Journal of Industrial Microbiology & Biotechnology (2001) 27, 386–392

www.nature.com/jim

Characterization of the biosynthetic gene cluster for the oligosaccharide antibiotic, Evernimicin, in *Micromonospora carbonacea* var. *africana* ATCC39149

TJ Hosted, TX Wang, DC Alexander and AC Horan

Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033, USA

Evernimicin (EV) belongs to the orthosomycin class of antibiotics and consists of several modified L- and D-deoxysugars containing unusual orthoester and glycosyl linkages and two orsellinic acid groups, one that is halogenated. The EV biosynthetic gene cluster from *Micromonospora carbonacea* var. *africana* ATCC39149 was localized by hybridization to a dTDP-D-glucose 4,6-dehydratase probe and a 120-kb region containing the EV biosynthetic cluster and surrounding regions has been sequenced. BLAST analysis has identified a type I polyketide synthase for orsellinic acid biosynthesis as well as enzymes required for L- and D-deoxyglucose and D-deoxymannose synthesis. In addition, genes involved in glycosyltransfer and resistance were identified. Insertional mutations in several biosynthetic genes blocked EV production, indicating a role for these genes in EV biosynthesis. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 386–392.

Keywords: *Micromonospora*; Evernimicin; oligosaccharide antibiotic; glycosyltransferase; antibiotic biosynthesis

Introduction

Evernimicin (EV), an oligosaccharide antibiotic characterized as an orthosomycin, is produced by *Micromonospora carbonacea* var. *africana* (ATCC39149, SCC1413). ATCC39149 also produces chloramphenicol and a novel glycosylated thiostrepton [28]. EV consists of eight deoxysugar residues, including both mannose- and glucose-derived sugars, attached to modified orsellinic acid (Figure 1) [13,35]. The orthosomycins, which include curamycins, avilamycins and flambamycins, contain at least one acidic phenolic hydrogen and two orthoester linkages associated with the deoxysugar residues [14,23,24]. EV is structurally similar to avilamycin-A produced by *Streptomyces viridochromogenes* Tu57, but differs by an additional orsellinic acid moiety, an unusual L-nitro sugar and alternate deoxysugar groups in the oligosaccharide backbone (Figure 1).

These lipophilic oligosaccharide antibiotics exhibit broad-spectrum biological activity against Gram-positive and some Gram-negative bacteria including glycopeptide-resistant enterococci, methicillin-resistant staphylococci and penicillin-resistant streptococci [11]. EV's antibiotic activity is due to inhibition of protein synthesis, specifically by high-affinity binding to the 50S ribosomal subunit [21]. Mutations in the ribosomal L16 of *Streptococcus pneumoniae* resulted in a reduced susceptibility to EV [1]. In addition, a newly described rRNA methyltransferase, AviRa, from the *S. viridochromogenes* Tu57 avilamycin gene cluster can confer avilamycin resistance when expressed in *S. lividans* [36].

Many secondary metabolites contain deoxysugars that are synthesized by the combined action of dTDP-D-glucose synthetase (GS) and dTDP-D-glucose 4,6-dehydratase (GDH) forming the common intermediate dTDP-4-keto-6-deoxyhexose. The dTDP-4-keto-6-deoxyglucose intermediate can be further modified to form structurally diverse sets of deoxysugars by isomerization, epimerization, deoxygenation, transamination and methylation [26,33]. GS- and GDH-encoding genes are often linked to secondary metabolic gene clusters producing glycosylated products [18,20,22,27]. We utilized degenerate polymerase chain reaction (PCR) primers to amplify a DNA fragment with GDH homology from ATCC39149 chromosomal DNA. The resulting PCR product was used as a probe to isolate cosmids with GDH homology that also contained EV biosynthetic genes. We report here the localization of the EV biosynthetic cluster, sequences analysis and insertional mutagenesis of EV biosynthetic genes.

Correspondence: Dr TJ Hosted, Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033, USA
Received 26 January 2001; accepted 16 July 2001

Materials and methods

Bacterial growth and fermentation conditions

ATCC39149- and ATCC39149-derived strains are listed in Table 1. For isolation of chromosomal DNA and preparation of ATCC39149 for conjugation, strains were grown in Trypticase soy broth (BRL, Bethesda, MD). For fermentation studies, strains were grown in FICo, containing 0.5% yeast extract, 4.0% PD 650 dextrin, 2.2% glucose, 0.6% meat peptone, 0.4% $CaCO_3$, 0.5% corn steep powder and 0.0103 M $CoCl_2$ (pH 7.0), and SIM-1, 0.3% beef extract, 0.5% tryptone, 0.1% cerelose, 2.4% potato dextrin, 0.1% $CaCO_3$ (pH 7.5) medium at 30°C. *Escherichia coli* strains were grown in either TY medium or Circlegrow media (Bio101, Vista, CA). Antibiotics were added to appropriate media for *E. coli*, ATCC39149- and ATCC39149-derived strains for apramycin (Am; 30 μg/ml), naladixic acid (Nal; 50 μg/ml), Ampicillin (Ap; 100 μg/ml), kanamycin (Km; 25 μg/ml) and chloramphenicol (Cm; 12.5 μg/ml).

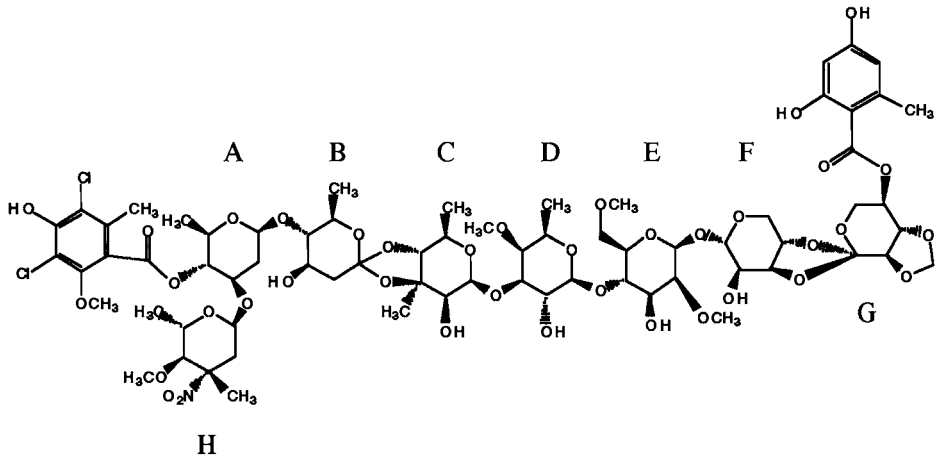

Figure 1 Structure of EV. Glycosyl groups are labeled with letters.

Determination of EV production

A mycelium stock of strain ATCC39149 was inoculated into the seed medium SIM-1 (2.5 ml) and incubated at 28°C and 300 rpm. A 5% inoculum was then added to FICo production medium (50 ml) and incubated at 28°C at 300 rpm for 96 h. A 10-ml aliquot of the fermentation broth was extracted with 20 ml of ethylacetate, and the organic phase was evaporated to dryness. After resuspension in 2 ml of methanol, 10 ml of the extract was subjected to high-performance liquid chromatography (HPLC) analysis on a YMC-pack ODS-A C-18 column (3 mm, 150×4.6 mm; Waters, Milford, MA). The column was equilibrated with 3 mM tetramethyl ammonium hydroxide (pH adjusted to 7.2 with glacial acetic acid) with 70% (vol/vol) methanol and developed with a 24-min linear gradient from 70% to 90% methanol in the same 3 mM tetramethyl ammonium hydroxide buffer at a flow rate of 0.7 ml/min. EV was detected at 270 nm by UV–Vis detection using an Agilent Series 1100 HPLC system (Agilent Technologies, Wilmington, DE).

DNA manipulations

The methods of Sambrook *et al* [31] were used for plasmid isolation, restriction enzyme digestion, random priming and Southern analysis. Restriction endonucleases and other enzymes

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source or reference
E. coli XL1-blue MFR′	General cloning host	Stratagene
E. coli XL10-Gold	General cloning host	Stratagene
E. coli ET12567	pUB307 containing strain for intergenic conjugation	[10]
ATCC39149	Wild type (WT) everninocin producer	This study
ATCC39149 SPH572	WT derivative with *evrJ* disrupted by insertion of pSPRX572	This study
ATCC39149 SPH570	WT derivative with *evrF* disrupted by insertion of pSPRX570	This study
ATCC39149 SPRA2377	WT derivative with *evrW* disrupted by insertion of pSPRA2377	This study
pNOTA	PCR fragment cloning vector	5′ 3′ Inc.
pBluescript II KS$^-$	Cloning vector	Stratagene
pSupercosII	Cosmid cloning vector	Stratagene
pJR255	ColE, AmR	[25]
p16R1	pAL5000 origin, HmR	[15]
pRL1058	ColE1, oriT from RK2	[37]
pSPRH825	1.1 kb *Nru*I–*Not*I HmR fragment from p16R1 ligated to *Ssp*I-digested, T4 polymerase-treated pUC19	This study
pSPRH826b	787 bp *Pst*I, T4 polymerase-treated oriT fragment from pRL1058 ligated to *Nde*I-digested, T4 polymerase-treated pSPRH825	This study
pSPRH900b	2.6 kb *Eam1 104*I PCR product amplified from pSPRH826b ligated to a 1.3-kb *Eam1 104*I PCR product amplified from pJR225	This study
pSPRA2350	*Pac*I adapter ligated to *Eco*RI-digested pSPRH900b	This study
pSPR131	520 PCR product amplified from ATCC39149 ligated to pNOTA	This study
pSPRX211	3.2 kb *Bam*HI fragment from pSPR192 ligated to *Bam*HI-digested pBluescript II KS$^-$	This study
pSPRX570	1.4 kb *Bam*HI fragment from pSPR192 ligated to *Bam*HI-digested pSPRH900b	This study
pSPRX572	1.8 kb *Bam*HI fragment from pSPR192 ligated to *Bam*HI-digested pSPRH900b	This study
pSPRA2377	0.7 kb PCR product internal to *evrW* amplified from pSPR192 ligated to *Pac*I–*Xba*I-digested pSPRA2350	This study

were used according to the manufacturer's recommendations. Strain ATCC39149 genomic DNA was isolated as described previously [16]. DNA sequence was determined using a Taq Dye Deoxy Terminator Cycle sequencing kit (SeqWright, Houston, TX). High-GC DNA PCR amplification was performed utilizing the GC Melt Kit (Clontech, Palo Alto, CA). Primers for PCR amplification were designed using the Oligo Primer Analysis software (National Biosciences, Plymouth, MN).

PCR amplifications, resistance markers and adapters

Degenerate PCR primers PR222 (5′ CSG GSG SSG CSG GST TCA TSG G 3′) and PR223 (5′ GGG WRC TGG YRS GGS CCG TAG TTG 3′) were used to amplify a 520 bp fragment internal to GDH from strain ATCC39149. Primers PRD5 (5′ GCCTCTT-CGATAGGAGTCAGGCAACTATGGATG 3′) and PRD6 (5′ GCCTCTTCGGTGTTGTCTCATGAGCGGATACAT 3′) were used to amplify a 2.6 kb fragment from pSPRH826b. Primers PRD11 (5′ AACTCTTCACACCCGCGTTCAGCCAGCATCT 3′) and PRD12 (5′ AACTCTTCATATAGACGTCGCGGTGAGTT-CAGG 3′) were used to amplify a 1.3 kb fragment containing AmR from pJR255 [25]. Primers PR1387 (5′ CCTCTTAAT-TAAACGTCCGCGACCTGGCCACCG 3′) and PR1388 (5′ GCCATCTAGAGCAGCCGCCCGGTCAGCTCCATG 3′) were used to amplify a 0.7 kb internal fragment of *evrW*. A *Pac*I site adapter (5′ AATTGTTAATTAAC 3′) was designed and synthesized. An *HmR* gene from p16R1 [15] and oriT from pRL 1058 [37] were used in plasmid constructions.

Transformation, electroporation and conjugation

Plasmids were introduced into *E. coli* strains by transformation [31]. Intergenic conjugation from *E. coli* ET12567(pUB307) into strain ATCC39149 was performed according to the method of Flett *et al* [10] on ASI plates buffered with 0.25 M TES pH 7.2, supplemented with a trace element solution.

Cosmid library preparation and isolation of the EV biosynthetic gene cluster

A strain ATCC39149 chromosomal DNA library was prepared in pSupercos II according to the manufacturer's recommendations using Gigapack II XL for packaging of the ligation products (Stratagene, La Jolla, CA). Packaged ligation product was transduced into *E. coli* XL1 Blue MFR′. Screening filters were prepared and probed as described previously [16]. A probe containing part of the dTDP-glucose 4,6-dehydratase gene amplified from strain ATCC39149 chromosomal DNA using PCR primers PR222 and PR223 was used to screen the cosmid library. Cosmid edge fragments were isolated by digestion of cosmid clones with *Bam*HI followed by self-ligation. These derivatives were digested with *Bam*HI–*Eco*RI to release left and right cosmid edge fragments for use as probes to screen the cosmid library for flanking cosmids.

Nucleotide sequence and data analysis

ORFs were determined by analysis of primary sequence by using Testcode and GC coding prediction programs from the Gene Inspector Kit (Textco, West Lebanon, NH). Amino acid sequence homology searches were performed using the BLAST server at the National Center for Biotechnology Information (Bethesda, MD) and non-redundant protein sequence databases [2].

Results

Cloning of the EV biosynthetic gene cluster

Three cosmids containing GDH-hybridizing sequences were identified by probing the cosmid library with a 520 bp fragment internal to the GDH gene amplified utilizing degenerate primers PR222 and PR223. Strain ATCC39149 genomic and cosmid DNA were digested with *Bam*HI and DNA fragments were separated by gel electrophoresis, blotted to nylon filters and analyzed by Southern hybridization using radiolabeled GDH probe. Both cosmid and strain ATCC39149 genomic DNA showed hybridization to a single 3.2 kb *Bam*HI fragment indicating a single copy of GDH on the chromosome. Sequence analysis of the 3.2 kb *Bam*HI fragment revealed ORFs with homology to GS and GDH.

To obtain the complete EV biosynthetic cluster, additional cosmids were identified by isolation of cosmid insert edge fragments for use as probes to screen the cosmid library and define the relative amount of cosmid overlap. Cosmids were mapped by partial digestion followed by Southern analysis using cosmid edge fragments as probes. In total, six overlapping cosmids, which extended over a continuous 190 kb of the ATCC39149 chromosome, were identified.

Sequence analysis of the EV biosynthetic cluster

The sequence data for a 120 kb DNA region were obtained by sequencing cosmids pSPR192, pSPRX262 and subclones derived from cosmids pSPRX272, pSPRX210 and pSPRX256. The overall GC content of the sequenced region was 72.4% and ORFs that had a characteristic high bias towards G or C in the third codon position characteristic of actinomycetes were identified [38]. BLAST analysis was performed to identify proteins of known function in the public database. Results of this analysis, for genes described in this work, are shown in Table 1. A map of ORFs identified within the EV biosynthetic cluster is shown in Figure 2.

Deduced functions of the proteins, deoxysugar biosynthetic genes

The gene product of *evrX* bears a close resemblance to GS proteins, such as StrD from the *S. griseus* streptomycin cluster [27]. The deduced protein for *evrW* shows homology to a family of secondary metabolism GDH proteins, such as AviE from *S. viridochromogenes* Tu57, the producer of avilamycin. These data suggest that EvrX forms dTDP-glucose and EvrW forms dTDP-glucose and 4-keto-6-deoxyglucose, both intermediates in deoxysugar biosynthesis. EvrD shows a high degree of similarity to a dGDP-D-mannose 4,6-dehydratases, such as *Nys*III from the *S. noursei* nystatin cluster [6]. Therefore, EvrD is predicted to form 4-keto-6-deoxymannose intermediates for the mannose-derived deoxysugar groups C and E in EV (Figure 1). The gene product of *evdB* shows a high degree of similarity to a family of dTDP-3-keto-6-deoxyhexose 3-aminotransaminases, such as DnrJ from the *S. peucetius* daunorubicin cluster [32]. Therefore, EvdB is presumably involved in transamination of the nitro-containing deoxysugar group.

Glycosyltransferases (GTases)

Five putative GTases, EvdD, EvdF, EvdH, EvdL and EvrS, are identified within the EV biosynthetic region. Four of the GTase genes, *evdD*, *evdF*, *evdH* and *evdL*, are closely linked, and the fifth

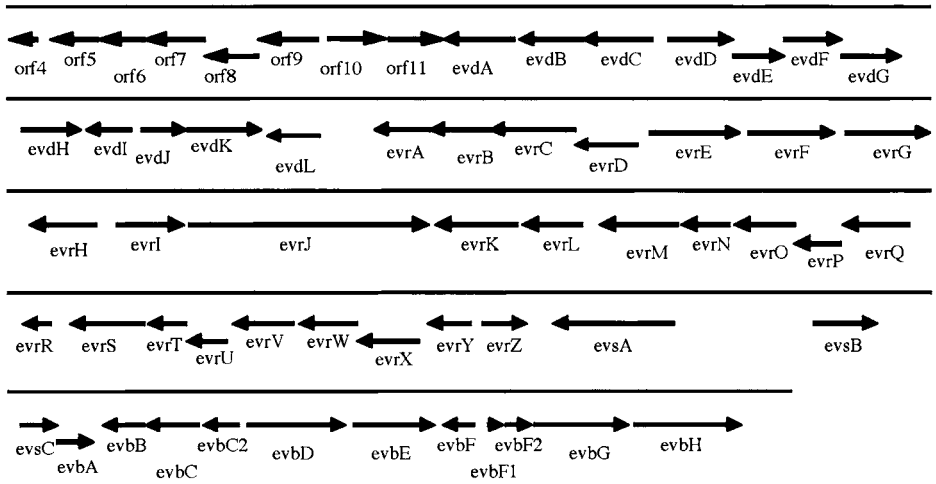

Figure 2 ORFs identified in the EV biosynthetic region.

GTase gene, *evrS*, is closely linked to *evrX* (Figure 2). BLAST analysis showed that the gene product of *evdF* showed homology to WbbL, a dTDP-L-rhamnosyl transferase involved in *Mycobacterium smegmatis* cell wall biosynthesis. The deduced protein of *evdL* shares homology to ExoM, a UDP-hexose transferase involved in *Bradyrhizobium japonicum* exopolysaccharide biosynthesis (Table 1) [5]. An alignment of GTases from several secondary metabolic clusters, ExoM from the *Rhizobium meliloti* lipopolysaccharide synthesis [5], WbbL from *M. smegmatis* cell wall biosynthesis, StrH from the *S. griseus* streptomycin cluster [7], and the EV GTases was performed and a phylogenetic tree was generated (Figure 3). Analysis of these results showed that the EV GTases clustered into a fourth class of secondary metabolism GTases and that EV GTases fall into two distinct groups.

Orsellinic acid synthesis

The gene product of *evrJ* shows a high degree of similarity to the family of iterative type I polyketide synthases (PKS), such as AviM from the *S. viridochromogenes* Tu57 avilamycin-A cluster [12]. Expression of AviM in *S. lividans* resulted in the synthesis of orsellinic acid [12]. The deduced EvrJ protein contains conserved PKS domains including a β-ketoacyl:ACP synthetase region with a highly conserved active site Cys-185 residue, an acyl-CoA acetyltransferase region containing a conserved AT domain motif (GVxPxxxGHSxGE) and an acyl carrier protein region containing a conserved Ser-1219 residue for attachment of the 4′-phosphopantheine prosthetic group. The deduced *evrI* gene product is closely related to a family of PKS acyl-carbon choice starter proteins, such as *dpsC* from the *S. peucetius* daunorubicin cluster [3,4]. These proteins are proposed to have β-ketoacyl:ACP synthetase III (KSIII) activity and initiate polyketide synthesis by condensation of an activated protein-bound acyl group with a PKS-bound acyl group. DpsC is specifically acylated at Ser-188 by propionyl CoA to form the activated DpsC-Ser-*O*-propionyl and is proposed to initiate daunorubicin synthesis by condensation with DpsG-ACP-malonyl [3,4]. EvrI contains a Cys-127 at the conserved acylation region for formation of EvrI-Cys-S-acyl. These data suggest that EvrI is acylated to form EvrI-Cys-S-acetyl and then initiates orsellinic acid synthesis by a KSIII type condensation with EvrJ-ACP-malonyl to form EvrI-ACP-acetoacetyl. The *evbD* gene product encodes a protein with a high degree of similarity to acyl-CoA carboxylases, such as AccA1 from *S. coelicolor*, suggesting that EvbD supplies malonyl-CoA precursor for orsellinic acid biosynthesis [29]. The deduced protein of *evrF* shows a high degree of similarity to a family of non-heme

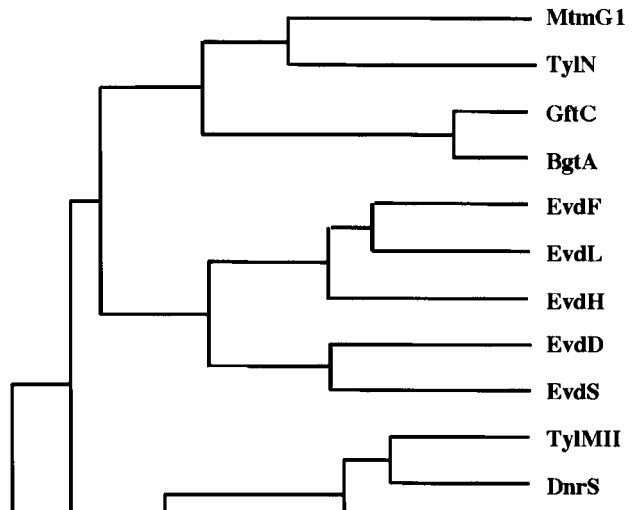

Figure 3 Phylogeny of amino acid sequences of actinomycete secondary metabolite GTases. GTases were aligned and a phylogenetic tree generated by the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) [30]. *S. argillaceus mithramycin* MtmG1 (AAC64927), *A. orientalis* GtfC (A82846), *S. fradiae* TylN (AAD12163), TylMII (CAA57472) and TylCV (AF147704), *S. peucetius* DnrS (AAD12163), *Saccharopolyspora erythraea* EryCIII (AAB84067), *S. avermitilis* AveB1 (AB032523), *A. mediterranei* BgtA(CAA76551), *B. japonicum* ExoM (AF039306) and *M. smegmatis* strain mc2155 WbbL (AF187550).

Table 2 Putative genes identified in the EV biosynthetic cluster

Gene designation	Size	Proposed function	Representative homolog	%ID/%SM
evrX	333	dTDP-glucose synthetase	A26984	62/77
evrW	355	dTDP-glucose 4,6-dehydratase	CAA72715	75/85
evrD	354	dGTP-mannose 4-6-dehydratase	AAF71765	65/78
evdB	474	hexose aminotransferase	B43306	70/78
evdD	390	glycosyltransferase	B70978	30/40
evdF	347	glycosyltransferase	B70978	30/40
evdL	304	glycosyltransferase	S39957	28/43
evdH	340	glycosyltransferase	CAA19930	24/37
evrS	423	glycosyltransferase	AAD15267	36/53
evrI	358	B-ketoacyl:ACP synthetase III activity	AAA65208	36/53
evrJ	1264	orsellinic acid synthetase	CAA72713	65/74
evbD	582	acyl-CoA carboxylase	CAB95892	66/73
evrF	229	non-heme halogenase	CAA11780	35/52
evrH	348	23S rRNA methylase	H75367	28/38
orf6	252	23S rRNA methylase	AF317789	49/59
evrE	492	MDR efflux pump	AAF00219	47/62
evbB	251	ABC type efflux pump	AAC443581	31/47
evbC	319	ATP binding protein	AAC44357	46/63
evrZ	250	1,4-Beta-*N*-acetylmuramidase	CAB61285	65/77

halogenases, such as pCZA361.26, from the *Amycolatopsis orientalis* vancomycin biosynthetic cluster [34].

Putative resistance genes

Within the EV biosynthetic cluster, several distinct classes of putative resistance genes were identified (Table 2). The gene product of *evrE* shares homology with a family of multidrug efflux transporters, such as UrdJ from *S. fradiae* Tu2717 [8]. The deduced proteins for *evbB* and *evbC*, which share strong homology to the family of ATP-binding cassette transporters, such as MtrA and MtrB from *S. argillaceus*, confer resistance to mithramycin [9]. The gene product of *orf6* shares homology to AviRa, a recently described rRNA methyltransferase from *S. viridochromogenes* Tu57 [36]. The gene product of *evrH* shares homology at the C-terminal region to rRNA methyltransferases. The gene product of *evrZ* shows a high degree of similarity to a family of 1–4 beta-*N*-acetylmuramidases, such as Acm from *S. globisporus* [19].

Insertional inactivation of the EV cluster

EvrJ (orsellinic acid synthetase), *evrF* (halogenase) and *evrW* (GDH) were disrupted in ATCC39149 *via* homologous recombination using the conjugative suicide vectors pSPRH900b or pSPRA2350. Internal fragments of *evrJ*, *evrF* and *evrW* were cloned into suicide vectors and these plasmids were inserted into the strain ATCC39149 chromosome by conjugation from *E. coli* ET12567 (pUB307) to yield mutant strains SPR572, SPR570 and SPRA2377, respectively. Southern analysis confirmed insertion into the correct chromosomal loci for each plasmid. Fermentation extracts from strain ATCC39149 and mutant strains SPH572, SPH570 and SPH2377 were analyzed by HPLC. EV production was abolished in all mutant strains, indicating that *evrJ*, *evrF*, and *evrW* are essential for EV biosynthesis.

Discussion

Based on the results derived from analysis of the genes found in the EV biosynthetic cluster, we can propose a model for biosynthesis of the major structural moieties, formation of glycosidic bonds and resistance mechanisms for EV in the producing organism ATCC39149.

Synthesis of orsellinic acid would begin by acetylation of EvrI by acetyl-CoA to form EvrI-Cys-acetyl. A FASIII type condensation between EvrI-Cys-acetyl and EvrJ-ACP-malonyl would form EvrJ-ACP-acetoacetyl. This reaction is directly analogous to the first step in fatty acid biosynthesis as carried out by FASIII where acetyl-CoA is condensed with ACP-malonyl-CoA to form acetoacetyl-ACP. Synthesis would proceed by condensation of two additional malonyl-CoA extender units directed by EvrJ, followed by cyclization and release of orsellinic acid. Acetyl-CoA would presumably be provided for by primary metabolic routes derived from glycolysis and fatty acid biosynthesis [17] and malonyl-CoA would be provided from carboxylation of acetyl-CoA by EvbD. EvrF halogenation of orsellinic acid to form a dichloroisoverninic acid moiety could occur prior to or after attachment of deoxysugar groups.

Synthesis of the glucose-derived deoxysugars in EV would begin by activation of 1-P-D-glucose by EvrX (GS) to form dTDP-D-glucose followed by conversion to dTDP-4-keto-6-deoxyglucose by EvrW (GDH). Mannose derived deoxysugar groups would be supplied from primary metabolism conversion of 6-P-D-mannose to 1-P-mannose through the action of a phosphomannomutase and activation to dGDP-mannose by a dGDP-D-mannose synthase. EvrD (dGDP-mannose 4,6-dehydratase) would then convert dGDP-D-mannose to dGDP-4-keto-6-deoxymannose. This is similar to the NysDIII biosynthesis of the mannose derived glycosyl group, mycosamine, in *S. noursei* ATCC 11455 nystatin biosynthesis [6]. Therefore, an interaction between primary and secondary metabolism is required for synthesis of mannose derived deoxysugar groups in EV. In addition, EV GTases would utilize both dGDP- and dTDP-activated deoxysugars.

EV contains several glycosidic linkages that include unusual linkages at deoxysugar non-reducing positions for attachment to the orsellinic acid moiety between deoxysugars G–H and B-1–4 linkages and between deoxysugars A–B and D–E that resemble cell wall glycosidic bonds. We have identified five EV linked

GTases; therefore, certain EV GTases may form multiple glycosidic linkages or GTases encoded outside the EV cluster are involved in EV glycosidic bond formation.

Interestingly, phylogenetic analysis indicates the EV GTases represent a fourth class of secondary metabolites distinct from previously described GTases (Figure 2). The EV GTases cluster with GTases involved in oligosaccharide biosynthesis in *B. japonicum* and *M. smegmatis*.

The presence of multiple resistance mechanisms linked to a secondary metabolic cluster is not unusual. We have identified two efflux mechanisms, an ABC transporter composed of EvrB and EvrC, and EvrE a MDR efflux pump that may export EV. The AviRa RNA methyltransferase from *S. viridochromogenes* confers avilamycin resistance presumably by blocking binding of avilamycin to the ribosome. In a similar manner, ORF6 may confer EV resistance by methylation of ribosomal rRNA. 1–4 Beta-*N*-acetylmuramidases hydrolyze the 1–4 beta-linkages between *N*-acetyl-D-glucosamine and *N*-acetylmuramic acid in peptidoglycan cell walls. Therefore, EvrZ may cleave the B-1–4 linkages between deoxysugars A and B or C and D in the EV oligosaccharide backbone (Figure 1). Thus, EvrZ may represent a novel class of resistance mechanisms that acts by cleavage of B-1–4 linkages in oligosaccharide antibiotics.

Identification of genes required for synthesis of the major precursors for EV should allow enhanced yield of EV by overexpression of precursor genes. In particular, overexpression of EvrX and EvrW would provide additional dNDP-deoxysugar precursor pools and overexpression of EvbD would provide additional malonylCoA for orsellinic acid biosynthesis. Analysis of the EV biosynthetic cluster provides a starting point for future modification of the biosynthetic cluster to produce EV analogs with enhanced or altered biological activity.

Acknowledgements

The authors thank Ron Mierzwa, Min Chu and Mahesh Patel of the Schering-Plough Research Institute, Natural Products Department, for their helpful discussions.

References

1 Adrian PV, W Zhao, TA Black, KJ Shaw, RS Hare and KP Klugman. 2000. Mutations in ribosomal protein L16 conferring reduced susceptibility to Evernimicin (SCH27899): implications for mechanism of action. *Antimicrob Agents Chemother* 44: 732–738.
2 Altschul SF, W Gish, W Miller, EW Myers and DJ Lipman. 1990. Basic local alignment search tool. *J Mol Biol* 215: 403–410.
3 Bao W, PJ Sheldon and CR Hutchinson. 1999. Purification and properties of the *Streptomyces peucetius* DpsC beta-ketoacyl:acyl carrier protein synthase III that specifies the propionate starter unit for type II polyketide biosynthesis. *Biochemistry* 38: 9752–9757.
4 Bao W, PJ Sheldon, E Wendt-Pienkowski and CR Hutchinson. 1999. The *Streptomyces peucetius dpsC* gene determines the choice of starter unit in biosynthesis of the daunorubicin polyketide. *J Bacteriol* 181: 4690–4695.
5 Becker A, A Kleickmann, M Keller, W Arnold and A Puhler. 1993. Identification and analysis of the *Rhizobium meliloti* exoAMONP genes involved in exopolysaccharide biosynthesis and mapping of promoters located on the exoHKLAMONP fragment. *Mol Gen Genet* 241: 367–379.
6 Brautaset T, ON Sekurova, H Sletta, TE Ellingsen, AR Strm, S Valla and SB Zotchev. 2000. Biosynthesis of the polyene antifungal antibiotic nystatin in *Streptomyces noursei* ATCC 11455: analysis of the gene cluster and deduction of the biosynthetic pathway [published erratum appears in *Chem Biol* 2000: 7(7): R176]. *Chem Biol* 7: 395–403.
7 Distler J, A Ebert, K Mansouri, K Pissowotzki, M Stockmann and W Piepersberg. 1987. Gene cluster for streptomycin biosynthesis in *Streptomyces griseus*: nucleotide sequence of three genes and analysis of transcriptional activity. *Nucleic Acids Res* 15: 8041–8056.
8 Faust B, D Hoffmeister, G Weitnauer, L Westrich, S Haag, P Schneider, H Decker, E Kunzel, J Rohr and A Bechthold. 2000. Two new tailoring enzymes, a glycosyltransferase and an oxygenase, involved in biosynthesis of the angucycline antibiotic urdamycin A in *Streptomyces fradiae* Tu2717. *Microbiology* 146: 147–154.
9 Fernandez E, F Lombo, C Mendez and JA Salas. 1996. An ABC transporter is essential for resistance to the antitumor agent mithramycin in the producer *Streptomyces argillaceus*. *Mol Gen Genet* 251: 692–698.
10 Flett F, V Mersinias and CP Smith. 1997. High-efficiency intergeneric conjugal transfer of plasmid DNA from *Escherichia coli* to methyl DNA-restricting streptomycetes. *FEMS Microbiol Lett* 155: 223–229.
11 Foster DR and MJ Rybak. 1999. Pharmacologic and bacteriologic properties of SCH-27899 (Ziracin), an investigational antibiotic from the everninomicin family. *Pharmacotherapy* 19: 1111–1117.
12 Gaisser S, A Trefzer, S Stockert, A Kirschning and A Bechthold. 1997. Cloning of an avilamycin biosynthetic gene cluster from *Streptomyces viridochromogenes* Tu57. *J Bacteriol* 179: 6271–6278.
13 Ganguly AK, OZ Sarre, D Greeves and J Morton. 1975. Letter: structure of everninomicin D-1. *J Am Chem Soc* 97: 1982–1985.
14 Ganguly AK, AK Bose and NF Cappuccino. 1979. Structural studies on curamycins. *J Antibiot (Tokyo)* 32: 1213–1216.
15 Garbe TR, J Barathi, S Barnini, Y Zhang, C Abou-Zeid, D Tang, R Mukherjee and DB Young. 1994. Transformation of mycobacterial species using hygromycin resistance as selectable marker. *Microbiology* 140: 133–138.
16 Hosted TJ, DA Rochefort and DR Benson. 1993. Close linkage of genes encoding glutamine synthetases I and II in *Frankia alni* CpI1. *J Bacteriol* 175: 3679–3684.
17 Hutchinson CR, H Decker, K Madduri, SL Otten and L Tang. 1993. Genetic control of polyketide biosynthesis in the genus *Streptomyces*. *Antonie Van Leeuwenhoek* 64: 165–176.
18 Ikeda H, T Nonomiya, M Usami, T Ohta and S Omura. 1999. Organization of the biosynthetic gene cluster for the polyketide anthelmintic macrolide avermectin in *Streptomyces avermitilis*. *Proc Natl Acad Sci USA* 96: 9509–9514.
19 Lichenstein HS, AE Hastings, KE Langley, EA Mendiaz, MF Rohde, R Elmore and MM Zukowski. 1990. Cloning and nucleotide sequence of the *N*-acetylmuramidase M1-encoding gene from *Streptomyces globisporus*. *Gene* 88: 81–86.
20 Liu W and B Shen. 2000. Genes for production of the enediyne antitumor antibiotic C-1027 in *Streptomyces globisporus* are clustered with the cagA gene that encodes the C-1027 apoprotein. *Antimicrob Agents Chemother* 44: 382–392.
21 McNicholas PM, DJ Najarian, PA Mann, D Hesk, RS Hare, KJ Shaw and TA Black. 2000. Evernimicin binds exclusively to the 50S ribosomal subunit and inhibits translation in cell-free systems derived from both Gram-positive and Gram-negative bacteria. *Antimicrob Agents Chemother* 44: 1121–1126.
22 Merson-Davies LA and E Cundliffe. 1994. Analysis of five tylosin biosynthetic genes from the tyllBA region of the *Streptomyces fradiae* genome. *Mol Microbiol* 13: 349–355.
23 Mertz JL, JS Peloso, BJ Barker, GE Babbitt, JL Occolowitz, VL Simson and RM Kline. 1986. Isolation and structural identification of nine avilamycins. *J Antibiot (Tokyo)* 39: 877–887.
24 Ninet L, F Benazet, Y Charpentie, M Dubost, J Florent, J Lunel, D Mancy and J Preud'Homme. 1974. Flambamycin, a new antibiotic from *Streptomyces hygroscopicus* DS 23 230. *Experientia* 30: 1270–1272.
25 Paget E and J Davies. 1996. Apramycin resistance as a selective marker for gene transfer in mycobacteria. *J Bacteriol* 178: 6357–6360.
26 Piepersberg W. 1994. Pathway engineering in secondary metabolite-producing actinomycetes. *Crit Rev Biotechnol* 14: 251–285.
27 Pissowotzki K, K Mansouri and W Piepersberg. 1991. Genetics of streptomycin production in *Streptomyces griseus*: molecular structure and putative function of genes strELMB2N. *Mol Gen Genet* 231: 113–123.

28 Puar MS, TM Chan, V Hegde, M Patel, P Bartner, KJ Ng, BN Pramanik and RD MacFarlane. 1998. Sch 40832: a novel thiostrepton from *Micromonospora carbonacea*. *J Antibiot (Tokyo)* 51: 221–224.
29 Rodriguez E and H Gramajo. 1999. Genetic and biochemical characterization of the alpha and beta components of a propionyl-CoA carboxylase complex of *Streptomyces coelicolor* A3(2). *Microbiology* 145: 3109–3119.
30 Saitou N and M Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4: 406–425.
31 Sambrook J, EF Fritsch and T Maniatis. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Press, Cold Spring Harbor, NY.
32 Stutzman-Engwall KJ, SL Otten and CR Hutchinson. 1992. Regulation of secondary metabolism in *Streptomyces* spp. and overproduction of daunorubicin in *Streptomyces peucetius*. *J Bacteriol* 174: 144–154.
33 Trefzer A, JA Salas and A Bechthold. 1999. Genes and enzymes involved in deoxysugar biosynthesis in bacteria. *Nat Prod Rep* 16: 283–299.
34 van Wageningen AM, PN Kirkpatrick, DH Williams, BR Harris, JK Kershaw, NJ Lennard, M Jones, SJ Jones and PJ Solenberg. 1998. Sequencing and analysis of genes involved in the biosynthesis of a vancomycin group antibiotic. *Chem Biol* 5: 155–162.
35 Weinstein MJ, GH Wagman, EM Oden, GM Leudemann, P Sloane, A Murawski and J Marquez. 1965. Purification and biological studies of everninomicin B. *Antimicrob Agents Chemother* 5: 821–827.
36 Weitnauer G, S Gaisser, A Trefzer, S Stockert, L Westrich, LM Quiros, C Mendez, JA Salas and A Bechthold. 2001. An ATP-binding cassette transporter and two rRNA methyltransferases are involved in resistance to avilamycin in the producer organism *Streptomyces viridochromogenes* Tu57. *Antimicrob Agents Chemother* 45: 690–695.
37 Wolk CP, Y Cai and J Panoff. 1991. Use of a transposon with luciferase as a reporter to identify environmental responsive genes in a cyanobacterium. *Proc Natl Acad Sci* 88: 5355–5359.
38 Wright F and MJ Bibb. 1992. Codon usage in the G+C-rich *Streptomyces* genome. *Gene* 113: 55–65.