Further Evidence for a Partially Folded Intermediate in Penicillinase Secretion by *Bacillus licheniformis*

GEORGE E. BETTINGER and J. O. LAMPEN

Institute of Microbiology, Rutgers, The State University of New Jersey, New Brunswick, New Jersey 08903

Received for publication 30 October 1974

Protoplasts of *Bacillus licheniformis* 749/C (a mutant constitutive for penicillinase production) continued to synthesize and release penicillinase in hypertonic growth medium in the presence of trypsin and chymotrypsin at 25 μg each per ml. When the protoplasts were stripped of about half of their membrane-bound penicillinase by pretreatment at pH 9.5 or with a higher level of trypsin, penicillinase activity no longer increased in the presence of the proteases. This effect was immediately eliminated after addition of soybean trypsin inhibitor. These proteases do not significantly inhibit general protein synthesis. Stripped protoplasts of strain 749/C and of uninduced strain 749 (unable to synthesize penicillinase) were incubated with 50 μg of chymotrypsin per ml, and the supernatant fluids were examined immunochemically for peptides derived from the penicillinase chain. Such fragments were found only with the protoplasts capable of synthesizing penicillinase (strain 749/C). The direct detection of the products of protease degradation of a susceptible form of penicillinase provides strong evidence that, in stripped protoplasts of *B. licheniformis* 749/C, penicillinase synthesis continues in the presence of trypsin or chymotrypsin and that, in these modified membranes, the protease is able to act on an early form of the enzyme that has not yet attained the protease-resistant conformation characteristic of the membrane-bound and exopenicillinases. This finding is discussed in terms of the current models of penicillinase secretion.

*Bacillus licheniformis* 749/C (11) produces two forms of penicillinase (beta-lactamase, EC 3.5.2.6), a hydrophilic exoenzyme (14) whose secretion by protoplasts is tightly coupled to synthesis (27), and a cell-bound, hydrophobic form (8, 29). This latter type can be further separated after protoplast formation into membrane penicillinase, sedimenting with intact protoplasts (26) or their membranes (29), and vesicle penicillinase, associated with tubular and vesicular membrane components believed to arise from mesosome eversion (26).

Sawai et al. (30, 30a) recently presented evidence that membrane penicillinase is a phospholipid-protein with an amino acid composition similar to that of exopenicillinase, although it has a less stable conformation when compared to the exoenzyme (8). Purified membrane penicillinase could be converted to an exo-type enzyme by trypsin or phospholipase D (30, 30a). Lampen (14) reported that trypsin can stoichiometrically convert the penicillinase in intact membranes to exoenzyme without loss of enzyme activity, and both the membrane-bound and exoenzymes are resistant to inactivation by either protease.

A mechanism for the secretion of penicillinase was proposed by Sargent and Lampen (28) in which the nascent penicillinase is extruded into the plasma membrane from bound polysomes in an incompletely folded form. The enzyme is thought to transit the membrane in this state (secretion) and then assume its functional protease-resistant conformation as either the hydrophilic (exo) or hydrophobic (bound) form. Turnover experiments have shown that, at least at pH 6.0 to 6.5, the majority of exopenicillinase does not originate from preexisting cell-bound enzyme, but is directly secreted, although a slight amount of bound enzyme is released as exoenzyme (7).

We have examined penicillinase secretion by *B. licheniformis* 749/C protoplasts based on the hypothesis that penicillinase traverses the membrane in a partially folded form which might be susceptible to degradation by trypsin or chymotrypsin before assuming its catalyt-
cally active conformation (2). Our data indicated that at some point during penicillinase secretion a protease-sensitive form exists and we interpreted this as evidence supporting the original hypothesis of Sargent and Lampen (28). We now report the detection of fragments of penicillinase formed by protease-treated protoplasts of strain 749/C under conditions wherein protein synthesis takes place at a normal rate, but no net increase in penicillinase activity occurs. (This work was part of a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree from Rutgers University by G. E. Bettinger.)

MATERIALS AND METHODS

*B. licheniformis* 749, penicillinase inducible, and its magnoconstitutive mutant strain 749/C were maintained on sporulation medium, and inocula were prepared as previously published (1). Cells were grown at pH 6.5 in casein hydrolysate-salts medium (11) containing 2 mg of maltose per ml to induce production of alpha-glucosidase (20) as outlined before (1). Cells (or protoplasts) for enzyme assay were sampled by dilution into cold buffer containing 40 µg of chloramphenicol per ml and supplemented with soybean trypsin inhibitor as needed (2). Bound enzyme was determined by sedimenting the cells or protoplasts at 15,000 × g for 5 min, and subtracting the amount present in the supernatant from the total found in the whole culture.

Assay. Penicillinase was assayed as described by Sargent (25), and alpha-glucosidase was assayed by the method of Pollock (20). Cell growth was monitored turbidimetrically and expressed as milligrams cell dry weight equivalent (d.w.e.) of organisms per milliliter (26), whereas cell number was estimated by direct count (5). Protein was estimated using the Folin phenol reagent (18) and peptides were estimated by the biuret method (16); values are relative to bovine serum albumin as standard. Total protein synthesis was determined from the incorporation of radioactivity from a 3H-labeled amino acid mixture (New England Nuclear, Boston, Mass.) into hot 5% (wt/vol) trichloroacetic acid-insoluble material (2).

Protoplast formation. Protoplasts, prepared as outlined by Sargent et al. (26) and monitored by phase contrast microscopy, were washed once in fresh protoplasting medium. Washed protoplasts were either directly suspended to 1 mg cell d.w.e./ml in a buffer medium [0.05 M 2-amino-2-(hydroxymethyl)-1,3-propanediol, 0.02 M KH₂PO₄, 0.001 M MgCl₂, and salts as described by Pollock (23)] adjusted to pH 7.5 and supplemented with 0.5% (wt/vol) Casitone (Difco) as a nitrogen and energy source and osmotically supported with 0.4 M sodium succinate (referred to as PrG [protoplast growth] medium) or suspended in the buffer medium at pH 9.5 with 0.75 M sucrose (termed PrS [protoplast stripping] medium). PrG medium supports penicillinase formation by the protoplasts (27), whereas approximately 50% of the bound penicillinase can be removed from intact protoplasts in PrS medium in 30 min without appreciably affecting their ability to synthesize penicillinase (2). However, if they were resuspended in pH 7.5 PrS medium having 0.5% (wt/vol) Casitone (a medium able to stabilize untreated protoplasts and permit penicillinase synthesis [26]), they immediately began to lyse. Lysis could be prevented by the addition of sufficient chloramphenicol (40 µg/ml) to prevent protein synthesis (data not shown).

Protoplasts incubated for 30 min in PrS medium were washed once in PrG medium and suspended in fresh PrG medium at 1 mg cell d.w.e./ml. Penicillinase synthesis typically commences 30 min after resuspension in PrG medium (2). Zero time in Fig. 5 is therefore actually 30 min following protoplast resuspension in PrG medium.

Liquid scintillation counting. Radioactivity was measured in nonsolvent samples using 10 ml of a toluene-based commercial scintillant (Omnifluor, New England Nuclear, Boston, Mass.). For aqueous samples this cocktail was mixed with Triton X-100 in a ratio of two parts cocktail to one part Triton X-100 (33). Counts were either used directly or converted into disintegrations.

Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed by the method of Shapiro et al. (31). Gels run with radioactive samples were cut into 1.8-mm slices, solubilized (12), and counted.

Preparation of chymotryptic peptides. Protoplasts (1 g of cell d.w.e.) of either *B. licheniformis* 749 or 749/C were treated in PrS medium as detailed above and suspended in 1 liter of PrG medium containing 75 mg of alpha-chymotrypsin and incubated for 4 h at 30 C. Protoplasts were sedimented, the culture supernatant was diluted fourfold, and the pH was lowered to pH 6.0 with 2 N HCl to stop chymotryptic activity. A bacteriostatic agent (NaN₃, 0.02% wt/vol) was added, and the entire contents were passed through a 39-cm² Amicon UM-10 ultrafiltrator (>10,000 molecular weight retained, Amicon Corp., Lexington, Mass.) at 10 C using 60 lb/in² N₂. The UM-10 filtrate was then put through an Amicon UM-2 ultrafiltrator (>1,000 molecular weight retained). Filtration was continued until 30 ml of retentate remained, and then 200 ml of 0.05 M sodium citrate (pH 5.0) was added and filtration resumed until 30 ml remained. The contents were washed in this way an additional two times, and the remaining retentate (chymotryptic peptides) was stored at −20 C.

Antibodies to penicillinase. Exopenicillins, purified as described by Pollock (23) and kindly supplied by Laura J. Crane, was used as antigen. Normal rabbit serum, drawn 1 week prior to the start of immunization, showed no antipenicillinase activity by either immobilodiffusion or inactivation of enzyme activity. Each thigh was injected intramuscularly with 0.5 ml of antigen, emulsified in Freund complete adjuvant (Difco) at 0.5 mg of penicillinase per ml and 1 ml was injected suprascapularly, commencing on day zero and on every 7th day thereafter. After four injections the rabbit was given 0.5 ml of antigen intravenously biweekly, 20 ml of blood was withdrawn
at the midpoint between injections, and the serum was separated and stored at -20 C.

Immunoglobulin (IgG) was isolated from the pooled sera (bleedings 6 and 7) as outlined by Levy and Sober (17), and found to be homogeneous by immunoelectrophoresis using goat anti-rabbit IgG serum (Cappel Laboratories, Johnstown, Pa.). Inactivation of penicillinase by the antiserum was determined by adding increasing volumes of the sera to tubes having 900 units of pure penicillinase in a final volume of 1.5 ml and incubating for 30 min at 30 C. Remaining activity was assayed, and the percent inhibition was expressed relative to the activity of the enzyme in the absence of serum.

Radioimmunoassay. The solid-phase, radioimmunoassay system of Sox and Mohit was used (32). Isolated IgG from serum having a unique penicillinase inactivation profile (described below) was conjugated to bromoacetyl cellulose (24) as outlined by Ungar-Waron et al. (34).

Radioactive exopenicillinase was isolated by affinity chromatography (8) from the supernatant fluid of a culture of B. licheniformis 749/C grown for 4 h in pH 7.5 CH/S medium containing 1.0 mCi of a 14C-labeled amino acid mixture per ml. This enzyme was 95% pure by gel electrophoresis (see Fig. 3). Penicillinase antigenic determinants were detected by their ability to inhibit the binding of the purified [14C]exopenicillinase to the cellulose-conjugated antibody.

RESULTS

Penicillinase antisera. The sera used in preparing the bromoacetyl-cellulose immunoglobulin conjugate were first tested for specificity by double immunodiffusion (Fig. 1). Sera from the 6th and 7th biweekly bleedings (in wells A and B) formed single precipitin bands to the purified exopenicillinase used as antigen (well 1). These bands showed a relationship of identity to the major band formed against a culture supernatant of strain 749/C (well 2) and the band to exopenicillinase purified by affinity chromatography (well 4). No band developed against material in the culture supernatant of strain 749/C that passed through the cephalosporin C affinity column (well 5). The precipitate which formed between wells 2 and 3 was not stable and soon disappeared. Well 6 contained a 10-fold concentrate of culture supernatant from stationary-phase B. licheniformis 749/C and did not contain penicillinase and having no penicillinase activity when assayed by the Sargent method (25). A faint band of partial identity to that formed at well 1 (exopenicillinase) developed against this concentrate.

Immune sera formed against penicillinase may either stimulate or inhibit penicillinase activity (22). The immune sera described above do not markedly inactivate penicillinase (Fig. 2), and the titration curves in fact show multiple slopes. The intersections of the slopes (E1, E2, E3) are considered by Pollock to be equivalence points (21); multiple equivalence points are interpreted as the expression of antibody populations directed against multiple determinants on the antigen (22). Immune sera capable of recognizing several determinants on the immunogen rather than strong inactivating antisera were chosen to increase the possibility of detecting one or more of the penicillinase determinants in the peptide fragments resulting from proteolysis.

Radioimmunoassay. The specificity of the penicillinase radioimmunoassay was established by testing various B. licheniformis proteins as potential inhibitors of the binding of pure [14C]exopenicillinase (Fig. 3) to cellulose-
coupled antibody (Table 1). The proteins in the 749/C culture filtrate not retained by the affinity column did not inhibit the binding of radioactive antigen to the bromoacetyl-cellulose IgG conjugate until the ratio of inhibitor protein to antigen was extremely large (31:1), and then inhibition was only 7%. Exopenicillinase purified by affinity chromatography gave 88% inhibition at a 1:1 weight ratio with the [14C]penicillinase.

Based on the findings discussed earlier (2, 28), we tested the chymotryptic peptides isolated from culture supernatants of protoplasts of strain 749 or 749/C as inhibitors in the immunooassay (Fig. 4).

Both peptide preparations acted as inhibitors, but the inhibition caused by the fragments from strain 749 reached a maximum of about 20% at 0.7 mg/ml. The fragments from 749/C protoplasts gave 40% inhibition at the highest level tested (1 mg/ml) without any indications of maximum inhibition. Due to the limited amount of peptides from strain 749/C remaining after necessary preliminary experiments, higher levels could not be tested.

**Synthetic capacity.** Protoplasts of *B. licheniformis* 749 and 749/C in PrG medium (pH 7.5) were compared in their ability to incorporate [14C]-labeled amino acids (0.62 µCi/}

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Inhibitor enzyme ratio</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nonlabeled pure exopenicillinase</td>
<td>1</td>
<td>88</td>
</tr>
<tr>
<td><em>B. licheniformis</em> 749/C exoproteins</td>
<td>6.3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>12.6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>18.9</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>25.2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>31.5</td>
<td>7</td>
</tr>
</tbody>
</table>

* The bromoacetyl-cellulose-coupled antipenicillinase IgG was preincubated for 60 min with the above antigen preparations (inhibitors) prior to adding [14C]exopenicillinase. Inhibition was measured by the decreased binding of [14C]exopenicillinase caused by the nonlabeled inhibitor.


* Purified by affinity chromatography (8).

* Proteins not retained by the affinity column.

![Graph](https://via.placeholder.com/150)

**Fig. 4.** Detection of antigenic chymotryptic penicillinase peptides. The chymotryptic peptides isolated were tested as inhibitors of the binding of [14C]exopenicillinase in the radioimmunoassay. Symbols: ●, fragments from protoplasts of *B. licheniformis* 749/C (penicillinase constitutive); △, fragments from protoplasts of *B. licheniformis* 749 (not induced for penicillinase).
ml) into hot 5% trichloroacetic acid-precipitable material in the total culture. Strain 749 had a rate of 28,000 counts per min incorporated per mg cell d.w.e. per h as compared to 25,000 counts per min incorporated per mg cell d.w.e. per h for 749/C.

**Penicillinase removal from protoplasts.** Removal of penicillinase by trypsin-chymotrypsin from intact protoplasts in pH 7.5 PrS medium was almost total within 30 min (Table 2) and without loss of enzyme activity (data not shown; reference 14). There was a slow rate of release due to pH alone. In modified PrG medium (no Casitone), differing from PrS only in osmotic support, penicillinase removal by protease at pH 7.5 was not as rapid as in PrS medium (only 36% removed in 1 h), although after 3 h 78% of the bound enzyme had been removed. In neither medium was there any release of alpha-glucosidase, an internal enzyme used as an indicator of membrane damage and lysis (20).

**Penicillinase synthesis.** We have shown that the ability of trypsin to prevent a net increase in penicillinase activity by *B. licheniformis* 749/C protoplasts is related to the amount of bound penicillinase previously removed (2). Since these protoplasts are able to replenish lost enzyme with newly formed penicillinase (2), it seemed possible that, at low levels of trypsin sufficient to affect penicillinase formation by pH 9.5-treated protoplasts, untreated protoplasts might be able to synthesize and replace membrane penicillinase faster than it could be removed, and that no net loss of bound enzyme occurred. This relationship might be altered by simply increasing the amount of trypsin added to protoplasts having a full complement of membrane penicillinase, so that now the rate of removal of bound enzyme was greater than its rate of replenishment. The increase in penicillinase activity would cease when a sufficient amount of bound enzyme had been removed.

Protoplasts of strain 749/C were preincubated in PrG medium for 30 min before the addition of increasing levels of trypsin (Fig. 5). Penicillinase activity and its distribution between bound and free forms were monitored. Net synthesis of penicillinase continued in the presence of trypsin until such time as the level of cell bound enzyme had been reduced to approximately 50% of that originally present, and then it abruptly ceased. Alpha-glucosidase activity was not lost to the medium; thus, no lysis had occurred.

**DISCUSSION**

Protoplasts of *B. licheniformis* 749/C continued to synthesize penicillinase in growth medium with a low level of protease (trypsin and chymotrypsin) at rates similar to control cultures (2; and Fig. 5). However, when the protease was added to protoplasts which had been stripped of half of their original bound penicillinase, there was no net increase in activity (2). This restriction was not due to lysis nor was the synthesis of internal protein affected. The effect was immediately annulled by the addition of trypsin inhibitor, and we suggested that the protease was degrading the nascent enzyme at some point during the secretion process before it could assume the usual resistant conformation. This would imply that peptides should be formed from the penicillinase chain during restriction by protease, and these might be detected immunochemically.

Chymotrypsin by itself is sufficient to restrict the increase in penicillinase activity in stripped protoplasts (unpublished data). On the basis of the primary sequence of penicillinase (19), chymotrypsin should form larger peptide fragments than would trypsin and, hopefully, some of these would retain antigenic determinants present on the intact enzyme. Chymotryptic peptides were isolated from incubation mixtures containing protoplasts of the constitutive strain 749/C or the uninduced strain 749 and were tested for their ability to inhibit the binding of [14C]exopenicillinase to antibodies to the exoenzyme.

The immunoassay utilized immunoglobulins to purified exopenicillinase (Table 1). The results of inhibition assays comparing the two peptide fractions are plotted in Fig. 4 as a

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Modified PrG medium</th>
<th>PrS medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trypsin + chymotrypsin</td>
<td>No additions</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>36</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>76</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>78 (0)</td>
<td>30 (0)</td>
</tr>
</tbody>
</table>

*Protoplasts were prepared in pH 6.5 protoplasting medium and resuspended at 1 mg cell d.w.e./ml in pH 7.5 modified PrG (0.4 M succinate, no Casitone) or PrS medium (0.75 M sucrose). Trypsin and chymotrypsin were added at 50 μg/ml each.*

*Results are expressed as the percent of the original membrane-bound enzyme (400 units/mg cell d.w.e.) liberated into the medium.*

*Values in parentheses are release of alpha-glucosidase (a measure of lysis).*
FIG. 5. Effect of trypsin on penicillinase synthesis. Protoplasts of B. licheniformis 749/C were resuspended in pH 7.5 PrG medium at 1 mg/ml, and the net increase in penicillinase (●) and percent of bound penicillinase (▲) were followed when the medium was supplemented with trypsin at (A) 200 μg/ml, (B) 100 μg/ml, (C) 50 μg/ml, (D) 10 μg/ml, and (E) no addition (control).

The rate of proteolytic removal of the mem-
brane penicillinase from intact protoplasts at pH 7.5 is dependent upon the medium used (Table 2). Thus, conversion of the bound penicillinase was 82% complete within 1 h (and typically by 30 min [2]) in PrS medium. In the same time span only 36% was released from protoplasts in modified PrG medium (no nitrogen source), although release reached 78% at 3 h. The catalytic activity of the proteases was the same in the two media (data not presented); hence, the slower rate of conversion in PrG is thought to reflect a stabilizing effect of succinate on the protoplast membrane. Sargent et al. (26) reported that protoplasts were not formed in succinate-stabilized medium and, as mentioned in Materials and Methods, succinate prevented the metabolic lysis of pH 9.5-treated protoplasts. The two carboxyl groups of the succinate may crosslink membrane components and thus provide increased structural stability.

Protoplasts tend to maintain a fixed level of bound penicillinase by preferentially retaining the nascent penicillinase as bound enzyme at the expense of exoenzymic formation (2, 15). In PrG medium with a level of trypsin just sufficient to prevent an increase in total penicillinase activity by stripped protoplasts, untreated protoplasts synthesize penicillinase faster (200 units per mg cell d.w.e. per h, Fig. 5E) than the trypsin removes the bound enzyme (110 units per mg cell d.w.e. per h, Table 2), and a constant level of membrane-bound enzyme is maintained. The restriction by protease of the accumulation of active enzyme is seen only when the amount of membrane enzyme has been substantially reduced (2); hence, at low protease levels the net synthesis of penicillinase by unstripped protoplasts continues unaffected.

One would expect, therefore, that since only the rate and not the eventual amount of penicillinase removal is reduced in PrG medium (Table 2), the restrictive effect could be produced with unstripped protoplasts by increasing the concentration of protease to a point at which net loss of bound enzyme would occur. Net increase of penicillinase activity should then halt. The results shown in Fig. 5 are in accord with this prediction. When unstripped protoplasts were incubated in PrG medium containing increasing amounts of trypsin, total penicillinase activity increased in each instance until the level of bound enzyme had been reduced by about half, at which point net increase in penicillinase activity abruptly ceased. We infer that a combination of bound penicillinase and other molecules in the outer face of the protoplast membrane ordinarily protects the initial form of penicillinase during its folding into the resistant conformation.

In B. licheniformis, apparently all the polyriboosomes are membrane associated (36) and we now envision penicillinase as being synthesized on membrane-bound polyribosomes and extruded directly into the membrane in a partially folded (protease-sensitive) conformation (28). While still in a protected environment, this initial form completes its folding to the protease-resistant conformation. Newly secreted enzyme may either be retained as membrane-bound penicillinase if a binding site is available (2, 15) or released as exoenzyme. Membrane penicillinase is less stable than exoenzyme to inactivation by heat or iodine (14, 31), indicating some conformational difference, although the hydrophobic nature of membrane penicillinase is probably due to a covalently bound phospholipid (9, 30, 30a).

Similar mechanisms have been postulated for the secretion of other prokaryotic exoenzymes (3, 4, 6), but the incompletely folded conformation has only been inferred, not directly demonstrated. The detection of the protease-sensitive form of penicillinase reported here represents the first positive identification of such a secretory form in a prokaryote.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-04572 from the National Institute of Allergy and Infectious Diseases. G. Bettinger held a postdoctoral traineeship under Public Health Service Training Grant GM-507 from the National Institute of General Medical Sciences.

LITERATURE CITED


