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Bacillus licheniformis β-lactamases: multiple forms and their roles

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B. licheniformis 749/C secretes a hydrophilic penicillinase (detected by immunoprecipitation) which is a precursor of the usually isolated 29500 molecular mass exoenzyme. This larger form carries an eight amino acid *N*-terminal extension with the sequence: Ser-Gln-Pro-Ala-Glu-Lys-Asn-Glu-exoenzyme (K. Izui, J. B. K. Nielsen, M. Caulfield & J. O. Lampen, unpublished results; K. Simons, personal communication). Translation of 749/C mRNA in an *in-vitro* protein synthesizing system from *Escherichia coli* yields an active hydrophobic penicillinase of molecular mass 34000–36000 with an *N*-terminal extension (C. N. Chang, K. Izui, G. Blobel & J. O. Lampen, unpublished results; M. Sarvas *et al.* (1978) *FEBS Lett.* **95**, 76). Partial sequence data show at least one Lys residue in the 16 residues adjacent to the *N*-terminal Lys of exoenzyme. Both sequences are incompatible with the relatively polar, Lys-free extension reported for the previously characterized 33000 molecular mass membrane-bound form (S. Yamamoto & J. O. Lampen (1976), *Proc. natn. Acad. Sci. U.S.A.* **73**, 1457–1461). The biosynthetic interrelations among the several forms are discussed.

About 10 years after Fleming's discovery of penicillin, Abraham & Chain (1940) reported that a strain of *Escherichia coli* that was relatively resistant to penicillin contained an enzyme that destroyed the antibiotic. It was soon evident that this enzyme can be an important element in clinical resistance to the β-lactams, especially in staphylococcal infections. Quite predictably, many microbiologists and biochemists have subsequently turned their attention to the properties of such enzymes and the factors controlling their biosynthesis.

Much of the basic work on the biochemistry and catalytic properties of the β-lactamases (penicillinases, cephalosporinases, etc.) has been carried out with the enzymes produced by *Bacillus cereus* or *Bacillus licheniformis*.

Strains of *B. licheniformis* freshly isolated from soil produce one or the other of two types of β-lactamase that are usually identified with the high-producing strains 749 and 6346. These enzymes are approximately the same size and show a high degree of sequence homology (Ambler & Meadway 1969); however, they differ in electrophoretic mobility and in their patterns of substrate specificity, although both are basically penicillinases (Pollock 1965). We have chosen to study the penicillinase of *B. licheniformis* 749 mainly because of one characteristic: only about one-half of the total penicillinase in an exponential phase culture is present in the culture fluid; the remainder is firmly bound to the cell, yet accessible to external substrate. Furthermore, strain 749 appears to contain a single structural gene for penicillinase and, consequently, for the production of the two distinct enzyme forms (Sherratt & Collins 1973).

The exoenzyme, as it has customarily been isolated, is a monomer of molecular mass 29500 with lysine residues at both *N*- and *C*-termini. It is extremely hydrophilic, lacks cysteinyl residues, is resistant to proteases in the native state, and refolds rapidly after denaturation by a variety of agents (Pollock 1965; Yamamoto & Lampen 1976*a*, *b*).

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The hydrophobic membrane-bound enzyme has an apparent molecular mass of about 33000 on the basis of Weber–Osborn sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis. It is eventually converted to the smaller extracellular form, although at acid pH the conversion is slower and a substantial fraction of the exoenzyme appears to be derived directly from the soluble amino acid pool and not from pre-existing membrane enzyme (Crane *et al.* 1973).

Nature of membrane penicillinase

Several years ago we characterized a form of the membrane penicillinase which differed from the 29500 exoenzyme in that it carried an additional 24 amino acid residues (which resembled a repeated tetrapeptide) and had a phosphatidylserine residue as its *N*-terminus (Yamamoto & Lampen 1976*c*). We cannot now detect this material in similar cultures, whether they are derived from current stock cultures or from stocks lyophilized as long ago as 1967. This is true for the inducible strain 749 and for the penicillinase-constitutive mutant 749/C. The material now isolated in Simons's laboratory (Simons *et al.* 1978), and in our own, lacks covalently bound phosphorus and the tetrapeptide-type chain extension. We have no specific basis for doubting the original results, but we cannot identify or reproduce the material that was sequenced 5 years ago. This problem remains under investigation.

The membrane penicillinase now being isolated is very similar to the 1976 enzyme: it is hydrophobic, has a molecular mass of about 32000 and its *N*-terminus is blocked. The purified material contains some phosphorus, but repeated chloroform–methanol extraction will bring the level down to about 0.2 atoms of phosphorus per molecule of enzyme. Treatment of this membrane penicillinase with trypsin releases several lysine residues rather than only one, as expected from the earlier sequence. In addition, a small amount of a phosphopeptide was isolated; this was probably a tightly bound impurity. An analogous phosphopeptide may have been present (in nearly stoichiometric amounts) in the earlier preparations.

Location of membrane penicillinase

The cell-bound penicillinase activity of *B. licheniformis* 749 appears to be associated with the outer surface of the plasma membrane. It is accessible to external substrate, and treatment of protoplasts with trypsin in the presence of chloramphenicol, can remove essentially all of the active enzyme. In addition, recent collaborative work with Davis' laboratory at Harvard (W. P. Smith, P.-C. Tai, B. D. Davis, K. Izui & J. O. Lampen, unpublished data) has revealed that the nascent penicillinase chains of strain 749/C protoplasts are accessible to a non-penetrating ^{125}I-labelled reagent. To identify these labelled chains, the membrane-bound polysomes were isolated, chain elongation was carried out in a typical run-off system, and the products were collected by precipitation with antibodies to purified exopenicillinase and separated by SDS gel electrophoresis. Labelled proteins corresponding in size to the exo- and membrane penicillinases and to a presumed precursor form (molecular mass *ca.* 36000) were obtained. Thus the nascent penicillinase chain spans the membrane during synthesis. After subsequent removal of an *N*-terminal segment, the resulting membrane enzyme remains attached to the outer membrane surface.

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Initial form secreted

The standard purified exoenzyme had been considered homogeneous but the newer discontinuous SDS gel procedures revealed two additional components larger than the characterized 29500 exoenzyme (apparent molecular masses of 30000 and 31000). To determine what form was initially secreted by the cell, exopenicillinase was harvested rapidly (by precipitation with specific antibodies) from cultures in early to mid-exponential growth phase. The only form detected was the hydrophilic 31000 molecule (K. Izui, J. B. K. Nielsen, M. Caulfield & J. O. Lampen, unpublished results). This material is slowly converted to the 29500 exoenzyme in older cultures or during the standard purification procedures. The 31000 exopenicillinase is produced by both inducible and constitutive strains and is released by chloramphenicol-treated protoplasts at pH 6.5–9.0. We conclude that this form, which we have named 'exo-large', is the initial species secreted. If a larger molecule is released, it must be broken down rapidly.

Exo-large penicillinase consists of the 29500 form that we now call 'exo-small' with eight additional amino acids at the *N*-terminus. The sequence as determined by automated Edman degradation is: Ser-Gln-Pro-Ala-Glu-Lys-Asn-Glu-[exo-small enzyme]. A minor component with glutamic acid rather than glutamine as the second residue appears to be identical with the 'exo-slow' obtained in Simon's laboratory (Simons *et al.* 1978). Most of the amino acids in this eight-residue extra piece are polar; consequently, exo-large is about as hydrophilic as the exo-small penicillinase.

In-vitro synthesis of penicillinase

To obtain direct information concerning the precursors of the several forms of penicillinase described so far, we turned to *in-vitro* synthesis of the enzyme. This was carried out in collaboration with C. N. Chang and G. Blobel of Rockefeller University. The system contained 30000 *g* supernatant (S_{30}) prepared from *E. coli* MRE600, RNA isolated from the constitutive penicillinase producer, *B. licheniformis* 749/C and, in some experiments, membrane vesicles prepared from strain 749/C as a source of processing enzymes. The reaction products were treated with antibody to purified exopenicillinase, adsorbed on staphylococcal cells and finally separated and detected by SDS gel electrophoresis and autoradiography. The major penicillinase formed is a hydrophobic protein of molecular mass approximately 35000 with f-Met as the *N*-terminal residue. This molecule is probably the primary translation product. Unfortunately it has not been possible to remove the formyl group selectively to permit direct sequencing. The translation product is, however, processed by the *B. licheniformis* vesicles to a hydrophilic form of molecular mass 31000 which includes a 16 amino acid extension beyond the *N*-terminus of exo-small enzyme.

Further information on the sequence of the translation product has been obtained from experiments with cells of *B. licheniformis* 749/C that had been treated briefly with phenylethyl alcohol at 0 °C to alter membrane fluidity. Such cells produce three forms of penicillinase: (1) a molecule the size of the primary translation product with methionine at the *N*-terminus but lacking the usual *N*-formyl group, (2) the hydrophobic membrane enzyme (molecular mass 32000), and (3) a hydrophilic form which is one residue shorter than the processed enzyme obtained with the *in-vitro* system.

On the basis of the available data, one can outline the general nature of the primary translation product of the penicillinase gene. The *N*-terminal region contains the f-Met and four

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lysines in the first 12 residues and is probably fairly hydrophilic. The catalytically active exo-small enzyme (270 residues) composes the bulk of the molecule and includes the *C*-terminus. Attached to exo-small is a 16 residue segment, 8 residues of which are present (and identified) in the exo-large form. The intervening section, of undetermined length, is almost certainly hydrophobic. A substantial portion of this region must be retained in the membrane enzyme, as isolated from the cell, if one is to account for its overall hydrophobic character.

Stages in processing of the primary translation product

The intact *B. licheniformis* cell has several pathways available for conversion of the initial translation product of the penicillinase structural gene to the ultimate form of the enzyme, i.e. the protease-stable exo-small penicillinase. Presumably synthesis occurs on membrane-associated ribosomes with concurrent insertion of the nascent chain into the membrane. Cleavage of the growing chain could yield either the hydrophilic 31000 enzyme (exo-small plus a 16 residue hydrophilic segment) or the typical membrane penicillinase (32000) which must retain an additional hydrophobic portion of the translation product. Membrane enzyme might in turn be cleaved to produce the 31000 enzyme, although there is no direct evidence for this. The existence of these two possible cleavage sites suggests that processing could bypass the membrane form. This might explain why pre-existing membrane enzyme was not a major precursor of new exopenicillinase molecules in cultures growing at pH 6.0 (Crane *et al.* 1973), but could readily be converted to exoenzyme at neutral or alkaline pH.

The 31000 enzyme is hydrophilic and might conceivably be secreted directly into the medium, although we have not been able to detect it there. Perhaps both this form and the membrane enzyme are processed in or at the surface of the plasma membrane to yield the exo-large enzyme (eight extra residues). The final step, i.e. conversion of the exo-large penicillinase to exo-small, takes place (in cultures of strain 749/C) only after a considerable amount of the exo-large enzyme has accumulated and coincides with the appearance of the appropriate hydrolytic activity in the culture fluid.

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