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Molecular Cloning and Expression in *Escherichia coli* of the *Bacillus licheniformis* Bacitracin Synthetase 2 Gene

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The structural genes for the entire bacitracin synthetase 2 (component II) and for a part of the putative bacitracin synthetase 3 (component III) from *Bacillus licheniformis* ATCC 10716 were cloned and expressed in *Escherichia coli*. A cosmid library of *B. licheniformis* DNA was constructed. The library was screened for the ability to produce bacitracin synthetase by in situ immunoassay using anti-bacitracin synthetase antiserum. A positive clone designated B-15, which has a recombinant plasmid carrying about a 32-kilobase insert of *B. licheniformis* DNA, was further characterized. Analysis of crude cell extract from B-15 by polyacrylamide gel electrophoresis and Western blotting (immunoblotting) showed that the extract contains two immunoreactant proteins with high molecular weight. One band with a molecular weight of about 240,000 comigrates with bacitracin synthetase 2; the other band is a protein with a molecular weight of about 300,000. Partial purification of the gene products encoded by the recombinant plasmid by gel filtration and hydroxyapatite column chromatography revealed that one gene product catalyzes L-lysine- and L-ornithine-dependent ATP-PP_i exchange reactions which are characteristic of bacitracin synthetase 2, and the other product catalyzes L-isoleucine-, L-leucine, L-valine-, and L-histidine-dependent ATP-PP_i exchange activities, suggesting the activities of a part of bacitracin synthetase 3. Subcloning experiments indicated that the structural gene for bacitracin synthetase 2 is located near the middle of the insert.

Bacitracin A (Fig. 1), produced by some strains of *Bacillus licheniformis*, is one of the antibiotic polypeptides whose biosynthetic mechanism has been intensively studied (6). It is synthesized by the three multifunctional enzymes, that is, bacitracin synthetases (BA) 1, 2, and 3, which were referred to as components I, II, and III, respectively, in our previous paper (9).

BA1 activates the five amino acids contained in the linear peptide part of the bacitracin molecule, and its molecular weight is about 335,000 (7). BA2 activates L-lysine and L-ornithine, and its molecular weight is about 240,000 (12). BA3 activates the remaining five amino acids contained in the cyclic peptide part of bacitracin, and its molecular weight is about 380,000 (9). Recently, several genes from *Bacillus* species encoding part of the antibiotic polypeptide synthetases were cloned in *Escherichia coli*. In the case of the tyrocidine synthetase 1 gene, the entire structural gene was cloned and expressed in *E. coli* (17). In gramicidin S (13) and bacitracin synthetase (12) genes, parts of the structural genes were cloned by using *E. coli* expression vectors. We have been interested in isolating the genes for bacitracin biosynthesis to study their organization and regulation. As the total molecular weight of bacitracin synthetase is about 10^6, a stretch of at least 26 kilobase pairs (kb) of DNA fragment is required to cover all of the synthetase genes if the genes for the three component enzymes are located in a cluster, as shown in genes for the production of antibiotics in *Streptomyces* species. Because of the instability in *Bacillus* cells of plasmid vectors having a large insert and the necessity of cloning large DNA fragments, we chose an *E. coli* cosmid vector as a cloning vehicle. In this paper, we report the cloning of a DNA fragment encoding the entire gene for BA2 and a part of the putative gene for BA3 from *B. licheniformis* and their expression in *E. coli*.

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MATERIALS AND METHODS

Bacterial strains and vectors. The bacitracin-producing microorganism *B. licheniformis* ATCC 10716 was used for the isolation of chromosomal DNA and bacitracin synthetase. The *E. coli* strains LE392 (16) and JM109 (23) were used as cloning hosts. pKY2662 (8) carrying an ampicillin resistance gene was used as a cosmid vector. pUC18 (23) was used as a vector for subcloning experiments.

Media and growth conditions. *B. licheniformis* was grown in a medium (pH 7.2) containing 10 g of meat extract, 10 g of peptone, and 2.5 g of NaCl per liter. *E. coli* LE392 and JM109 were cultured in LB broth (16) or on LB agar. Transductants of LE392 were selected on LB agar containing 50 μg of ampicillin per ml. Transformants of JM109 were selected as white colonies on LB agar containing 50 μg of ampicillin per ml, 0.1 mM isopropylthiogalactoside, and 40 μg of 5-bromo-4-chloroindolyl-β-D-galactoside per ml.

Isolation of chromosomal and plasmid DNAs. Chromosomal DNA was isolated from *B. licheniformis* as described by Rodriguez and Tait (20). Cosmid and recombinant plasmid DNAs were prepared by the method of Marko et al. (19).

Preparation of cosmid library. *B. licheniformis* DNA was partially digested with *Sau*3AI to achieve an average fragment size of 30 to 40 kb. These fragments were ligated into the *Bam*HI site in the pKY2662 cosmid vector, packaged in vitro, and transfected to *E. coli* LE392 by the method described by Maniatis et al. (16).

Purification of antigen (BA3 protein) and preparation of antiserum. Bacitracin synthetase was partially purified from *B. licheniformis* cells through the hydroxyapatite column chromatography step of the purification procedure described previously (10). The peak 2 fraction, which contained BA2 and BA3, from the hydroxyapatite column chromatography was further purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (14). After electrophoresis, the gel was stained with

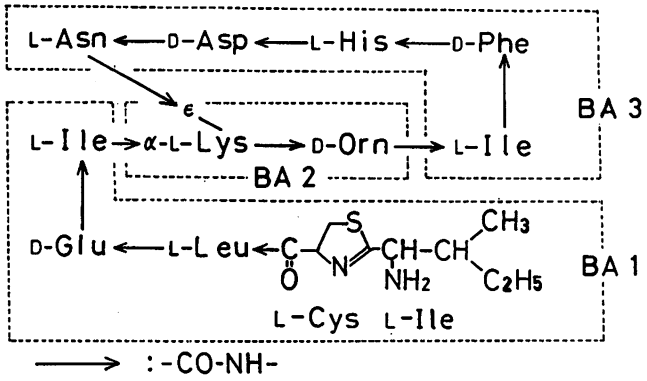

FIG. 1. Structure of bacitracin A. The broken lines box the amino acids activated by the three complementary BAs 1, 2, and 3.

Coomassie brilliant blue, destained in 5% methanol containing 10% acetic acid, and washed with 50 mM potassium phosphate buffer (pH 7.2). Then, the region containing the BA3 protein band was cut out from the gel and homogenized in water in a Potter homogenizer. About 1 ml of homogenized gel suspension containing about 0.1 mg of protein was emulsified in 1 ml of Freund adjuvant (Difco Laboratories). A rabbit was immunized by multiple injections, first into the toe pads with complete Freund adjuvant (Difco) and then subcutaneously with incomplete Freund adjuvant (Difco). Booster injections were administered five times at about 3-week intervals. Blood was collected 1 week after the last injection and serum was clarified by centrifugation at 8,000 × *g* for 20 min. The antibody raised against BA3 protein cross-reacted with BA1 and BA2 proteins (Fig. 2D).

Screening of transductants. Transductants were screened for the production of BA antigens by using the antiserum and ^{125}I-protein A (specific activity, 40 mCi/mg; Amersham Corp.). Transductants were grown overnight at 37°C on a nitrocellulose filter (TM-2, Toyo) overlaid on LB agar containing ampicillin (50 μg/ml). The filters were removed and placed for 15 min in an atmosphere saturated with $CHCl_3$ vapor to lyse the cells. The filters were immersed in 50 mM Tris hydrochloride (pH 8.0) containing 100 mM NaCl, 10 mM EDTA, and lysozyme (5 mg/ml) for 30 min at room temperature. After being blotted with a paper towel, the filters were incubated for 30 min at 37°C with 20 mM Tris hydrochloride (pH 8.0) containing 10 mM $MgCl_2$ and DNase I (1 μg/ml). The filters were incubated for 30 min at room temperature in TSS solution (20 mM Tris hydrochloride [pH 7.5] containing 0.1 M NaCl, 0.01% NaN_3) containing 5% skim milk and then treated for 3 h at room temperature with the antiserum diluted 1:50 with the same solution. After the filters were washed five times with TSS solution containing 0.5% skim milk, the bound antibody was allowed to react with 1 μCi of ^{125}I-labeled protein A in 3 ml of TSS solution containing 5% skim milk. After 1 h of incubation, the filters were washed five times in TSS solution containing 0.5% skim milk at room temperature and were subjected to autoradiography.

Preparation of crude cell extracts. *E. coli* cells were grown overnight at 37°C in 5 ml of LB broth containing 50 μg of ampicillin per ml, washed once, and suspended in 0.15 ml of 50 mM Tris hydrochloride (pH 7.2) in an Eppendorf tube. The crude cell extract (about 9 mg of protein per ml) was prepared by sonication for 30 s at 10 W with the microtip of a Sonifier (Branson Sonic Power Co.) and centrifugation at 10,000 × *g* for 20 min.

SDS-PAGE and Western blotting (immunoblotting). Proteins were analyzed by SDS-PAGE (5% gel) as described by Laemmli (14). Gels were stained with Coomassie brilliant blue. For Western blotting, proteins on gels were transferred electrophoretically (0.8 mA/cm^2, 4 h) to nitrocellulose filters with a Semi Dry Electroblotter (Sartorius). Immunodetection was carried out by using the antiserum and anti-rabbit immunoglobulin G alkaline phosphatase conjugate as described by the supplier (Promega Biotec).

Labeling of probes and Southern blot hybridization. ^{32}P-labeled probe DNAs were prepared by the random primer extension method (5) with [α-^{32}P]dCTP (Amersham).

DNA samples were digested with the appropriate restriction enzymes, subjected to electrophoresis on a 0.7% agarose gel, and transferred to a Gene-Screen Plus membrane (Dupont, NEN Research Products) under alkaline conditions (2). The membrane was hybridized at 65°C for 16 h in 1 M NaCl containing 0.05 M Tris hydrochloride (pH 7.5),

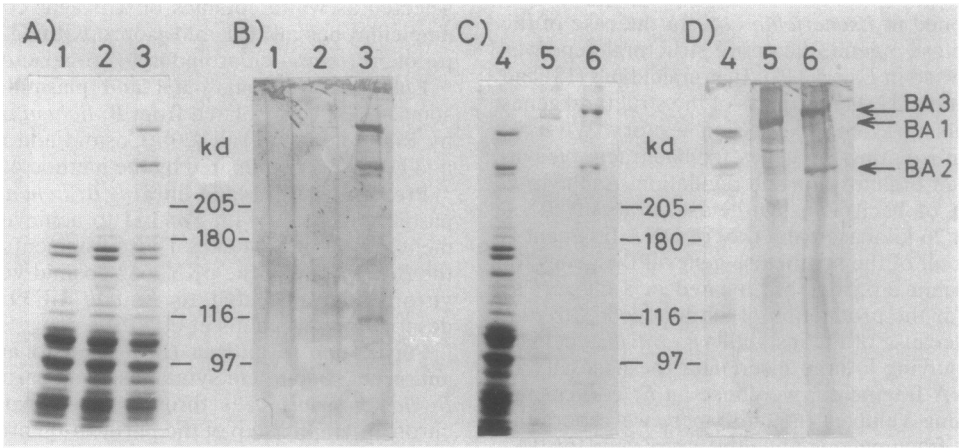

FIG. 2. Coomassie brilliant blue staining on SDS-PAGE (A, C) and corresponding Western blot (B, D) of proteins in crude cell extracts of *E. coli* cells (lanes 1 to 4) and of partially purified BAs 1, 2, and 3 (lanes 5 and 6). Samples were subjected to electrophoresis on a 5% polyacrylamide gel for 4 h at 10 mA. Lanes: 1, *E. coli* LE392; 2, control strain C-46; 3 and 4, strain B-15; 5, partially purified BA1, peak 1 fraction from the hydroxyapatite column chromatography (9); 6, partially purified BA2 and BA3, peak 2 fraction from the same chromatography. kd, Kilodaltons.

10× Denhardt solution (3), 1% SDS, 0.1% sodium pyrophosphate, 100 μg of sonicated heat-denatured salmon sperm DNA per ml, and a probe DNA. The membrane was washed successively in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature for 5 min twice, in 2× SSC containing 1% SDS at 65°C for 30 min twice, and in 0.1× SSC containing 1% SDS at 65°C for 1 h; dried; and subjected to autoradiography.

Partial purification of BA2 expressed in *E. coli*. Portions (2 ml) of an overnight culture of *E. coli* carrying the recombinant plasmid were transferred to five 500-ml shaking flasks, each containing 100 ml of LB broth with 50 μl of ampicillin per ml. Cells were grown at 37°C for 8 h, collected by centrifugation, washed twice with 50 mM Tris hydrochloride (pH 7.6) containing 10 mM $MgCl_2$, and resuspended in the same buffer (6 ml of buffer per g of wet cells). Crude extract was obtained by sonication for 90 s in 3-ml portions as described above and centrifugation at 10,000 × *g* for 20 min. The extract was brought to 50% saturation with solid $(NH_4)_2SO_4$. After centrifugation, the precipitate was dissolved in 3 ml of buffer A (0.01 M potassium phosphate buffer [pH 7.2] containing 1 mM $MgCl_2$, 2 mM 2-mercaptoethanol, and 2% glycerol). The enzyme solution (3 ml) was applied to a Sephadex G-200 column (2.5 by 45 cm) equilibrated with buffer A, which was used for elution. Fractions (4.1 ml) were collected, and their protein concentrations and amino acid-dependent ATP-PP_i exchange activities were assayed. In some experiments, a smaller Sephadex G-200 column (1.4 by 27 cm) was used and fractions (1.3 ml) were collected and assayed as described above. The fractions (12 mg of protein) containing BA2 activity were pooled and applied to a hydroxyapatite column (1.2 by 8 cm; Bio-Gel HTP; Bio-Rad Laboratories) equilibrated with 0.01 M potassium phosphate buffer (pH 7.2). The column was washed with 30 ml of the same buffer, and the enzyme was eluted with a linear salt gradient from 0.05 (pH 7.2, 40 ml) up to 0.25 (pH 7.2, 40 ml) M potassium phosphate buffer. Fractions (1.9 ml) were collected and assayed for protein concentration and enzyme activities.

Assay of ATP-PP_i exchange activity. Amino acid activation activities were assayed by the amino acid-dependent ATP-$^{32}PP_i$ exchange reaction as described previously (9) except for the reaction mixture. The reaction mixture contained the following in a total volume of 0.25 ml: 50 mM potassium phosphate buffer (pH 7.2), 10 mM $MgCl_2$, 2 mM ATP, 2 mM potassium [^{32}P]pyrophosphate (10^4 to 10^5 cpm), 5 mM amino acid, and the enzyme solution to be assayed.

Solid-phase radioimmunoassay. The antigenic activities of fractionated gene products after the hydroxyapatite column chromatography were detected by solid-phase radioimmunoassay as described by Romani et al. (21). Wells of microdilution plates were coated with 100 μl of each fraction from the chromatography. The antigen adsorbed was detected by the antiserum and ^{125}I-labeled protein A.

Miscellaneous methods. Restriction mapping was performed by the standard procedure using double enzyme digestion. Protein concentration was assayed by the method of Lowry et al. (15). Bovine serum albumin was used as a standard.

RESULTS

Cloning in *E. coli* of a DNA fragment encoding a part of bacitracin synthetase from *B. licheniformis*. A recombinant DNA library of *B. licheniformis* was constructed in the *Bam*HI site of the cosmid vector pKY2662. A *Sau*3AI partial digest of *B. licheniformis* chromosomal DNA was fractionated on a 0.5% agarose gel, and partial digestion products ranging from 30 to 40 kb were isolated. The vector and the chromosomal DNA were ligated. The ligation mixture was then packaged into bacteriophage lambda in vitro, and the resulting phage particles were transduced to *E. coli* LE392. The library was screened without amplification. Colonies producing BA antigen were detected by an in situ colony immunoassay using anti-BA antiserum and ^{125}I-labeled protein A.

Screening of about 300 ampicillin-resistant colonies gave 15 colonies that were found to react with the antiserum. Crude cell extracts from each strain were analyzed by SDS-PAGE (5% gel) with Coomassie blue staining. The 15 strains were separated into two groups by the electrophoretic pattern. One group of five strains gave two protein bands of high molecular weight in the area where no protein band was detected for the control strains as shown below. As the smaller protein band of these strains showed the same electrophoretic mobility as the BA2 protein, one of these strains, designated B-15, was further characterized. The other group of 10 strains gave one immunoreactive protein band of about 280,000 molecular weight. These strains were not further characterized.

Detection of immunoreactive gene products. Analysis of the crude cell extract from strain B-15 by SDS-PAGE and Western blotting revealed that the extract contained two conspicuous immunoreactive protein bands with high molecular weights (Fig. 2). One protein band with a molecular weight of about 240,000 comigrated with the BA2 protein from *B. licheniformis*, and the other band had a molecular weight of about 300,000. No protein bands of these sizes were detected in the control strains C-46 and LE392. The C-46 strain was one of the transductants of strain LE392 by a λ phage particle carrying recombinant cosmid DNA and was thus resistant to ampicillin but did not produce proteins immunoreactive with anti-BA antiserum (Fig. 2A and B).

Analysis of the recombinant plasmid DNA. A recombinant plasmid designated pBA15 was isolated from strain B-15, and its molecular size and cleavage pattern were investigated with various restriction enzymes. Plasmid pBA15 is about 40 kb in size, of which about 32 kb of *B. licheniformis* chromosomal DNA is inserted into the *Bam*HI site of the 8.7-kb vector pKY2662. A restriction map of pBA15 is shown in Fig. 3. The insert contains no site for the endonucleases *Xba*I and *Kpn*I.

To confirm that the cloned fragment was really derived from the genome of *B. licheniformis*, restriction patterns of the inserted DNA and the corresponding gene region obtained from *B. licheniformis* DNA were compared by Southern blot hybridization. The same hybridization patterns were obtained in both the cloned fragment and the chromosomal DNA (Fig. 4). Thus, these results indicate that a 25-kb stretch of the 32-kb insert of pBA15 has the same DNA arrangement as that of the genomic DNA of *B. licheniformis*.

Detection of BA2 activity in *E. coli*. Activity of each of the enzymes which constitute bacitracin synthetase can be detected by the ATP-$^{32}PP_i$ exchange reaction dependent on the substrate amino acid specific to the enzyme. Since the molecular weights of the gene products encoded by the recombinant plasmid were considerably high as described above, catalytic activities related to BA2 were expected to be easily separated by the gel filtration on Sephadex G-200 from almost all aminoacyl-tRNA synthetases which are present in the *E. coli* host and catalyze the same exchange reaction.

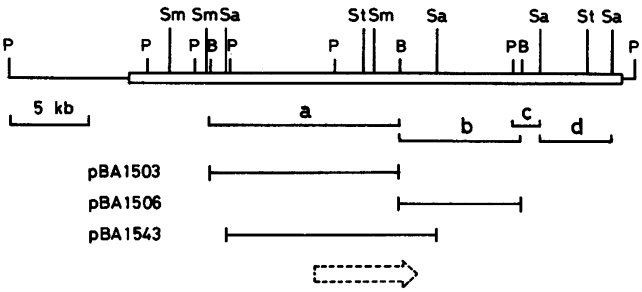

FIG. 3. Restriction map of plasmid pBA15 and location of the gene for BA2. The top line shows the restriction map of pBA15 which contains an approximately 32-kb *B. licheniformis* DNA fragment (double line) inserted into the *Bam*HI site of the cosmid vector pKY2662 (single line). Restriction sites are abbreviated as follows: B, *Bam*HI; P, *Pst*I; Sa, *Sal*I; Sm, *Sma*I; St, *Stu*I. Lines below the map show the locations of the fragments used as probes a to d in the Southern blot hybridization shown in Fig. 4. The locations of the fragments subcloned in the pUC18 vector are indicated by bars. The approximate position of the gene for BA2 is shown by the arrow.

The soluble extract obtained from the B-15 strain by sonication and subsequent centrifugation was subjected to $(NH_4)_2SO_4$ fractionation. The proteins precipitated by 50% saturation with $(NH_4)_2SO_4$ were then separated by gel filtration on a Sephadex G-200 column, and ATP-PP_i exchange activities dependent on amino acids were assayed (Fig. 5A). The C-46 strain was used as a control (Fig. 5B). Further, exchange activities dependent on each of the bacitracin-constituting amino acids were assayed with the peak fraction of L-ornithine-dependent exchange activity of the B-15 strain and with the corresponding fraction of the control strain. As was expected, clear ATP-PP_i exchange activities dependent on several bacitracin-constituting amino acids were detected in the high-molecular-weight fractions, whereas no exchange reaction except for that of D-phenylalanine was observed in the control strain (Table 1). The presence of exchange activities dependent on the nonprotein amino acid L-ornithine and L-lysine in the fractions from the B-15 strain gives conclusive evidence for the expression of the cloned BA2 gene. The exchange activities found in a low-molecular-weight region in the two strains are due to aminoacyl-tRNA synthetases present in the *E. coli* host.

To ascertain further whether L-ornithine-activating activity is due to the same protein as L-lysine-activating activity, we pooled the peak fractions of L-ornithine-activating activity by Sephadex G-200 gel filtration and chromatographed them on a hydroxyapatite column. The results are shown in Fig. 6. The peak of L-ornithine-activating activity exactly coincides with that of L-lysine-activating activity and elutes one fraction after the peak of L-isoleucine-activating activity (a part of the putative BA3 enzyme as described below). Radioactivities in solid-phase radioimmunoassay coincided in position with these two enzymes. Furthermore, from SDS-PAGE and Western blotting of proteins of the peak fraction (fraction 36) showing both the antigen activity and the L-ornithine-dependent exchange activity shown in Fig. 6, it was demonstrated that this fraction contains the gene product that comigrates with the BA2 protein and is immunoreactive with anti-BA antibody (Fig. 7B, lane 2). On the other hand, the peak fraction (fraction 14) of D-phenylalanine-dependent exchange activity in Fig. 6 did not contain the high-molecular-weight immunoreactive gene product (Fig. 7B, lane 1). These results indicate that the enzyme

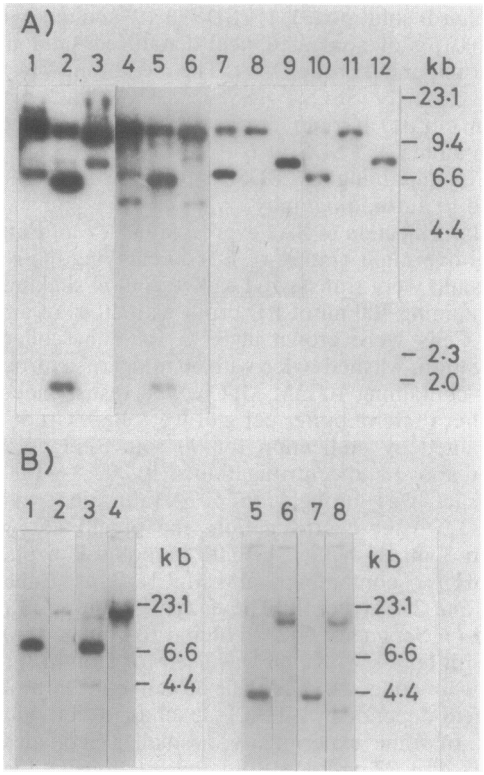

FIG. 4. Southern blot hybridization of restriction fragments of pBA15 and chromosomal DNA of *B. licheniformis*. (A) pBA15 (lanes 1 to 3 and 7 to 9) and *B. licheniformis* chromosomal (lanes 4 to 6 and 9 to 12) DNA fragments obtained by digestion with *Sal*I (lanes 1, 4, 7, and 10), *Pst*I (lanes 2, 5, 8, and 11), and *Bam*HI (lanes 3, 6, 9, and 12) were hybridized with probe a (lanes 1 to 6) and probe b (lanes 7 to 12), respectively. Probes a and b were *Bam*HI fragments of pBA15. (B) pBA15 (lanes 1, 2, 5, and 6) and *B. licheniformis* chromosomal (lanes 3, 4, 7, and 8) DNA fragments obtained by digestion with *Sal*I (lanes 1, 3, 5, and 7) and *Stu*I (lanes 2, 4, 6, and 8) were hybridized with probe c (lanes 1 to 4) and probe d (lanes 5 to 8), respectively. Probes c and d are *Pst*I-*Sal*I and *Sal*I fragments of pBA15, respectively. The locations of the DNA probes a to d with respect to the physical map of pBA15 are shown in Fig. 3.

activities activating both L-lysine and L-ornithine are due to BA2 protein synthesized in *E. coli* carrying the cloned gene for BA2.

In hydroxyapatite column chromatography, L-histidine-, L-leucine-, and L-valine-dependent ATP-PP_i exchange activities were detected in coincidence with L-isoleucine-activating activity (data not shown). L-Leucine and L-valine may be activated by the L-isoleucine-activating site of the enzyme because of low substrate specificity, which is a characteristic feature of enzymes participating in bacitracin biosynthesis (10). Furthermore, the fraction (fraction 36) having these activities in the hydroxyapatite column chromatography contains a 300,000-molecular-weight protein immunoreactive with anti-BA antibody (Fig. 7B, lane 2). On the basis of the findings that both L-isoleucine and L-histidine are activated by the same protein fraction and the protein is immunoreactive with anti-BA antibody, it is suggested that a part of the BA3 enzyme is synthesized in the B-15 strain and, therefore, a part of the gene for BA3 is included in the insert of the pBA15 plasmid.

A marked ATP-PP_i exchange activity dependent on D-phenylalanine, which is a constituent amino acid of bacitra-

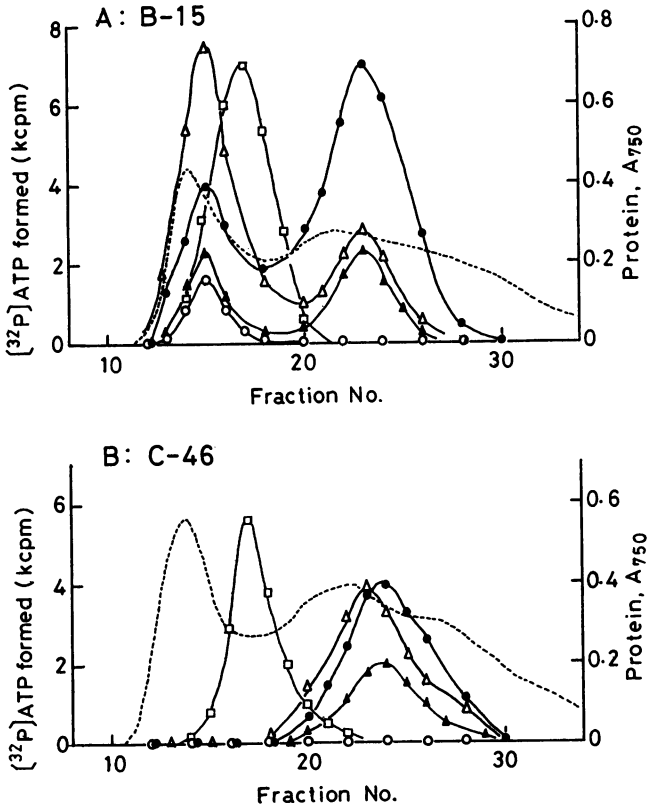

FIG. 5. Amino acid-dependent ATP-PP_i exchange activities of fractions obtained by Sephadex G-200 gel filtration. A 0 to 50% $(NH_4)_2SO_4$ fraction (48 [A] or 55 [B] mg of protein) of the crude extract from strain B-15 (A) or control strain C-46 (B) was applied to a column (2.5 by 45 cm) and eluted with buffer A as described in the text. Fractions of 4.1 ml each were collected. - - - - -, Protein (A_{750}); other lines show L-lysine (●)-, L-ornithine (○)-, L-isoleucine (△)-, L-histidine (▲)-, and D-phenylalanine (□)-dependent ATP-PP_i exchange activities.

cin, was detected in the fraction of the Sephadex G-200 gel filtration (Table 1), but the enzyme activity did not coincide in elution position with the L-isoleucine- and L-histidine-activating activities in both the gel filtration and the hydroxyapatite column chromatography (Fig. 5A and 6). The D-phenylalanine-dependent exchange activity is detected also in the control strain (Fig. 5B) and wild-type strain (data not shown) of *E. coli* K-12. Since both isomers of phenylalanine are shown to be activated by the same enzyme fraction (Fig. 6) as reported in the activation of the isomers of tyrosine by tyrosyl-tRNA synthetase of *E. coli* (1), these activities can be attributed to phenylalanyl-tRNA synthetase in *E. coli*.

Localization of the gene encoding the BA2 protein. To map an approximate position of the gene encoding the BA2 protein in the cloned DNA segment, we subcloned various restriction fragments into the pUC18 vector, and the crude extracts from *E. coli* JM109 cells bearing these recombinant plasmids were analyzed by SDS-PAGE (5% gel). Among the recombinants, pBA1543 carrying a 13.6-kb *Sal*I fragment (Fig. 3) directed the synthesis of a protein with the same mobility as that of the BA2 protein on SDS-PAGE (Fig. 8, lane 3). Amino acid-dependent ATP-PP_i exchange activities in the fraction obtained by Sephadex G-200 gel filtration from cells bearing pBA1543 were assayed (Fig. 9B). Apparent exchanges dependent on both L-lysine and L-ornithine, which are characteristic of the BA2 enzyme, were detected in the high-molecular-weight fraction. In the control clone pBA1506 carrying the 7.5-kb *Bam*HI fragment (Fig. 3), neither activity was detected in the same region. A clone pBA1503 carrying 12 kb of *Bam*HI fragment (Fig. 3) directed the synthesis of a protein with a molecular weight of 200,000 (Fig. 8, lane 2). This protein catalyzed only the L-lysine-dependent ATP-PP_i exchange (data not shown). These results indicate that the gene encoding BA2 is located near the middle of the insert and the direction of transcription is from left to right, as shown in Fig. 3.

DISCUSSION

The results obtained in this study show that the entire structural gene for BA2, one of the three multifunctional enzymes constituting the BA, was cloned into the cosmid vector and expressed in *E. coli*. This conclusion was based on the following observations. (i) *E. coli* cells carrying the plasmid pBA15 produced a protein which is identical in electrophoretic mobility to the 240,000-molecular-weight BA2 protein on SDS-PAGE and is immunoreactive with anti-BA antibody. (ii) The gene product encoded by pBA15 catalyzed the ATP-PP_i exchange reactions dependent on both L-lysine and L-ornithine which are characteristic of the BA2 enzyme. Both the enzyme activities were copurified in the same fraction during partial purification of the enzyme. (iii) Most of the 32-kb insert of the recombinant plasmid pBA15 has the same arrangement as that of the genomic DNA of *B. licheniformis*, and the structural gene for BA2 is located in this region.

Recent studies on the organization of the structural genes for the enzymes involved in antibiotic biosynthesis in the genus *Streptomyces* revealed that these genes are clustered (4). In the case of peptide antibiotics produced by bacteria of the genus *Bacillus*, the organization of the genes for the peptide antibiotic synthetases is not yet clarified, because until now genes for only one component enzyme of the multicomponent synthetases have been cloned. In this respect, it is of interest that pBA15 carries the gene encoding a part of the putative BA3 in addition to the BA2 gene. This result suggests that the genes for peptide antibiotic syn-

TABLE 1. Amino acid-dependent ATP-PP_i exchange activities of the Sephadex G-200 fractions from B-15 and control strain C-46

Amino acid added	[^{32}P]ATP formed (nmol/min per mg of protein)[a]	
	B-15	C-46
None	25	25
L-Ile	296	31
L-Cys	29	26
L-Leu	99	28
L-Glu	36	29
L-Lys	186	31
L-Orn	91	31
D-Phe	307	117
L-His	138	37
L-Asp	35	32
L-Asn	33	28
L-Val	68	30

[a] Assay conditions: 2 mM ATP; 2 mM $^{32}PP_i$ (2.4×10^4 cpm per assay); 5 mM amino acid; protein concentration, 50 μg per 250-μl assay. For each enzyme assay, fraction 15 on the Sephadex G-200 gel filtration shown in Fig. 5 was used.

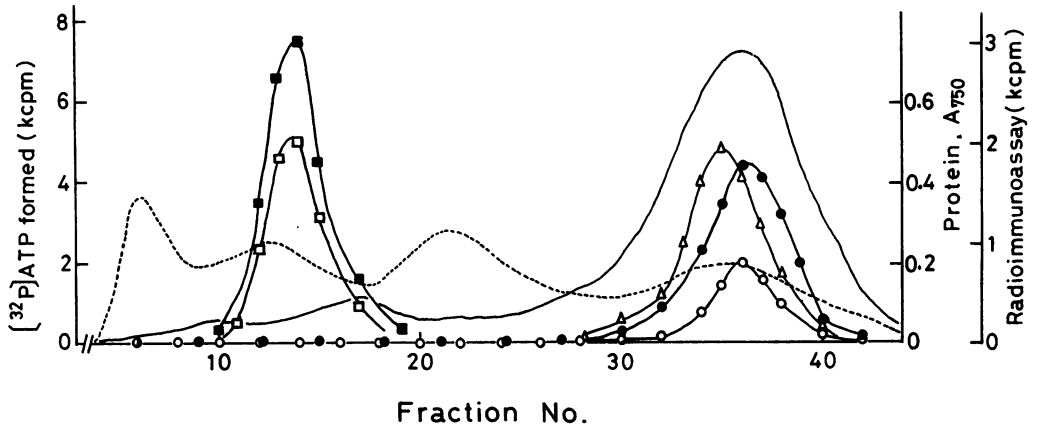

FIG. 6. Amino acid-dependent ATP-PP_i exchange activities and solid-phase radioimmunoassay of fractions obtained by hydroxyapatite column chromatography. The pooled Sephadex G-200 fraction (12 mg of protein) from strain B-15 was applied to a column (1.2 by 8 cm) and eluted with a linear gradient of potassium phosphate buffer (pH 7.2) from 0.05 to 0.25 M as described in the text. - - - - -, Protein (A_{750}); —— solid-phase radioimmunoassay; other lines show L-lysine (●)-, L-ornithine (○)-, L-isoleucine (△)-, D-phenylalanine (□)-, and L-phenylalanine (■)-dependent ATP-PP_i exchange activities.

thetases also are clustered in the genomes. Thus, the gene for BA1 is probably located near the BA2 gene, and then the whole stretch of the genes could be cloned by using DNA fragments from either end of pBA15 as probes for screening a genomic library ("chromosomal walk"). Experiments along this line are now in progress.

In gramicidin S synthetase, from the analyses of enzyme fragments produced by limited proteolysis and of the products of mutants carrying defects in enzyme structure, it was shown that the sequence of amino acid-activating units in the multienzyme polypeptide chain is identical with the amino acid sequence of the product (11). A putative BA3 fragment synthesized by strain B-15 activated L-isoleucine and L-histidine but not D-phenylalanine. This suggests that the same rule regarding the arrangement of amino acid-activating units is not applicable to the putative BA3 protein. However, it is not certain at present whether the D-phenylalanine-activating unit is completely absent in the gene product encoded by pBA15 or whether this unit is functionally inactive though present, because a certain downstream part of the polypeptide chain may be required for the enzyme activity. To answer this question, it is necessary to isolate the entire structural gene for BA3 and to analyze the gene products synthesized by various subclones with deletions in the 3′ end of the coding sequence.

The cosmid vector pKY2662 is not an expression vector, and as mentioned above, the gene encoding BA2 lies near the middle of the cloned 32-kb fragment of *B. licheniformis* DNA. Furthermore, four other *E. coli* clones which give the same gene products on SDS-PAGE as the B-15 strain have been isolated independently from the unamplified cosmid library. These results show that the cloned BA2 gene is transcribed from a promoter located in the insert DNA. However, taking account of the fact that fortuitous promoters are used in *E. coli* as a transcriptional starting point in some *Bacillus* genes, e.g., *B. thuringiensis* toxin gene (22)

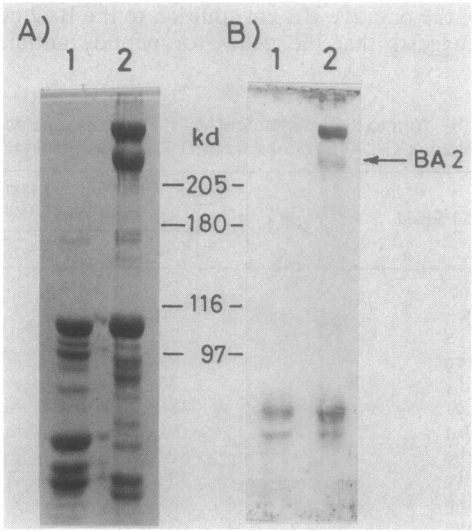

FIG. 7. Coomassie brilliant blue staining on SDS-PAGE (A) and corresponding Western blot (B) of proteins in fraction 14 (lane 1) and fraction 36 (lane 2) on hydroxyapatite column chromatography (Fig. 6). Electrophoresis was performed as described in the legend to Fig. 2 and Materials and Methods. kd, Kilodaltons.

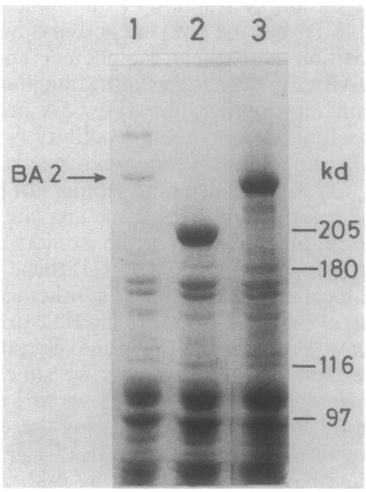

FIG. 8. Coomassie brilliant blue staining on SDS-PAGE of proteins in crude cell extracts of *E. coli* LE392 bearing pBA15 (lane 1) and JM109 bearing pBA1503 (lane 2) or pBA1543 (lane 3). pBA1503 and pBA1543 have the inserts in the opposite direction from that of the *lacZ* gene of the pUC18 vector. kd, Kilodaltons.

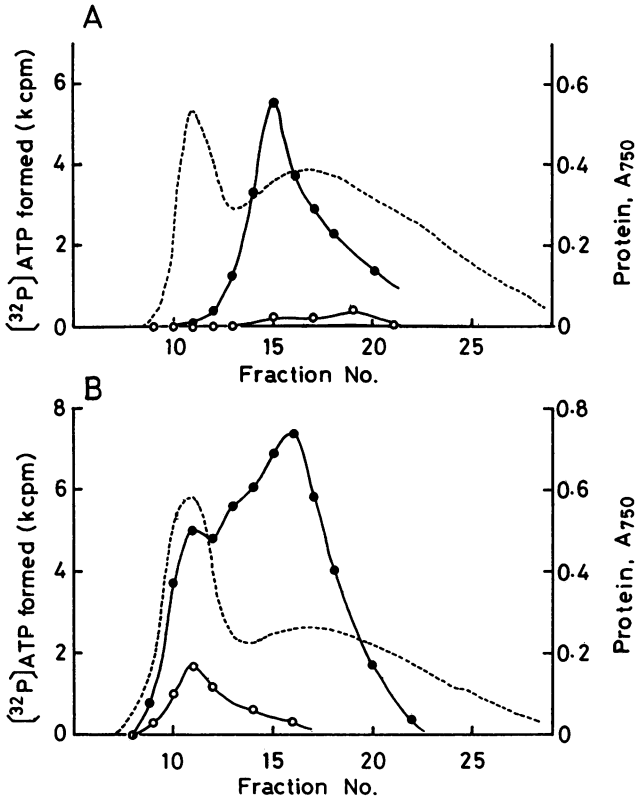

FIG. 9. Amino acid-dependent ATP-PP_i exchange activities of fractions obtained by Sephadex G-200 gel filtration. A 0 to 50% $(NH_4)_2SO_4$ fraction (4.5 mg of protein) of the crude extract from cells bearing pBA1506 (A, control) or pBA1543 (B) was applied to a column (1.4 by 27 cm), and the column was eluted with buffer A as described in Materials and Methods. Fractions of 1.3 ml each were collected. - - - - -, Protein (A_{750}); other lines show L-lysine (●)- and L-ornithine (○)-dependent ATP-PP_i exchange activities.

and *B. brevis* tyrocidine synthetase I gene (18), we cannot tell whether the transcription of the BA2 gene in *E. coli* is initiated from the promoter utilized in *B. licheniformis* or from another promoter in the inserted DNA.

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