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The Penicillinase of *Bacillus licheniformis* Is an Outer Membrane Protein in *Escherichia coli*

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The cloned gene coding for *Bacillus licheniformis* penicillinase (*penP*) was introduced into *Escherichia coli* in a heat-inducible λ *Q*am vector. After induction, significant amounts of penicillinase were synthesized in the new host. The cellular location of the penicillinase was found to be almost exclusively the outer membrane fraction of *E. coli*, and virtually no soluble penicillinase was found. According to sodium dodecyl sulfate-gel electrophoresis, the size of the penicillinase from *E. coli* was identical to that of the membrane-bound form of the *B. licheniformis* penicillinase. Gel filtration in the presence of Triton X-100 suggested that the penicillinase from *E. coli* had amphiphilic properties, as does *B. licheniformis* membrane penicillinase. These results show that the export of the penicillinase to the outer membrane of *E. coli* involves the cleavage of the signal peptide from the prepenicillinase, giving an outer membrane component indistinguishable from the membrane penicillinase of *B. licheniformis*.

The penicillinase of *Bacillus licheniformis* has unique features, which make it an interesting model for studying the mechanism and structural requirements for protein export. Like most secretory or membrane proteins, it is initially synthesized as a precursor molecule, prepenicillinase, with an *N*-terminal signal sequence (most probably 26 amino acid residues long) and translocated through the cytoplasmic membrane (CM) with concomitant cleavage of the signal peptide (2, 14, 20, 22). This processed penicillinase remains membrane bound as an amphiphilic membrane protein from which soluble exopenicillinase is formed after proteolytic cleavage(s), removing at least eight amino acid residues from the *N* terminus (2, 9, 14, 21, 25). The *N*-terminal cysteine of the membrane penicillinase is modified with diglyceride (10, 15), which is apparently responsible for its hydrophobic properties. Such a structure was first described for *Escherichia coli* lipoproteins (6, 7). A similar modification has recently been reported to be present in the membrane penicillinases of some gram-positive bacteria (16) and even in some membrane proteins of bacilli other than penicillinase (17). These unusual chemical reactions must require specific cellular enzymes, which are not likely to be involved in the export of most other secretory or membrane proteins.

The structural gene (*penP*) of the *B. licheniformis* penicillinase has been cloned and shown to be expressed in *E. coli* (1). We used this cloned gene to study the cellular location and properties of the penicillinase synthesized in *E. coli*. We show that the *B. licheniformis* penicillinase is not secreted as a soluble protein but is found in the outer membrane (OM) as an amphiphilic protein whose properties are indistinguishable from those of *B. licheniformis* membrane penicillinase.

MATERIALS AND METHODS

Bacteria, phages, and plasmids. The bacterial strains used are listed in Table 1. λ *pen* is a derivative of the replacement vector λNM574 of the genotype λ Δ(*srI* λ 1-2) *cI857* Δ*srI* λ4 *nin-5* Δ*srI* λ5 (1). γ 369 is λ *pen att*$^+$ Δ*srI*3 *cI857* *Q*am73, phage L404 is λΔ (*srI*λ 1-2) Δ(*srI* λ 3) *cI857* Δ*srI* λ4 *nin-5* Δ*srI* λ5, and phage L405 is λ Δ(*srI* λ 1-2) Δ*srI* λ3 *cI857* Δ*srI* λ4 *nin*$^+$ *Q*am73. These phages were kindly given by W. Brammar, University of Leicester, Leicester, United Kingdom, and λ *cI857* was given by A. Klein, Max Planck Institute for Medicine, Heidelberg, Federal Republic of Germany.

Media and growth conditions. The complete medium was either L-broth composed of 1% tryptone (Difco Laboratories, Detroit, Mich.), 0.5% yeast extract (Difco), 1% NaCl; the pH was adjusted to 7.0. The minimal medium was Davies minimal medium (19) supplemented with 0.2% glucose. Liquid cultures were grown in Erlenmeyer flasks aerated with vigorous shaking (about 200 rpm) on a rotary shaker-incubator. Batches larger than 5 liters were grown in a New Brunswick Magnaferm fermentor aerated at 12 liters per min. Agar plates contained either L-broth or CH/S medium (21). The heat induction of strains lysogenic to λ *cI857* derivatives was performed as described below for the OM and CM.

Isolation of CM and OM. The isolation of the OM

TABLE 1. *E. coli* K-12 strains

Strain	Genotype/relevant features	Source/reference
JC9366	*recA13 thr-1 leu-6 proA2 his-4 thyA299 argE3 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 tsx-33 supE44* λ^-	No. 5181 in CGSC[a]
EH223	JC9366 (λ *pen* λcI857)	
WB956	W3350, φ369	W. J. Brammar
WB984	W3350, L405	

[a] *Coli* Genetics Stock Center, Yale University, New Haven, Conn.

and the CM was done by the procedure of Osborn et al. (18). WB956 was grown at 28°C on a rotary shaker in Davies minimal medium. At a density of 30 Klett units, the culture was shifted into a rotary shaker-water bath at 42°C for 15 min, 0.5 mCi of [^{35}S]methionine (>500 Ci/mmol; Amersham Corp., Amersham, United Kingdom) was added, and the incubation was continued at 37°C for 45 min (up to 65 Klett units, red filter) before harvesting. EH223 was grown in L-broth at 30°C.

Other methods. Penicillinase from *B. licheniformis* was purified as described by Simons et al. (21), and the membrane-bound penicillinase from *E. coli* WB956 was purified as follows. A culture of 5 liters of L-broth was grown to the density of 80 Klett units (red filter) at 30°C. Then, 5 liters of L-broth at 54°C was added, and the culture was maintained at 42°C for 15 min, shifted to 37°C for 2 h, and then rapidly cooled by being mixed with 2 kg of crushed ice. Bacteria (15 g [wet weight]) were collected by centrifugation and suspended in 20 ml of 20 mM Tris (pH 8.0). After being frozen and thawed, the suspension was sonicated with an MSE sonicator for 3 min in an ice bath, and the cell envelopes were collected by centrifugation at 100,000 × *g* for 1 h. Penicillinase was extracted with Triton X-100 from the cell envelopes and purified as described above for the penicillinase from *B. licheniformis*.

The particulate fraction of the bacteria was prepared by breaking the cells by ultrasonic treatment for about 3 min at 0°C in 10 mM Tris-hydrochloride–30 mM $MgCl_2$ (pH 8.0) and then centrifuging at 100,000 × *g* for 1 h at 4°C. The pellet and the supernatant were designated the particulate and soluble fractions, respectively.

Penicillinase activity was determined with the chromogenic cephalosporin Nitrocefin (Glaxo Research Ltd.) (21). One unit of penicillinase hydrolyzes 1 μmol of penicillin G in 1 h at 30°C (21).

Immunoprecipitations were performed as described by Sarvas et al. (20) except that antigen-antibody-protein A-Sepharose complexes were washed only once.

Polyacrylamide gel electrophoresis was performed on slab gels measuring 30 cm by 20 cm by 1.5 mm (the lower gel) as described elsewhere (21). They contained a gradient of 12 to 18% (wt/wt) of acrylamide, either with or without 8 M urea. For fluorography, the gels were treated with En^{3}Hance solution (New England Nuclear Corp., Boston, Mass.) as recommended by the manufacturer. For exposure, Kodak X-Omatic AR film was used.

RESULTS

Production of penicillinase by *E. coli* K-12 strains carrying λ *pen* phages. The *penP* gene of *B. licheniformis* has been cloned in a λ phage vector and shown to be expressed in *E. coli* K-12 (1). To study more carefully this penicillinase in *E. coli*, we used two *E. coli* K-12 strains carrying the *penP* in two different vectors.

The first one, strain EH223, was a *recA* mutant lysogenic to phages λ *pen* (1) and λ *c*I857 (Table 1). It was constructed as follows. *recA* mutant JC9366 was grown overnight in L-broth at 30°C with aeration, harvested, washed once with 26 mM Na_2HPO_4–22 mM KH_2PO_4–70 mM NaCl–1 mM $MgSO_4$, suspended in one half of the original volume of 10 mM $MgSO_4$, and incubated on a shaker at 25°C for 40 min. A 50-μl amount of this suspension, 200 μl of phage λ *c*I857, and 200 μl of λ *pen* (each ca. 2×10^9 PFU/ml) and 500 μl of 10 mM $MgSO_4$ were then mixed. After an incubation of 20 min at 25°C, dilutions of the suspension were plated onto L-agar plates. After overnight growth, single colonies were streaked onto L-agar plates containing 100 μg of penicillin G per ml. Resistant colonies were tested for penicillinase production. One positive colony was picked up and designated EH223.

Table 2 shows that in agreement with the findings of Brammer et al. (1), strain EH223 produced a low level of penicillinase (5 to 10 U/10^9 cells). This is about 0.5% of the amount produced by the magnoconstitutive *B. licheniformis* strain 749C, the source of the cloned *penP* gene.

The second strain was WB956, which is lysogenic to the hybrid phage λ *pen* *c*I857 *Q*am. As described elsewhere (1), at a nonpermissive temperature, up to 1,000 copies of the phage genome

TABLE 2. Penicillinase produced by *E. coli* K-12 strains containing the *penP* gene of *B. licheniformis*[a]

Strain	Lysogenic phage	Cell-bound penicillinase (U/10^9 cells)[b]
EH223	λ *pen*, λ *c*I857	5–10
WB956	φ369 (*penP* *Q*am *c*I857)	15–30 4,000[c]
JC9366	—[d]	<0.5
WB984	L405 (*Q*am *c*I857)	<0.1[c]

[a] The bacteria were grown in L-broth on a rotary shaker at 28°C to a density of about 100 Klett units (red filter), harvested by centrifugation, and broken by sonication in 50 mM K-phosphate buffer, pH 7.0. The growth temperature was 37°C (28°C in the case of WB956).

[b] Range of three to four batches.

[c] At 2.5 h after induction.

[d] —, None.

per cell are produced, with a concomitant increase in the amount of penicillinase, but without lysis of the cells. In our case, heat induction increased the level of penicillinase in the culture of strain WB956 from 30 to 4,000 U/10^9 cells in 3.5 h (Table 2). This is slightly more than the amount produced by *B. licheniformis* 749C in the exponential phase of growth.

The parent strains, JC9366 and WB984 (devoid of *penP*), produced less than 0.5 U of penicillinase per 10^9 bacteria (Table 2).

Absence of soluble penicillinase in *E. coli* (λ *pen*) derivatives. If *B. licheniformis* penicillinase were secreted from *E. coli* as it is from *B. licheniformis*, we would expect to find soluble penicillinase in the periplasmic space of *E. coli* (λ *pen*) strains. However, previous studies (5, 8) have already shown that most of the penicillinase of *E. coli* strains carrying *penP* is associated with the cell envelope. Similarly, after sonic disruption, we found that more than 90% of the penicillinase activity of strain EH223 (lysogenic to λ *pen*) was in the particulate fraction. Only 10% was released by osmotic shock (Table 3), not significantly more than that of the cytoplasmic marker enzyme β-galactosidase. We found no penicillinase activity in the growth medium.

A similar distribution of penicillinase was found in strain WB956 (λ *pen* Qam) after heat induction. One hour after the induction, no lysis of cells was detected by phase-contrast microscopy and sodium dodecyl sulfate (SDS)-gel electrophoresis of proteins from the growth medium, but the amount of cell-bound penicillinase had already increased to 1,000 U/10^9 cells. At that time, less than 0.01% of the total penicillinase was found in the medium. Again, most of the penicillinase (85%) sedimented with the particulate fraction after lysozyme treatment and sonic disruption (Table 3).

To study more carefully the nature of the penicillinase in the particulate fraction and that released by sonication but not sedimented by centrifugation (Table 3), we performed gel filtration on Sephadex G-150 in the presence of Triton X-100. Membrane penicillinase from *B. licheniformis* elutes at void volume under these conditions, whereas the soluble hydrophilic exopenicillinase elutes after ovalbumin marker (21). The penicillinase from the particulate fraction of strain WB956 also eluted at void volume (Fig. 1A), suggesting that it is an amphiphilic protein like the *B. licheniformis* membrane penicillinase. Unexpectedly, only 15% of the small amount of penicillinase activity which was not sedimentable (6 to 14%) eluted at the position of exopenicillinase (Fig. 1B). Most of the soluble penicillinase also eluted at void volume and thus seems to be similar to the amphiphilic penicillinase in the particulate fraction. This was also confirmed by its size in SDS-gel electrophoresis (see below). Thus, no more than 2 to 3% of the total penicillinase activity from *E. coli* (λ *pen*) was in the form of water-soluble, hydrophilic protein, analogous to the exopenicillinase of *B. licheniformis*. Most of the penicillinase in the soluble fraction is probably amphiphilic penicillinase associated with membrane fragments or vesicles.

TABLE 3. Localization of penicillinase in different cell compartments of *E. coli* K-12 strains EH223 (λ *pen* lysogen) and WB956 (λ *pen* Qam induced)

Source of enzyme	Enzymatic activity (% of total activity)			
	Penicillinase[a]		Alkaline phosphatase[b] (EH223)	β-Galactosidase[b] (EH223)
	EH223	WB956[c]		
Culture medium	<1	<1	<1	<1
Periplasmic proteins[b]	10		83	6
Sonicated bacteria				
Soluble fraction	6	14	95	90
Particulate fraction	94	86	5	10

[a] The total amount of penicillinase activity was 5 and 1,000 U/10^9 bacteria in strains EH223 and WB956, respectively. Similar results were obtained with EH223 grown in L-broth and in low-phosphate medium (11).

[b] The bacteria were grown, and the enzymes were assayed as described elsewhere (11). The total activities of alkaline phosphatase and β-galactosidase were 2×10^{-3} and 40 U/10^9 bacteria, respectively (units as described in references 11 and 13).

[c] Released by osmotic shock as described in reference 11.

[d] The bacteria were harvested after heat induction.

The heat induction of strain WB956 rendered it fragile, and the osmotic shock always released large amounts of cytoplasmic proteins (data not shown). The distribution of soluble penicillinase between the cytoplasmic and periplasmic compartments of this strain was therefore not studied.

Localization of penicillinase in OM of *E. coli* (λ *pen*). The experiments described above strongly suggest that essentially all of the bacillar penicillinase made in *E. coli* is bound to the cell envelope. To study the distribution of the penicillinase between the CM and OM fractions, we isolated these from strain WB956 after heat induction as described elsewhere, (18). Figure 2A shows that penicillinase activity was found only in the heavy membrane fraction, which by its density and the presence of ketodeoxyoctonate (Fig. 2B) clearly represents the OM (18). No penicillinase activity was found in the light CM fraction (Fig. 2).

To rule out the possibility that the induction

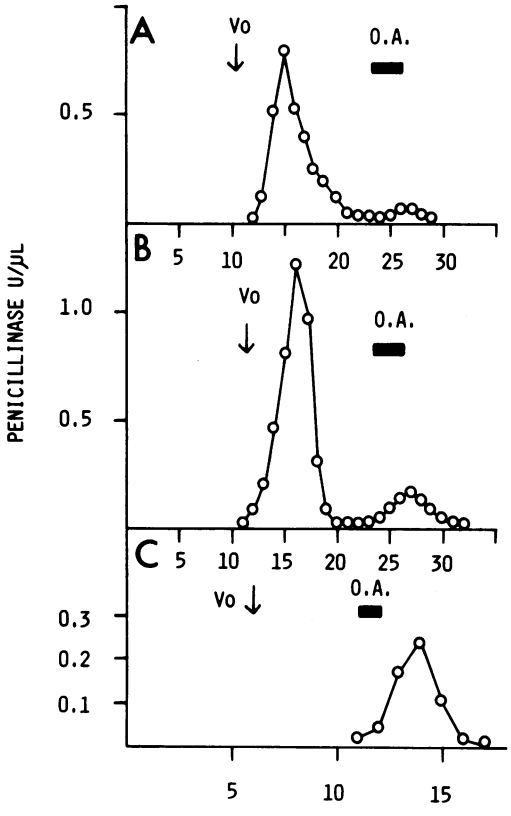

FIG. 1. Gel filtration of penicillinase from *E. coli* WB956 (λ *pen Q*am) on Sephadex G-150 in the presence of Triton X-100. (A) Penicillinase (2,000 U) solubilized from the particulate fraction. (B) Penicillinase (40 μl, about 2,000 U) of the soluble fraction of WB956 (Table 3). (C) Purified penicillinase (400 U) from *E. coli* digested with 0.2 mg of trypsin per ml for 45 min at pH 7.5. V_0 shows the void volume of the Sephadex column. O.A. marks the position where ovalbumin eluted. The elution buffer was 0.1 M Tris-hydrochloride–0.1 M NaCl–0.05% Triton X-100 (pH 7.5). Ovalbumin (3 mg) was added as a marker; its position was located by quantitative protein determination.

and subsequent replication of λ *pen Q*am phage in strain WB956 had affected the localization of penicillinase, we also isolated the OM and CM from strain EH223, lysogenic to phage λ *pen*. Again, penicillinase activity was found only in the heavy OM fraction (data not shown).

Characterization of penicillinase protein made in *E. coli*. Using the method described elsewhere (21) for the purification of the membrane penicillinase from *B. licheniformis*, we solubilized with Triton X-100 and then purified the penicillinase from the particulate fraction of *E. coli* WB956. Purified penicillinase was found to have the same specific activity as *B. licheniformis* membrane penicillinase (about 350 U/μg of protein). It can thus be calculated from the data in Table 2 that, after heat induction, strain WB956 produced up to 12 μg of penicillinase protein per 10^9 bacteria. This suggests that penicillinase is a prominent cell envelope and OM protein under these conditions.

Antiserum against *B. licheniformis* penicillinase readily precipitated one protein from the Triton X-100-solubilized particulate fraction of WB956 (Fig. 3, lane 6). This protein migrated identically with the *B. licheniformis* membrane penicillinase in an SDS-acrylamide gradient gel (Fig. 3, lanes 4 and 6), which can separate the three forms of *B. licheniformis* penicillinase, the two forms of exopenicillinase (exoS, exoL; 9, 21), and membrane penicillinase (Fig. 3, lanes 1 to 4). A protein comigrating with the membrane penicillinase was also immunoprecipitated from the soluble fraction obtained after sonic disruption (Fig. 3, lane 5), compatible with the hydrophobic properties of this penicillinase suggested by its gel filtration profile in the presence of Triton X-100 (Fig. 1). Nothing was precipitated after heat induction from control strain WB956 (λ *c*I857 *Q*am) or from the cell envelope from WB956 with an unspecific antiserum (data not shown).

SDS-gel electrophoresis showed that in the OM fraction of WB956, purified after heat induction, a prominent band comigrated with the membrane penicillinase of *B. licheniformis* (Fig. 4, lane 5). This protein was also precipitated with anti-penicillinase serum (Fig. 4, lane 2). Nothing was precipitated with this serum from the CM fraction (Fig. 4, lane 1), nor was any band seen at the position of membrane penicillinase in the CM preparation (Fig. 4, lane 6).

The hydrophilic, exoS-like domain of the *B. licheniformis* membrane penicillinase is cleaved off by the trypsin treatment of protoplasts or membrane preparations (21, 25). The trypsin treatment of the purified OM fraction of WB956 similarly converted the penicillinase to a protein comigrating with trypsin-treated membrane penicillinase from *B. licheniformis* (Fig. 4, lane 8), suggesting a mode of insertion into the membrane in *E. coli* similar to that in *B. licheniformis*.

DISCUSSION

Two major groups of *E. coli* proteins are exported outside the CM: soluble periplasmic proteins and hydrophobic OM proteins (for a review, see reference 12). Both types of proteins are initially synthesized as preproteins with a signal peptide at their amino terminus (12). The secretion of bacillar exoproteins is closely comparable to that of soluble periplasmic proteins in *E. coli*. Indeed, when the gene coding for α-amylase was cloned from *B. coagulans* and

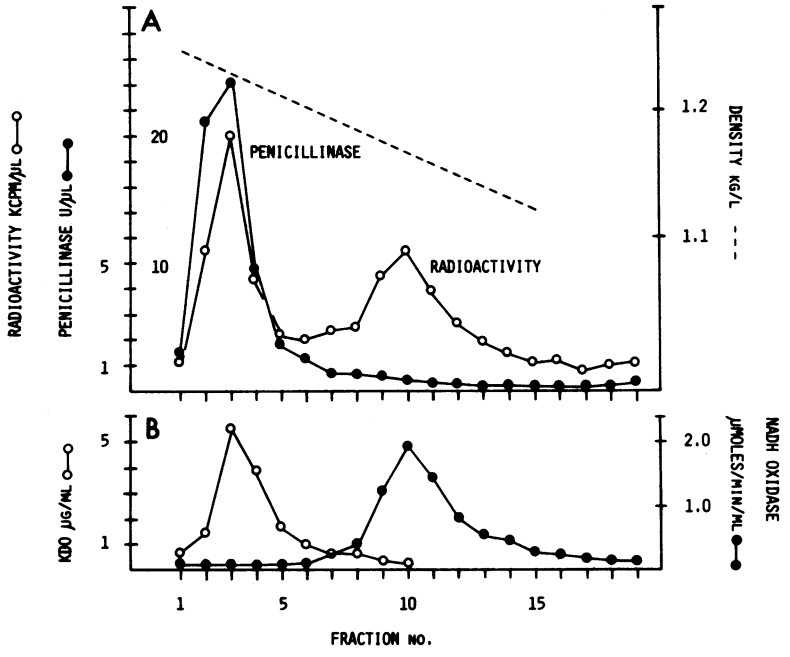

FIG. 2. Localization of penicillinase of WB956 in the OM fraction. (A) Penicillinase activity and ^{35}S label. (B) Ketodeoxyoctonate (KDO) and NADH oxidase activities. Ketodeoxyoctonate and NADH oxidase were assayed as described in reference 18.

transferred into *E. coli*, the α-amylase was secreted into the periplasmic space (3). The same result has also been obtained with cDNA clones coding for some eucaryotic secretory proteins (4, 23, 24).

When gene *penP* of *B. licheniformis* was introduced into *E. coli*, it was expressed, but penicillinase remained in the particulate fraction (5, 8). Our analysis of the cellular location of *B. licheniformis* penicillinase in *E. coli* showed, unexpectedly, that it was found almost exclusively in the OM fraction. The penicillinase was a prominent OM protein when an inducible λ *pen Q*am vector was used. The location of the penicillinase was also the same when its amount was much lower, presumably with minimal disturbance of the assembly of the cell envelope (λ *pen* lysogen, strain EH223). It thus seems that the association of the penicillinase with the OM is specific rather than an artifact of the disturbed synthesis of the cell envelope after the induction of the lysogenic λ.

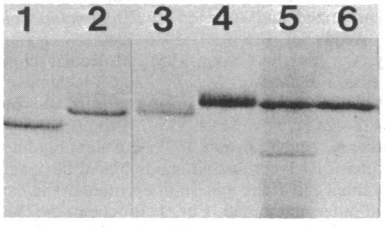

FIG. 3. SDS-gel electrophoresis of particulate-bound and soluble penicillinase from *E. coli* WB956 in SDS-acrylamide gradient gel. The bacteria were grown, and the particulate and soluble fractions were prepared as described for the purification of penicillinase from *E. coli* WB956. Penicillinase was immunoprecipitated and electrophoresed in an SDS-slab gel with an acrylamide gradient of 12 to 18%. Lane 1, Membrane penicillinase from *B. licheniformis* treated with trypsin; lanes 2 and 3, purified exoL penicillinase from *B. licheniformis*; lane 4, membrane penicillinase from *B. licheniformis*; lane 5, immunoprecipitate of penicillinase from the soluble fraction of *E. coli* WB956; lane 6, immunoprecipitate of the particulate fraction of WB956. Lanes 1 and 2 and 3 to 6 are from two similar gels run in parallel.

The apparent M_r of the penicillinase from the OM of *E. coli* was indistinguishable from that of *B. licheniformis* membrane penicillinase. This suggests that the export of the penicillinase to the OM involved the cleavage of the signal peptide in this heterologous host also (Fig. 4). Judged by its ability to bind Triton X-100 (Fig. 1), the penicillinase from *E. coli* was amphiphilic, similar to the *B. licheniformis* membrane penicillinase. This is in good agreement with the finding that the *B. licheniformis* penicillinase also contains covalently bound glyceryl-cysteine and fatty acids when made in *E. coli* (10).

The location of the penicillinase in the OM suggests that it is specifically recognized as an

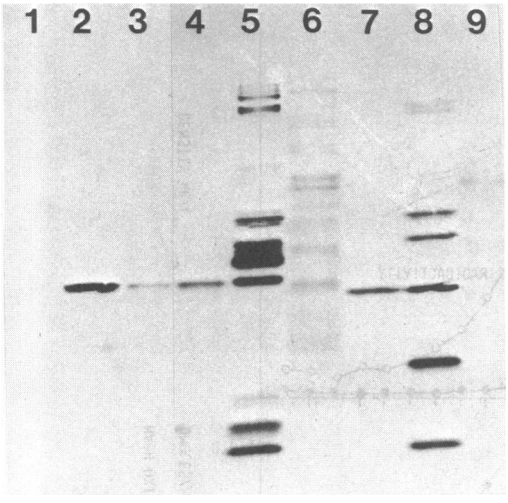

FIG. 4. SDS-slab gel electrophoresis of the OM and CM fractions of *E. coli* WB956. An SDS-slab gel with an acrylamide gradient of 12 to 18% containing 8 M urea was used. Lane 1, CM fraction immunoprecipitated with anti-penicillinase serum; lane 2, OM fraction immunoprecipitated as in lane 1; lanes 3 and 4, purified membrane penicillinase from *B. licheniformis* labeled with [^{14}C]leucine; lane 5, OM fraction; lane 6, CM fraction; lanes 7 to 9, like lanes 4 to 6, respectively, but digested with 0.3 mg of trypsin per ml at 37°C for 45 min.

OM protein in *E. coli*. This would imply the presence of structures similar to those determinants of OM proteins which destine them for their cellular location. It is striking that the *N*-terminal part of the penicillinase of *B. licheniformis* resembles the *N* terminus of the most abundant OM protein of *E. coli*, the Braun lipoprotein. The lipoprotein also has *N*-terminal glyceryl-cysteine substituted for with ester and amide-linked fatty acids (2, 10, 15). Furthermore, the prepenicillinase and prolipoprotein have an identical tetrapeptide at the cleavage site of the signal peptide (2, 14). It is tempting to relate these lipoprotein-like structures of the penicillinase molecule to the export and insertion of this foreign protein into the OM in *E. coli*. However, some other determinants or elements could also be involved. Several minor lipoproteins are found in *E. coli*, all containing glyceryl-cysteine (7). Some of these proteins are clearly located in the CM, indicating that the presence of glyceryl-cysteine alone does not destine a protein for the OM.

Although most steps of translocation, processing, and modification of the *B. licheniformis* penicillinase seem to take place similarly in *E. coli* and in *B. licheniformis*, virtually no soluble penicillinase was found in *E. coli* (Fig. 1). In *B. licheniformis*, the soluble exopenicillinase is cleaved from the membrane-bound form by a proteolytic enzyme(s). Also, in *E. coli* the membrane-bound penicillinase seems to be accessible to proteolytic enzymes, because penicillinase similar to *B. licheniformis* exopenicillinase was also released by trypsin from an *E. coli* OM preparation (Fig. 4). It thus seems that *E. coli* lacks the proper proteolytic enzymes to cleave the membrane-bound lipoprotein form of this penicillinase.

The lack of soluble penicillinase in *E. coli* would further point to the dissimilarity between this protein and its mode of processing and most secretory proteins of the bacilli. α-Amylases are major secretory proteins of the bacilli. In addition to the lack of a lipoprotein form (17), soluble α-amylases are found in the periplasmic space when the cloned gene has been transferred into *E. coli* (3; I. Palva, submitted for publication).

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