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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 (*Sl*PVA), 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 *Sl*PVA, 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 extracellularly homogeneous 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 *Sl*PVA, 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 (*Sl*PVA) 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 *Sl*PVA 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 *Sl*PVA is an effective industrial enzyme, provided that it can be obtained in large amounts.

Here, we describe the heterologous overproduction of *Sl*PVA 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. lavendulae* 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 thiostrepton in liquid medium or 25 μg/ml thiostrepton 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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This work is dedicated to María Pilar Castillón on her retirement.

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TABLE 1 List of bacterial strains, plasmids and primers used

Strain, plasmid, or primer	Relevant genotype, description, or sequence (5′ to 3′)[a]	Reference and/or source
Escherichia coli strains		
DH5α	F$^-$ λ$^-$ ϕ80d*lacZ*ΔM15 *endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*)*U169*	20
XL10 Gold	Δ(*mcrA*)*183*Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA lac*, Hte, Tet^r, Cam^r	Stratagene
XL1 red competent cells	*endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutT* Tn*10*, Tet^r	Stratagene
Actinomycetes		
Streptomyces lavendulae ATCC 13664	Penicillin V acylase producer	ATCC; 7
Streptomyces lividans 1326 (NRRL B-16148)	Tsr^s	ARS culture collection (NRRL)
Rhodococcus sp. strain T104KACC 21099	Kn^s	Korean Agricultural Culture Collection; 18
Plasmids		
pGEM-T Easy Vector	Lineal, T at the ends, Ap^r, f1, *orilacZ*, T3p, T7p, 3 kb	Promega
pEM4	Shuttle vector for *E. coli*/*Streptomyces*, Ap^r, Tsr^r, P*ermE**, pUCori, pWHM4ori, 7.9 kb	16
pNV19	Shuttle vector for *E. coli*/*Rhodococcus*, Kn^r, pAL5000ori, *lacZ*, CoE1ori, 4.4 kb	17
pEPA1F	pEM4 derivative containing 2,445-bp XbaI/EcoRI *pva* gene from *S. lavendulae* ATCC 13664, *E. coli* RBS, ATG start codon and signal peptide sequence	This work
pNPVA1F	pNV19 derivative containing 2,249-bp HindIII/EcoRI fragment from pEPA1F including *pva* gene from *S. lavendulae* ATCC 13664, *E. coli* RBS, ATG start codon, signal peptide sequence, and strong constitutive P*ermE** promoter from *Saccharopolyspora erythraea*	This work
Primers		
STREPTOP2	5′-CCGGAATTCAC(C/G)GAGGC(C/G)GGCATCCC(G/C)CA-3′	This work
STREPTOD2	5′-CCGGAATTC(C/G)GGGCCGTA(C/G)CG(C/G)GTCCACCACTG-3′	This work
STREPTOG1	5′-CCGGAATTCCG(C/G)GGCCT(C/G)CT(C/G)CT(C/G)GGCAACCC(C/G)CA-3′	This work
STREPTOG3	5′-CCGGAATTCGCC(C/G)CGGAA(C/G)GC(C/G)AC(C/G)GCGTT-3′	This work
NsacII	5′-CCGGAATTCCCGCGGTGGAGACCGCCGCGT-3	This work
CsacII	5′-CCGGAATTCCCGCGGGCACCGCCGCCGCC-3′	This work
FcβD2	5′-AGCCGGGGCGCGCTGCTCTT-3′	This work
FcβI2	5′-AGGCCGCCGACACGT(C/T)(G/A)AC-3′	This work
PVA-1	5′-TCTA*GAGGG*TATTAATA**ATG**ACCTTCCGTAACCGCCTCAGACTG-3′	This work
PVA-F	5′-CCGGAATTCC**CTA**CCGCCGCTCGTGCACCCG-3′	This work

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

(Km^r, *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).

Production and purification of *Sl*PVA from *S. lavendulae*. *Sl*PVA 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 *Sl*PVA subunits, 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 (Qbiogene, 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 Pcr6 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*. Two EcoRI restriction sites were included at both ends of the primers to facilitate the cloning of the resulting DNA fragments (Table 1). PCR amplification 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, containing 0.25 mM each deoxynucleoside triphosphate in the buffer provided by the supplier and 5% dimethyl sulfoxide (Merck, Germany). The NCsacII DNA fragment containing the 5′ noncoding upstream *pva* gene region, including the *Ppva* promoter and the downstream 3′ noncoding region of *pva*, were obtained by inverse PCR using the Pcr68 conditions (see Table S1) and the NsacII and CsacII primers and the FcβD2 and FcβI2 primers (Table 1). SacII and HincII sites were selected to digest the chromosomal DNA before its recircularization after testing different restriction enzymes by

Southern blotting experiments. Inverse PCR experiments were performed as described previously (25). In brief, 8 μg of DNA from *S. lavendulae* was digested with SacII 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 performed inverse PCRs. Chromosomal DNA from *S. lavendulae* was used as the template to amplify the complete *pva* gene by PCR using the *PFU*-T-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 *PFU*-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 *pva* gene then was rescued as an XbaI-EcoRI fragment for subcloning into the EcoRI-XbaI site of pEM4 in the right orientation, i.e., downstream of the promoter P*ermE** of the erythromycin resistance gene from *Saccharopolyspora erythraea* (26, 27). The resulting recombinant plasmid, pEPA1F, harboring the engineered *pva* gene, was used to transform *S. lividans* 1326 to obtain the recombinant strain *S. lividans*(pEPA1F).

Activity assays and kinetic constants. The acylase activity was routinely assayed using PV as the substrate. The acylase activity present in the culture supernatants of *S. lavendulae* ATCC 13664 and 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 released 6-APA was monitored with fluorescamine (7, 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://homepage.ntlworld.com/john.easterby/software.html). Substrate saturation kinetics curves were fitted to equation $v = (V_{max} \times S)/(K_m + S)$, 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 C_{18} 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 *Sl*PVA. For the production and purification of recombinant *Sl*PVA, 400 ml of tryptone soya broth (TSB) with thiostrepton (5 μg/ml) was inoculated with 2×10^6 spores/ml of recombinant *S. lividans*(pEPA1F) and incubated aerobically under submerged conditions at 30°C and 250 rpm for 96 h. Cell-free culture supernatants then were 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 *Sl*PVA.

Spectrophotometric determinations. Fluorescence emission spectra of both pure wild-type and recombinant *Sl*PVA were monitored at 25°C using a Sim-Aminco 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 ($deg \cdot cm^2 \cdot dmol^{-1}$), assuming a residue molecular mass of 110. Secondary structure information of *Sl*PVA 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 PVA 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 pNPVA1F plasmid derived from pNV19 (17) and pEPA1F (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 pEPA1F 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 pEPA1F, 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 P*ermE** 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 Hβ23D). 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 AAU09670.1.

RESULTS AND DISCUSSION

Isolation and cloning of the *pva* gene from *S. lavendulae*. To characterize and overexpress the *pva* gene encoding the *Sl*PVA from *S. lavendulae* ATCC 1366, the gene was isolated by following a classical reverse strategy, i.e., by purifying and sequencing the

TABLE 2 Primers used in *pva* site-directed mutagenesis studies and effect of mutations on r*Sl*PVA catalytic activity

Rhodococcus sp. strain	Mutation	Synthetic oligonucleotide and sequence[a]	Remaining activity[b] (%)
Control pNPAATG			100
pNPAATGS1C	Sβ1C	*PVAS1C1*; 5′GCAGAACGCCGACATGGGGCTGCAACGCGGTCGCCTTCCGGGGG3′ *PVAS1C2*; 5′CCCCCGGAAGGCGACCGCGTTGCAGCCCCATGTCGGCGTTCTGC3′	0
pNPAATGS1A	Sβ1A	*PVAS1A1*; 5′GCAGAACGCCGACATGGGGCGCCAACGCGGTCGCCTTCCGGGGG3′ *PVAS1C2*; 5′CCCCCGGAAGGCGACCGCGTTGGCGCCCCATGTCGGCGTTCTGC3′	0
pNPAATGS1D	Sβ1D	*PVAS1D1*; 5′GCAGAACGCCGACATGGGGCGATAACGCGGTCGCCTTCCGGGGG3′ *PVAS1D2*; 5′CCCCCGGAAGGCGACCGCGTTATCGCCCCATGTCGGCGTTCTGC3′	0
pNPAATGS1K	Sβ1K	*PVAS1K-1*; 5′GCAGAACGCCGACATGGGGCAAGAACGCGGTCGCCTTCCGGGGG3′ *PVAS1K-2*; 5′CCCCCGGAAGGCGACCGCGTTCTTGCCCCATGTCGGCGTTCTGC3′	0
pNPAATGS1H	Sβ1H	*PVAS1H-1*; 5′GCAGAACGCCGACATGGGGCCACAACGCGGTCGCCTTCCGGGGG3′ *PVAS1H-2*; 5′CCCCCGGAAGGCGACCGCGTTGTGGCCCCATGTCGGCGTTCTGC3′	0
pNPAATGHQ	Hβ23Q	*PVAH23Q-1*; 5′GGGCTGCTCCTCGGCAACCCGCAGTATCCGTGGGAGGGCGGCCGC3′ *PVAH23Q-2*; 5′GCGGCCGCCGTCCCACGGATACTGCGGGTTGCCGAGGAGCAGCCC3′	0
pNPAATGHD	Hβ23D	*PVAH23D-1*; 5′GGGCTGCTCCTCGGCAACCCGGACTATCCGTGGGAGGGCGGCCGC3′ *PVAH23D-2*; 5′GCGGCCGCCGTCCCACGGATAGTCCGGGTTGCCGAGGAGCAGCCC3′	0
pNPAATGVA	Vβ70A	*PVAV70A-1*; 5′GTGGCCTGGAGCCACACGGCCGCGACCGGCGTCACGCTG3′ *PVAV70A-2*; 5′CAGCGTGACGCCGGTCGCGGCCGTGTGGCTCCAGGCCAC3′	0.25
pNPAATGVD	Vβ70D	*PVAV70D-1*; 5′CGTGGCCTGGAGCCACACGGACGCGACCGGCGTCACGCTG3′ *PVAV70D-2*; 5′CAGCGTGACGCCGGTCGCGTCCGTGTGGCTCCAGGCCACG3′	0.15
pNPAATGNQ	Nβ272Q	*PVAN272Q-1*; 5′CGCCCCGTACGTCGAGAACTCCCAGGACAGCGCCTGGCTGACC3′ *PVAN272Q-2*; 5′GGTCAGCCAGGCGCTGTCCTGGGAGTTCTCGACGTACGGGGCG3′	0.35
pNPAATGND	Nβ272D	*PVAN272D-1*; 5′CGCCCCGTACGTCGAGAACTCCGACGACAGCGCCTGGCTGACC3′ *PVAN272D-2*; 5′GGTCAGCCAGGCGCTGTCGTCGGAGTTCTCGACGTACGGGGCG3′	0.33
pNPAATGNV	Nβ272V	*PVAN272V-1*; 5′CGCCCCGTACGTCGAGAACTCCGTCGACAGCGCCTGGCTGACC3′ *PVAN272V-2*; 5′GGTCAGCCAGGCGCTGTCGACGGAGTTCTCGACGTACGGGGCG3′	0

[a] Mutated codons are underlined.
[b] Activity was determined under standard conditions using penicillin V as the substrate.

enzyme. *Sl*PVA 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 affect by the proteolytic effect. The analyses of the N-terminal residues of the subunits provided the sequences GGGLSATVRYTEAGIPHIVAK (α-subunit), SNAVAFRGSTT ANGRGLLLGNPHY (β-subunit), and AAPVTRTQWWTRYG PVVTSLGA (truncated β-subunit).

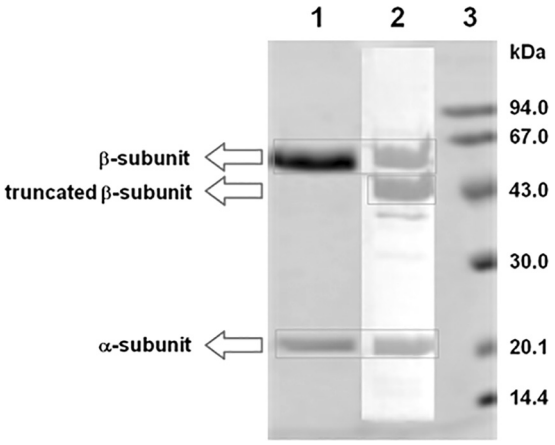

FIG 1 SDS-PAGE analysis of pure wild-type PVA produced by *S. lavendulae*. Lane 1, pure *Sl*PVA stained with Coomassie blue; lane 2, pure *Sl*PVA, after storage at 4°C for 1 month, stained with Amido black after being transferred to PVDF membrane for sequencing; lane 3, standard molecular mass markers.

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 STREPTOP2/STREPTOG3 and STREPTOG1/STREPTOD2 (Table 1), used as primers, were designed according to the N-terminal sequences of the α-subunit (STREPTOP2; codified for TEAGIPH), β-subunit (STREPTOG3 and STREPTOG1; codified for NAVAFRG and RGLLLGNPH, respectively), and truncated β-subunit (STREPTOD2; 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.

FIG 2 Signal peptide sequence of *Sl*PVA. Different amino acid regions are indicated with a color code. Black, N-terminal end (Nt); dark gray, C-terminal end (Ct); light gray, hydrophobic region.

Analysis of the DNA sequence deposited in GenBank as AAU09670.1 showed that the complete open reading frame (ORF) for *pva* contains 2,420 bp, with a TTG start codon and a TGA stop codon. The putative ribosome binding site (RBS), GGAGG, is located 7 bp upstream of the start codon (see Fig. S1 in the supplemental material). The gene encodes a protein of 806 amino acids, where the N-terminal residue of the α-subunit is located at position 40, suggesting that the first 39 N-terminal residues constitute the *Sl*PVA signal peptide (see Fig. S1). This signal peptide shows an amino acid sequence characteristic of the signal peptides found in the extracellular proteins of *Streptomyces* (Fig. 2), i.e., a short N-terminal sequence with basic residues, followed by a short chain of hydrophobic amino acids and a C-terminal signal peptide end containing a typical hydrolytic target sequence (36, 37).

According to the N-terminal sequences of *Sl*PVA determined by SDS-PAGE, the sizes of the α-subunit and β-subunit were 220 and 556 amino acid residues, respectively. Nevertheless, taking into account that the molecular mass of the α-subunit determined by MALDI-TOF was 18.79 kDa, corresponding to 177 amino acid residues, we suggest the existence of a dispensable linker peptide of 25 amino acid residues located between the C terminus of the α-subunit and the N terminus of the β-subunit.

Sequence analysis of *Sl*PVA. The isolated *Sl*PVA shows the characteristic polypeptide organization found in the PA family (i.e., signal peptide, α-subunit, spacer peptide, and β-subunit). Accordingly, the native *Sl*PVA is an αβ heterodimer originating from an inactive single polypeptide precursor containing a signal peptide at its N terminus. The activation of the precursor most probably consists of three hydrolytic steps, i.e., cleavage of the signal peptide by the secretion machinery, followed by two autoproteolytic cleavages releasing the internal linker and the α-subunit (18.79 kDa) and β-subunit (60.09 kDa). Although this complex activation process has been described for other PAs (4, 38–42), the structure of *Sl*PVA 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 *Sl*PVA 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 *Sl*PVA 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, *Sl*PVA 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, *Sl*PVA shares similarity with other acyl homoserine lactone (AHL) acylases from Gram-negative bacteria, such as AHL acylase from *Pseudomonas aeruginosa* PAO1 (52%) (50). In addition, *Sl*PVA shows homology with the aculeacin A acylase (AAC) from *Actinoplanes utahensis* (*Au*AAC) (41% identity) (51), *Deinococcus radiodurans* (41% identity) (52), and *Shewanella oneidensis* MR-1 (32% identity) (53).

Overproduction and purification of r*Sl*PVA. To overproduce a recombinant *Sl*PVA (r*Sl*PVA), 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, pEPA1F, was used to transform *S. lividans* to render the recombinant *S. lividans*(pEPA1F). The highest r*Sl*PVA 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 r*Sl*PVA, which is a 5-fold higher yield than that found under the best production conditions of *S. lavendulae* (7). As expected, r*Sl*PVA is secreted in *S. lividans* as a homogeneous heterodimeric form (Fig. 3) that can be purified by a single

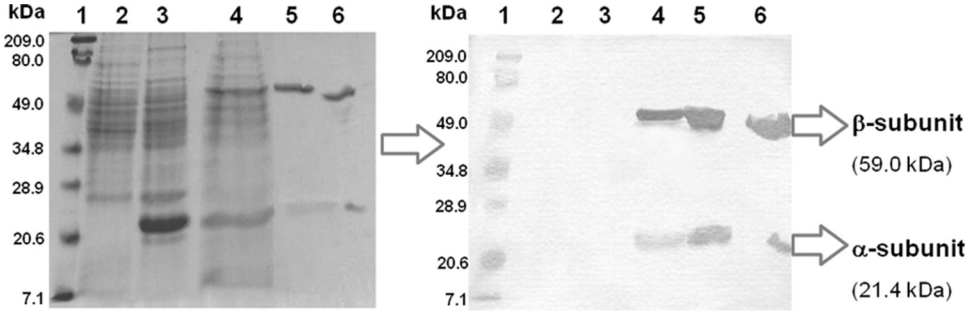

FIG 3 (Left) SDS-PAGE analysis of r*Sl*PVA produced by *S. lividans*(pEM4). (Right) Western blot analysis of each purification step. Lane 1, molecular mass markers; lane 2, fermentation broth of *S. lividans* (control); lane 3, fermentation broth of *S. lividans*(pEM4); lane 4, cell extract of the fermentation broth of *S. lividans*; lane 5, pure r*Sl*PVA after S-Sepharose chromatography; lane 6, pure *Sl*PVA (control).

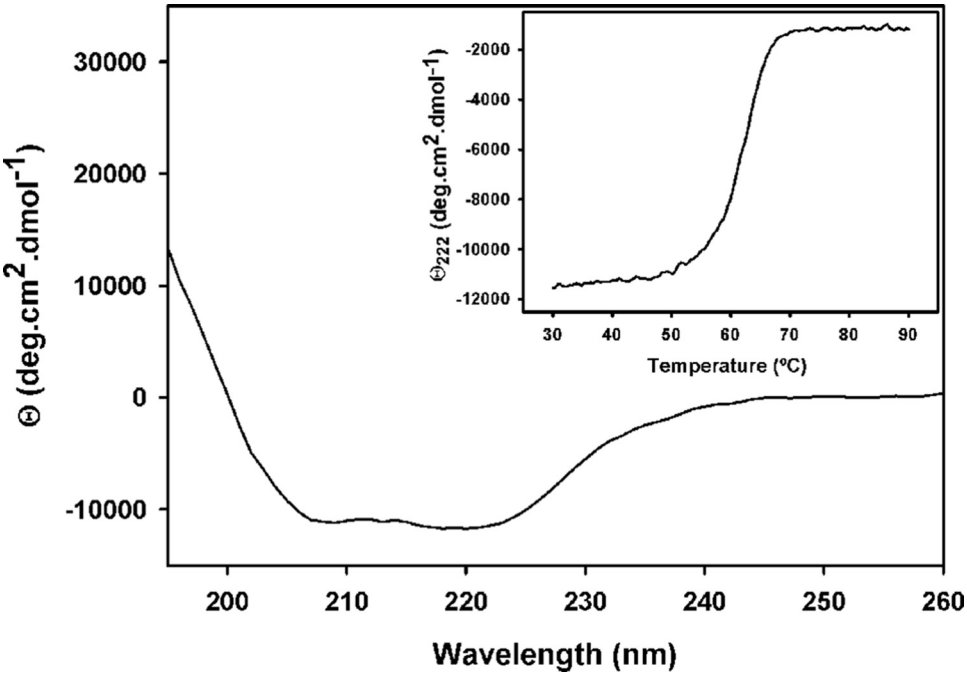

FIG 4 Far-UV-visible CD spectra of r*Sl*PVA. Spectra were recorded between 195 and 260 nm with 0.28 mg/ml r*Sl*PVA in 10 mM potassium phosphate buffer, pH 7.0, at 25°C under thermostated conditions using a Jasco J-715 spectropolarimeter (Jasco, Japan). The inset shows thermal unfolding of r*Sl*PVA studied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

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 *Sl*PVA in *S. lavendulae* instead of a putative secondary autoproteolytic process.

Structural and biochemical characterization of the recombinant *Sl*PVA. The purified r*Sl*PVA was used to determine several structural and biochemical properties of this enzyme. The deconvolution of CD spectrum in the far-UV region of r*Sl*PVA (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 αββα fashion as described for the N-terminal nucleophile (Ntn hydrolase) superfamily (54). Remarkably, CD thermal denaturation experiments revealed that the r*Sl*PVA global structure was thermostable, showing a melting temperature (T_m) of 62°C (Fig. 4, inset).

On the other hand, the fluorescence spectra of wild-type and recombinant *Sl*PVA 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 *Sl*PVA three-dimensional (3D) structure, and also confirming that the recombinant protein is folded as the native enzyme.

It could be argued that the recognized *Sl*PVA activity on natural penicillins is due to the partial contamination of the original enzyme preparation, but we have found that the r*Sl*PVA 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 r*Sl*PVA 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 *Sl*PVA (13), showing the highest bimolecular constant (specificity constant) value, k_{cat}/K_m = 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, r*Sl*PVA 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-

TABLE 3 Kinetic parameters of recombinant *Sl*PVA assayed on different natural penicillins

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
Penicillin V	3.68 ± 0.18	77.92 ± 4.2	21.17 ± 0.1
Penicillin K	0.14 ± 0.007	35.58 ± 1.1	244.11 ± 5.41
Penicillin dihydro-F	0.82 ± 0.05	14.75 ± 0.4	17.93 ± 1.7
Penicillin F	1.39 ± 0.07	3.33 ± 0.19	2.40 ± 0.02
Penicillin G	58.18 ± 5.23	10.95 ± 0.87	0.18 ± 0.009

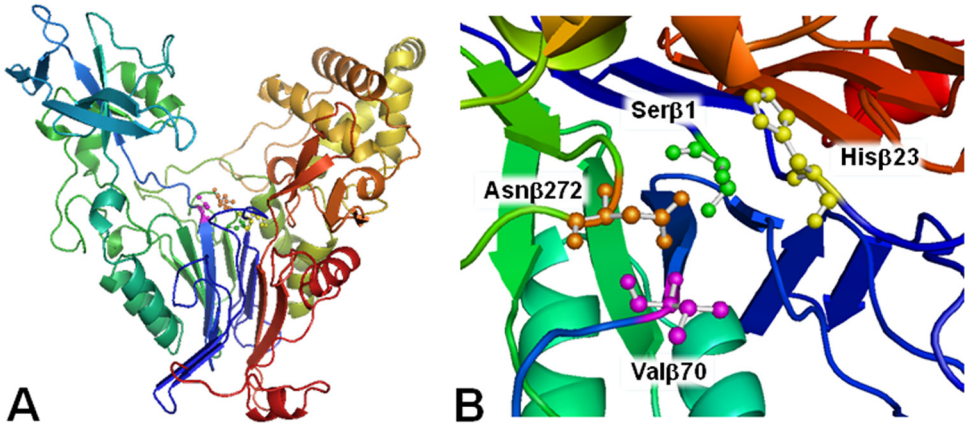

FIG 5 3D structure model of β-subunit of *Sl*PVA. (A) Complete model. This structure was modeled by the Phyre2 server (62), available at www.sbg.bio.ic.ac.uk/phyre2, which combines homology and *ab initio* modeling algorithms. These figures were rendered using Discovery Studio 3.1 software (Accelrys Software Inc.). The best fit was obtaining 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.

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 *Sl*PVA. 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 *Sl*PVA. These residues are conserved in many PAs, excluding the PGAs from *E. coli* and *Kluyvera citrophila* (57), where Hisβ23 and Valβ70 are replaced by Glnβ23 and Alaβ69, respectively. Previous studies on the chemical mechanism of *Sl*PVA 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 *Sl*PVA 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 *Sl*PVA 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 *Sl*PVA. Moreover, the 3D model of the β *Sl*PVA subunit (Fig. 5) revealed the presence of a hydrophobic pocket involved in the binding of the phenoxyacetyl 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 suggest 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).

Therefore, the amino acids in the *Sl*PVA substrate binding pocket could be Alaα154, Glyα158, Tyrβ24, Argβ31, Trpβ33, Leuβ50, Serβ53, Serβ57, Ileβ58, Serβ61, and Valβ186, which are different from those related to the substrate specificity of acylases from *E. coli* and *P. diminuta* (40, 55). Surprisingly, seven of these amino acid residues found in *Sl*PVA are identical to those of PVA from *Streptomyces mobaraensis* (4), CLA from *Streptomyces* sp. strain FERM BP-5809 (GenBank accession no. AB158476) (48), and AhlM from *Streptomyces* sp. strain M664 (49), and five are identical to those of AAC from *Actinoplanes utahensis* (51) (see Fig. S2 in the supplemental material). Interestingly, these five enzymes show similar substrate specificity with respect to the acyl moiety, because they catalyze the deacylation of substrates with long hydrophobic acyl moieties (4, 13, 48, 49, 60, 61). In this sense, we have shown in this work that *Sl*PVA is able to hydrolyze not only penicillins but also aculeacin A; in addition, r*Sl*PVA hydrolyzes AHLs (data not shown), raising new questions about the genuine role of *Sl*PVA in nature. Taking these results together with the fact that *Au*AAC is able to hydrolyze aliphatic penicillins as *Sl*PVA (61), we suggest that *Sl*PVA must be classified as a representative member of a new acylase subfamily showing the same physiological role involved in the hydrolysis of substrates carrying long hydrophobic acyl moieties (49). Therefore, our findings suggest that the physiological role of *Sl*PVA is not related to the hydrolysis of penicillins, as previously envisioned.

In summary, on the basis of the structural analysis and the studies of the catalytic mechanism carried out here, we propose that *Sl*PVA 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-

ena, like quorum-quenching processes or the interruption of quorum-sensing signal processing in bacteria, that remain to be identified.

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