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Molecular Cloning of *Bacillus sphaericus* Penicillin V Amidase Gene and Its Expression in *Escherichia coli* and *Bacillus subtilis*

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The *Bacillus sphaericus* gene coding for penicillin V amidase, which catalyzes the hydrolysis of penicillin V to yield 6-aminopenicillanic acid and phenoxyacetic acid, has been isolated by molecular cloning in *Escherichia coli*. The gene is contained within a 2.2-kilobase *Hin*dIII-*Pst*I fragment and is expressed when transferred into *E. coli* and *Bacillus subtilis*. The expression in *B. subtilis* carrying the recombinant plasmid is approximately two times higher than in the original *B. sphaericus* strain. A comparison of the purified enzyme from *B. sphaericus* and the expressed gene product in *E. coli* minicells suggests that the native enzyme consists of four identical subunits, each with a molecular weight of 35,000.

Penicillin amidases (EC 3.5.1.11) are enzymes that catalyze the hydrolysis of penicillins and are used in the production of semisynthetic penicillins on a 1,000-ton (ca. 1,000,000-kg) annual scale (24). Obviously, there is great interest in enhancing the production of these enzymes by genetic approaches. This has been previously described for the production of penicillin G amidase from *Escherichia coli* (17), and in this paper we describe the cloning of the gene encoding penicillin V amidase from *Bacillus sphaericus* and the expression of the cloned gene in *E. coli* and *Bacillus subtilis*. Studies have also been performed to characterize the enzyme.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used are listed in Table 1.

Media and buffers. NY medium (16), LA plates (16), and DM3 plates (7) were prepared as previously described. Antibiotic-resistant clones were selected on LA plates containing 70 μg of ampicillin (Astra, Södertälje, Sweden) per ml, 10 μg of tetracycline (Pfizer Inc., New York, N.Y.) per ml, or 10 μg of chloramphenicol (Sigma Chemical Co., St. Louis, Mo.) per ml. Penicillin V, 6-aminopenicillanic acid (6-APA), and phenoxyacetic acid were obtained from Fermenta, Strängnäs, Sweden; [^{35}S]methionine was from New England Nuclear Corp., Boston, Mass.

Preparation of DNA. Chromosomal DNA from *B. sphaericus* was prepared by total lysis with lysozyme and sodium dodecyl sulfate (SDS) in a neutral buffer followed by extraction with phenol. The DNA was fractionated after digestion with restriction enzyme on a sucrose gradient (10 to 30% [wt/vol]) in TE buffer (16) containing 1 M NaCl. Plasmid DNA from *E. coli* was prepared by the alkaline lysis method as described by Birnboim and Doly (1). Plasmids from *B. subtilis* were prepared with a modified alkaline lysis method essentially as described by Kieser (11). A 5-ml overnight culture was centrifuged, and the pellet was suspended in a total volume of 500 μl of lysozyme solution (0.3 M sucrose, 25 mM Tris-hydrochloride [pH 8.0], 0.02% bromphenol blue, 25 mM EDTA, 2 mg of lysozyme per ml). The suspension was incubated for 30 min at 37°C. After the addition of 250 μl of NaOH-SDS solution (0.3 M NaOH, 2% SDS), the mixture was incubated at 65°C for 20 min. The solution was then extracted at room temperature with 80 μl of phenol-chloroform (500 g of phenol, 500 ml of chloroform, 200 ml of water), the phases separated, and the aqueous phase containing plasmid DNA precipitated with 0.3 M sodium acetate and 1 volume of isopropanol. The DNA was digested with restriction enzymes as suggested by the suppliers (New England Biolabs, Beverly, Mass.; Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

Transformation of cells. The transformation of competent *E. coli* was performed as described by Morrison (19). Protoplasts of *B. subtilis* were transformed as described by Chang and Cohen (7).

Screening for penicillin V amidase-positive clones. *E. coli* clones carrying *B. sphaericus* DNA were replicated to LA plates and incubated overnight. They were then overlaid with 5 ml of soft agar containing *Serratia marcesens* (0.5 ml of an overnight culture per 100 ml) and K-penicillin V (4 mg/ml). The plates were incubated overnight at 28°C, and positive clones were detected as described by Mayer (17). An alternative and quick method of detecting positive clones was also developed. The cells were suspended in a 2 mM solution of 6-nitro-3-phenylacetamido-benzoic acid, and the hydrolysis was measured. The product of this reaction, 5-amino-2-nitrobenzoic acid, was determined quantitatively by recording the increase in optical density at 405 nm or qualitatively by visual inspection. This substrate was also used for enzymatic staining in the gradient gel electrophoresis. 6-Nitro-3-phenoxyacetamido-benzoic acid was synthesized as previously described by Kutzbach and Rauenbusch (13), except that the phenylacetate was replaced by phenoxyacetic acid. The product was finally purified on a Florisil column.

Purification and labeling of minicells. Minicells were purified from the *E. coli* strain M2141 by two sucrose gradient centrifugations as described by Kennedy et al. (10). Labeling of minicells with [^{35}S]methionine was carried out in minimal medium supplemented with glucose, thiamine, and methionine assay medium as described by Molin et al. (18).

Assay of penicillin V amidase activity. Cells were grown in liquid medium containing the appropriate antibiotic and harvested after overnight growth. The cells were suspended in 1/10 volume of 0.1 M sodium citrate buffer (pH 5.8) and disrupted by sonication. The homogenate was centrifuged, and the supernatant was used for the assay of penicillin V

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TABLE 1. Bacterial strains and plasmids

Strains and plasmids	Relevant characteristics	Source or reference
Escherichia coli HB101	Host for gene constructions	Boyer and Roulland-Dussoix (3)
Bacillus sphaericus	Penicillin V amidase producer	ATCC 14577
Serratia marcescens	6-APA sensitive	ATCC 27177
Bacillus subtilis 168	Type strain	ATCC 6051
Escherichia coli M2141	Minicell producer	Dougan and Shenatt (8)
pTR262	Positive selection vector	Roberts et al. (21)
pC194	Cm^r	Horinouchi and Weisblum (9)
pBR322	Ap^r; Tc^r	Bolivar et al. (2)
pUN101	Ap^r	Nilsson et al. (20)
pOH3	Tc^r; penicillin V amidase positive	This paper
pOH31	Tc^r; penicillin V amidase negative	This paper
pOH35	Tc^r; penicillin V amidase positive	This paper
pOH36	Tc^r; penicillin V amidase negative	This paper
pOH38	Tc^r; Cm^r; penicillin V amidase positive	This paper

amidase activity. The standard assay mixture (total volume, 500 μl) contained 3% (wt/vol) K-penicillin V (potassium salt) in 0.1 M sodium citrate buffer (pH 5.8). The reaction was started by adding enzyme (50 to 250 μl), and the mixture was incubated at 37°C for up to 50 min. The reaction was terminated by heating in a boiling water bath for 90 s. The amount of 6-APA was determined as described by Kornfeld (12). The reaction product, 6-APA, was also identified by thin-layer chromatography (15). The pH optimum was determined by using 0.1 M sodium citrate (pH 4.5 to 6.2) and 0.1 M sodium phosphate (pH 5.8 to 8.0) buffers. The determination of the kinetic parameters was carried out in a pH stat in which the reaction rate was measured by titration with 10 mM NaOH.

Isolation of penicillin V amidase from *B. sphaericus*. *B. sphaericus* was grown in a 12-liter fermentor (Chemoferm) in NY medium without regulation of pH, with an aeration of 1 volume/volume per min, stirring at 700 rpm, and at a temperature of 30°C. After 10 to 11 h of growth, the cells were harvested in a CEPA centrifuge. The yield was 85 g (wet weight) from 10 liters of culture medium. Cell paste was suspended (16 g [wet weight]/60 ml) in 50 mM potassium phosphate buffer (pH 6.8), and the cells were disrupted by sonication three times for 45 s each at 0°C (70 W). The extract was centrifuged at 15,000 × *g* for 30 min at 4°C, and the supernatant used for purification of the enzyme was essentially as described by Carlsen and Emborg (6). After a 40% ammonium sulfate precipitation, the enzyme was precipitated with 70% ammonium sulfate. The precipitate was suspended in a small volume of 0.1 M Tris-hydrochloride–10 mM EDTA (pH 8.0) and placed on a Sephadex G-200 gel column equilibrated with 0.1 M Tris-hydrochloride–10 mM EDTA (pH 8.0). The column was eluted with the same buffer, and fractions with high activity were pooled. This step removed some proteolytic activity, and this stabilized the solution. The pooled fraction was immediately chromatographed on a DEAE-Sephadex A-50 column. This preparation was then used to determine the pH optimum and the kinetic properties of the enzyme.

Analysis of the purified enzyme. The protein concentration was measured by the method of Bradford, with bovine albumin as the standard (4). SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (14). Isoelectric focusing was carried out by using LKB ampholine polyacrylamide gel plates (pH 4.0 to 6.5). The molecular weight of the native enzyme was determined by high-pressure liquid chromatography with an LKB 2135 Ultropac TSK column packed with G3000 SW gel. The solvent was 50 mM potassium phosphate buffer (pH 7.0) with 0.2 M NaCl.

RESULTS

Purification and characterization of penicillin V amidase from *B. sphaericus*. The penicillin V amidase from *B. sphaericus* was purified, and the results of the purification procedure are summarized in Table 2. The purity of the final preparation was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 1), revealing a major band with a molecular weight of ca. 35,000 which corresponded to more than 95% of the total protein content. The molecular weight of the native enzyme was estimated by two independent methods. Gradient electrophoresis followed by enzyme staining gave one band with a molecular weight of 140,000, and gel filtration in a high-pressure liquid chromatography system gave a molecular weight of 135,000 (data not shown).

The isoelectric point of the native enzyme was determined by isoelectric focusing, which gave a major band corresponding to a pI of 4.8. The enzyme activity at various pH values was measured and gave a pH profile with an optimum of ca. 5.8 (Fig. 2A). The kinetic properties are shown in Fig. 2B. The K_m of 11 mM is significantly lower than that previously published (6). As shown in Fig. 2B, both enzyme products

TABLE 2. Summary of the purification of penicillin V amidase from *B. sphaericus*

Purification step	Vol (ml)	Total activity (U)[a]	Total protein (mg)[b]	Sp act (U/mg)	Purification factor	Yield (%)
Sonication	44	1,540	1,170	1.31	1	100
Ammonium sulfate	16	874	333	2.62	2.0	56
Sephadex G-200	120	328	121	2.71	2.1	21
DEAE-Sephadex	81	139	6.89	20.2	15.4	9

[a] One unit (U) is defined as the amount of enzyme which catalyzed the formation of 1 μmol of 6-APA per minute. Determined as described by Kornfeld (12).
[b] Protein determined as described by Bradford (4).

inhibit the reaction. Phenoxyacetic acid is a noncompetitive inhibitor with a K_i of 25 mM, and 6-APA acts as a competitive inhibitor with a K_i of 50 mM.

We also investigated the ability of whole cells of *B. sphaericus* to convert 10% (wt/vol) K-penicillin V to 6-APA and phenoxyacetic acid at various pH values and found a pH optimum for full conversion (>90%) of 6.4 (data not shown), which is slightly higher than the optimum for the initial reaction rate.

Cloning and expression of the gene for penicillin V amidase. A gene bank of *B. sphaericus* DNA was constructed. Chromosomal DNA was partially digested with *Mbo*I and fractionated by sucrose gradient centrifugation (10 to 30%). Fractions containing ca. 10-kilobase fragments were mixed and ligated with *Bcl*I-cleaved pTR262 and used to transform *E. coli* HB101. About 2,200 tetracycline-resistant clones were transferred to microtiter plates, grown overnight, and stored in a freezer at −80°C. Approximately 1,000 clones were screened with the *Serratia* overlay technique, and two positive clones, pOH2 and pOH3, were found. A restriction map of pOH3, the smaller of the two plasmids, is shown in Fig. 3.

The size of the insert in pOH3 was shown to be 9.1 kilobases. Various subclones were constructed to determine the minimum size of inserted DNA that is necessary for amidase activity (Fig. 3). The plasmid pOH31 obtained by digestion with *Hin*dIII and religation has no functional amidase activity (Table 3), in contrast to plasmid pOH35 containing a 2.2-kilobase fragment obtained by digestion with *Pst*I and religation. When the *Cla*I fragment was removed from pOH35, the activity was lost, indicating that all or part of the gene is within this fragment. For unknown reasons, the resulting plasmid pOH36 was, in all clones isolated, found to be exclusively a dimer (Fig. 3). A shuttle vector for *B. subtilis* and *E. coli* was constructed by inserting

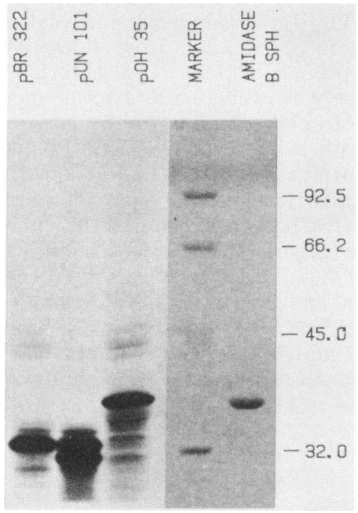

FIG. 1. SDS-polyacrylamide gel electrophoresis of minicell preparations and purified penicillin V amidase from *B. sphaericus*. The samples were applied to the same gel, and the gel was divided into two halves (bands visualized by autoradiography and Coomassie blue staining, respectively). Lanes 1 to 3, Plasmid-encoded ^{35}S-labeled proteins from pBR322 (lane 1), pUN121 (lane 2), pOH35 (lane 3); lane 4, size marker; and lane 5, purified penicillin V amidase from *B. sphaericus*.

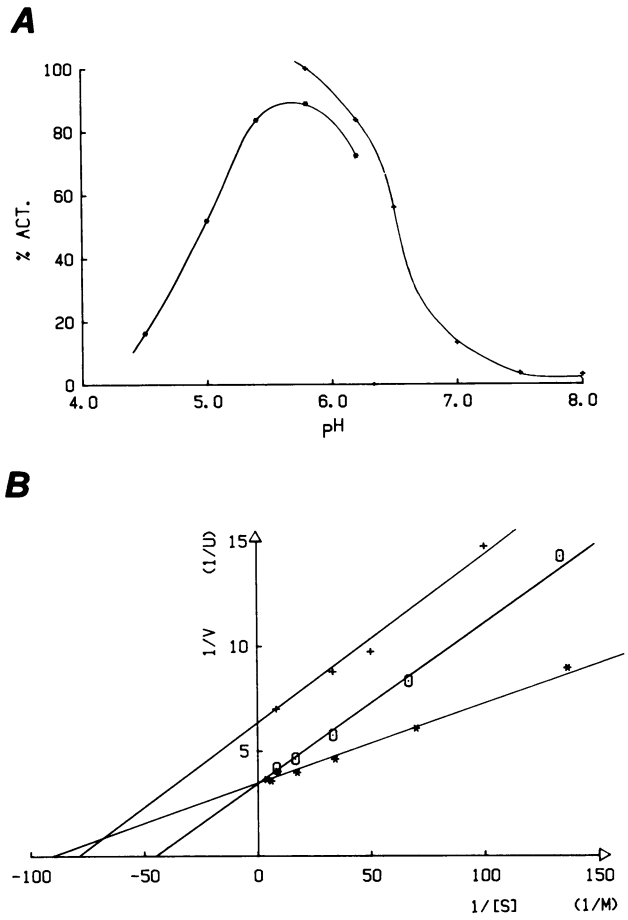

FIG. 2. (A) pH optimum of penicillin V amidase. The buffers used were 0.1 M sodium citrate (pH 4.5 to 6.2) and 0.1 M sodium phosphate (pH 5.8 to 8.0). (B) Lineweaver-Burk plot for penicillin V amidase (*), with 50 mM 6-APA (○) and 20 mM phenoxyacetic acid (+).

*Hin*dIII-cleaved pC194 (which has a gene for chloramphenicol acetyltransferase) in the *Hin*dIII site of pOH35. Protoplasts of *B. subtilis* were transformed with the resulting plasmid pOH38. Transformants were selected on regeneration plates containing chloramphenicol. Plasmid purification and enzyme digestion showed that all chloramphenicol-resistant clones contained pOH38. Expression of amidase activity was confirmed in cell extracts.

The specific amidase activity in cell extracts of *E. coli* and *B. subtilis* is shown in Table 3. The activities in the various *E. coli* clones are lower than those in *B. sphaericus*. *B. subtilis* (pOH38) gives an expression that is two times higher than in *B. sphaericus*.

Identification of plasmid-coded penicillin V amidase. The plasmids pOH35, pBR322, and pUN101 were used to transform the *E. coli* minicell strain M2141. The minicells were labeled with [^{35}S]methionine, and the plasmid-coded polypeptides were identified after separation on SDS-polyacrylamide gel electrophoresis (Fig. 1). A protein coded by pOH35 and not by the controls pBR322 and pUN101 comigrates with purified penicillin V amidase from *B. sphaericus*. The band common to pBR322 and pUN101 corresponds to β-lactamase (23), and the lower band in pUN101 corresponds to the cI repressor protein (22).

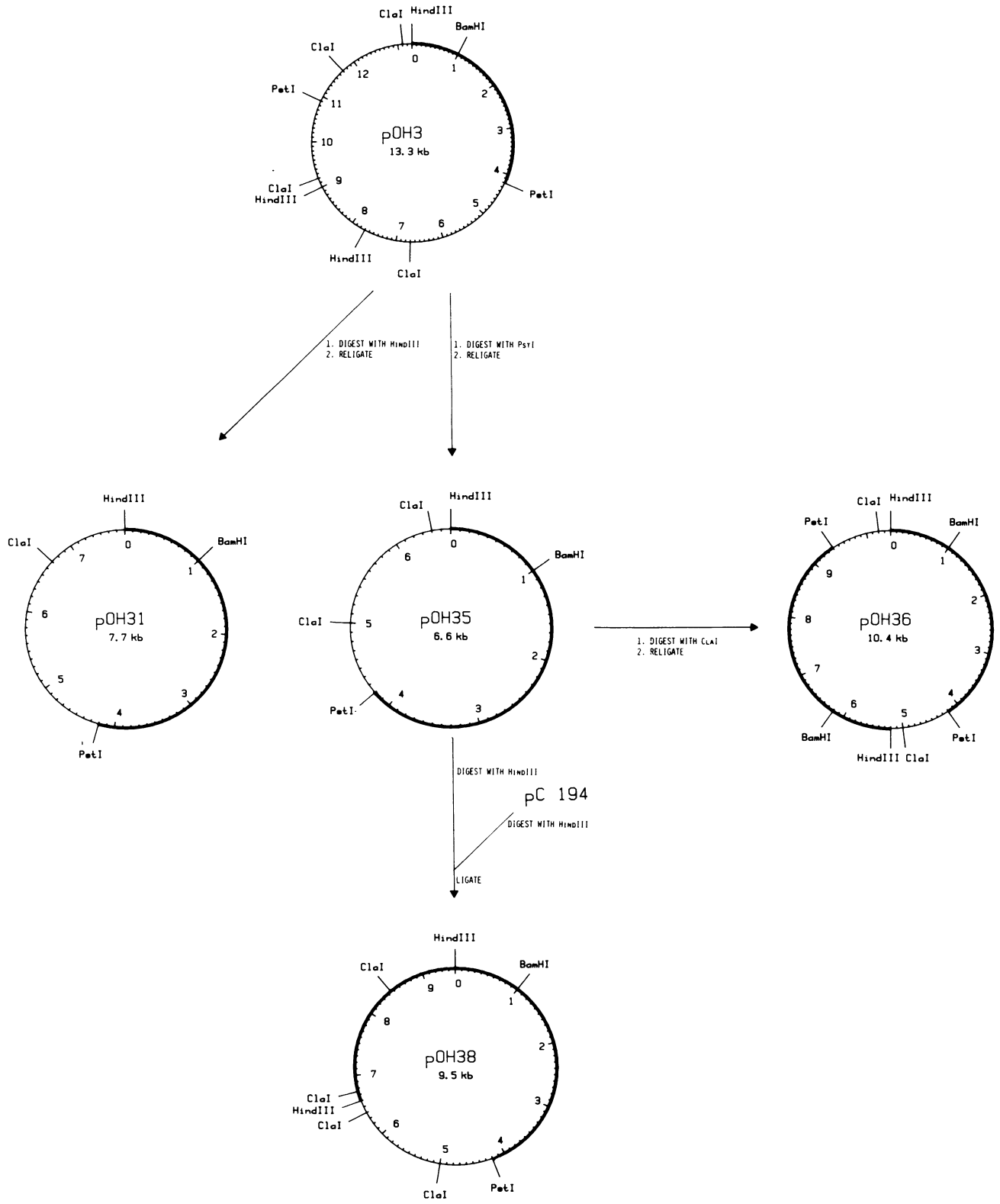

FIG. 3. Schematic presentation of plasmid constructions used to subclone the gene coding for penicillin V amidase. Thick lines indicate plasmid vectors and thin lines indicate inserted fragment. Relevant restriction sites are indicated.

TABLE 3. Specific penicillin V amidase activity of various strains and clones

Strain	Plasmid	Sp act (U/g)[a]
Escherichia coli HB101	pOH3	110
	pOH31	0
	pOH35	110
	pOH36	0
	pOH38	82
Bacillus subtilis	pOH38	410
	pC194	0
Bacillus sphaericus		240

[a] Determined as described by Kornfeld (12). Zero values correspond to less than 1 U/g.

DISCUSSION

In this paper, we have described the cloning of the gene encoding penicillin V amidase from *B. sphaericus* and the production of the enzyme in various hosts. Although this penicillin V amidase has been described earlier (5, 6), it has not been characterized extensively at a biochemical or structural level. Therefore, in addition to the genetic cloning, we have also characterized the enzyme to investigate its suitability for industrial production of 6-APA from penicillin V.

The results from gel electrophoresis and high-pressure liquid chromatography studies of various preparations of purified penicillin V amidase suggest a native enzyme with a molecular weight of ca. 140,000 consisting of four identical subunits.

Characterization of the enzyme revealed a pI of 4.8, a pH optimum of 5.8, and a K_m of 11 mM. This is significantly different from previous studies (6) which showed a pH optimum of 6.8 and a higher K_m value (140 mM). Both the reaction products inhibit the reaction (Fig. 2B). However, the K_i values are completely different at pH 5.8 and 6.8. The kinetic parameters suggest that the enzyme could be used for industrial purposes; this is further supported by the high yield of conversion with whole cells of *B. sphaericus*.

The penicillin V amidase gene was cloned in *E. coli*. Due to its sensitivity to the reaction product 6-APA, *S. marscecens* was used to detect the production of the enzyme. By using a vector with two different replicons (pOH38), it was possible to transfer the gene between *E. coli* and *B. subtilis*.

Although the replicons used gave a high copy number of plasmids in these species, no dramatic increase in the level of expression was observed. In fact, *E. coli* cells containing the plasmid-coded gene produce less enzyme than the original strain containing only one copy of the gene. It is at present unknown whether the reduced expression is restricted at the level of transcription, translation, or post-translation. However, in *B. subtilis*, which is closely related to the donor organism, the amount of enzyme produced is approximately twice as much as in the original strain. This suggests that *B. subtilis* could be a suitable host for the production of penicillin V amidase, but the reasonable yields in both species indicate that the enzyme is relatively stable in these heterologous hosts. This is confirmed by the expression in minicells of *E. coli* (Fig. 1), which reveal little or no degradation of the enzyme.

The present investigation has demonstrated that the penicillin V amidase of *B. sphaericus* could be suitable for large-scale conversion of penicillin V to 6-APA. The cloned gene is expressed both in *E. coli* and *B. subtilis* in reasonable yields, indicating that any of these heterologous hosts could be used for production of the enzyme by genetic approaches. Therefore, future work will be focused on the complete characterization of the gene by sequence analysis to study the structural features of the enzyme as well as the regulatory signals needed for the expression in *B. sphaericus*. This would also facilitate the production of the enzyme by inserting the gene into suitable expression vectors designed for *E. coli* or *B. subtilis*.

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