

Gene Cluster Responsible for Validamycin Biosynthesis in *Streptomyces hygroscopicus* subsp. *jinggangensis* 5008

Yi Yu,¹ Linqun Bai,¹ Kazuyuki Minagawa,² Xiaohong Jian,¹ Lei Li,¹ Jialiang Li,¹
Shuangya Chen,¹ Erhu Cao,¹ Taifo Mahmud,² Heinz G. Floss,³
Xiufen Zhou,¹ and Zixin Deng^{1*}

Bio-X Life Science Research Center and School of Life Science & Biotechnology, Shanghai Jiaotong University, Shanghai 200030, People's Republic of China¹; College of Pharmacy, Oregon State University, Corvallis, Oregon 97331-3507²; and Department of Chemistry, University of Washington, Seattle, Washington 98195-1700³

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A gene cluster responsible for the biosynthesis of validamycin, an aminocyclitol antibiotic widely used as a control agent for sheath blight disease of rice plants, was identified from *Streptomyces hygroscopicus* subsp. *jinggangensis* 5008 using heterologous probe *acbC*, a gene involved in the cyclization of D-sedoheptulose 7-phosphate to 2-*epi*-5-*epi*-valiolone of the acarbose biosynthetic gene cluster originated from *Actinoplanes* sp. strain SE50/110. Deletion of a 30-kb DNA fragment from this cluster in the chromosome resulted in loss of validamycin production, confirming a direct involvement of the gene cluster in the biosynthesis of this important plant protectant. A sequenced 6-kb fragment contained *valA* (an *acbC* homologue encoding a putative cyclase) as well as two additional complete open reading frames (*valB* and *valC*, encoding a putative adenylyltransferase and a kinase, respectively), which are organized as an operon. The function of ValA was genetically demonstrated to be essential for validamycin production and biochemically shown to be responsible specifically for the cyclization of D-sedoheptulose 7-phosphate to 2-*epi*-5-*epi*-valiolone in vitro using the ValA protein heterologously overexpressed in *E. coli*. The information obtained should pave the way for further detailed analysis of the complete biosynthetic pathway, which would lead to a complete understanding of validamycin biosynthesis.

Filamentous gram-positive *Streptomyces* strains produce myriads of secondary metabolites with diversified structures and biological activities, including antibacterial, antifungal, antitumor, immunosuppressant, pesticidal, and herbicidal effects (5). The recently published genome sequences of *Streptomyces coelicolor* A3(2) (2) and *Streptomyces avermitilis* (20) further verified their position as a most abundant source of biosynthetic gene clusters encoding various bioactive compounds.

Streptomyces hygroscopicus subsp. *jinggangensis* 5008 (*S. hygroscopicus* 5008 or strain 5008 hereafter), isolated from the Jinggang Mountain area of China in 1974 (27), produces at least two antibiotics of agricultural importance. Jinggangmycin, a weakly basic water-soluble aminocyclitol antibiotic, which was later proven to be identical to validamycin A (Fig. 1) produced by *S. hygroscopicus* var. *limoneus* IFO 12703 (13), has been widely used as a prime control reagent against sheath blight disease of rice plants and dumping-off of cucumber seedlings in China and many other eastern Asian countries. Upon treatment with validamycin, normal extension of the main hyphae is switched to an abnormal branching at the tips and further development of the growing fungi is severely repressed (23). Furthermore, voglibose, a valiolamine derivative produced from validamycin by bioconversion and chemical modifications (10), is widely used for the treatment of diabetes.

The other antibiotic, jingsimycin, is an acidic polypeptide similar to saramycetin and has activity against various fungi.

A structural comparison between validamycin A and acarbose (Fig. 1), a compound used for the treatment of type II insulin-independent diabetes, revealed that they contain an identical C₇N aminocyclitol moiety (16), valienamine, whose structure and stereochemistry resemble that of D-glucose and which is responsible for the strong inhibitory activity of acarbose against α -glucosidases. Based on feeding experiments with isotopically labeled precursors, 2-*epi*-5-*epi*-valiolone, 5-*epi*-valiolone, valienone, and validone were suggested to be the intermediates of the validamycin A biosynthetic pathway by Floss and coworkers (7) (Fig. 1).

The incorporation patterns of various putative intermediates seemed to be different between the validamycin-producing *S. hygroscopicus* var. *limoneus* and the acarbose-producing *Actinoplanes* sp. strain SE50/110, but 2-*epi*-5-*epi*-valionone was found to be efficiently incorporated into both compounds, suggesting that both biosynthetic pathways share the same initial cyclization reaction catalyzed by common enzymes with similar activities. AcbC, an enzyme closely related to 3-dehydroquininate synthetases (AroB proteins), was proven to be involved in the cyclization of D-sedoheptulose 7-phosphate to 2-*epi*-5-*epi*-valionone in acarbose biosynthesis (25). The encoding gene (*acbC*) was thus used as a heterologous probe for the cloning of the validamycin biosynthetic genes from *S. hygroscopicus* 5008, which is reported in this paper. The direct involvement of *valA*, an *acbC* homologue, in validamycin biosynthesis was confirmed in vivo by gene inactivation as well as in

* Corresponding author. Mailing address: Bio-X Life Science Research Center, Shanghai Jiaotong University, Shanghai 200030, China. Phone: 86 21 62933404. Fax: 86 21 62932418. E-mail: zxdeng@sjtu.edu.cn.

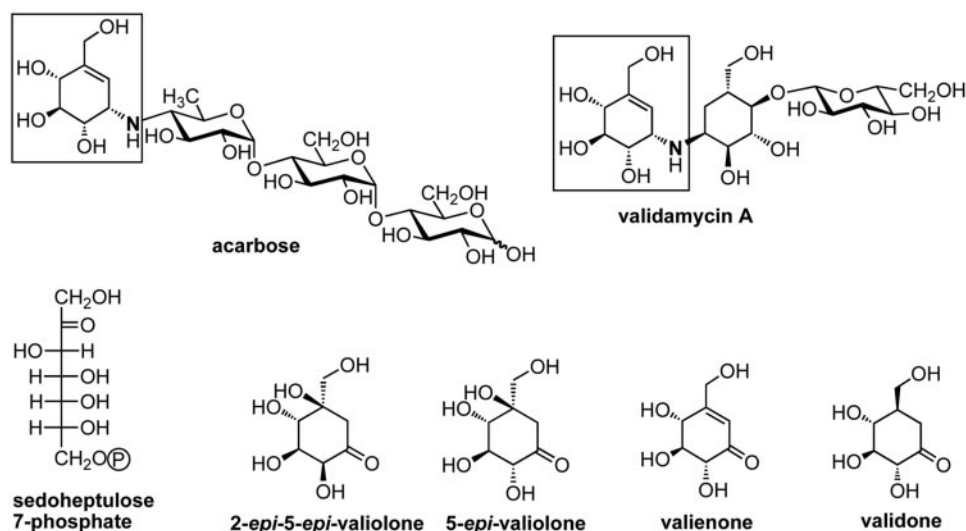


FIG. 1. Chemical structures of acarbose and validamycin A (top), and the initial intermediates proposed to be involved in validamycin A biosynthesis (bottom) (7). Boxed regions show the valienamine moiety shared by both compounds.

vitro by biochemical characterization of the reaction catalyzed by the ValA protein heterologously overexpressed in *E. coli*.

MATERIALS AND METHODS

Bacterial strains, phage, plasmids, and cosmids. Bacterial strains, phage, plasmids, and cosmids are described in Table 1.

Culture techniques, transformation, and conjugation. *S. hygroscopicus* 5008 and its derivatives were grown on SFM agar plates or in TSB liquid medium supplemented with 10.3% (wt/vol) sucrose and 1% (wt/vol) yeast extract (15) at 28°C for growth of mycelia, isolation of total DNA, or conjugation. FM-II liquid medium (containing [per liter] soluble starch, 60 g; sucrose, 30 g; beef extract, 35 g; NaCl, 0.75 g; MgSO₄ 2 g; CaCO₃ 8 g; initial pH, 7.8.) was used for fermentation. Protoplast preparation and transformation were performed according to Hopwood et al. (9). *Escherichia coli* strains were cultured according to Sambrook et al. (22). Cosmid clones were selected after infection of *E. coli* LE392 on LB agar containing 100 µg ampicillin ml⁻¹ or 30 µg apramycin ml⁻¹. For selection of *Streptomyces* transformants, apramycin and thiostrepton were both used at 30 µg ml⁻¹ in SFM agar medium and at 5 µg ml⁻¹ in liquid media.

Cloning techniques. Plasmid and total DNA was isolated from *Streptomyces* strains according to Kieser et al. (15). Restriction enzymes, T4 DNA ligase, *Taq* polymerase, and alkaline phosphatase were purchased from various companies (New England Biolabs, Takara, and MBI Fermentas). The gel recovery kit (Shanghai Watson and QIAGEN) was used for DNA recovery from agarose gels. For the generation of cosmid libraries, total DNA samples were partially digested with MboI, dephosphorylated with calf intestinal alkaline phosphatase, and size-fractionated by sedimentation analysis using a sucrose gradient (9). DNA fragments between 30 and 40 kb were mixed in a 1:1 molar ratio with BamHI-digested cosmid vector pHZ1358 and ligated at ca. 200 µg ml⁻¹ DNA. Packaging was done with λ packaging mixes prepared according to Sambrook et al. (22).

DNA probes, PCR primers, and Southern hybridization. An NdeI-EcoRI fragment carrying the *acbC* gene from *Actinoplanes* sp. strain SE50/110 producing acarbose was excised from plasmid pAS8/7 (25) and used as a heterologous probe. The two oligonucleotide primers used for PCR amplification were ValA-F (5'-GGATCCACATATGACCATGACCAAG-3') and ValA-R (5'-GAATTCACACCCCATGTCC-3'). For Southern hybridization experiments, *S. hygroscopicus* 5008 genomic DNA was cleaved with restriction enzymes, separated on 0.8% agarose gels, and transferred onto Hybond-N⁺ nylon membrane (Amersham-Pharmacia). α-[³²P]dCTP-labeled radioactive probes using a random priming kit (Roche) were used for both Southern blots and in situ colony hybridization.

Construction of pHZ2236 for targeted deletion of 30-kb region within the ca. 70-kb contig. Complete digestion of cosmid 3G8 DNA by BamHI and religation resulted in the construction of pHZ2234, in which an internal ca. 30 kb (marked by a solid bar in Fig. 2) of 3G8 was found to be deleted and the two 2.1-kb

flanking fragments were connected. Then a 1.4-kb BamHI fragment carrying *aac(3)IV* (apramycin resistance gene) was inserted between the two 2.1-kb fragments with the same transcriptional direction as *valA*, which generated pHZ2236 for subsequent conjugation.

Construction of pJTU519 for targeted deletion of 563 bp of DNA internal to *valA*. A 1.33-kb XbaI-EcoRI fragment overlapping one side of the *valA* gene on the ca. 6-kb sequenced BamHI fragment (Fig. 2) in pHZ2229 was excised from pJTU690 and inserted into the corresponding site of pBluescript II SK(+), resulting in pJTU504. A 1.4-kb EcoRI fragment carrying the apramycin resistance gene *aac(3)IV* was inserted into the EcoRI site of pJTU504 to generate pJTU514, which was subsequently digested with EcoRV and HindIII to accept a 1.5-kb PvuII-HindIII fragment of pJTU696 overlapping the other end of the *valA* on the ca. 6-kb sequenced BamHI fragment (Fig. 2) to generate pJTU515. A total of 4.23 kb of DNA linking 1.33 kb (left arm)-1.4 kb *aac(3)IV*-1.5 kb (right arm), was transferred as an XbaI fragment to the corresponding site of pIJ2925 to construct pJTU516, for the final excision as a BglII fragment to be inserted into the unique BamHI site of pHZ1358, resulting in pJTU519 as a final construct for the targeted replacement of a 563-bp DNA fragment internal to *valA* by the 1.4-kb *aac(3)IV*, in the wild-type strain 5008.

Antibiotic assay. Production of validamycin by *S. hygroscopicus* 5008 and its derivatives was detected using a bioassay and high performance liquid chromatography (HPLC). For the bioassay, 1 milliliter of fermentation supernatant extracted with chloroform was mixed with 14 ml of melted agar (8 g of agar, 1 liter of water). An agar plug with *Pellicularia sasakii* (Shanghai Jiaotong University Stock Collection, Shanghai Jiaotong University, Shanghai), which is sensitive to validamycin, was transferred to the center of the agar plate and after 24 h incubation at 30°C the diameter of the colony was measured, which is inversely related to the inhibitory potency. For HPLC analysis, the strains were cultured in 40 ml of FM-II fermentation medium in 250-ml baffled flasks at 37°C and 220 rpm for 6 days. The fermentation broth was centrifuged at 12,000 rpm for 5 min followed by chloroform extraction. The extracted supernatant was directly loaded onto a Nucleosil C18 column (250 mm by 4.6 mm, Sigma-Aldrich) for HPLC analysis (Waters 220). The mobile phase (0.005 M sodium phosphate buffer-acetone, 97:3) was applied with the flow rate of 1 ml min⁻¹ at room temperature. The elute was monitored at 210 nm with a Waters 996 photodiode array detector and the data were analyzed with a Waters Millennium Chromatography Manager.

Sequence analysis. DNA sequencing was done at Shanghai Sangon Ltd. using pUC18 as the vector. Sequencing reactions were carried out using the Amersham Thermosequencing kit containing fluorescent dye terminators and an Applied Biosystems model 377 automated DNA sequencer. Sequence analysis was performed with the Lasergene DNA analysis tools (DNASTAR) (Madison, MI). Open reading frames and ribosome binding sites were predicted with FramePlot (11). Nucleotide and amino acid sequence comparisons against public databases were done using the BLAST program (1).

Cloning and heterologous overexpression of recombinant His₆-tagged ValA.

TABLE 1. Strains, phage, plasmids, and cosmids used in this study

Strain, phage, or plasmid	Relevant characteristics ^a	Reference or source
<i>S. hygrosopicus</i> strains		
5008	Wild-type producer of validamycin	27
YU-1	Nonproducer of validamycin generated by gene replacement of a ca. 30-kb region including <i>acbC</i> homolog with <i>aac(3)IV</i>	This work (Fig. 3)
JXH-1	As above, but replacing a 563-bp DNA fragment internal to <i>valA</i>	This work (Fig. 5)
<i>S. lividans</i> 66 ZX1	<i>rec-46 str-6 pro-2</i>	30
<i>E. coli</i> strains		
DH10B	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>dlacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>deoR</i> <i>recA1 endA1 araΔ139 D(ara, leu)1697 galU galK λ⁻rpsL nupG</i>	GIBCO-BRL
ET12567(pUZ8002)	<i>dam dcm hsdS/pUZ8002</i>	21
LE392	<i>supE44 supF58 hsdR514</i> , used for infection with in vitro-packaged cosmids	3
BL21Gold(DE3)/pLysS XL-1-Blue	F ⁻ <i>ompT hsdS_B (r_B⁻ m_B⁻) gal dcm(DE3)</i> pLysS (Cm ^r) <i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i>	18 Stratagene
<i>Pellicularia sasakii</i>	Fungal indicator strain sensitive to validamycin A	SJUSC
Plasmids		
pAS8/7	pIJ6021 with a 1,264-bp NdeI-EcoRI fragment containing <i>acbC</i> in the corresponding sites	25
pBlueScript II SK(+) pHZ1358	<i>bla lacZ orifl</i> Cosmid used for the construction of 5008 genomic library; pIJ101 derivative; <i>tsr Ltz⁻ sti⁺ oriT</i>	Stratagene 26
pHZ2229	pBlueScript II SK(+) carrying a ca. 6-kb sequenced BamHI fragment (Fig. 2) from cosmid 3G8	This study
pHZ2234	Construct after religation of BamHI-digested 3G8	This study
pHZ2236	Insertion in BamHI site of pHZ2234 of an <i>aac(3)IV</i> gene sandwiched between the two 2.1-kb genomic DNA fragments	This study
pIJ2925	<i>bla lacZ</i>	12
pJTU504	Insertion of a 1.33-kb XbaI-EcoRI fragment from pJTU690 in pBlueScript II SK(+)	This study
pJTU514	pJTU504 derivative with the insertion of <i>aac(3)IV</i> in EcoRI site	This study
pJTU515	Insertion of 1.5-kb PvuII-HindIII fragment from pJTU696 into EcoRV-HindIII sites of pJTU514	This study
pJTU516	Insertion of a 4.23-kb BglII fragment from pJTU515, which contains a linked 1.33-kb (left arm)-1.4-kb <i>aac(3)IV</i> -2.1-kb (right arm) for <i>valA</i> inactivation, in BamHI site of pHZ1358	This study
pJTU690	Part of the 6-kb sequenced BamHI fragment (Fig. 2) including the 1.33-kb XbaI-EcoRI fragment inserted into pBlueScript II SK(+)	This study
pJTU696	Part of the 6-kb sequenced BamHI fragment (Fig. 2) including the 1.5-kb PvuII-HindIII fragment inserted into pBlueScript II SK(+)	This study
pKMW5	pUC18 derivative carrying the <i>strD</i> and <i>strE</i> genes of the streptomycin pathway	24
pRSET-B pUC18	<i>bla rep^{UIC}</i> , T7 promoter <i>bla lacZ</i>	Invitrogen 28
Phage		
φC31	Wild type; <i>c⁺ attP⁺ int⁺</i>	31

^a *Ltz* (lethal zygotosis), pock formation caused by plasmid transfer; *mel*, tyrosinase gene for melanin production; *oriT*, origin of transfer of plasmid RK2; *tsr*, thiostrepton resistance gene; *aac(3)IV*, apramycin resistance gene; *sti*, origin for second-strand synthesis of the multicopy plasmid pIJ101 (6); *vph*, viomycin resistance gene; *xylE*, catechol 2,3-dioxygenase from *Pseudomonas putida*; Cm^r, chloramphenicol resistance gene.

The *valA* gene was amplified by PCR with Platinum Pfx DNA polymerase (Invitrogen) using the cosmid clone 3G8 as the template and primers ValA-F3, 5'-GAAGATCTGCATATGACCAAGCAGAGTTCCTTATCC-3' (BglII and NdeI), and ValA-R2, 5'-GGAATTCTCACACCCCATGTCCACGGCACC G-3' (EcoRI). PCR amplification was done in a Thermocycler (Eppendorf, Mastercycler gradient) under the following conditions: 33 cycles of 90 s at 95°C, 45 s at 60°C, and 45 s at 72°C. The PCR products were digested with BglII and EcoRI, and subsequently ligated into BamHI- and EcoRI-digested pRSET-B. The constructs were transformed into *E. coli* XL-1-Blue and plated on LB agar plates containing 100 μg ml⁻¹ ampicillin.

The plasmid DNA was isolated and introduced by heat-pulse transformation

into *E. coli* BL21Gold(DE3)/pLysS (Stratagene), which was then plated onto LB agar plates containing 100 μg ml⁻¹ ampicillin and 25 μg ml⁻¹ chloramphenicol. The transformants were grown in 20 ml LB medium containing ampicillin and chloramphenicol at 37°C to an optical density at 600 nm of 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM and the incubation was continued at 28°C for 24 h. The cells were harvested by centrifugation at 3,500 rpm for 15 min and stored frozen at -80°C until further use.

Preparation of cell extracts and purification of His₆-tagged ValA. Cells were thawed and resuspended in disruption buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0). The suspension was sonicated three times for 25 s

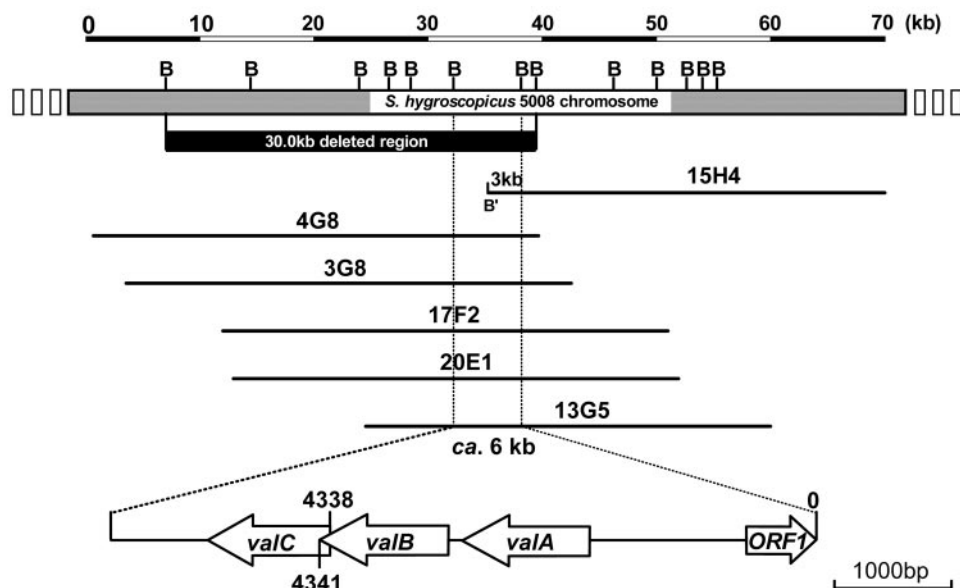


FIG. 2. Overlapping cosmids covering genes for validamycin biosynthesis and gene organization of the 6-kb sequenced region. Top: BamHI restriction map of the ca. 70-kb contig for validamycin biosynthesis. The solid bar indicates the 30-kb deleted region in Fig. 3, which abolished validamycin production. Bottom: 6-kb sequenced region including the *acbC* homologue (*valA*). B: BamHI site. B': regenerated BamHI site from BamHI (vector) and MboI (insert) sites.

each and cell debris was removed by centrifugation at 10,000 rpm for 10 min. The protein solution was applied to a Ni-nitrilotriacetic acid spin column (QIAGEN) and centrifuged at 2,000 rpm for 2 min. The column was washed with washing buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 20 mM imidazole, pH 8.0, and 50 mM NaH_2PO_4 , 300 mM NaCl, 100 mM imidazole, pH 8.0). The His₆-tagged protein was eluted with elution buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 500 mM imidazole, pH 8.0) and dialyzed for 24 h against 1 liter of dialysis buffer (20 mM potassium phosphate [pH 7.4], 0.05 mM CoCl_2 , 2 mM KF and 0.5 mM dithiothreitol). Protein concentration was measured by the Bradford protein microassay with bovine serum albumin as the standard.

Enzyme assay. The enzyme assay was carried out at 30°C for 3 to 12 h in a 100- μl volume of 20 mM potassium phosphate (pH 7.4), 0.05 mM CoCl_2 , 2 mM KF, 1 mM NAD^+ , 5 mM sedoheptulose 7-phosphate, and 50 μl of protein solution (2.4 mg/ml protein). The reaction progress was monitored by thin-layer chromatography analysis. The reaction mixture was lyophilized and the reaction products were extracted with methanol. This extract was then dried and a few drops of SIGMA-SIL-A (SIGMA) were added. The solvent was removed in a flow of argon gas and the products were reextracted with *n*-hexane and injected for gas chromatography-mass spectroscopy (GC-MS) (Hewlett Packard 5890 series II gas chromatograph).

Nucleotide sequence accession number. The DNA and deduced protein sequences reported in this paper have been deposited in GenBank under accession number AY753181.

RESULTS

***S. hygroscopicus* 5008 contains a homologue of the *acbC* gene required for acarbose biosynthesis in *Actinoplanes*.** Probing a Southern transfer of BamHI-digested genomic DNA of 5008 with the labeled *acbC* gene (a 1,264-bp NdeI-EcoRI fragment carrying a gene involved in the cyclization of D-sedoheptulose 7-phosphate to 2-*epi*-5-*epi*-valiolone of the acarbose biosynthetic pathway) of *Actinoplanes* sp. strain SE50/110 gave a weak signal at ca. 6 kb at low stringency in 6 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 65°C (data not shown). To isolate the hybridizing fragment, further hybridization with the same probe in a pHZ1358 library was carried out with the same stringency. Six of the 39 initial isolates were confirmed positive by successive hybridization experiments.

Five (4G8, 3G8, 17F2, 20E1, and 13G5, Fig. 2) out of the six positive overlapping cosmids shared a common 6-kb fragment hybridizing to the above-mentioned probe, while the sixth cosmid (15H4) gave a hybridization signal to a fragment of only about 3 kb (Fig. 2).

The BamHI digestion patterns of the six cosmids resulted in a contig spanning ca. 70 kb, without apparent genomic rearrangement. The different hybridization signal originating from BamHI-digested 15H4 was found to have resulted from the regeneration of a BamHI (designated B' in Fig. 2) site between the cloning site (BamHI) of the vector and an accidental MboI site of the insert during library construction, as this fragment was found to lie at the very end of the cloned fragment in cosmid 15H4.

Cosmid 3G8 contains genes involved in validamycin biosynthesis. One of the six pHZ1358-derived (26) bifunctional cosmids, 3G8 (Fig. 2), carrying a *tsr* gene suitable for selection in *Streptomyces* and a 34.2-kb insert under the control of pIJ101 origin of replication (6), was proven to be extremely unstable (ca. 98% loss after one round of nonselective growth on SFM medium) in strain 5008. For targeted gene replacement, ca. 30 kb of the strain 5008 DNA insert containing the *acbC* homologue in cosmid 3G8 was replaced by a 1.4-kb apramycin resistance (*Apr*^r) determinant, *aac(3)IV*, which resulted in pHZ2236 for subsequent conjugation.

pHZ2236 was transferred by conjugation from *E. coli* ET12567(pUZ8002) into strain 5008. About 10⁻⁸ exconjugants per donor were obtained which were initially selected to be *Apr*^r. Surprisingly, all four randomly selected exconjugants were found to be sensitive to thiostrepton. A Southern transfer of total DNA (Fig. 3B) from the four *Apr*^r Thio^s exconjugants (YU-1-1 to YU-1-4) together with wild-type 5008 was probed with the labeled 5.6-kb insert from pHZ2236 (Fig. 3A). As

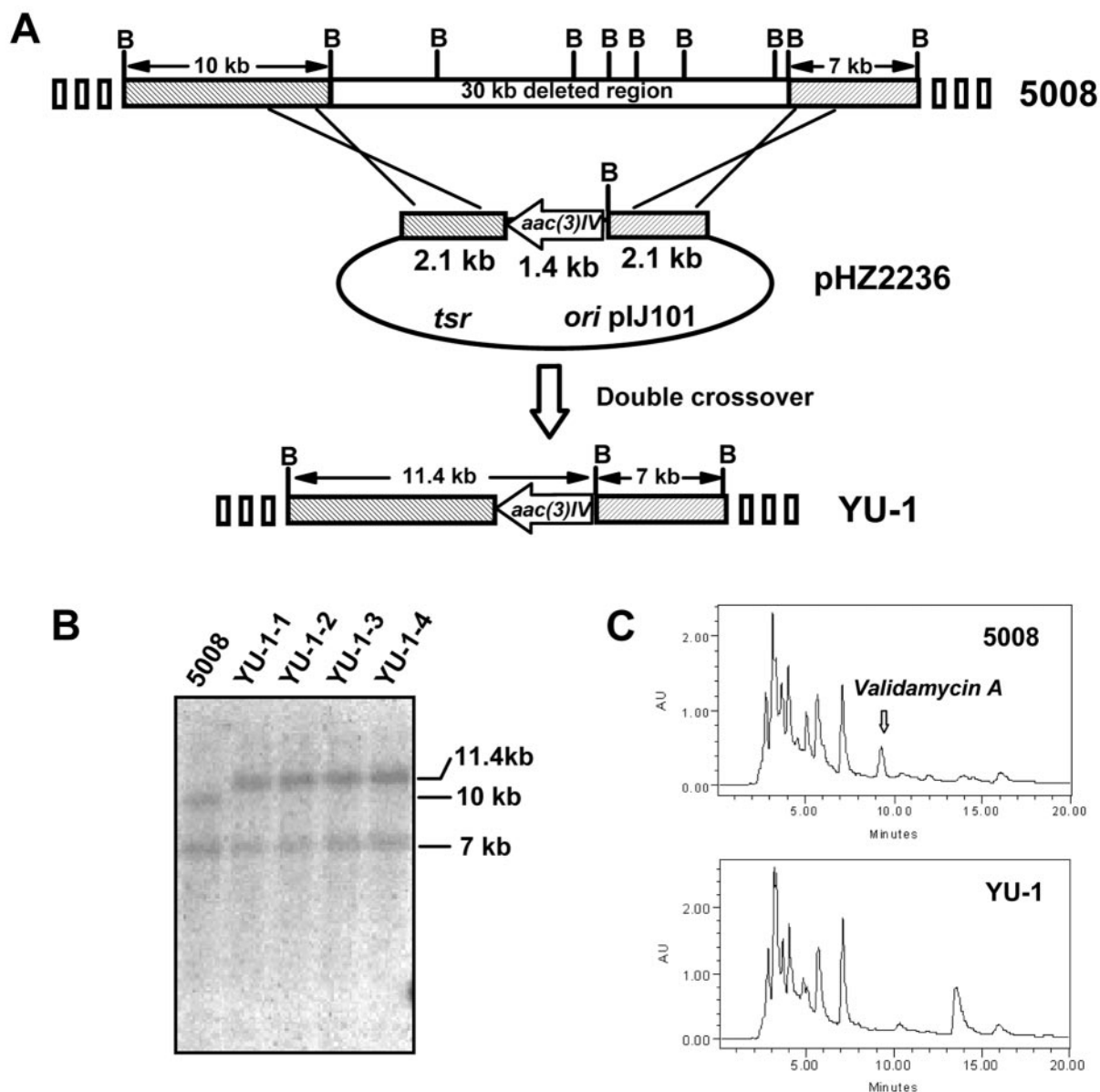


FIG. 3. Replacement of a 30-kb region containing the *acbC* homologue by *aac(3)IV* in strain 5008. (A) Schematic representation of the replacement of the 30 kb of DNA mediated by 2.1-kb genomic fragments flanking both sides of the 1.4-kb *aac(3)IV* apramycin resistance determinant in pHZ2236. The mutants generated by double crossover (YU-1-1 to YU-1-4) had a fusion of the 10-kb leftward BamHI fragment with the BglII end of the 1.4-kb *aac(3)IV* to form an 11.4-kb new BamHI fragment, but keep the rightward 7-kb fragment unchanged. (B) Southern hybridization using the α - ^{32}P dCTP-labeled 5.6 (2.1 + 1.4 + 2.1)-kb insert from pHZ2236 as the probe after genomic DNAs of the wild-type 5008 and its derivatives (YU-1-1 to YU-1-4) were digested with BamHI. (C) HPLC analysis demonstrating that validamycin A is produced by wild-type strain 5008, but not by YU-1.

expected, the ca. 30-kb fragment in the 5008 chromosome was found to be replaced by a 1.4-kb fragment in the YU-1 mutants, resulting in a fusion of the 10-kb leftward BamHI fragment with the BglII end of the 1.4-kb *aac(3)IV* to form a new 11.4-kb BamHI fragment (Fig. 3A), which is distinguishable from the 10-kb BamHI fragment of the wild-type 5008 in Fig. 3B.

All four mutants were tested for their ability to inhibit the growth of *Pellicularia sasakii*, a plant pathogen causing rice sheath blight disease. While *P. sasakii* stopped growing on agar plates containing the fermentation supernatant of strain 5008 (as a positive control), the fungus continued to grow on plates

containing the fermentation supernatants of each of the four candidate strains (YU-1-1 to YU-1-4). HPLC analysis revealed that the mutant candidates did not produce validamycin like the wild-type strain. Unlike with the wild-type strain 5008, no peak corresponding to validamycin A (retention time of 9.5 min) was found in samples obtained from the individual mutant candidates (Fig. 3C). This is consistent with the presumption that genes essential for validamycin biosynthesis including the *acbC* homologue have been deleted.

Sequencing analysis of a DNA fragment including the *acbC* homologue. The common 6-kb BamHI fragment shared by the

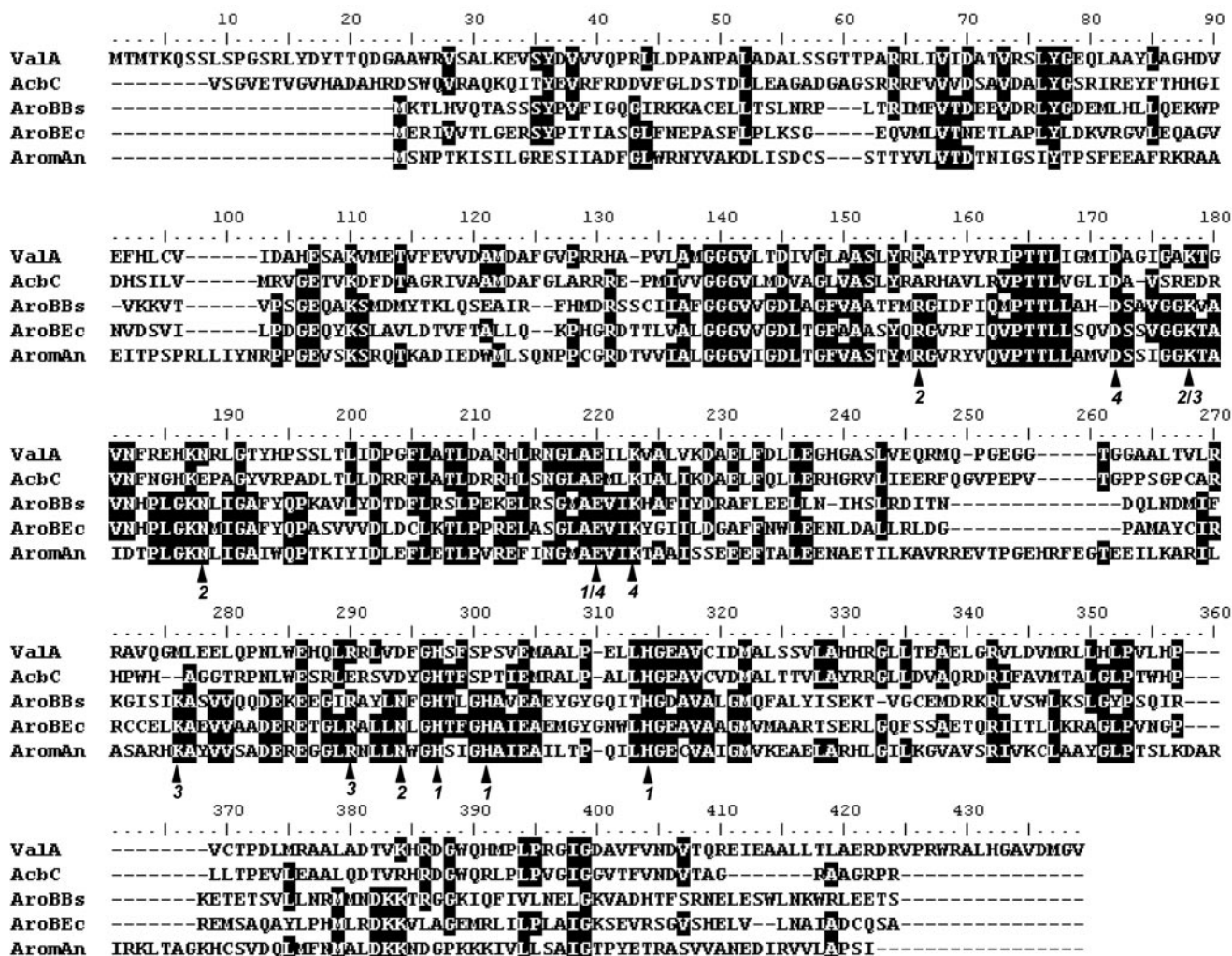


FIG. 4. Alignment of ValA with AcbC and three AroB proteins. Deduced amino acid sequences are from the following organisms: AcbC, *Actinoplanes* sp. (Y18523.3); AroBBs, *Bacillus subtilis* (M80245); AroBEc, *E. coli* (X03867); and AromAn, *Emericella* (formerly *Aspergillus*) *nidulans* (395-amino-acid DHQS domain at the N terminus of the pentafunctional AROM protein; X05204). A functional attribution of the amino acid residues indicated with black arrows, based on the analysis of the three-dimensional structure of the dehydroquinase (DHQS) domain of *E. nidulans* (4), is given below the alignment by the following code: 1 = Co²⁺ binding (Zn²⁺ in the fungal protein instead); 2 = heptulose phosphate group binding; 3 = C-1 hydroxyl fixation; and 4 = C-4 hydroxyl fixation.

five cosmids (4G8, 3G8, 17F2, 20E1, and 13G5, Fig. 2) including the *acbC* homologue was cloned into pBluescript II SK(+) and sequenced. The overall G+C content of the sequenced region was 67.9%, lower than that of *S. coelicolor* A3(2), which showed an overall G+C content of 72.1%. FramePlot (11) analysis revealed three open reading frames (*valA*, *valB*, and *valC*) transcribed in the same direction, with *valA* separated from *valB* by 5 bp, while *valB* and *valC* overlap by 3 bp, suggesting that they are transcribed into one polycistronic mRNA (Fig. 2).

The *valA* gene seems to encode a polypeptide of 412 amino acids in length, with a putative ribosome binding site (GTGA) 10 bp preceding the putative translational start codon (ATG). The nucleotide sequence of *valA* has 59.5% identity with the *Actinoplanes acbC* gene, while the deduced ValA protein has 48% identity with the AcbC protein (25) (Fig. 4). The ValA protein also showed significant similarity (37% identity) to the

AroB protein from *Emericella nidulans* (Fig. 4), which is known to be responsible for the cyclization of 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) to dehydroquinase (4).

The deduced amino acid sequence (373 amino acids) of ValB shows 33% identity with GlgC, a glucose-1-phosphate adenylyltransferase isolated from *Bacillus halodurans* C-125 (8). ValC (351 amino acids) shows 30% identity to AcbM, the 2-*epi*-5-*epi*-valiolone 7-kinase from the acarbose biosynthetic gene cluster, whose demonstrated function is to phosphorylate 2-*epi*-5-*epi*-valiolone to form 2-*epi*-5-*epi*-valiolone 7-phosphate. Additionally, ValC also shows weaker homology with glucokinase from a *Bacillus* species (29% identity and 43% similarity).

There is an incomplete open reading frame (tentatively designated ORF1) at the 5' end of the fragment, which is transcribed in the opposite direction and shows 31% identity to the N-terminal amino acids of dTDP-4-dehydrorhamnose reductase, RmlD, whose known function is to reduce dTDP-6-deoxy-

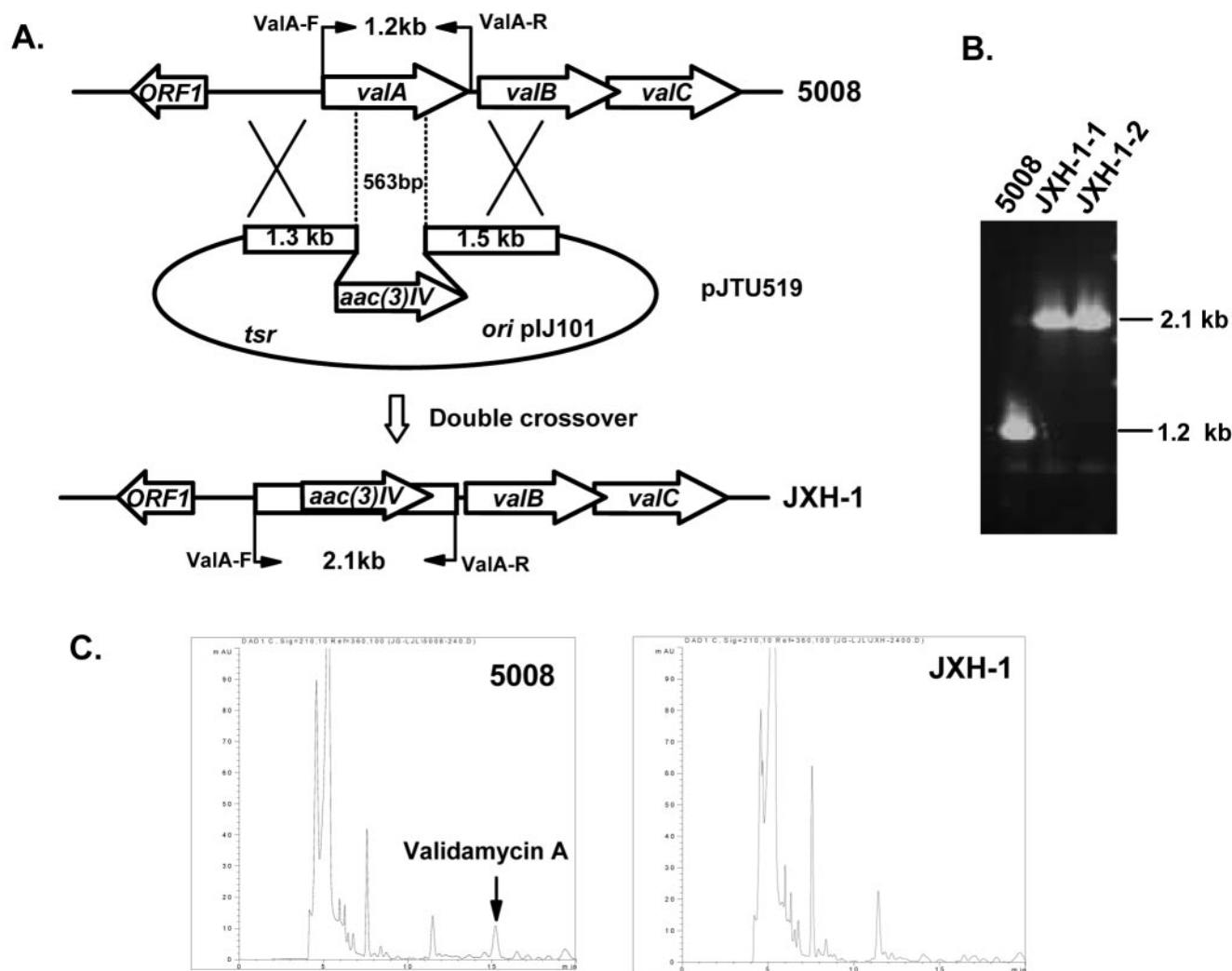


FIG. 5. Inactivation of *valA* of the validamycin biosynthetic gene cluster. (A) Schematic representation of the replacement of a 563-bp internal fragment of *valA* with the 1.4-kb *aac(3)IV*. In shuttle plasmid pJTU519, *aac(3)IV* was inserted between 1.3-kb and 1.5-kb genomic fragments originally flanking the deleted 563-bp region. While wild-type 5008 should give a 1.2-kb PCR-amplified product, mutant JXH-1 should yield a 2.1-kb product using a pair of primers (ValA-F and ValA-R) designed for amplification of full-length *valA*. (B) PCR analysis of wild-type 5008 and mutant JXH-1. (C) HPLC comparison between 5008 and JXH-1. The peak corresponding to validamycin A is absent from mutant JXH-1.

L-xylo-4-hexulose to dTDP- L-rhamnose in *Geobacillus stearothermophilus* (19).

DNA replacement in *valA* is abolished validamycin biosynthesis. Direct evidence for the involvement of *valA* in the biosynthesis of validamycin came from the replacement of a 563-bp DNA fragment internal to *valA* with *aac(3)IV* (Fig. 5A). This was performed by using a pHZ1358-derived plasmid (pJTU519, detailed in Materials and Methods), in which *aac(3)IV* (apramycin resistance gene) was sandwiched between sequences of 1.33 kb flanking to the left, and 1.5 kb flanking to the right of the 563-bp DNA to be deleted (Fig. 5A). JXH-1, a 5008 derivative, was obtained after introduction of pJTU519 into wild-type strain 5008 by conjugation from *E. coli* ET12567(pUZ8002), initial selection by thiostrepton, and further screening for the Thio^s Apr^r phenotype. Total DNA from the two independent Thio^s Apr^r exconjugants (JXH-1-1 and JXH-1-2) and from the wild-type strain 5008 was used as tem-

plate for PCR amplification using two oligonucleotide primers (ValA-F and ValA-R) (Fig. 5B).

The 5008 DNA gave a 1.2-kb PCR product, while both JXH-1-1 and JXH-1-2 gave an expected 2.1-kb PCR product (Fig. 5B), which confirmed that a 563-bp DNA fragment internal to *valA* (Fig. 5A) has been removed from the chromosome of these mutants. No inhibition of *Pellicularia sasakii* could be detected in the bioassay, as in the case of YU-1 reported above, and no peak corresponding to validamycin A could be detected in the JXH-1-1 sample by HPLC analysis (Fig. 5C) as opposed to that of 5008, confirming the complete loss of validamycin production in JXH-1-1.

Characterization of ValA activity using heterologously over-expressed protein. In order to confirm the function of its gene product as a 2-*epi*-5-*epi*-valiolone synthase, *valA* was heterologously expressed in *E. coli*. A 1.24-kb DNA fragment containing the *valA* gene was amplified from cosmid 3G8 by PCR,

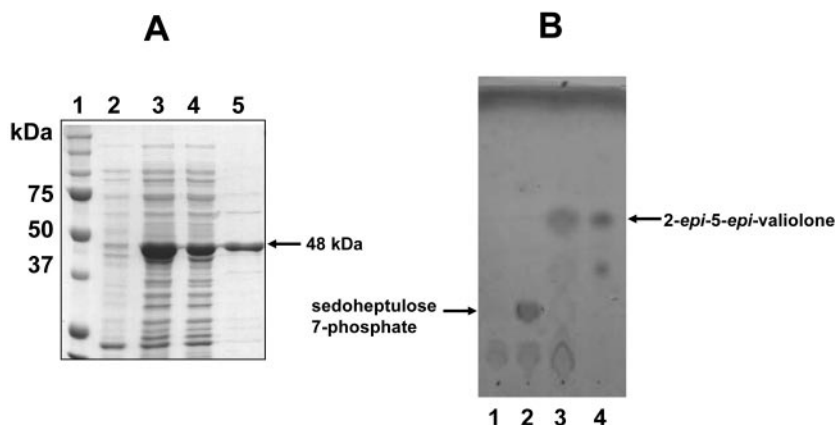


FIG. 6. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the His₆-tagged ValA protein. Lanes: 1, molecular weight markers; 2, soluble protein of cell extract of *E. coli* BL21Gold(DE3)pLysS/*valA* before induction with IPTG; 3, total protein of cell extract of *E. coli* BL21Gold(DE3)pLysS/*valA* after induction with IPTG; 4, soluble protein of cell extract of *E. coli* BL21Gold(DE3)pLysS/*valA* after induction with IPTG; 5, purified His₆-tagged ValA. (B) Thin-layer chromatography analyses of ValA assay (solvent system, *n*-butanol:ethanol:H₂O, 9:7:4). Lane 1, ValA without substrate; lane 2, boiled ValA with sedoheptulose 7-phosphate; lane 3, ValA with sedoheptulose 7-phosphate; lane 4, 2-*epi*-5-*epi*-valiolone.

introducing BglIII and NdeI restriction sites with the forward primer and an EcoRI site with the reverse primer. The PCR product was subcloned into the expression vector pRSET-B as a BglIII/EcoRI fragment, transformed into *E. coli* XL-1-Blue. The correct plasmids were subsequently transformed into *E. coli* BL21Gold(DE3)pLysS. Expression of *valA* under the control of the T7 promoter was induced by isopropyl- β -D-thiogalactopyranoside (IPTG), which gave rise to a 48-kDa soluble polyhistidine-tagged protein. Affinity purification on a Ni-nitrilotriacetic acid spin column (QIAGEN) or a BD TALON column gave a protein that was >80% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 6A).

To test the catalytic activity of ValA, the enzymatic reaction was carried out using sedoheptulose 7-phosphate as the substrate under reaction conditions used previously for the AcbC protein of acarbose biosynthesis (25). The reaction extracted with methanol gave rise to a product which has the same *R_f* value as authentic 2-*epi*-5-*epi*-valiolone on thin-layer chromatography (Fig. 6B). On the other hand, incubation with cell extracts of *E. coli* harboring the empty pRSET-B vector gave no product.

The product was extracted from the lyophilized reaction mixture by methanol, converted to its trimethylsilylated derivative, and analyzed by GC-MS (Fig. 7). An isotopically labeled authentic sample, 2-*epi*-5-*epi*-[6-²H₂]valiolone, chemically synthesized from D-mannose (25) was used for comparison (Fig. 7A). The enzyme product was detected as a tetratrimethylsilyl derivative of 2-*epi*-5-*epi*-valiolone [*m/z* 480 (M⁺)] with major fragment ions at *m/z* 335, 276, 217, 147, and 73 (Fig. 7D). This fragmentation pattern is consistent with that of the authentic sample, which showed *m/z* 482 (M⁺), 335, 278, 217, 147, and 73 (Fig. 7C).

DISCUSSION

Considerable effort has been made in our laboratories during the last few years to clone biosynthetic genes for the aminocyclitol antibiotic validamycin, which is commercially produced on a large scale in China and some other eastern Asian

countries and is widely used for the protection of rice seedlings against fungal sheath blight. Genetic manipulation of the structural and/or regulatory genes is expected to be of great help for strain improvement, e.g., to increase yield or the relative abundance of the validamycin A component in the fermentation broth, and possibly to make new validamycin derivatives.

A first effort by heterologous expression in *Streptomyces lividans* 66, based on the proposed biosynthetic pathway (7) that implied that the validamycin gene cluster may possess a limited number of genes, failed in an initial trial. A second attempt using radioactively labeled *strD* encoding dTDP-glucose synthase of the streptomycin biosynthetic gene cluster of *Streptomyces griseus* as a probe also yielded no signal, which suggests that the activation of glucose in validamycin biosynthesis is catalyzed by an enzyme different from StrD, which seems to be specific for 6-deoxyhexose pathways.

The success of using the *acbC* gene from the acarbose cluster of *Actinoplanes* to probe for the validamycin biosynthetic gene cluster was not surprising, as both compounds contain the same valienamine moiety in their structures, whose initial biosynthetic step involves cyclization of D-sedoheptulose 7-phosphate to form 2-*epi*-5-*epi*-valiolone. The significant sequence homologies between ValA and ValC of the validamycin pathway and AcbC and AcbM (40) of the acarbose pathway strongly support the idea that they have similar functions. This suggests that 2-*epi*-5-*epi*-valiolone in validamycin biosynthesis has the same fate of being phosphorylated (by ValC) as was demonstrated in acarbose biosynthesis (29).

It was proposed that all of the intermediates from 2-*epi*-5-*epi*-valiolone to valienone in the acarbose biosynthetic pathway are phosphorylated (29), which explains the lack of incorporation of the nonphosphorylated putative intermediates, such as 5-*epi*-valiolone and valienone. On the contrary, nonphosphorylated 5-*epi*-valiolone, valienone, and validone were found to be efficiently incorporated into validamycin A (7). If ValC has a role in phosphorylating the intermediates in the validamycin pathway similar to that of AcbM in acarbose biosynthesis, its substrate specificity or catalytic ability to phosphorylate all of

the cyclitol intermediates, including 2-*epi*-5-*epi*-valiolone, remains to be explored.

Both ValA and AcbC show significant similarities to AroB-related DHQS proteins from diverse organisms (4, 8, 17), whose catalytic function is known to be cyclization of DAHP to dehydroquinone. Based on the analysis of the three-dimensional structure of the DHQS domain of the functional AroM protein of the filamentous fungus *Emericella nidulans*, a total of 13 amino acid residues were identified to be important for catalysis and as being involved in Zn²⁺ binding (Co²⁺ in bacteria instead; indicated as 1 in Fig. 4), heptulose phosphate group binding (indicated as 2 in Fig. 4), C-1 hydroxyl fixation (indicated as 3 in Fig. 4), or C-4 hydroxyl fixation (indicated as 4 in Fig. 4) (4). In ValA, three of the four identified residues for Co²⁺ binding (1), three of the four for heptulose phosphate group binding (2), two of the three for C-1 hydroxyl fixation (3), and all three for C-4 hydroxyl fixation (4), are conserved. Therefore, ValA seems to be more related to AroB proteins than AcbC.

Preceding *valC* is a cotranscribed gene, *valB*, encoding a putative adenylyltransferase (ValB). Conceivably, this activity could be involved in the conversion of glucose 1-phosphate, possibly from primary metabolism, into dTDP-glucose. Incorporation of activated glucose has been proven through feeding experiments with validoxylamine A to be the final step in validamycin biosynthesis (14). Alternatively, ValB may be involved in the activation of one of the cyclitol intermediates, e.g., 1-*epi*-valienol 1-phosphate, to its nucleotide derivative, setting the stage for the coupling reaction that leads to a pseudodisaccharide intermediate.

The combination of the results of the previous feeding experiments (7) with the genetic and biochemical information obtained, especially the results of the in vivo gene inactivation and in vitro enzymatic characterization of ValA, strongly suggests that the identified genes are involved in validamycin biosynthesis. From the overlapping cosmid contig we know that the flanking DNA to the left and right of the 6-kb sequenced region covering the complete *valA*, *valB*, and *valC* genes extends to 29 kb and 35 kb, respectively, likely to cover the complete set of genes necessary for validamycin formation. We can thus expect a detailed understanding of the biosynthesis of validamycin, which is critical for more targeted strain improvement or generating novel validamycin derivatives by combined genetic and biochemical approaches. This will become possible when the entire gene cluster has been completely sequenced and the genes have been individually characterized, work that is now in progress.

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