

Genes Involved in Formation and Attachment of a Two-Carbon Chain as a Component of Eurekanate, a Branched-Chain Sugar Moiety of Avilamycin A

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Eurekanate belongs to the important class of branched-chain carbohydrates present in a wide variety of natural sources. It is a component of avilamycin A, a potent inhibitor of bacterial protein synthesis targeting the 50S ribosomal subunit. The present work provides experimental proof for the function of two genes of the avilamycin biosynthetic gene cluster, *aviB1* and *aviO2*, that are both involved in avilamycin structure modification. The functions of both genes were identified by gene inactivation experiments and nuclear magnetic resonance analyses of extracts produced by the mutants. We suggest that both *AviO2* and *AviB1* are involved in the biosynthesis of eurekanate within avilamycin biosynthesis. Moreover, two other genes (*aviO1* and *aviO3*) have been inactivated, resulting in a breakdown of avilamycin production in the mutants ITO1 and ITO3, which clearly shows the essential role of both enzymes in avilamycin biosynthesis. The exact functions of both *aviO1* and *aviO3* remained unknown.

Avilamycin A and avilamycin C (Fig. 1), both belonging to the orthosomycin class of antibiotics, are the major compounds produced by *Streptomyces viridochromogenes* Tü57 (6). Avilamycin A is currently used as a growth promoter in animal feed, but the European Community decided to prohibit its further use beginning in January of 2006. Like other orthosomycins, avilamycin A inhibits the growth of multidrug-resistant gram-positive bacteria (29). The orthosomycin evernimicin (also called SCH27899 or ziracin) is structurally very similar to avilamycin A. It has been tested in clinical trials as a potential drug for the treatment of serious infectious diseases (11). Both avilamycin A and evernimicin bind exclusively to the 50S ribosomal subunit (19) to inhibit protein synthesis. Recently we reported the characterization of the avilamycin resistance determinants *aviRa* and *aviRb*, encoding two rRNA methyltransferases that specifically target 23S rRNA (27). Present evidence indicates that the site of action of the orthosomycins is physically distinct from that of other drugs targeting the 50S ribosomal subunit (18) that makes orthosomycins interesting for the pharmaceutical industry. A complete assignment of the ¹H and ¹³C resonances of avilamycin A, which is an important requirement for biosynthetic studies, has been performed recently. Based on these studies, we were able to elucidate the functions of five methyltransferase genes involved in the biosynthesis of avilamycin A by generating mutants of the wild-type strain. One of these mutants was named *S. viridochromogenes* GW4 (28). It was producing avilamycin derivatives, named gavibamycin A1 and A3, without a methoxy group at

the orsellinic acid moiety. Structural features common to members of the orthosomycin class of antibiotics produced by various actinomycetes are a terminal dichloroisovernic acid unit, several deoxysugars, and an eurekanate moiety associated by an unique orthoester linkage (29). The eurekanate moiety belongs to an important class of carbohydrates, the branched-chain sugars, containing a two-carbon side chain at position C4.

The biosynthesis of branched-chain sugars with one carbon side chain is well understood. Several genes encoding *S*-adenosyl-L-methionine-dependent enzymes have been found in different strains known to catalyze the formation of mostly methylated sugars. Much less is known about the biosynthesis of branched-chain sugars with two carbons. In *Yersinia pseudotuberculosis* YerE, a thiamine pyrophosphate-dependent flavoprotein catalyzing the conversion of 3,6-dideoxy-4-keto-D-glucose to 3,6-dideoxy-4-acetyl-D-glucose, has been characterized by Liu and coworkers (8). YerE, a bifunctional protein, converts pyruvate to an acetyl carbanion, which then gets attached to the sugar moiety.

To identify genes involved in the biosynthesis and attachment of the two-carbon side chain of eurekanate, we inactivated four genes of the avilamycin biosynthetic gene cluster. As candidates we first chose *aviB1*, encoding an enzyme resembling the α -chain of several E1 components from different pyruvate dehydrogenase (PDH) multienzyme complexes and *aviO1*, *aviO2*, and *aviO3*, all encoding 2-oxoglutarate-dependent enzymes suggested to be involved in the formation of either C-C or O-C linkages during avilamycin biosynthesis.

Supported by nuclear magnetic resonance (NMR) analysis, the involvement of two gene products (*AviO2* and *AviB1*) in eurekanate biosynthesis was able to be elucidated, while the function of the other two gene products (*AviO1* and *AviO3*) remained unknown.

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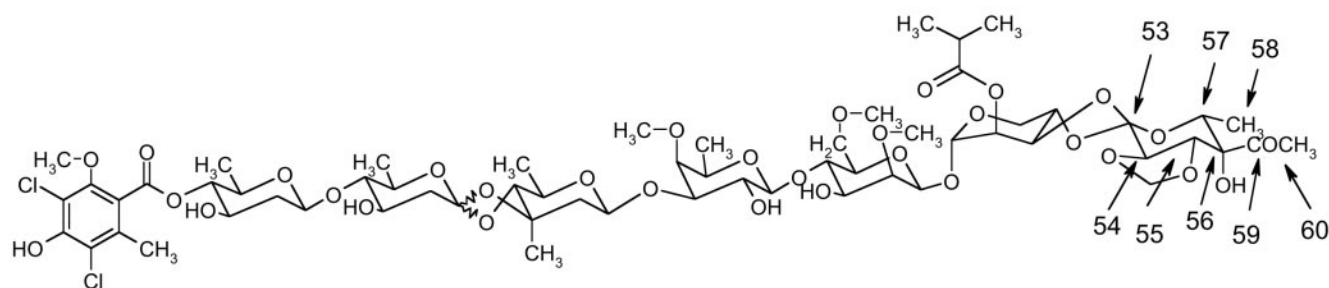


FIG. 1. Structure of avilamycin A and avilamycin C.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *S. viridochromogenes* Tü57 was grown on 1% malt extract, 0.4% yeast extract, 0.4% glucose, and 1 mM CaCl₂, adjusted to pH 7.2 (HA medium) at 37°C. For avilamycin production, *S. viridochromogenes* Tü57 and all mutants were grown at 28°C in production medium containing 2% glucose, 1% soy-peptone, 0.2% CaCO₃, 0.23% L-valine, and CoCl₂ (1 mg/liter) adjusted to pH 7.2 (SG medium). DNA manipulation was carried out using *Escherichia coli* XL1-Blue MRF' (Stratagene) as a host strain. Before transforming *S. viridochromogenes* Tü57, plasmids were propagated in *E. coli*

ET12567 (*dam*⁻ *dcm*⁻ *hsdS* Cm^r) (10) to obtain unmethylated DNA. *E. coli* strains were grown on Luria-Bertani agar or liquid medium containing the appropriate antibiotic. pBluescript SK(-) (pBSK-) was from Stratagene. Plasmid pSP1(23) conferring erythromycin resistance was a gift from S. Pelzer, Combinature Biopharm (Berlin, Germany), and pSET152 (3) conferring apramycin resistance was obtained from Eli Lilly and Co. (Indianapolis, Ind.). Plasmid pKC1218 was kindly provided by José A. Salas. Medium components and other chemicals, including antibiotics, were from Roth. Carbenicillin (50 µg ml⁻¹) and apramycin (100 µg ml⁻¹) were used for selective growth of recombinant strains. The ¹³C-labeled L-methionine and pyruvate for the feeding experiments were from Euriso-Top.

General genetic manipulation, PCR, and sequence analysis. Standard molecular biology procedures were performed as described by Sambrook et al. (24a). Isolation of plasmids was carried out with ion-exchange columns (Nucleobond AX kits; Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Southern hybridization was performed on a Hybond-N nylon membrane (Amersham-Pharmacia, Freiburg, Germany) with a digoxigenin-labeled probe using the digoxigenin high-prime DNA labeling and detection starter kit II (Roche Molecular Biochemicals). Isolation of DNA fragments from agarose gel and purification of PCR products were carried out with the NucleoSpin 2 in 1 Extract kit (Macherey-Nagel). *Streptomyces* protoplast formation, transformation, and protoplast regeneration were performed as described previously (16). PCR was carried out using an Applied Biosystems GeneAmp 9700 thermal cycler and either *Taq* or *Pfu* DNA polymerase (Amersham-Pharmacia) according to the supplier's recommendations. DNA sequencing was carried out by 4baseLab, Rüttingen, Germany. All oligodeoxynucleotides used for PCR in this study are listed in Table 2.

Construction of gene inactivation plasmids. (i) *IkaviO1*. *aviO1* was obtained using a 2.2-kb *SacI* fragment derived from F4E6 (Table 1; Fig. 2). The fragment was ligated into the *SacI* site of pSP1 to generate pSP1-O1/E6S22. A unique *NcoI* restriction site inside the gene *aviO1* was chosen for targeted inactivation by shifting the reading frame. After *NcoI* restriction, treatment with T4 DNA polymerase, and religation, the intended alteration (correct fill-in) was confirmed by DNA sequencing.

(ii) *IkaviO2*. *aviO2* was amplified by PCR using the oligodeoxynucleotides *aviO2F* and *aviO2R* (Table 2). The resulting 1.0-kb PCR fragment was ligated into *EcoRI* and *BamHI* sites of pBSK-, forming *aviO2-SK*-. A unique *NdeI* restriction site inside the gene *aviO2* was chosen for targeted inactivation by shifting the reading frame in a way similar to that described for *aviO1*. The inactivation plasmid was named *IkaviO2*.

(iii) *IkaviO3*. *aviO3* was generated by PCR using the oligodeoxynucleotides *aviO3F* and *aviO3R* (Table 2). The 1.2-kb PCR fragment was ligated into *EcoRI* and *XbaI* sites of pBSK-, forming *aviO3SK*-. A unique *NcoI* restriction site inside the gene *aviO3* was chosen for targeted inactivation by shifting the reading frame in a way similar to that described above. The inactivation plasmid was named *IkaviO3*.

(iv) *IkaviB1*. *aviB1* was obtained using a 2.0-kb *SacI* fragment of plasmid H4S112 from the avilamycin biosynthesis cluster (Fig. 2). The fragment containing *aviB1* was ligated into the *SacI* site of pSP1 to generate *AviB1pSP1*. A unique *NcoI* restriction site inside the gene *aviB1* was chosen for targeted inactivation by shifting the reading frame. The inactivation plasmid was named *IkaviB1*.

Generation of mutants. The inactivation plasmids were introduced into *S. viridochromogenes* Tü57 by protoplast transformation using DNA isolated from *E. coli* ET12567. The integration of the inactivated genes was analyzed by PCR using *Taq* DNA polymerase. Screening for the integration of the defective *aviB1* gene into the chromosome of GW4-ITB1 was carried out using the oligode-

TABLE 1. List of strains and plasmids used in this study

Strain or plasmid	Description and/or reference(s)
Strains	
<i>S. viridochromogenes</i> Tü57Producer of avilamycins; 6
<i>S. viridochromogenes</i> GW4 <i>aviG4</i> -defective mutant, producer of gavigamycins; 27
<i>S. viridochromogenes</i> GW4-ITB1 <i>aviG4</i> - and <i>aviB1</i> -defective mutant
<i>S. viridochromogenes</i> ITO1 <i>aviO1</i> -defective mutant
<i>S. viridochromogenes</i> ITO2 <i>aviO3</i> -defective mutant
<i>S. viridochromogenes</i> ITO3 <i>aviO3</i> -defective mutant
<i>E. coli</i> XL1-Blue MRF'Stratagene
<i>E. coli</i> ET 12567DNA-methylase-negative strains; 9
Plasmids	
pBSK-Stratagene
pSP1Inactivation plasmid; 22
pSET1cermExpression plasmid (integrative); 2, 3
pKC1218Expression plasmid (replicative)
pSP1-O1/E6S222.2-kb <i>SacI</i> fragment containing <i>aviO1</i> ligated into pSP1
AviO2-SK- <i>aviO2</i> ligated into the <i>EcoRI</i> and <i>BamHI</i> sites of pBSK-
AviO3-SK- <i>aviO3</i> ligated into the <i>EcoRI</i> and <i>XbaI</i> sites of pBSK-
AviB1-pSP12.0-kb <i>SacI</i> fragment containing <i>aviB1</i> ligated into pSP1
<i>IkaviB1</i>Defective <i>aviB1</i> gene ligated into pSP1
<i>IkaviO1</i>Defective <i>aviO1</i> gene ligated into pSP1
<i>IkaviO2</i>Defective <i>aviO2</i> gene ligated into pSP1
<i>IkaviO3</i>Defective <i>aviO3</i> gene ligated into pSP1
O1pSET <i>aviO1</i> ligated into pSET1cerm
O1pSET <i>aviO1</i> ligated into pSET1cerm
O1pSET <i>aviO1</i> ligated into pSET1cerm
B1pKC <i>aviB1</i> ligated into pKC1218
H4S1122.0-kb <i>SacI</i> fragment containing <i>aviB1</i> ligated into pBSK-
F4E66.2-kb <i>EcoRI</i> fragment containing <i>aviO1</i> ligated into pBSK-
P2P139.7-kb <i>PstI</i> fragment containing <i>aviO2</i> ligated into pBSK-
H4S1098.1-kb <i>SacI</i> fragment containing <i>aviO3</i> ligated into pBSK-

TABLE 2. List of primers used in this study

Primer	Sequence
Primers used to generate inactivation plasmids	
aviO2F	AAGCACGCCGAATTCTGGCAGGGC
aviO2R	TCCGGGGAGGATCCTATCGTTAC
aviO3F	CCACGAATCCGCGACTACG
aviO3R	CAGGTTCTAGACCGCCAGTAC
S3A	CTGCTGTGGCGCTACTCG
S3B	GAGCTGGCACGGGTAGTC
Primers used to screen for mutants	
O1Fn	CTCAGCGTCGACGTGCGCTGGCTGG
O1Rn	CATCCGAAAGACTCCCTGACCTGCC
O2aF	CTACGGGCGAATTCTTCGACATCG
O2aR	GGCGGCTAGACGGTGAGATTGG
O3Fn	CGCCTCCCTCGCGGTCCCGAATG
O3Rn	CACCACCGCCAGTACTCTCGTGC
Primers used to generate complementation plasmids	
aviO2FK	GACGAGGTCTAGAGTGGCGGGTCC
aviO2RK	GACGCCATGGAATTCGGCTACCG
aviO3KF-Eco	GTCGACGAATTCGGCTACACGCTG
aviO3KR-Xba	GGAGCGCTCGGTCTAGACCTCTG

oxynucleotides S3A and S3B. For analyzing mutant ITO1, the primer pair O1Fn-O1Rn was used to screen for the integration of the defective *aviO1* gene (Table 2). For analyzing mutant ITO2, we used the primer pair O2aF-O2aR. Mutant ITO3 was screened by PCR using the primer pair O3Fn-O3Rn. All oligonucleotides used for generating constructs and for analyzing the mutants are listed in Table 2.

Construction of complementation plasmids. For the generation of the complementation plasmid O1pSET, plasmid pSP1-O1/E6S22 was digested with EcoRI and XbaI, resulting in a 2.2-kb fragment containing *aviO1*. This fragment was ligated into pSET-1cerm (2) previously incubated with MunI and XbaI to generate plasmid O1pSET. For complementation of the *S. viridochromogenes* mutants ITO2 and ITO3, *aviO2* and *aviO3* were amplified by PCR using *Pfu* polymerase and chromosomal DNA of the wild type as a template. The two suitable restriction sites, EcoRI and XbaI, were introduced using the primer pairs aviO2FK-aviO2RK and aviO3KF-Eco-aviO3KR-Xba (Table 2), respectively. PCR fragments were ligated into the MunI and XbaI restriction sites of pSET-1cerm to generate the integration plasmids O2pSET and O3pSET. For the complementation of *S. viridochromogenes* GW4-ITB1, *aviB1* was cloned from H4S112 (Fig. 2) of the avilamycin biosynthesis gene cluster using the restriction sites PvuII and BamHI. It was ligated into the EcoRV and BglII sites of the replicative plasmid pKC1218 (Table 1). The resulting ligation product was called B1pKC and transferred into *S. viridochromogenes* GW4 by protoplast transformation.

Production and analysis of secondary metabolites. *S. viridochromogenes* Tü57, *S. viridochromogenes* GW4, *S. viridochromogenes* ITO1, *S. viridochromogenes* ITO2, *S. viridochromogenes* ITO3, and *S. viridochromogenes* GW4-ITB1 were cultivated as described above. Cultures were filtered, and the filtrate (200 ml) was extracted with an equal volume of ethylacetate. After evaporation of the solvent, the residue was dissolved in 1 ml of 20% methanol in water and applied to a solid-phase extraction column (SepPak C₁₈; Waters). The column was eluted using a 10 to 100% methanol gradient in water. Avilamycin derivatives could be detected in fractions containing 60 to 80% methanol. After evaporation of the solvent, the avilamycin-containing fractions were redissolved in 0.1 ml of methanol, and 2 μ l was analyzed by thin-layer chromatography on silica gel plates (silica gel 60 F₂₅₄; Merck) with methylene chloride-methanol (9:1, vol/vol) as a solvent. Avilamycin derivatives were detected after treatment with anisaldehyde/H₂SO₄.

HPLC-ESI-MS analysis. High-performance liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS) was performed on an Agilent 1100 Series System with an electrospray chamber and a quadrupole detector. HPLC analysis was carried out on a Zorbax SB-C18 column (4.6 by 150 mm; 5- μ m particle size; Agilent). A 20 to 70% nonlinear gradient of acetonitrile in 0.5% acetic acid over 22 min at a flow rate of 0.5 ml/min was used. The column temperature was 23°C, and the UV detection wavelength was 451 nm. The mass selective detector chamber settings were as follows: drying gas flow, 12 liters/min; nebulizing pressure, 50 lb/in²/g; drying gas temperature, 350°C. The samples were analyzed in a negative scan mode with a mass range of 300 to 1,500 Da.

NMR analysis. The following experiments were performed with dimethyl sulfoxide-d₆ at 295 K on a Bruker DMX 750-MHz spectrometer: ¹H-1D, ¹³C-1D, correlated spectroscopy (COSY) (24), total correlation spectroscopy (1, 5), nuclear Overhauser effect spectroscopy (15), heteronuclear single-quantum coherence (HSQC) (4), heteronuclear multiple-bond correlation (26), heteronuclear multiquantum coherence-COSY (21), HSQC-total correlation spectroscopy (7), and ¹³C-filtered nuclear Overhauser effect spectroscopy (mixing time, 200 ms) (22). The spectrometer is part of the Bavarian NMR Center in Garching, Germany. All spectra were assigned using the program SPARKY 3 (13).

Bioassay. Antibacterial activities of the new avilamycin A derivatives were tested using *Bacillus subtilis* COHN ATCC6051 from the American Type Culture Collection (20a).

Computer-assisted sequence analysis. The DNASIS software package (Hitachi Software Engineering, San Bruno, Calif.) and the BLAST program were used for sequence analysis and homology searches of the GenBank database, respectively.

RESULTS

Generation of gene replacement mutants. To inactivate *aviB1*, *aviO1*, *aviO2*, and *aviO3*, protoplasts of *S. viridochromogenes* GW4 (*aviB1*) and *S. viridochromogenes* Tü57 (*aviO1*, *aviO2*, and *aviO3*) were transformed using the inactivation plasmids *IkaviB1*, *IkaviO1*, *IkaviO2*, and *IkaviO3*. Several erythromycin-resistant colonies were obtained in each case.

After screening for loss of erythromycin resistance, double-crossover mutants were obtained. PCR fragments obtained from these mutants using primers listed in Table 2 could not be cleaved by NcoI (*aviB1*, *aviO1*, and *aviO3*) and NdeI (*aviO2*),

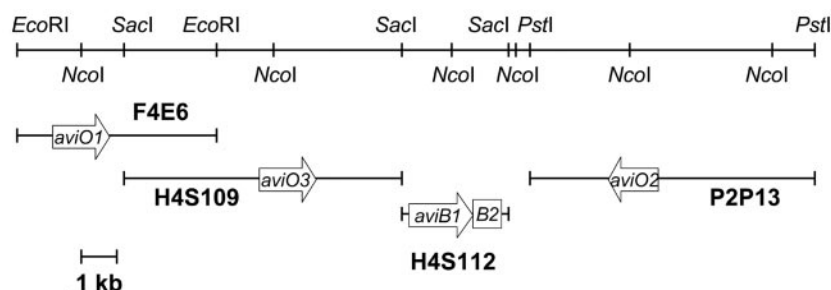


FIG. 2. Region of the avilamycin biosynthesis gene cluster containing *aviO1*, *aviO2*, *aviO3*, *aviB1*, and *aviB2*. Only genes investigated during this study are shown as arrows. Fragments containing each individual gene as well as important restriction sites are indicated.

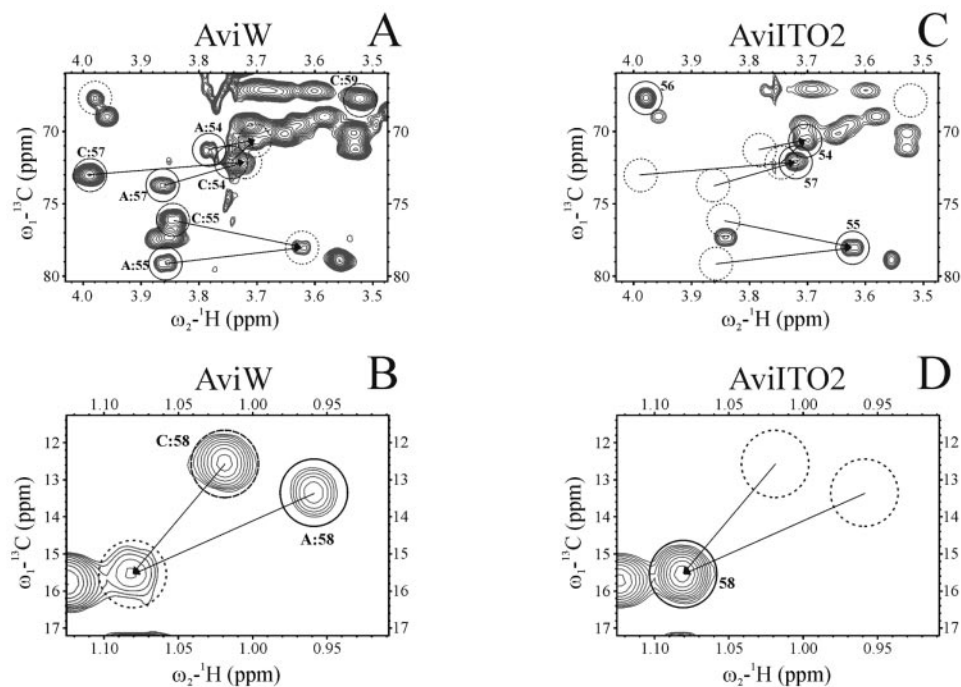


FIG. 3. Sections of the HSQC spectra of extracts of *S. viridochromogenes* Tü57 (AviW) (A and B) and *S. viridochromogenes* ITO2 (AviITO2) (C and D). Interesting peaks are named and encircled. A: and C: indicate signals of avilamycin A and C response. Solid circles: avilamycin A (A and B), avilamycin C (A and B); dashed circles: nonacetylated avilamycins (C and D), position of corresponding peaks in Fig. 4 A and B (C and D).

whereas the PCR fragments obtained from *S. viridochromogenes* GW4 (*aviB1*) and *S. viridochromogenes* Tü57 (*aviO1*, *aviO2*, and *aviO3*) were clearly cut by the enzymes in each case.

aviB1. The frameshift mutation within *aviB1* was confirmed by Southern hybridization. As expected, a 1.4- and a 4.6-kb fragment of NcoI-cleaved chromosomal DNA of *S. viridochromogenes* GW4 were hybridizing with a 2-kb gene probe, while a 6-kb fragment was hybridizing with chromosomal DNA of mutant *S. viridochromogenes* GW4-ITB1 (Fig. 2).

aviO1. The frameshift mutation within *aviO1* was also confirmed by Southern blot analysis. When DNA from the wild-type *S. viridochromogenes* Tü57 cleaved by both NcoI and EcoRI was hybridized with a 2.2-kb probe, two fragments (3.8 and 2.3 kb) were detected. As expected, mutant *S. viridochromogenes* ITO1 gave only one signal at 6.1 kb (Fig. 2).

aviO2. The complete digestion of chromosomal DNA by NdeI is not a reliable process. Therefore, the mutant *S. viridochromogenes* ITO2 was confirmed not by Southern hybridization but by amplifying the disrupted *aviO2* gene by use of PCR with the primers O2aF and O2aR. The resulting PCR fragments were cloned into pBluescript SK-. Twenty-five recombinant plasmids were characterized by restriction analysis, and none of these plasmids could be restricted by NdeI. In addition, five plasmids were sequenced, and in all cases the defect within *aviO2* was able to be confirmed (Fig. 2)

aviO3. When NcoI-treated chromosomal DNA of the *S. viridochromogenes* ITO3 mutant was probed against a 1.2-kb fragment, a 10-kb signal was obtained. In contrast to this, two fragments of 5.4 and 4.6 kb were obtained using DNA of the wild-type strain (Fig. 2).

Complementation of *S. viridochromogenes* Tü57 mutants ITO1, ITO2, and ITO3 and *S. viridochromogenes* GW4-ITB1.

To determine clearly that the mutation events in the gene replacement mutants affected only the desired genes, *aviO1*, *aviO2*, *aviO3*, and *aviB1* were ligated behind the *ermE* promoter, cloned into either the integration plasmid pSET152 (*aviO1*, *aviO2*, and *aviO3*) or pKC1218 (*aviB1*), and introduced by protoplast transformation into the corresponding mutants. Avilamycin A and C production (*aviO1*, *aviO2*, and *aviO3*) and gavibamycin A1 and A3 production (*aviB1*) were restored as confirmed by HPLC-ESI-MS. Thus, we were able to rule out any upstream or downstream effects.

Identification of new avilamycin derivatives produced by the defective mutants by HPLC-MS. *S. viridochromogenes* Tü57, *S. viridochromogenes* GW4, and the four mutants *S. viridochromogenes* GW4-ITB1, *S. viridochromogenes* ITO1, *S. viridochromogenes* ITO2, and *S. viridochromogenes* ITO3 were grown under the conditions described in Materials and Methods. Extracts resulting from culture supernatants were analyzed by thin-layer chromatography, HPLC with UV detection, and HPLC-ESI-MS. As main products, all measured in the negative ion modus ($[M]^-$), avilamycin C ($[M]^- = 1,403$) was detected in extracts of *S. viridochromogenes* Tü57, and gavibamycin A3 ($[M]^- = 1,389$) was detected in extracts of *S. viridochromogenes* Tü57-GW4. No avilamycin derivative could be detected in the *S. viridochromogenes* mutants ITO1 and ITO3. Avilamycin derivatives could be detected in extracts of the *S. viridochromogenes* mutants GW4-ITB1 and ITO2. The mass of the main product of *S. viridochromogenes* ITO2 was an $[M]^-$ value of 1,359, while the mass of the main product produced by

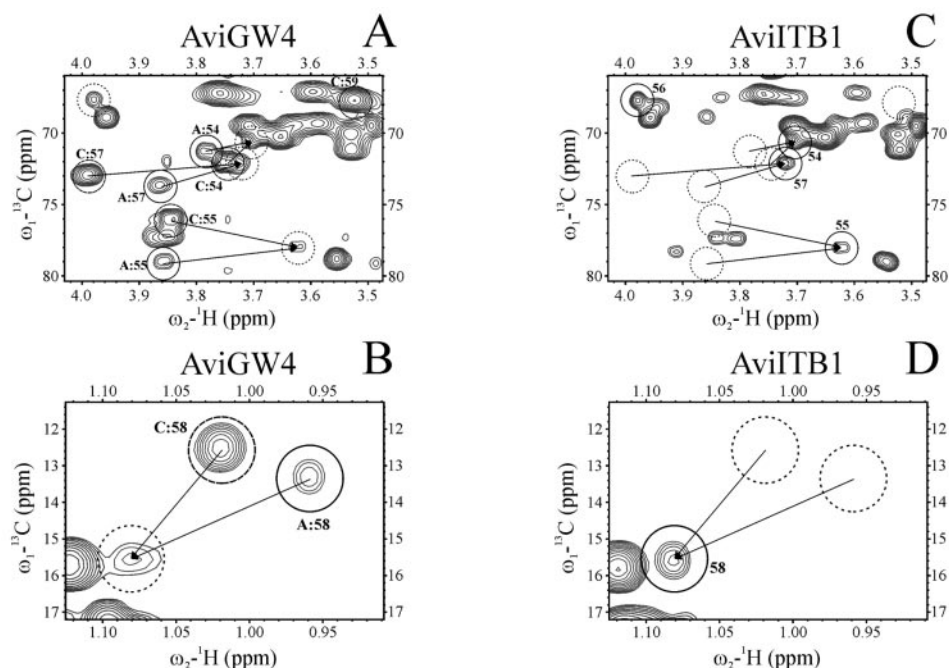


FIG. 4. Sections of the HSQC spectra of *S. viridochromogenes* GW4 (AviGW4) (A and B) and *S. viridochromogenes* ITB1 (AviITB1) (C and D). Interesting peaks are named and encircled. A: and C: indicate signals of GW4 derivative of avilamycin A and C response. Solid circles: GW4 derivative of avilamycin A (A and B), AviITB1 (C and D); dashed circles: GW4 derivative of avilamycin C (A and B); dotted circles: nonacetylated GW4 derivative of avilamycin (C and D), position of corresponding peaks in Fig. 5 top and bottom (C and D).

S. viridochromogenes GW4-ITB1 was an $[M]^-$ value of 1,345. These results are in accordance with avilamycin derivatives lacking the acetyl residue at position C4 of the eurekanate moiety. The mass difference of 14 Da can be explained by the fact that *S. viridochromogenes* ITO2 was obtained from the wild-type strain and *S. viridochromogenes* GW4-ITB1 was obtained from *S. viridochromogenes* GW4 already lacking a methyl group at the dichloroisoverminic acid moiety.

Identification by NMR of avilamycin derivatives produced by the defective mutants. NMR analysis was now performed to demonstrate clearly that both *S. viridochromogenes* mutants ITO2 and GW4-ITB1 are lacking the acetyl residue at position C4 of the eurekanate moiety (Fig. 3 and 4). The HSQC spectra of the major compounds produced by *S. viridochromogenes* Tü57 (AviW) and *S. viridochromogenes* GW4 (AviGW4) show the signals of 54, 55, 57, and 59 (Fig. 3A and Fig. 4A) and 58 and 60 (Fig. 3B and Fig. 4B), corresponding to avilamycin A and C (AviW) and gavibamycin A1 and A3 (AviGW4). These two sets of signals are united in the spectra of AviITO2 (Fig. 3C and D) and AviITB1 (Fig. 4C and D). (The parts of the HSQC spectra with the signals of 56 and 60 have been omitted [for space considerations], but the corresponding results are shown.)

DISCUSSION

AviB1 and AviB2, components of an incomplete PDH complex. AviB1 shows convincing sequence similarities to thiamine PP_i (TPP)-dependent enzymes. Enzymes that use TPP as a cofactor are found in all organisms, where they catalyze reactions involving the formation or cleavage of a carbon-carbon bond adjacent to an oxo group. The biologically active form of

vitamin B1 is an essential cofactor in biocatalysis and is involved in numerous metabolic pathways, such as the oxidative and nonoxidative decarboxylation of α -keto acids (pyruvate decarboxylase, PDH), the formation of amino acid precursors (acetoxyhydroxyacid synthase), electron transfer reactions (pyruvate oxidase), and ketol transfer between sugars (transketolase) (25). In all thiamine-dependent enzymes, there is a common sequence motif of about 30 residues that generates a common structural motif involved in the binding of the cofactor (14). Within the amino acid sequence of AviB1, we found the highly conserved TPP fingerprint binding region GDG and the highly conserved sequence EXXXXAXXXXXPXXX NNKY. This motif is found in alpha chains of E1 components from PDH multienzyme complexes of several bacteria. In the avilamycin biosynthetic gene cluster, we also found *aviB2*. The deduced amino acid sequence of *aviB2* resembles beta chains of E1 components of PDH complexes. Depending on the organism, the E1 component is an $E1\alpha_2$ homodimer typical for gram-negative bacteria or an $E1\alpha_2\beta_2$ heterotetramer common in gram-positive bacteria. It was recently reported that the $E1\beta$ subunit of the heterotetrameric type of E1 components is not functional in the absence of its α -subunit counterpart since it cannot bind cofactors or assemble into the appropriate quaternary structure (9, 17). Multiple sequence alignments of heterotetrameric E1 components indicated a common site that appeared to be conserved in $E1\alpha$ subunits (12). This region lies about 50 amino acids downstream of the TPP binding motif. When analyzing the amino acid sequence of AviB1, we found this consensus sequence, indicating that AviB1 and AviB2 form a heterotetrameric E1 ($\alpha_2\beta_2$) complex.

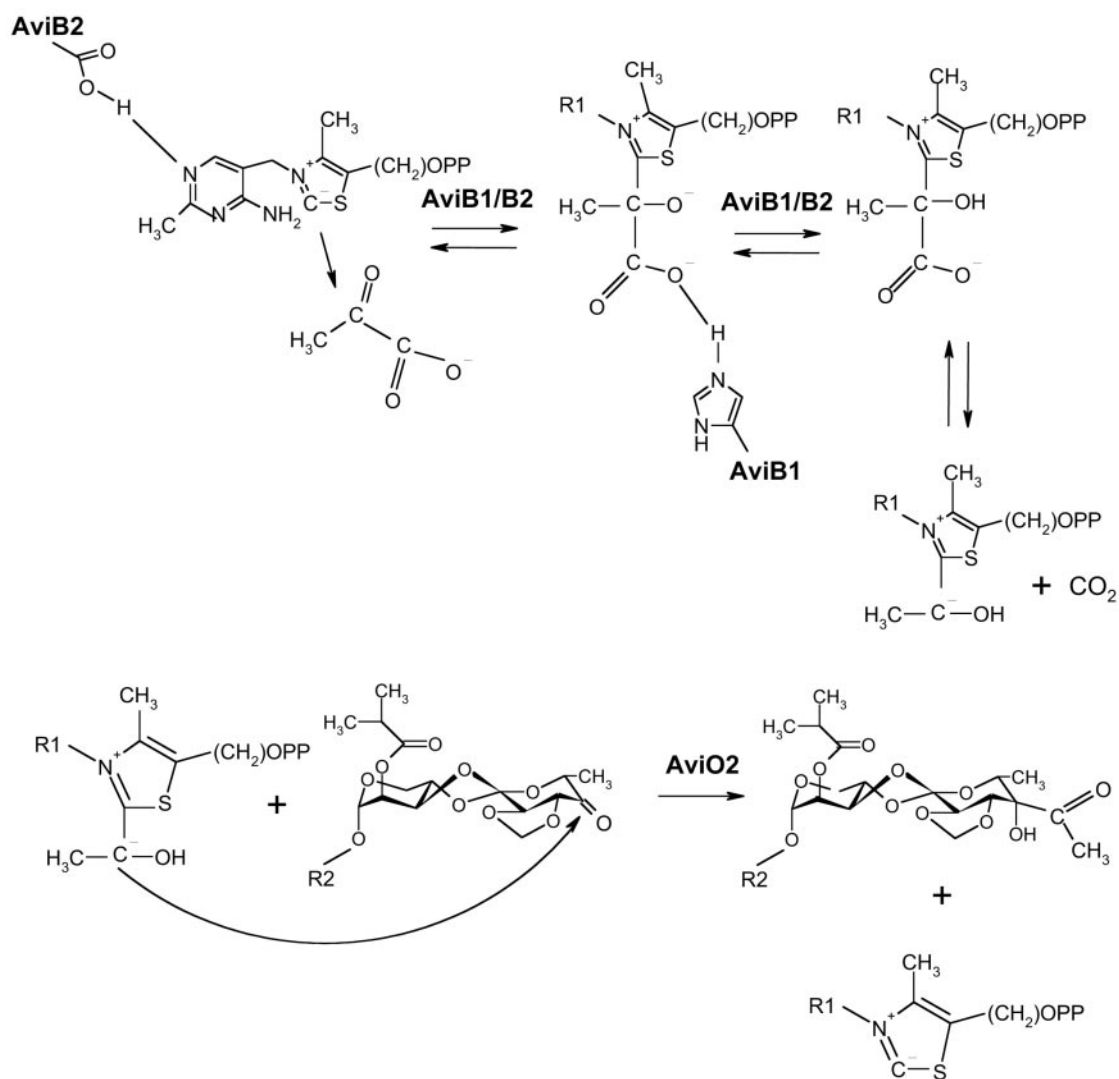


FIG. 5. Hypothetical mechanism of the enzymatic activation and attachment of a two-carbon branch to the eurekanate moiety. (Top) AviB1 and AviB2 are involved in the formation of an acetyl carbanion which is bound to thiamine diphosphate. (Bottom) AviO2 is involved in the attachment of the acetyl carbanion to form the branched-chain sugar moiety of avilamycin A.

AviO1, AviO2, and AviO3 are similar to α -ketoglutaric acid-dependent enzymes. AviO1, AviO2, and AviO3 show some degree of similarity to α -ketoglutaric acid-dependent, non-heme iron-requiring enzymes. These enzymes contain two conserved histidine-signature sequences determined as His-1 and His-2 motifs (20). These motifs, named the 2-His-1-carboxylate facial triad, are thought to play a crucial role in accommodating a variety of catalytic reactions as desaturative cyclization or oxidative ring expansion.

Characterization of compounds produced by mutants ITO2 and GW4-ITB1. The inactivation of the avilamycin biosynthetic genes *aviO2* and *aviB1* resulted in the production of novel avilamycin derivatives. Based on MS data, we could conclude that the acetyl group at the eurekanate moiety (ring H) was missing in extracts of both mutants. Based on our complete assignment of wild-type avilamycin A, the signals of the interesting atoms in ring H of the samples AviW and GW4 could be assigned. Starting from the characteristic signals of methylene

group 61 in the spectra of AviITO2 and AviITB1, unambiguous nuclear Overhauser effect contacts to adjacent hydrogens 54 and 55 were found. Based on these assignments, the COSY spectra showed a yet-unassigned five-spin system that could be identified as C54H-C55H-C56H-C57H-C58H₃. This spin system was already visible in the spectra of AviW and GW4 as an impurity not yet assigned. Therefore, the two reference samples contained both the normal and nonacetylated compounds. As indicated by our NMR results, *S. viridochromogenes* mutants ITO2 and ITB1 produce only nonacetylated avilamycin derivatives. The presence of nonacetylated derivatives in the reference samples leads to the assumption that in these cases the biosynthesis was aborted, and the molecules therefore were not completed. Additionally, sample AviW contains both avilamycin A and C, whereas sample AviGW4 contains the corresponding derivatives lacking the methyl group at the orsellinic acid moiety. As the difference between avilamycin A and C (or between the corresponding derivatives) is located in the inter-

esting residue connected to C56, the signals of the atoms of ring H show two sets of different chemical shifts, one with the normal resonance frequencies for avilamycin A and another with slightly perturbed frequencies and an additional proton signal. These belong to avilamycin C, and the new signal is that of the hydrogen at C59. The two sets of signals collapse into one for AviTO2 and AviTB1, since they both lack the residue attached to C56.

Function of AviB1/AbiB2 and AviO2. The *aviB1* mutant accumulates avilamycin derivatives lacking the acetyl group at position C4 of the eurenkate. In view of this result as well as sequence homology, the *aviB1*- and *aviB2*-encoded proteins must catalyze the conversion of pyruvate to an acetyl carbanion. The disruption of *aviO2* also led to a mutant producing nonacetylated avilamycin derivatives. Based on this result, we speculate that AviO2 attaches the acetyl carbanion to the sugar moiety (Fig. 5). However, at present, we cannot exclude the possibility that AviO2 is involved in the oxidation of the 4-OH group to initiate the condensation step catalyzed by AviB1/AviB2. Surprisingly, derivatives produced by both the *aviB1* and the *aviO2* mutants lack the keto group at position C4 and contain a hydroxyl group instead. From the mechanistic point of view, the 4-keto group is required for the attachment of the carbanion. We believe that the 4-keto derivatives become reduced after the condensation step by a so-far-unrecognized, possibly unspecific ketoreductase. The functions of neither AviO1 nor AviO3 could be determined within this study. Inactivation of *aviO1* or *aviO3* resulted in a complete breakdown of avilamycin biosynthesis, and extracts of both mutants did not show any antibiotic activity, indicating an essential function of both genes for avilamycin A biosynthesis or its regulation.

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