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Research Paper

Biosynthesis of the orthosomycin antibiotic avilamycin A: deductions from the molecular analysis of the *avi* biosynthetic gene cluster of *Streptomyces viridochromogenes* Tü57 and production of new antibiotics

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Abstract

Background: *Streptomyces viridochromogenes* Tü57 is the producer of avilamycin A. The antibiotic consists of a heptasaccharide side chain and a polyketide-derived dichloroisoeverninic acid as aglycone. Molecular cloning and characterization of the genes governing the avilamycin A biosynthesis is of major interest as this information might set the direction for the development of new antimicrobial agents.

Results: A 60-kb section of the *S. viridochromogenes* Tü57 chromosome containing genes involved in avilamycin biosynthesis was sequenced. Analysis of the DNA sequence revealed 54 open reading frames. Based on the putative function of the gene products a model for avilamycin biosynthesis is proposed. Inactivation of *aviG4* and *aviH*, encoding a methyltransferase and a halogenase, respectively, prevented the mutant strains from producing the complete dichloroisoeverninic acid moiety resulting in the accumulation of new antibiotics named gavibamycins.

Conclusions: The avilamycin A biosynthetic gene cluster represents an interesting system to study the formation and attachment of unusual deoxysugars. Several enzymes putatively responsible for specific steps of this pathway could be assigned. Two genes encoding enzymes involved in post-PKS tailoring reactions were deleted allowing the production of new analogues of avilamycin A.

Keywords: Avilamycin; Gavibamycin; Orthosomycin; Methyltransferase; Halogenase

1. Introduction

The emergence of pathogenic bacteria, which are resistant to multiple antibiotics, represents a growing threat to human health and has given additional impetus to the search for new drugs [1]. Fewer and fewer new drugs have been found in target screening programs during the past two decades, and scientists have started to look for new technologies to generate new compounds. One new promising technology is named combinatorial biosynthesis, which uses biosynthetic genes as tools to develop new drugs [2]. The orthosomycins are a prominent class of antibiotics produced by various actinomycetes [3]. Members of this class are active against a broad range of Gram-positive pathogenic bacteria including glycopeptide-resistant enterococci, methicillin-resistant staphylococci and penicillin-resistant streptococci [4–6]. Prominent examples of orthosomycins are the avilamycins [7] (Fig. 1) and the everninomicins [8] produced by *Streptomyces viridochromogenes* Tü57 and *Micromonospora carbonacea*, respectively. Structural features common to these antibiotics

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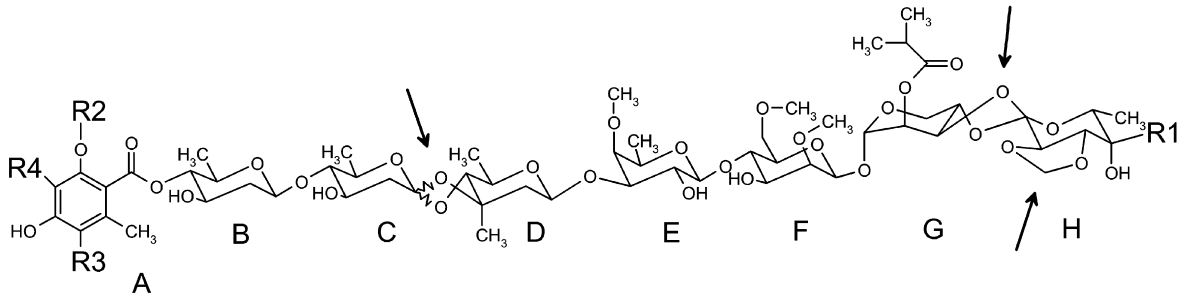
Table 1
Deduced functions of the ORFs in Fig. 2

Polypeptide (gene)	Sequence similarity	Proposed function	Accession number	Reference
AviX1	GTP-binding protein from *M. tuberculosis*	Unknown	AL021897	[40]
AviX2	Protein of unknown function from *S. coelicolor* A3(2)	Unknown	Y17736	[14]
AviX3	Protein of unknown function from *S. coelicolor* A3(2)	Unknown	Y17736	[14]
AviX4	–	Unknown		
AviX5	–	Unknown		
AviRb	rRNA-methyltransferase from *Chlamydia trachomatis*	Resistance	AF317790	[10]
AviX6	–	Unknown		
AviX7	–	Unknown		
AviX8	–	Unknown		
AviRa	–	Resistance		[10]
AviQ1	UDP-glucose 4-epimerase from *T. maritima*	Sugar biosynthesis	AE001727	[17]
AviGT2	Glycosyltransferase (Cps2J) from *S. suis*	Biosynthesis of the heptasaccharide chain	AF118389	[28]
AviX9	Hypothetical protein (SCF55.28c) from *S. coelicolor* A(3)2	Unknown	AL132991	[14]
AviC1	Putative two-component response regulator from *S. aureus*	Regulation	AB035448	[37]
AviC2	Putative response regulator from *S. noursei*	Regulation	AF263912	[38]
AviX10	–	Unknown		
AviX11	–	Unknown		
AviG1	TylCIII from *S. fradiae*	Sugar-biosynthesis (2-deoxy-D-evalose)	AF147704	[25]
AviJ	Putative Na^+/H^+ antiporter from *Amycolatopsis orientalis*	Antibiotic transport	D90902	[36]
AviN	DpsC from *Streptomyces peucetius* ATCC29050	Orsellinic acid biosynthesis	L35560	[9]
AviM	6-methylsalicylic acid synthase from *Penicillium patulum*	Orsellinic acid biosynthesis	X55776	[9]
AviD	dTDP-glucose synthetase from *S. griseus*	Sugar biosynthesis (D-olivose, 2-deoxy-D-evalose)	A26984	[9]
AviE1	dTDP-glucose 4,6-dehydratase from *S. spectabilis*	Sugar biosynthesis (D-olivose, 2-deoxy-D-evalose)	AF128272	[9]
AviQ2	UDP-glucose 4-epimerase from *T. maritima*	Sugar biosynthesis	AE001727	[17]
AviG5	Putative methyltransferase from *Aeropyrum pernix* K1	Modification (methylation)	AP000064	[52]
AviO1	L-proline 4-hydroxylase from *Dactylosporangium* sp.	C–O–C formation	D78338	[32]
AviGT1	Glycosyltransferase from *S. nogalater*	Biosynthesis of the heptasaccharide chain	AF077869	[24]
AviE2	dTDP-glucose 4,6-dehydratase from *S. coelicolor* A(3)2	Sugar biosynthesis	AL034492	[14]
AviG2	Putative methyltransferase from *D. radiodurans*	Modification (methylation)	AE001866	[29]
AviZ1	Putative oxidoreductase from *P. aeruginosa* PA01	Sugar biosynthesis	AE004542	[16]
AviG6	L-isoaspartate *O*-methyltransferase from *A. pernix* K1	Modification (methylation)	AP000064	[52]
AviO3	SnoK from *S. nogalater*	C–O–C formation	AF187532	[34]
AviG3	Protein of unknown function from *Rhodococcus erythropolis*	Modification (methylation)	U17129	[53]
AviX12	EryCV from *S. erythraea*	Unknown	U77459, Y11199	[38,39]
AviABC1	DrrA from *S. peucetius*	Antibiotic transport	M73758	[9]
AviABC2	DrrB from *S. peucetius*	Antibiotic transport	M73758	[9]
AviB1	Pyruvate dehydrogenase (α chain) from *C. magnum*	Modification	L31844	[27]
AviB2	Pyruvate dehydrogenase (β chain) from *C. magnum*	Modification	L31844	[27]
AviGT3	Putative rhamnosyltransferase from *Pyrococcus abyssi*	Biosynthesis of the heptasaccharide chain	AJ248286	[29]
AviGT4	Putative mannosyltransferase (WbaZ-2) from *A. fulgidus*	Biosynthesis of the heptasaccharide chain	AE001063	[31]
AviO2	L-proline 4-hydroxylase from *Dactylosporangium* sp.	C–O–C formation	D78338	[32]
AviP	*p*-nitrophenyl phosphatase from *Halobacterium* sp. NRC-1	Sugar biosynthesis (L-lyxose)	AE005027	[26]
AviQ3	SpcI from *S. flavopersicus*	Sugar biosynthesis	Z95436	[18]
AviH	PltA from *P. fluorescens* Pf-5	Modification (halogenation)	AF081920	[12]
AviX13	–	Unknown		
AviG4	*O*-Demethylpuromycin *O*-methyltransferase from *S. alboniger*	Modification (methylation)	M74560	[11]
AviE3	dGDP-mannose-4,6-dehydratase from *S. noursei* ATCC 11455	Sugar biosynthesis (4-*O*-methyl-D-fucose)	AF317224	[15]
AviS	Putative 2,3-dehydratase from *S. argillaceus*	Sugar biosynthesis (D-olivose, 2-deoxy-D-evalose)	AJ007932	[20]
AviT	Putative 3-ketoreductase from *S. argillaceus*	Sugar biosynthesis (D-olivose, 2-deoxy-D-evalose)	AJ007932	[20]
AviZ3	Putative 4-ketoreductase (SnoG) from *S. nogalater*	Sugar biosynthesis (D-olivose, 2-deoxy-D-evalose)	AJ224512	[24]
AviZ2	Putative oxidoreductase from *S. lividans*	Sugar biosynthesis	Y18817	[14]
AviX14	Putative helicase from *S. coelicolor* A(3)2	Unknown	AL132824	[14]
AviX15	Putative DNA-binding protein from *S. coelicolor* A(3)2	Unknown	AL035212	[14]
AviX16	Alkaline serine protease from the marine bacterium *Alteromonas* sp. strain O-7	Unknown	AL359949	[42]



Name of the compound	R1	R2	R3	R4	gene(s) inactivated
Avilamycin A	$COCH_3$	CH_3	Cl	Cl	-
Avilamycin C	$CH(OH)CH_3$	CH_3	Cl	Cl	-
Gavibamycin A1	$COCH_3$	H	Cl	Cl	*aviG4*
Gavibamycin A3	$CH(OH)CH_3$	H	Cl	Cl	*aviG4*
Gavibamycin B1	$COCH_3$	H	H	H	*aviG4+aviH*
Gavibamycin B3	$CH(OH)CH_3$	H	H	H	*aviG4+aviH*

Fig. 1. Structure of avilamycin A, avilamycin C, gavibamycin A1, gavibamycin A3, gavibamycin B1 and gavibamycin B3. (A) Dichloroisoeverninic acid, (B, C) D-olivoses, (D) 2-deoxy-D-evalose, (E) 4-*O*-methyl-D-fucose, (F) 2,6-di-*O*-methyl-D-mannose, (G) 2-*O*-isobutyryl-L-lyxose, (H) methyleurekanate. Orthoester linkages and one methylene bridge are marked by arrows.

are a terminal dichloroisoeverninic acid unit (residue A in Fig. 1), D-olivose moieties (residues B and C), a 2-deoxy-D-evalose moiety (residue D), a D-fucose moiety (residue E), a D-mannose moiety (residue F), a L-lyxose moiety (residue G) and a methyleurekanate moiety (residue H) [3]. These residues are partly associated by orthoester linkages. Early genetic work on the avilamycin biosynthetic gene cluster revealed the presence of a NDP-glucose synthase gene (*aviD*), a NDP-glucose 4,6-dehydratase gene (*aviE1*) and a polyketide synthase gene (*aviM*). The deduced amino acid sequence of *aviM* showed that this polyketide synthase is an unusual multifunctional protein belonging to iterative type I polyketide synthases. Expression of *aviM* in *Streptomyces lividans* led to the formation of orsellinic acid, an intermediate in the biosynthesis of dichloroisoeverninic acid [9]. Recently, we reported the cloning and characterization of two rRNA-methyltransferase genes (*aviRa* and *aviRb*) and the ABC transporter genes *aviABC1* and *aviABC2* which confer avilamycin resistance when expressed in *S. lividans* [10]. The sequence and the deduced functional analysis of the complete biosynthetic cluster as well as the characterization of two genes *aviG4* and *aviH* is the subject of the present report.

2. Results

2.1. Cloning and sequencing of approximately 60 kb of the avilamycin cluster

Earlier work demonstrated that the NDP-glucose 4,6-dehydratase gene *aviE1* and the orsellinic acid synthase gene *aviM* are involved in the biosynthesis of avilamycin A [9]. Given the precedent for the clustering of related biosynthetic genes in actinomycetes we proceeded to isolate and sequence DNA flanking the *aviE1* and *aviM* genes in order to identify the entire avilamycin biosynthetic gene cluster. A 17.6-kb section of the avilamycin biosynthetic gene cluster located upstream of *aviM* and a 35.9-kb section located downstream of *aviE1* were sequenced. Sequenced genes and the actual or putative function of their gene products are listed in Table 1. Fig. 2 shows the genetic organization of the avilamycin biosynthetic gene cluster. The boundaries of the *avi* gene cluster have not been determined by experiments, but it is reasonable to propose that the cluster is flanked by an avilamycin resistance gene (*aviRb*) and a putative deoxysugar biosynthetic gene (*aviZ2*).

2.2. Genes putatively involved in dichloroisoeverninic acid biosynthesis

AviM is responsible for orsellinic acid formation during avilamycin biosynthesis. *AviN*, located upstream of *aviM*, might encode an enzyme controlling the starter unit for orsellinic acid synthesis [9]. As the biosynthesis of dichloroisoeverninic acid (residue A in Fig. 1) from orsellinic acid requires methylation and di-halogenation, it was reasonable to suggest that AviG4, resembling DmpM, an *O*-demethylpuromycin *O*-methyltransferase from *Streptomyces alboniger* [11] (44% identical aa) and AviH, which is similar to PltA, a halogenase from *Pseudomonas fluorescens* Pf-5 involved in pyoluteorin biosynthesis [12] (39% identical aa), are responsible for modifying orsellinic acid.

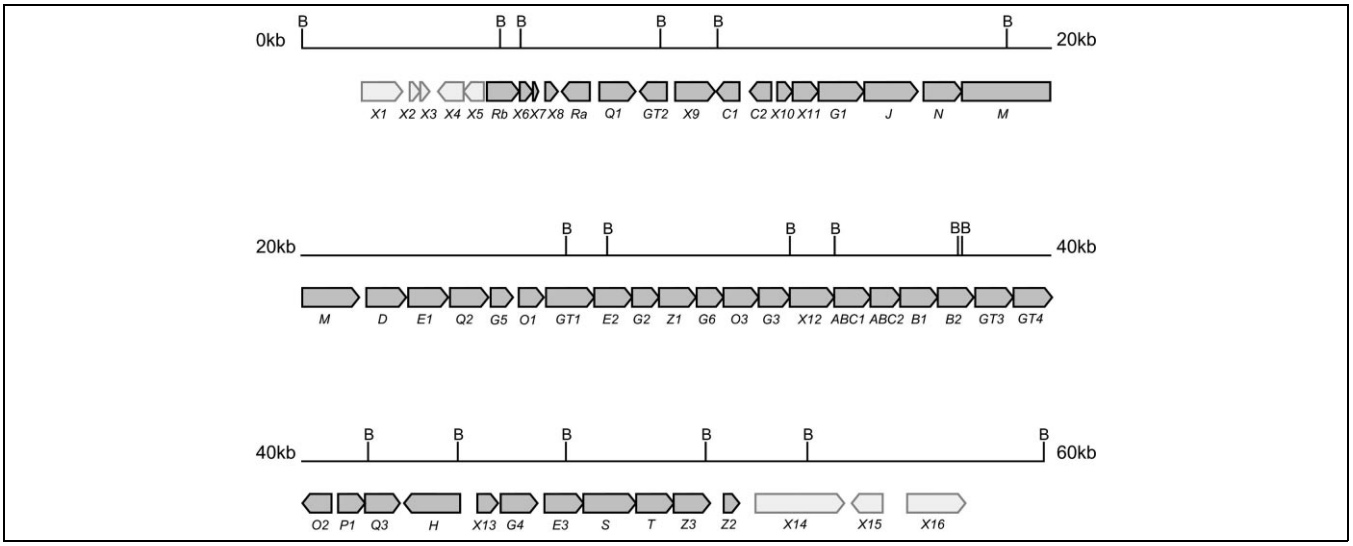


Fig. 2. Genetic organization of the avilamycin biosynthetic gene cluster. Genes, belonging to the cluster, are indicated as dark arrows orientated in the direction of transcription. Genes, probably located outside the cluster, are shown as light arrows (B, *Bam*HI).

2.3. Putative deoxysugar biosynthetic genes

Surprisingly, the avilamycin biosynthetic gene cluster contains six genes (*aviE1*, *aviE2*, *aviE3*, *aviQ1*, *aviQ2*, *aviQ3*) that appear to encode either putative NDP-hexose 4,6-dehydratases or putative NDP-hexose epimerases. The gene product of *aviE1* is most similar to a dTDP-glucose 4,6-dehydratase from *Streptomyces spectabilis* [13] (59% identical aa), and AviE2 is resembling a dNDP-glucose 4,6-dehydratase from *Streptomyces coelicolor* [14] (54% identical aa). AviE3 shares 61% identical aa with NysDIII from *Streptomyces noursei* [15] and 51% identical aa with a dGDP-mannose 4,6-dehydratase from *Pseudomonas aeruginosa* [16]. The proteins encoded by *aviQ1* and *aviQ2* are most similar to an UDP-glucose 4-epimerase from *Thermotoga maritima* [17] (AviQ1: 43% identical aa; AviQ2: 38% identical aa), and AviQ3 resembles a putative epimerase (SpcI) from *Streptomyces flavopersicus* [18] (44% identical aa). In the case of AviE2, AviQ1, AviQ2, and AviQ3 the exact function is difficult to predict. The gene product of *aviE1* shows the highest degree of similarity to NDP-hexose 4,6-dehydratases known to be involved in the formation of 6-deoxysugars as components of antibiotics. Therefore we expect that AviE1, together with AviD, which is 62% identical to a NDP-glucose synthetase from *Streptomyces griseus* [19], is involved in the early steps of D-olivose (residue B and residue C in Fig. 1) and 2-deoxy-D-evalose biosynthesis (residue D in Fig. 1). The product of the *aviS* reading frame resembles proteins identified as NDP-hexose 2,3-dehydratases in various species. Highest homology is found to a putative NDP-hexose 2,3-dehydratase from *Streptomyces argillaceus* [20] (53% identical aa). Downstream of *aviS* we found *aviT*. Homology of the deduced 337 amino acids gene product suggests that *aviT* encodes an oxidoreductase, since significant similarity is found to the product of *lanT*, an oxidoreductase homologue of *Streptomyces cyanogenus* (52% identical aa) [21], to Gra-orf26 of *Streptomyces violaceoruber* (50% identical aa) [22], and to an oxidoreductase from *Amycolatopsis mediterranei* (49% identical aa) [23]. Database comparison of AviZ3 revealed close similarity to proteins of different origin which act as (or presumably are) NDP-hexose 4-ketoreductases. Highest homology is found to SnoG, a NDP-hexose 4-ketoreductase homologue from *Streptomyces nogalater* [24] (44% identical aa). AviS, AviT and AviZ3 are most likely to be involved in the conversion of NDP-4-keto-6-deoxy-D-glucose into NDP-D-olivose. 2-Deoxy-D-evalose differs from D-olivose in a methyl group at position C3. We assume that NDP-4-keto-2,6-dideoxy-D-glucose is a key intermediate in the biosynthesis of this methylated deoxysugar. Methylation by AviG1, mostly resembling TylCIII, a 3-C-methyltransferase from *Streptomyces fradiae* [25] (54% identical aa), and ketoreduction by either AviZ1 or AviZ2, which both resemble putative oxidoreductases (AviZ1 is most similar to a putative oxidoreductase from *P. aeruginosa* (38% identical aa) [16] and AviZ2 resembles a putative oxidoreductase from *S. coelicolor* A3(2) (70% identical aa) [14]), are completing the biosynthesis. GDP-D-mannose (residue F in Fig. 1) most probably derives from L-fructose-6-phosphate. Genes encoding enzymes involved in the conversion of L-fructose-6-phosphate to GDP-D-mannose are not located within the avilamycin cluster, and this has also been reported for the biosynthesis of the nystatin deoxysugar moiety, mycosamine [15]. AviE3 might convert GDP-mannose to GDP-4-keto-6-deoxy-D-mannose as the first intermediate of GDP-D-fucose biosynthesis. Further conversion to GDP-D-fucose might then be catalyzed by AviZ1 or AviZ2 (4-ketoreduction) and AviQ1, AviQ2 or AviQ3 (2-epimerization). The biosynthesis of L-lyxose might start from D-ribose-5-phosphate which is a product of the pentose phosphate pathway. Conversion of D-ribose-5-phos-

phate to L-lyxose requires dephosphorylation and epimerization. The gene product of *aviP1* resembles Pho2, a *p*-nitrophenyl phosphatase from *Halobacterium* sp. NRC-1 (34% identical aa) [26] and a putative hydrolase from *S. coelicolor* A3(2) (28% identical aa) [14]. Therefore AviP1 might be the responsible dephosphatase involved in D-lyxose biosynthesis. The epimerization steps might then be catalyzed by either AviQ1, AviQ2 or AviQ3. Residue H can also be seen as a D-fucose moiety which again could be derived from GDP-D-mannose. After 2-epimerization by AviQ1, AviQ2 or AviQ3 and 4,6-dehydration (AviE2) GDP-4-keto-6-deoxy-D-glucose might be produced. The final step of methyleurekanate biosynthesis might then be accomplished by AviB1 and AviB2 which both resemble enzyme components of various 2-oxo-acid dehydrogenase complexes. The gene product of *aviB1* mostly resembles the α-chain (45% identical aa), and AviB2 the β-chain of an acetoin dehydrogenase (42% identical aa) from *Clostridium magnum* [27]. We assume AviB1 and AviB2 to be catalyzing the oxidative decarboxylation of pyruvate to yield acetate and also the attachment of acetate to eventually produce this sugar derivative.

2.4. Genes putatively involved in the formation of the heptasaccharide chain

We have identified four putative glycosyltransferase genes in the cluster. The deduced protein of *aviGT1* is similar to glycosyltransferases known to be involved in antibiotic biosynthesis. It mostly resembles SnoZ, a glycosyltransferase from *Streptomyces nogalater* [24] (41% identical aa). The deduced amino acid sequences of all other putative glycosyltransferases found in the cluster were similar to glycosyltransferases assigned to the biosynthesis of O-antigen oligosaccharides. AviGT2 is similar to a putative glycosyltransferase (Cps2J) from *Streptococcus suis* serotype 2 [28] (25% identical aa). The *aviGT3* gene product shows significant similarity to putative rhamnosyltransferases. Most similar are a rhamnosyltransferase from *Deinococcus radiodurans* R1 (31% identical aa) [29] and a rhamnosyltransferase from *Streptococcus pneumoniae* type 19B (29% identical aa) [30]. *AviGT4* appears to encode a mannosyltransferase as it resembles putative mannosyltransferases from different species. Most similar is WbaZ-2 from *Archaeoglobus fulgidus* (29% identical aa) [31]. Three genes in the cluster (*aviO1*, *aviO2* and *aviO3*) all resemble a putative α-ketoglutarate-dependent L-proline 4-hydroxylase from *Dactylosporangium* sp. [32], HtxA, an α-ketoglutarate-dependent hypophosphite dioxygenase from *Pseudomonas stutzeri* WM88 [33] and SnoK from *S. nogalater* [34]. AviO1 and AviO2, which share 36% identical aa, are most similar to the L-proline oxygenase (AviO1: 31% identical aa; AviO2: 29% identical aa), and AviO3 mostly resembles SnoK (30% identical aa). All enzymes contain the motif HxD strongly conserved in α-ketoglutarate-dependent enzymes. We speculate that AviO1, AviO2 and AviO3 are involved in the formation of both orthoester linkages and the O–CH_2–O group (residue H in Fig. 1) of avilamycin A.

2.5. Genes putatively involved in tailoring the heptasaccharide chain

Apart from *aviG1*, *aviG4*, *aviRa* and *aviRb* four further putative methyltransferase genes (*aviG2*, *aviG3*, *aviG5* and *aviG6*) were found in the cluster. They were identified as putative methyltransferase genes since either their products resemble methyltransferases from different organisms or they contain motifs which are found in various methylating proteins [35]. Several avilamycin derivatives, which do not contain methyl groups at different positions of the molecule, are produced by our strain indicating that methylation occurs at a very late stage of the biosynthesis. AviG2, AviG3, AviG5 and AviG6 might incorporate methyl groups at the D-fucose moiety (residue E in Fig. 1), D-mannose moiety (residue F in Fig. 1) and methyleurekanate moiety (residue H in Fig. 1) of avilamycin A.

2.6. Regulatory and resistance genes

As described recently *aviRa*, *aviRb, aviABC1* and *aviABC2* are involved in avilamycin resistance or avilamycin transport [10]. A further putative transporter gene is *aviJ*. Its deduced amino acid sequence shows homology to putative integral membrane ion transporters. Highest resemblance was found to a Na^+/H^+ antiporter from *Synechocystis* sp. Strain PCC6803 [36] (49% identical aa). Two putative regulatory genes were found in the cluster. The deduced amino acid sequence of *aviC1* and *aviC2* are similar to transcriptional activators of the response regulator type. AviC1 closely resembles a response regulator from *Staphylococcus aureus* [37] (31% identical aa), and AviC2 is most similar to Orf4 from *S. noursei* ATCC 11455 [15] (27% identical aa).

2.7. Genes of unknown function

AviX8 resembles a hypothetical protein from *S. coelicolor* with 34% identical aa [14]. AviX12 shows a significant degree of similarity to EryCV, a protein with unknown function located in the erythromycin biosynthetic gene cluster from *Saccharopolyspora erythraea* [38,39] (28% identical aa). The putative gene products of short open reading frames (ORFs) *aviX4*, *aviX5*, *aviX6*, *aviX7*, *aviX8*, *aviX10*, *aviX11* and *aviX13* do not resemble any protein in the databases.

2.8. Genes putatively located outside the cluster

Five putative ORFs (*aviX1*, *aviX2*, *aviX3*, *aviX4*, *aviX5*) were found to reside close to the margin on one side of the cluster and three putative ORFs (*aviX14*, *aviX15*, *aviX16*)

near the opposite margin of the cluster. They probably do not belong to the avilamycin biosynthetic gene cluster. AviX1 is a GTP-binding protein with 64% identical aa to a GTP-binding protein from *Mycobacterium tuberculosis* [40]. AviX2 and AviX3 resemble proteins found in the genome of *S. coelicolor* [14]. These ORFs with unknown function are clustered in *S. coelicolor* and are located downstream of a putative NAD^+ synthase gene (*nadE*) and upstream of a putative glutamate-ammonia-ligase adenyltransferase gene (*glnE*) [41]. The deduced amino acid sequence of *aviX14* is similar to a putative helicase from *S. coelicolor* [14] (32% identical aa), *aviX15* encodes a putative DNA-binding protein similar to a protein from *S. coelicolor* [14] (52% identical aa), and AviX16 might be an alkaline serine protease very similar to a protease from the marine bacterium *Alteromonas* sp. strain O-7 [42] (43% identical aa).

2.9. Generation of an aviG4 gene replacement mutant

For inactivation of *aviG4* plasmid pMIKG4E3 was constructed allowing the replacement of the wild-type gene by a mutated allele. After *S. viridochromogenes* Tü57 protoplasts were transformed with plasmid pMIKG4E3, erythromycin-resistant colonies were obtained. The transformation efficiency was about 10 colonies per μg plasmid DNA. Numerous colonies were grown without erythromycin to select for loss of resistance. Several sensitive colonies were obtained suggesting that they were the consequence of a double cross-over. Two mutants, named G4/24/20 and G4/24/30, were further examined. PCR-fragments, amplified from G4/24/20 and G4/24/30 DNA using primers aviG4F and aviG4R, could not be digested by *Nco*I, whereas PCR-fragments obtained from the wild-type DNA could be cut by this enzyme. To confirm the presence of the deletion within *aviG4* Southern blot analysis was performed as follows. *Nco*I-digested chromosomal DNA was prepared from G4/24/20, G4/24/30 and the wild-type strain. When probed with a 1.9-kb fragment containing the entire gene *aviG4* a 11-kb fragment was detected while the expected 5-kb and 6-kb fragments could be identified in the *S. viridochromogenes* Tü57 strain (data not shown). Mutant G4/24/30, now named *S. viridochromogenes* GW4, was used for further experiments.

2.10. Generation of an aviG4–aviH double gene replacement mutant

To knock out the *aviH* gene the inactivation plasmid pSP1S2Nar was constructed. *S. viridochromogenes* GW4 protoplasts were transformed with this plasmid. About 20 erythromycin-resistant colonies per μg DNA were obtained. Some were cultivated and screened for loss of erythromycin resistance indicating a double cross-over event. Mutant *S. viridochromogenes* GW4-AM1 was chosen for further investigations. A 1.34-kb PCR-fragment

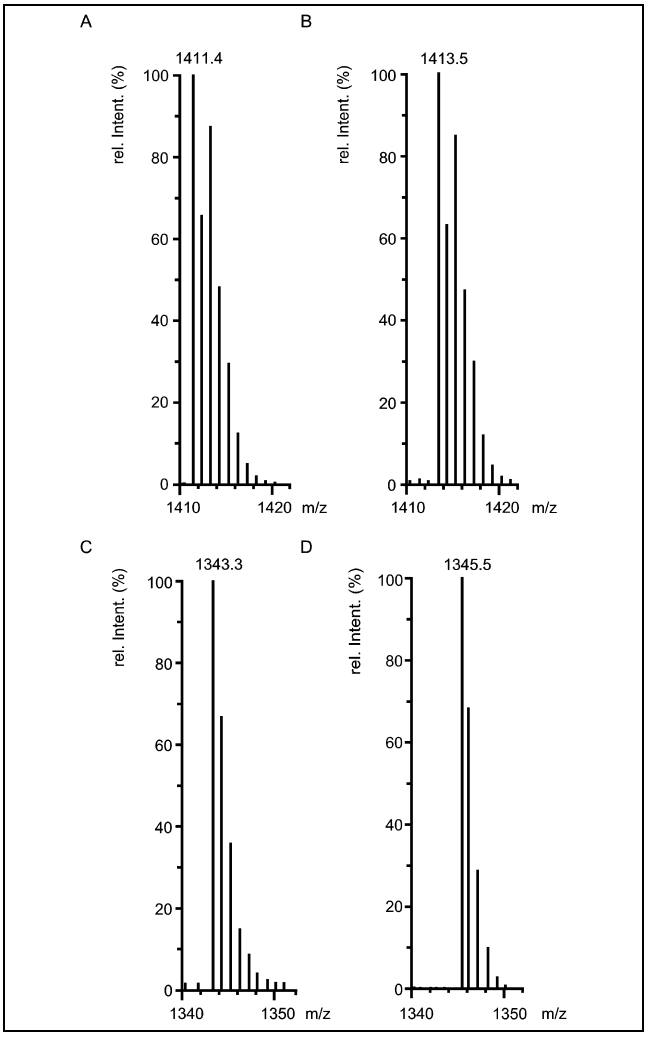


Fig. 3. Isotopic patterns of the main new derivatives produced by mutant *S. viridochromogenes* GW4. (A) Gavibamycin A1, (B) gavibamycin A3 and *S. viridochromogenes* GW4-AM1, (C) gavibamycin B1, (D) gavibamycin B3.

obtained from *S. viridochromogenes* GW4-AM1 using primers S2A and S2B could not be digested by *Nar*I, whereas the PCR-fragment obtained from *S. viridochromogenes* GW4 could be digested by the enzyme. The deletion within *aviH* was confirmed by Southern blot analysis. Chromosomal DNA from *S. viridochromogenes* GW4-AM1 was digested with *Nar*I and hybridized with a 3.7-kb probe containing the gene *aviH*. As result a 5.7-kb fragment was detected, while chromosomal DNA from *S. viridochromogenes* GW4 showed the expected fragments of 4.3 and 1.4 kb (data not shown).

2.11. Complementation of S. viridochromogenes GW4 and S. viridochromogenes GW4-AM1

To determine clearly that the mutation event only affected the desired genes and not other genes, *aviG4* and *aviH* were ligated behind the *ermE** promoter, cloned into the integration plasmid pSET152 and introduced by pro-

toplast transformation into the corresponding mutants. Avilamycin A and C production and gavibamycin A1 and A3 production respectively were restored. Thus, we could rule out any upstream or downstream effects.

2.12. Formation of new avilamycin derivatives by S. viridochromogenes GW4 and S. viridochromogenes GW4-AM1

Avilamycin A ($[M+Na]^+$: 1425) and avilamycin C ($[M+Na]^+$: 1427) were detected in extracts of *S. viridochromogenes* Tü57 by liquid chromatography (LC)-ESI-MS analysis. They were detected as sodium adduct ions ($[M+23]^+$). The observed distribution of the isotopic pattern of the pseudomolecular ions was in accordance with calculated values. With the assignment of molecular masses avilamycin C was produced as main compound. Due to their characteristic isotopic patterns higher resolved mass spectra showed that both compounds contain two chlorine atoms. The molecular masses of the pseudomolecular ions detected as the respective sodium adduct ions of two major compounds produced by *S. viridochromogenes* GW4 were $[M+Na]^+ = 1411$, and $[M+Na]^+ = 1413$ (Fig. 3) indicating that *aviG4* indeed encodes a methyltransferase. Based on the fact that AviG4 mostly resembles an *O*-demethylpuromycin methyltransferase we concluded that AviG4 is involved in methylation of the orsellinic acid moiety. The major products of mutant *S. viridochromogenes* GW4 were isolated, ethylated by treatment with ethyliodide, hydrolyzed using methanol and hydrochloric acid, and treated with diazomethane (Fig. 4). Reaction products were analyzed by GC-MS. The peak at *m/z* 436 corresponds to the D-olivosyl-ester of dichloro-di-*O*-ethyl-orsellinic acid and most of further peaks (*m/z* 405, *m/z* 275, *m/z* 247) correspond to fragments derived from the orsellinic acid moiety (Fig. 5). When extracts of *S. viridochromogenes* Tü57 were treated with ethyliodide a molecular mass peak of *m/z* 422 was detected (data not shown). This leads to the conclusion that the difference between avilamycin A (C) and the new derivative named gavibamycin A1 (A3) (Fig. 1) results from a change in the structure of the orsellinic acid moiety.

Extracts of *S. viridochromogenes* GW4-AM1 were also analyzed by LC-ESI-MS. The mass of the two major avilamycin derivatives was $[M+Na]^+ = 1343$ and $[M+Na]^+ = 1345$ (Fig. 3) which was leading to the conclusion that the difference between the mass of these new derivatives and the mass of the major compounds produced by *S. viridochromogenes* GW4 resulted from the absence of two chlorine atoms. This was confirmed by comparing the isotopic patterns of the main derivatives from mutant *S. viridochromogenes* GW4 with the isotopic patterns of the main products of mutant *S. viridochromogenes* GW4-AM1 (Fig. 3). The new derivatives were named gavibamycin B1 (avilamycin A analogue) and gavibamycin B3 (avilamycin C analogue) (Fig. 1).

2.13. Biological properties of gavibamycin A3 and gavibamycin B3

The antimicrobial spectra of gavibamycin A3 (the major compound produced by mutant *S. viridochromogenes* GW4) was determined and compared to the antimicrobial spectra of avilamycin A by the broth microdilution method following the procedures outlined by the National Committee for Clinical Laboratory Standards [43]. Both metabolites Gavibamycin A3 as well as avilamycin A showed antibiotic activity against *Bacillus subtilis*, *S. aureus* ATCC6538, *S. aureus* ATCC6538P, *S. aureus* ATCC29213, a chinolon-resistant *S. aureus* Q48-1.2.1, *Enterococcus faecalis* ATCC29212, a glycopeptide-resistant *E. faecalis* H-7-6 and *S. pneumoniae* ATCC49619.

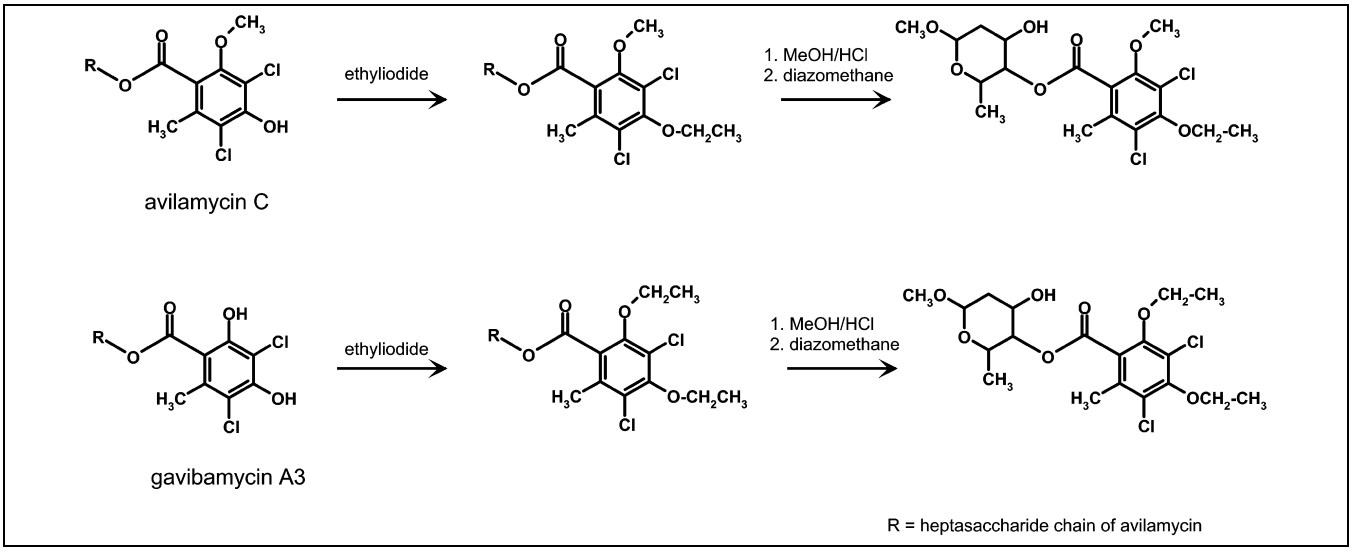


Fig. 4. Reaction scheme for the treatment of avilamycin derivatives and gavibamycin derivatives for GC-MS analysis.

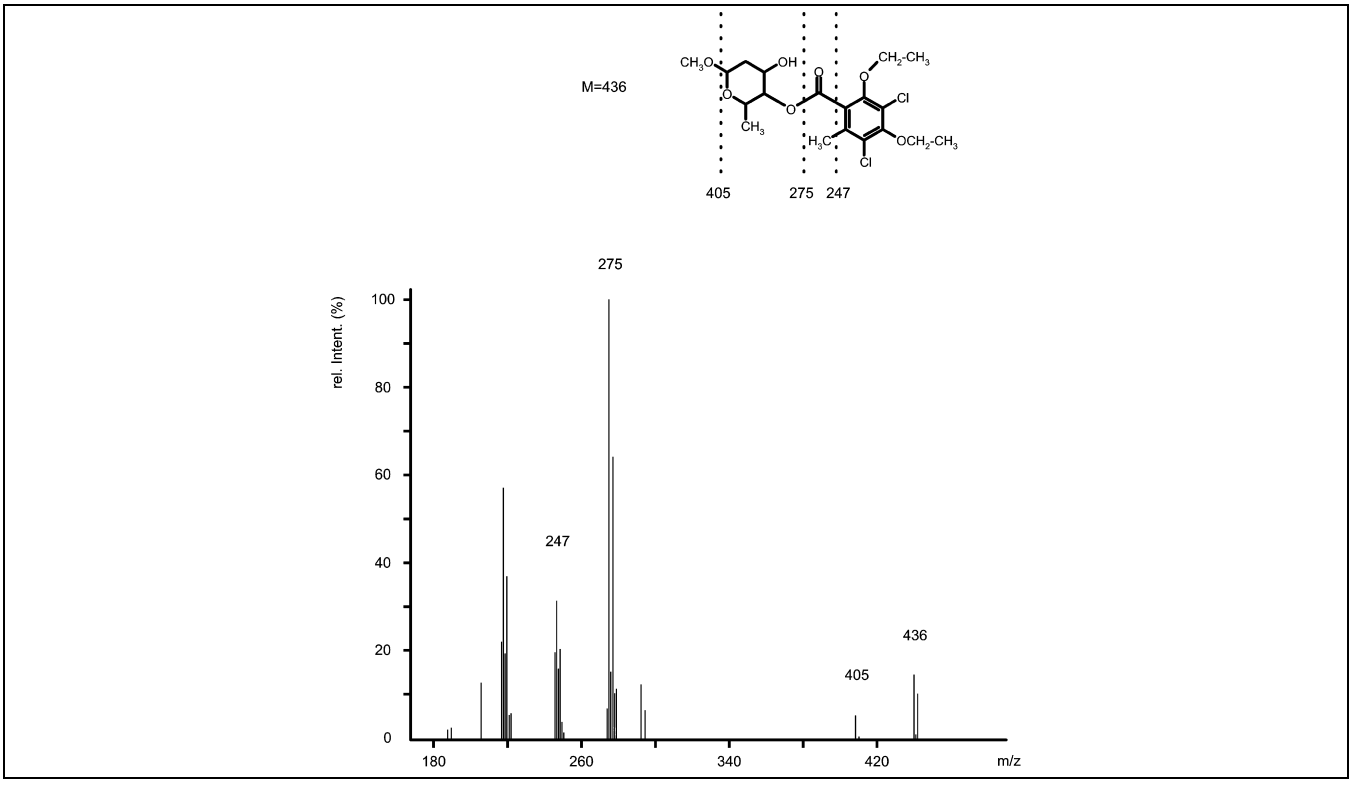


Fig. 5. EI mass spectrum of the D-olivosyl ester of dichloro-di-*O*-ethyl-orsellinic acid.

Gavibamycin B3, the major compound produced by *S. viridochromogenes* GW4-AM1 was tested against *B. subtilis* using the agar diffusion test. Antibiotic activity could be detected.

3. Discussion

In the work presented here we report the sequence of the avilamycin biosynthetic gene cluster that spans about 60 kb and involves at least 46 ORFs. A detailed sequence analysis of the *avi* gene set revealed several features that support a proposed model of the biosynthetic pathway to this complex oligosaccharide antibiotic (Fig. 6). The putative function of some of the sugar biosynthetic genes can be explained as their deduced amino acid sequences resemble proteins known to be involved in the biosynthesis of D-olivose in other organisms. As described for the biosynthesis of D-olivose in *S. violaceoruber* Tü22 (granaticin producer) [44] and *S. fradiae* (urdamycin producer) [45] the biosynthesis starts from glucose-1-phosphate which is converted to dTDP-D-olivose and dTDP-2-deoxy-D-evalose by several enzymes (Fig. 6). A novel feature in the avilamycin biosynthetic pathway involves the occurrence of three different NDP-hexose 4,6-dehydratase genes. On the basis of sequence homology AviE1 is a dTDP-glucose 4,6-dehydratase, and AviE3 is a GDP-mannose 4,6-dehydratase indicating that the biosynthesis of some of the basic sugar building blocks starts from different nucleotide-linked hexose pools. Based on the structure of avilamycin A and supported by the putative function of some gene products apparently the biosynthesis of L-lyxose even starts off from a third sugar pool as it is a product of the pentose phosphate pathway. Residue H (Fig. 1) of avilamycin A has originally been described as methyleurekanate derived from 2,3-di-*O*-methylene-4,5-dihydroxyhexanoic acid [3]. However, the sequence analysis presented here suggests that methyleurekanate is also the product of a sugar biosynthetic pathway. Based on the putative function of genes detected in the gene cluster a model for the synthesis of each sugar component of avilamycin A can be proposed (Fig. 6). However, the whole pathway and especially the exact order for individual biosynthetic steps has to be determined by future experiments.

Taking into account the number of sugar moieties in the avilamycin molecule six glycosyltransferase genes are expected. Interestingly, only four have been found. A conceivable explanation could be the involvement of one or more glycosyltransferases in several attachment steps or the involvement of glycosyltransferases encoded outside the cluster. Three of the four glycosyltransferases resemble rather glycosyltransferases for the biosynthesis of O-antigen structures or cell wall polysaccharides which can be explained by the polysaccharide-like structure of avilamycin A.

The *avi* pathway exposes even more interesting features: two orthoester linkages and one methylene bridge (marked by arrows in Fig. 1). Considering the oxidative nature of

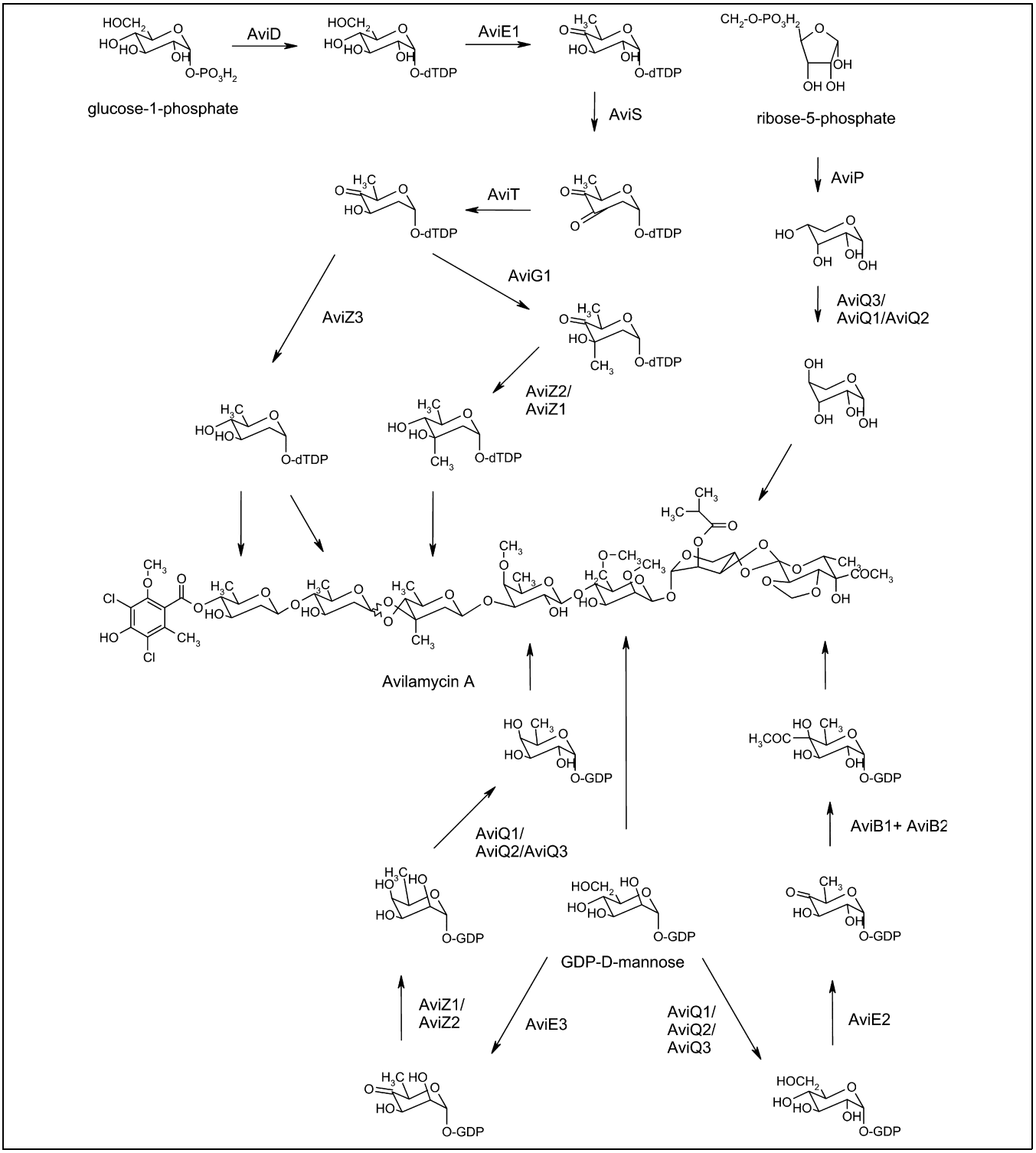


Fig. 6. Proposed biosynthetic pathway of avilamycin A.

these C–O–C arrangements, the putatively α-ketoglutarate-dependent oxygenases AviO1, AviO2 and AviO3 may catalyze the formation of these unique bonds. We propose such enzymes to use molecular oxygen as the direct electron acceptor for oxidation by using α-ketoglutarate as a cosubstrate producing the final C–O–C bonds, succinate and CO_2. The avilamycin A heptasaccharide is modified by methylation, attachment of acetate, attachment of dichloroisoeverninic acid and attachment of an isobutyryl moiety. Six methyltransferase genes appear to be present in the cluster which is the adequate number for avilamycin biosynthesis, whereas genes required for the attachment of the other residues have not been located yet. Interestingly, we found *aviB1* and *aviB2* encoding en-

zymes similar to the α and the β chain of components I of 2-oxo acid dehydrogenase complexes. These complexes are usually composed of three enzymatic units, which are TPP-dependent dehydrogenases, heterotetramers ($\alpha_2\beta_2$), dihydrolipoamide acetyltransferases (homomultimers) and dihydrolipoamide dehydrogenases (homodimers) [27]. ORFs encoding the latter components of these complexes are either not located inside the cluster or are not required for the biosynthesis of avilamycin A at all.

Members of the orthosomycin antibiotics are powerful agents against many Gram-positive pathogenic microorganisms, and some of them are promising antibiotics. Anyway, they have not been developed so far as antibiotics against human infectious diseases for clinical use because of some disadvantageous pharmacokinetic characteristics. Structural modification of antibiotics has proven to impart significant improvement in oral bioavailability and more persistent serum/tissue levels of pharmaceuticals. Therefore we decided to apply molecular genetic methods to alter the structure of the avilamycins with the aim of generating new analogues. Two of the putative modifying genes, *aviG4* and *aviH*, were inactivated by gene replacement. New antibiotics named gavibamycins were produced by the corresponding mutants (Fig. 1). These antibiotics consist of the entire heptasaccharide chain and an orsellinic acid moiety which can occur in a chlorinated (*aviG4*-mutant) or non-chlorinated form (*aviG4–aviH* double mutant). *S. viridochromogenes* Tü57 produces several avilamycin derivatives which differ in their methylation pattern. However, a compound missing the methyl group or both chlorine atoms at the aromatic polyketide has never been described. Gavibamycin A3, major compound of mutant *S. viridochromogenes* GW4, and gavibamycin B3, major compound of mutant *S. viridochromogenes* GW4-AM1, were tested against several bacterial strains. Both compounds were active indicating that the loss of the methyl group and both chlorine atoms did not prevent the new derivatives from acting as antibiotics.

In addition gavibamycin A3 and gavibamycin B3 seem to be much more hydrophilic than avilamycin A as indicated by Rf-values obtained by TLC and by HPLC analysis. Our future work will revolve around creating new avilamycin derivatives by targeted gene deletion or combinatorial biosynthesis approaches. This may eventually lead us to pharmacokinetically improved members of this promising antibiotic class.

4. Significance

The avilamycin biosynthetic gene cluster encodes a novel pathway that yields the structurally interesting orthosomycin antibiotic avilamycin A. Functional assignment to the proteins encoded within the cluster suggests a model for the complex avilamycin biosynthetic pathway. We demonstrated that genetic manipulation of the avilamycin cluster results in the production of new antibiotically active avilamycin derivatives.

5. Materials and methods

5.1. Bacterial strains, plasmids and culture conditions

S. viridochromogenes Tü57 [7] was grown on 1% malt extract, 0.4% yeast extract, 0.4% glucose and 1 mM $CaCl_2$, pH adjusted to 7.2 (HA medium) at 37°C. For avilamycin production *S. viridochromogenes* Tü57 and all mutants were grown at 28°C in NL19+ medium containing 2% D-mannitol, 2% soy flour and 20 mM L-valine adjusted to pH 7.5. DNA manipulation was carried out using *Escherichia coli* XL-1 Blue MRF′ (Stratagene) as host strain. Before transforming *S. viridochromogenes* Tü57, plasmids were propagated in *E. coli* ET 12567 (*dam*$^-$, *dcm*$^-$, *hsd*S, Cm^R) [46] to obtain unmethylated DNA. *E. coli* strains were grown on Luria–Bertani (LB) agar or liquid medium containing the appropriate antibiotic. pBluescript SK- (pBSK-) and pBC-SK- were from Stratagene. Plasmids pUC19 and pSP1 [9], conferring erythromycin resistance, were a kind gift of Dr. S. Pelzer, Tübingen and pSET152 [47], conferring apramycin resistance, was obtained from Eli Lilly and Co. (Indianapolis, IN, USA).

5.2. General genetic manipulation, PCR and DNA sequencing/sequence analysis

Standard molecular biology procedures were performed as described [48]. Isolation of *E. coli* plasmid DNA, DNA restriction, DNA modification and Southern hybridization were performed following the protocols of the manufacturers of kits, enzymes and reagents (Amersham-Pharmacia, Roche Diagnostics, Promega, Stratagene). *Streptomyces* protoplast formation, transformation and protoplast regeneration were performed as described [49]. PCR was carried out using a Perkin Elmer GeneAmp 2400 thermal cycler. The conditions were as described [50]. Oligonucleotide primers used were AviG4F (5′-GGACGCCTATCTGTGCCACCCCTTCCTGGT-3′), AviG4R (5′-TGAGCGCTCGCCTAGACAGAATCATCTCCC3′), S2A (5′-GCGTCCATCTTGCCGGGA-3′) and S2B (5′-CGTGGATCCCGCCGGCCC-3′). Nucleotide sequences were determined by the dideoxy chain-termination method using an automatic laser fluorescence sequencer (Perkin Elmer ABI). Sequencing reactions were done using a thermosequenase cycle sequencing kit with 7-deaza-dGTP (Amersham) and standard primers (M13 universal and reverse, T3, T7). Computer-aided sequence analysis was done with the DNASIS software package (version 2.1, 1995; Hitachi Software Engineering), database searches were performed with the BLAST 2.0 program [51] on the server of the National Center for Biotechnology Information, Bethesda MD, USA. The sequence reported here has been deposited in the GenBank data base under the accession number AF333038.

5.3. Construction of gene inactivation plasmids

aviG4: A unique *Nco*I restriction site inside the gene *aviG4*, which is located on a 1.9-kb fragment ligated into the *Sac*I and *Eco*RI sites of pBSK- was chosen for targeted inactivation by shifting the reading frame. The 1.9-kb fragment was digested with *Sac*I and *Kpn*I and was ligated into the gene inactivation plasmid pSP1. After *Nco*I restriction, treatment with the Klenow fragment of *E. coli* DNA-polymerase I and religation, the intended alteration (correct fill-in) was confirmed by DNA sequencing. The generated plasmid was named pMIKG4E3.

*avi*H: The unique *Nar*I site inside the *aviH* gene, which is located on a 3.7-kb *Sac*I fragment ligated into pBCSK-, was altered by *Nar*I restriction and subsequent treatment as described for *aviG4*. Sequencing of several plasmids revealed correct alteration. The 3.7-kb fragment was cloned into pSP1 to generate the gene inactivation plasmid pSP1S2Nar.

5.4. Construction of complementation plasmids

pUWLurdGT1c [54] was subjected to a *Kpn*I/*Xba*I restriction digestion to obtain a 1.6 kb fragment that covers the complete reading frame *urdGT1c* as well as the *ermE** promoter sequence. This fragment was inserted in the multiple cloning site of pUC19 cleaved with the same enzymes. This plasmid was named pUC19-1cerm. Then, *urdGT1c* and the *ermE** promoter were excised by *Eco*RI restriction and ligated into pSET152 to create plasmid pSET-1cerm. Both genes, *aviG4* and *aviH*, were amplified using PCR. Suitable restriction sites (*aviG4*, *Mun*I and *Xba*I; *aviH*, *Eco*RI and *Xba*I) were introduced using oligonucleotide primers G4F (5′-AAGGACAATTGGCGACGAGA-3′), G4R (5′-GCTCCTCTAGACGGTGAAAT-3′), aviHA (5′-GTATTTCAGAATTCCCCGTGCCGG-3′) and aviHB (5′-GCCCGGTCTAGACGCTGTGATCAGGG-3′) (restriction sites are underlined). Plasmid pSET-1cerm was digested with *Mun*I and *Xba*I to remove *urdGT1c*, and *aviG4* and *aviH*, respectively, were cloned behind the *ermE** promoter in place of *urdGT1c* to create integration plasmids pSET-G4erm and pSET-Herm. After transformation of mutants *S. viridochromogenes* GW4 and *S. viridochromogenes* GW4-AM1 with the corresponding integration plasmids several apramycin-resistant colonies were obtained and grown at 28°C in liquid medium as described above. Extracts of cultures were analyzed by TLC or LC-ESI-MS.

5.5. Analysis of new derivatives of avilamycin A

5.5.1. TLC analysis

S. viridochromogenes Tü57 and mutants *S. viridochromogenes* GW4 and *S. viridochromogenes* GW4-AM1 were incubated for 3 days. Cultures were filtered and the filtrate was applied to a solid-phase extraction cartridge (SepPak®C_{18}, Waters). The cartridge was eluted using a gradient from 10 to 100% methanol in water. Avilamycin derivatives could be detected in fractions containing 60–70% methanol. After ethyl acetate extraction and evaporation of the solvent avilamycin derivatives were redissolved in methanol and analyzed by TLC on silica gel plates (silica gel 60 F_{254}, Merck) with methylene chloride/methanol (9:1, v/v) as solvent. Avilamycin derivatives could be detected after treatment with anisaldehyde/H_2SO_4.

5.5.2. HPLC-UV analysis

Analytical HPLC-UV was performed on a Hewlett Packard 1090 liquid chromatograph with a photodiode array detector and an HP-ODS-Hypersil 5 μm, 200×2 mm column. The detection wavelength was 210 nm. The solvent system was as follows: solvent A, 0.04 M $(NH_4)_2HPO_4$ pH 7.0 buffer; solvent B, 100% methanol; non-linear gradient, 30–62% B in 25 min at a flow rate of 0.2 ml/min.

5.5.3. HPLC-ESI-MS analysis

For HPLC-ESI-MS analysis avilamycin derivatives were separated by HPLC (HP1110, Hewlett-Packard) with a HP-ODS-Hypersil C_{18} column (100×2.1 mm; 5 μm) at a flow rate of 0.1 ml/min, detection at 220 nm and following gradient: 0–5 min, linear gradient to 20% B; 5.0–120 min, linear gradient to 90% B (solvent A, H_2O: MeOH 3:2; solvent B, MeOH). Mass spectra were recorded on a Bruker Esquire-LC 1.6n mass spectrometer (Bruker Daltonik) equipped with an electrospray ion source (positive ion mode) (ESI). The scan-range was from 200 to 1800 *m/z* with nominal mass resolution.

5.5.4. GC-MS analysis

Analysis of new gavibamycin derivatives produced by mutant *S. viridochromogenes* GW4 was performed by GC-MS analysis after ethylation. The derivatives were dissolved in a mixture of DMSO and acetonitrile (3:40). After addition of ethyliodide and K_2CO_3 the reaction was incubated over night. After evaporation of the solvent avilamycin derivatives were hydrolyzed by HCl/methanol at 115°C for 15 min. The solvent was evaporated again and compounds were treated with diazomethane in ethanol (30 min at 20°C). After evaporation of the solvent derivatives were extracted with diethylether and analyzed by GC-MS. An Hewlett Packard 5973 MSD system was used to obtain EI (electron impact) spectra (column: SE54, 12 m×0.25 mm; d_f = 0.125 μ). The column temperature was programmed as follows: 50°C for 2 min; 25°C/min to 100°C; 5°C/min to 250°C.

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