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Penicillinase from Bacillus licheniformis: nucleotide sequence of the gene and implications for the biosynthesis of a secretory protein in a Gram-positive bacterium

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ABSTRACT

The gene for the penicillinase from *B. licheniformis* has been cloned in a functional state on a 1.5 kb DNA fragment and its nucleotide sequence has been determined. A sequence of 307 amino acid residues is infered for the penicillinase precursor. Of these 34 amino acids precede the sequence of the secreted form of the enzyme. This peptide extension shows the features of a signal for secretion and also provides the hydrophobic anchor for the membrane-bound form of the enzyme.

INTRODUCTION

Many secretory proteins are synthesized as a larger hydrophobic precursor which is cleaved by membrane-bound proteases to the mature gene product (for reviews, see 1 and 2). The penicillinase from *B. licheniformis* provides an interesting system for studying such a processing pathway since several functionally active forms of this enzyme appear to be synthesized from a common larger precursor via successive proteolytic cleavage steps (2, 3, 4). Amino acid sequencing has revealed the structure of two closely related forms of the secreted exo-penicillinase: An "exo-small" enzyme of 265 amino acid residues (5, 6) and an eightamino acids larger "exo-large" form (7, 8). However, until very recently contradicting results were obtained as to the structure and the processing reactions of the larger membrane-bound form of the enzyme and of its primary precursor molecule. In order to avoid the difficulties encountered in analysing the highly hydrophobic and possibly modified terminal segments of these proteins we have determined the structure of the primary precursor indirectly from the nucleotide sequence of the cloned penicillinase gene. This provides for the first time the amino acid sequence of the precursor for a secretory protein from a Gram-positive bacterium.

MATERIAL AND METHODS

Bacterial strains and phages. *B. licheniformis* 749/C a constitutive mu-

tant of the penicillinase (9) and phage λ pen (15) were obtained from K. Simons. Phage fd11 (10) was used as single-stranded cloning vector. *E. coli* C600 m_K^+ r_K^- (Cold Spring Harbor collection) was used for DNA transformation and *E. coli* KB35 (N.D. Zinder) for phage fd multiplication.

Enzymes. Restriction enzymes were purchased from either commercial sources or prepared in our laboratory. Restriction reactions were carried out under conditions described by the suppliers, EcoRI* digestion in 25 mM Tris-HCl pH 8.5, 2 mM $MgCl_2$, 25 % glycerol (11).

DNA isolation. Single-stranded DNA from viral particles and double-stranded DNA from phage infected cells were prepared as described previously (10).

Construction of fd pen phages. 0.3 pmol of the 4.3 kb DNA fragment isolated from λ pen DNA and 0.1 pmol EcoRI digested fd11 DNA were ligated in 10 µl reaction mixture under standard conditions (10). Ampicillin transducing fd phages were isolated and tested for the relative orientations of the inserted 4.3 kb fragment as described in detail by Herrmann et al (10). An apparently identical 4.3 kb EcoRI fragment was cloned also directly from *B. licheniformis* chromosomal DNA in a shot gun experiment into a *B. subtilis/E. coli* vector plasmid (17). The 4.3 kb EcoRI fragment from both sources (λ pen and *B. licheniformis* DNA) was shortened by EcoRI* digestion and recloned into fd11. To enrich for fd pen phages with shortened genomes, which were expected to replicate faster (19), *E.coli* C600 cells were transformed with the ligation mixtures. Phage particles were collected from an over night culture and passaged three times in *E. coli* KB35 under ampicillin selection. Single isolates were plaque-purified from the phage pool and checked for the size of the DNA insert, possible EcoRI sites and for orientation of the penicillinase gene. All of the 20 isolated clones were found to be identical by these criteria. Thus, only one of them, designated fd pen1540, was used for further analysis. The shortened phage DNA possesses one EcoRI site upstream from the penicillinase gene which had been created from the EcoRI* site by cloning into the

Table 1: fd pen phages constructed and used for DNA sequencing

Ap-transducing fd phage	vector	insertion length (bp)	orientation*	source of the cloned penicillinase gene
fd pen4300-11	fd11	4300	+	λ pen
fd pen4300-12	fd11	4300	-	λ pen
fd pen1540-1	fd11	1540	+	*B. licheniformis* DNA

*plus (or minus) signs indicate that the penicillinase gene is transcribed in the same (or in the opposite) direction as the fd genes (see. Fig. 1).

EcoRI site of the vector DNA.

DNA fragments for restriction mapping and sequencing. Taking advantage of the available single strands of the penicillinase gene in the fd pen phages DNA fragments were often prepared from hybrid molecules between the DNA from phages which carry the gene in opposite orientation as shown in Fig. 1 for fd pen4300-11 and fd pen4300-12. Equal amounts of both phage DNAs were mixed in 50 mM Tris pH 8.0, 0.2 M NaCl to a concentration of 1 mg DNA/ml sealed in a capillary tube and heated to 100° C for 1 min. The capillary tube was then transferred into a beaker of water at 80° C and allowed to cool to room temperature within 30 - 60 min. After desalting by ethanol precipitation the double-stranded part of the DNA molecule could be cut from the hybrid with EcoRI or S1 nuclease or directly cleaved by many restriction enzymes. At a high DNA concentration (1 mg/ml) Hpa II selectively digested only the double-stranded part of the hybrid DNA (without loss of efficiency due to the presence of single-stranded DNA), whereas HaeIII attacked the whole molecule. To remove the single-stranded DNA fragments, the DNA solution was adjusted to 0.5 M NaCl, 50 mM EDTA and filtered through two layers nitrocellulose filters (Schleicher & Schüll, MF15, Ø 25 mm for 200µg of DNA). Using this method the cloned DNA insert or sub-fragments thereof can be prepared in large amounts and free from contaminating cellular DNA or vector DNA fragments.

Restriction mapping was carried out according to the method of Smith &

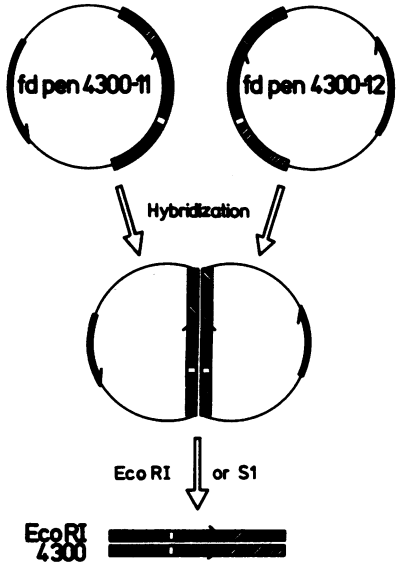

Fig. 1: The structure of the fd pen4300 phages and isolation of the double-stranded insert of the fd pen phage DNAs. The top of the diagram shows the viral DNAs of the fd pen phages. The cloned EcoRI 4300 fragments are indicated by the boxed sections. The open part corresponds to the NH_2-terminal peptide extension, the dark area to the exo-enzyme. The directions of transcription of the penicillinase gene and of the fd genome are indicated by the arrows. To prepare the EcoRI 4300 fragment the single-stranded viral DNAs were allowed to form a hybrid over the complementary insert. The double-stranded DNA segment was then cut out by EcoRI or S1 nuclease (see METHODS).

Birnstiel (12), which analyses the distribution of partially digested DNA fragments with only one labeled 5'-end, usually prepared by secondary cleavage of a double-stranded, 5'-labeled DNA fragment. To establish restriction patterns of the EcoRI 4300 fragment or sub-fragments thereof, we again took advantage of the available cloned single-strands of the insert in the viral DNA of the fd pen4300 phages. Instead of recutting the doubly end-labeled DNA fragment to be mapped we hybridized it, after partial restriction, to the fd pen4300-1 (or fd pen4300-12) DNA as described above. Thus, only the DNA fragments from one labeled DNA strand remained free and could be sized on a 4 % polyacrylamide gel, whereas the fragments of the complementary DNA strand, bound as hybrid molecules, stayed at the top of the gel. Depending on the DNA strand present in the phage DNA (fd pen4300-11 or -12) the restriction pattern from either 5'-end of the DNA fragment being analysed could be resolved in the gel. Using this method, the information from a single polyacrylamide gel was sufficient to establish the HaeIII map of the entire 4.3 kb EcoRI fragment.

DNA sequencing was carried out as described by Maxam & Gilbert (13). DNA fragments used for sequencing were isolated from the DNA inserts of the fd pen phages listed in Table 1. Nucleotide sequences from the individual sequence runs were stored, matched and processed using the computer programs of Osterburg & Sommer (14).

RESULTS

Cloning. The penicillinase gene has been cloned previously as part of a 4.3 kb DNA fragment from EcoRI digested *B. licheniformis* DNA in a λ phage vector (15). To simplify its analysis we recloned the DNA fragment in fd11, a small single-stranded fd vector, with a unique EcoRI cut in its fully sequenced genome (10, 16). fd pen phages, identified by their ability to transduce resistance to ampicillin, were isolated and shown to carry the 4.3 kb DNA fragment in either orientation (see METHODS, Fig. 1, and Table 1). Two phages with oppositely oriented inserts were isolated and designated fd pen4300-11 and fd pen4300-12 (Table 1). DNA from these phages was used for initial mapping of restriction sites within the cloned DNA fragment and for initial sequencing experiments (16).

In addition, the same 4.3 kb DNA fragment was cloned directly from EcoRI fragmented *B. licheniformis* DNA (17). This fragment was shortened further by EcoRI* digestion (18) and again cloned into the EcoRI site of fd11. Twenty ampicillin resistant subclones were isolated and characterized by sizing and

restriction analysis. All were found to contain the identical 1.5 kb DNA fragment in the same orientation as in phage fd pen4300-11 (17). This indicates that there exists a selection against clones containing the penicillinase gene in opposite orientation to the genes of the vector. This had also been noted as an enhanced lability of the DNA insert in fd pen4300-2 (16). In spite of the different sizes and orientations of the various cloned DNA fragments no significant differences were observed with regard to the low expression of the penicillinase gene in *E. coli* (15, 16). High levels of expression were obtained with all DNA fragments (4.3 and 1.5 kb) upon transfer into a *B. subtilis* host vector system (17). Since this result was independent from the orientation of the DNA inserts it indicates that the information contained in the 1.5 kb fragment suffices for expression of the penicillinase gene and also for formation of the exo-enzyme in bacillus.

Restriction mapping. Initial experiments were carried out with the fd pen 4300 phages. Using a modification of the Smith & Birnstiel procedure (12, and METHODS) a HaeIII map (Fig. 2) was established first since single-stranded

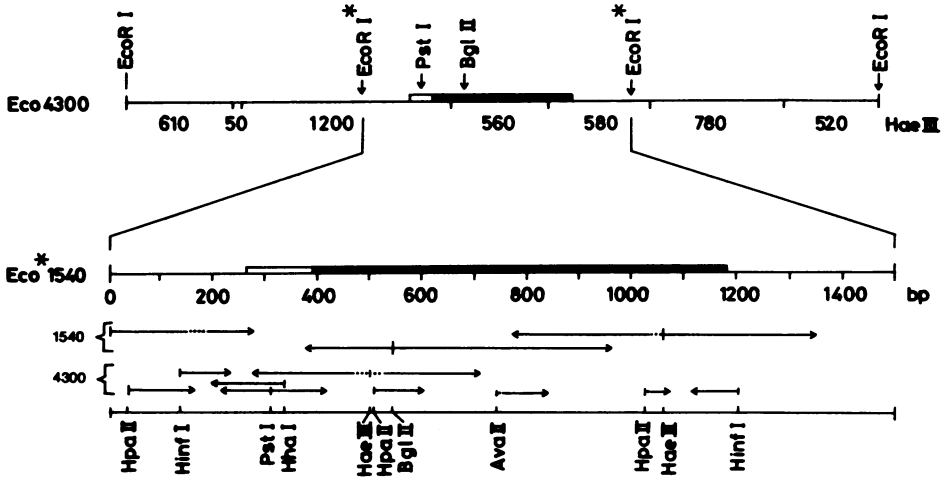

Fig. 2: Restriction map of the penicillinase gene region and a diagram of the sequenced DNA fragments. Vertical bars below the top line represent the HaeIII cleavage sites and the lengths of the fragments in base pairs. The penicillinase gene is indicated by a box, the shaded section corresponding to the exo-enzyme and the open section to the additional peptide extension at the NH_2-terminal part of the gene. The middle part of the figure delineates the gene on the shortened EcoRI*1540 fragment. The stretches of DNA sequenced are indicated by full arrows, dotted lines show non-sequenced regions. Nucleotide sequences derived from the EcoRI 4300 insert and from the shortened insert, EcoRI*1540, are distinguished.

DNA fragments created by this nuclease can be used directly for rapid DNA sequencing experiments. In addition, some unique cleavage sites, such as PstI and BglII, were mapped in the 4.3 kb DNA insert. For the 1.5 kb fragment a more detailed map of many different restriction sites was determined (16) some of which are included in Fig. 2.

DNA sequencing. Sequencing (13) was started using the HaeIII fragmented DNA single-strands from the fd pen4300 phages for 5' end-labeling which made secondary cleavage of the labeled DNA fragments unneccessary. This approach provided preliminary sequence information from various parts of the 4.3 kb DNA and allowed the rapid mapping of the penicillinase gene. A match observed between the known amino acids sequence at the NH_2-terminal part of exo-fast penicillinase (6) and the nucleotide sequence at the 5'-end of fragment HaeIII 1200 from fd pen4300-12 established the position and the orientation of the gene in the fd pen phages as shown in Figs. 1 and 2. This located the structural gene in the center of the 4.3 kb insert and also within the subsequently subcloned 1.5 kb EcoRI* DNA fragment. Further DNA sequencing was therefore confined to this region of the cloned *B. licheniformis* DNA.

A map of the cleavage sites used for 5' end-labeling and for sequencing, and the extent of the individual sequence runs obtained is shown in Fig. 2. Most of the sequence was either determined in both DNA strands (Fig. 2) or is confirmed by the known acid sequence of the exo-enzyme. Although parts of the sequence were determined using DNA inserts with a different history of isolation (see Table 1 and Fig. 2) no differences were observed between the separately derived sequences. This is consistent with the recent observation that the functional differences that exist between the seemingly identical 4.3 kb DNA fragments from λ pen and from *B. licheniformis* DNA reside outside of the 1.54 kb EcoRI* DNA fragment (Sprengel et al., in prep.). Altogether a continuous sequence of 1300 base pairs was established. The resulting composite nucleotide sequence is presented in Fig. 3.

DISCUSSION

We have cloned the penicillinase gene from *B. licheniformis* 749/C in a fully functional state on an 1.5 kb EcoRI fragment and have determined the nucleotide sequence of 1300 base pairs from this cloned DNA. Within this sequence starting from nucleotide 368 an amino acid sequence can be deduced which is in perfect agreement with the sequence determined for the 265 amino acids of the exo-small penicillinase (5,6) and for the additional 8 amino acids of the exo-large enzyme (7,8). As shown in Fig.3

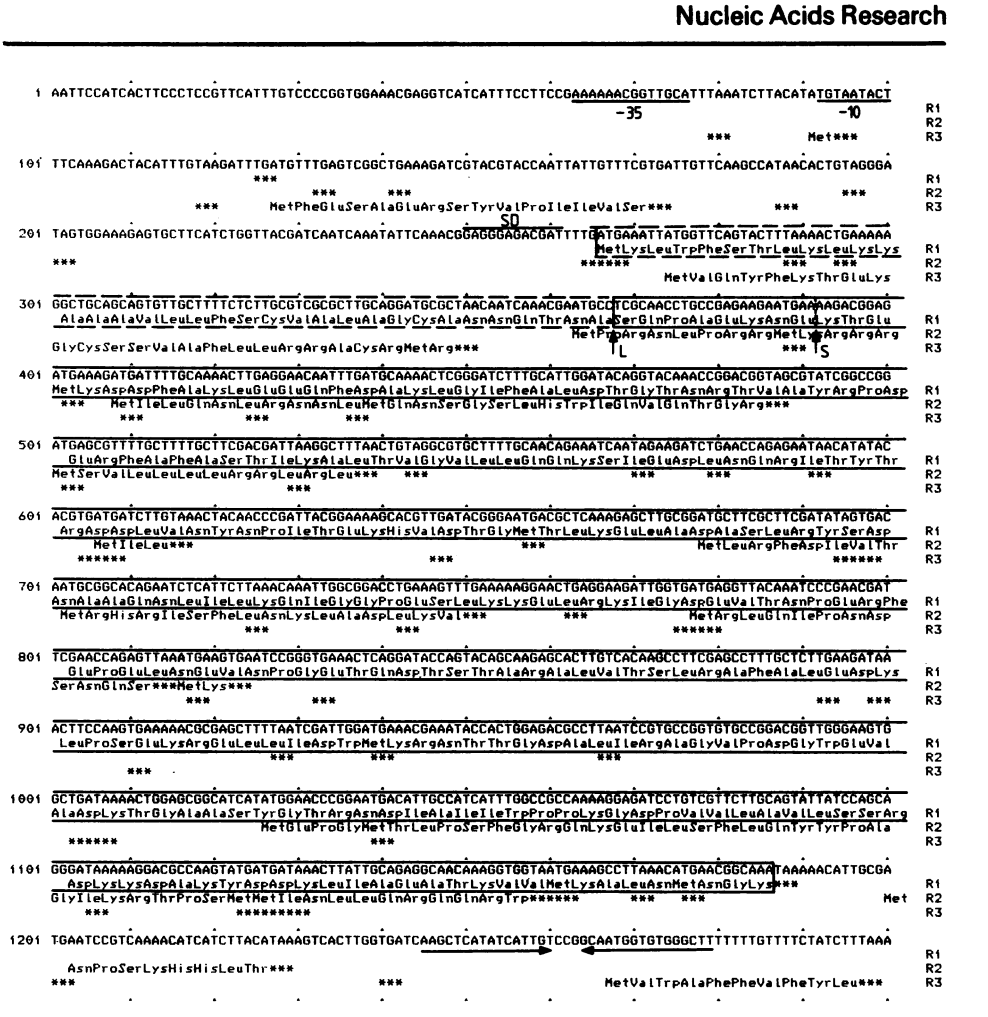

Fig. 3: Nucleotide sequence of the penicillinase gene and of adjacent DNA regions. Proteins derived from the nucleotide sequence are listed in frames R1, R2, and R3. The penicillinase gene and its gene products are boxed: a solid line borders the exo-enzymes, a broken line the NH_2-terminal peptide extension. The arrows indicate the processing sites for the primary (L) and secondary (S) exo-penicillinase cleavage products (7, 8). Nucleotide sequences homologous to regulatory signals for transcription and translation are indicated (see DISCUSSION).

and discussed below these secreted exo-enzymes appear to be derived from a precursor molecule containing 34 additional amino acid residues. No gene products of significant size appear to be encoded within the sequences that precede and that follow the structural gene. Instead, these regions contain sequences with the typical features of regulatory signals for gene

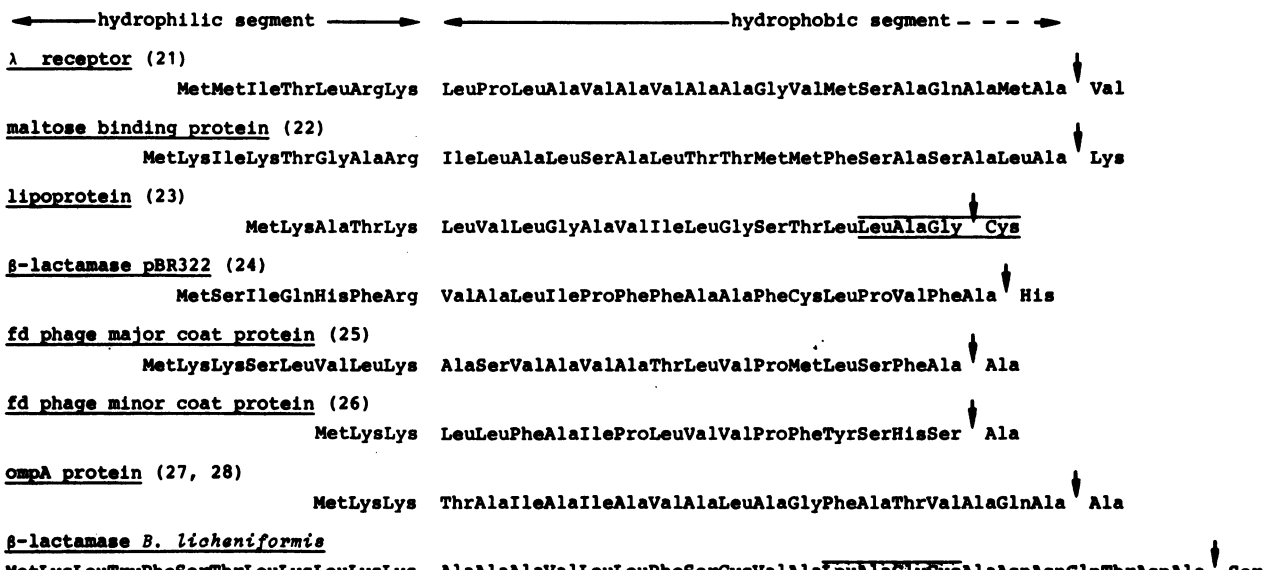

Fig. 4: Signal sequences of procaryotic proteins. The sequences are spaced according to the characteristic parts of the signal sequences, a charged hydrophilic segment followed by a hydrophobic one. Vertical arrows indicate the cleavage sites of the signal sequences. An identical amino acid sequence present in the signal sequences of the *E. coli* lipoprotein and of the β-lactamase from *B. licheniformis* (20) is framed.

expression in procaryotes: A long run of purine nucleotides around nucleotide 255 fulfils the requirements of a good Shine-Dalgarno (SD) sequence for translational initiation (29,30), sequences around nucleotide 100 show sequence homologies to the -10 and -35 consensus sequences of bacterial promoters (31), whereas an inverted repeat/oligo dT sequence around position 1250 shows all structural characteristics expected for a transcriptional termination signal (31). These data are in accordance with the observation that the 1540 bp DNA segment serves as an autonomous unit for expression of the penicillinase gene (17) and contradict the model of a polycistronic reading unit which has been suggested from genetic experiments (32).

Our sequence data also eliminate the hypothesis that processing at the carboxy-terminus of the precursor protein could be involved in the formation of the exo-enzyme (33), since the coding sequence for the penicillinase (Fig. 3) does not extend beyond the known carboxy-terminal amino acid sequence of the mature enzyme (6). Instead, our nucleotide sequence strongly suggests that the penicillinase is synthesized as a precursor protein with an NH_2-terminal peptide extension, as has been also suggested by the analysis of the products from *E. coli in vitro* system (3). The initiation of the primary gene product must occur at the ATG codon in position 266 since this is the only start codon that is in phase with the reading frame of the structural gene of the enzyme. Direct experimental evidence for this prediction has been obtained by S.N. Chang et al. (pers. comm.) who have analysed the positions of the lysine residues at the amino terminus of the precursor protein by Edman degradation of a radiolabeled *in vitro* product. The positions determined in this independent analysis (2, 9, 11, and 12) were found to agree exactly with those predicted from our nucleotide sequence. We therefore conclude that the penicillinase from *B. licheniformis* is synthesized as a precursor molecule with an extension of 34 amino acids at the NH_2-terminus of the exo-large penicillinase and that this extension is the target for all processing reactions that lead to the different forms of catalytically active enzyme. The existence of an NH_2-terminal signal peptide has been generally accepted on the basis of the structural analysis of the membrane penicillinase (4, 7) and of the precursor protein (3). However, attempts to determine its amino acid composition or its sequence did not give conclusive results and their interpretation has been the subject of very controversal views (2, 4, 7). The determination of the coding sequence for the signal peptide eliminates these uncertainties[1] and provides an unambiguous basis for a more direct analysis of the amphiphilic forms of the penicillinase.

Our results represent the first known amino acid sequence of a signal peptide that is involved in the export of a protein from Gram-positive bacteria. As peptide extensions from other secretory systems (1, 2) it starts with a hydrophilic segment (amino acid residues 1 - 12) followed by a hydrophobic segment (residues 13 - 28). However, as depicted in Fig. 4, the penicillinase signal sequence differs significantly from those from Gram-negative bacteria: The hydrophilic NH_2-terminal segment is longer and contains the exceptionally high number of four basic amino acids. In addition there is also a peptide extension beyond the hydrophobic core segment (residues 29 - 34) which other than in all other known bacterial systems separates the hydrophobic segment from the processing site. Similar features have very recently also been noticed in the signal sequence of the α-amylase from *B. amyloliquefaciens,* another secretory protein from the genus *Bacillus* (34). It seems therefore likely that these structural variations reflect modifications of the mechanism used for the secretion of proteins in Gram-positive or in Gram-negative bacteria: Firstly, the long, highly charged NH_2-terminus (see Fig. 4) may be needed to attach the growing penicillinase molecule to the inner surface of the cytoplasmic membrane as proposed by the "loop-mechanism" for transport of proteins across membranes (1), but it may be cleaved off at a later stage during the formation of the membrane-bound enzyme. Secondly, a similar dual role can be envisaged for the hydrophobic core segment of the signal peptide (residues 13 - 38) which in addition to its function as a guide for secretion may serve as an anchor of the membrane-bound protein. This view is supported by the hydrophobic properties of the membrane-bound penicillinase (2, 3, 4), which suggest that the hydrophobic part of the N-terminal extension is conserved in the membrane-bound molecule. Finally, the segment of the signal sequence spacing the hydrophobic core from the processing-site of the mature exo-penicillinase may be essential in Gram-positive bacteria to allow the proteolytic release of the soluble exo-form. This mode of biosynthesis implicates that the membrane-bound form serves as an essential intermediate for the secreted forms of the exo-penicillinase.

The penicillinase gene is only poorly expressed in *E. coli* (15, 16). This is surprising, since it functions quite well in a coupled *in vitro* system prepared from this microorganism (3). Furthermore, as discussed above, good initiation sequences for transcription and translation are present in appropriate distances from the NH_2-terminus of the structural gene. Finally, a high expression of an additional gene has been observed upon insertion into the penicillinase gene (unpublished results). One explanation for its never-

theless low expression is the finding that the penicillinase precursor is modified in *E. coli* at a site which shows sequence homology to the processing and modification site of the *E. coli* lipoprotein precursor (see Fig. 4) which leads to the export of a yet unidentified form of the protein to the *E. coli* outer membrane (20). In any case, there remains enough catalytically active enzyme to allow to use the penicillinase gene as a selective marker.

The expression of the penicillinase gene in *E. coli* and *B. subtilis* (17, 35) and the knowledge of its nucleotide sequence with several unique restriction sites makes the gene an attractive element for a double-vector system for these microorganisms. Together with the recent finding that the *B. licheniformis* penicillinase is synthesized and secreted in high yields from *B. subtilis* cells (17, 35) this should allow to construct expression vectors for the export of a fused gene product from a bacterial cell.

[1]The amino acid sequence of the signal peptide was first presented during a meeting on "Membrane Biogenesis", Cold Spring Harbor Laboratory, Cold Spring Harbor, USA, May 1979.

ABBREVIATIONS

bp, base pairs; Ap, ampicillin; pen, penicillinase; kb, kilobases.

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