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Deciphering Biosynthesis of the RNA Polymerase Inhibitor Streptolydigin and Generation of Glycosylated Derivatives

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SUMMARY

The biosynthetic gene cluster for the dienoyltetramic acid streptolydigin was identified and characterized from the producer organism *Streptomyces lydicus* NRRL2433. Sequence analysis of an 80.8 kb DNA region revealed the presence of 38 ORFs, 29 of which are probably involved in streptolydigin biosynthesis and would code for all activities required for its biosynthesis. Six insertional inactivation mutants were generated in the sequenced region to prove its involvement in streptolydigin biosynthesis, to define the boundaries of the cluster, to functionally characterize some genes, and to generate novel derivatives. A model for streptolydigin biosynthesis is proposed that includes a probable domain skipping in the streptolydigin PKS and the participation of a free-standing adenylation domain protein. Some bioactive derivatives of streptolydigin with altered glycosylation pattern have been produced by combinatorial biosynthesis showing a certain degree of flexibility of the L-rhodinosyl transferase SlgG for the recognition of 2,3,6-trideoxyhexoses and 2,6-dideoxyhexoses, both in D- and L-configuration.

INTRODUCTION

Streptolydigin (Figure 1), produced by *Streptomyces lydicus* (Deboer et al., 1955), is a member of the tetramic acids family, which includes compounds with a large spectrum of biological activities such as antibiotic, antiviral, antifungal, and antitumor (Royles, 1995). Streptolydigin is a potent antibiotic inhibitor of bacterial RNA polymerase (RNAP), which interferes with RNA chain elongation process and thus inhibits DNA transcription (Siddhikol et al., 1969; von Meyenburg et al., 1978). Interestingly, streptolydigin exhibits only limited cross-resistance with rifampicin (Campbell et al., 2001) and no cross-resistance with other inhibitors of RNAP such as microcin J25 (Yuzenkova et al., 2002; Adelman et al., 2004) and sorangicin (Campbell et al., 2005). Recent studies have allowed the unraveling of the structural basis of inhibition of bacterial RNAP by streptolydigin and a definition of the "streptolydigin pocket" (Tuske et al., 2005; Temiakov et al., 2005). In addition, early reports showed that streptolydigin and structural analogs are potent inhibitors of terminal deoxynucleotidyl transferase (TdT), an enzyme poorly expressed in normal leukocytes but found in large amounts in leukocytes from patients with acute lymphoblastic leukemia or with rare cases of acute and chronic myelocytic leukemia (Dicioccio and Srivastava, 1976; Dicioccio et al., 1980). These reports point to a putative therapeutic value for these streptolydigin structural analogs for the treatment of leukemia exhibiting abnormal TdT activity. This positive effect has been shown by nucleoside analog cordycepin, which inhibits the activity of TdT and, in addition, shows cytotoxic activity against TdT^+ rather than TdT^- leukemic cells in vitro (Kodama et al., 2000; Foss, 2000). TdT activity in leukemia is associated with a poor prognosis in chemotherapy and survival time.

Several studies regarding the biosynthesis of streptolydigin have shown the incorporation of propionate, acetate, methionine, and glutamic acid in the form of β-methylaspartate in the main structure of the compound (Pearce et al., 1980; Pearce and Rinehart, 1983; Chen and Harrison, 2004; Chen et al., 2006). These studies point to a hybrid polyketide synthase nonribosomal peptide synthetase (PKS-NRPS) system for the biosynthesis of streptolydigin, which has been followed by the identification of a type II thioesterase gene (usually associated to type I PKS systems) in *S. lydicus* and demonstrated to be involved in streptolydigin biosynthesis (Yu et al., 2006). In addition, the biosynthesis of streptolydigin has been recently linked to fatty acid biosynthesis through inactivation experiments of *fabCF* genes in *S. lydicus* encoding an acyl carrier protein (ACP) and a beta-ketoacyl-ACP synthase II, respectively, belonging to a type II fatty acid synthase, that led to streptolydigin-nonproducing mutants (Zhao et al., 2009).

Here we report the cloning and characterization of the complete gene cluster for streptolydigin biosynthesis from *S. lydicus* NRRL2433. Insertional inactivation experiments provide evidence for the involvement of the identified genes in streptolydigin biosynthesis and the boundaries of the gene cluster. In addition, several glycosylated derivatives were generated by combinatorial biosynthesis after expressing deoxysugar biosynthesis genes in a mutant in which all genes specifically involved in the biosynthesis of L-rhodinose were deleted.

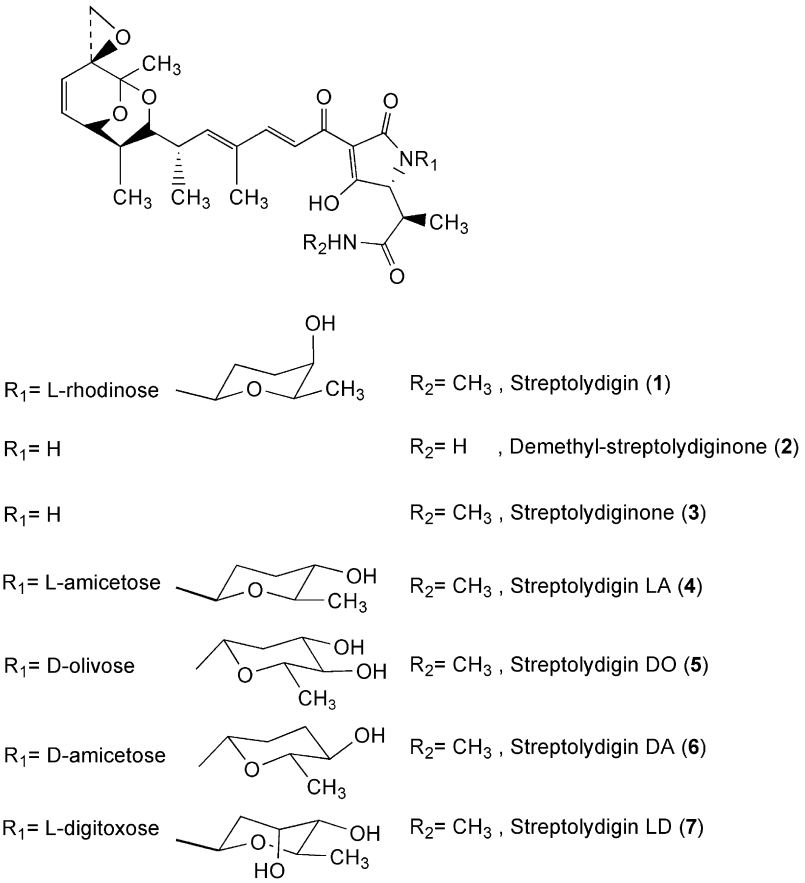

Figure 1. Structures of Streptolydigin, Streptolydigin Aglycon, and Novel Glycosylated Derivatives

RESULTS

Isolation and Characterization of the Streptolydigin Gene Cluster

A library of *S. lydicus* genomic DNA in cosmid pWE15 was screened by in situ colony hybridization using as probes: (i) borrelidin PKS coding genes from *Streptomyces parvulus* Tü4055 (Olano et al., 2004), (ii) *urdZ3* and *urdQ* genes from *Streptomyces fradiae* (Hoffmeister et al., 2000), and (iii) a gene encoding a thioesterase II (TEII) from *S. lydicus* (Yu et al., 2006). Using the PKS probe, 12 positive clones were isolated and verified by Southern hybridization. All clones initially identified overlapped and defined a unique chromosomal region. Two of them, Slg9C7 and Slg6G6 (sharing a region of 2.9 kb), were selected for further analysis. A 1.2 kb internal fragment to a PKS gene (as deduced from partial sequencing) and shared by both cosmids (Figure 2A) was subcloned into pOJ260 and the resultant construct pOJ961 used for gene disruption in *S. lydicus* leading to mutant SLM961. Ultra-performance liquid chromatography (UPLC) analysis of cultures of *S. lydicus* wild-type strain and SLM961 mutant revealed the latter to be a streptolydigin-nonproducer mutant (Figure 2B). Using the second probe (*urdZ3* and *urdQ* genes), another group of nine cosmid clones was identified, some of them previously isolated when using the PKS probe. In addition, the use of the third probe (TEII probe) against both groups of cosmids revealed the presence of this gene in cosmids Slg4A8 and Slg9C7. To test the involvement of Slg4A8 in streptolydigin biosynthesis, a 627 bp BamHI fragment internal to a PKS gene (Figure 2A) was used for gene disruption in *S. lydicus* leading to mutant SLM4C1. This mutant was also confirmed by UPLC analysis to be a streptolydigin-nonproducer mutant (Figure 2B). These experiments demonstrated that the cloned DNA region mapped in cosmids Slg4A8, Slg9C7, and Slg6G6 was involved in streptolydigin biosynthesis. However, sequence analysis showed the need for further extension to the left-hand side of the region because the biosynthetic gene cluster was not completed in Slg4A8 (Figure 2A). A 3 kb BamHI fragment from the border of Slg4A8 was used to rescreen the library leading to the identification of five new overlapping clones, one of which (Slg6E5) was selected for further sequencing. Finally, the nucleotide sequence of an 80,894 bp region was determined (EMBL accession number FN433113). Sequence analysis and alignment with related sequences in databases revealed the presence of 38 open reading frames (ORFs), all of them showing high G+C content and G+C bias in the third codon position that is characteristic of *Streptomyces* genes. Analysis of the deduced products of the different ORFs revealed the similarities shown in Table 1.

Delimiting the Boundaries of Cluster

Twenty-nine ORFS covering a region of 74.5 kb (from *slgR2* to *slgA1*), putatively assigned as streptolydigin biosynthesis genes, are flanked by nine ORFs (two at the left-hand side and seven at the right-hand side) presumably involved in primary metabolism and housekeeping (Figure 2A and Table 1). From these, *orf1* to *orf6* showed by BLAST analysis significant similarities to related proteins from *S. coelicolor* A3(2) (SCO1090, SCO1092, SCO1100, SCO1101, SCO1102, and SCO1103) and *S. avermitilis* MA-4680 (SAV1492, SAV1494, SAV1499, SAV1500, SAV1501, and SAV1502). Furthermore, they also share the same genetic organization as in these two streptomycetes. *orf7, orf8,* and *orf9* deduced gene products did not show similarities to proteins in databases that would suggest a role for these proteins in streptolydigin biosynthesis (Table 1 and Figure 2A). Taken together all this information, it was quite probable that all these *orfs* were not participating in streptolydigin biosynthesis. In order to unequivocally determine the boundaries of the cluster, *orf2* on the left-hand side and *orf3* on the right-hand side were inactivated by insertional inactivation. The resulting mutants SLM2A (*orf2*) and SLMA3 (*orf3*) were found to synthesize streptolydigin at the same level as *S. lydicus* (data not shown). These results provide evidence that *orf2* lies at the upstream end and *orf3* at the downstream end of the streptolydigin gene cluster (Figure 2A).

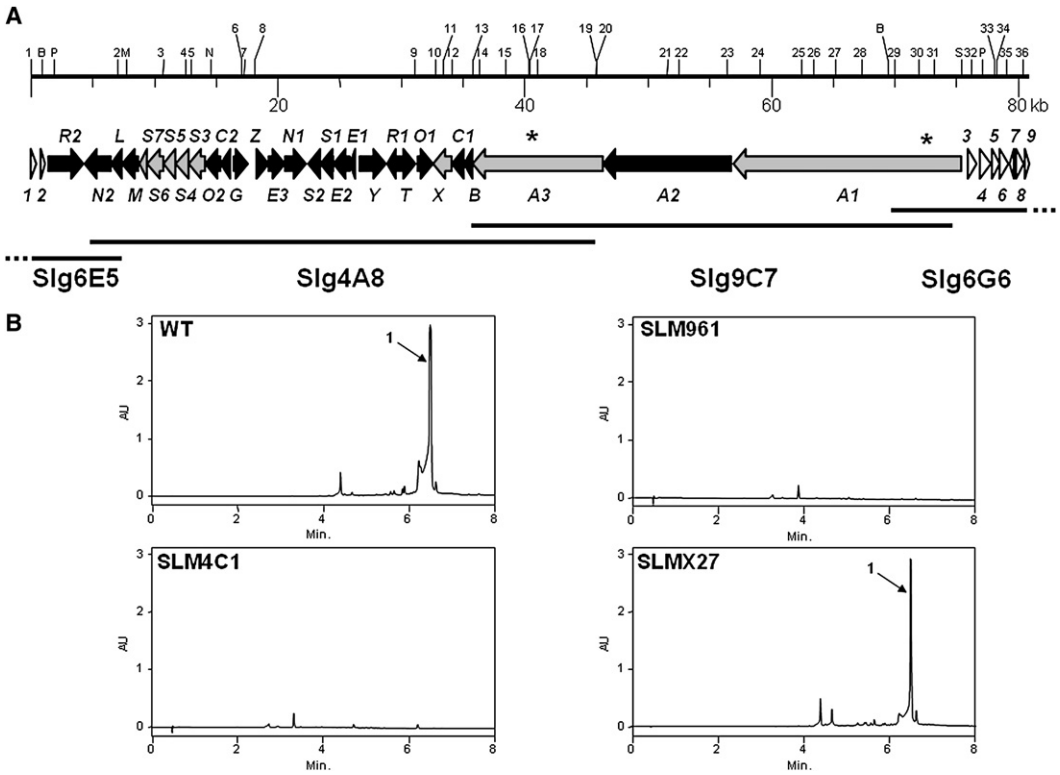

Figure 2. Genetic Organization of the Streptolydigin Gene Cluster and UPLC Analysis of *S. lydicus* Strains

(A) Genetic organization of the streptolydigin gene cluster. White arrows indicate genes not involved in streptolydigin biosynthesis. Black and gray arrows indicate genes involved in streptolydigin biosynthesis. Gray arrows indicate genes deleted in mutant SLM7H13 and disrupted in mutants SLMX27, SLM4C1, and SLM961. Slg6E5, Slg4A8, Slg9C7, and Slg6G6 represent cosmids containing the sequenced region. Asterisks show the BamHI fragments used for disruption in mutants SLM4C1 and SLM961. BamHI sites are numbered. B: BglII, M: MfeI, N: NruI P: PstI and S: StuI sites used for cloning purposes.

(B) UPLC analysis of *S. lydicus* wild-type (WT) and mutant SLM961, SLM4C1, and SLMX27. AU: arbitrary units. 1: Streptolydigin.

Hybrid PKS-NRPS System and Related Genes

Three ORFs, *slgA1*, *slgA2,* and *slgA3*, arranged head-to-tail and transcribed in the same direction, would code for a type I PKS containing a total of 34 individual domains organized into a loading module and seven extender modules (Table 2 and Figure 3). The *slgA1* gene would code for the loading and three extension modules. Because the loading module contains a KSQ domain, the biosynthesis of streptolydigin might start with a malonyl-CoA, which subsequently would be decarboxylated (Bisang et al., 1999). The *slgA2* and *slgA3* genes would code for the remainder streptolydigin PKS, SlgA2 and SlgA3, containing two modules each (Table 2 and Figure 3). All PKS extension modules contain conserved KS and ACP domains. There are DH domains at modules 3, 5, and 6 with the conserved active site (Table 2). The AT domains for these PKS extension modules display the active site motif and also contain the expected motifs for the selection of either malonyl-CoA or methylmalonyl-CoA as shown in Table 2 and Figure 3. All KR domains, except that from module 7, contain the conserved LDD motif characteristic for the B-type KRs (Caffrey, 2003). In addition, this KR7 lacks the characteristic Lx(S,G)RxG motif (Scrutton et al., 1990), possibly rendering this domain inactive.

Two ORFs, *slgN1* and *slgN2*, that would code for proteins with high similarity to NRPS, were found in the streptolydigin cluster. SlgN2 shows high similarity to LipNrps from *Streptomyces aureofaciens* involved in the biosynthesis of the tetramic acid α-lipomycin (Bihlmaier et al., 2006), and it also contains three domains, condensation (C), adenylation (A), and peptidyl carrier protein (PCP) (Figure 3). The C domain contains three of seven core motifs described for these domains, C2 (RHDxLRTxF), C5 (IGxHVNTLPxR), and particularly C3 (VHHxVFDTVS), which is the best-conserved core motif in all NRPSs (Marahiel et al., 1997). In addition, the thiolation motif is also present in the PCP domain (AxFFxLGGHSL). However, as it happens in LipNrps, the A domain is shorter than previously described for other NRPSs and it contains only four of ten core motifs described for these domains: A3 (VACxFTSGSTGxPKG), A4 (FVxS), A7 (YRRTGEL), and A8 (GRxDxRVTVHGxPVQLDRLE) (Marahiel et al., 1997). In particular, motif A2, especially conserved in NRPSs, and motifs A6 and A9, involved in adenylation, are absent in SlgN2 A domain. The absence of most of the A domain core motifs make it impossible to predict the constituents of the substrate binding pocket by comparison to other NRPS systems present in the available databases. *slgN1* would code for a discrete NRPS A domain like those described in leinamycin, clorobiocin, and coumermycin A_1 gene clusters (Tang et al., 2004a; Garneau et al., 2005). SlgN1 contains eight of the ten core motifs mentioned before: A1 (LTYxAL), A2 (LKAGxGYTMLDP), A5 (NxYGPAE), A6 (GELxVxGxGLAHGYV), A7 (YRTGDL), A8 (GRxDxQVKIRGxRVEPGEVE), A9 (LPxYMVP),

Table 1. Proposed Functions for Streptolydigin Biosynthesis Genes

Gene	aa[a]	Proposed Function in Streptolydigin Biosynthesis	Closest Similar Protein, Origin, (% Identity / Similarity), Accession Number
orf1	145[b]		Putative glycerophosphoryl diester phosphodiesterase SAV1492, *S. avermitilis* MA-4680, (72/80), BAC69202
orf2	128		Hypothetical protein SCO1092, *S. coelicolor* A3(2), (78/88), CAB95979
slgR2	943	LuxR family transcriptional regulator	ORF4, *Amycolatopsis orientalis,* (39/54), ABM47005
slgN2	707	NRPS domains C, A, T	LipNrps, *S. aureofaciens*, (43/54), ABB05101
slgL	280	Unknown	Hypothetical protein LipX2, *S. aureofaciens*, (38/53), ABB05100
slgM	426	N-methyl transferase	Acid7565, *Solibacter usitatus* Ellin6076, (34/50), ABJ88473
slgS7	218	dTDP-hexose 3,5-epimerase	RmlC, *Saccharopolyspora erythraea* NRRL 2338, (53/65), CAM05586
slgS6	434	dNDP-hexose 3,4-dehydratase	UrdQ, *S. fradiae*, (76/85), AAF72550
slgS5	340	dNDP-4-keto-6-deoxyhexose reductase	med-ORF14, *Streptomyces sp.* AM-7161, (44/53), BAC79033
slgS4	338	dNDP-hexose 3-ketoreductase	SchS1, *Streptomyces sp.* SCC 2136, (56/66), CAF31369
slgS3	450	dNDP-hexose 2,3-dehydratase	Francci3_4134, *Frankia sp.* CcI3, (55/66), ABD13482
slgO2	413	Cytochrome P450	SACE5814, *Saccharopolyspora erythraea* NRRL 2338, (54/67), CAM04998
slgC2	284	Unknown	Putative secreted hydrolase SAMR0843, *S. ambofaciens* ATCC 23877, (30/47), CAJ88552
slgG	391	Glycosyl transferase	SnogD, *S. nogalater*, (40/53), AAF01811
slgZ	326	Aspartate-ammonia ligase	PRK06462, *Pyrobaculum aerophilum str.* IM2, (52/69), AAL64277
slgE3	440	Ferredoxin-dependent glutamate synthase	CmaqDRAFT1154, *Caldivirga maquilingensis* IC-167, (39/56), EAZ64261
slgN1	567	NRPS domain A	CY0110_07826, *Cyanothece sp.* CCY0110, (43/63), EAZ87983
slgS2	328	dNDP-glucose 4,6 dehydratase	GilE, *S. griseoflavus*, (72/83), AAP69571
slgS1	355	dNDP-D-glucose synthase	StrD, *S. griseus subsp. griseus*, (71/84), CAH94331
slgE2	449	Glutamate mutase E-chain	VinI, *S. halstedii*, (63/72), BAD08366
slgE1	153	Glutamate mutase S-chain	VinH, *S. halstedii*, (63/77), BAD08365
slgY	699	Unknown	ATP/GTP-binding protein SAV4016, *S. avermitilis* MA-4680, (64/75), BAC71728
slgR1	220	TetR-family transcriptional regulator	SAML0351, *S. ambofaciens* ATCC 23877, (60/74), CAJ89338
slgT	515	Transport integral membrane protein	SAV3957, *S. coelicolor* A3(2), (55/69), CAC04255
slgO1	414	Putative cytochrome P-450	RubU, *S. collinus*, (51/62), AAM97370
slgX	515	Unknown	Ent-copalyl diphosphate synthase Ent-cdps, *Streptomyces sp.* KO-3988, (41/56), BAD86797
slgC1	300	Unknown	Putative secreted hydrolase SCO0787, *S. coelicolor* A3(2), (36/50), CAC14352
slgB	260	Type II thioesterase	LeuTE, *S. lydicus*, (94/95), AAZ20309
slgA3	3477	PKS modules 6 and 7	Orf9, *S. aizunensis*, (54/65), AAX98184
slgA2	3479	PKS modules 4 and 5	CONE, *S. neyagawaensis*, (54/66), AAZ94390
slgA1	6171	PKS loading domain and modules 1-3	Orf11, *S. aizunensis*, (52/64), AAX98186
orf3	246		Putative integral membrane protein SCO1100, *S. coelicolor* A3(2), (41/45), CAB95987
orf4	311		Putative integral membrane protein SAV1500, *S. avermitilis* MA-4680, (70/81), BAC69210
orf5	220		Putative integral membrane protein SAML1091, *S. ambofaciens* ATCC 23877, (56/69), CAJ90077
orf6	242		Putative hydrolase SAV1502, *S. avermitilis* MA-4680, (67/75), BAC69212
orf7	132		Putative lyase SCO1893, *S. coelicolor* A3(2), (79/85), CAB46394
orf8	205		Putative TetR-family transcriptional regulator Orf1006, *S. kanamyceticus*, (85/90), BAE95420
orf9	142[b]		Conserved hypothetical protein SAMR0656, *S. ambofaciens* ATCC 23877, (71/82), CAJ88366

[a] Amino acids of the deduced protein.
[b] Incomplete.

Table 2. Conserved Motifs Found in PKS Proteins Involved in Streptolydigin Biosynthesis

KS	Motif	AT	Motif	DH	Motif	KR	Motif	ACP	Motif
LD	DTAQSSG	LD	GxSxG…HAFH					LD	LGxDS
M1	DTACSSS	M1 (p)	GxSxG…YASH			M1	LxSRxG…LDD…GxGxxAxxxA	M1	LGxDS
M2	DTACSSS	M2 (a)	GxSxG…HAFH			M2	LxSRxG…LDD…GxGxxAxxxA	M2	LGxDS
M3	DTACSSS	M3 (p)	GxSxG…YASH	M3	LxxHxxxGxxxxP	M3	LxSRxG…LDD…GxGxxNxxxA	M3	LGxDS
M4	DTACSSS	M4 (p)	GxSxG…YASH			M4	GxGRxV..LDD…GxGxxGxxxA	M4	LGxDS
M5	DTGCSSS	M5 (p)	GxSxG…YASH	M5	LxxHxxxGxxxxP	M5	LxSRxA…LDD…GxGxxAxxxA	M5	LGxDS
M6	DTACSSS	M6 (a)	GxSxG…HAFH	M6	LxxHxxxGxxxxP	M6	LxSRxA…LDD…GxGxxAxxxA	M6	LGxDS
M7	DTACSSS	M7 (a)	GxSxG…HAFH			M7[a]	np np GxAxxAxxxA	M7	lGxAS
Con.[b]	**DTACSSS**	**Con.**[c]	**GxSxG…HAFH**	**Con.**[d]	**LxxHxxxGxxxxP**	**Con.**[e]	**LxSRxG…LDD…GxGxxGxxxA**	**Con.**[f]	**LGxDS**
			Y S				**G A**		

KS: ketosynthase, AT: acyl transferase, a: malonyl-CoA specific, p: methylmalonyl-CoA specific, DH: dehydratase, KR: ketoreductase, ACP: acyl carrier protein, np: not present.

[a] Inactive domain.

[b] Consensus sequence as determined by Aparicio et al. (1996).

[c] Consensus sequence as determined by Haydock et al. (1995) and Reeves et al. (2001).

[d] Consensus sequence as determined by Bevitt et al. (1992).

[e] Consensus sequence as determined by Scrutton et al. (1990) and Caffrey (2003).

[f] Consensus sequence as determined by Wakil (1989).

and A10 (NGKLDR), lacking motifs A3 and A4, which implies that a portion of the region between core motifs A3 and A6, thought to accommodate the binding pocket (Marahiel et al., 1997), is absent. That is probably the reason why the signature sequence found for the binding pocket (DALQIGGGFK) predicts the use of valine as substrate with a similarity of 80% with previously described sequences (Rausch et al., 2005).

In the streptolydigin gene cluster there are two ORFs, *slgB* and *slgL* (Figure 2A), coding for enzymes related to the PKS-NRPS system. The gene *slgL* would code for a protein with high similarity to LipX2 from *S. aureofaciens* (Table 1) and to hypothetical proteins from *Streptomyces verticillus*, *Streptomyces collinus,* and *Streptomyces griseus* subspecies *griseus*. LipX2 has been proposed to be involved in the adenylation of glutamic acid

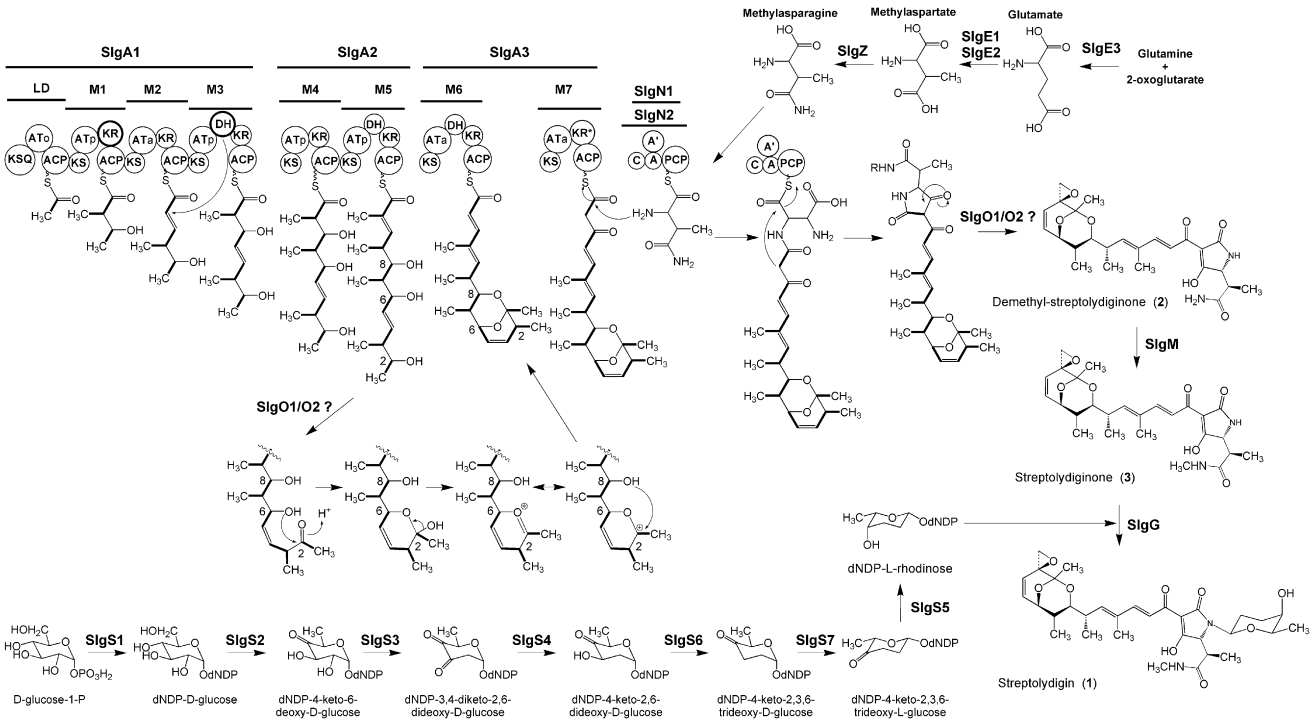

Figure 3. Proposed Pathway for Streptolydigin Biosynthesis

In bold, PKS domains that differ from the predicted model. LD: loading module, M: module, KS: ketosynthase, AT: acyl transferase, a: malonyl-CoA specific, p: methylmalonyl-CoA specific, ACP: acyl carrier protein, DH: dehydratase, KR: ketoreductase, C: condensation, A: adenylation, PCP: peptidyl carrier protein.

before it is bound to the PCP of LipNrps during the biosynthesis of α-lipomycin (Bihlmaier et al., 2006). The gene *slgB* would code for a type II thioesterase, which contains the characteristic GHSMG-130 aa-PGGHF motif at the catalytic center (Kotowska et al., 2002), and shows similarities to type II thioesterases from other *Streptomyces,* in particular LeuTE from *S. lydicus* AS 4.2501 (Table 1) involved in the biosynthesis of streptolydigin (Yu et al., 2006). Type II thioesterases have been proposed to rid PKS and NRPS of aberrant acyl and peptidyl chains that might otherwise block the enzyme complex (Heathcote et al., 2001; Schwarzer et al., 2002).

Precursor Supply

Three genes in the cluster, *slgE1*, *slgE2,* and *slgE3*, are probably involved in the biosynthesis of β-methylaspartic acid (Figure 3), putative substrate for the NRPS (Chen and Harrison, 2004; Chen et al., 2006). The amino acid sequences of SlgE1 and SlgE2 showed significant similarities to VinH and VinI, S and E subunits of a coenzyme B12-dependent mutase from *Streptomyces halstedii.* (Ogasawara et al., 2005). SlgE1 and SlgE2 are also similar to S and E subunits of glutamate mutases from *Actinoplanes friuliensis* and *Streptomyces ansochromogenes*, respectively (Heinzelmann et al., 2003; Li et al., 2005). SlgE3 shows high similarity to ferredoxin-dependent glutamate synthases and α subunits of NADPH-dependent glutamate synthases from different archaeas. SlgE3 might be involved in the channeling of glutamate specifically for the biosynthesis of streptolydigin.

Sugar Biosynthesis and Transfer

Several genes have been identified that might be involved in L-rhodinose biosynthesis (Table 1, Figure 3). Two of them, *slgS1* and *slgS2*, that are located together and transcribed in the same direction, would code for a dNDP-D-glucose synthase and a dNDP-D-glucose 4,6-dehydratase respectively. SlgS1 and SlgS2 contain ADP-glucose pyrophosphorylase (PDOC00638) and short-chain dehydrogenase/reductases (PDOC00060) conserved family signatures, respectively. In a different operon and apparently transcribed together are five additional genes *slgS3*, *slgS4*, *slgS5*, *slgS6,* and *slgS7*. SlgS3 shows strong similarity to dTDP-2,3-glucose dehydratases, SlgS4 shows similarity to 3-ketoreductases, which render hydroxyl groups at C-3 with an equatorial configuration, SlgS5 is similar to different 4-ketoreductases. SlgS6 shows strong similarity to dTDP-3,4-glucose dehydratases and SlgS7 resembles proteins that contain conserved domains for dTDP-sugar-isomerases (Pfam00908) and are proposed to act as 5- or 3,5-epimerases required for the D-hexose to L-hexose switch (Salas and Méndez, 2005). In another different operon there is an additional gene, *slgG*, encoding a glycosyltransferase based in its similarities with other known enzymes from the glycosyltransferase family 28 (Coutinho et al., 2003). SlgG might be responsible for the transfer of L-rhodinose to the streptolydigin aglycon.

Tailoring Modification

There are four genes in the cluster, *slgO1*, *slgO2*, *slgZ,* and *slgM,* that would code for enzymes that might be involved in oxygenation, amidation, and methylation steps required during the biosynthesis of streptolydigin (Figure 3). SlgO1 and SlgO2 are similar to cytochrome P450 enzymes. Both contain the conserved O_2 binding site, LLxAGxxT, and the C-terminal heme binding domain, FGxGxHxCxG, including the invariant cysteine residue that is the heme iron ligand (PDOC00081) associated with these enzymes (Mendes et al., 2005). SlgZ shows similarity to archaeal asparaginyl-tRNA synthetase-like enzymes and, in a similar manner to those proteins, it contains the catalytic core of asparaginyl-tRNA synthetases but lacks the N-terminal anticodon-binding site. This kind of truncated form of asparaginyl-tRNA synthetases have been shown to produce asparagine by amidation of aspartic acid (Roy et al., 2003). Based on the similarities found in protein databases, SlgM might be the methyltransferase responsible for methylation of the amino group present in the side chain of the acyltetramic moiety after its introduction by SlgZ. SlgM contains the methyltransferase-11 signature domain (pfam08241) present in SAM-dependent methyltransferases of this family of enzymes.

Regulation and Secretion

Two putative regulatory genes are located in the streptolydigin gene cluster, *slgR1*and *slgR2.* SlgR1 is similar to TetR-family transcriptional regulators from different streptomycetes. It contains the characteristic N-terminal helix-turn-helix (HTH) DNA binding domain signature present in all members of this family of regulatory proteins (pfam00440). These regulatory proteins generally act as repressors (Ramos et al., 2005), but there are examples of TetR-positive regulators like AtrA from *S. griseus* (Hirano et al., 2008). SlgR2 shows high similarity to PikD and LipReg4 regulatory proteins from pikromycin and α-lipomycin gene clusters in *S. venezuelae* and *S. aureofaciens*, respectively (Wilson et al., 2001; Bihlmaier et al., 2006). These proteins are members of the *l*arge *A*TP-binding regulators of the *L*uxR family (LAL) characterized by the presence of an ATP-binding domain represented by discernible Walker A, GxxGxGK, and Walker B, R/K-x(7-8)-h(4)-D, motifs (Walker et al., 1982), and a C-terminal LuxR type HTH DNA binding motif (PDOC00542). In addition, there are two TTA codons in *slgR2,* which might imply an upper level of regulation during streptolydigin production by some homolog to the pleiotropic regulatory gene *bldA* encoding a tRNA-Leu (Chater and Chandra, 2008).

One gene in the cluster, *slgT*, is a candidate thought to be involved in streptolydigin secretion outside the cell. SlgT is similar to transmembrane efflux proteins and contains 14 putative transmembrane-spanning helices. It shows high similarity to LipEx1, proposed to be involved in the export of tetramic acid α-lipomycin (Bihlmaier et al., 2006).

Unknown Function

Four genes in the cluster would code for proteins with an unclear role in streptolydigin biosynthesis. The *slgC1* and *slgC2* gene products contain the characteristic glycosyl hydrolase family 16 signature domain EIDVxExxGxxP and EMDVxExxGxxP (PDOC00794), respectively. They show similarity to putative secreted hydrolases and endo-1,3-β-glucanases from different *Streptomyces* sp. SlgX contains a terpene synthase signature (PDOC00825) and shows similarity to an ent-copalyl diphosphate synthase from *Streptomyces* sp. KO-3988 (Table 1) and other terpene cyclases. However, it lacks the characteristic DDxxD or DxDD motif present in the A or B type of isoprenoid

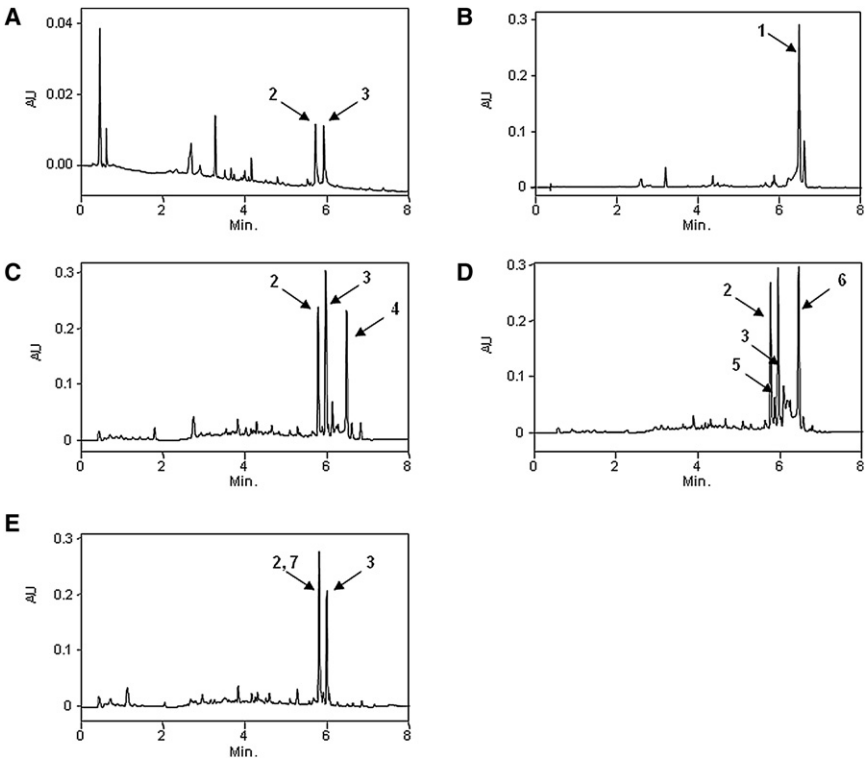

Figure 4. UPLC Analysis of *S. lydicus* Strains

UPLC analysis of *S. lydicus* strain SLM7H13 (A) and expressing pEM4T8-12 (B), pFL844T (C), pFL845T (D), and pLNBIVT (E). AU: arbitrary units.

cyclases, which are proposed to mediate substrate binding by chelating a divalent metal ion (Dairi, 2005). Inactivation of *slgX* was performed to verify its possible involvement in streptolydigin biosynthesis. The resultant mutant SLMX27 was able to produce the same yields of streptolydigin than *S. lydicus* (Figure 2B), indicating that this gene is not essential for streptolydigin biosynthesis in this strain. The *slgY* product shows similarity to ATP/GTP-binding proteins that belong to superfamily I of DNA and RNA helicases (COG3973). SlgY, like the other members of this family, contains an ATP-binding domain composed of Walker A and B motifs.

Rational Design of New Glycosylated Streptolydigins

Experiments were carried out to attempt the generation of novel streptolydigin derivatives. For this purpose, the *S. lydicus* mutant SLM7H13 was generated, causing the simultaneous deletion of most of all genes involved in L-rhodinose biosynthesis, genes *slgS3* to *slgS7* (Figure 3). Analysis of the products accumulated by mutant SLM7H13 (Figure 4A) showed two novel compounds with UPLC retention times of 5.7 min (compound **2**) and 5.8 min (compound **3**), with masses of 473 and 487 m/z ($[M+H]^+$), respectively. According to these masses, these compounds might lack the deoxyhexose moiety, as anticipated. The structural elucidation of both compounds was carried out using one-dimensional (1D) ^1H, two-dimensional (2D) ^1H correlated spectroscopy (COSY), ^1H, ^{13}C heteronuclear single quantum coherence (HSQC)-edited and heteronuclear multiple bond correlation (HMBC) nuclear magnetic resonance (NMR) experiments. In the case of compound **3**, we found analogous chemical shifts and atom connectivities to the NMR data of reference compound streptolydigin (**1**), but without resonances from the sugar moiety. This allowed us to identify compound **3** as streptolydiginone, the streptolydigin aglycon (Figure 1; see Table S1 available online). Product **2** has an identical NMR spectrum to molecule **3**, but lacking the resonance signal of the methyl group located in the streptolydigin acyltetramic moiety side chain, which allowed its characterization as a demethyl derivative of streptolydiginone (Figure 1 and Table S2), which is in agreement with the observed mass of 473 m/z ($[M+H]^+$). Production of streptolydigin in mutant SLM7H13 was restored by introduction of plasmid pEM4T8-12 containing genes *slgS3* to *slgS7* under the control of *ermE** promoter (Figure 4B).

Mutant SLM7H13, lacking all L-rhodinose specific biosynthesis genes but still possessing an active SlgG glycosyltransferase, was now used as recipient host to independently express plasmids directing the biosynthesis of different deoxyhexoses: pFL844T (L-amicetose), pFL845T (D-amicetose), pLN2T (L-olivose), pLNRT (D-olivose), and pLNBIVT (L-digitoxose). With two of these plasmids (pLN2T and pLNRT) no modification of the UPLC pattern was observed (data not shown). In contrast, analysis of strains SLM7H13/pFL844T, SLM7H13/pFL845T, and SLM7H13/pLNBIVT showed the appearance of several new UPLC peaks (Figure 4). Strain SLM7H13/pFL844T (Figure 4C) showed the production of compounds **2** and **3**, and the novel compound **4** with a UPLC retention time of 6.48 min and masses of 601 and 487 m/z $[M+H]^+$, ions corresponding to the unfragmented compound and the aglycon fragment, respectively. Structural elucidation by NMR of compound **4** (Figure 1 and Table S3) showed a streptolydigin derivative containing L-amicetose instead of L-rhodinose (streptolydigin LA, Figure 1). Signals characteristic of the amicetose sugar are observed in the 1D ^1H proton spectrum, and consistent connectivities were found in the 2D COSY, HSQC, and HMBC spectra. The presence of the anomeric proton at 5.3 ppm correlating with the amicetose proton 31 proves the union of the sugar moiety to the amide group of the tetramic ring. The coupling constant between 30 and the axial proton 31 is 12 Hz, which clearly corresponds to an axial-axial interaction. This means that the anomeric proton is at an axial position, while the streptolydigin unity has to be at an equatorial position, as expected for sterical reasons. Strain SLM7H13/pFL845T (Figure 4D) showed the production of compounds **2** and **3** and two novel compounds, **5** and **6**, with UPLC retention times of 5.73 and 6.48 min and masses of 617 and 601 m/z $[M+H]^+$, respectively. In both cases, molecular ions corresponding to fragments of those compounds lacking

the sugar moiety were detected with a mass of 487 m/z $[M+H]^+$. Due to the low production of compound **5,** we were unable to characterize this compound at the structural level. However, according to the observed masses, it might correspond to a streptolydigin derivative containing a 2,6-dideoxyhexose, probably D-olivose (streptolydigin DO, Figure 1), because it has been described that pFL845 can direct the biosynthesis of this sugar in addition to D-amicetose (Pérez et al., 2005). Instability of compound **6** allowed only partial structural elucidation. Thus, the NMR spectra showed similar patterns and molecular connectivity to compound **4**, including the anomeric proton at 5.3 ppm and other sugar-related signals, which allowed to identify **6** as a streptolydigin derivative probably containing D-amicetose instead of L-rhodinose (streptolydigin DA, Figure 1). This would be in agreement with the expected deoxyhexose synthesized by this plasmid, D-amicetose (Pérez et al., 2005). Analysis of the products accumulated by strain SLM7H13/pLNBIVT showed the production of compounds **2** and **3,** and a novel compound **7** with the same retention time as **2** (Figure 4E) and masses of 617 and 487 m/z $[M+H]^+$ corresponding to the unfragmented compound and the aglycon fragment, respectively. We were unable to separate compounds **7** and **2** for structural elucidation. However, according to the masses observed, compound **7** might correspond to a streptolydigin derivative containing a 2,6-dideoxyhexose, probably L-digitoxose (streptolydigin LD, Figure 1), because it is the deoxyhexose whose biosynthesis is directed by pLNBIV (Fischer et al., 2002).

After structural elucidation, compounds **3** (streptolydiginone) and **4** (streptolydigin LA) were tested for their antibacterial activity against *S. albus* in comparison with streptolydigin used as a control. Streptolydiginone was weakly active, showing only a narrow inhibition halo. In contrast, streptolydigin LA showed an inhibition halo similar to that of streptolydigin, indicating that this compound has similar antibacterial potency (Figure 5).

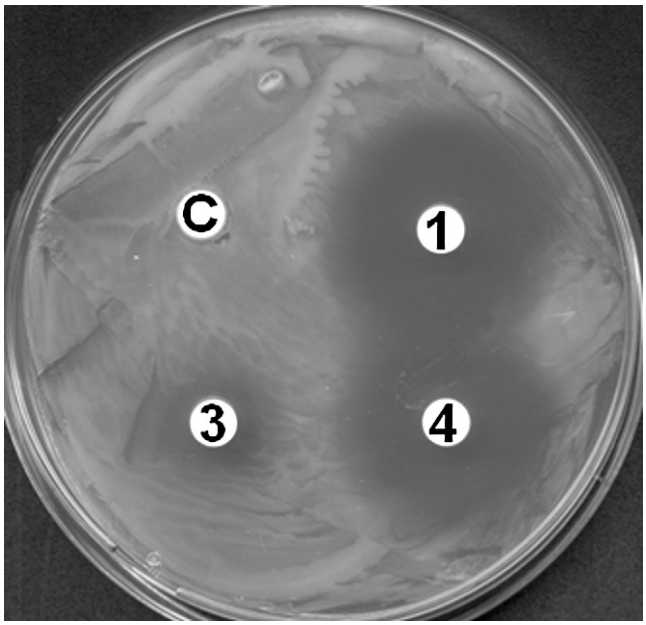

Figure 5. Bioactivity Testing of Streptolydigin (1), Streptolydiginone (3), and Streptolydigin LA (4) against *S. albus*
Each paper disk was soaked with 2 μg of the corresponding compound dissolved in 15 μl methanol. Negative control (**C**) contained 15 μl methanol and no antibiotic.

DISCUSSION

The *S. lydicus* streptolydigin gene cluster consists of 29 ORFs: 19 coding for structural proteins, 2 for pathway regulatory proteins, 3 involved in NRPS substrate supply, 1 involved in streptolydigin secretion, and 4 with unknown function. These genes are organized in at least eight transcriptional units. Genes encoding all the activities that are required for streptolydigin biosynthesis were identified within the gene cluster. The confirmation of the involvement of this cluster in streptolydigin biosynthesis was achieved by inactivation of *slgA1* and *slgA3* genes, coding for PKS enzymes. In addition, the boundaries of the gene cluster were defined by inactivation of *orf2* and *orf3,* leading to the generation of mutants still producing streptolydigin at the same level as the wild-type strain.

According to streptolydigin polyketide structure (Figure 1), 32 PKS domains were initially predicted, arranged in eight modules, but 34 were found (Figure 3), one of which was inactive (KR at module 7) (Table 2). The presence of a KSQ module in SlgA1 was predicted based on feeding experiments that showed the incorporation of malonyl-CoA instead of acetyl-CoA as starter unit (Chen and Harrison, 2004). There are two discrepancies in the PKS, taking in consideration streptolydigin structure. SlgA1 contains an unexpected KR domain at module 1 that dramatically changes the reduction pattern that might be necessary for the correct cyclization to form the bicyclic ketal (Figure 3) (Chen et al., 2006). In addition, DH domain in module 3 was expected in module 2. This DH domain is supposed to be active, because it contains the conserved active-site motif (Table 2). We have initially considered the possibility that this DH domain acts during module 2 and 3 extension cycles by a module-skipping mechanism, as shown before in the case of epothilone PKS (Tang et al., 2004b), thus introducing two double bonds because the KR domains in modules 2 and 3 seem active even though there is a nonconservative replacement in NADPH binding motif in module 3 (Table 2). The presence of these double bonds might require the introduction of a hydroxyl group at C-6 and the oxidation of the C-2 hydroxyl to a keto group by one of the SlgO monooxygenases. This would allow the correct cyclization to form the bicyclic ketal by C-6 hydroxyl group attack over C-2 to form a hemi-ketal. Loss of water and attack of the C-8 hydroxyl group on the resulting oxonium ion might give the bicyclic ketal as proposed before (Chen et al., 2006) (Figure 3). However, this mechanism does not agree with the previously reported biosynthetic experiments using oxygen-18 that clearly showed retention of both the C-6 and C-8 oxygen bonds from propionate and acetate, respectively, in the ketal (Chen et al., 2006). For this reason, the most plausible mechanism implies the DH3 activity only in module 2, which will provide at the end of M3 a product containing an intact C-O bond from acetate at C-6 and a *cis* double bond between C-4 and C-5, and the oxidation of the C-2 hydroxyl group to a ketone by the activity of SlgO1 or SlgO2 (Figure 3). This mechanism is consistent

with the labeling experiments and the cyclization model reported before (Chen et al., 2006).

The next step during the biosynthesis of streptolydigin requires the incorporation of β-methylaspartic acid by a NRPS module (Figure 3). Two proteins containing NRPS domains were found in the streptolydigin gene cluster, SlgN2 containing C, A, and PCP domains, and SlgN1 containing only an A domain. SlgN1 and SlgN2 lack some of the predicted A domain core motifs (Marahiel et al., 1997), but all the motifs are present in both proteins, which opens the possibility of cooperation between these proteins for the introduction of β-methylaspartic acid on the growing PKS chain. Additional proteins could be involved in the adenylation process, like SlgL due to its similarity to LipX2, involved in the biosynthesis α-lipomycin at the level of adenylation of glutamic acid before it is bound to the PCP of LipNrps (Bihlmaier et al., 2006). The involvement of SlgL in the biosynthesis of streptolydigin by perhaps a ternary complex together with SlgN1 and SlgN2 is an issue that would require further experiments. The type II thioesterase SlgB is in addition involved in the biosynthesis of streptolydigin, probably with a PKS-NRPS editing role, as previously proposed for other analogous enzymes (Heathcote et al., 2001; Schwarzer et al., 2002), because its inactivation in *S. lydicus* AS 4.2501 sharply reduced the production of streptolydigin (Yu et al., 2006).

β-Methylaspartic acid derived from glutamate has been suggested to be a precursor in streptolydigin biosynthesis (Chen and Harrison, 2004; Chen et al., 2006), and therefore could be the substrate for the NRPS. β-Methylaspartic acid might originate from glutamic acid by the glutamate mutase system composed of SlgE1 and SlgE2, similar to that described in the biosynthesis of friulimycin, nikkomycin, and vicenistatin (Heinzelmann et al., 2003; Li et al., 2005; Ogasawara et al., 2005). The putative ferredoxin-dependent glutamate synthase coded by *slgE3* gene could be in charge of the specific supply of glutamic acid from primary metabolism-derived 2-oxoglutarate and glutamine (Kameya et al., 2007) to the biosynthesis of streptolydigin. SlgZ could be involved in the introduction of an amino group based on its high homology to archaeal asparaginyl-tRNA synthetase-like enzymes that, like SlgZ, contain the catalytic core of asparaginyl-tRNA synthetases but lack the N-terminal anticodon-binding site. These kind of truncated forms of asparaginyl-tRNA synthetases have been shown to produce asparagine by amidation of aspartic acid (Roy et al., 2003). By analogy, SlgZ might carry out the biosynthesis of 3-methylasparagine by amidation of 3-methylaspartate, as it has been shown before for the biosynthesis of asparagine in *Pyrococcus abyssi* (Roy et al., 2003). Then, the NRPS could incorporate β-methylaspartate as a precursor of streptolydigin. After the release of the nascent compound from the NRPS and its probable spontaneous cyclization to form the tetramic acid moiety following a direct Claisen-like process—because a reductase domain was not found in the streptolydigin gene cluster, which precludes an aldol-like cyclization (Chen et al., 2006)—further modifications might occur. The biosynthesis might proceed through the introduction of an epoxide group by a cytochrome P450, either SlgO1 or SlgO2, followed by *N*-methylation by SlgM (Figure 3). The production of demethyl-streptolydiginone and streptolydiginone, compounds **2** and **3**, respectively (Figure 1), by strain SML7H13 (Figure 4A) lacking genes *slgS3* to *slgS7* (Figure 2A), points to the epoxidation and methylation occurring before glycosylation takes place. The involvement of cytochrome P450 in epoxidation pathways has been previously showed for OleP and PimD during oleandomycin and pimaricin biosynthesis, respectively (Shah et al., 2000; Mendes et al., 2005). The *N*-methylation process occurring after the introduction of the epoxide moiety is supported by the isolation of compound **2** (Figure 1). However, the introduction of the *N*-methyl group on β-methylaspartate once it is bound to the NRPS and before its attachment to the nascent polyketide cannot be excluded, because a similar situation has been proposed to occur to glutamic acid during the biosynthesis of α-lipomycin (Bihlmaier et al., 2006).

Final steps in the biosynthesis of streptolydigin should require the introduction of an L-rhodinose moiety in the aglycon (Figure 3). All genes required for the biosynthesis of this deoxysugar are present in the cluster together with the glycosyltransferase SlgG responsible for the attachment of this 2,3,6-trideoxyhexose to the streptolydigin aglycon. The participation of SlgC1 and SlgC2, putative endo-1,3-β-glucanases, in the glucose supply for the biosynthesis of L-rhodinose cannot be discarded. Recently the involvement of similar enzyme VldI, a 1,4-α-D-glucan glucohydrolase, in the biosynthesis of validamycin has been reported in *Streptomyces hygroscopicus* (Singh et al., 2007).

The expression of different sets of genes involved in the biosynthesis of several deoxyhexoses into a strain lacking all genes involved in the biosynthesis of L-rhodinose led to the generation of new glycosylated forms of streptolydigin. SlgG has therefore been shown to possess a certain degree of substrate flexibility regarding the deoxysugar, because it was able to transfer, in addition to its usual substrate L-rhodinose, some 2,3,6-trideoxyhexoses (L-amicetose and D-amicetose) and, less efficiently, some 2-6-dideoxihexoses (probably D-olivose and L-digitoxose). Nevertheless, SlgG does not seem to be so flexible and efficient as other glycosyltransferases such as ElmGT (Rodríguez et al., 2002; Lombó et al., 2004; Pérez et al., 2005, 2006; Ramos et al., 2009), StfG (Olano et al., 2008), AraGT (Luzhetskyy et al., 2007), LanGT3 (Zhu et al., 2007), or UrdGT2 (Trefzer et al., 2002). This apparent lack of efficiency of SlgG could be influenced by the low production of unglycosylated compounds observed in mutant SLM7H13 (Figure 4A). The complementation of this mutant with L-rhodinose biosynthetic genes in *trans* restored the production of streptolydigin but not at the level of the wild-type strain (Figure 4B). This decrease in production has been observed in other *S. lydicus* mutants defective in genes involved in streptolydigin biosynthesis (data not shown), but not in mutants SLMX27 (Figure 2B), SLM2A, and SLM3A defective in genes not required for streptolydigin biosynthesis in *S. lydicus*.

Bioactivity testing of the compounds purified in this work showed that the removal of the deoxyhexose (streptolydiginone) clearly decreases the antibacterial activity of the compound. In contrast, substitution of L-rhodinose by L-amicetose does not greatly affect the antibacterial activity, suggesting that the change in the spatial orientation of the hydroxyl group at C4 of the sugar skeleton does not affect the interaction with the RNA polymerase.

SIGNIFICANCE

Streptolydigin is a potent inhibitor of bacterial RNA polymerase that shows limited cross-resistance with other inhibitors of this enzyme. This effect makes streptolydigin a good candidate for the development of new derivatives with improved antibiotic activity because of the emergence of multiresistant bacterial strains for which antibiotics in current clinical use have limited efficacy. In addition, because streptolydigin has no effect on eukaryotic RNA polymerase and it was also described as a potential antitumor agent, the opportunity for developing derivatives with enhanced antitumor activity seems promising. The characterization of the streptolydigin biosynthetic gene cluster from *S. lydicus* NRRL2433 has shown that its tetramic acid moiety is synthesized by a hybrid type I PKS-NRPS system with some streaking features as the participation of a free-standing adenylation domain protein. The identification of streptolydigin cluster has allowed, in addition, the production of several new derivatives, unglycosylated and glycosylated, with alternative deoxyhexoses, through combinatorial biosynthesis and opens up a way for future studies aimed at engineering more potent analogs of this compound.

EXPERIMENTAL PROCEDURES

Strains, Culture Conditions, and Plasmids

The bacterial strains used in this work were *S. lydicus* NRRL2433, streptolydigin producer, *Escherichia coli* DH10B (Invitrogen), and ET12567 (pUB307) (Kieser et al., 2000), used for subcloning, and *E. coli* XLI-Blue MR (Stratagene), used to propagate a cosmid library. The growth medium for *S. lydicus* and mutants was tryptone soya broth (TSB); A medium was used for sporulation (Fernández et al., 1998) and R5A as production medium (Fernández et al., 1998). Conjugation of *Streptomyces* mycelium was performed following standard procedures (Kieser et al., 2000). The *E. coli* media were those described in the literature (Sambrook et al., 1989). When plasmid-containing clones were grown, the medium was supplemented with the appropriate antibiotics: 100 μg/ml ampicillin, 20 μg/ml tobramycin, 25 μg/ml apramycin, 50 μg/ml thiostrepton, 50 μg/ml hygromycin, 10 μg/ml tetracycline, 25μg/ml chloramphenicol, or 50 μg/ml nalidixic acid.

Plasmids used in this work were pSL1180 (Amersham Pharmacia) for routine cloning; pWE15 (Stratagene) for constructing the cosmid library; and pOJ260 (Bierman et al., 1992), pOJ260P (Olano et al., 2004), and pEM4T (Menéndez et al., 2006) for gene replacement and gene expression, respectively. pCR-BLUNT (Invitrogen) was used for cloning polymerase chain reaction (PCR) products. pEFBA (Lozano et al., 2000) was the source of apramycin resistance gene *aac3(IV)*. pAR15AT (Lombó et al., 2006) was the source of apramycin resistance gene *aac3(IV)*, *oriT, attB* bacterial attachment site, and *int* gene specifying site-integration function from bacteriophage ϕC31. pLHyg (Olano et al., 2004) was used as donor of the hygromycin resistance gene *hyg*. Plasmids pFL844 and pFL845 (Pérez et al., 2005), pLN2 and pLNR (Rodríguez et al., 2002), and pLNBIV (Fischer et al., 2002) direct the biosynthesis of deoxysugars L-amicetose, D-amicetose, L-olivose, D-olivose, and L-digitoxose, respectively.

DNA Manipulation

DNA manipulations were performed according to standard procedures for *E. coli* (Sambrook et al., 1989) and *Streptomyces* (Kieser et al., 2000). A cosmid library of *S. lydicus* NRRL2433 genomic DNA was constructed. DNA fragments obtained from a partial digestion with MboI were ligated to cosmid pWE15 digested with BamHI and in vitro packaged using the Gigapack III Gold Packaging Extract Kit according to the manufacturer's handbook (Stratagene). The resulting *E. coli* transductants were picked and transferred to 96-well microtiter plates containing Luria broth medium and ampicillin. Clones were replica plated onto Luria agar plates containing ampicillin. After overnight growth at 37°C, colonies were transferred to nylon membrane filters for in situ colony hybridization analysis according to published methods (Sambrook et al., 1989) and screened using labeled probes that were generated using the DIG DNA labeling and detection kit (Roche).

DNA Sequencing and Analysis of the Streptolydigin Gene Cluster

Streptolydigin gene cluster was located in a DNA region delimited by a BamHI fragment of 6.9 kb from cosmid Slg6E5 (sites 1–2, Figure 2A), cosmids Slg4A8 and Slg9C7, and an EcoRI-BglII fragment of 10.7 kb from cosmid Slg6G6, being the EcoRI site from pWE15. DNA sequencing of this region (Figure 2A) was performed on double-stranded DNA templates with the dideoxynucleotide chain termination method (Sanger et al., 1977) and the Cy5 Autocycle Sequencing Kit (Pharmacia Biotech). An Alf-express automatic DNA sequencer (Pharmacia) was used. Computer-aided database searching and sequence analysis were carried out with the University of Wisconsin Genetics Computer Group software (Devereux et al., 1984) and the BLAST program (Altschul et al., 1997). Analysis of PKS and NRPS predicted proteins were carried out using programs ASMPKS (Tae et al., 2007) and NRPSpredictor (Rausch et al., 2005). Analysis of transmembrane regions in putative membrane proteins were carried out using the program TMHMM v. 2.0 (Krogh et al., 2001).

Construction of Plasmids for Gene Inactivation and Complementation of Mutants

Involvement of PKS genes in streptolydigin biosynthesis was achieved by gene disruption. For this purpose, two constructions, pOJ4C1 and pOJ961, were generated in the *Streptomyces*-suicide vector pOJ260 (Bierman et al., 1992). Plasmid pOJ4C1 and pOJ961 were obtained by cloning a 627 bp BamHI fragment from cosmid Slg4A8 (sites 17–18, Figure 2A) and a 1.2 kb BamHI fragment from cosmid Slg9C7 (sites 30–31, Figure 2A), respectively. Plasmids pOJ4C1 and pOJ961 were used for generation of *S. lydicus* strains SLM4C1 and SLM961.

The boundaries of the gene cluster were determined by inactivation of *orf2* and *orf3* by gene replacement. For inactivation of *orf2,* a 1.5 kb BamHI-PstI fragment from cosmid Slg6E5 was subcloned into the same sites of pHyg leading to pHyg2. After BglII digestion of pHyg2 (BglII restriction site is internal to *orf2*, Figure 2A) and treatment with the Klenow fragment, the *aac(3)IV* gene from pEFBA was subcloned as a SmaI-EcoRV fragment leading to pHyg2A. For inactivation of *orf3,* a 1.9 kb StuI-PstI fragment from cosmid Slg6G6 was subcloned into EcoRV-PstI-digested pHyg leading to pHyg3. After BamHI digestion of pHyg3 (BamHI restriction site is internal to *orf3*, site 32, Figure 2A) and treatment with the Klenow fragment, the *aac(3)IV* gene from pEFBA was subcloned as a SmaI-EcoRV fragment leading to pHyg3A. Finally, a 1 kb XbaI-SpeI fragment from pOJ260 containing the *oriT* was subcloned into XbaI-digested pHyg2A and pHyg3A to obtain plasmids pHyg2AT and pHyg3AT, respectively, that were used for generation of *S. lydicus* strains SLM2A and SLM3A.

Inactivation of gene *slgX* coding for a putative ent-copalyl diphosphate synthase of unknown function was accomplished by amplification of a *slgX* internal fragment of 700 bp by PCR from cosmid Slg4A8 using oligoprimers CRIS23 (5′-AAA AAG CTT ACG CAC GGT TGG CCG CCC-3′, HindIII underlined) and CRIS24 (5′-AGA ATT CTG CTG CCG CTC CC CCA GC-3′, EcoRI site underlined). PCR conditions used were 97°C, 5 min; 30 cycles of 95°C, 30 s, 50°C, 45 s, and 68°C, 1 min, and a final extension cycle at 68°C, 10 min. Pfx DNA polymerase (Invitrogen) and 2.5% dimethylsulfoxide (DMSO) were used for all amplifications. The PCR product was cloned into pCR-BLUNT and then sequenced. The resultant plasmid was digested HindIII-EcoRI and the 700 bp fragment cloned into pOJ260 to obtain plasmid pOJX27 used for generation of *S. lydicus* strain SLMX27.

For deletion of genes *slgS3* to *slgS7*, all presumably involved in the biosynthesis of the deoxysugar L-rhodinose, plasmid pOJ7H13 was constructed. For that purpose, gene *slgO2* was amplified by PCR as a 1.2 kb fragment using oligoprimers CRIS13 (5′-AAG GAT CCG GCT CCG CGA TGA GCG AG-3′, BamHI site underlined) and CRIS14 (5′-AGA ATT C**AT GCA T**GG TGG TCA TCC GCC GCC-3′, EcoRI site underlined and NsiI site in bold). Gene *slgM* was amplified by PCR as a 1.3 kb fragment using oligoprimers CRIS17

(5′-AAG GAT CCA CCG AAC CCG GAG GGT CG-3′, BamHI site underlined) and CRIS18 (5′-AGA ATT C**AC TAG T**TC CTC GCC GGG CGT CAC-3′, EcoRI site underlined and SpeI site in bold). PCR conditions for both amplifications were as described above. Both PCR products were cloned in pCR-BLUNT and sequenced. Afterwards, gene *slgM* was cloned as a BamHI-EcoRI fragment into BamHI-EcoRI-digested pOJ260P (Olano et al., 2004), where it will be located downstream of *erm*E* promoter leading to plasmid pOJPM. Gene *slgO2* was then cloned into BamHI-PstI-digested pLHyg as a BamHI-NsiI fragment leading to construct pL7H, which was later on digested with HindIII and SpeI for cloning a HindIII-SpeI fragment of 1.5 kb from pOJPM, containing *slgM* and *erm*E* promoter, leading to construct pL7H13. In the former plasmid, *hyg* gene is flanked by *slgO2* upstream and *slgM* downstream, being *slgM* under the control of *erm*E* promoter in order to avoid possible polar effects in mutant strain SLM7H13. Finally, plasmid pL7H13 was digested with NheI and SpeI and the resultant 4.3 kb fragment cloned into XbaI-digested pOJ260, leading to construct pOJ7H13, which was used to generate *S. lydicus* strain SLM7H13.

Plasmid pEM4T8-12 was constructed for complementation of *S. lydicus* strain SLM7H13 by cloning a NruI-MfeI fragment of 6.9 kb, containing genes *slgS3* to *slgS7* from cosmid Slg4A8, into a SmaI-MfeI-digested pSL1180 leading to plasmid pSL8-12, which was digested with EcoRI and MfeI and the resultant fragment cloned into EcoRI-digested pEM4T. In construct pEM4T8-12, genes *slgS3* to *slgS7* are under the control of *erm*E* promoter.

Plasmids pFL844T, pFL845T, pLN2T, pLNRT, and pLNBIVT are the integrative version of plasmids pFL844, pFL845, pLR2, pLNR, and pLNBIV. These integrative plasmids were constructed by cloning in an XbaI site of plasmids pFL844, pFL845, pLR2, pLNR, and pLNBIV a 6.2 kb SpeI fragment from plasmid pAR15AT (Lombó et al., 2006) containing the *aac3(IV)* and *int* genes, the *attB* site, and the *oriT* RK2 region. Before SpeI digestion, the *ori*p15A region from pAR15AT was deleted by removing a 700 bp PstI-BglII blunt-ended fragment followed by religation.

Generation of *S. lydicus* Mutant Strains

Constructs pOJ4C1, pOJ961, pHyg2AT, pHyg3AT, pOJX27, and pOJ7H13 were introduced into *S. lydicus* by intergeneric conjugation from *E. coli* ET12567 (pUB307). Transconjugant *S. lydicus* strains SLM4C1, SLM961, and SLMX27 were selected for resistance to apramycin, and integration in the *S. lydicus* chromosome was verified by Southern hybridization. For generation of *S. lydicus* strain SLM7H13, a single-crossover strain, apramycin and hygromycin resistant, was cultured in absence of selection and then screened for the lost of apramycin resistance, keeping hygromycin resistance because of a double recombination event. The deletion of genes *slgS3* to *slgS7* was verified by Southern hybridization. For the generation of *S. lydicus* strains SLM2A and SLM3A, a single-crossover strain, apramycin and hygromycin resistant, was cultured in absence of selection and then screened for the loss of hygromycin resistance, keeping apramycin resistance because of a double recombination event. The inactivation of *orf2* in SLM2A and *orf3* in SLM3A was verified by Southern hybridization. Plasmids pEM4T, pEM4T8-12, pFL844T, pFL845T, and pLNBIVT were introduced in *S. lydicus* SLM7H13 by intergenic conjugation and transconjugants were selected for resistance to thiostrepton.

Analysis of Streptolydigin Production by UPLC and LC-MS

Streptolydigin production was assessed by growing *S. lydicus* wild-type strain or mutant strains on solid R5A medium. After 7 days at 30°C, agar plugs containing 1.5 ml agar media were extracted with 1 ml ethyl acetate and the presence of streptolydigin in the extract analyzed by UPLC as described below. In liquid cultures, strains were grown as a seed culture in TSB (30 ml in a 250 ml Erlenmeyer flask). After 2 days incubation in a rotary incubator (30°C; 250 rpm), 2.5% v/v of the cultures was used to inoculate 30 ml R5A liquid medium. After 7 additional days of incubation with shaking, the cultures were harvested for analysis. One milliliter aliquots of fermentation broths were removed and 300 μl ethyl acetate was added to the sample and then mixed vigorously for 30 min. The phases were separated by centrifugation in a microfuge and then the ethyl acetate removed by evaporation using a Speed-Vac. Residues were resuspended in methanol and clarified by centrifugation. Analysis of streptolydigin production was performed by reversed phase chromatography on Acquity UPLC equipment with a BEH C18 column (2.1 × 100 mm, Waters), with acetonitrile and 0.05% trifluoroacetic acid (TFA) as solvents. Samples were eluted with 10% acetonitrile for 1 min, followed by a linear gradient from 10% to 80% acetonitrile over 7 min, at a flow rate of 0.5 ml/min and a column temperature of 30°C. For HPLC-MS analysis, an Alliance chromatographic system coupled to a ZQ4000 mass spectrometer and a Symmetry C18 (2.1 × 150 mm, Waters) was used. Solvents were the same as above and elution was performed with an initial isocratic hold with 10% acetonitrile for 4 min followed by a linear gradient from 10% to 88% acetonitrile over 26 min, at 0.25 ml/min. MS analyses were done by electrospray ionization in the positive mode, with a capillary voltage of 3 kV and a cone voltage of 20 V. Detection and spectral characterization of peaks was performed in both cases by photodiode array detection and Empower software (Waters), extracting bidimensional chromatograms at 360 nm.

Isolation of Streptolydigin Novel Derivatives

For structural characterization compounds **2**, **3**, **4**, and **6** were purified from 10 l cultures of the corresponding *S. lydicus* mutants following the culture conditions described above. The extracts were dissolved in 5 ml of a mixture of DMSO and methanol (1:1) and centrifuged, and the resulting upper lipid layer was removed. The first purification step was done by chromatography in an XTerra PrepRP18 column (19 × 300 mm, Waters) with acetonitrile and 0.05% TFA in water as solvents. A linear gradient from 30% to 100% acetonitrile in 7 min followed by a 3 min isocratic hold with 100% acetonitrile was used, at a flow rate of 15 ml/min. Peaks of interest were collected in 0.1 M phosphate buffer (pH 7.0). The solutions obtained were partially evaporated in rotavapor to reduce the acetonitrile concentration and then applied to a solid-phase extraction cartridge (Sep-Pak C18, Waters), washed with water to remove salts, and eluted with methanol. Further purifications were performed in isocratic conditions with a Symmetry C18 column (7.8 × 300 mm, Waters), using mixtures of acetonitrile and 0.05% TFA in water optimized for each peak, at a flow rate of 7 ml/min. As described above, peaks were always collected on buffer, desalted by solid-phase extraction, and finally lyophilized.

NMR Spectroscopy

Final identification was carried out by ^{1}H- and ^{13}C-NMR experiments. Sample preparation was done by dissolving 100–500 μg pure products in 200 μl DMSO-d_6 and transferred to a 3mm NMR tube. Solvent signals were used as internal reference. NMR spectra were recorded at 300 K using a Bruker Avance Ultrashield Plus 600 spectrometer equipped with a 5 mm TCI cryoprobe. Homonuclear 2D ^{1}H, ^{1}H COSY and heteronuclear ^{1}H, ^{13}C HSQC/HMBC experiments were acquired. Typical parameters for 2D experiments were as follows: COSY, 256 and 2048 points in F1 and F2, respectively, 16 transients each; HSQC, 256 and 2048 points in F1 and F2, respectively, 48 transients each; HMBC, 512 and 2048 points in F1 and F2, respectively, 64 transients each. NMR spectra were processed using the program Topspin 1.3 (Bruker GmbH, Karlsruhe, Germany).

The information regarding structural characterization of compounds **2**, **3**, and **4** is supplied as Tables S1, S2, and S3.

Bioactivity Testing

The antibiotic activity of streptolydigin, streptolydiginone (compound **3**), and streptolydigin LA (compound **4**) was analyzed via antibiotic disc diffusion assay. To do this, a spore suspension of *S. albus* was spread in a Petri dish containing tryptone soy agar medium (Merck). Paper disks of 5 mm in diameter were used. Each disk contained a solution of 2 μg of each compound dissolved in 15 μl methanol. Fifteen microliters of methanol were used as negative control. The antibiotics were allowed to diffuse from the disk at 4°C during 2 hr and then the plates were incubated at 30°C for 24 hr.

SUPPLEMENTAL DATA

Supplemental Data include three tables and can be found with the article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00325-1.

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