Overexpression of Penicillin V Acylase from *Streptomyces lavendulae* and Elucidation of Its Catalytic Residues

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The *pva* gene from *Streptomyces lavendulae* ATCC 13664, encoding a novel penicillin V acylase (SIPVA), has been isolated and characterized. The gene encodes an inactive precursor protein containing a secretion signal peptide that is activated by two internal autoproteolytic cleavages that release a 25-amino-acid linker peptide and two large domains of 18.79 kDa (α-subunit) and 60.09 kDa (β-subunit). Based on sequence alignments and the three-dimensional model of SIPVA, the enzyme contains a hydrophobic pocket involved in catalytic activity, including Serβ1, Hisβ23, Valβ70, and Asnβ272, which were confirmed by site-directed mutagenesis studies. The heterologous expression of *pva* in *S. lividans* led to the production of an extracellular homogenous heterodimeric enzyme at a 5-fold higher concentration (959 IU/liter) than in the original host and in a considerably shorter time. According to the catalytic properties of SIPVA, the enzyme must be classified as a new member of the Ntn-hydrolase superfamily, which belongs to a novel subfamily of acylases that recognize substrates with long hydrophobic acyl chains and have biotechnological applications in semisynthetic antifungal production.

Penicillin acylase (PA; penicillin amidohydrolase; EC 3.5.1.11) catalyzes the hydrolysis of penicillins into 6-aminopenicillanic acid (6-APA) and the corresponding organic acid. The classification of PAs is based on their substrate specificity, i.e., penicillin G acylases (PGA) or penicillin V acylases (PVA), that preferentially cleave phenylacetyl penicillin (penicillin G [PG]) or phenoxymethyl penicillin (penicillin V [PV]), respectively (1, 2). The relevance of these enzymes lies in the fact that semisynthetic penicillins currently are industrially produced by the enzymatic hydrolysis of PG or PV.

PVA is widely distributed among several microorganisms, being intra- and extracellularly produced (2–6). PVA from *Streptomyces lavendulae* ATCC 13664 (SIPVA) is an extracellular enzyme which has been exhaustively characterized (7–10) and immobilized (11, 12) due to its ability to hydrolyze very efficiently PV and other natural aliphatic penicillins that contaminate PV and usually reduce 6-APA yield at the end of the process. The broad substrate specificity of SIPVA allows this enzyme to hydrolyze several penicillins with aliphatic acyl chains, e.g., 3-hexenoyl-penicillin (penicillin F [PF]), hexanoyl-penicillin (penicillin dihydro-F [PdF]), and octanoyl-penicillin (penicillin K [PK]), as the catalytic constant for PK was even higher than that for PV (13). These observations indicate SIPVA is an effective industrial enzyme, provided that it can be obtained in large amounts.

Here, we describe the heterologous overproduction of SIPVA in *Streptomyces lividans* and the characterization of its catalytic residues by site-directed mutagenesis.

**MATERIALS AND METHODS**

**Materials.** Penicillin V (potassium salt), penicillin G (potassium salt), phenoxyacetic acid, phenylacetic acid, aculeacin A, and fluorescamine were from Sigma-Aldrich (St. Louis, MO). 6-APA and natural aliphatic penicillins (penicillin K, penicillin F, and penicillin dihydro-F) were provided by Antibióticos S.A. (León, Spain). All other reagents and products were from Merck (Darmstadt, Germany).

**Microorganisms, culture conditions, and plasmids.** The bacterial strains, plasmids, and oligonucleotides used are listed in Table 1. *S. laven-

dulae* ATCC 13664 was used as a PVA producer and DNA source (7, 8). *Escherichia coli* DH5α was used as the host for subcloning experiments, and *S. lividans* 1326 was used as the host for gene expression. For sporulation on solid medium, the actinomycetes were grown at 30°C on plates containing SFM (mannitol soya flour agar) medium (14). For protoplast preparation or DNA extraction, *S. lavendulae* and *S. lividans* were cultured in liquid YEMEG medium (YEME [yeast extract, malt extract] plus 0.5% glycine) to allow dispersed growth at 30°C and 250 rpm (14). *E. coli* cells were cultured at 37°C in Luria-Bertani medium, and the transformation was carried out by standard procedures (15). pGEM-T Easy vector (Promega) was used for subcloning experiments and DNA sequencing. The *Streptomyces-E. coli* shuttle vector pEM4 (16) was used for gene expression in *S. lividans*. *S. lividans* transformants were cultured in media containing 5 μg/ml thiostrcptin in liquid medium or 25 μg/ml thiostrptcin in solid medium. *E. coli* transformants were cultured and selected in medium containing ampicillin at 100 μl/ml. The shuttle vector pNV19

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Production and purification of SPPa from *S. lavendulae*. SPPa from *S. lavendulae* ATCC 13664 was produced as previously described (7, 8) and purified with slight modifications, using hydroxyapatite for the last step instead of Ultrogel AcA44. Protein concentration was determined according to Bradford (21). Electrophoresis on 0.1% SDS was carried out on a polyacrylamide slab gel (12.5%) with 25 mM Tris-HCl buffer, pH 8.6 (22). To determine the N-terminal sequences of the protein bands, the protein bands were separated by SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) (23), and sequenced by automatic Edman degradation.

**DNA manipulation and sequencing.** Total DNA from *S. lavendulae* ATCC 13664 was purified as described previously (14). Plasmid DNA preparations, restriction endonuclease digestions, ligations, and other DNA manipulations were carried out according to the standard procedures used for *E. coli* (15) and for *Streptomyces* (14). The preparation of *S. lividans* protoplasts and the transformation and selection of transformants were performed as described previously (14). DNA sequences were determined by the dideoxy chain termination method (24) with an automated ABI Prism 3730 DNA sequencer (Applied Biosystems).

**PCR conditions.** Amplifications were carried out in a Mastercycler gradient thermocycler (Eppendorf, Germany), and all PCR products were purified by GeneClean (Qiagen, USA) and subcloned into pGEM-T Easy vector. The DNA fragments of 600 and 336 bp, carrying sequences of *pva*, were obtained by PCR amplification of the *S. lavendulae* chromosomal DNA using the degenerated primers STREPTOP2/STREPTOG3 and STREPTOG1/STREPTOD2, respectively (Table 1), and the PCR conditions (see Table S1 in the supplemental material). The PCR primers were designed according to the N-terminal sequences determined in this work, taking into account the codon usage of *Streptomyces* Easy vector. The PCR amplifications standard conditions were as follows. Template DNA (0.5 μg) was mixed with 0.8 μM each primer and 2.5 U of *Pfu* DNA polymerase (Promega, USA) in a total reaction volume of 100 μl. Amplification was performed using an automatic thermal cycleer (Eppendorf, Germany). The following reaction conditions were used: initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 1.5 minutes, followed by final extension at 72°C for 3 minutes. Following amplification, the DNA products were separated on a 1% agarose gel and the DNA fragment of 600 bp was purified by the electroelution method (25) and digested with appropriate restriction enzymes for cloning into the *E. coli* host vector pGEM-T Easy (Promega, USA). The PCR products were ligated into the vector pGEM-T Easy using T4 DNA ligase (Promega, USA) and used to transform *E. coli* DH5α competent cells, according to the manufacturer’s instructions (24). The transformants were selected on LB plates containing 100 μg/ml ampicillin and 100 μg/ml X-Gal and the presence of the recombinant plasmid pGEM-T Easy vector was confirmed by digestion with appropriate restriction enzymes and confirmed by sequencing (24), using an ABI Prism 3730 DNA sequencer (Applied Biosystems).

**TABLE 1 List of bacterial strains, plasmids and primers used**

<table>
<thead>
<tr>
<th>Strain, plasmid, or primer</th>
<th>Relevant genotype, description, or sequence (5’ to 3’)</th>
<th>Reference and/or source</th>
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<tr>
<td>DH5α</td>
<td>F− λ− ϕ80dlacZΔM15 endA1 recA1 hsdR17 (rK− mK−) supE44 thi-1 gyrA96 relA1 Δ(lac2Z Ya-argF)U169</td>
<td>Stratagene</td>
</tr>
<tr>
<td>XL10 Gold</td>
<td>Δ(mcrA)18SΔ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA lac, Hte, Tet, Cam</td>
<td>Stratagene</td>
</tr>
<tr>
<td>XL1 red competent cells</td>
<td>endA1 gyrA96 thi-1 hsdR17 supE44 relA lac mutD5 mutS mutT Tn10, Tet</td>
<td>Stratagene</td>
</tr>
<tr>
<td>Actinomycetes</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces lavendulae</em></td>
<td>Penicillin V acylase producer</td>
<td>ATCC; 7</td>
</tr>
<tr>
<td>ATCC 13664</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces lividans</em></td>
<td>Tsr*</td>
<td>ARS culture collection (NRRL)</td>
</tr>
<tr>
<td>1326 (NRRL B-16148)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Rhodococcus</em> sp. strain</td>
<td>Km*</td>
<td>Korean Agricultural Culture Collection; 18</td>
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<tr>
<td>T104KACC 21099</td>
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<td></td>
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<tr>
<td>Plasmids</td>
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<td></td>
</tr>
<tr>
<td>pGEM-T Easy Vector</td>
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<td>Promega</td>
</tr>
<tr>
<td>pEM4</td>
<td>Shuttle vector for <em>E. coli/Streptomyces</em>, Ap’, Tsr’, PermE*, pUCori, pWHM4ori, 7.9 kb</td>
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</tr>
<tr>
<td>pNV19</td>
<td>Shuttle vector for <em>E. coli/Rhodococcus</em>, Kat’, pAL5000ori, lacZ, ColE1ori, 4.4 kb</td>
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<tr>
<td>pPEA1F</td>
<td>pEM4 derivative containing 2.445-bp HindIII/EcoRI fragment from pEPA1F</td>
<td>This work</td>
</tr>
<tr>
<td>pNPV1A1F</td>
<td>pNV19 derivative containing 2.249-bp HindIII/EcoRI fragment from pEPA1F including <em>pva</em> gene from <em>S. livendulae</em> ATCC 13664, <em>E. coli</em> RBS, ATG start codon, signal peptide sequence, and strong constitutive Per<em>E</em> promoter from <em>Saccharopolyspora erythraea</em></td>
<td>This work</td>
</tr>
<tr>
<td>Primers</td>
<td></td>
<td></td>
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<tr>
<td>STREPTOP2</td>
<td>5’-CCGGAATTC(A/C/G)GGGCGCTA(G/C)CG(C/G)GTCGGACACGTG-3’</td>
<td>This work</td>
</tr>
<tr>
<td>STREPTOD2</td>
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<td>This work</td>
</tr>
<tr>
<td>STREPTOG1</td>
<td>5’-CCGGAATTC(C/G)GGGCGCTA(C/G)CG(C/G)GTCGGACACGTG-3’</td>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>CsaII</td>
<td>5’-CCGGAATTCGCCGGGCCGCGCGCGCGCGCGCG-3’</td>
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</tr>
<tr>
<td>FcbD2</td>
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<td>This work</td>
</tr>
<tr>
<td>FcbD2</td>
<td>5’-AGGCCGGGCGCGCTGCCTT-3’</td>
<td>This work</td>
</tr>
<tr>
<td>PVA-1</td>
<td>5’-TCTAGAGGCTATTAAATATGACCCTCGTAACCGCTCAGACTG-3’</td>
<td>This work</td>
</tr>
<tr>
<td>PVA-F</td>
<td>5’-CCGGAATTCCTACCGCCGCTGCGACCGACGCG-3’</td>
<td>This work</td>
</tr>
</tbody>
</table>

**a** Engineered endonuclease sites on the oligonucleotides are underlined, RBS are in italics, and start and stop codons are in boldface.

(Km*, lacZ) (17) and *Rhodococcus* sp. strain T104 KACC 21099 (18) were used for site-directed mutagenesis studies. *Rhodococcus* sp. strain T104 cells were grown in 2× YTG (yeast extract–Bacto tryptone–NaCl) medium supplemented with glucose (5 g/liter) (19).
Southern blotting experiments. Inverse PCR experiments were performed as described previously (25). In brief, 8 μg of DNA from *S. lavendulae* was digested with ScaI or HincII endonuclease according to the specifications of the supplier (Pharmacia). Restriction digestions were stopped by DNA isopropanol precipitation and resuspended in 20 μl Tris-EDTA (TE) buffer. For circularization, restriction fragments were ligated by T4 DNA ligase (USB) in a total reaction volume of 300 μl during 16 h at 4°C using the buffer recommended by the supplier. DNA purified by ethanol precipitation was resuspended in 25 μl TE buffer, and 5 μl of circularized DNA solution was used as the template to perform inverse PCRs. Chromosomal DNA from *S. lavendulae* was used as the template to amplify the complete pva gene by PCR using the PEUT-d PCR conditions (see Table S1) and PVA-1 and PVA-F primers (Table 1) designed according to the pva gene sequence determined in this work.

**Construction of *S. lividans* strains overexpressing the pva gene.** The *pva* gene, including its signal peptide coding sequence, was amplified by PCR using total DNA from *S. lavendulae* as the template. The PCR primers PVA-1 and PVA-F (Table 1) were designed according to the DNA sequence of *pva* determined in this work. The *XbaI* and EcoRI restriction sites were included in the primers to facilitate the subcloning of the PCR fragment. An *E. coli* ribosome binding site (RBS) consensus sequence and the ATG start codon were included in the PVA-1 primer. PCR amplification was carried out as described above using PEUT-t-d conditions (see Table S1 in the supplemental material). The purified PCR product (2.44 kb) was cloned into pGEM-T Easy vector. The recombinant plasmids were sequenced to confirm the absence of mutations, and the engineered *S. lividans* 1326 to obtain the recombinant strain *S. lividans* p(PEA1F).

**Activity assays and kinetic constants.** The acylase activity was routinely assayed using PV as the substrate. The acylase activity present in the recombinant *S. lividans* strains was determined according to the method described previously (8). Crude protein samples were prepared by growing the cells in the appropriate production media. Mycelia then were removed by centrifugation at 3,500 × g for 30 min, and the supernatants were used for assaying the acylase activity. Supernatant (135 μl) was incubated with 150 μl of 30 mM PV and 15 μl of 1 M potassium phosphate buffer, pH 8.0, at 40°C for 20 min. The reaction was stopped by the addition of 0.9 ml of 20% acetic acid water solution in an ice bath. After centrifugation of the samples, the amount of released 6-APA was determined with p-dimethylaminobenzaldehyde (28). One unit of PA activity was defined as the amount of enzyme required to produce 1 μmol of product per min under standard conditions. To determine substrate specificity, pure recombinant enzyme (0.5 μg) was incubated with increasing concentrations of different penicillins (e.g., PV, PK, PF, PdF, and PG) in 100 mM potassium phosphate buffer, pH 8.0, at 40°C for 20 min in a final volume of 100 μl. The reaction was stopped by addition of 400 μl of 0.5 M sodium acetate. After centrifugation of the samples, the amount of released 6-APA was determined with p-dimethylaminobenzaldehyde (29). The reaction was linear under these assay conditions. Values of kinetic constants were determined by fitting initial velocity data to the Hanes-Woolf equation by using a hyperbolic regression program (Hyper.exe 1.01, 2003; J. S. Easterby; http://homepages.ntlworld.com/john.easterby/software.html). Substrate saturation kinetics curves were fitted to equation \( v = \frac{v_{\text{max}} \times S}{(K_m + S)} \), where \( v \) is initial velocity and \( S \) is substrate concentration.

In the case of aculeacin A used as the substrate, the activity was measured by estimating the amount of aculeacin A in the reaction mixture (50 μl), which contained 100 mM phosphate buffer, pH 8.0, 1 M NaCl, and 20% (vol/vol) dimethylsulfoxide (DMSO), after 10 min at 60°C and 250 rpm. The aculeacin A that was not hydrolyzed was quantified by high-performance liquid chromatography (HPLC), as described previously (30), using a Kromasil100 C18 column (5 μm; 300 by 4.6 mm; Teknokroma, Spain) and acetonitrile (65%–water (35%) as the mobile phase. All PA activity determinations were performed in triplicate.

**Purification of recombinant SIPVA.** For the production and purification of recombinant SIPVA, 400 ml of tryptone soy broth (TSB) with thioacetamide (5 μg/ml) was inoculated with 2 × 10^6 spores/ml of recombinant *S. lividans* (p(PEA1F)) and incubated aerobically under submerged conditions at 30°C and 250 rpm for 96 h. Cell-free culture supernatants were then adjusted to pH 7.0 and applied to an S-Sepharose fast flow column (Amersham Biosciences, United Kingdom) equilibrated with 10 mM sodium phosphate buffer, pH 7.0. The column was washed with the same buffer, and bound proteins were eluted with a linear gradient of 0 to 1 M NaCl in the same buffer. The fractions containing PA activity were pooled and analyzed by SDS-PAGE as described above. Likewise, N-terminal sequences of α- and β-subunits were determined as described for wild-type SIPVA.

**Spectrophotometric determinations.** Fluorescence emission spectra of both pure wild-type and recombinant SIPVA were monitored at 25°C using a Sim-Amino 8000 fluorescence spectrophotometer with thermostated 0.4-cm- and 1-cm-path-length quartz cells of excitation and emission, respectively. The excitation and emission slit width was 5 nm. The scan rate was 60 nm/min. Two excitation wavelengths were used, 275 nm and 295 nm. Protein concentration was 0.1 mg/ml in 50 mM potassium phosphate buffer, pH 7.0. Circular dichroism (CD) spectra were recorded using a Jasco J-715 (Japan) spectropolarimeter with a thermostated 1-mm-path-length quartz cell in the far-UV region. The protein concentration was 0.28 mg/ml in 50 mM potassium phosphate buffer, pH 7.0. The CD readings were expressed as the mean residue molar ellipticity (deg cm^2 /dmol^1), assuming a residue molecular mass of 110. Secondary structure information of SIPVA was obtained from CD spectra by using the CCA (31) and CDNN V2.0.3.188 (32) programs. Furthermore, protein secondary structure was deduced from the amino acid sequence of SIPVA by using PSIpred (33), PHD (34), and JUFO (35) programs.

**Site-directed mutagenesis studies.** In order to identify the amino acids involved in the active site of the enzyme, the residues Serβ1, Hisβ23, Valβ70, and Asnβ272 were modified by site-directed mutagenesis. These residues were selected based on the comparison with other PAs by a multiple-sequence alignment. The recombinant pNPV1A1F plasmid derived from pNV19 (17) and pPEA1F (this work) was used as the template for mutagenesis by the QuiKchange II XL site-directed mutagenesis kit (Stratagene), using the primers listed in Table 2. For that purpose, both pNV19 and pPEA1F plasmids were digested with HindIII–EcoRI endonucleases, and the purified HindIII–EcoRI-linearized pNV19 plasmid was ligated by T4 DNA ligase with the HindIII–EcoRI fragment from pPEA1F, which contains an engineered pva gene, including the PVA signal peptide coding sequence, the E. coli RBS, the ATG start codon, and the strong constitutive Permeα promoter from *S. erythraea* (16, 19). The resulting mutant constructions (Table 2) were sequenced to confirm the mutations and then transferred to *Rhodococcus* sp. strain T104 by electroporation for protein expression as described previously (19). Serine β1 was exchanged for cysteine (mutant Sβ1C), alanine (mutant Sβ1A), aspartic acid (mutant Sβ1D), histidine (mutant Sβ1H), or lysine (mutant Sβ1K). Histidine β23 was exchanged for glutamine (mutant Hβ23Q) or aspartic acid (mutant Hβ23A). Valine β70 was exchange for alanine (mutant Vβ70A) or aspartic acid (mutant Vβ70D). Asparagine β272 was exchanged for glutamine (mutant Nβ272Q), valine (mutant Nβ272V), or aspartic acid (mutant Nβ272D).

**Nucleotide sequence accession number.** The gene sequence described in the present study has been deposited in the GenBank database under accession number AAU096760.1.

**RESULTS AND DISCUSSION**

**Isolation and cloning of the pva gene from *S. lavendulae*.** To characterize and overexpress the pva gene encoding the SIPVA from *S. lavendulae* ATCC 1366, the gene was isolated by following a classical reverse strategy, i.e., by purifying and sequencing the
enzyme. SLPVA is a heterodimer composed of two subunits, α and β, with molecular masses of 21.4 kDa and 59.0 kDa (Fig. 1), or 18.79 kDa and 60.09 kDa, respectively, when determined by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) (data not shown). Remarkably, the analysis by SDS-PAGE showed that the β-subunit loses a fragment of 11 kDa when the enzyme is stored at 4°C (Fig. 1), although the enzyme remains active, indicating that the 11-kDa fragment remains attached to the enzyme via noncovalent bonds and that the structure of PVA is not affected by the proteolytic effect. The analyses of the N-terminal residues of the subunits provided the sequences GGGGLSATVRYTEAGIPHIVAK (α-subunit), SNAVAFRGSTTANGRGLLLGNPHY (β-subunit), and AAPVTRTQWWTRYGPPVTSGLA (truncated β-subunit).

These N-terminal sequences were used to isolate the pva gene from S. lavendulae by several subcloning steps using a PCR approach. First, two DNA fragments of 600 and 336 bp carrying sequences of pva were obtaining by PCR amplification of the S. lavendulae chromosomal DNA as described in Materials and Methods. The degenerated oligonucleotides STREPTOS2 (Table 1), used as primers, were designed according to the N-terminal sequences of the α-subunit ([STREPTOS2; codified for TEAGIPH]), β-subunit ([STREPTOS3 and STREPTOSG1; codified for NAVAFLRG and RGGLLLGNP], respectively), and truncated β-subunit (STREPTOS2; codified for QWWTRYGP) isolated by SDS-PAGE.

Subsequently, a 1,500-bp NCsacII-DNA fragment containing the 5’ noncoding upstream pva gene region, including the Ppva promoter, was obtained by inverse PCR as described in Materials and Methods. Likewise, the DNA fragment containing the downstream 3’ noncoding region of pva was obtained by inverse PCR using the FcβD2 and FcβI2 primers designed from the 3’ sequence NCsacII DNA fragment. Finally, the complete pva gene was amplified by PCR from the chromosomal DNA of S. lavendulae using two primers designed according to the DNA sequences of the partial fragments cloned before.

### Table 2: Primers used in pva site-directed mutagenesis studies and effect of mutations on rSLPVA catalytic activity

<table>
<thead>
<tr>
<th>Rhodococcus sp. strain</th>
<th>Mutation</th>
<th>Synthetic oligonucleotide and sequence*</th>
<th>Remaining activityb (%)</th>
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<tr>
<td>Control pNPAATG</td>
<td></td>
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<td>100</td>
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<td>pNPAATG51C</td>
<td>SB1C</td>
<td>PVAS1C1; 5’GCACAACGCCGACATGGGGCTGCCACGCGCCTTGCTCGTGCTCGTCGGGGGG3’</td>
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<td>PVAN272V-1; 5’GGGCCTGCTCTCGGCAACCCCGCAGTCATCGGTTGGGAGGGGAGGGGGC3’</td>
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</tr>
</tbody>
</table>

*a Mutated codons are underlined.

b Activity was determined under standard conditions using penicillin V as the substrate.

![FIG 1 SDS-PAGE analysis of pure wild-type PVA produced by S. lavendulae. Lane 1, pure SLPVA stained with Coomassie blue; lane 2, pure SLPVA, after storage at 4°C for 1 month, stained with Amido black after being transferred to PVDF membrane for sequencing; lane 3, standard molecular mass markers.](http://aem.asm.org/.../http://aem.asm.org/aem.asm.org)
peptides found in the extracellular proteins of Bacillus subtilis has been described for other PAs (4, 36–42), the structure of SlPVA is very different from that of PVA from Bacillus subtilis and Bacillus sphaericus, homotetrameric proteins that currently are the only PVAs whose structures have been elucidated (43, 44). A Gly-Ser pair representing the cleavage site between the spacer peptide and the β-subunit is well conserved (see Fig. S2 in the supplemental material), which renders the catalytic N-terminal serine of the β-subunit. Moreover, the generation of the free N-terminal nucleophile serine allows us to propose that SlPVA belongs to the N-terminal nucleophile (Ntn)-hydrolase superfamily characterized by an N-terminal nucleophile that acts as the main catalytic residue.

The comparison of SlPVA to other members of the PA family by BLAST (45) revealed that the protein shares 68%, 23%, and 32% sequence identity with PVA from Streptomyces mobaraensis (4), PGA from Escherichia coli (46), and glutaryl 7-amino cephalosporanic acid acylase from Pseudomonas diminuta (47) (see Fig. S2 in the supplemental material). In contrast, SlPVA does not show homology with PVAs from Bacillus subtilis and Bacillus sphaericus (43, 44). More interestingly, the highest similarities are seen with the cyclic lipopeptide acylase (CA) from Streptomyces sp. strain FERM BP-5809 (GenBank accession no. AB158476; 92%) (48) and acyl homoserine lactone acylase (AhlM) from Streptomyces sp. strain M664 (93%) (49) (see Fig. S2); likewise, SlPVA shares similarity with other acyl homoserine lactone (AHL) acylases from Gram-negative bacteria, such as AHL acylase from Pseudomonas aeruginosa PAO1 (52%) (50).

**Overproduction and purification of rSlPVA.** To overproduce a recombinant SlPVA (rSlPVA), the pva gene was overexpressed in a heterologous host, like S. lividans, since all attempts to express it in E. coli have been unsuccessful so far (data not shown). The gene was engineered to include its original signal peptide, an RBS consensus sequence, and an ATG start codon instead of the TTG originally starting the pva codon. The modified pva was cloned into the expression vector pEM4, and the resulting plasmid, pPEA1F, was used to transform S. lividans to render the recombinant S. lividans (pPEA1F). The highest rSlPVA production was reached after 96 h of incubation, in contrast to the 275 h required by the native S. lavendulae strain (7). The recombinant strain produced 950 IU/liter of rSlPVA, which is a 5-fold higher yield than that found under the best production conditions of S. lavendulae (7). As expected, rSlPVA is secreted in S. lividans as a homogeneous heterodimeric form (Fig. 3) that can be purified by a single
chromatographic step on S-Sepharose with a high yield, 8 mg of protein and 665 UI per liter of culture (about 70% recovery). The N-terminal sequences of the α- and β-subunits of the recombinant enzyme were identical to that of the native enzyme, indicating a correct maturation in the heterologous host. It is also worth mentioning that the β subunit of the purified recombinant enzyme did not suffer the proteolysis of the 11-kDa β subunit fragment when stored at 4°C for long periods, suggesting that this proteolysis was due to the presence of traces of contaminant proteases which were copurified with SipVA in S. lavendulae instead of a putative secondary autoproteolytic process.

Structural and biochemical characterization of the recombinant SipVA. The purified rSipVA was used to determine several structural and biochemical properties of this enzyme. The deconvolution of CD spectrum in the far-UV region of rSipVA (Fig. 4) revealed that the protein contains 20 to 30% of α-helix, 16% of β-sheet, 15 to 20% of β-turns, and 30% of random coil arranged in a αβαβ fashion as described for the N-terminal nucleophile (Ntn hydrolase) superfamily (54). Remarkably, CD thermal denaturation experiments revealed that the rSipVA global structure was thermostable, showing a melting temperature (Tm) of 62°C (Fig. 4, inset).

On the other hand, the fluorescence spectra of wild-type and recombinant SipVA showed a single maximum emission peak at 326 nm, which was shifted to 346 nm in the presence of 9 M urea (data not shown), suggesting that tryptophans are the main residues responsible for this emission and that all of them must be hidden inside the SipVA three-dimensional (3D) structure, and also confirming that the recombinant protein is folded as the native enzyme.

It could be argued that the recognized SipVA activity on natural penicillins is due to the partial contamination of the original enzyme preparation, but we have found that the rSipVA behaves as the native purified acylase (7, 8, 13). In this sense, the enzyme showed the highest activity at 55°C and pH 9.5 to 10.0 when PV was used as the substrate. Likewise, the activity was stable up to 45°C and between pH 7.0 and 10.5. The activity was slightly enhanced at 0.6 M NaCl, reached 100% activity at 1 M NaCl, and was gradually inhibited by higher NaCl concentrations, retaining 60% activity at 3 M NaCl. Moreover, the enzyme retained 100% activity in the presence of 10 mM EDTA or EGTA, and the same effect was observed in the presence of 125 mM β-mercaptoethanol or 150 mM dithiothreitol (DTT). Furthermore, the kinetic parameters for the rSipVA hydrolysis of different natural β-lactam antibiotics, i.e., PV, PK, PF, PdF, and PG, have been determined (Table 3). The values were similar to that reported for the original SipVA (13), showing the highest bimolecular constant (specificity constant) value, kcat/Km = 244.11 mM⁻¹ s⁻¹, with penicillin K as the substrate, indicating that the recombinant enzyme is processed and matured as in S. lavendulae. Remarkably, rSipVA is able to hydrolyze aculeacin A, showing a specific activity of 13 μmol/min/mg. To date, no other PA enzyme described in the literature displays such hydrolytic capability, opening new and interesting bio-

![Figure 4](http://aem.asm.org/)

**FIG 4** Far-UV-visible CD spectra of rSipVA. Spectra were recorded between 195 and 260 nm with 0.28 mg/ml rSipVA in 10 mM potassium phosphate buffer, pH 7.0, at 25°C under thermostated conditions using a Jasco J-715 spectropolarimeter (Jasco, Japan). The inset shows thermal unfolding of rSipVA studied by CD variation at 222 nm in the range of 25 and 90°C and scanned at 20°C/h.

### Table 3 Kinetic parameters of recombinant SipVA assayed on different natural penicillins

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km (mM)</th>
<th>kcat (s⁻¹)</th>
<th>kcat/Km (mM⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin V</td>
<td>3.68 ± 0.18</td>
<td>77.92 ± 4.2</td>
<td>21.17 ± 0.1</td>
</tr>
<tr>
<td>Penicillin K</td>
<td>0.14 ± 0.007</td>
<td>35.58 ± 1.1</td>
<td>244.11 ± 5.41</td>
</tr>
<tr>
<td>Penicillin dihydro-F</td>
<td>0.82 ± 0.05</td>
<td>14.75 ± 0.4</td>
<td>17.93 ± 1.7</td>
</tr>
<tr>
<td>Penicillin F</td>
<td>1.39 ± 0.07</td>
<td>3.33 ± 0.19</td>
<td>2.40 ± 0.02</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>58.18 ± 5.23</td>
<td>10.95 ± 0.87</td>
<td>0.18 ± 0.009</td>
</tr>
</tbody>
</table>
technological applications of this enzyme as a biocatalyst in the industrial production of semisynthetic β-lactam antibiotics as well as semisynthetic antifungals.

**Active site of SIPVA.** Based on the structure of the active centers of glutaryl-7-amino cephalosporin acylase from *P. diminuta* (55, 56) and acyl-homoserine lactone acylase from *Pseudomonas aeruginosa* (50), we predicted that Serβ1, Hisβ23, Valβ70, and Asnβ272 would be responsible for the catalytic activity of SIPVA. These residues are conserved in many PAs, excluding the PGAs from *E. coli* and *Klyvera citrophila* (57), where Hisβ23 and Valβ70 are replaced by Glnβ23 and Alaβ69, respectively. Previous studies on the chemical mechanism of SIPVA have suggested the involvement of an essential serine for the catalytic activity and the possible participation of an α-amino nitrogen in the reaction (58). The optimum pH for the hydrolysis of penicillin V by SIPVA was 8, which is consistent with a requirement for the α-amino group to remain uncharged upon ligand binding (8, 58).

The catalytic role of the SIPVA residues identified above has been tested by constructing different *pva* site-directed mutants that were expressed and assayed for their residual activity (Table 2). All mutants on Serβ1 and Hisβ23 yielded inactive enzymes, and mutations of Valβ70 and Asnβ272 led to a drastic decrease of activity. These results demonstrate that these four residues are essential for activity and reinforce the hypothesis that they constitute part of the active center of SIPVA. Moreover, the 3D model of the β SIPVA subunit (Fig. 5) revealed the presence of a hydrophobic pocket involved in the binding of the phenoxacyclato moiety of penicillin V, showing the spatial proximity of these four residues and reinforcing their putative role in catalysis, where Serβ1 is located in the bottom of the hydrophobic site. This observation correlates with the active center of the acylases from *E. coli*, *P. diminuta*, and *P. aeruginosa*, where their catalytic residues have been located in a deep hydrophobic binding pocket (40, 41, 50, 55, 56). Furthermore, since essential residue Hisβ23 is adjacent to Serβ1, it could be suggested that these residues form a catalytic diad similar to that described for other serine proteases (59), where the imidazole ring would act as a basic group to enhance the nucleophilicity of catalytic serine, functioning as a general base catalyst, in contrast to that described for PGA from *E. coli*, which has a Glnβ23 and where the nucleophilicity of catalytic serine is enhanced by its α-amino group. Likewise, the oxyanion hole would be formed by the nearby δ nitrogen of Asnβ272 and nitrogens of the main chain of Valβ70 and Hisβ23, which stabilize the negatively charged tetrahedral intermediate by hydrogen bonds in a manner similar to that described for acylase from *P. diminuta* (55).

![FIG 5 3D structure model of β-subunit of SIPVA. (A) Complete model. This structure was modeled by the Phyre2 server (62), available at www.sbg.bio.ic.ac.uk/phyre2, which combines homology and ab initio modeling algorithms. These figures were rendered using Discovery Studio 3.1 software (Accelrys Software Inc.). The best fit was obtained with acyl homoserine lactone acylase from *P. aeruginosa* as the template. (B) Detail of the active-site catalytic amino acids. Serβ1, the putative nucleophile, is indicated in green. Hisβ23 (yellow), Valβ70 (pink), and Asnβ272 (orange) are indicated as the putative oxyanion hole residues.](http://aem.asm.org/)

In summary, on the basis of the structural analysis and the studies of the catalytic mechanism carried out here, we propose that SIPVA is a novel Ntn-hydrolase that belongs to a new acylase subfamily. Thus, these studies not only pave the way to use this enzyme for biotechnological applications at an industrial scale but, even more importantly, also open the possibility of studying the implication of these enzymes in other physiological phenom-
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