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Molecular Cloning and Structure of the Gene for 7β-(4-Carboxybutanamido)cephalosporanic Acid Acylase from a *Pseudomonas* Strain

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A *Pseudomonas* strain produced an enzyme capable of deacylating 7β-(4-carboxybutanamido)cephalosporanic acid to 7-aminocephalosporanic acid in response to glutaric acid. The gene for the enzyme was cloned within the *PstI* site of pBR325 as a 7.35-kilobase-pair DNA segment from a mutant of this strain whose enzyme is produced constitutively. The gene expression in the primary clone appeared to be low in *Escherichia coli* but was significantly enhanced by reducing the size of the initial segment coupled with *E. coli* promoters. Subsequent subcloning resulted in localization of the gene to a 2.45-kilobase-pair fragment. Three clone-specific polypeptides with molecular weights of ca. 16,000, 54,000, and 70,000 were shown by maxicell analysis. The former two corresponded to the small and large subunits of the purified enzyme from the *Pseudomonas* strain, and the third polypeptide was suggested to be their precursor. This was supported by DNA sequence study together with amino acid sequencing of the amino terminus of both subunits: the sequences for the small and large subunits were localized contiguously in this order on the structural gene without termination codons between them. The nucleotide sequence also disclosed the presence of a signallike sequence preceding that for the small subunit, consistent with the previous observation that the enzyme might be periplasmic in the *Pseudomonas* strain. Those results suggest a process for the formation of an active enzyme complex from a precursor through two steps of processing.

An enzyme capable of deacylating 7β-(4-carboxybutanamido)cephalosporanic acid (glutarylamidocephalosporanic acid, GL-7ACA) to 7-aminocephalosporanic acid (7ACA) was found in a few strains of *Pseudomonas* species by Shibuya et al. (24), and further studies were made on its properties and application to an industrial process for 7ACA preparation (12, 13). The enzyme, designated GL-7ACA acylase, seems unique among so-called cephalosporin acylases in that it specifically hydrolyzes cephalosporin compounds with an aliphatic dicarboxylic acid, especially glutaric acid, moiety in the acyl side chain and does not attack those having aromatic side chains which are substrates for other known acylases (28). One of the enzyme-producing organisms, *Pseudomonas* sp. SY-77-1, requires glutaric acid for the maximum production of the enzyme, which appears to be transported into the periplasmic space of the cell. However, its physiological role in the producing organism has not been understood with this evidence. This question has been left unanswered not only with this particular enzyme but also with any other penicillin or cephalosporin acylases so far investigated. To facilitate such a study, we undertook to reveal the structure of genes for this enzyme. Here we report the molecular cloning of a gene from a mutant of *Pseudomonas* sp. SY-77-1 that codes for GL-7ACA acylase, its expression in *Escherichia coli*, and a partial DNA sequence of the gene.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this investigation are listed in Table 1. *Pseudomonas* sp. GK16 was grown in nutrient broth (Difco Laboratories) at 32°C with aeration. L-broth was used as the basal medium for *E. coli* strains, which were grown at 37°C. Antibiotics were included to select for cells carrying plasmids: tetracycline hydrochloride, 10 μg/ml; sodium ampicillin, 50 μg/ml; chloramphenicol, 25 μg/ml; and kanamycin sulfate, 40 μg/ml.

Chemicals. All antibiotics and 5-bromo-4-chloro-3-indolyl-β-D-galactoside were purchased from Sigma Chemical Co. 7ACA was obtained from Asahi Chemical Industry Co., Ltd., and GL-7ACA was synthesized from 7ACA by the method previously described (24). Radioisotopes were obtained from Amersham Corp.

Recombinant DNA techniques. Chromosomal DNA from *Pseudomonas* sp. GK16 was prepared essentially as described by Marmur (19). Plasmid DNA was isolated by the cleared lysate method (10), followed by ethidium bromide-cesium chloride density gradient centrifugation. A small-scale plasmid isolation technique (4) was also employed for screening purposes. Restriction endonucleases, T4 DNA ligase, DNase I, and DNA polymerase I large fragment were purchased from Bethesda Research Laboratories, Inc. Bacterial alkaline phosphatase was from Takara Shuzo Co. All enzymes were used in the buffers and under conditions recommended by the suppliers. *E. coli* strains were transformed by the modified method of Mandel and Higa (18) as described by Lederberg and Cohen (17).

Detection of GL-7ACA-acylase-positive clone. Colonies of *E. coli* C600 transformed with a DNA library of *Pseudomonas* sp. GK16 carried in pBR325 were grown on a selection plate (L-broth and chloramphenicol) for 24 h. Then, each colony on the plate was exposed to chloroform vapor for 15

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genetic markers	Reference or source
E. coli		
C600	F⁻ *thi-1 thr-1 leuB6 lacY1 tonA21 supE44*	1
MM294	F⁻ *thi-1 hsdR17 supE44*	2
CSR603	F⁻ *thi-1 thr-1 leuB6 proA2 argE3 recA1 uvrA6 rpsL31 gyrA98*	23
Pseudomonas sp. GK16		12
Plasmid		
pBR322	Ap[ra] Tc[r]	8
pBR325	Ap[r] Tc[r] Cm[r]	7
pSK12	Tc[r] Cm[r]	This laboratory
pSY343	Km[r]	29
pRZ4024	Ap[r]	W. S. Reznikoff, unpublished work
pRZ4026	Ap[r]	W. S. Reznikoff, unpublished work

[a] Ap[r], Phenotype for ampicillin resistance.

min, scraped with a toothpick, and suspended in 100 μl of GL-7ACA (1 mg of GL-7ACA per ml of 0.1 M phosphate buffer [pH 7.0]) in a well of a microtiter dish. The mixture was incubated at 37°C for 60 min, and the reaction was terminated by addition of 120 μl of acetic acid–0.25 M NaOH (2:1), followed by addition of 40 μl of *p*-dimethylaminobenzaldehyde (0.5% in methanol). *p*-Dimethylaminobenzaldehyde makes a yellow condensation product with 7ACA by a mechanism described by Balasingham et al. (3) for its reaction with 6-aminopenicillanic acid.

GL-7ACA acylase assay. Cells of an overnight culture were suspended in 0.1 M phosphate buffer (pH 7.0) and broken by sonic oscillation. The sonicate thus obtained was directly applied to the enzyme assay. The GL-7ACA acylase assay with *p*-dimethylaminobenzaldehyde was carried out as previously described (12). One unit of the enzyme activity was defined as the amount which liberated 1 μmol of 7ACA per min. Detection of 7ACA by high-pressure liquid chromatography was also previously described (13).

DNA hybridization. Nick translation of plasmid to obtain a ^{32}P-labeled DNA probe and subsequent hybridization to total DNA from *Pseudomonas* sp. GK16 were carried out as described by Southern (27).

Maxicell analysis. Identification of plasmid-encoded proteins in maxicells was carried out by the method of Sancar et al. (23). Protein electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gel and fluorography were performed as previously described (15, 16).

Nucleotide sequence determination. Labeling of 5′ ends with polynucleotide kinase (Bethesda Research Laboratories) and [γ-^{32}P]ATP after alkaline phosphatase treatment and labeling of 3′ ends with DNA polymerase I large fragment and [α-^{32}P]dCTP were accomplished by the procedure of Maxam and Gilbert (20). The labeled DNA fragments were digested with a restriction enzyme, and each fragment was separated by 5% polyacrylamide gel electrophoresis. All sequence determinations were done by the method of Maxam and Gilbert (20).

Isolation and amino-terminal sequencing of subunit proteins. The GL-7ACA acylase protein purified as previously described (13) was dissolved in 62.5 mM Tris hydrochloride (pH 6.8) containing 2% SDS and 10% glycerol, heated at 80°C for 3 min, and applied to a preparative SDS-polyacrylamide gel (15) that led to dissociation of the enzyme into nonidentical subunits. The protein bands were located by staining a small vertical strip of gel with Coomassie blue, and horizontal strips containing each subunit were excised from the remaining gel. The protein was then eluted by overnight incubation in 10 mM phosphate buffer (pH 7.0) containing 0.1% SDS, dialyzed against deionized water, and concentrated in vacuo. The amino-terminal sequencing of the subunit proteins was performed by an automated Edman degradation on a gas-phase sequenator (470A protein sequencer; Applied Biosystems). The phenylthiohydantoin derivatives were identified by high-pressure liquid chromatography on a Senshu Pack SEQ-4 column (4.6 by 300 mm; Senshu Scientific Co.).

RESULTS

Isolation of an *E. coli* clone carrying GL-7ACA acylase gene. GL-7ACA acylase synthesis by the original strain (SY-77-1) is induced by glutaric acid (24). As a DNA donor for the acylase gene, however, we used a mutant strain (GK16) which produces the enzyme constitutively, expecting to reduce a possible difficulty in detecting the enzyme activity in *E. coli*. Total DNA of GK16 was partially digested with *Pst*I, and fragments of 4 to 9 kilobase pairs (kb) were isolated from agarose gels by electroelution. Then, the size-fractionated DNA was ligated with the *Pst*I-cut and alkaline phosphatase-treated pBR325 and transformed into *E. coli* C600. Two thousand colonies from the gene library thus obtained were screened for the ability to hydrolyze GL-7ACA to 7ACA by a technique with *p*-dimethylaminobenzaldehyde. As a result, a single positive colony was detected. The colony was found to harbor a plasmid capable of transforming the plasmid-free *E. coli* to the acylase-positive one. Deacylation of GL-7ACA by the clone was further confirmed by detecting 7ACA with high-pressure liquid chromatography (data not shown).

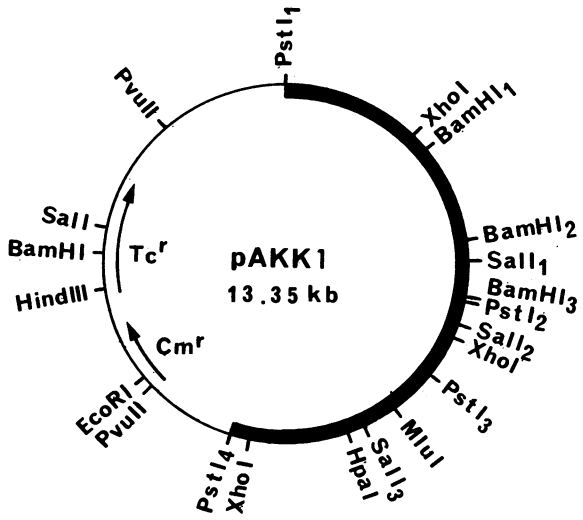

FIG. 1. Restriction cleavage map of pAKK1. Sites for some restriction enzymes in the cloned segment (thick black line) are numbered clockwise from the top.

The plasmid designated pAKK1 was isolated from the *E. coli* clone, and a preliminary restriction cleavage map was obtained (Fig. 1). The plasmid was found to have a 7.35-kb fragment inserted in the *Pst*I site of pBR325, and this segment carried no site for *Eco*RI, *Hin*dIII, *Kpn*I, *Pvu*II, or *Cla*I. To confirm that the inserted fragment originally came from the *Pseudomonas* DNA, the ^{32}P-labeled pAKK1 DNA was hybridized to the total DNA of GK16 by Southern blotting. pAKK1 hybridized to the *Pst*I-digested GK16 DNA on three bands which exactly corresponded in size to the three fragments generated by *Pst*I from the inserted DNA in the plasmid (Fig. 2); only one large fragment in the *Hin*dIII-digested *Pseudomonas* DNA was shown to have a certain homology to pAKK1.

Gene localization. To localize the acylase gene on the 7.35-kb DNA fragment, a series of subclonings and plasmid modifications were made as described below (Fig. 3). The plasmids thus obtained were examined for their ability to produce the enzyme in *E. coli* C600. For the convenience of following studies, the whole 7.35-kb fragment in pAKK1 was transferred into the *Pst*I site of pBR322 to give rise to a plasmid designated pAKK11. Subsequent subclonings were carried out by using a vector derived from pBR325 having an inactive β-lactamase gene otherwise; the pBR325-encoded β-lactamase might hamper the detection of the GL-7ACA acylase activity. This plasmid, designated pSK12, was isolated by accident during an experiment involving digestion of pBR325 with *Pst*I and religation with T4 DNA ligase. pSK12 was found to have lost the *Pst*I site within the β-lactamase gene without changing its gross molecular size.

(i) pAKKB1 and pAKKB1'. pAKK11 was digested partially with *Bam*HI and ligated with the *Bam*HI-cleaved pSK12. By transformation of the mixture into *E. coli* C600, pAKKB1 from chloramphenicol- and tetracycline-resistant (Cm^r Tc^r) transformants and pAKKB1' from Cm^r and tetracycline-sensitive (Tc^s) colonies were obtained. From the enzyme activity of the cells carrying pAKK1 or pAKK11 as compared with that in *Pseudomonas* sp. GK16, the acylase gene appeared to be expressed at quite a low level in the primary clone (Table 2). Subsequently, an increase of a factor of approximately five in the acylase activity was exhibited by the size-reduced plasmid pAKKB1. However, pAKKB1' carrying the same DNA insertion as pAKKB1 but in the opposite orientation gave no detectable enzyme activity. The results suggest that the acylase gene on pAKKB1 is expressed by readthrough from the Tc^r promoter on the vector, and therefore the direction of transcription is from the *Pst*I-1 to *Pst*I-4 sites in Fig. 1.

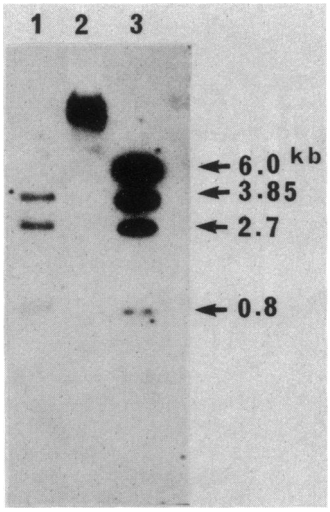

FIG. 2. Southern blot hybridization between ^{32}P-labeled pAKK1 and total DNA of *Pseudomonas* sp. GK16 digested with *Pst*I (lane 1) and *Hin*dIII (lane 2). Lane 3, Hybridization with the *Pst*I-digested pAKK1 (as a control). Sizes of fragments were determined from λ DNA-*Hin*dIII fragments (shown in kilobases) used as markers.

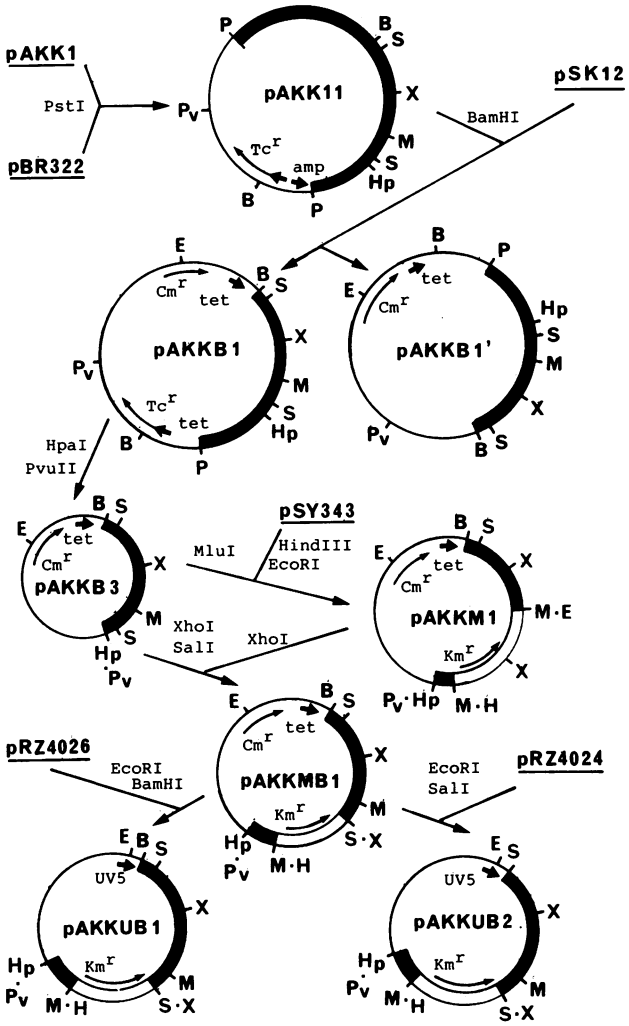

FIG. 3. Diagram of subcloning of the GL-7ACA acylase gene. The only restriction sites shown are those used in the cloning experiments: P, *Pst*I; B, *Bam*HI; S, *Sal*I; X, *Xho*I; M, *Mlu*I; Hp, *Hpa*I; Pv, *Pvu*II; E, *Eco*RI; H, *Hin*dIII. Thick black and open lines represent the DNA segments from *Pseudomonas* sp. GK16 and pSY343, respectively. Promoters (⧫) to be noted are indicated as: tet, Tc^r; amp, Ap^r; UV5, lacL8UV5. Details of the plasmid construction are provided in the text.

(ii) pAKKB3. pAKKB1 was digested completely with *Hpa*I and partially with *Pvu*II and religated. The resultant shortened plasmid, pAKKB3, was recovered from Cm^r Tc^s transformants and gave a slightly higher enzyme activity than pAKKB1.

(iii) pAKKM1 and pAKKMB1. A 2.2-kb *Eco*RI-*Hin*dIII fragment containing a kanamycin-resistant (Km^r) gene was isolated from pSY343 (29) for introduction of the Km^r gene into pAKKB3. The fragment and the *Mlu*I-cleaved pAKKB3

TABLE 2. GL-7ACA acylase activity of *E. coli* C600 harboring pAKK1 and its derivatives

Plasmid clone	Enzyme activity (10^{-2} U/mg [dry wt] of cells)
Pseudomonas sp. GK16[a]	12
pAKK1	0.7
pAKK11	0.7
pAKKB1	3.6
pAKKB1'	ND[b]
pAKKB3	4.8
pAKKM1	ND
pAKKMB1	5.3
pAKKUB1	20
pAKKUB2	ND

[a] Value for *Pseudomonas* sp. GK16 is given for comparison.
[b] ND, Not detectable.

were treated with DNA polymerase I large fragment to make blunt ends and ligated. The resultant plasmid, pAKKM1, was isolated from Cm^r Km^r transformants and exhibited no enzyme activity. This indicates that the acylase gene was disrupted by the insertion of the above fragment. Then, the disrupted gene was repaired by replacing the small *Xho*I fragment in pAKKM1 with the 1.5-kb *Xho*I-*Sal*I fragment of pAKKB3. The resultant plasmid, pAKKMB1, gave enzyme activity comparable to that of pAKKB3. The result indicates that the region between the *Sal*I-3 and *Pst*I-4 sites is dispensable for gene expression.

(iv) pAKKUB1 and pAKKUB2. The lacUV5 promoter was introduced into the region containing the Tc^r promoter on pAKKMB1. Plasmids pRZ4024 and pRZ4026 as the donors of the lacUV5 promoter are pBR322 derivatives harboring the 0.2-kb *Hae*III fragment containing this promoter and cloned into the *Eco*RI and *Sal*I sites or the *Eco*RI and *Bam*HI sites, respectively (W. S. Reznikoff, unpublished work). The 0.2-kb *Eco*RI-*Bam*HI fragment isolated from pRZ4026 was ligated with pAKKMB1 which was digested completely with *Eco*RI and partially with *Bam*HI. The recombinant was transformed into *E. coli* MM294, and pAKKUB1 was obtained from blue colonies on the L-agar plate containing 5-bromo-4-chloro-3-indolyl-β-D-galactoside and kanamycin. pAKKUB1 exhibited a significantly higher enzyme activity than pAKKMB1, supporting the above proposal on the direction of transcription. When the 0.2-kb *Eco*RI-*Sal*I fragment of pRZ4024 was placed at the region between the *Eco*RI and *Sal*I (*Sal*I-1 in Fig. 1) sites in pAKKMB1, the resultant plasmid, pAKKUB2, gave no enzyme activity. This suggests the presence of the translation initiation site between the *Bam*HI-2 and *Sal*I-1 sites.

The above results in a series of subclonings indicate that the 2.45-kb fragment between the *Bam*HI-2 and *Sal*I-3 sites contains a structural gene for the functional acylase.

Characterization of proteins produced by clone. The acylase protein has been purified from *Pseudomonas* sp. GK16, and molecular weight (M_r) in the active form was estimated as ca. 130,000 by gel filtration (13). SDS-polyacrylamide gel electrophoresis of the purified enzyme yielded two subunits, with M_rs of 16,000 and 54,000 (Fig. 4). These results suggest that the active enzyme complex is composed of two molecules of each of the two nonidentical subunits. To characterize the acylase gene product, the maxicell analysis was applied to the *E. coli* clones. pAKKB3 gave three major bands of polypeptide, with M_rs of 16,000, 54,000, and ca. 70,000, besides products coded on the vector (Fig. 4). pAKK1, the original clone, also gave a band of a 54,000-M_r polypeptide, while the other two bands given by pAKKB3 were detected with intensities too weak to be visualized in the picture, probably reflecting the low expression of the acylase gene. The polypeptides with M_rs of 16,000 and 54,000 may correspond to the small and large subunits of the acylase. The 70,000-M_r polypeptide was not found in the purified enzyme, but it was presumed to be a product of the acylase gene and possibly the precursor of both subunits for the following reasons. (i) The *Pseudomonas* DNA segment (2.7 kb) in pAKKB3 was not capable of coding for the above three polypeptides without any overlapping, suggesting that the 70,000-M_r polypeptide is encoded on the region containing the acylase gene. (ii) pAKKM1 carrying an insertion of a Km^r gene in a region near the 3' end of the acylase gene gave none of the above three bands but instead gave a polypeptide with an M_r of ca. 90,000 (Fig. 4, lane 3). This suggests that a single polypeptide is produced by translation of the acylase gene.

Gene structure of acylase. A part of the nucleotide sequence of the fragment containing the acylase gene was determined (Fig. 5). The sequence disclosed the presence of three possible initiation codons (ATG) within a region between the *Bam*HI-2 and *Sal*I-1 sites. Since a sequence (GAGG) regarded as a ribosome-binding site was found at 9 base pairs upstream from the first ATG, an open reading frame was proposed from this initiation codon. The proposed reading frame was confirmed by isolation of each subunit of the purified enzyme from *Pseudomonas* sp. GK16 and determination of its amino-terminal sequence by a method with Edman degradation. Sequences of 17 amino acids from the amino termini of both subunits were revealed and showed a perfect match with two regions of the proposed reading frame: the sequences of the small and large subunits were consistent with those from amino acid 30 (Glu)

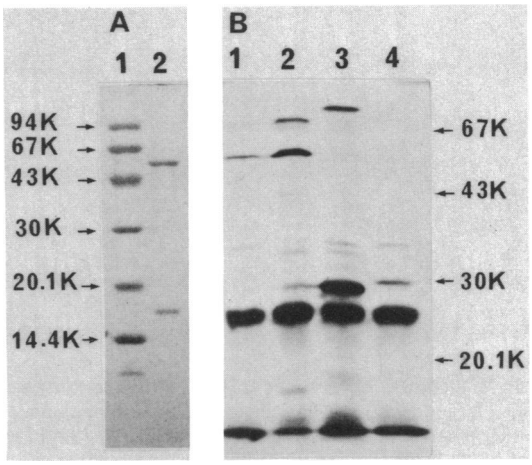

FIG. 4. SDS-polyacrylamide gel electrophoresis patterns of the purified acylase and proteins from the plasmid clones. (A) The *Pseudomonas* acylase protein. The denatured acylase protein was electrophoresed on an SDS-15% polyacrylamide gel and visualized with Coomassie blue (lane 2). M_rs of marker proteins used (lane 1) are: phosphorylase *b*, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,100; α-lactalbumin, 14,400. (B) Fluorogram of [^{35}S]methionine-labeled maxicells of *E. coli* CSR603 harboring pAKK1 (lane 1), pAKKB3 (lane 2), pAKKM1 (lane 3), or pBR325 (lane 4). Electrophoresis was performed on an SDS-12.5% polyacrylamide gel.

FIG. 5. Nucleotide sequence of a part of the GL-7ACA acylase gene. The sequence of the ca. 1.05-kb *Bam*HI-2–*Sal*I-2 fragment is presented. The predicted amino acid sequence is shown under the nucleotide sequence, and the amino-terminal amino acid sequences of the small and large subunits determined by the Edman method are underlined; one which was supposed to be Trp or Cys but was not identified is marked by an asterisk. The putative ribosome-binding site is indicated by a box.

to 46 (Ser) and from amino acid 199 (Ser) to 215 (Leu), respectively. The result indicated that the region coding for the small subunit preceded that coding for the large subunit on the structural gene, and it predicted a precursor polypeptide for both the subunits, since no termination codon was found in the sequence upstream of that for the large subunit on the proposed reading frame. The frame also predicted a sequence of 29 amino acids immediately upstream from the small subunit. This appears to be a signal peptide sequence because the coded peptide contained several positively charged amino acids, followed by a run of hydrophobic residues, as generally described on procaryotic secreted proteins (22). Furthermore, the acylase activity was found in periplasm in the *E. coli* clone (data not shown), corresponding to the previous observation that the enzyme appeared to be periplasmic in *Pseudomonas* sp. GK16 (13).

DISCUSSION

A plasmid designated pAKK1 containing the GL-7ACA acylase gene was isolated from a gene library for the total DNA of *Pseudomonas* sp. GK16. Despite the multicopy nature of the plasmid, the enzyme activity of *E. coli* carrying pAKK1 was much lower than that of GK16. When the size-reduced fragments were placed at a region downstream of the *E. coli* promoters, they gave a significant increase in the enzyme activity. We are not sure whether the acylase gene is transcribed by its own promoter in pAKK1, but the above observation suggests some difficulty for the *Pseudomonas* gene to be expressed in *E. coli*, as indicated by several studies (9, 11, 14, 21). A series of subcloning experiments localized the structural gene on the 2.45-kb fragment. The final segment may not contain the endogeneous promoter since the gene was not expressed without the help of an *E. coli* promoter.

The maxicell analysis of gene products by the clone disclosed the existence of an additional polypeptide with an M_r of ca. 70,000 besides those corresponding to two subunits of the purified acylase. We suspect that this polypeptide is the immediate precursor of the two subunits, although more evidence to prove it remains to be seen. If this is the case, the polypeptide itself may be generated from a precursor, with an estimated M_r of ca. 74,000, containing a signal sequence which is predicted from the DNA sequence.

Finally, the DNA sequence study together with the amino-terminal sequencing of the two subunit proteins localized the regions coding for the two subunits in the right order (small to large) on the structural gene. The result strongly supports the presence of a common precursor for both subunits since no termination codon was found between the sequences for the amino termini of the small and large subunits in the predicted reading frame. We also found a signallike sequence immediately upstream of the amino terminus of the small subunit. A preliminary study, together with the previous observation with *Pseudomonas* sp. GK16, suggests that this is functional as a signal sequence in *E. coli*. The nucleotide sequence disclosed the presence of a possible ribosome-binding site at 9 base pairs upstream of the putative initiation codon. Since this sequence is complementary to the 3′ end of 16S rRNA both in *E. coli* and *Pseudomonas aeruginosa* (25, 26), the presumed barrier for expression of the acylase gene may not be the step for initiation of translation.

From the results obtained in this study, a possible process for formation of the active acylase might be proposed as: (i) synthesis of a polypeptide containing sequences for the signal peptide and the small and large subunits as the translation product from the acylase gene, (ii) translocation of the product into periplasm and processing of the signal sequence to give rise to the 70,000-M_r polypeptide, and (iii) processing of the 70,000-M_r polypeptide to the small and large subunits and formation of an active enzyme complex by association of both the subunits. The details and order of each step must await further studies for confirmation of the proposed model. Recently, we have learned that some of these steps are quite similar to those in the formation of a penicillin acylase originated from *E. coli* ATCC 11105 (5, 6); the enzyme consists of two nonidentical subunits which appear to be processed from a common precursor. It will be of interest to see whether the proposed process is common to the penicillin or cephalosporin acylases.

The genetic nature for the induction of acylase synthesis by glutaric acid remains to be elucidated, since we used the DNA donor which produces the enzyme constitutively. To answer the question, investigations are currently in progress to clone the acylase gene from the original strain (SY-77-1).

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