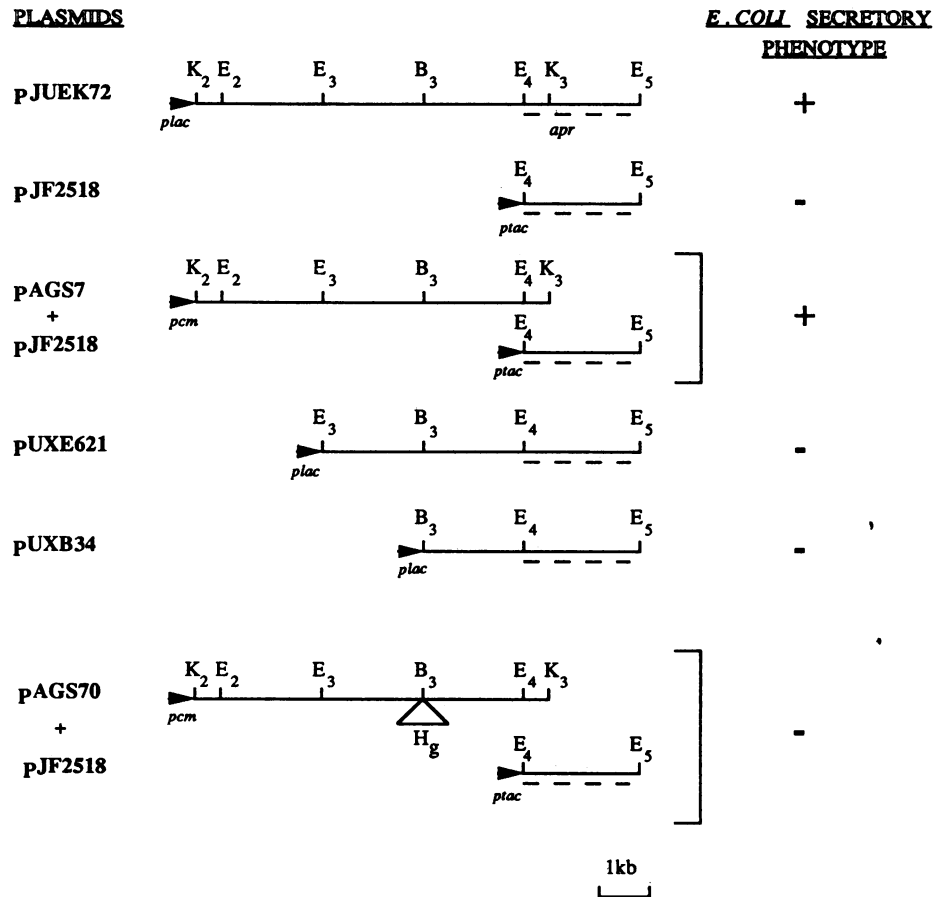


FIG. 1. Restriction endonuclease map of the insert in pJUEK72 (12) and subclones derived from it and the ability of recombinant plasmids to give a secretory phenotype to *E. coli*. The proteolytic phenotype was determined by halo formation on skim milk agar plates. Only pertinent restriction sites are indicated. The nomenclature is from a previous description of the original cosmid isolated by Guzzo et al. (12). Plasmid DNA is represented by solid lines. The arrowheads indicate the direction of transcription from the *lac*, *tac*, and *cm* promoters. The open triangle indicates where the Ω -Hg interposon was inserted. The location of the *apr* gene on the various plasmids is indicated by dashed lines. K, *KpnI*; E, *EcoRI*; B, *BamHI*.

6.2-kb fragment adjacent to the APR structural gene encodes proteins involved in protease secretion. On the basis of the results described above, it is clear that pJUEK72 must carry a gene(s) that encodes determinants of the secretion function(s) and that this gene(s) is operational in an *E. coli* background.

In order to check this hypothesis, *KpnI*-2-*KpnI*-3, the 7-kb fragment carrying the entire DNA sequence adjacent to *apr*, was subcloned into pACYC184 as described in Materials and Methods. In the recombinant plasmid, pAGS7, *KpnI*-2-*KpnI*-3 was inserted under the control of the *Cm* promoter (Fig. 1). When pAGS7 was introduced into *E. coli* cells harboring pJF2518, APR was secreted and was active (Fig. 1). When we measured overnight cultures, large amounts of enzyme were found in the medium (Table 2). Secretion appeared to be specific since no cell lysis was observed (data not shown). The amount of protease synthesized was higher than the amount observed with pJUEK72. This was probably due to the higher copy number of the plasmid and also due to the fact that, in this construction, the *apr* gene was directly under the control of *ptac* and not at the distal end of a long DNA fragment. Only a faint band was

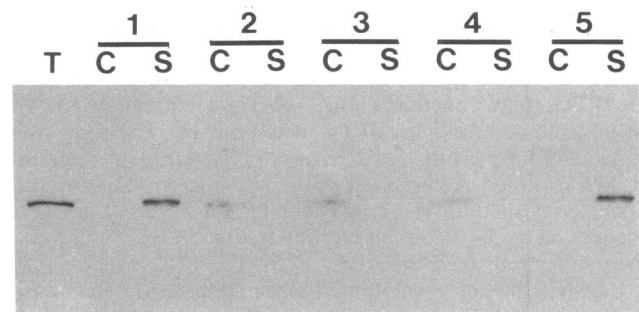


FIG. 2. Immunodetection of APR in culture supernatants and cellular extracts of *E. coli* carrying various plasmids. *E. coli* cultures were grown on LB medium containing 1 mM IPTG. The cells were harvested when they reached an A_{600} of 4. Supernatants and crude extracts were subjected to an SDS-PAGE analysis, transferred to nitrocellulose, and analyzed by immunodetection. S, extracellular medium; C, cellular extract. Lane T contained 100 ng of purified APR. Lanes 1, *E. coli*(pJUEK72); lanes 2, *E. coli*(pUXE621); lanes 3, *E. coli*(pJF2518); lanes 4, *E. coli*(pAGS70); lanes 5, *E. coli*(pAGS7, pJF2518).

TABLE 2. Secretion of APR by *E. coli* TG1 harboring various plasmids^a

Plasmid(s)	Gene(s)	Protease activity (U/ml/OD, 10 ⁻²)	
		Extra-cellular	Intra-cellular
pJUEK72	<i>aprA</i> + secretion gene(s)	ND ^b	30
pJF2518	<i>aprA</i>	ND	ND
pJF2518 + pAGS7	<i>aprA</i> + secretion gene(s)	ND	94

^a *E. coli* TG1 cells harboring various plasmids were grown overnight at 37°C in LB medium containing IPTG. Supernatants and cellular extracts were prepared as described in Materials and Methods. In each case, cell lysis was estimated by using β -galactosidase assays (data not shown).

^b ND, activity not detected within the limits of the bioassay.

detected intracellularly, indicating that secretion was efficient (Fig. 2, lane 5).

Thus, we concluded that pAGS7 encodes at least one diffusible product that is specifically involved in APR secretion.

Deletion analysis and mutagenesis with an interposon were carried out to determine whether all of this DNA fragment encoded genes that were essential for APR secretion. Plasmids were then tested for their ability to promote APR secretion in *E. coli* cells. When plasmid pJUEK72 was truncated (*KpnI*-2-*EcoRI*-3 deletion, plasmid pUXE621 [Fig. 1]), APR was no longer secreted (Fig. 2, lanes 2S and 2C). This result provided evidence that all or part of one gene that is essential for protease secretion is located on *KpnI*-2-*EcoRI*-3. Of course, an extended deletion (pUXB34) (Fig. 1) produced the same result (data not shown).

Interposon Ω -Hg (6) was inserted into the *Bam*HI site of pAGS7 (Fig. 1). It is widely accepted that a transposon insertion has polar effects on genes located downstream from, and in the same operon as, the gene into which it is inserted. This insertion into *Bam*HI completely abolished the ability of pAGS7 to promote protease secretion (Fig. 2, lanes 4S and 4C). Thus, we concluded that all or part of this region of the cloned DNA between *Bam*HI and *KpnI*-3 is also required for protease secretion.

Taken together, all of the results described above showed that two different DNA regions of pAGS7, which are 2 kb apart, are required for protease secretion.

Identification of the products of the genes carried by plasmid pJUEK72. Proteins encoded by plasmid pJUEK72 were analyzed by using the *E. coli* minicell system, which allows specific labeling of plasmid-encoded proteins. Minicells were prepared from strain AR1062 transformed with plasmid pJUEK72 and labeled with [³⁵S]methionine. Proteins were analyzed by SDS-PAGE and autoradiography. After IPTG induction, minicells carrying pJUEK72 expressed four proteins in addition to APR (Fig. 3, lane 2). The molecular weights of these proteins were 54,000, 45,000, 42,000, and 40,000. APR appeared on the gel at a molecular weight of 50,000 (Fig. 3, lane 4); the molecular weight of the purified enzyme was found to be 48,000 (24). The levels of expression of each of the proteins (after induction by IPTG) were not equal; the 42-kDa protein was underexpressed compared with the other three.

When plasmid pJUEK72 was truncated on the left side (*KpnI*-2-*EcoRI*-3 deletion, plasmid pUXE621) the 54-kDa protein disappeared (Fig. 3, lane 3). Despite repeated attempts, we could not identify the proteins which were encoded by pUXB34 and pAGS70 (data not shown). The

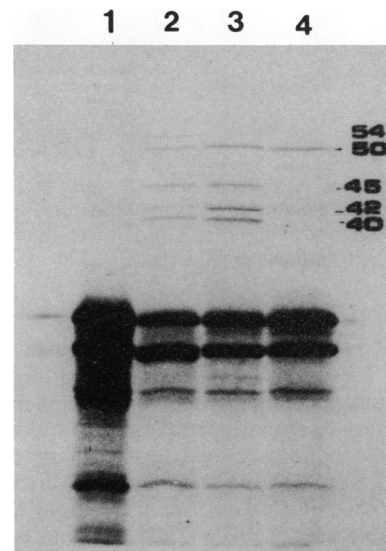


FIG. 3. ³⁵S-labeled proteins synthesized in *E. coli* minicells containing pUC19 (lane 1), pJUEK72 (lane 2), pUXE621 (lane 3), and pJUE25 (lane 4). pJUE25 carried the 2.5-kb *EcoRI* insertion from pJF2518 in pUC19. Minicells were labeled in the presence of 1 mM IPTG. The numbers indicate the positions of molecular mass standards (in kilodaltons).

failure to detect these proteins in minicells may have been due to altered stability of the proteins when they were not produced together.

Export of APR from cells. In order to estimate the time required for export of newly synthesized APR and the amount of APR associated with cells compared with the amount exported, cells were pulse-labeled with [³⁵S]methionine for 30 s and chased for the times indicated in the legend to Fig. 4. Each sample was fractionated into cell pellet and culture supernatant and treated as described in Materials and Methods. At 1 min after the end of the pulse, most of the protease was still within the cells and was the same size as the secreted form (Fig. 4). After 6 min of chase, only a faint band of APR was still detected in the cell extracts (Fig. 4). It should also be noted that the secreted APR was subsequently degraded in the medium (Fig. 4).

Two conclusions were drawn from the results of this experiment. (i) In previous studies, we reported that cell-bound protease could not be detected enzymatically and could be detected only faintly by Western blotting. Labeling of the cells with radioactive amino acids revealed an intracellular protein that could be immunoprecipitated and that was the same size as the mature protein (exoprotein). (ii) There was a slight delay between the appearance of mature APR in the cells and its appearance in the supernatant; the time necessary for the release of one-half of the newly synthesized APR was approximately 2 min.

Search for an intracellular protease precursor form. We first investigated whether APR was synthesized as a precursor with a signal sequence. Signal sequences are present in precursors of nearly all exported proteins and are processed later by signal peptidases with different specificities (29). Repeated experiments in which cells were pulsed briefly (30 s) failed to demonstrate the involvement of a higher-molecular-weight precursor in the synthesis of APR (Fig. 4). It is well known that precursors are very often difficult to identify, even with very short pulse-labeling. In some instances

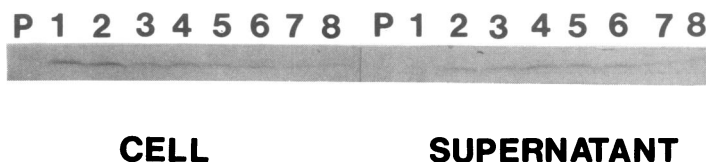


FIG. 4. Export of APR from cells. *E. coli* TG1(pJUEK72) cells were pulse-labeled as described in Materials and Methods with [35 S]methionine (200 μ Ci/ml) for 30 s, and then unlabeled methionine was added to a final concentration of 100 μ g/ml. Samples of the culture were split into cells and supernatants and treated as described in Materials and Methods at the following times after the beginning of the pulse (P): 30 s (lane 1), 60 s (lane 2), 90 s (lane 3), 120 s (lane 4), 150 s (lane 5), 180 s (lane 6), 240 s (lane 7), and 360 s (lane 8).

chemicals are known to inhibit processing of proforms of proteins that are secreted through membranes. Phenethyl alcohol is a local anesthetic that acts on membranes and impairs secretion of several proteins in *E. coli* (13).

Phenethyl alcohol was added to a growing culture of *E. coli*(pJUEK72) at a concentration of 0.5%; this was followed after 3 min by the addition of [35 S]methionine. After 2 min of incubation, the total culture was divided into two parts and immunoprecipitated with various antisera (anti-OmpA and anti-APR). The only form detected for APR was the mature form (Fig. 5A, lane 2). As a control for the efficiency of phenethyl alcohol treatment, we also observed the maturation of OmpA, a major protein of the *E. coli* outer membrane. Under these experimental conditions, most of the OmpA was found in a precursor form (Fig. 5A, lane 4). Thus, we concluded that APR was apparently not synthesized in a precursor form with a cleavable signal sequence.

We also searched for the existence of a proenzyme. Most secreted proteases are synthesized as inactive precursors which are subsequently cleaved to give fully active mature

proteases (31). The size of the propeptide is variable and can be as small as 16 amino acids in the case of *Erwinia chrysanthemi* protease B (3). As explained above, short pulses failed to provide evidence for the existence of a higher-molecular-weight precursor of any kind (Fig. 4). In several cases, cleavage of the propeptide is part of the secretion process itself (31). It has been reported previously that *E. chrysanthemi* proteases B and C are synthesized as zymogens which are secreted into the external medium, where activation occurs by autoproteolytic cleavage (3). The presence of 300 μ M EDTA in the growth medium inhibits this processing (3). APR is a metalloprotease that is inhibited by EDTA (24). We speculated that a proenzyme might be observed if the cells were grown in the presence of 300 μ M EDTA to slow down maturation, as is the case for *Erwinia* proteases (3). *E. coli*(pJUEK72) cells were grown in minimal medium, and EDTA was added 5 min before the pulse. Cells were pulse-labeled (30 s) with 14 C-labeled amino acids and chased. APR synthesized under such conditions was the same size as the extracellular protease (Fig. 5B).

Thus, once again we were unable to detect any precursor form in the cells. Either such a precursor does not exist or its half-life is so short that we missed it. However, it should be kept in mind that maturation of zymogen is a fairly slow process (31).

When the secretion process is inhibited, the cell-bound protease is very unstable. We mentioned above that when APR was synthesized in *E. coli* without any secretion functions [*E. coli*(pJF2518)], only small amounts of protease were detected in cellular extracts, even though *apr* gene expression was controlled by the *tac* promoter (Fig. 2).

By using pulse-chase experiments, we showed that protease was synthesized but was degraded very rapidly within the cells. *E. coli*(pJF2518) cells were pulsed with [35 S]methionine for 30 s and chased for different times (Fig. 6). The maximum level of protease labeling was reached 75 s after the pulse (Fig. 6). This was probably due to the time required for complete elongation of nascent chains (30). Then the level of APR decreased with time, and complete disappearance occurred within 450 s of the chase. The half-life of APR was about 160 s (Fig. 6).

Thus, it appears that in the absence of interactions with the secretion machinery, the protease is degraded.

Secretion process is independent of SecA. Bacterial proteins destined for either the periplasmic or the outer membrane compartment of the *E. coli* envelope possess characteristic N-terminal signal sequences. Besides signal sequences, several other proteins are necessary, which constitute a complex general export system. SecB, GroEL, SecY, and SecA are some of these proteins that have been well characterized (29).

To investigate whether APR uses this general export pathway when it is secreted in *E. coli*, we studied APR

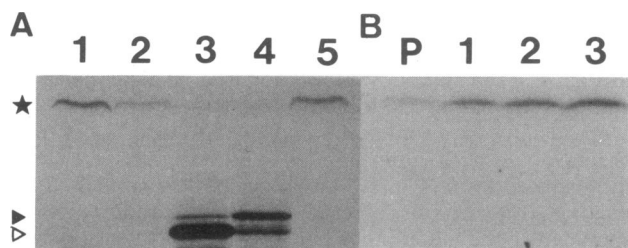


FIG. 5. Search for an intracellular protease precursor form. (A) Treatment with phenethyl alcohol. *E. coli* TG1(pJUEK72) was grown at 37°C on M9 minimal medium until the early logarithmic phase. After induction with 1 mM IPTG for 30 min, phenethyl alcohol was added at a final concentration of 0.5%. At 3 min after phenethyl alcohol addition, the cells were labeled with [35 S]methionine (50 μ Ci/ml) for 2 min. Samples were immediately frozen and treated as described in Materials and Methods. Lanes 1 and 2, samples immunoprecipitated with anti-APR serum (lane 1, control; lane 2, phenethyl alcohol present); lanes 3 and 4, samples immunoprecipitated with anti-OmpA serum (lane 3, control; lane 4, phenethyl alcohol present). Lane 5 contained secreted APR. The star, open arrowhead, and solid arrowhead indicate the positions of APR, OmpA, and proOmpA, respectively. (B) Pulse-labeling with 14 C-labeled amino acids in presence of EDTA (300 μ M). *E. coli* TG1(pJUEK72) was grown at 37°C on M9 minimal medium without methionine assay medium, as it was normally used. After induction with IPTG and preincubation at 25°C, EDTA was added to the culture 5 min before the pulse. Cells were pulse-labeled with 14 C-labeled amino acids (0.11 mCi/ml) for 30 s, and then unlabeled amino acids were added to a final concentration of 10 mg/ml. Samples were taken at different times and treated as described in Materials and Methods. Lane P, pulse; lane 1, 30-s chase; lane 2, 60-s chase; lane 3, 120-s chase.

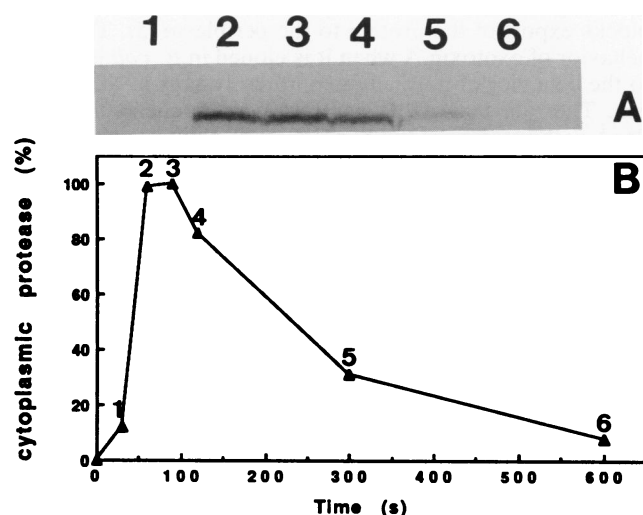


FIG. 6. Effect of nonsecretion conditions on accumulation of APR inside cells. *E. coli* TG1(pJF2518) was grown at 37°C in M9 minimal medium until the early logarithmic growth phase. After induction with 1 mM IPTG and preincubation at 25°C for 20 min, the cells were pulsed-labeled with [³⁵S]methionine (500 μCi/ml). After 30 s, unlabeled methionine (100 μg/ml) was added, and the cells were incubated for 600 s. Samples were taken at different times and quickly centrifuged. The cell pellets were immediately frozen and treated as described in Materials and Methods. (A) APR immunoprecipitates after 30 s (lane 1), 60 s (lane 2), 90 s (lane 3), 120 s (lane 4), 300 s (lane 5), and 600 s (lane 6) of chase. Only the relevant part of the fluorogram is shown. (B) Plot showing the relative percentages of radioactive APR found in cells as a function of length of chase. The relative percentages of immunoprecipitated products were evaluated from a densitometer analysis of suitably exposed fluorograms.

secretion in a strain carrying a defective component of the protein export apparatus, SecA. Plasmid pJUEK72 was introduced in strain MM52, which carried a temperature-sensitive mutation, *secA51*. At 41°C, strains carrying this mutation accumulate unsecreted cytoplasmic precursors for a number of exported proteins (25). Wild-type *E. coli* and *secA*(Ts) mutant strains carrying pJUEK72 were grown at 30°C to an A_{600} of 0.5. After 3 h at 37°C and induction by IPTG (1 mM), cells were pulsed-labeled with ¹⁴C-labeled amino acids for 30 s and chased for the times indicated in the legend to Fig. 7. Each sample was fractionated into a cell pellet and culture supernatant and was treated as described in Materials and Methods. We found that the kinetics of secretion were the same in the mutant as in the wild type (Fig. 7A); no intracellular accumulation of APR was observed, and protease was secreted as rapidly in MM52 as in MC4100 (Fig. 7A). As a control, maturation of the OmpF protein was examined; proOmpF accumulated under the restrictive conditions in the mutant (Fig. 7B). These results suggest that APR secretion in *E. coli* does not depend on the SecA pathway to undergo translocation across the inner membrane.

DISCUSSION

We previously reported that when an 8.8-kb DNA fragment carrying the *apr* gene was expressed in *E. coli*, active protease was secreted into the medium (12). In this study we found that structural gene *apr* does not contain all of the information necessary to translocate APR across both mem-

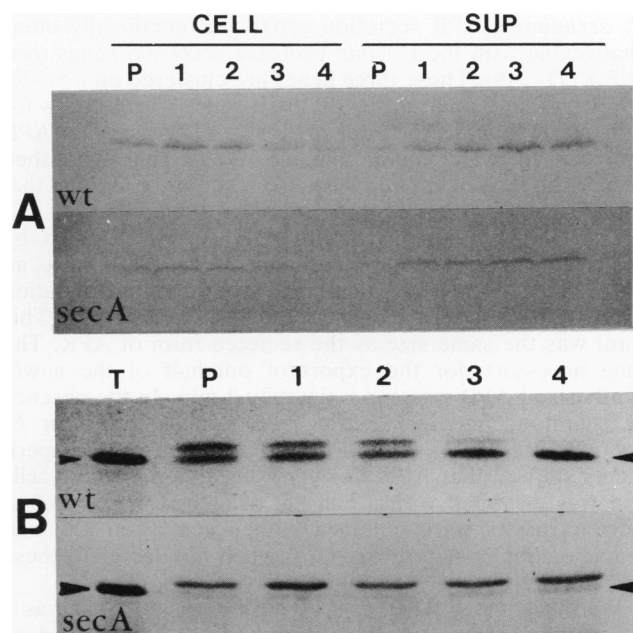


FIG. 7. Effect of *secA* inactivation on secretion of APR in *E. coli*. Wild-type strain MC4100 (wt) and *secA*(Ts) mutant MM52 (*secA*) carrying pJUEK72 were grown at 30°C in M9 minimal medium to an A_{600} of 0.5. The cultures were shifted to 37°C, and 1 mM IPTG was added. After preincubation for 3 h at 37°C, the cells were pulsed-labeled with ¹⁴C-labeled amino acids (0.11 mCi/ml). After 30 s, unlabeled amino acids (10 mg/ml) were added, and the cells were incubated for a total of 300 s. Samples were taken at different times, fractionated into supernatants (SUP) and cellular extracts (CELL), and treated as described in Materials and Methods. Lanes P, pulse; lanes 1, 30-s chase; lanes 2, 60-s chase; lanes 3, 120-s chase; lanes 4, 300-s chase. (A) APR immunoprecipitates. (B) OmpF immunoprecipitates. Lane T, long-term labeling to show the migration of mature OmpF (arrowheads).

branes in *E. coli*. When *apr* is cloned alone in *E. coli* containing plasmid pJF2518, APR is immunodetected only in cellular extracts. Protease-secreting *E. coli* can be obtained by transformation of *E. coli*(pJF2518) with a recombinant plasmid carrying the *KpnI*-2-*KpnI*-3 DNA fragment. When genes carried by this flanking DNA are expressed, APR is efficiently secreted into the growth medium; thus, all or part of the genes present on this *KpnI* DNA fragment are required for secretion of APR. The cloning and expression in *E. coli* of *apr* and all of the *P. aeruginosa* genes required for APR secretion (either on one plasmid [pJUEK72] or on two plasmids [pAGS7 and pJF2518]) have greatly facilitated our analysis of the secretion pathway, since the entire secretion process is indistinguishable from the process which occurs in *P. aeruginosa* (12).

Expression of plasmid pJUEK72 in a minicell-producing *E. coli* strain enabled us to identify four proteins in addition to APR; these proteins had molecular weights of 54,000, 45,000, 42,000, and 40,000. Deletion analysis and interposon mutagenesis showed that two DNA regions (*KpnI*-2-*EcoRI*-3 and *BamHI*-3-*EcoRI*-4), which are 2 kb apart, are required for protease secretion. Given the size of the gene products identified in minicells, none of the genes carried by pJUEK72 could overlap a distance of 2 kb. Thus, it is possible to conclude that pAGS7 carries at least two different genes involved in APR secretion. On the other hand, we have shown, by transcomplementation studies, that the

P. aeruginosa APR secretion genes are functionally interchangeable with the *Erwinia* protease secretion genes (*prt-D,E,F*) (11, 19). These three genes are clustered on a 5.5-kb DNA fragment adjacent to the *prtB* gene, which codes for protease B. Each of the three *prt* genes is necessary for APR secretion since any mutation in one of these genes abolished secretion (11, 19). In view of these results, we assume that pAGS7 probably carries three secretion functions.

The synthesis and secretion of APR were studied by performing pulse-labeling experiments. After a 30-s pulse, an intracellular form was identified by immunoprecipitation with antibodies raised against purified exoprotease. This form was the same size as the secreted form of APR. The time necessary for the export of one-half of the newly synthesized APR was approximately 2 min. In the absence of secretion functions, little APR was detected in *E. coli*(pJF2518) by Western blot analysis. Pulse-chase experiments showed that APR was very unstable inside the cells and was degraded with a half-life of approximately 160 s. Similar results were obtained with α -hemolysin (26) and immunoglobulin A protease (16); when not secreted, these proteins were rapidly degraded.

We investigated whether APR was first synthesized as a proenzyme. Many secreted proteases are synthesized as inactive precursors (zymogens) which are subsequently cleaved to give fully active mature proteases (31). No proenzyme was identified by very short pulse-labeling of the protease under conditions where it was not secreted (pJF2518) or under conditions where possible autoproteolytic cleavage was inhibited by the addition of EDTA.

We also searched for the presence of a signal sequence. Most of the proteins exported by gram-negative bacteria are synthesized with a signal peptide that is cleaved off upon translocation across the inner membrane (29). Many secreted proteins are also synthesized with such a signal sequence (28). In the presence of phenethyl alcohol, a substance known to interfere with the maturation of exoproteins, the intracellular form identified was not larger than the extracellular form. Thus, APR was apparently not synthesized in a precursor form with a cleavable signal sequence. This result was not unexpected if we consider what is presently known about the secretion mechanism of *E. chrysanthemi* proteases B and C. So far, it has been proven that these proteases are secreted without a signal peptide and that the secretion signal is located at the C terminus (19). Three secretion functions are necessary for secretion to the extracellular medium, and these functions are significantly homologous to those of *E. coli* α -hemolysin (19). We previously showed that these three secretion functions could replace those of *P. aeruginosa* in order to get APR secretion by *E. coli* (11). Thus, the absence of a cleavable signal sequence in APR is quite consistent with our working hypothesis that APR is secreted from cells by a molecular mechanism homologous to the *Erwinia* protease secretion pathway and to the *E. coli* α -hemolysin pathway (11). This working hypothesis is supported by another result. We found that APR secretion in *E. coli* did not depend on the SecA pathway to undergo translocation across the inner membrane. The same result was found for the secretion of α -hemolysin in *E. coli* (10). SecA, the translocation ATPase (29), is part of the general export pathway and is necessary for export of most proteins with signal sequences (29). Exotoxin A, another protein secreted by *P. aeruginosa*, possesses a signal sequence (9). Expression of the exotoxin A gene in *E. coli* yields native exotoxin A which is localized within the periplasmic space (5). A mutation in the *secA* gene

blocks export of the protein to the periplasm (5). Thus, the behavior of exotoxin A when it is cloned in *E. coli* is similar to the behavior of normally exported proteins in this organism. The fact that APR secretion was independent of the SecA pathway favors the hypothesis of a different secretion mechanism that is independent of the general export pathway.

We are currently working on molecular characterization of the APR secretion functions with a view to improving our understanding of the similarities among secretion mechanisms in *P. aeruginosa*, *E. chrysanthemi*, and *E. coli*, bacteria which are not phylogenetically related.

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