Analysis of a C-methyltransferase gene (*aviG1*) involved in avilamycin biosynthesis in *Streptomyces viridochromogenes* Tü57 and complementation of a *Saccharopolyspora erythraea* *eryBIII* mutant by *aviG1*

G. Weitnauer,¹ S. Gaisser,² L. Kellenberger,² P. F. Leadlay² and A. Bechthold¹

Author for correspondence: A. Bechthold. Tel: +49 761 2038371. Fax: +49 761 8383.
e-mail: andreas.bechthold@uni-freiburg.de

¹ Albert-Ludwigs-Universität Freiburg, Institut für Pharmazeutische Biologie, Stefan-Meier Str. 19, 79104 Freiburg, Germany

² Cambridge Centre for Molecular Recognition and Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1GA, UK

***Streptomyces viridochromogenes* Tü57 is the principal producer of avilamycin A. *aviG1*, a putative methyltransferase gene, was detected in the avilamycin biosynthetic gene cluster. To determine the function of *aviG1*, a targeted gene inactivation experiment was performed. The resulting chromosomal mutant, carrying an in-frame deletion in *aviG1*, was deficient in avilamycin production. *aviG1* was used to complement an *eryBIII* mutant of the erythromycin A producer *Saccharopolyspora erythraea* [Gaisser, S., Böhm, G. A., Doumith, M., Raynal, M. C., Dhillon, N., Cortes, J. & Leadlay, P. F. (1998). *Mol Genet* 258, 78–88]. The presence of erythromycin A in the culture supernatant of the complemented mutant indicated that L-mycarose biosynthesis could be restored and that *AviG1* could take over the function of the C-methyltransferase *EryBIII*.**

Keywords: avilamycin A, erythromycin, deoxysugar biosynthetic genes, methyltransferase

INTRODUCTION

Avilamycin A and avilamycin C (Fig. 1) are oligosaccharide antibiotics belonging to the orthosomycin group (Buzzetti *et al.*, 1968). Like ziracin, another orthosomycin, which has been tested in clinical trials against human infectious diseases (Foster & Rybak, 1999; Fuchs *et al.*, 1999; Jones *et al.*, 1999; Lin *et al.*, 2000; Wang *et al.*, 2000), avilamycin is a potent antibiotic with excellent activity against Gram-positive bacteria (Wright, 1979).

The biosynthesis of avilamycin A requires the formation of a polyketide moiety and its attachment to a heptasaccharide chain consisting of D-olivose, 2-deoxy-D-avalose, 4-O-methyl-D-fucose, 2,6-di-O-methyl-D-mannose, L-lyxose and (methyl) eurenkanate. As for many antibiotics, the cloning of the avilamycin biosynthetic genes has been facilitated by the fact that they are organized in a cluster. The complete avilamycin biosynthetic gene cluster has been sequenced and several genes

have been characterized (Gaisser *et al.*, 1997; Weitnauer *et al.*, 2001). Expression of one of these genes, encoding a polyketide synthase, in *Streptomyces lividans* resulted in the production of orsellinic acid. It was also shown that insertional inactivation of a putative dNDP-glucose 4,6-dehydratase gene (*aviE1*) abolished avilamycin A production. Targeted gene disruption of two other genes, *aviG4* and *aviH*, encoding a methyltransferase and a halogenase, respectively, led to the production of avilamycin derivatives with an incomplete dichloroisoevreninic moiety.

In contrast to avilamycin A, in which a long sugar side chain is connected to a small polyketide moiety, erythromycin is a macrolide polyketide, to which only two separate sugar residues (L-mycarose and D-desosamine) are transferred (Fig. 1). The biosynthesis of erythromycin is well understood and many mutants containing deletions in single biosynthetic genes are available (Paulus *et al.*, 1990; Haydock *et al.*, 1991; Weber *et al.*, 1991; Stassi *et al.*, 1993; Lambalot *et al.*, 1995; Summers *et al.*, 1997; Salah-Bey *et al.*, 1998; Gaisser *et al.*, 1998). *eryB* genes have been shown to be involved in dTDP-mycarose biosynthesis. One of these

Abbreviation: ES-MS, electrospray mass spectrometry.

The GenBank accession number for the sequence reported in this paper is AF333038.

Fig. 1. Structure of avilamycin A, avilamycin C and erythromycin A.

genes, *eryBIII*, encodes a C-methyltransferase involved in methylation at position C3 during dTDP-L-mycarose biosynthesis (Gaisser *et al.*, 1998).

For better understanding of the biosynthesis of avilamycin A we have initiated the characterization of another gene (*aviG1*) of the avilamycin biosynthetic gene cluster located upstream of the avilamycin polyketide synthase biosynthetic gene. The deduced amino acid sequence of *aviG1* was very similar to EryBIII. The involvement of *AviG1* in the biosynthesis of avilamycin A was demonstrated by targeted gene disruption and complementation of an *eryBIII* mutant of *Saccharopolyspora erythraea*.

METHODS

Bacterial strains, plasmids and culture conditions and materials. *Streptomyces viridochromogenes* Tü57 (Buzzetti *et al.*, 1968), obtained from the Department of Microbiology, University of Tübingen, Germany, was grown on 1% malt extract, 0.4% yeast extract, 0.4% glucose and 1 mM $CaCl_2$, pH 7.2 (HA medium) at 37 °C. For production of avilamycins, spores of *Str. viridochromogenes* Tü57 were transferred to NL19+ medium containing 2% mannitol, 2% soya flour and 20 mM L-valine (pH 7.2) and were grown at 28 °C in 500 ml baffled flasks filled with 100 ml medium at 180 r.p.m. for 48 h. The erythromycin-producing red variant strain of *Sac. erythraea* NRRL2338 (Hessler *et al.*, 1997) was kindly provided by J. M. Weber, FermaLogic, Chicago, IL, USA. For optimal production of erythromycin A and its derivatives, *Sac. erythraea* NRRL2338, the *eryBIII* mutant 335 and the *eryBIII* mutant 335 containing *aviG1* were grown at 30 °C as described previously (Gaisser *et al.*, 1998). DNA manipulation was performed in *Escherichia coli* XL-1 Blue MRF from Stratagene. Plasmids were passed through *E. coli* ET12567 (*dam*[−] *dcm*[−] *hsdS*[−] *Cm*⁺) (Flett *et al.*, 1997) to generate unmethylated DNA before being transferred into *Str. viridochromogenes* Tü57. *E. coli* strains were grown under standard conditions. pBluescript SK− (pSK−) was obtained from Stratagene, plasmids pUC18, pUC19 and pSP1, carrying the erythromycin resistance gene (Pelzer *et al.*, 1997), were provided by S. Pelzer, University of Tübingen, Germany, and pSET152 (Biermann *et al.*, 1992) was obtained from Eli Lilly. The construction of the

expression plasmid pSG142 has been described previously (Gaisser *et al.*, 2000). Plasmids used in this study are listed in Table 1.

Medium components were purchased from Hartge Ingredients and restriction enzymes were from Amersham Pharmacia. Avilamycin A was a gift from Eli Lilly, carbenicillin was from Roth, and apramycin and erythromycin were from Fluka. All other chemicals were from Roth.

General genetic manipulation. Isolation of *E. coli* plasmid DNA, digestion of DNA with restriction endonucleases and Southern hybridization were carried out according to the directions of the suppliers of kits, enzymes and reagents (Amersham Pharmacia). Southern hybridization was performed with Hybond-N nylon membranes (Amersham Pharmacia). Probes were labelled with digoxigenin (DIG) by using a DIG labelling and detection kit (Roche). Restriction mapping and other routine molecular biology methods were performed as described by Sambrook *et al.* (1989). Protoplast formation, transformation and regeneration of protoplasts from *Str. viridochromogenes* Tü57 were carried out by standard procedures (Hopwood *et al.*, 1985). PCR was carried out using a Perkin Elmer GeneAmp 2400 thermal cycler. The conditions were as described by Bechthold & Floss (1994).

DNA sequencing and computer-assisted sequence analysis. DNA was sequenced by the dideoxynucleotide chain-termination method using thermosequencase (Amersham Pharmacia). Universal and reverse primers (Amersham Pharmacia) were used. Sequencing reactions were performed on an automated sequencer (Vistra 725) from Molecular Dynamics and on an ABI sequencer from 4-Base Lab. DNA sequences were analysed using the DNASIS software package (version 2, 1995; Hitachi Software Engineering). BLASTX analysis (Altschul *et al.*, 1997) was used to search the GenBank CDC translations + PDB + SWISS-PROT + Spupdate + PIR, release 2.0 for matching sequences.

Generation of a chromosomal *aviG1* mutant of *Str. viridochromogenes* Tü57. For generation of a chromosomal *aviG1* mutant of *Str. viridochromogenes* Tü57 by homologous recombination, plasmid pMIK1 was constructed. A 7.7 kb *Bam*HI fragment containing the entire methyltransferase gene was cloned into pBluescript SK− to create plasmid B7. B7 was digested with *Bgl*II and *Spe*I (the *Spe*I site is located within the polylinker of pBluescript SK−), treated with Klenow frag-

Table 1. Most important plasmids used in this study

Plasmid	Description	Reference or source
pBluescript SK –	Cloning vector	Stratagene
pUC19	Cloning vector	New England Biolabs
pUC18	Cloning vector	Pharmacia
pSET152	Integrating vector	Biermann <i>et al.</i> (1992)
pSP1	Non-replicating vector	Pelzer <i>et al.</i> (1997)
pUWL201	<i>E. coli</i> – <i>Streptomyces</i> shuttle vector, used for expression experiments in <i>Streptomyces</i>	Trefzer <i>et al.</i> (2000)
B7	7.7 kb <i>Bam</i> HI fragment containing <i>aviG1</i> cloned into pBluescript SK –	This study
pMIK1a	Religation product of plasmid B7 after restriction by <i>Bgl</i> II and <i>Spe</i> I and treatment with Klenow fragment	This study
pSP-MIK	2.5 kb <i>Sac</i> I fragment of pMIK1a containing <i>aviG1</i> cloned into pSP1	This study
pMIK1	pSP-MIK after deletion of an <i>Nco</i> I fragment within <i>aviG1</i>	This study
pUWLurdGT1c	pUWL201 containing <i>urdGT1c</i>	Trefzer <i>et al.</i> (2000)
pUC19-1cerm	pUC19 containing <i>urdGT1c</i> and the <i>ermE</i> promoter	Hoffmeister <i>et al.</i> (2001)
pSET-1cerm	pSET152 containing <i>urdGT1c</i> and the <i>ermE</i> promoter	Hoffmeister <i>et al.</i> (2001)
pSET-G1erm	pSET152 containing <i>aviG1</i> and the <i>ermE</i> promoter	This study
pUCB	pUC18 containing <i>aviG1</i>	This study
pSG142		Gaisser <i>et al.</i> (2000)
pSGaviG1	pSG142 containing <i>aviG1</i>	This study

Fig. 2. Organization of a part of the avilamycin biosynthetic gene cluster. The region described in this work is shown in greater detail. Restriction sites used to generate the inactivation plasmid are indicated. Abbreviations: B, *Bam*HI; Bg, *Bgl*II; S, *Sac*I; N, *Nco*I.

ment and religated to generate pMIK1a. A 2.5 kb *Sac*I fragment of pMIK1a, containing *aviG1*, was ligated into the corresponding sites of pSP1, generating pSP-MIK (Fig. 2). pSP-MIK was digested with *Nco*I to delete a 285 bp fragment within *aviG1*, resulting in plasmid pMIK1. pMIK1 was used to transform protoplasts of *Str. viridochromogenes* Tü57. Selection of primary transformants was performed on erythromycin-containing plates. For characterization of transformants by Southern hybridization, an internal 1.7 kb *Sma*I fragment of the *ermE* gene of pSP1 and the internal 2.2 kb *Sac*I fragment of pMIK1 were used as probes. After screening for erythromycin-sensitivity, a double cross-over mutant, named *Str. viridochromogenes* GW1, was obtained. Chromosomal DNA from this mutant was analysed by Southern hybridization using the 2.2 kb *Sac*I fragment as probe. The result of Southern hybridization was confirmed by PCR using oligonucleotide primers HaloF (5'-GCCGAGC-AAAGCTTGGAGAATCAT-3') and HaloR (5'-TGGTGG-CATGCGATGTCACCTCC-3').

Complementation of mutant *Str. viridochromogenes* GW1. For complementation of mutant *Str. viridochromogenes* GW1, plasmid pSET-G1erm was constructed. pUWLurdGT1c (Trefzer *et al.*, 2000) 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. The *urdGT1c* gene and the *ermE* promoter were excised by *Eco*RI restriction and ligated into pSET152 to create plasmid pSET-1cerm. *aviG1* was PCR-amplified to introduce *Mun*I and *Xba*I restriction sites by using oligonucleotide primers AviG1F (5'-AGATGTCAAT-TGCTGGCTCA-3') and AviG1R (5'-TGGTGTCTAGAGATGTCACCTCC-3' (restriction sites are underlined). Plasmid pSET-1cerm was digested with *Mun*I and *Xba*I to remove *urdGT1c*, and *aviG1* was cloned behind the *ermE* promoter in place of *urdGT1c* to create the integration plasmid pSET-G1erm. After transformation of mutant *Str. viridochromogenes* GW1 by pSET-G1erm, several colonies were obtained and grown at 28 °C in liquid medium as described above. Extracts of cultures were analysed by TLC, bioassay and HPLC-UV (see Fig. 4).

Complementation of the erythromycin *eryBIII* mutant 335. For complementation of the *eryBIII* mutant 335, a *Hind*III restriction site 5' to the ribosome-binding site and an *Sph*I restriction site 3' to the termination codon were introduced

into *aviG1* by using PCR. The fragment was cloned into the *Hind*III and *Sph*I sites of pUC18 to generate pUCB, which was digested with *Hind*III, followed by a fill-in reaction (Sambrook *et al.*, 1989). After *Bam*HI digestion, the fragment harbouring *aviG1* was ligated into pSG142, previously digested with *Nde*I, followed by treatment with Klenow polymerase and *Bgl*II digestion. The cloning region around the former *Nde*I site in the resulting plasmid, pSGaviG1, was sequenced, revealing the deletion of 9 nt at the *Nde*I/*Hind*III fusion site. Plasmid pSGaviG1 was used to transform the *Sac. erythraea eryBIII* mutant 335 as described by Gaisser *et al.* (1998). The presence of the *eryBIII* mutation was confirmed using chromosomal DNA of a *Sac. erythraea eryBIII* mutant and the transformed strain containing *aviG1* as template. PCR reactions were carried out as described previously (Gaisser *et al.*, 1998) and the sequence of the PCR fragments was confirmed. The PCR fragment obtained with chromosomal DNA of *Sac. erythraea eryBIII* mutant as template was also used as a probe in Southern hybridization experiments with *Bam*HI-digested genomic DNA of *Sac. erythraea* NRRL2338, *eryBIII* mutant 355 and *eryBIII* mutant 355 harbouring *aviG1*. No difference in the pattern of the labelled DNA bands was detected. These results indicated that the *eryBIII* gene was not involved in a recombination event.

Analysis of avilamycin and erythromycin derivatives. For avilamycin production, *Str. viridochromogenes* Tü57 and mutant *Str. viridochromogenes* GW1 were grown as described above. Cultures were extracted with an equal volume of ethyl acetate. After evaporation of the solvent the dried extracts were redissolved in methanol. TLC analysis was carried out on silica gel plates (silica gel 60 F254, Merck) with $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ (9:1, v/v) as solvent. Avilamycin derivatives could be detected after treatment with anisaldehyde/ H_2SO_4 (Braun, 1995).

HPLC analysis was performed on a Waters Alliance HT Liquid Chromatograph with a diode-array detector and a ProC18, 50×4 mm column. The detection wavelength was 220 nm. The solvent system was as follows: Solvent A, acetonitrile/ $[\text{H}_2\text{O}/\text{H}_3\text{PO}_4$ (99.9:0.1)], 5:95 (v/v); solvent B: acetonitrile/ $[\text{H}_2\text{O}/\text{H}_3\text{PO}_4$ (99.9:0.1)], 42:58 (v/v); non-linear gradient, 0–100% solvent B over 16 min at a flow rate of 1.2 ml min^{-1} . Avilamycin A was identified by comparison with an authentic sample. For erythromycin A production, culture supernatants were treated as described previously and the extracts were analysed using electrospray MS (ES-MS; Gaisser *et al.*, 1998).

RESULTS AND DISCUSSION

Sequence analysis

Having cloned and sequenced the avilamycin biosynthetic gene cluster (Gaisser *et al.*, 1997; Weitnauer *et al.* 2001), our work now was aimed at characterizing further genes involved in avilamycin biosynthesis. Database comparison with the deduced product of *aviG1* using BLASTP and BLASTX revealed similarities between *AviG1* and several C-methyltransferases. The most similar proteins found were TylCIII from *Streptomyces fradiae* (66% identical amino acids; Bate *et al.*, 2000) and EryBIII from *Sac. erythraea* (64% identical amino acids; Haydock *et al.*, 1991; Gaisser *et al.*, 1998). Homology was also found to ORF14 from the chloroeremomycin producer *Amycolatopsis orientalis* (29% identical amino acids; Chen *et al.*, 2000). The C-methyltrans-

	1		50
EryBIII	-----M	IFLVGLGKCR	ICGNHDESV LHLGQALTG VFPNRDEIV
TylCIII	VPAVPREDQM	I...ISACR	VCGNRELLPV LDLGEQALTG VFPRTREETV
AviG1	-----MSTT	GHSTVIDRCR	ICDNTELLPV LDLGPQALTG VFPRTRGEDV
ORF14	-----	MSVTSQCR	ICDGT.VKEF IDFGRLPLSD AFVVPGEDEK
	51		100
EryBIII	PSVPLELVKC	APPGCGLVQL	RESADFLGMY NEGGYGRSGI RPFMINHLHG
TylCIII	PSIPLELVKC	SPQGCGLVQL	RHTPDPGLMY GEGYGRSGI RPFMIGHLRR
AviG1	PYVPLELVRC	SPGGCELVQL	RHTADFLGMY GEGYGRSSL NRSMADHLRG
ORF14	EFF.FRLATG	ICDSCTMVQL	MEEVPRDLMF HDAYPYLSSG SAFMRTHFNE
	101		150
EryBIII	KVAKLRGMVP	VGPDDLVDI	GSNDSTLLRG YLPDAPKLAG HDLVGEKFRD
TylCIII	KVAARELVLD	LGPDDLVDI	GSNDATLLKA Y.PEGPRLVG IDPSGDKFRE
AviG1	KVAATIGLVD	LGPDDLVDI	GSNDSTLLAA YPADGPRLVG VDPAAITVFAA
ORF14	LAKRLLTTEL	TGEDPFI	VEL GCNDGIMLKA MAEAGVRQLG VEPGSGVADL
	151	motif I	200
EryBIII	LYPEADLVLT	GFF.SADAFE	ERYGERRAKV VTSIAMFYDL PEPMRFRMDV
TylCIII	LYPPHAELIA	EYF.SRDVFT	ARFGTRRARV ITSIAFYDL PDPLAFMRDV
AviG1	SYPPGVLELIP	DFP.AYDLL.	...GGRRAKV VTSIAMFYDL PRPMEFMREV
ORF14	AAAKGIRVRK	DFEEBATAAD	IRETDGPADV IYAANTLCHI PYMDSILKGV
	201		250
EryBIII	HDILADDGLW	LMEQSYLPSM	LDAGAYDVVC HEHLEYALA QIEWMAQRVG
TylCIII	HDVLADDGIW	VMEQSYLPAM	LEADAYDVVC HEHLEYALQ QIEWMAERAG
AviG1	GRLLTDDGIW	VTEQSYLPAM	LHACAYDVVC HEHLDYGLR QIEWMAERTG
ORF14	TTLLGPNGVF	VFEDPYLGD	I VERTSFQDIY DEHFFFTAR SVQEMAKRNG
	251	motif II	300
EryBIII	LKVVDABITD	VYGGSLCAVL	AKQSGHPVD EAGLERIRAR EAAAKLDTMA
TylCIII	LTVLRAELTD	VYGGSLCVTL	ARASSPHPRD EAGPARIRAR ETEAKINTMA
AviG1	LKVVDABITP	VYGGSLSLVL	ARRGSSRQVN EPALARIRAG ETD....L
ORF14	LELVDFVERIP	VHGGEVRYTL	ALAGARQPT E..AVAEALLW EAERKLAEYA
	301	motif III	350
EryBIII	PYEAFARETE	RQRDQLLEFL	AKSRAEGKLT LGYGASTKGN VILQYCGLTE
TylCIII	PFEFARRVE	HQRDALRDFL	DRSRAAGRLT LGYGASTKGN VILQYCGIGE
AviG1	PYAEFARTE	ESRDRLLEFL	TASRDKGLHT LGYGASTKGN VILQYCGLDE
ORF14	TLERFATNVK	KIKDDLIALL	TKLRAEGKRV VGYGATAKSA TVTNFCGITP
	351		400
EryBIII	QDLPCIGEV	PEKSGCYTPG	TGIPIVSEEE AKSRRPDQLL VLPWIYRDGF
TylCIII	RDLPCIGEV	PEKAGRFTPG	TGIPIVSEED AKAMRPDQLL VLPWIYREGF
AviG1	TLLPCIAEVN	EDKFGCYTPG	TNIPIVSEEE ARALEPDQLL VLPWIYRDAM
ORF14	DLVEFISDTT	PAQNRLSPG	QHVPREPKE FAADYPDYAL LFAWNHADEI
	401		425
EryBIII	VEREQEFLAG	GKGLIFPLPR	LEVV~
TylCIII	VERERDFLAG	GGRLVFPLPR	LDVV*
AviG1	VARERDFLAS	GGSLVFPLPT	LEVV*
ORF14	MNAEQAFRDA	GGQWILYVPN	VHVS*

Fig. 3. Comparison of the *aviG1* gene product with the known C-methyltransferases EryBIII, TylCIII and ORF14. Motifs I–III (Kagan & Clarke, 1994) are indicated.

ferase activity of this enzyme has been demonstrated. *AviG1*, *TylCIII*, *EryBIII* and *ORF14* contain three sequence regions which are usually found in *S*-adenosylmethionine-dependent methyltransferases (Kagan & Clarke, 1994) (Fig. 3). Motif I, which has been defined as a nine residue block with the consensus sequence (V/I/L)(L/V)(D/E)(V/I)G(G/C)G(T/P)G, is located at position 111–120 in *AviG1* (VVDIGSNDG) with a conserved glycine residue at position 5. This motif has been suggested to be a possible component of the *S*-adenosylmethionine-binding site (Schluckebier *et al.*,

Fig. 4. HPLC chromatograms of extracts of the wild-type strain *Str. viridochromogenes* Tü57 (a), mutant *Str. viridochromogenes* GW1 (b) and the complemented mutant *Str. viridochromogenes* GW1 (c), showing characteristic signals for avilamycin A and avilamycin C. In the chromatogram of mutant *Str. viridochromogenes* GW1, no signal corresponding to avilamycin derivatives could be detected.

1995). An aspartate as a conserved acidic residue is found 17 residues C-terminal to this motif, which is characteristic for methyltransferases and which, as the crystal structures indicate, makes contact with bound S-adenosylmethionine (Schluckebier *et al.*, 1995). Putative second and third motifs are indicated in Fig. 3.

Inactivation of the *aviG1* gene

To assay the role of *aviG1* in avilamycin biosynthesis, pMIK1 was constructed containing *aviG1* with an in-frame deletion. After transformation of *Str. viridochromogenes* Tü57 by pMIK1, several erythromycin-resistant colonies were obtained. Integration of pMIK1 into the chromosome at the expected position was verified by Southern hybridization (data not shown). To allow for the second recombination event, integrants were screened for erythromycin sensitivity. The chromo-

somal mutation in mutant *Str. viridochromogenes* GW1, which showed erythromycin sensitivity, was analysed by Southern hybridization (data not shown). The internal 2.2 kb *SacI* fragment of pMIK1 was used to probe *EcoRI*-digested chromosomal DNA. Analysis of the wild-type *Str. viridochromogenes* Tü57 showed one signal consisting of the two expected fragments of 1.7 kb after hybridization. When chromosomal DNA from clone *Str. viridochromogenes* GW1 was treated in the same way, two hybridization signals were obtained, one of 1.7 kb and one of 1.5 kb, indicating that the 285 bp *NcoI* fragment was deleted. To confirm the presence of the deletion within the chromosome of *Str. viridochromogenes* GW1, chromosomal DNA of the mutant and the wild-type strain was subjected to PCR analysis. The size of the amplified fragment (0.9 kb) detected in the mutant strain was identical to that generated with the inactivation construct pMIK1, while the wild-type strain gave a PCR product of the expected higher size (1.2 kb) (data not shown). The products accumulated by this mutant were also analysed for antibacterial activity. After cultivation of the mutant, culture supernatants were extracted as described in Methods and the extract was tested for antibiotic activity against *Bacillus subtilis*. No inhibitory effect on the growth of *B. subtilis* was observed for the pMIK1 mutant extract, but extracts of the wild-type showed inhibitory effects. In addition, TLC and HPLC analysis of the extract of mutant *Str. viridochromogenes* GW1 did not reveal the presence of any avilamycin derivatives (Fig. 4). Assuming that AviG1 is responsible for methylation at position C3 during 2-deoxy-D-evalose biosynthesis, an explanation for the breakdown of avilamycin biosynthesis could be that either the synthesis of evalose or the attachment of this sugar is blocked in the methyltransferase mutant. We also did not detect any orsellinic acid derivative in the mutant. We assume that the expression of *aviM*, encoding an orsellinic acid synthase, is suppressed in the absence of the saccharide moiety.

Complementation of mutant *Str. viridochromogenes* GW1

To determine clearly that the mutation event only affected the desired gene and not other genes, *aviG1* was ligated downstream of the *ermE* up-promoter, cloned into the integration plasmid pSET152 and introduced by protoplast transformation into the corresponding GW1 mutant. Analysis of extracts of complemented mutants by TLC and HPLC-UV clearly showed that avilamycin C and avilamycin A production was restored (Fig. 4). Thus, we could rule out any upstream or downstream effects.

Complementation of the erythromycin *eryBIII* mutant 335

To demonstrate the complementation of the *Sac. erythraea* *eryBIII* mutant 335, plasmid pSGaviG1 was isolated which places the expression of *aviG1* under the control of the activator ActII-ORF4 (Rowe *et al.*, 1998).

Fig. 5. ES-MS spectra of extracts of *eryBIII* mutant 335 (a) and *eryBIII* mutant 335 containing *aviG1* (b), showing signals characteristic for 3''-C-desmethyl erythromycin A (m/z 720.7) and erythromycin A (m/z 734.7).

Extracts of culture supernatants of the wild-type strain, *eryBIII* mutant 355 and *eryBIII* mutant 355 containing *aviG1* were analysed by ES-MS. Extracts of the wild-type strain gave the expected signal for erythromycin A at m/z 734.7. Mutant 355 gave a signal at m/z 720.7, which indicates the formation of 3''-C-desmethyl erythromycin A (Gaisser *et al.*, 1998). The MS data obtained with the *eryBIII* mutant 355 containing *aviG1* showed peaks at m/z 720.7 and 734.7, indicating that erythromycin A as well as 3''-C-desmethyl erythromycin A are formed (Fig. 5). To confirm that methylation

occurred at 3''-C of L-mycarose, MS/MS analyses of the peaks at m/z 734.7 and m/z 720.7 were performed. In both cases, a daughter ion at m/z 576.5 was detected unambiguously, indicating the loss of the 3''-C sugar (data not shown). From these data we can conclude that the expression of *aviG1* did allow complementation of the *eryBIII* mutation. *AviG1* seems to be able to participate in L-mycarose biosynthesis, providing further support for the proposal that *AviG1* encodes a C-methyltransferase capable of acting on position C3 of an activated sugar. Since L-mycarose and 2-deoxy-D-evaiose differ mainly in their stereochemistry at position C5, *AviG1* is therefore most likely involved in the synthesis of 2-deoxy-D-evaiose. However, results using bioassays revealed that the expression of *aviG1* restored erythromycin production just to a very small extent (data not shown). No reproducible difference could be detected between the size of the haloes of *Sac. erythraea* 335 and *Sac. erythraea* 335 expressing *aviG1* (data not shown).

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Received 18 May 2001; revised 13 September 2001; accepted 15 October 2001.