JOURNAL OF BACTERIOLOGY, Jan. 1981, p. 422-428
0021-9193/81/010422-07$02.00/0

Vol. 145, No. 1

Molecular Cloning and Expression of *Bacillus licheniformis* β-Lactamase Gene in *Escherichia coli* and *Bacillus subtilis*

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The chromosomal β-lactamase (penicillinase, *penP*) gene from *Bacillus licheniformis* 749/C has been cloned in *Escherichia coli*. The locations of the target sites for various restriction enzymes on the 4.2-kilobase *Eco*RI fragment were determined. By matching the restriction mapping data with the potential nucleotide sequences of the *penP* gene deduced from known protein sequence, we established the exact position of the *penP* gene on the fragment. A bifunctional plasmid vector carrying the *penP* gene, plasmid pOG2165, was constructed which directs the synthesis of the heterologous β-lactamase in both *E. coli* and *Bacillus subtilis* hosts. The protein synthesized in *E. coli* and *B. subtilis* is similar in size to the processed β-lactamase made in *B. licheniformis*. Furthermore, the β-lactamase made in *B. subtilis* is efficiently secreted by the host into the culture medium, indicating that *B. subtilis* is capable of carrying out the post-translational proteolytic cleavage(s) to convert the membrane-bound precursor enzyme into the soluble extracellular form.

The inducible enzyme β-lactamase (penicillinase; EC 3.5.2.6) made by *Bacillus licheniformis* 749/C is found in two forms under normal growth conditions; more than half of the enzyme is in a secreted form (exoenzyme), and the rest is in a membrane-bound precursor form (29). The exoenzyme consists of at least two protein species which differ from each other at their amino-terminal regions. Simons et al. (34) have analyzed these two exoenzymes on sodium dodecyl sulfate-polyacrylamide gels and determined the amino-terminal sequences of the purified proteins; the fast-migrating protein (ExoF) appears to be the same as the 265-residue exoenzyme characterized by Ambler and Meadway (1). The slow-migrating protein (ExoS) contains eight additional amino acids at the amino terminus of the protein. The membrane-bound form of the enzyme has a hydrophobic leader sequence which is processed by proteolytic cleavage(s) to the soluble exoenzymes (30). Due to the difficulties of working with the insoluble hydrophobic leader peptide, its primary sequence has yet to be established unambiguously (10, 34). By molecular cloning and nucleotide sequencing of the β-lactamase gene, it should be possible to deduce the primary structure of the leader peptide from the DNA sequence (2, 37).

The β-lactamase of *B. licheniformis* is encoded by a single chromosomal gene. Dubnau and Pollock (14) isolated and characterized a large number of mutants with altered synthesis of β-lactamase. Genetic data (14, 33) indicate that the expression of the structural gene (*penP*) is regulated by the gene *penI*, which encodes for the repressor protein. These two genes are probably transcribed as a single polycistronic mRNA (19). The constitutive mutant *penI*(C), strain 749/C, shows high-level synthesis of β-lactamase and has been widely used for the study of protein secretion in bacteria (10).

We report here the molecular cloning and characterization of the *B. licheniformis* chromosomal fragment containing the *penP* gene. We also describe the functional expression of the cloned gene, as well as the post-translational processing of the β-lactamase protein in *Escherichia coli* and *Bacillus subtilis*.

MATERIALS AND METHODS

Strains, culture conditions, and transformation. *E. coli* CS412, an *hsdR* derivative of C600, was obtained from A. C. Y. Chang. *B. licheniformis* strains 749 and 749/C [*penI*(C)] (33), *B. subtilis* BD224 (*trpC2 thr-5 recE4*) (16), QB127 [*leu-8 trpC2 sacU200*(H)] (20), *E. coli* minicell-producing strain DS410 (13), *E. coli* plasmid pSC101 (8), and *B. subtilis* plasmids pC194 and pUB110 (16) have been described previously. Broth medium used was 2× LB (22) supplemented with thiamine hydrochloride to 5 μg/ml after autoclaving. Plates were prepared from Difco Penassay agar. Unless otherwise specified, antibiotics were supplemented to media for selection at the following final concentrations: ampicillin, 20 μg/ml; tetracycline, 15 μg/ml; chloramphenicol (Cm) and kanamycin (Km), 20 μg/ml each for *E. coli* cells and 5 μg/ml each for *B. subtilis*. Transformation of *E. coli* was carried out as described by Cohen et al. (9). The original plasmid transformation protocol for *B. subtilis* using protoplasts (6) was employed with modifications

made on the regeneration medium. Mannitol-based regeneration medium (A. Docherty, personal communication) consisted of: Casamino Acids, 0.5%; yeast extract, 0.5%; agar, 1.2%; gelatin, 2%; mannitol, 0.5 M; glucose, 0.5%; K_2HPO_4, 0.5%; $MgCl_2$, 20 mM. When needed, chloramphenicol was supplemented to 5 μg/ml and kanamycin was supplemented to 7.5 μg/ml in the regeneration medium for direct selection of *B. subtilis* transformants.

DNA manipulation. Plasmid DNA from *E. coli* and *B. subtilis* was prepared essentially as described (21). Restriction endonucleases were purchased from New England Biolabs and used according to the supplier's recommendations. T4 DNA ligase was prepared from *E. coli* carrying the cloned T4 ligase gene (23). Ligation of DNA by using phage T4 DNA ligase (23), agarose gel electrophoresis of DNA fragments (32), and recovery of DNA fragments from agarose gels (7) were as previously described.

Protein analyses. Plasmid-encoded proteins were labeled and prepared from *E. coli* minicells as described (13) except that the minimal medium of Vogel and Bonner (38), supplemented with required nutrients, was used. The same medium supplemented with 0.1% (vol/vol) broth medium and 0.1% (vol/vol) Novick's trace-metal solution (24) was used to grow *B. subtilis* and *B. licheniformis* for labeling exoproteins. L-[^{35}S]methionine (20 μCi) was added at mid-log phase, and cells were grown for 18 h to ensure incorporation of label into the exoenzyme. Cell-free culture fluid (2.5 ml) containing secreted proteins was dialyzed against deionized water and concentrated in the dialysis bag with externally applied molecular sieve granules (Fisher Co.). Rabbit antibody was raised against purified *B. licheniformis* β-lactamase exoprotein. Immunoprecipitation using heat-killed, formalin-fixed *Staphylococcus aureus* Cowan I (18), sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and autoradiography of the dried gels (26) were as described. The β-lactamase activity of bacterial colonies was measured on plates containing polyvinyl alcohol according to Sherratt and Collins (33); enzyme activity in solution was determined spectrophotometrically using nitrocefin (chromogenic cephalosporin 87/312, kindly provided by Glaxo Research Ltd.) by the method of O'Callaghan et al. (25).

RESULTS

Molecular cloning of the *B. licheniformis* β-lactamase gene in *E. coli*. From the known amino acid sequence of the *B. licheniformis* β-lactamase (1, 34), we were able to determine, using a computer program, that the recognition sequence of the restriction endonuclease *Eco*RI must be absent from the coding region of the gene. Based on the observation of Chang and Cohen (5) that the cloned *Staphylococcus* β-lactamase gene can be functionally expressed in *E. coli*, it was anticipated that transformants harboring the cloned *B. licheniformis* β-lactamase gene could be directly selected as ampicillin-resistant clones.

Endonuclease *Eco*RI-digested chromosomal DNA of *B. licheniformis* 749/C was mixed with *Eco*RI-digested *E. coli* plasmid pSC101, at a ratio of 2:1 (by weight), and ligated using T4 DNA ligase (23). Competent *E. coli* CS412 cells were prepared (9) and transformed using 1 μg of the ligated DNA. After 90 min of growth to allow for expression of plasmid-encoded proteins, samples were plated and scored for transformation efficiency; it was calculated that a total of 2.2×10^4 tetracycline-resistant clones were obtained. The remaining portion of the transformed culture was grown in broth containing 10 μg of ampicillin per ml. After overnight incubation, ampicillin-resistant cells were streaked onto plates containing ampicillin (20 μg/ml), and four ampicillin-resistant clones were selected at random. Gel analysis of the *Eco*RI-digested plasmid DNAs prepared from these clones showed that they all contained inserts of identical size. One of the plasmids (pTB2) was studied further. Upon retransformation into *E. coli* CS412, pTB2 was found to confer upon the host resistance to both tetracycline and ampicillin, indicating that the ampicillin-resistant phenotype is associated with the inserted fragment. We were able to detect low-level production of β-lactamase by the ampicillin-resistant transformants by using the polyvinyl alcohol-plate method for assaying the enzyme (33) (Fig. 1).

Characterization of the cloned *B. licheniformis* β-lactamase gene. A restriction map of the 4.2-kilobase (kb) *Eco*RI fragment in pTB2 was determined by using various hexanucleotide-recognizing restriction endonucleases (Fig. 2 and 3). Restriction endonucleases *Bal*I,

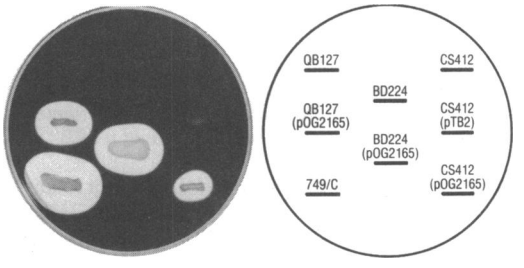

FIG. 1. *Assay for β-lactamase activity on polyvinyl alcohol plate. Bacterial cells from different strains were streaked onto a polyvinyl alcohol plate at the locations shown. After overnight incubation at 37°C, the plate was flooded sequentially with iodine and penicillin. Penicillin is converted enzymatically to penicilloic acid, which specifically bleaches the dark-colored iodine-polyvinyl alcohol complex. The presence of a clearing zone surrounding the colony indicates the production of β-lactamase enzyme by the strain (33).*

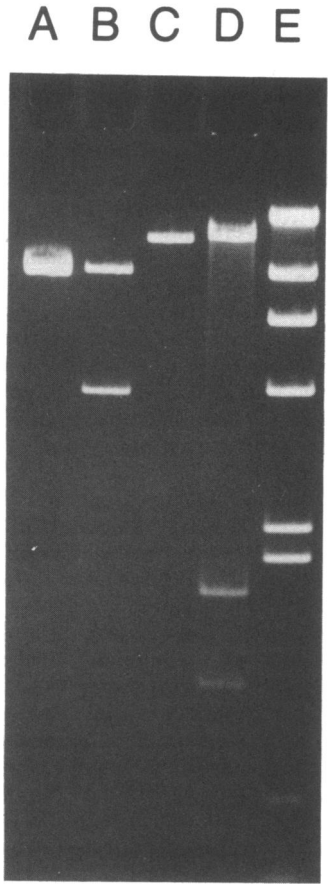

FIG. 2. *Restriction endonuclease analyses of various plasmid DNAs by agarose gel electrophoresis. Fragments generated by endonuclease EcoRI from plasmids (A) pSC101 and (B) pTB2 and by endonuclease PstI from plasmids (C) pTB2 and (D) pOG2054 were separated on a 1% agarose gel using Tris-acetate buffer for 16 h at 30 V. Lane (E) is HindIII-digested λ cI857 S7 DNA as molecular weight markers. Plasmid pOG2054 is an ampicillin-sensitive derivative of pTB2 containing three PstI fragments of coliphage λ inserted at the PstI site of pTB2.*

*Bam*HI, *Sal*I, *Sst*II, and *Xho*I did not cleave this fragment.

The known sequence of the exoprotein contains the dipeptides Gly-Pro and Trp-Pro at positions 125–126 and 230–231, respectively (amino acid positions are numbered from the first amino-terminal residue of the ExoS protein as reported by Simons et al. [34]). The corresponding coding sequences, G-G-N-C-C-N and T-G-G-C-C-N, must contain the restriction sites for *Sau*96I (G-G-N-C-C) and *Hae*III (G-G-C-C), respectively. The dipeptide Ser-Thr at position 52–53 could be encoded by a nucleotide sequence containing a *Taq*I site (T-C-G-A), and the tripeptide Asp-Leu-Asn at position 69–71 could be encoded by a nucleotide sequence containing a *Bgl*II site (A-G-A-T-C-T). We therefore digested pTB2 with these enzymes and mapped the *Sau*96I, *Hae*III, and *Taq*I sites adjacent to the unique *Bgl*II site. As shown in Fig. 3, the locations of the mapped sites are in excellent agreement with the distances predicted from the protein sequence. We recently obtained partial sequencing data of 60 nucleotides from the coding region, TTA GAG GAA CAA TTT GAT GCA AAA CTC GGG ATC TTT GCA TTG GAT ACA GGT ACA TAC CGG (Kroyer and Chang, unpublished data), which corresponds to the sequence of the 20 amino acids at position 19–38 of the β-lactamase exoenzyme. From these data, we were able to locate and determine the orientation of the cloned *B. licheniformis* β-lactamase gene (Fig. 3).

Construction of plasmid vectors containing the *B. licheniformis* β-lactamase gene in *E. coli* and *B. subtilis*. The 3.5-kb *Eco*RI-*Sst*I fragment containing the β-lactamase gene was purified and ligated with a 2.1-kb *Eco*RI-*Sst*I fragment containing the replication function of the *E. coli* plasmid pOPIΔ6 (15). Upon transformation into CS412 cells and a subsequent 90 min of growth to allow expression, ampicillin-resistant transformants were obtained. Plasmids from three clones were prepared and found to have the identical size of 4.3 kb, indicating that a deletion of 1.3 kb had occurred in each case. One of the plasmids, pOG2110, was further characterized. The locations of the expected restriction sites and the observed sites are shown in Fig. 4. The deletion covers the *Eco*RI site at the junction and the two *Pvu*II sites flanking the *Hin*dIII site in the *B. licheniformis* fragment.

To allow for replication of pOG2110 in *B. subtilis*, it was used to construct a bifunctional replicon using pOG2110 and the *B. subtilis* plasmid pOG1196. The construction of pOG1196 is summarized in Fig. 4. We initially made a chimeric plasmid (pCS832) containing the entire sequences of plasmids pC194 (Cm) and pUB110 (Km) by ligating the two *Mbo*I fragments of pC194 with *Bam*HI-digested pUB110. The resulting plasmid, which carries both the chloramphenicol resistance gene from pC194 and the kanamycin (neomycin) resistance gene from pUB110, has a size of 7.5 kb. A spontaneous deletion mutant (plasmid pCS1006) was obtained from one of the subclones. It had lost the *Hpa*II site originated in pC194, which is known to be located in the pC194 replication region (6), but still retained the replication function of pUB110 and the two resistance markers. By

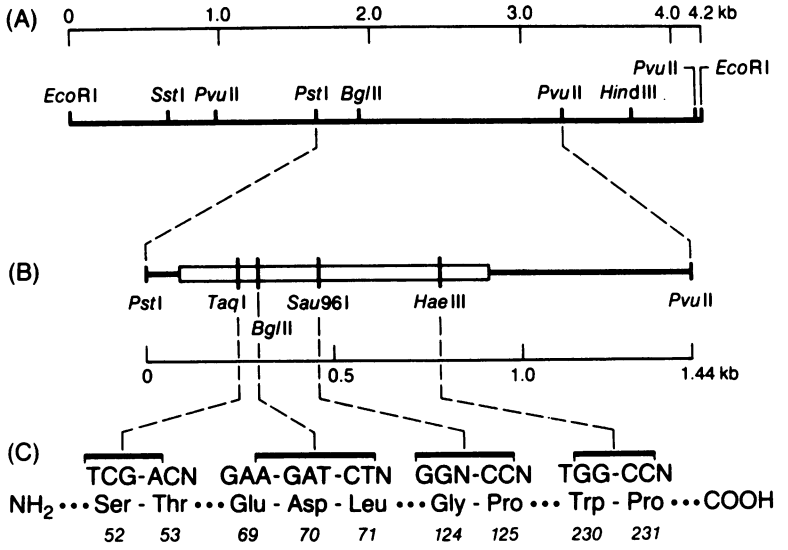

FIG. 3. *Restriction map of the β-lactamase fragment cloned in plasmid pTB2. (A) Map location of target sites for various hexanucleotide-recognizing endonucleases on the fragment. (B) Location of the TaqI, Sau96I, and HaeIII sites adjacent to the unique BglII site; the boxed area represents the coding region for the β-lactamase exoprotein. (C) Regions of the nucleotide sequences in the coding region, of which each matches both the corresponding amino acid sequences in the β-lactamase molecule and the recognition sequences of the respective restriction endonucleases (underlined sequence).*

recircularizing the largest *Hpa*II fragment (3.6 kb) of pCS1006, we obtained plasmid pOG1196, which confers only chloramphenicol resistance and possesses the replication function derived from plasmid pUB110. The map of pOG1196 is shown in Fig. 4.

E. coli plasmid pOG2110 and *B. subtilis* plasmid pOG1196 contain two and three *Pvu*II sites, respectively. Equal amounts of *Pvu*II-digested pOG2110 and pOG1196 plasmid DNA were ligated and used to transform *E. coli* CS412. Chloramphenicol-resistant clones were selected; all were also ampicillin resistant. The composite plasmid pOG2165, isolated from one of the chloramphenicol- and ampicillin-resistant transformants, was studied further. A map of this 7.5-kb plasmid is shown in Fig. 4. Plasmid pOG2165 replicates in both *E. coli* and *B. subtilis* and confers upon either host both chloramphenicol and ampicillin resistance.

Analysis of β-lactamase production in *E. coli* and *B. subtilis*. *B. subtilis* and *E. coli* cells harboring plasmid pOG2165 are resistant to ampicillin as a result of the production of the *B. licheniformis* β-lactamase enzyme. This can be shown by using the polyvinyl alcohol-plate assay (Fig. 1). The β-lactamase produced in *E. coli* minicells was immunoprecipitated by using rabbit antiserum and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (see Materials and Methods). As shown in Fig. 5A, a 29-kilodalton protein is made in *E. coli* (lane A-III) which is similar in size to the 749/C β-lactamase exoenzyme (lane A-II). A slightly higher-molecular-weight form protein was also detected in *E. coli* minicells when the 15-min chase step after the labeling was omitted (not shown). This could be the precursor β-lactamase with the leader sequence on it (31).

When pOG2165 was propagated in *B. subtilis* BD224, both the membrane-bound and the secreted form of the heterologous β-lactamase were synthesized. The amount produced by this strain is variable and depends on the growth conditions used. In early-stationary-phase culture containing 2×10^8 colony-forming units per ml, we were able to measure β-lactamase activity comparable to 10 μg/ml. From 60 to 80% of the enzyme activity was detected in the medium. This level represents 10^6 molecules per colony-forming unit. Thus *B. subtilis* cells can efficiently process the membrane-bound precursor of the *B. licheniformis* β-lactamase and secrete it into the medium. Lampen (personal communication) has found, in *B. subtilis* cell extracts, protease activities which can carry out the proteolytical cleavage of the *B. licheniformis* β-lactamase precursor to the secreted form. These protease activities observed in vitro may be also responsible for the conversion of the β-lactamase precursor to the secreted form in vivo. We have also tested the production of *B. licheniformis*

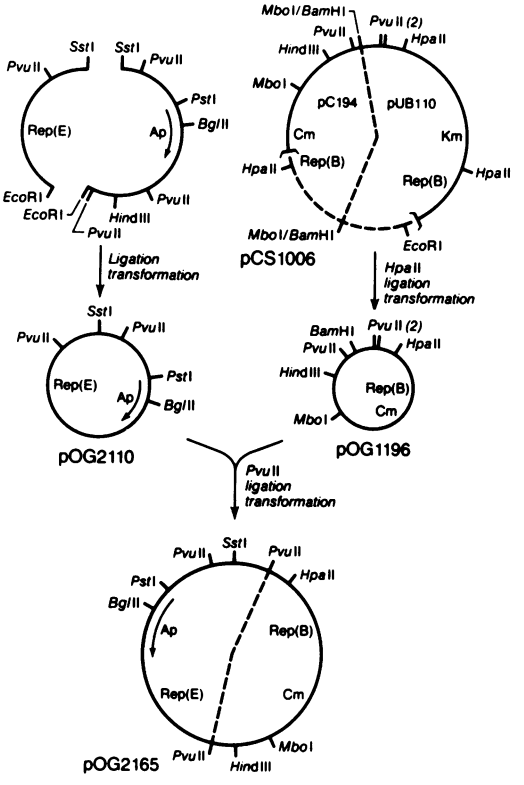

FIG. 4. *Schematic illustration of the construction of the bifunctional plasmid pOG2165. E. coli plasmid pOG2110 (4.3 kb), B. subtilis plasmid pOG1196 (3.6 kb), and the bifunctional plasmid pOG2165 (7.5 kb) were constructed as described in the text. The locations of target sites for various hexanucleotide-recognizing enzymes are marked; only some of the MboI and HpaII sites are shown here. Regions coding for plasmid replication functions in E. coli and B. subtilis are marked as Rep(E) and Rep(B), respectively. The arrows indicate the location and the direction of transcription of the β-lactmase gene. Dashed line in brackets shows the region deleted from pCS832 in vivo giving rise to plasmid pCS1006 (see the text).*

β-lactamase in *B. subtilis* strain QB127 (20, 36), a strain with the *sacU*(H) mutation which causes overproduction of several exoenzymes such as levansucrase, α-amylase, and extracellular proteases. The level of β-lactamase detected in the culture of QB127(pOG2165) was similar to that in BD224(pOG2165) culture under the same conditions.

The secreted β-lactamase made by *B. subtilis* strain BD224(pOG2165) was also immunoprecipitated and analyzed on a sodium dodecyl sulfate-polyacrylamide gel. Two protein species were barely resolved; the fast-migrating one, produced by *B. subtilis*, had an electrophoretic mobility similar to the minor form of the secreted β-lactamase produced by *B. licheniformis* 749/C (Fig. 5B). The respective slow-migrating β-lactamase bands produced in these two bacilli showed different electrophoretic mobilities which probably reflect the differences in the

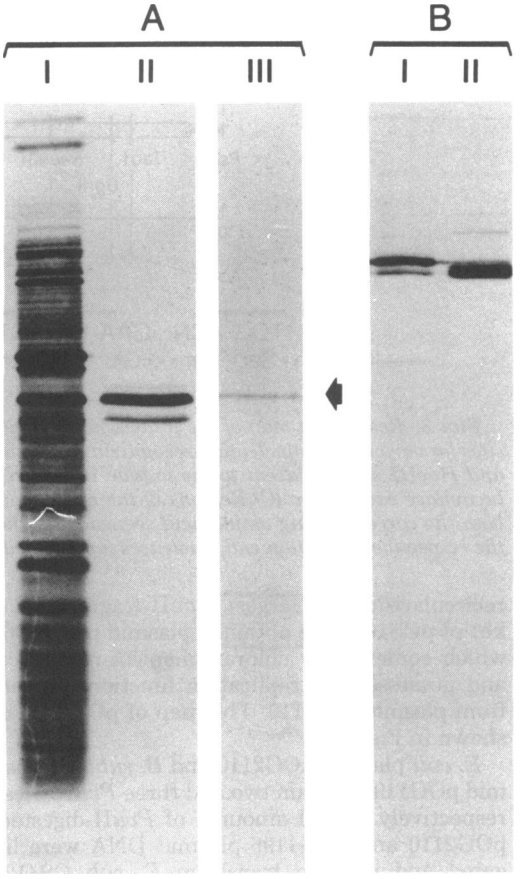

FIG. 5. *Gel electrophoresis and autoradiography of [^{35}S]methionine-labeled protein synthesized in vivo. The position of the unlabeled β-lactamase exoprotein (indicated by the arrow) was detected by staining with Coomassie brilliant blue R-250. Lane A-I is the total labeled exoprotein prepared from B. licheniformis 749/C as described in the text. Lane A-II shows the immunoprecipitate obtained from the sample in lane A-I by using antibody against β-lactamase exoprotein. Lane A-III shows a similar immunoprecipitate obtained from labeled protein prepared from minicells of E. coli DS410(pOG2110). Lane B-I contains the same sample as in Lane A-II. Lane B-II shows the immunoprecipitate obtained from labeled exoprotein prepared from BD224(pOG2165). Control serum did not precipitate detectable amounts of β-lactamase protein from B. licheniformis 749/C, BD224(pOG2165), or DS410(pOG2165) samples. Slab gels of 15% acrylamide-sodium dodecyl sulfate were used to resolve the bands. Lane A-III is derived from the same gel as A-I and A-II except that it was exposed longer than other samples due to the presence of lower amounts of labeled material in this track.*

specificity of the protease(s) involved in the cleavage process.

DISCUSSION

We report here the molecular cloning and expression of the β-lactamase (*penP*) gene of *B. licheniformis* 749/C in both *E. coli* and *B. subtilis*. From the mapping data on the location of various restriction sites within the cloned DNA fragment and the preliminary DNA sequence data, we were able to assign the specific position of the *penP* coding sequence on the fragment. Using genomic hybridization technique (35), we have found that the cloned fragment on pTB2 is similar in size to the chromosomal *Eco*RI fragment containing the gene. Brammar et al. recently reported the cloning of the *B. licheniformis Eco*RI fragment containing the β-lactamase gene in phage λ (4). Based on the reported size of the cloned λ *pen* fragment, it appears to be identical to the one which we obtained. On the *B. licheniformis* chromosome, the repressor gene *penI* is known to be closely linked to *penP*. Because frameshift mutation at the carboxy terminal of *penP* gene exerts a strong polar effect on the expression of the *penI* gene, both genes are likely transcribed into a single polycistronic mRNA from *penP* to *penI* (19). With the availability of the present clone as a probe, it should be possible to study this operon more closely.

The cloned β-lactamase gene is found to be functionally expressed in *B. subtilis*. *B. subtilis* is a powerful producer of a number of important extracellular enzymes (27). These proteins are secreted by the cells in the true sense since they can be recovered directly from cell-free culture media, whereas most proteins "secreted" by the gram-negative *E. coli* are actually trapped in the periplasmic space due to the differences in cell envelope structure (28). Indeed, we observed that the majority of the heterologous β-lactamase molecules made in *B. subtilis* cells are translocated across the membrane and subsequently processed proteolytically to form the exoenzyme. Based on the facts that the enzyme is produced in large quantity and that a major fraction of β-lactamase is present as soluble enzyme, *B. subtilis* cells apparently carry out the translation/translocation and the post-translational processing events with very high efficiency.

Protein translocation and secretion across membranes is an important biological phenomenon (reviewed in reference 11). Based on the signal hypothesis proposed by Blobel and Dobberstein (3), a discrete portion of the peptide sequence (signal sequence) contains the information for the translocation event. To explain the mechanism in detailed molecular terms, Steiner et al. (β-transposition hypothesis; 35a) and Inouye et al. (loop model; see references 12 and 17) independently postulated the specific sequences and structural features that the signal peptide must posses in order to accomplish the translocational event. We are currently using the cloned fragment to determine the DNA sequence of the amino-terminal coding region, including the signal (leader) peptide. The peptide sequence deduced from these data should provide further insight towards better understanding of the mechanisms involved in protein secretion.

The bifunctional plasmid pOG2165 possesses unique sites for the restriction enzymes *Sst*I, *Hin*dIII, *Pst*I, and *Bgl*II. Insertion of DNA into the *Bgl*II and *Pst*I sites leads to inactivation of the *B. licheniformis* β-lactamase gene and provides an easily recognizable phenotype for identifying clones carrying inserts. In addition, knowing the exact reading frame of the DNA sequence to be inserted, it is possible to create a fused protein containing the leader sequence and the first 71 amino acid residues of the β-lactamase exoenzyme by cloning into the *Bgl*II site; the fused proteins made this way may thus be secreted by *Bacillus* cells due to the presence of the leader sequence at the amino terminal. These features make pOG2165 a useful vector for the cloning and efficient expression of heterologous genes and the subsequent secretion of the gene products in *B. subtilis*.

ACKNOWLEDGMENTS

The technical assistance of H. Baney at the initial stage of this work is acknowledged. We are grateful to our colleagues for their help, constructive comments, and encouragement, and to E. Jarvis for her excellent secretarial assistance. Jack Nunberg made many helpful suggestions for improvement of the manuscript.

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