Brief report

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

Cristina Gómez,1,2 Dina H. Horna,1,2 Carlos Olano,1,2 Carmen Méndez1,3 and José A. Salas1,2*

1Departamento de Biología Funcional, Universidad de Oviedo, 33006 Oviedo, Spain.
2Instituto Universitario de Oncología del Principado de Asturias (I.U.O.P.A), Universidad de Oviedo, 33006 Oviedo, Spain.

Summary

Two genes of the streptolydigin gene cluster in *Streptomyces lydicus* 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., 2011). 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 β-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...
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. 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 coelicolorA3(2) (NP_625089); SAV7442 from Streptomyces avermitilisMA-4880 (NP_629818). x: any amino acid.

**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, K₂SO₄ 0.25 g l⁻¹, MgCl₂–6H₂O 10.12 g l⁻¹, glucose 10 g l⁻¹, casamino acids 0.1 g l⁻¹, yeast extract 5 g l⁻¹, yeast extract 5 g l⁻¹, yeast extract 5 g l⁻¹, and 2 ml l⁻¹ of trace element solution (ZnCl₂ 40 mg l⁻¹; FeCl₃–6H₂O 200 mg l⁻¹; CuCl₂–2H₂O 10 mg l⁻¹; MnCl₂–4H₂O 10 mg l⁻¹; Na₂B₄O₇–10H₂O 10 mg l⁻¹; (NH₄)₆Mo₇O₂₄–4H₂O 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 pEM4TslgC2 (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 (A₆0₀) following the diphenylamine assay method (Méndez et al., 1985). Streptolydigin production monitored by UPLC was corrected in each case by the A₆0₀ value.
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

Table 1. Oligonucleotides used in this study.

| Primer  | Sequence (5'→3') | Characteristics and comments |
|---------|------------------|-----------------------------|
| CRIS19  | AATCTAGATGGAGGAGCACACCGACG | XbaI, to generate pOJPC1 |
| CRIS20  | AGAATTCCTGGGTAAGCCCGAGGCC | EcoRI, to generate pOJPC1 |
| CRISE1A | GATATC GGGTGGTACCGGAACGT | EcoRV, to generate pDslgC2Hyg |
| CRISE1B | GGATCC GCGATGAGCGAGCGGA | BamHI, to generate pDslgC2Hyg |
| CRISE2A | ATGCAT GGGATCGGCTGTCGTCATC | NsiI, to generate pDslgC2Hyg |
| CRISE2B | ACTAGT GCGGCAGATAGCGCCATCGG | SpeI, to generate pDslgC2Hyg |
| CRIS40  | GGATCC GACGACTGCGGACGCTCG | BamHI, cloning of slgC1 to create pEM4TslgC1 |
| CRIS41  | GAATTC AGGTCCTGTGCGGTGGGC | EcoRI, cloning of slgC1 to create pEM4TslgC1 |
| CRIS34  | AAGATCCTGGGTAAGCCCGAGGCC | For slgC2 RT-PCR analysis |
| CRIS35  | AGAATTCCTGGGTAAGCCCGAGGCC | For slgC1 RT-PCR analysis |
| CRIS19  | AATCTAGATGGAGGAGCACACCGACG | For slgC2 RT-PCR analysis |

Endonuclease restriction sites are given in boldface.

Fig. 3. A. Production of streptolydigin by S. lydicus (WT), SLMC1 (C1-), SLMC2 (C2-), and S. lydicus carrying pEM4T (Ct), pEM4TslgC1 (C1) or pEM4TslgC2 (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, MgSO₄ 0.6 g l⁻¹, CaCl₂ 5 mg l⁻¹, MnCl₂ 1 mg l⁻¹, ZnSO₄ 1 mg l⁻¹, FeSO₄ 5 mg l⁻¹, K₂HPO₄ 1.75 g l⁻¹, NH₄Cl 1.6 g l⁻¹ and fumarate 2%, pH 6.8, in deionized water. Laminarin and starch were purchased form Sigma.

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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 Vldl supplies glucose with the hydrolysis of α-1,4-D-glucan for the production of validamycin. Inactivation of vldl in *Streptomyces hygroscopicus* subsp. *limoneus* decreases validamycin production (Singh et al., 2007). Vldl, 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. SCI 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 streptolydigin 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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**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::pPJ260P              | This study  |
| SLMC2            | slgC2::acc(3)IV             | This study  |
| **Escherichia coli** |                             |            |
| DH10B            | F-, mcrA, Δ(mrr-hsdRMS-mcrBC), Φ01lacZM15, lacX74, recA1 endA1, araD139 Δ(ara leu) 7697 galU, galK, rpsL, nupG, λ::Invitrogen |
| ET12567 (pUB307) | F-, dam13::Tn9, dcmE, hsdM hsdR, recF143, zij201::Tn10, galK2 galT22, ara14, lacY1, xyl5, leuB5 thi1, tonA31, pslL136, hisG4, tss78 mtT1, glnV44 | Kieser et al. (2000) |
| **Plasmids**     |                             |            |
| Slg4A8           | Source of slgC1 and slgC2   | Olano et al. (2009) |
| pCR-BLUNT       | lacZu, ccdB, pUC19         | Invitrogen |
| pOJ260P          | acc(3)IV, oriT, lacZu, ermE’p | Olano et al. (2004) |
| pEM4T            | tsr, bla, oriT, ermE’p     | Menéndez et al. (2006) |
| pLHyg            | LITMUS18 derivative bearing hhy | Olano et al. (2004) |
| pEFBAoriT        | bla, oriT, acc(3)IV        | Horna et al. (2011) |
| pOJPC1           | pOJ260P derivative used to generate mutant strain SLMC1 | This study |
| pEllgC2Hyg       | 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, thioestrepton 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.
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