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Molecular Cloning of the Penicillin G Acylase Gene from *Arthrobacter viscosus*

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Penicillin G acylase was purified from the cultured filtrate of *Arthrobacter viscosus* 8895GU and was found to consist of two distinct subunits with apparent molecular weights of 24,000 (alpha) and 60,000 (beta). The partial N-terminal amino acid sequences of the alpha and beta subunits were determined with a protein gas phase sequencer, and a 29-base oligonucleotide corresponding to the partial amino acid sequence of the alpha subunit was synthesized. An *Escherichia coli* transformant having the penicillin G acylase gene was isolated from an *A. viscosus* gene library by hybridization with the 29-base probe. The resulting positive clone was further screened by the *Serratia marcescens* overlay technique. *E. coli* carrying a plasmid designated pHYM-1 was found to produce penicillin G acylase in the cells. This plasmid had an 8.0-kilobase pair DNA fragment inserted in the *Eco*RI site of pACYC184.

Penicillin acylase (EC 3.5.1.11) occurs intracellularly and extracellularly in many kinds of microorganisms (20). It catalyzes the deacylation of beta-lactam antibiotics, and the resulting product, 6-aminopenicillanic acid (6-APA), is a widely used intermediate for organic synthesis of a variety of semisynthetic penicillins (24). Much effort has been made toward enhancing enzyme productivity through genetic manipulation. An alternative approach is gene cloning. Penicillin acylase genes have been cloned from *Escherichia coli* (17), *Bacillus sphaericus* (19), *Proteus rettgeri* (7), *Pseudomonas* sp. strain GK16 (16), and *Kluyvera citrophila* (8). These penicillin acylases are intracellular enzymes (11, 19, 24). In our laboratories, a microorganism which produces penicillin acylase extracellularly has been found and deposited in the American Type Culture Collection as *Arthrobacter viscosus* ATCC 15294 (Takeda et al.). *A. viscosus* secretes a large amount of penicillin acylase into the culture medium, thus providing a convenient means of obtaining the purified enzyme in large quantity. In addition, a secretion vector system which contains the *A. viscosus* penicillin acylase gene secretory leader might also be useful for the production and secretion of other valuable proteins into the culture medium. We were interested in isolating the penicillin G (Pc-G) acylase gene from *A. viscosus*, to improve enzyme productivity as well as to develop a novel secretion vector system composed of the promoter and signal sequence regions of the gene.

In this report, we describe the purification and characterization of the *A. viscosus* Pc-G acylase, the isolation of alpha and beta subunits of the enzyme, the partial N-terminal amino acid sequences of these subunits, and the use of a 29-base oligonucleotide corresponding to the partial N-terminal amino acid sequence of the alpha subunit to isolate the *A. viscosus* Pc-G acylase gene.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this work are listed in Table 1. *A. viscosus*, *Serratia marcescens*, and *E. coli* HB101 were grown with aeration in L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.0) at 28, 30, and 37°C, respectively. *A. viscosus* was also grown for enzyme preparation in M-6 medium, which consisted of 0.3% meat extract, 0.005% $CuSO_4 \cdot 5H_2O$, 0.00001% Nonion, and 5.4% corn steep liquor (pH 7.0) with 0.25% phenylacetic acid.

Reagents. Pc-G and 6-APA were the products of Banyu (Tokyo, Japan). Restriction endonucleases were obtained from Toyobo (Osaka, Japan) and Takara Shuzo (Kyoto, Japan). T4 DNA ligase and T4 polynucleotide kinase were obtained from Takara Shuzo. Calf intestinal alkaline phosphatase was purchased from Boehringer Mannheim (Mannheim, Federal Republic of Germany). Enzyme reactions were all performed following the recommendations of the supplier.

Characterization of penicillin acylase. Penicillin acylase activity was determined by measuring the 6-APA liberated from Pc-G according to the method of L. P. Marrelli (15). One unit of the enzyme was defined as the amount required to liberate 1 μmol of 6-APA per min at 37°C. Protein concentration was measured by the method of Lowry et al. (12). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was carried out by the method of Weber and Osborn (25). The molecular weight of the purified enzyme was estimated by SDS-PAGE and Sephacryl S-200 gel filtration. The marker proteins used were cytochrome *c* (molecular weight [MW], 12,500), chymotrypsin (MW, 25,000), ovalbumin (MW, 45,000), bovine serum albumin (MW, 68,000), and aldolase (MW, 158,000).

N-terminal amino acid sequencing of alpha and beta subunits. The partial N-terminal amino acid sequences of alpha and beta subunits were determined by automated Edman degradation on an Applied Biosystems 470A protein gas phase sequencer (Applied Biosystems, Inc.). For N-terminal

TABLE 1. Bacterial strains and plasmids

Strains and plasmids	Relevant characteristics	Source or reference
E. coli HB101	Host for transformation	4
A. viscosus (wild type)	Penicillin acylase producer	ATCC 15294
A. viscosus 8895GU	Hyperproducing mutant of *A. viscosus* ATCC 15294	This paper
S. marcescens	Pc-G resistant, 6-APA sensitive	ATCC 27177
pACYC184	Cm^r Tc^r	5
pHYM-1	Tc^r, penicillin acylase positive	This paper
pHYM-2	Tc^r, penicillin acylase positive	This paper

amino acid sequencing, 24 μg of the isolated alpha subunit and 74 μg of the isolated beta subunit were used.

Oligonucleotide synthesis. The 29-base probe was synthesized by a beta-cyanoethyl phoshoramidite method on an Applied Biosystems 380A DNA synthesizer (Applied Biosystems, Inc.) and purified by high-pressure liquid chromatography (column, TSK DEAE-2SW; solvent A, 0.2 M $HCOONH_4$–20% CH_3CN; solvent B, 1.0 M $HCOONH_4$–20% CH_3CN; elution, a linear gradient of 35 to 100% solvent B).

Construction of the *A. viscosus* 8895GU gene library. The *A. viscosus* 8895GU gene library was constructed by insertion of *Eco*RI-cleaved *A. viscosus* chromosomal DNA into the *Eco*RI site of the plasmid pACYC184. The chromosomal DNA from *A. viscosus* was prepared according to the method of Marmur (14) and completely digested with *Eco*RI. The plasmid DNA of pACYC184 from *E. coli* was prepared by the alkaline lysis method (1). The vector DNA was completely digested with *Eco*RI, and the 5′ end was dephosphorylated with calf intestinal alkaline phosphatase. The dephosphorylated vector DNA (2.0 μg) and the *A. viscosus* *Eco*RI-digested DNA (1.0 μg) were mixed and incubated with T4 DNA ligase in a 42-μl reaction volume at 16°C for 20 h and then were transformed into *E. coli* HB101 by the method of Maniatis et al. (13). The *E. coli* transformants were spread directly onto agar plates (100-mm diameter) containing L broth supplemented with 10 μg of tetracycline per ml.

Screening for Pc-G acylase-positive clones. The L agar plates were incubated at 37°C overnight, and then the colonies were transferred to nitrocellulose filters. Duplicate filters were made by gently pressing a second filter onto the first nitrocellulose filter. The filters were hybridized with the 29-base probe and 5′ labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (specific activity, $>10^8$ cpm/μg) for 44.5 h at 50°C in a solution containing 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium acetate; pH 7.0), 50 μg of denatured salmon sperm DNA per ml, 10× Denhardt solution (1× Denhardt solution is 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin), and 5×10^6 cpm of denatured probe DNA per ml (10). After hybridization the filters were washed three times at room temperature in 4× SSC–0.1% SDS for 20 min and then washed for 1.0 min in 4× SSC–0.1% SDS at 50°C. After drying in air, the filters were exposed to X-ray film at −70°C for 50 h with an intensifying screen. The resulting positive clones were further screened by an *S. marcescens* overlay technique to identify the Pc-G acylase-positive clones.

***S. marcescens* overlay technique.** Pc-G acylase-positive clones were isolated using the *S. marcescens* overlay technique (18). The *S. marcescens* strain is resistant to Pc-G but sensitive to 6-APA. Colonies of *E. coli* transformants on petri dishes were overlaid with 5 ml of soft agar containing 25 μl of an overnight culture of *S. marcescens* and 4 mg of Pc-G per ml. Inhibition plaques were scored after overnight culture at 30°C.

TABLE 2. Summary of purification of *A. viscosus* Pc-G acylase

Procedure	Total protein (mg)	Total activity (U)	Sp act (U/mg of protein)	Yield (%)
Extracts from enzyme cake	766.7	17,020	22.2	100
Ammonium sulfate	435.5	13,543	31.1	79.6
Hydroxyapatite	245.9	10,033	40.8	58.9
CM-Sephadex	42.8	1,874.3	43.8	11.0
Sephacryl S-200	39.2	1,629.5	41.6	9.6
DE52-cellulose	26.0	1,205.6	46.3	7.1
Sephacryl S-200	22.2	1,139.3	51.3	6.7

Construction of pHYM-2. pHYM-1 was digested completely with *Eco*RI, extracted with phenol, and precipitated with ethanol. The *Eco*RI-digested DNA was circularized with T4 DNA ligase to construct pHYM-2. The orientation of the DNA inserted in pHYM-2 was opposite to that in pHYM-1.

RESULTS

Purification and characterization of *A. viscosus* Pc-G acylase. *A. viscosus* 8895GU was grown in M-6 medium containing 0.25% phenylacetic acid at 28°C for 96 to 120 h with aeration. After cultivation, Pc-G acylase in the filtered culture medium (100 liters) was adsorbed on calcium phosphate gel (2.57 liters) containing Celite (3.57 kg), and 7.25 kg of enzyme cake was obtained. This immobilized-enzyme cake (200 g) was extracted with 500 ml of 0.75 M phosphate buffer (pH 7.0) by a batch method and washed with 500 ml of the same buffer. The extract solution was precipitated at 80% saturated ammonium sulfate. The precipitate was subjected to column chromatography on hydroxyapatite, CM-Sephadex, and DEAE-cellulose, followed by gel filtration on Sephacryl S-200. Three active enzyme fractions were obtained after CM-Sephadex column chromatography (data not shown). The first fraction was found in the wash (0.1 M sodium-potassium phosphate buffer, pH 6.0), and the second and third fractions were eluted with the same buffer containing 0.1 and 0.25 M NaCl, respectively, in a linear elution gradient (0 to 0.5 M NaCl). The third fraction (about 25% of the total activity) was used for further purification. The enzyme purification procedures are summarized in Table 2. The partially purified enzyme contained two major bands (MW, 24,000 and 60,000) and some minor bands on SDS-PAGE (see Fig. 1), and it was used for isolation of the alpha and beta subunits.

The MW of the partially purified Pc-G acylase was estimated to be 81,000 by Sephacryl S-200 gel filtration in 0.05 M sodium-potassium phosphate buffer (pH 7.0) (data not shown).

The *A. viscosus* acylase hydrolyzed mainly Pc-G, cephalothin, and cephalexin, but did not hydrolyze penicillin V and cephalosporin C (data not shown). The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) for penicillin G were 0.42 mM and 62 μmol of 6-APA per min per mg of protein, respectively. The optimum pH values and temperature for enzymatic reaction were pH 6.0 to 7.5 and 45 to 50°C with 10 min of incubation. The enzyme was stable

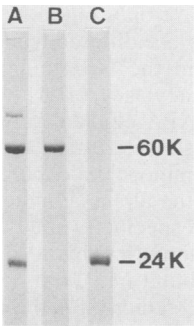

FIG. 1. SDS-PAGE of the purified *A. viscosus* Pc-G acylase (A), the isolated beta subunit (B), and the isolated alpha subunit (C).

in the pH range from 5.5 to 10 with 1 h of incubation at 37°Cand was rapidly inactivated above 50°C with 1 h of incubation at pH 7.0.

Isolation of alpha and beta subunits. The *A. viscosus* acylase was found to consist of two distinct polypeptide subunits with apparent MW of 24,000 (alpha) and 60,000 (beta) by SDS-PAGE analysis (Fig. 1A). For separation of the subunits, 0.20 ml of the enzyme preparation (4.3 mg of protein) was incubated overnight at 30°C with an equal volume of 0.02 M sodium phosphate buffer (pH 7.0), containing 2% 2-mercaptoethanol and 2% SDS, and applied to a Sephacryl S-200 gel filtration column (1.3 by 91 cm) previously equilibrated with 0.05 M sodium-potassium phosphate buffer (pH 7.0) containing 0.1% SDS. Elution was performed with the same buffer. The large (MW 60,000) and the small (MW 24,000) peptide fractions were pooled separately, concentrated by ultrafiltration, and rechromatographed on a Sephacryl S-200 column (1.3 by 91 cm) in the same SDS-containing buffer. The yields of the large and small peptide fractions were 1.60 and 0.92 mg of protein, respectively. As shown in SDS-PAGE (Fig. 1B and C), the isolated large peptide fraction contained a single band (MW, 60,000), and the small peptide fraction had a single band (MW, 24,000). The purified subunit polypeptides were used for determination of the N-terminal amino acid sequence.

N-terminal amino acid sequences of alpha and beta subunits. The N-terminal 38-amino-acid residues of alpha and beta subunits of *A. viscosus* Pc-G acylase were sequenced (Fig. 2). Two distinct N-terminal sequences were found in the alpha subunit, and they differed in length by three amino acid residues. The first sequence started from Asp-1, and the other (shorter) started from Glu-4 (Fig. 2). Forty-one (38 + 3) amino acid residues were sequenced in the alpha subunit. Their molar ratio (longer/shorter) was approximately 1/2.

Synthesis of the 29-base oligonucleotide. The 29-base oligonucleotide was synthesized based upon the partial amino acid sequence of the alpha subunit as follows, where I is deoxyinosine:

	27	28	29	30	31	32	33	34	35	36
Amino acids	Tyr	Glu	Ala	Tyr	Gly	Tyr	Val	Met	Ala	Lys
mRNA	UAU	GAA	GCU	UAU	GGU	UAU	GUU	AUG	GCU	AAA
	C	G	C	C	C	C	C		C	G
			A		A		A		A	
			G		G		G		G	

29-base probe 3′-ATGCTTCGI ATGCCI ATGCAI TACCGI TT-5′

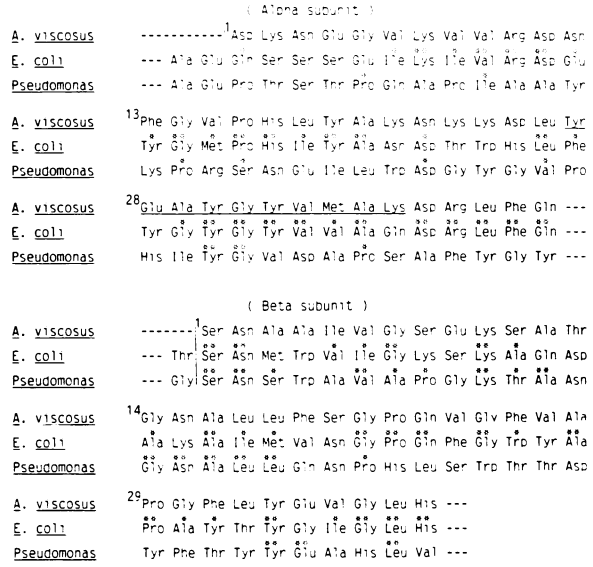

FIG. 2. Alignments of the partial amino acid sequences of alpha and beta subunits derived from *A. viscosus*, *E. coli*, and *Pseudomonas* acylases. The amino acid sequences of *E. coli* and *Pseudomonas* acylases are deduced from these nucleotide sequences (16, 21). *E. coli* acylase sequences are aligned from Ala-26 to Gln-68 and from Thr-289 to His-327 (21). *Pseudomonas* acylase sequences are aligned from Ala-29 to Tyr-71 and from Gly-198 to Val-237 (16). Identical and chemically related residues are indicated by ** and *, respectively. The partial amino acid sequence corresponding to the 29-base probe is underlined. The alpha subunit of *A. viscosus* acylase had two N-terminal amino acid sequences, one at Asp-1 and the other at Glu-4.

The 10-amino-acid stretch (Tyr-27 to Lys-36) was selected from the fewest number of combinations of genetic codons in the 41-amino-acid sequences of the alpha subunit. The choice of codons for Tyr and Glu was based upon the preferable codon usage in bacteria (9). The third positions of codons for Ala, Gly, and Val were substituted for deoxyinosine, which can pair with any of the four natural bases (23).

Isolation of the *A. viscosus* Pc-G acylase gene. Approximately 9,000 colonies from the *A. viscosus* gene library were screened by colony hybridization with the 5′-^{32}P-labeled 29-base probe. Positive clones were further screened by the *S. marcescens* overlay technique. As a result, a single positive colony was isolated and found to produce Pc-G acylase (Fig. 3). This transformed *E. coli* clone harbored a plasmid designated pHYM-1, and a restriction endonuclease

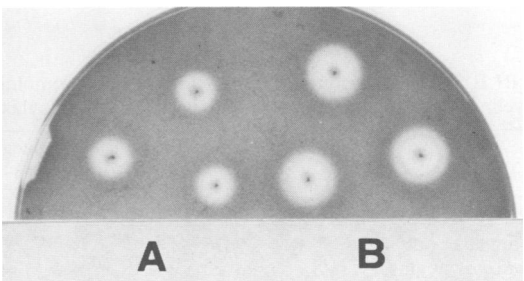

FIG. 3. Plaques of *S. marcescens* growth inhibition by the *E. coli* transformant carrying pHYM-1 (A) and the *E. coli* transformant carrying pHYM-2 (B).

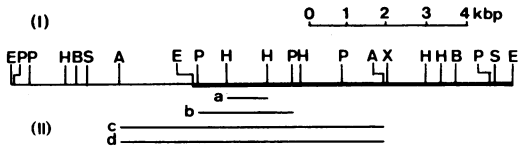

FIG. 4. Restriction endonuclease map of pHYM-1 (I) and DNA fragments hybridized with the 29-base probe (II). In line I, the thin line represents the pACYC184 vector and the thick line represents the cloned DNA fragment containing *A. viscosus* Pc-G acylase. For line II, Southern blotting was carried out using the ^{32}P-labeled 29-base probe, and pHYM-1 was digested with *Eco*RI and *Hin*dIII (a), *Pvu*II (b), *Ava*I (c), and *Ava*I and *Bam*HI (d), respectively. Restriction sites: E, *Eco*RI; P, *Pvu*II; H, *Hin*dIII; B, *Bam*HI; S, *Sal*I; A, *Ava*I; X, *Xho*I.

map of the plasmid was constructed (Fig. 4). The plasmid pHYM-1 was found to have an 8.0-kilobase-pair (kbp) DNA fragment which contained the *A. viscosus* Pc-G acylase gene in the *Eco*RI site of pACYC184.

The plasmid pHYM-2, which has the same 8.0-kbp DNA fragment inserted in the opposite orientation, transformed a plasmid-free *E. coli* strain to a Pc-G acylase-positive one (Fig. 3). To localize the Pc-G acylase gene on the 8.0-kbp *Eco*RI DNA fragment, the ^{32}P-labeled 29-base DNA probe was hybridized to pHYM-1 plasmid DNA digested with various restriction endonucleases (22). The 29-base probe hybridized to a 1.0-kbp *Hin*dIII, a 2.5-kbp *Pvu*II, and a 6.7-kbp *Ava*I DNA fragment (Fig. 4).

DISCUSSION

In this report, we describe the purification and characterization of *A. viscosus* Pc-G acylase, the partial N-terminal amino acid sequences of alpha and beta subunits, and the molecular cloning of the gene encoding Pc-G acylase from *A. viscosus*.

The *A. viscosus* acylase has an apparent MW of 81,000 and consists of two distinct (alpha and beta) subunit polypeptides with respective MWs of 24,000 and 60,000. Similarly, other penicillin acylases produced by *E. coli* ATCC 11105 (2), *P. rettgeri* ATCC 31052 (6), and *Pseudomonas* sp. strain GK16 (11) also have an alpha-beta-subunit structure with respective MWs of 20,500 and 69,000 for *E. coli* (2), 24,500 and 65,000 for *P. rettgeri* (6), and 16,000 and 54,000 for *Pseudomonas* sp. (11).

The partial N-terminal amino acid sequences of *A. viscosus* alpha and beta subunits were compared with the corresponding sequences of *E. coli* Pc-G acylase (21) and *Pseudomonas* sp. strain GK16 acylase (16) (Fig. 2). *A. viscosus* acylase was similar to *E. coli* acylase in the N-terminal amino acid sequences of both subunits and similar to *Pseudomonas* acylase only in the beta subunit (Table 3). The *Pseudomonas* acylase differed from the *A. viscosus* and the *E. coli* acylases in the N-terminal sequences of their alpha subunits. The acylases of *A. viscosus* and *E. coli* hydrolyze Pc-G to 6-APA efficiently, while the *Pseudomonas* enzyme deacylates 7-beta-(4-carboxybutanamido)cephalosporanic acid to 7-aminocephalosporanic acid (11). These findings indicate that the alpha subunit of penicillin acylase may regulate substrate specificity and that the beta subunit is required for acylase activity. This idea is supported by the fact that the alpha subunit of *P. rettgeri* acylase contains a domain that imparts specificity for the penicillin side chain, while the beta subunit contains a phenylmethylsulfonyl fluoride-sensitive residue which is essential for enzyme activity (6).

TABLE 3. Comparison of amino acid sequence homology between *A. viscosus* and *E. coli* and *Pseudomonas* acylases[a]

Enzyme compared	Amino acid sequence homology (%) with *A. viscosus* acylase:	
	Alpha subunit	Beta subunit
E. coli acylase	76	66
Pseudomonas acylase	22	47

[a] Homology was calculated from identical and conservative amino acids in the N-terminal 41- and 38-amino-acid sequences of their alpha and beta subunits (Fig. 2).

The proteolytic processing of the *E. coli* acylase has been reported as follows (3, 21): (i) the acylase gene is translated as a single precursor polypeptide in which a signal, an alpha peptide, a spacer, and a beta peptide are sequentially linked; (ii) the signal peptide is removed, and the resulting polypeptide is translocated through the cytoplasmic membrane; (iii) the spacer peptide is then removed during the course of processing to the alpha and beta subunits; and (iv) the catalytically active enzyme is released into periplasm. The two nonidentical subunits of *A. viscosus* acylase might be processed from a common precursor like those of *E. coli* (3) and *Pseudomonas* (16) acylases because (i) the homology between the *A. viscosus* and *E. coli* alpha and beta N-terminal amino acid sequences is relatively high, and (ii) the first two amino acids (Ser-Asn-) of the beta subunits for the *A. viscosus*, *E. coli*, and *Pseudomonas* acylases are identical (Fig. 2).

The alpha subunit purified from the *A. viscosus* acylase was found to have two cleavage sites at the N terminus of the polypeptide (Fig. 2). Therefore, the signal peptidase from *A. viscosus* might act at two distinct cleavage sites, or the polypeptide produced by signal peptidase might be further cleaved by other proteases. Thus the processing mechanism for the alpha subunit is still unclear.

The *A. viscosus* Pc-G acylase gene was isolated by colony hybridization using the 29-base probe and the *S. marcescens* overlay technique. The cloned Pc-G acylase gene would be expressed primarily from its own promoter, not by read-through from the Cm^r promoter on the vector, because pHYM-1 and pHYM-2 were both capable of transforming plasmid-free *E. coli* to Pc-G acylase positive (Fig. 3). Southern blot analysis using the 29-base probe indicated that the acylase gene exists around a 1.0-kbp *Hin*dIII DNA fragment (Fig. 4). This information will be useful to determine the minimum size of the inserted DNA for Pc-G acylase activity.

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