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Amino Acid Precursor Supply in the Biosynthesis of the RNA Polymerase Inhibitor Streptolydigin by *Streptomyces lydicus*▽†

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Biosynthesis of the hybrid polyketide-nonribosomal peptide antibiotic streptolydigin, 3-methylaspartate, is utilized as precursor of the tetramic acid moiety. The three genes from the *Streptomyces lydicus* streptolydigin gene cluster *slgE1-slgE2-slgE3* are involved in 3-methylaspartate supply. SlgE3, a ferredoxin-dependent glutamate synthase, is responsible for the biosynthesis of glutamate from glutamine and 2-oxoglutarate. In addition to *slgE3*, housekeeping NADPH- and ferredoxin-dependent glutamate synthase genes have been identified in *S. lydicus*. The expression of *slgE3* is increased up to 9-fold at the onset of streptolydigin biosynthesis and later decreases to ~2-fold over the basal level. In contrast, the expression of housekeeping glutamate synthases decreases when streptolydigin begins to be synthesized. SlgE1 and SlgE2 are the two subunits of a glutamate mutase that would convert glutamate into 3-methylaspartate. Deletion of *slgE1-slgE2* led to the production of two compounds containing a lateral side chain derived from glutamate instead of 3-methylaspartate. Expression of this glutamate mutase also reaches a peak increase of up to 5.5-fold coinciding with the onset of antibiotic production. Overexpression of either *slgE3* or *slgE1-slgE2* in *S. lydicus* led to an increase in the yield of streptolydigin.

The vast majority of antibiotic and antitumor drugs belong either to the polyketide or the nonribosomal families of natural products. A related family comprises hybrid compounds containing polyketide and nonribosomal peptide moieties. Their biosynthesis involves the participation of a modular polyketide synthase (PKS) for the condensation of acyl coenzyme A (acyl-CoA) precursors and a nonribosomal peptide synthetase (NRPS) that condenses amino acids after their activation to an aminoacyl-AMP precursor. Both type I PKSs and NRPSs are multifunctional enzymes that are organized into modules and use a similar strategy for the assembly of these short carboxylic and amino acid building blocks. The minimal set of domains in a type I PKS includes ketosynthase (KS), acyltransferase, and acyl-carrier protein activities responsible for the catalysis of one cycle of polyketide chain elongation. These PKS modules can contain further domains such as ketoreductase (KR), dehydratase (DH), or enoylreductase to reduce the keto groups generated during the condensation process (9). In a similar way, a typical minimal NRPS module consists of condensation, adenylation, and peptidyl carrier protein (PCP) domains (9).

Streptolydigin (compound 1) (Fig. 1) is an inhibitor of bacterial RNA polymerase β-subunit produced by *Streptomyces lydicus* (27, 29) and a potent inhibitor of eukaryotic DNA polymerase terminal deoxynucleotidyltransferase (6, 7). The streptolydigin biosynthetic gene cluster has been isolated and characterized from the producer organism (21). Streptolydigin belongs to the hybrid polyketide-nonribosomal peptide family of natural products. The streptolydigin type I PKS, composed of one loading domain and seven extension modules distributed over three polypeptides, would condense four units of malonyl-CoA and four units of methyl-malonyl-CoA and is proposed to generate the polyketide core. The formation of the tetramic acid moiety of the molecule involves the participation of an NRPS system composed of at least two polypeptides. Early biosynthetic studies using labeled precursors have shown the incorporation of propionate, acetate, methionine, and glutamic acid (suggested to be in form of β-methylaspartate) into the main structure of streptolydigin (3, 4, 22, 23). In addition, recent work in our laboratory has demonstrated the involvement of SlgZ, an asparaginyl-tRNA synthetase-like enzyme, in the 3-methylaspartate tailoring process by amidation of a 3-methylaspartatyl-NRPS bound to generate 3-methylasparaginyl-NRPS (11). This activity might be followed by the methylation of NRPS-bound 3-methylasparagine by SlgM to obtain *N*-methyl-3-methylasparaginyl-NRPS that then would be condensed with the polyketide chain synthesized by the streptolydigin PKS (11) (Fig. 1). Three other genes in the cluster are predicted to encode two subunits of a glutamate mutase (GM) (*slgE1* and *slgE2*) and a ferredoxin-dependent glutamate synthase (GS) (*slgE3*). We report here the characterization of these three genes and their role in supplying 3-methylaspartate as the precursor for streptolydigin biosynthesis. We have also identified two housekeeping GSs, dependent, respectively, on NADPH and ferredoxin. Deletion of the

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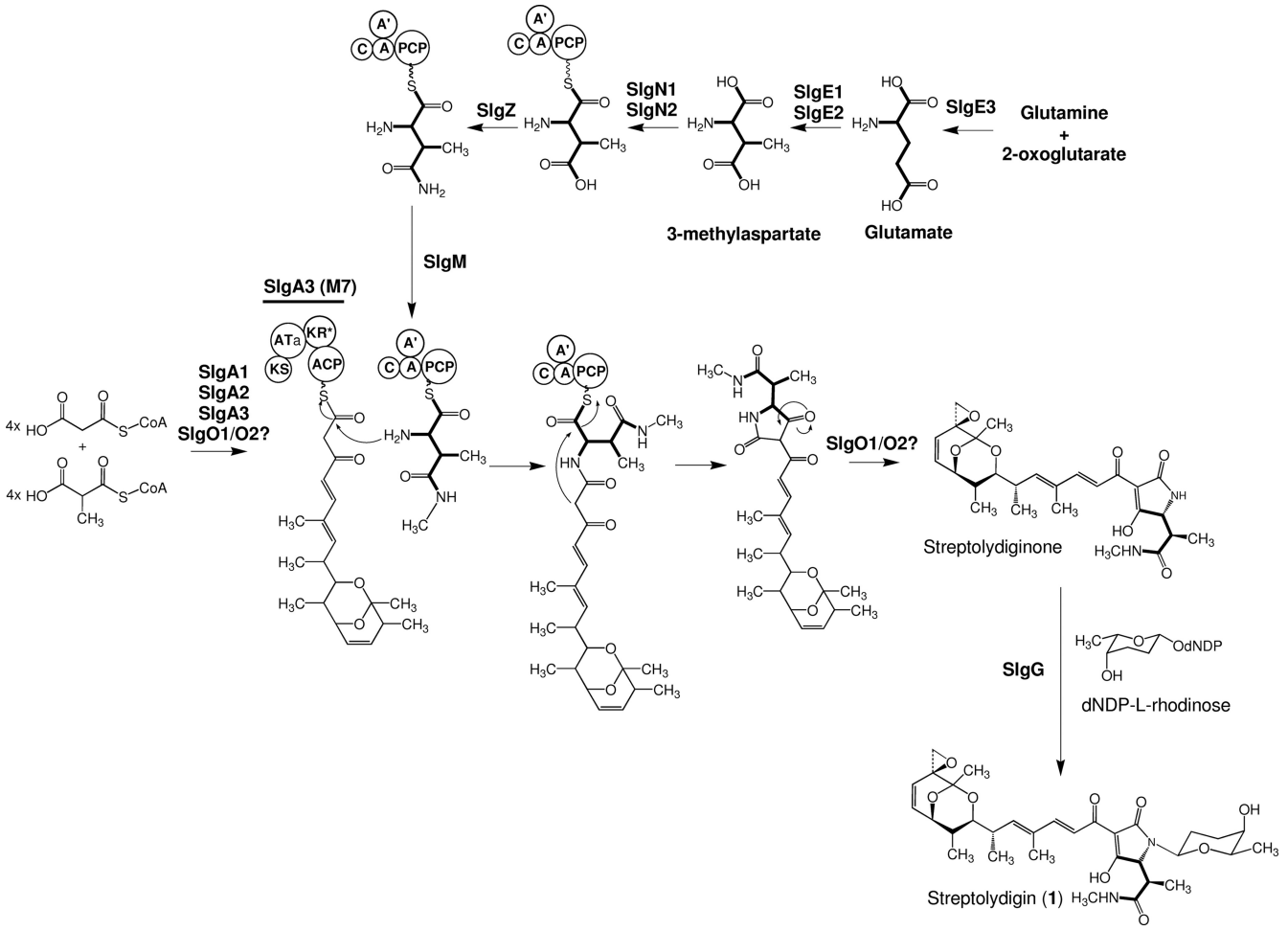

FIG. 1. Proposed pathway for the biosynthesis of streptolydigin (compound 1). The incorporation of glutamate, in the form of 3-methylaspartate, to generate the tetramic acid lateral side chain is shown in thick lines. M7, PKS module 7; KS, ketosynthase; ATa, acyl transferase specific for malonyl-CoA; ACP, acyl carrier protein; KR*, inactive ketoreductase; C, condensation domain; A', SlgN1 adenylation domain; A, SlgN2 adenylation domain; PCP, peptidyl carrier protein.

GM genes *slgE1* and *slgE2* led to the production of a novel streptolydigin derivative.

MATERIALS AND METHODS

Strains and culture conditions. The bacterial strains used in the present study were *S. lydicus* NRRL2433, a streptolydigin producer; *Escherichia coli* DH10B (Invitrogen); and ET12567(pUB307) (12). The growth medium for *S. lydicus* and mutants was tryptone soy broth. For sporulation, MA medium was used and R5A was the streptolydigin production medium (8). Growth of *S. lydicus* in R5A liquid medium was monitored by measuring absorbance at 600 nm using the diphenylamine assay method for determining DNA content (2, 16). Culture conditions were those previously described (21). Intergeneric conjugation of *Streptomyces-E. coli* was performed according to standard procedures (12). The *E. coli* media were as described previously (25). When plasmid-containing clones were grown, the medium was supplemented with the appropriate antibiotics: 100 μg of ampicillin/ml, 20 μg of tobramycin/ml, 25 μg of apramycin/ml, 50 μg of thiostrepton/ml, 50 μg of hygromycin/ml, 10 μg of tetracycline/ml, 25 μg of chloramphenicol/ml, or 50 μg of nalidixic acid/ml.

DNA manipulation and plasmids. DNA manipulations were performed according to standard procedures for *E. coli* (25) and *Streptomyces* (12). Platinum *Pfx* DNA polymerase (Invitrogen) and 2.5% dimethyl sulfoxide (DMSO) were used for all PCR amplifications. The PCR conditions used were as follows: 97°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 45 s, and 68°C for 1 min, with a final extension cycle at 68°C for 10 min. All of the PCR products were cloned into pCR-BLUNT (Invitrogen) and then sequenced. Other plasmids used in the present study included pSL1180 (Amersham Pharmacia) for routine cloning, pEFBAoriT (11) for gene replacement, and pEM4T (17) for gene expression. pLHyg (20) was the source of the hygromycin resistance gene *hyg*.

Construction of plasmids for gene replacement and complementation of mutants. For deletion of *slgE3* two DNA fragments of 1.0 and 1.1 kb, respectively, were amplified by PCR from cosmid Slg4A8 (21) using oligoprimers HEI9/HEI10 (PCR A) and HEI11/HEI12 (PCR B) (Table 1). The PCR A fragment (Fig. 2A) containing 34 bp from the *slgE3* 5′ end was cloned as a SpeI-NsiI fragment into SpeI-NsiI-digested pEFBAoriT. The resultant plasmid was digested with BamHI-EcoRVI, and the PCR B fragment (Fig. 2A), containing 22 bp from the *slgE3* 3′ end and digested with the same restriction enzymes, was cloned, yielding plasmid pΔslgE3. In this construct 1,267 bp of the *slgE3* coding region have been substituted by the apramycin resistance gene *aac3(IV)*. Deletion of genes *slgE1* and *slgE2* was accomplished by amplification of two DNA fragments (1.0 and 1.1 kb, respectively) from cosmid Slg4A8 by using the oligoprimers HEI15/HEI16 (PCR C) and HEI17/HEI18 (PCR D) (Table 1). The PCR C fragment (Fig. 2D), containing 18 bp from the *slgE2* 3′ end, was cloned as a SpeI-NsiI fragment into SpeI-NsiI-digested pEFBAoriT. The resultant plasmid was digested with BamHI-EcoRVI, and the PCR D fragment (Fig. 2D), containing 45 bp from the *slgE1* 5′ end and digested with the same restriction enzymes, was cloned, yielding plasmid pΔslgE1E2. In this construct, 1,744 bp of the *slgE1-slgE2* coding region were substituted by the apramycin resistance gene *aac3(IV)*. Finally, pΔslgE3 and pΔslgE1E2 were digested with XbaI, and the gene *hyg* from pLHyg was subcloned as a SpeI-NheI fragment to obtain plasmids

TABLE 1. Primers used in this study

Primer	Sequence (5′–3′)[a]	Description
HEI9	AACTAGTCCCCGTCAGCACCCCACG	SpeI
HEI10	AAAATGCATACCCCGGGGCAGACAGCC	NsiI
HEI11	AAGGATCCGACGACGAGGGAGCCCTC	BamHI
HEI12	AAGATATCTGAAGCCCATGCTCTCGG	EcoRV
HEI15	AACTAGTTGTGGTCGCCGAGGACGA	SpeI
HEI16	AAAATGCATGGCGGTCGAAGACCGATG	NsiI
HEI17	AAGGATCCGCTGTGCCGTGCGTCGTC	BamHI
HEI18	AAGATATCTGCAGACCGACCGCGGTC	EcoRV
HEI13	AAGGATCCGAAGATCCCCGGCGTGGT	BamHI
HEI14	AGAATTCGAACGGATTGCTCACGAG	EcoRI
CCL22B	AAGGATCCGATGGGCAAGGCAATGGA	BamHI
CCL21MF	AACAATTGCAGCACGAGTGCTCTCAT	MfeI
HEI-I	GTCGCCTCCGGCCGCTTC	Forward for *gltS*-α
HEI-J	SCCGACCGGGCAGGTGTC	Reverse for *gltS*-β
HEI-F	TTCTGCGTCCGCAACTCC	Forward for *gltS*-α and -β
HEI-C	CAGTCSGCRCCSGTGTCR	Reverse for *gltS*-α and -β
HEI-FR1	CCSTACGACATGGCSYTGCT	Forward for *gltS*-FD
HEI-FR2	CTTGCACACSGCGAGGAACT	Reverse for *gltS*-FD
NDGA	AGCGAGTACCTCGTCAACTCCGA	Forward for *gltS*-α[b]
NDGB	CATCATGACGCAGCCGGAGA	Reverse for *gltS*-α
NDPA	AGCGCGAGATCGCCAAGACC	Forward for *gltS*-β
NDPB	CGCTTGCGCAGCTTCTTGGC	Reverse for *gltS*-β
FDA	GACATGGCCCTGCTGAACGTCTC	Forward for *gltS*-FD
FDB	GGAGCCGACGCAGAGCTTGAAC	Reverse for *gltS*-FD
FDSLGE3A	TGGTGCCGCCGGTGTTCAT	Forward for *slgE3*
FDSLGE3B	GGATCTCCTCGGTGACGGTGC	Reverse for *sglE3*
CRIS3	ATCGCGCTCAACGGCTAC	Forward for *sglE2*
CRIS4	TAGGGGATGTCCGACGAC	Reverse for *sglE2*
RTNADG1	ACCACGACATCTACTCCATCGAG	Forward for *gltS*-α[c]
RTNADG2	CAGCTTCACGTGGATGCG	Reverse for *gltS*-α
RTNADP1	TCAAGATGGAGAAGCGCCA	Forward for *gltS*-β
RTNADP2	CGTACGGAACTTGGTGCCC	Reverse for *gltS*-β
RTGS1	TCCGAGTTCGCCGACAA	Forward for *gltS*-FD
RTGS2	GGTTTCAGGGAGACGCACTTGAT	Reverse for *gltS*-FD
RTGSSLG1	CCAGCAGATCCGCTTCAT	Forward for *slgE3*
RTGSSLG2	AAGAGCTTCACCCACACCC	Reverse for *sglE3*
RTGMA	GATTCACCACCTCGAAACCGT	Forward for *sglE2*
RTGMB	TGATCGTCAGCAGTCTCGCTT	Reverse for *sglE2*
HRDBqRT1	CAACCCAGTGGAAGAACGTT	Forward for *hrdB*
HRDBqRT2	TGCGGCACTGACCATCAG	Reverse for *hrdB*

[a] Restriction sites are underlined in the primer sequences.
[b] This primer and the following one were used for RT-PCR studies.
[c] This primer and the following one were used for qRT-PCR studies.

pΔslgE3Hyg and pΔslgE1E2Hyg used for the generation of *S. lydicus* strains SLME3 and SLME1E2, respectively.

Plasmids pEM4TslgE3 and pEM4TslgE1E2 were constructed for complementation of *S. lydicus* strains SLME3 and SLME1E2, respectively. The *slgE3* gene was amplified by PCR from cosmid Slg4A8 by using the oligoprimers HEI13 and HEI14. The resultant 1.3-kp product was cloned as a BamHI-EcoRI fragment into BamHI-EcoRI-digested pEM4T, yielding plasmid pEM4TslgE3. The *slgE1-slgE2* genes were amplified by PCR from cosmid Slg4A8 by using the oligoprimers CCL22B and CCL21MF. The resultant 1.8-kp product was cloned as a BamHI-MfeI fragment into BamHI-EcoRI-digested pEM4T, yielding plasmid pEM4TslgE1E2. In both constructs, the genes *slgE3* and *slgE1-slgE2* are under the control of the *ermE** promoter.

Generation of *S. lydicus* mutant strains. Constructs pΔslgE3Hyg and pΔslgE1E2Hyg were introduced into *S. lydicus* by intergeneric conjugation from *E. coli* ET12567(pUB307). For the generation of the *S. lydicus* strains SLME3 and SLME1E2, a single-crossover strain, which was apramycin and hygromycin resistant, was cultured in the absence of selection and then screened for the loss of hygromycin resistance and the retention of apramycin resistance because of a double recombination event. The deletion of *slgE3* in SLME3 and *slgE1-slgE2* in SLME1E2 were verified by Southern hybridization and PCR amplification by using the oligoprimers HEI9/HEI12 or HEI15/HEI18, respectively. Plasmids pEM4T, pEM4TslgE3, and pEM4TslgE1E2 were introduced into *S. lydicus* wild-type strain or mutants SLME3 and SLME1E2 by intergeneric conjugation and transconjugants were selected for resistance to thiostrepton.

UPLC, LC-MS, and NMR spectroscopy methods. Streptolydigin production in *S. lydicus* wild-type or mutant strains grown on R5A medium was analyzed by ultrahigh-performance liquid chromatography (UPLC) and liquid chromatography-mass spectrometry (LC-MS) using previously described procedures (21). For structural elucidation streptolydigin C was purified and characterized by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) experiments. The information regarding structural characterization of streptolydigin C is given in Table S1 in the supplemental material.

Bioactivity testing. The antibiotic activity of streptolydigin C (compound 3, Fig. 3A) was assayed via antibiotic disc diffusion assay against *Streptomyces albus*. Streptolydigin was used as reference compound. In both cases, 2 μg of each compound was utilized according to a previously described procedure (21).

Identification of *S. lydicus* housekeeping glutamate synthases. PCR amplification of glutamate synthase-encoding regions was performed using degenerate oligoprimers (Table 1) and total DNA from *S. lydicus* NRRL2433. The oligoprimers HEI-I and HEI-J were designed to locate the presence of an internal region of an NADPH-dependent glutamate synthase α subunit gene (*gluS*-α). They were derived from conserved amino acid sequences present in enzyme SAV6189 from *S. avermitilis* MA-4680, enzyme SCO2026 from *S. coelicolor* A3(2), and enzymes SACE3998 and SACE5742 from *Saccharopoly-*

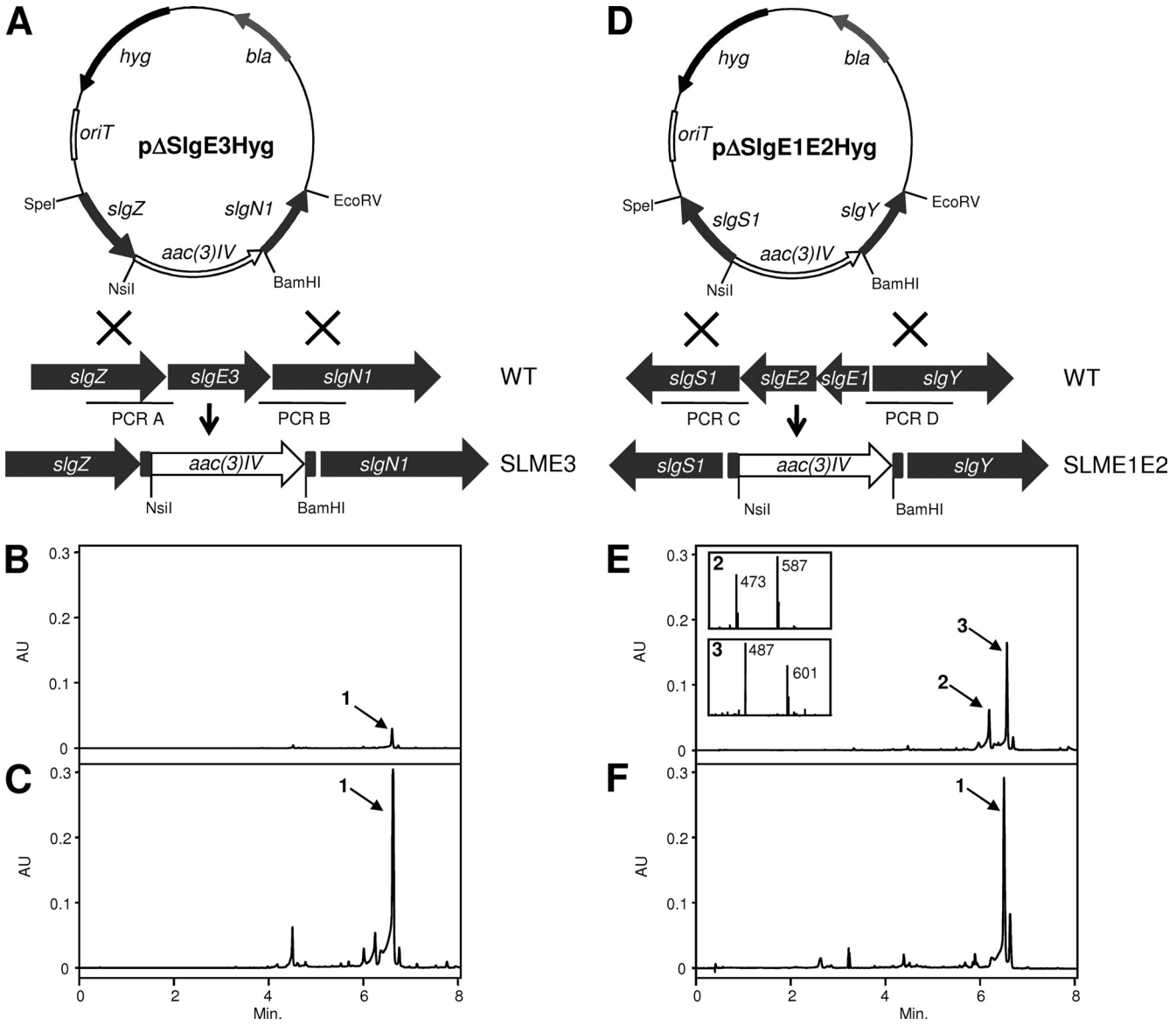

FIG. 2. (A) Scheme representing the replacement in the chromosome of the wild-type *slgE3* gene by a version mutated through the insertion of an apramycin resistance cassette. (B) UPLC analysis of mutant SLME3. (C) UPLC analysis of mutant SLME3 complemented by pEM4TslgE3. (D) Scheme representing the replacement of the wild-type *slgE1-E2* genes by one mutated through the insertion of an apramycin resistance cassette. (E) UPLC analysis of mutant SLME1E2 and MS analysis of compounds 2 and 3. (F) UPLC analysis of mutant SLME1E2 complemented by pEM4TslgE1E2. *aac(3)IV*, apramycin resistance gene; *hyg*, hygromycin resistance gene; *bla*, β-lactamase gene; 1, streptolydigin; 2, streptolydigin B; 3, streptolydigin C; AU, arbitrary units.

spora erythraea NRRL2338 (see Fig. 5B). Oligoprimers HEI-F and HEI-C were designed to locate the presence of the 5′ end of an NADPH-dependent glutamate synthase α subunit (*gluS*-α) and the 3′ end of an NADPH-dependent glutamate synthase β subunit (*gluS*-β) genes. Their sequences were designed from previously mentioned enzymes (see Fig. 5B) and from NADPH-dependent glutamate synthase β subunits SAV6190 and SAV6258 from *S. avermitilis* MA-4680, SCO1977, and SCO2025 from *S. coelicolor* A3(2) and SACE3997 and SACE5741 from *Saccharopolyspora erythraea* NRRL2338 (see Fig. 5C). Oligoprimers HEI-FR1 and HEI-FR2 were designed to locate the presence of an internal region of a housekeeping ferredoxin-dependent glutamate synthase gene (*gluS*-FD). These primers were designed using the enzymes SAV954 and SAV1232 from *S. avermitilis* MA-4680, SSEG09946 from *S. sviceus* ATCC 29083, and SSAG01435 from *Streptomyces* sp. strain Mg1 (see Fig. 5D). DNA sequencing of PCR product obtained was performed on double-stranded DNA templates with the dideoxynucleotide chain termination method (26) and the Cy5 Autocycle sequencing kit (GE Healthcare). An Alf-express automatic DNA sequencer (GE Healthcare) was used. Computer-aided database searching and sequence analysis were carried out with the University of Wisconsin Genetics Computer Group software (5) and the BLAST program (1).

Isolation of total RNA. Mycelium corresponding to cultures from *S. lydicus* NRRL2433 was obtained at 12, 24, 48, and 72 h during growth in R5A liquid medium. Mycelium corresponding to cultures from *S. lydicus* SLME3 and SLME1E2 was obtained at 72 h during growth in R5A liquid medium. Samples (15 ml) of each culture were mixed with 2 volumes of RNAprotect bacteria reagent (Qiagen) and, after vortexing, mycelia were harvested by centrifugation and immediately frozen at −70°C according to the manufacturer's instructions. The total RNA was extracted from frozen mycelium using the lysis method from the Kirby procedure (12) and purified with a RNeasy Midi kit (Qiagen) according to the manufacturer's instructions. RNA preparations were subjected to

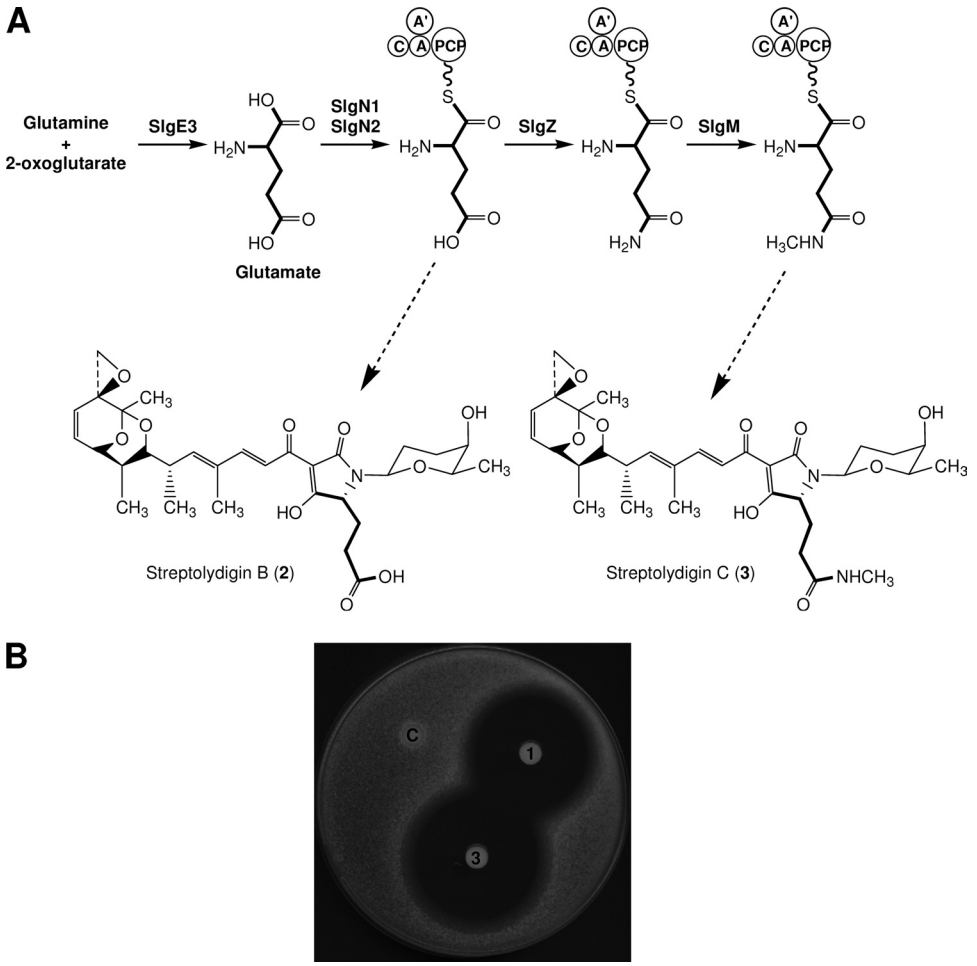

FIG. 3. (A) Structures of streptolydigin B (compound 2) and novel derivative streptolydigin C (compound 3) showing the proposed origin of tetramic acid lateral side chain from glutamate (thick lines). C, condensation domain; A′, SlgN1 adenylation domain; A, SlgN2 adenylation domain; PCP, peptidyl carrier protein. (B) Antibiotic activity of streptolydigin (area 1) and streptolydigin C (area 3) against *S. albus*. Each paper disk was soaked with 2 μg of the corresponding compound. Control without antibiotic (area C).

additional DNase I treatments (RNase-Free; Qiagen) to eliminate possible chromosomal DNA contamination. The RNA concentration was determined by measuring the absorbance at 260 nm.

Gene expression analysis by reverse transcriptase PCR (RT-PCR). Transcript detection analysis was carried out by using the SuperScript One-Step RT-PCR with Platinum *Taq* DNA polymerase (Invitrogen) with 100 ng of total RNA as a template. DMSO (5% [vol/vol], final) was added to all reactions, along with RNAguard RNase inhibitor (Amersham Biosciences; 32.2 U per reaction). The conditions were as follows: first-strand cDNA synthesis at 50°C for 30 min, followed by 94°C for 2 min, and then 30 amplification cycles of 95°C for 1 min, 61°C for 1 min, and 68°C for 2 min. Primers (18- to 23-mers; melting temperature [T_m], 65°C) (Table 1) were designed with the aid of the software Primer Express v.2.0 (Applied Biosystems) to generate PCR products of ~600 bp. The exception was the *slgE2*-specific primers CRIS3/CRIS4, which were designed to give a product of 840 bp. Negative controls for each pair of primers were carried out with Platinum *Taq* DNA polymerase (Invitrogen) in the absence of reverse transcriptase to confirm that amplified products were not due to the presence of contaminating chromosomal DNA in RNA preparations. The oligonucleotide primers HRDB-GB1-F and HRDB-GB2-R for *hrdB*, encoding the constitutively expressed housekeeping sigma factor, were used as an internal control to assess the quality of RNA (24). The amount of RNA used for *hrdB* detection was 300 ng. The RT-PCR analysis was carried out at least three times for each pair of primers, and the RT-PCR products were separated in agarose gels and visualized by ethidium bromide staining. The identity of the PCR products was verified by direct sequencing with one of the amplification primers.

Gene expression analysis by qRT-PCR. Transcript detection analysis by quantitative RT-PCR (qRT-PCR) was performed with 0.2 μg of RNA. cDNA was generated with the iScript cDNA synthesis kit (Bio-Rad). PCR amplification was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems, Warrington, United Kingdom). The data were analyzed with the software provided by the supplier. Amplification was carried out in 25 μl containing 0.5 μg of cDNA, 1× Power SYBR green (Applied Biosystems), and each primer (Table 1) at a concentration of 0.4 μM. After incubation at 95°C for 10 min, amplification proceeded with 40 cycles of 95°C for 15 s and 60°C for 1 min. The efficiencies of the primer sets were measured using a dilution series of cDNA. The raw threshold cycle (C_T) values were converted to relative expression levels by the $2^{-\Delta\Delta CT}$ method (15) to quantify the relative gene expression. Based on the sequence of *hrdB* amplified using primers HRDB-GB1-F and HRDB-GB2-R, two new oligonucleotides (HRDBqRT1 and HRDBqRT2) were designed and used for amplification of the *hrdB* transcript as an internal control to quantify the relative expression of target genes.

RESULTS

Insertional inactivation of glutamate synthase *slgE3*. SlgE3 shows high similarity to ferredoxin-dependent GSs and to alpha subunits of NADPH-dependent GSs from different microorganisms with the highest similarity scores being shown by the

GSs from the archaean bacteria *Caldivirga maquilingensis* IC-167 (ZP_01710164), *Thermofilum pendens* Hrk 5 (YP_920540), and *Methanothermobacter thermautotrophicus* strain Delta H (NP_275248). The involvement of SlgE3 in streptolydigin biosynthesis was demonstrated by deleting *slgE3* from *S. lydicus* (Fig. 2A). Analysis of the products accumulated by mutant SLM3 (Fig. 2B) showed the production of very small amounts of streptolydigin with a UPLC retention time of 6.48 min. Analysis of this peak by MS showed two ions with masses of 601 and 487 *m/z* $[M+H]^+$ corresponding to the unfragmented compound and the aglycone fragment, respectively. The mutant SLM3 produced only 2 to 5% of the level of streptolydigin produced by the wild type. Production of streptolydigin in mutant SLME3 was fully restored by the introduction of pEM4TslgE3 containing *slgE3* under the control of the *ermE** promoter (Fig. 2C). These experiments indicate that the active participation of SlgE3 is essential for the usual production yields of streptolydigin in the wild-type strain. In addition, the results also suggest that a second GS can partially sustain the supply of glutamate for the biosynthesis of the antibiotic.

Insertional inactivation of glutamate mutase *slgE1* and *slgE2*. SlgE1 and SlgE2 show significant similarities to VinH and VinI, respectively, S and E subunits of coenzyme B12 (adenosylcobalamin)-dependent mutase from *S. halstedii* (19). They are also similar to the pairs NikU-NikV, SanU-SanV, and GlmA-GlmB, S and E subunits of GMs from *S. tendae*, *S. ansochromogenes*, and *Actinoplanes friuliensis* involved in the biosynthesis of nikkomycin (13, 14) and friulimycin (10), respectively. The participation of *slgE1* and *slgE2* in streptolydigin biosynthesis was assessed by the simultaneous deletion of both genes from *S. lydicus*, thus generating mutant SLME1E2 (Fig. 2D). Analysis of the products accumulated by this mutant showed the production of two compounds (compounds 2 and 3) with UV spectra characteristic of streptolydigin (Fig. 2E). Compound 2 showed a UPLC retention time of 6.10 min and masses of 587 and 473 *m/z* $[M+H]^+$, ions corresponding to the unfragmented compound and the aglycone fragment, respectively. This compound was identical to streptolydigin B (Fig. 3A), previously characterized form *S. lydicus* mutant SLMZ (11). Compound 3 presented UPLC retention time of 6.50 min and masses of 601 and 487 *m/z* $[M+H]^+$. According to these masses, this compound could correspond to streptolydigin. However, after purification of compound 3 and coinjection with authentic samples of streptolydigin in UPLC, these compounds did not comigrate. Structural elucidation of compound 3 was carried out using one-dimensional ^1H, two-dimensional ^1H COSY, ^1H, ^{13}C HSQC-edited, and HMBC NMR experiments (see Table S1 in the supplemental material). This allowed the identification of this compound as a novel streptolydigin derivative carrying a tetramic acid lateral side chain directly derived from glutamate instead of 3-methyl-aspartate and decorated by amidation of the carboxyl group and subsequent methylation of the amino group. We designated this compound streptolydigin C (Fig. 3A). The production of streptolydigin by mutant SLME1E2 was restored by the introduction of pEM4TslgE1E2 containing *slgE1* and *slgE2* under the control of the *ermE** promoter (Fig. 2F).

Streptolydigin C, when tested for its antibacterial activity against *S. albus*, showed a halo of inhibition slightly bigger than that of streptolydigin (4.0 versus 3.2 cm in diameter) (Fig. 3B) or streptolydigin LA (3.0 cm in diameter) (21) and showed considerably more activity than that of streptolydigin B (2.5 cm in diameter) reported previously (11).

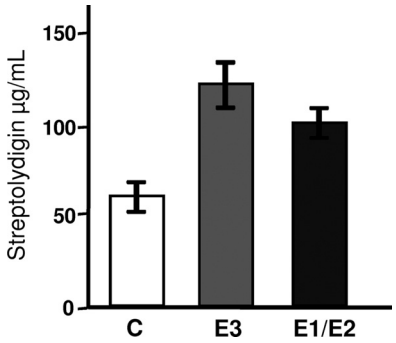

FIG. 4. Effect of *slgE3* or *slgE1-slgE2* overexpression in *S. lydicus* on streptolydigin production. Cultures were performed on R5A solid medium, and streptolydigin production was determined by high-pressure liquid chromatography analysis. Experiments were run in triplicate. C, *S. lycidus*/pEM4T; E3, *S. lydicus*/pEM4TslgE3; E1/E2, *S. lydicus*/pEM4TslgE1E2.

Expression of *slgE3* and *slgE1-slgE2* in *S. lydicus*. The effect of *slgE3* and *slgE1-slgE2* on the production of streptolydigin was also assessed by expressing these genes in *S. lydicus* wild type by using constructs pEM4TslgE3 and pEM4TslgE1E2. In both cases the genes were under the control of the *ermE** promoter. Overexpression of *slgE3* led to a 2-fold increase in streptolydigin production compared to the control *S. lydicus*/pEM4T (Fig. 4). Overexpression of *slgE1-slgE2* led to a 1.6-fold increase in streptolydigin production compared to *S. lydicus*/pEM4T (Fig. 4).

Identification of housekeeping glutamate synthases. The reduced amount of streptolydigin produced by mutant SLME3 implies the possible participation of another GS, different from SlgE3, in supplying glutamate for streptolydigin biosynthesis. To identify additional GS-encoding genes in the chromosome of *S. lydicus*, we used the PCR and oligoprimers derived from consensus amino acid sequences obtained by comparison of NADPH- and ferredoxin-dependent GS from different actinomycetes (Table 1 and Fig. 5). The PCR product obtained using the oligoprimers HEI-I and HEI-J was a fragment of 620 bp. Sequencing of this fragment and comparison of its deduced amino acid sequence with proteins in databases showed that it would code for an internal region of an NADPH-dependent GS α subunit (*gluS*-α) (Fig. 5A and B). The highest similarity score obtained was with a putative NADPH-dependent GS large subunit from *Streptomyces* sp. strain C (ZP_05506017) (99% identity, 100% similarity). The sequence of the 1,316-bp PCR product obtained using oligoprimers HEI-F and HEI-C showed 437 bp coding for the C-terminal end of an NADPH-dependent GS α subunit (*gluS*-α) and 887 bp for the N-terminal end of an NADPH-dependent GS β subunit (*gluS*-β) (Fig. 5A to C). The highest scores were with an NADPH-dependent GS large subunit from *Streptomyces flavogriseus* ATCC 33331 (ZP_04995602) (84% identity, 89% similarity) and an NADPH-dependent GS small subunit from *Streptomyces* sp. strain ACTE (ZP_06271158) (80% identity, 85% similarity). A PCR product of 520 bp was obtained using the oligoprimers HEI-FR1 and HEI-FR2, which could code for an internal region of

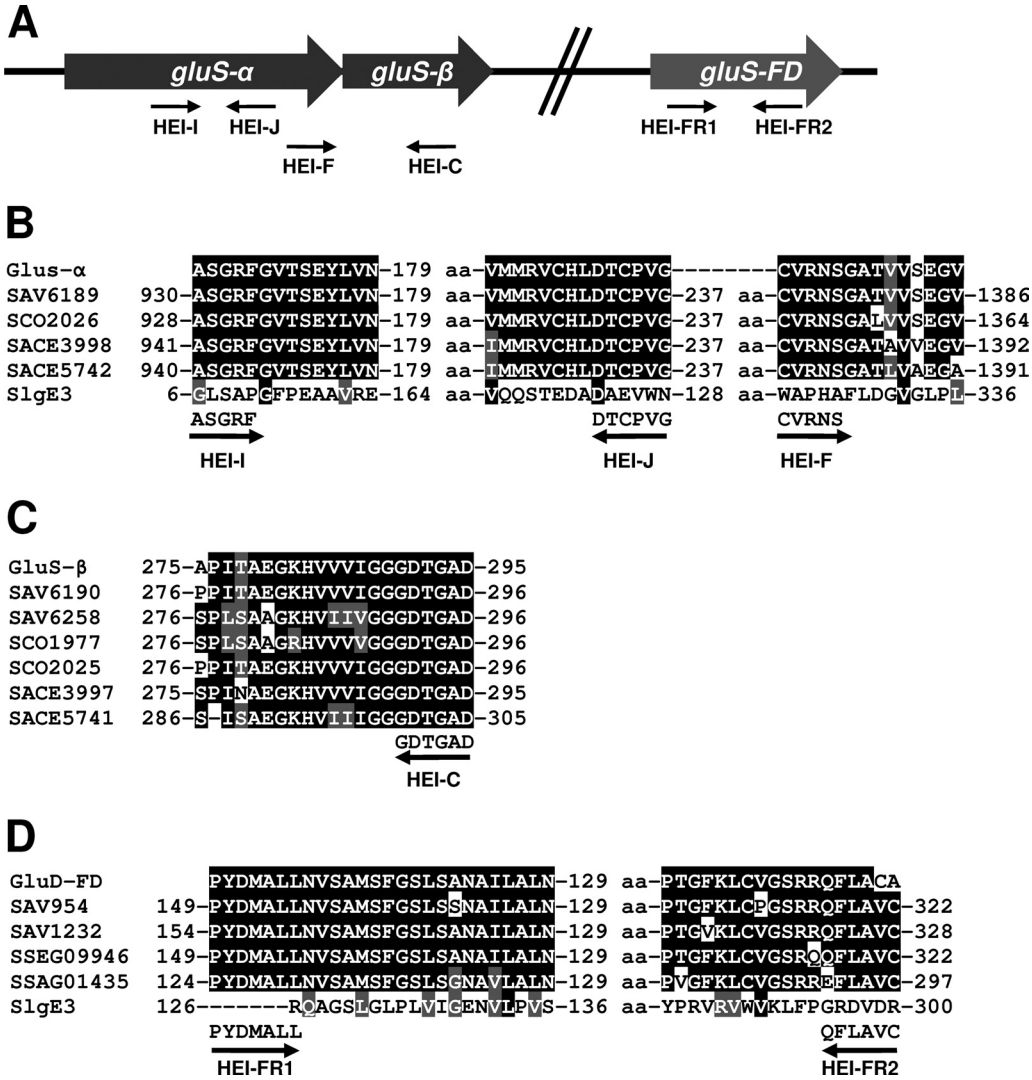

FIG. 5. (A) Scheme representing PCR amplification of glutamate synthase *gluS*-α, *gluS*-β, and *gluS*-FD from the chromosome of *S. lydicus* NRRL2433. (B) Sequence alignments of NADPH-dependent glutamate synthase α subunits showing the conserved regions used for designing degenerated the oligoprimers HEI-I, HEI-J, and HEI-F. GluS-α, *S. lydicus* NRRL2433; SAV6189, *S. avermitilis* MA-4680; SCO2026, *S. coelicolor* A3(2); SACE3998 and SACE5742, *Saccharopolyspora erythraea* NRRL2338. (C) Sequence alignments of NADPH-dependent glutamate synthase β subunits showing the conserved regions used for designing degenerated oligoprimer HEI-C. GluS-β, *S. lydicus* NRRL2433; SAV6190 and SAV6258, *S. avermitilis* MA-4680; SCO1977 and SCO2025, *S. coelicolor* A3(2); SACE3997 and SACE5741, *Saccharopolyspora erythraea* NRRL2338. (D) Sequence alignments of ferredoxin-dependent glutamate synthase showing the conserved regions used for designing degenerated oligoprimers HEI-FR1 and HEI-FR2. SlgE3 and GluS-FD, *S. lydicus* NRRL2433; SAV954 and SAV1232, *S. avermitilis* MA-4680; SSEG09946; *S. sviceus* ATCC 29083; SSAG01435, *Streptomyces* sp. strain Mg1. aa, amino acids.

a ferredoxin-dependent GS (*gluS*-FD) (Fig. 5). The best match was a ferredoxin-dependent GS from *Streptomyces* sp. strain AA4 (ZP_05479083) (85% identity, 91% similarity). The nucleotide sequences of the three PCR products have been deposited in the EMBL database with accession codes FR819656, FR819657, and FR819658, respectively. These results suggest the existence of at least two additional GS in *S. lydicus*: an NADPH-dependent GS constituted by two subunits (GluS-α and GluS-β) and a ferredoxin-dependent GS formed by a single polypeptide (GluS-FD).

We also attempted the identification of additional GM encoding genes in the chromosome of *S. lydicus* using a similar approach. Oligoprimers were designed from consensus amino acid sequences obtained after comparison of GM from different actinomycetes (data not shown). Furthermore, *slgE1* and *slgE2* were also used as probes for Southern hybridization. None of these approaches was successful, indicating that probably only the GM encoded by *slgE1* and *slgE2* is present in the streptolydigin producer organism, which would account for the results obtained by deleting these genes in *S. lydicus* (see above).

Gene expression analysis of glutamate synthases and glutamate mutase. The expression of GS genes *slgE3*, *gluS*-α, *gluS*-β, and *gluS*-FD and GM gene *slgE2* was analyzed in *S. lydicus* and in mutants SLME3 and SLME1E2 by RT-PCR in order to determine their temporal expression patterns in con-

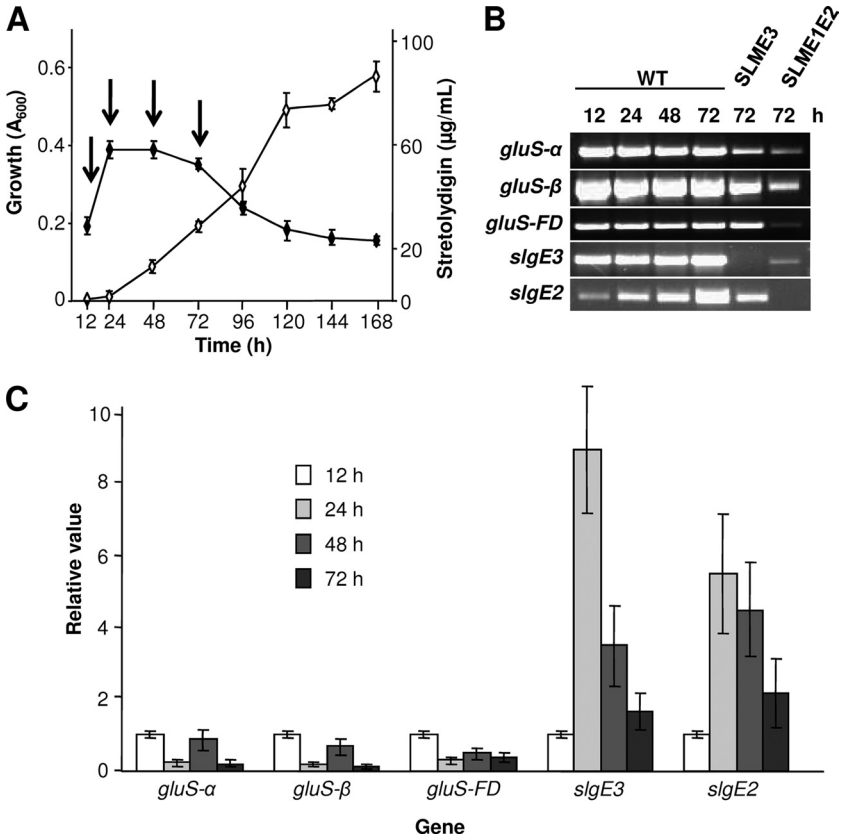

FIG. 6. (A) Growth and streptolydigin production by *S. lydicus* wild-type strain in R5A medium. Growth was monitored by measuring the absorbance at 600 nm for determining DNA content by the diphenylamine assay. Streptolydigin production was spectrophotometrically quantified at 360 nm by peak area integration (◇). Experiments were run in triplicate. The arrows show time points when total RNA was isolated. (B) Transcriptional analysis of *S. lydicus* GSs (*slgE3*, *gluS*-α, *gluS*-β, and *gluS*-FD) and GM (*slgE2*) by RT-PCR. RNA samples were obtained, during growth in R5A liquid medium, at 12, 24, 48, and 72 h from *S. lydicus* wild type and at 72 h from *S. lydicus* SLME3 and SLME1E2. (C) Quantification of gene expression by qRT-PCR. The *hrdB* gene was used as an internal control to quantify the relative expression of target genes. The expression level of each gene at 12 h was taken as the calibrator. Error bars were calculated from three independent determinations of mRNA abundance in each sample.

nection with the biosynthesis of streptolydigin. When monitored in R5A liquid medium, biosynthesis of streptolydigin occurred in a growth-phase-dependent manner and the antibiotic was first detected at 24 h when cultures entered the stationary phase (Fig. 6A). Total RNA was isolated at 12, 24, 48, and 72 h from cultures of the *S. lydicus* wild-type strain and at 72 h from cultures of the mutant strains. The expression of all four GSs was apparently constitutive, including the expression of *slgE3*. As expected, the three housekeeping GS genes were expressed in both streptolydigin-nonproducing mutants SLME3 and SLME1E2. In addition, expression of *slgE3* was also confirmed in GM mutant SLME1E2, but it was absent from GS mutant SLME3. Expression of GM *slgE2* increased after streptolydigin biosynthesis. This gene was also expressed in GS mutant SLME3 (Fig. 6B).

Expression of GSs and GM was quantified by qRT-PCR using the same RNA samples. The value obtained for the 12 h sample was fixed arbitrarily at 1 for reference (Fig. 6C). The expression level of the housekeeping GSs (*gluS*-α, *gluS*-β, and *gluS*-FD) decreased to one-fourth, coinciding with the entry into the stationary phase (24-h sample). However, expression of the streptolydigin-related GS *slgE3* and GM *slgE2* considerably increased at that time (24 h) 9- and 5.5-fold, respectively (Fig. 6C), coinciding with the onset of streptolydigin production (Fig. 6A). Afterward, at 48 and 72 h, the expression of both genes decreased but kept at 72 h a 2-fold expression with respect to the 12-h sample.

DISCUSSION

Streptolydigin biosynthesis requires the incorporation of a 3-methylasparagine moiety. Several genes from the streptolydigin biosynthesis gene cluster have been proposed to encode enzymes that participate in 3-methylaspartate biosynthesis. SlgE3 has been proposed to be involved in the specific supply of glutamate derived from 2-oxoglutarate and glutamine. SlgE1 and SlgE2 have been proposed to catalyze the conversion of glutamate into 3-methylaspartate. This amino is thought to be activated and loaded onto the SlgN2 NRPS PCP domain by catalyzed by the SlgN1 and SlgN2 adenylation domains acting together. Once generated, the NRPS-bound substrate would be further processed by the activity of 3-methylasparagine synthetase SlgZ, followed by N methylation by SlgM to generate NRPS-bound

N-methyl-3-methylasparagine (Fig. 1) (11). *N*-Methyl-3-methylasparagine would be condensed with the preformed streptolydigin polyketide chain, followed by the remaining biosynthetic steps: release and cyclization of the polyketide-nonribosomal peptide to generate the tetramic acid moiety, epoxidation to obtain streptolydiginone (streptolydigin aglycon), and finally attachment of L-rhodinose to obtain the final product (Fig. 1) (21).

The participation of streptolydigin GS and GM enzymes in the biosynthetic process has been confirmed by inactivation of the corresponding genes and their expression in *S. lydicus* wild type. Deletion of *slgE3* reduced the production of streptolydigin to low levels, indicating the importance of ferredoxin-dependent GS SlgE3 in supporting antibiotic biosynthesis by supplying glutamic acid. On the other hand, expression of *slgE3* in *S. lydicus* enhanced antibiotic production. Furthermore, the viability of mutant SLME3 is not compromised by the absence of SlgE3 since three other GS encoding genes have been identified in *S. lydicus*. Two of these genes (*gluS*-α and *gluS*-β) encode the α and β subunits of an NADPH-dependent GS, while the third (*gluS*-FD) encodes a ferredoxin-dependent GS. These housekeeping GSs, which support primary metabolism, have been shown to be expressed throughout *S. lydicus* growth, their expression decreasing when the strain reaches the stationary phase. In contrast, the expression of *slgE3* sharply increases coinciding with the onset of streptolydigin biosynthesis.

Deletion of *slgE1* and *slgE2* abolished streptolydigin production leading to the accumulation of two streptolydigin derivatives streptolydigin B and streptolydigin C both containing glutamate but differing in an *N*-methyl group present in streptolydigin C and absent in streptolydigin B. The production of streptolydigin B has been observed before in an SLMZ mutant affected in ammonia-dependent 3-methylasparagine synthetase *slgZ*, which implies that the streptolydigin NRPS is flexible enough to accept glutamate instead of 3-methylaspartate as a substrate for the biosynthesis of streptolydigin or streptolydigin B (11). In addition, the production of streptolydigin C, with a tetramic lateral side chain modified by amidation and methylation, implies the flexibility of both SlgZ and SlgM for amidating and methylating glutamyl-NRPS and glutaminyl-NRPS intermediates, respectively. A similar effect has been shown by disruption of a GM gene involved in the biosynthesis of vicenistatin. Inactivation of *vinI* from *S. halstedii* led to abolition of vicenistatin production and to the accumulation of desmethyl-vicenistatins (18). On the other hand, the expression of *slgE1* and *slgE2* in *S. lydicus* improved streptolydigin production, a positive effect that has also been observed in the production of nikkomycin by *S. ansochromogenes* after the expression of *sanU* and *sanV* (14). In accordance with its proposed involvement in streptolydigin biosynthesis, an increase in *slgE2* expression was observed coinciding with the production of the tetramic acid.

Comparison of the antibacterial activity of streptolydigin, streptolydigin B, and streptolydigin C gives some clues to a structure-function relationship. Substitution of 3-methylaspartate (as in streptolydigin) by glutamate (as in streptolydigin C) apparently increases the antibacterial activity. In addition, the presence of an acetamide moiety (as in streptolydigin C) clearly improves the biological activity in comparison with a compound lacking this group (as in streptolydigin B). This acetamide moiety has been shown to be important for the interaction of streptolydigin with the RNA polymerase (28).

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