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Brief report

Participation of putative glycoside hydrolases SlgC1 and SlgC2 in the biosynthesis of streptolydigin in *Streptomyces lydicus*

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Summary

Two genes of the streptolydigin gene cluster in *Streptomyces lydicus* cluster encode putative family 16 glycoside hydrolases. Both genes are expressed when streptolydigin is produced. Inactivation of these genes affects streptolydigin production when the microorganism is grown in minimal medium containing either glycerol or D-glucans as carbon source. Streptolydigin yields in *S. lydicus* were increased by overexpression of either *slgC1* or *slgC2*.

Streptomyces species are Gram-positive bacteria widely distributed in terrestrial and aquatic ecosystems (McCarthy and Williams, 1992; Stach and Bull, 2005; Gao and Gupta, 2012). They exhibit diverse physiological and metabolic properties, such as the production of a wide variety of secondary metabolites (Berdy, 2005). In addition, they are important for soil biodegradation and humus formation by decomposing and recycling complex mixtures of polymers in dead plant, animal and fungal materials using extracellular enzymes (McCarthy and Williams, 1992; Chater *et al*., 2010). Among these polymers are polysaccharides such as cellulose, chitin, β-glucans, starch, glycogen, inulin, pullulan and xylan, which can be degraded by hydrolytic enzymes (Guillén *et al*., 2010).

Streptolydigin (Fig. 1A) is a potent inhibitor of bacterial RNA polymerase and eukaryotic terminal deoxynucleotidyl transferase produced by *Streptomyces lydicus* NRRL 2344 (Olano *et al*., 2009; Sánchez-Hidalgo *et al*., 2010). The structure of streptolydigin is composed of a tetramic acid moiety, derived from a polyketide non-ribosomal peptide backbone to which an L-rhodinose moiety derived from D-glucose is attached. The gene cluster for streptolydigin biosynthesis has been characterized and several streptolydigin novel derivatives have been obtained by combinatorial biosynthesis (Olano *et al*., 2009; Gómez *et al*., 2011; 2012; Horna *et al*., 2011). In addition, streptolydigin yields have been improved by overexpression of some genes of the cluster (Gómez *et al*., 2011; Horna *et al*., 2011). The streptolydigin gene cluster contains two genes of unknown function, *slgC1* and *slgC2*, encoding putative glycoside hydrolases. Their deduced gene products contain the characteristic glycoside hydrolase family 16 signature domain and show similarities to several putative secreted endo-1,3-β-glucanases (Fig. 1B). Homologues to *slgC1* and *slgC2*, *tamE* and *trdE*, have been also found in the biosynthesis gene clusters of tetramic acid compound tirandamycin (Carlson *et al*., 2010; Mo *et al*., 2011). Signal peptide has not been detected either in SlgC1 or in SlgC2 and subcellular location prediction using PSORTb v3.0 (Yu *et al*., 2010) points to a cytoplasmic location. In addition, SlgC1 and SlgC2 lack a discernible carbohydrate-binding module (CBM) necessary to confer carbohydrate-binding activity (Hong *et al*., 2002; 2008; Shi *et al*., 2010). Occasionally, CBMs can also be found isolated as single proteins (Guillén *et al*., 2010) but in the streptolydigin biosynthesis cluster there are not genes encoding free-standing CBMs (Olano *et al*., 2009).

Since *slgC1* and *slgC2* are initially not expected to have a role in the biosynthesis of the streptolydigin chemical structure, we were interested in getting inside a possible role for these genes in streptolydigin production. Expression of *slgC1* and *slgC2* in *S. lydicus* was determined by RT-PCR and detected at 72 h (Fig. 2A and Table 1) when streptolydigin was actively being produced as determined previously (Horna *et al*., 2011). The role of these genes was further investigated through their inactivation in *S. lydicus* following the procedure previously described (Olano *et al*., 2009). Streptolydigin production by mutants SLMC1 and SLMC2 (Table 2) was assessed following the method

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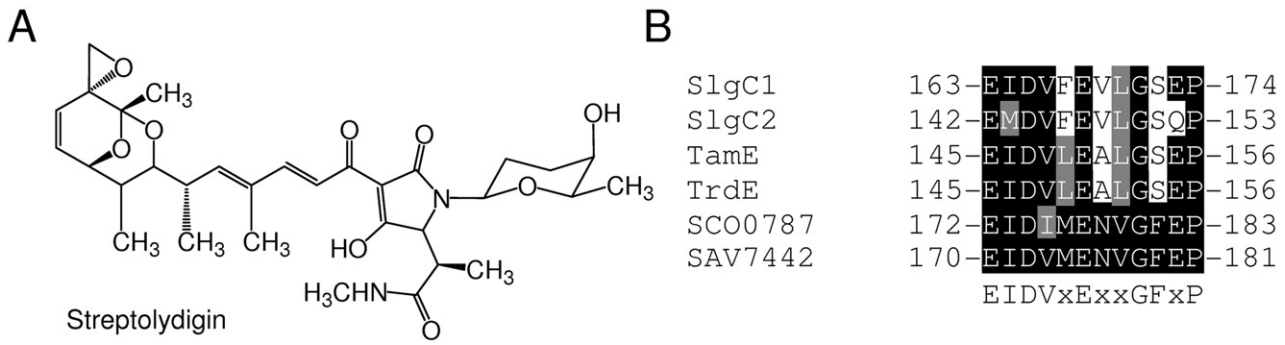

Fig. 1. A. Structure of streptolydigin. B. Sequence alignment of SlgC1 and SlgC2 with putative hydrolases showing the glycoside hydrolase family 16 signature (PDOC00794): E-[LIV]-D-[LIVF]-x(0,1)-E-x(2)-[GQ]-[KRNF]-x-[PSTA]. TamE from *Streptomyces* sp. 307-9 (Accession No. ADC79643); TrdE from *Streptomyces* sp. SCSIO1666 (ADY38537); SCO0787 from *Streptomyces coelicolor*A3(2) (NP_625089); SAV7442 from *Streptomyces avermitilis*MA-4680 (NP_828618). x: any amino acid.

previously described (Olano *et al.*, 2009), showing that streptolydigin is produced in both cases at the same level than in the *S. lydicus* wild-type strain (Fig. 2B). This result suggests that SlgC1 and SlgC2 are not essential for streptolydigin biosynthesis. However, when *slgC1* and *slgC2* were independently expressed in *S. lydicus*, using plasmids pEM4TslgC1 or pEM4TslgC2 (Table 2), 12- and 3-fold increase of streptolydigin production were observed respectively (Fig. 2C).

Streptomycetes are usually soil living microorganisms. Therefore, we wonder if the presence of *slgC1* and *slgC2* genes in the streptolydigin cluster could be an evolutionary

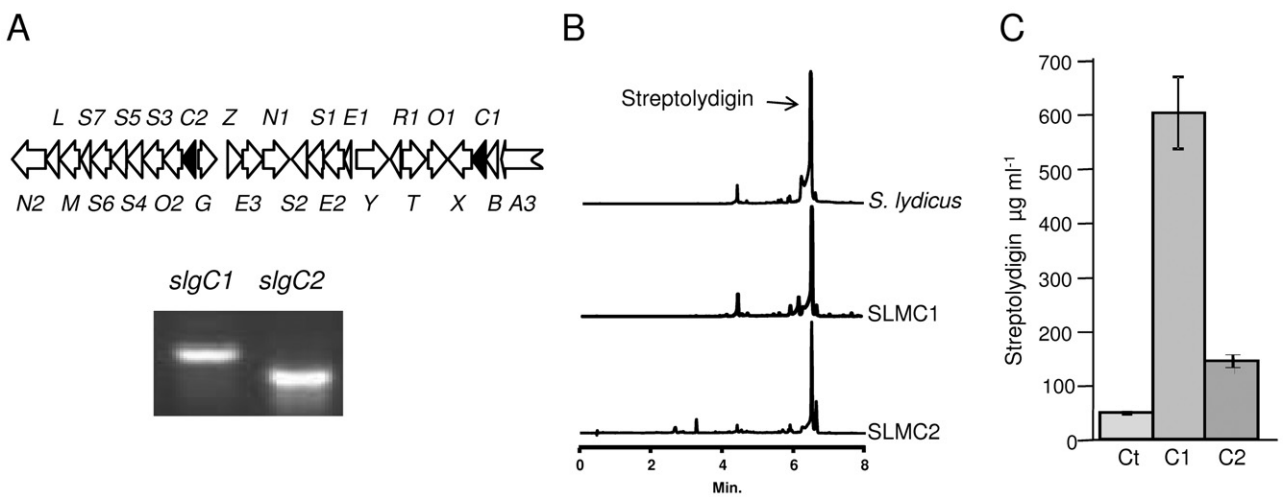

Fig. 2. A. Detail of streptolydigin biosynthesis gene cluster showing the location of *slgC1* (C1) and *slgC2* (C2) as black arrows and expression of *slgC1* and *slgC2* in *S. lydicus*. PCR products were obtained by RT-PCR using 150 ng of total RNA samples of *S. lydicus*, extracted during growth at 72 h in R5A [sucrose 103 g l^{-1}, K_2SO_4 0.25 g l^{-1}, $MgCl_2$–$6H_2O$ 10.12 g l^{-1}, glucose 10 g l^{-1}, casamino acids 0.1 g l^{-1}, yeast extract 5 g l^{-1}, MOPS 21 g l^{-1} and 2 ml l^{-1} of trace element solution ($ZnCl_2$ 40 mg l^{-1}; $FeCl_3$–$6H_2O$ 200 mg l^{-1}; $CuCl_2$–$2H_2O$ 10 mg l^{-1}; $MnCl_2$–$4H_2O$ 10 mg l^{-1}; $Na_2B_4O_7$–$10H_2O$ 10 mg l^{-1}; $(NH_4)_6Mo_7O_{24}$–$4H_2O$ 10 mg l^{-1}), pH 6.8, in deionized water] (Fernández *et al.*, 1998) and primers CRIS19/CRIS20 and CRIS21/CRIS22 to verify the expression of *slgC1* and *slgC2*. The expression of *hrdB* was monitored as a control to normalize RNA samples as described before (Gómez *et al.*, 2011). The absence of contaminating DNA in the samples was verified in PCR-negative controls containing DNA polymerase but lacking reverse transcriptase, in which amplified products were not detected with any of the primers. The analyses were carried out in triplicate for each pair of primers and the identity of each amplification product was authenticated by direct sequencing. B. UPLC analysis of *S. lydicus* and mutants SLMC1 and SlMC2 using an Acquity UPLC equipment with a BEH C18 Waters column of 2.1 × 100 mm. Detection and spectral characterization of peaks was performed by photodiode array detection and Empower software (Waters), extracting bidimensional chromatograms at 360 nm. C. Production of streptolydigin by *S. lydicus* carrying pEM4T (Ct), pEM4TslgC1 (C1) or pEM4slgC2 (C2) grown in R5A liquid medium. Production of streptolydigin was assessed by growing the corresponding recombinant strains in R5A liquid medium using square deep-well plates (Siebenberg *et al.*, 2010) consisting of 24 wells of 3 ml culture volume each. Pre-cultures of *S. lydicus* were prepared in square deep-well plates containing 3 ml of TSB medium. Cultivation was carried out at 30°C and 250 r.p.m. for 2 days. Then, square deep-well plates were inoculated at 1:80 ratio and cultivated at 30°C and 300 r.p.m. for 7 days. Growth of *S. lydicus* was monitored by determining the DNA content through measuring absorbance (A_{600}) following the diphenylamine assay method (Méndez *et al.*, 1985). Streptolydigin production monitored by UPLC was corrected in each case by the A_{600} value.

Table 1. Oligonucleotides used in this study.

Primer	Sequence (5′→3′)	Characteristics and comments
CRIS19	AA**TCTAGA**TGGAGGAGCACACCGACG	XbaI, to generate pOJPC1
CRIS20	A**GAATTC**CTGCCGATGATGACGGCC	EcoRI, to generate pOJPC1
CRISE1A	AA**GATATC**GGTGGTGTACCGGAACGT	EcoRV, to generate pΔslgC2Hyg
CRISE1B	AAA**GGATCC**GCGATGAGCGAGCGGA	BamHI, to generate pΔslgC2Hyg
CRISE2A	AA**ATGCAT**GGGATCGGCTGTCGTCATC	NsiI, to generate pΔslgC2Hyg
CRISE2B	A**ACTAGT**GCGGCAGATAGCGCCATCGG	SpeI, to generate pΔslgC2Hyg
CRIS40	AA**GGATCC**GACGACTGCGGACGCTCG	BamHI, cloning of *slgC1* to create pEM4TslgC1
CRIS41	A**GAATTC**AGGTCCTGTGCGGTGGGC	EcoRI, cloning of *slgC1* to create pEM4TslgC1
CRIS34	AA**GGATCC**GTGAGTCCGGTGAATGCA	BamHI, cloning of *slgC2* to create pEM4TslgC2
CRIS35	A**GAATTC**CTGCATGAAGTCGGCGTC	EcoRI, cloning of *slgC2* to create pEM4TslgC2
CRIS19	AATCTAGATGGAGGAGCACACCGACG	For *slgC1* RT-PCR analysis
CRIS20	AGAATTCCTGCCGATGATGACGGCC	For *slgC1* RT-PCR analysis
CRIS21	AATCTAGAACCGCCACCCGCGACGCC	For *slgC2* RT-PCR analysis
CRIS22	AGAATTCCCGCCCGGGCTGCCGATG	For *slgC2* RT-PCR analysis

Endonuclease restriction sites are given in boldface.

advantage for the producer microorganism by facilitating sugar supply for streptolydigin biosynthesis in its natural soil environment. To evaluate the potential participation of SlgC1 and SlgC2 enzymes in the degradation of β- and α-glucans, cultures of *S. lydicus*, mutants SLMC1 and SLMC2 and *S. lydicus* carrying pEM4T, pEM4TslgC1 or pEM4TSlgC2 were performed on three minimal media using different carbon sources: glycerol (MMG), β-glucan laminarin (MML) and α-glucan starch (MMS). Differences in the production of streptolydigin were observed in MMG between *S. lydicus* wild type and mutants SLMC1 and SLMC2. Streptolydigin yields in these mutants decreased 1.6- and 12-fold respectively. In contrast, a positive effect of the expression of *slgC1* or *slgC2 in S. lydicus* was observed. In this medium, production of streptolydigin increased 5.5- and 5-fold respectively, when compared with *S. lydicus*/pEM4T used as a control (Fig. 3A). Total production yields of streptolydigin by *S. lydicus* decreased when grown on MML or MMS (Fig. 3B and C). Inactivation of *slgC1* or *slgC2* also conducted to a clear decrease in streptolydigin yields when mutant strains SLMC1 and SLMC2 were grown in MML (2.8- and 5-fold respectively) or in MMS (3.3- and 13-fold respectively) (Fig. 3B and C). In addition, expression of *slgC1* or *slgC2* in *S. lydicus* also conducted to increase production yields in both MML (1.3- and 7-fold respectively) and MMS (3- and 10-fold respectively) (Fig. 3B and C). In both cases, yields of streptolydigin were higher when *slgC2* was expressed in *S. lydicus* than when *slgC1* was, being the streptolydigin titres higher than in the wild-type strain when grown in MML, or similar to the wild-type strain when grown in MMS. Curiously, the presence of the empty vector in *S. lydicus*

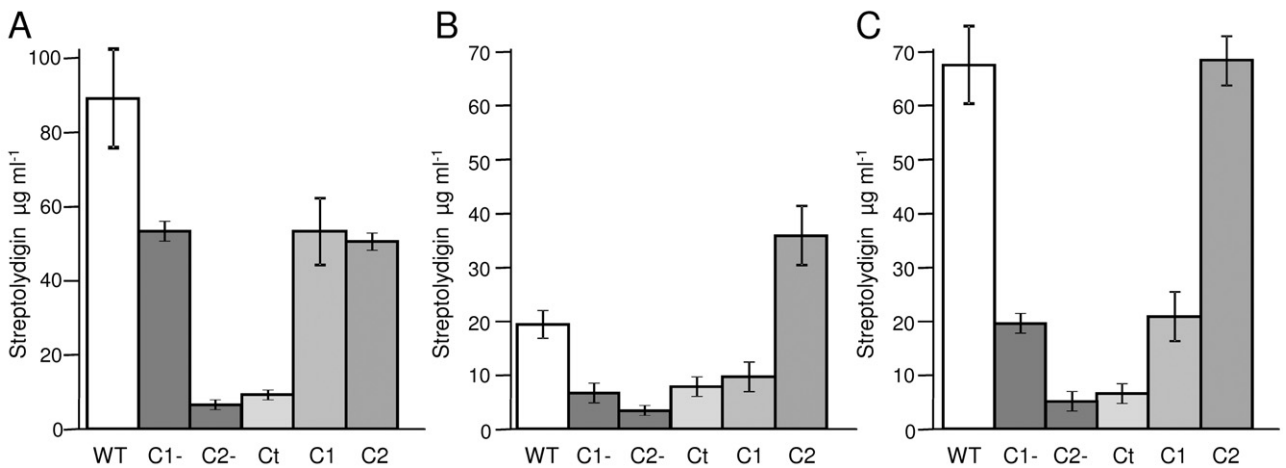

Fig. 3. A. Production of streptolydigin by *S. lydicus* (WT), SLMC1 (C1-), SLMC2 (C2-), and *S. lydicus* carrying pEM4T (Ct), pEM4TslgC1 (C1) or pEM4slgC2 (C2) grown in MMG (MM containing glycerol at 10 g l^{-1}). B. Production of streptolydigin in MML (MM containing laminarin at 10 g l^{-1}). C. Production of streptolydigin in MMS (MM containing starch at 10 g l^{-1}). In all cases streptolydigin production was determined by UPLC analysis. Experiments were run in triplicate. Minimal medium MM is composed of MOPS 21 g l^{-1}, $MgSO_4$ 0.6 g l^{-1}, $CaCl_2$ 5 mg l^{-1}, $MnCl_2$ 1 mg l^{-1}, $ZnSO_4$ 1 mg l^{-1}, $FeSO_4$ 5 mg l^{-1}, K_2HPO_4 1.75 g l^{-1}, NH_4Cl 1.6 g l^{-1} and fumarate 2%, pH 6.8, in deionized water. Laminarin and starch were purchased form Sigma.

Table 2. Bacterial strains and plasmids used in this study.

Strain or primer	Relevant characteristic(s)	References
Strains		
S. lydicus		
NRRL2433	Streptolydigin producer	Olano *et al.* (2009)
SLMC1	*slgC1*::pOJ260P	This study
SLMC2	*slgC2*::*acc(3)IV*	This study
Escherichia coli		
DH10B	*F–, mcrA*, Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*Δ*M15*, Δ*lacX74*, *recA1 endA1*, *araD139* Δ(*ara leu*) 7697 *galU*, *galK*, *rpsL*, *nupG*, λ⁻	Invitrogen
ET12567 (pUB307)	*F–, dam13::Tn9, dcm6, hsdM hsdR, recF143, zjj201::Tn10, galK2 galT22, ara14, lacY1, xyl5, leuB6 thi1, tonA31, rpsL136, hisG4, tsx78 mtli, glnV44*	Kieser *et al.* (2000)
Plasmids		
Slg4A8	Source of *slgC1* and *slgC2*	Olano *et al.* (2009)
pCR-BLUNT	*lacZα, ccdB*, pUC*ori*	Invitrogen
pOJ260P	*acc(3)IV, oriT, lacZα, ermE*p*	Olano *et al.* (2004)
pEM4T	*tsr, bla, oriT, ermE*p*	Menéndez *et al.* (2006)
pLHyg	LITMUS 18 derivative bearing *hyg*	Olano *et al.* (2004)
pEFBAoriT	*bla, oriT, acc(3)IV*	Horna *et al.* (2011)
pOJPC1	pOJ260P derivative used to generate mutant stain SLMC1	This study
pΔslgC2Hyg	pEFBAoriT derivative used to generate mutant strain SLMC2	This study
pEM4TslgC1	pEM4T derivative bearing *slgC1*	This study
pEM4TslgC2	pEM4T derivative bearing *slgC2*	This study

Antibiotics were used, where appropriate, at the following concentrations: ampicillin 100 μg ml^{-1}, tobramycin 20 μg ml^{-1}, apramycin 25 μg ml^{-1}, thiostrepton 50 μg ml^{-1}, tetracycline 10 μg ml^{-1}, chloramphenicol 25 μg ml^{-1} and nalidixic acid 50 μg ml^{-1}. *Streptomyces lydicus* strains were generated by intergeneric conjugation from *E. coli* ET12567 (pUB307). SLMC1 transconjugants were selected for resistance to apramycin. To isolate SLMC2 transconjugants an apramycin and hygromycin resistant strain was grown in the absence of antibiotic selection and then colonies were screened for the loss of hygromycin resistance and retention of apramycin resistance, as a consequence of a double recombination event.

used as control dramatically reduced the amount of streptolydigin produced in all media tested. This effect has been observed previously in *S. lydicus* (Gómez *et al.*, 2011; Horna *et al.*, 2011). In the present and previous works all strains were grown in the same conditions including the antibiotic for selection. However, the lower production observed in the control culture cannot be solely attributed to the presence of the antibiotic for selection (data not shown).

Several conclusions can be drawn from these experiments. First, streptolydigin biosynthesis can be supported using as unique carbon source a glucan, in particular starch. Second, SlgC1 and SlgC2 can exert an influence on the production yields of streptolydigin under certain culture conditions, probably by facilitating D-glucose or glucose-1-phosphate supply as primary precursor for the biosynthesis. Since both SlgC1 and SlgC2 lack signal peptide, their role in precursor supply might be intracellular, probably related to the mobilization of accumulated internal polysaccharides. Other glycoside hydrolases have been shown to be involved in precursor supply for antibiotic production. Glucoamylase VldI supplies glucose with the hydrolysis of α-1,4-D-glucan for the production of validamycin. Inactivation of *vldI* in *Streptomyces hygroscopicus* subsp. *limoneus* decreases validamycin production (Singh *et al.*, 2007). VldI, as SlgC1 and SlgC2, lacks the CBM and is predicted to be intracellular. On the other hand, glycoside hydrolase TrdE has been recently shown to have a structural role in the biosynthesis of tirandamycin. Interestingly, inactivation of *trdE* in *Streptomyces* sp. SCIO 1666 led to the accumulation of pre-tirandamycin, a biosynthetic intermediate of tirandamycin C. The conversion of pre-tirandamycin into tirandamycin C by TrdE was also verified *in vitro* (Mo *et al.*, 2012). However, inactivation of neither *slgC1* nor *slgC2* in *S. lydicus* led to the accumulation of putative streptolydigin precursors detectable at different wavelengths or by LC-MS (only streptolydigin is accumulated), which suggest a different role for glycoside hydrolases in streptolydigin and tirandamycin pathways.

Different genetic approaches have been used for improving the production of secondary metabolites produced by actinomycetes. Among these approaches are the modification of metabolic flux distribution of precursors, the deregulation of the biosynthetic pathway or the overexpression of structural genes coding for enzymes involved in the biosynthesis of the metabolite (Olano *et al.*, 2008). In the case of steptolydigin production, in addition to *slgC1* and *slgC2*, other genes involved in precursor supply and modification (*slgE1-E2-E3*, *slgZ* and *slgM*) have been shown to improve its production (Gómez *et al.*, 2011; Horna *et al.*, 2011).

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