ORIGINAL PAPER

Cloning and expression of the *sco2127* gene from *Streptomyces coelicolor* M145

Adán Chávez · Yolanda García-Huante · Beatriz Ruiz · Elizabeth Langley · Romina Rodríguez-Sanoja · Sergio Sanchez

Abstract It is known that *Streptomyces peucetius* var. *caesius* mutants resistant to 2-deoxyglucose (Dog[R]) exhibit glucose transport deficiency, low glucose kinase (Glk) activity and insensitivity to carbon catabolite repression (CCR). This phenotype can be pleiotropically complemented by a 576-bp gene encoding SCO2127 from *Streptomyces coelicolor*, suggesting the participation of this protein in the CCR process. In the present work, the *sco2127* region was subcloned into pQE30 and its transcription product (SCO2127-His$_6$) overexpressed. This procedure allowed purification of SCO2127 (with a Ni-sepharose resin) and production of polyclonal antibodies. In western blot assays, the antibodies gave a positive reaction against protein extracts from both *S. coelicolor* and *S. peucetius* var. *caesius*, appearing as a single band of 34 kDa. No protein was detected using extracts from a *S. coelicolor* mutant lacking the *sco2127* gene (Δ*sco2127*). In agreement with its possible involvement in the CCR process, SCO2127 was detected during the logarithmic growth phase of *S. coelicolor* grown in minimal medium supplemented with 50 and 100 mM glucose. In addition, when 50 mM glucose was utilized, SCO2127 and residual glucose concentration simultaneously decreased at later stages of the microbial growth.

Keywords SCO2127 · *Streptomyces coelicolor* · Carbon catabolite repression

A. Chávez · Y. García-Huante · B. Ruiz · E. Langley ·
R. Rodríguez-Sanoja · S. Sanchez (✉)
Instituto de Investigaciones Biomédicas,
Universidad Nacional Autónoma de México,
04510 Mexico D.F., Mexico
e-mail: sersan@biomedicas.unam.mx

Introduction

Streptomyces coelicolor mutants, isolated by their ability to utilize glycerol or lactose for growth in the presence of the glucose analog, 2-deoxyglucose (DOG), show insensitivity to glucose carbon catabolite repression (CCR) as well as to the utilization of several other carbon sources [1]. The insensitivity of these mutants (Dog[R]) to CCR seems to be due to both a failure to utilize glucose and low glucose kinase (Glk) activity [1, 2], which prevents production of glucose catabolites [3]. The Dog[R] phenotype can be restored to a wild type phenotype by transforming the mutants with a 2.9-kb *Bcl*I fragment of *S. coelicolor* DNA containing two complete open reading frames encoding SCO2126 (Glk) and protein SCO2127 of unknown function [4]. A derivative of the temperate phage ΦC31 containing *glkA* alone partially restores the wild type phenotype when used to lysogenize a *S. coelicolor glk* null mutant with the Dog[R] phenotype. Complete restoration to the wild type phenotype is observed with the temperate phage ΦC31 KC896 containing both *glkA* and *sco2127* genes [5].

In addition to the effect of *glkA* on *S. coelicolor* Dog[R] mutants, the effect of *glkA* and *sco2127* has been analyzed in a Dog[R] mutant of the doxorubicin producer *Streptomyces peucetius* var. *caesius* (deficient in glucose transport, low Glk activity and insensitive to CCR) [6, 7]. A partial reversion of the Dog[R] phenotype is observed when transforming the mutant with *glkA*. However, a complete reversion is obtained when transforming the mutant with the *sco2127* region alone [8, 9]. This result was unexpected since *sco2127* does not seem to encode for Glk [5], nor for a glucose permease (GlcP1) [10], and also lacks DNA binding motifs. Dot blot analysis of the *S. peucetius* var. *caesius* Dog[R] mutant suggested that *sco2127* encodes for a protein that stimulates transcription of *glk* and probably that of the

glucose permease gene as well [8]. In spite of the possible role of SCO2127 in the CCR processes of both *Streptomyces* species, there are no published data on the expression and activity of *sco2127* in *S. coelicolor* [2], a model species for an industrially important group of bacteria [11]. Therefore, the aim of this work was to clone and express the *sco2127* gene from *S. coelicolor*, determining its expression along the fermentation process in a chemically defined medium supplemented with glucose.

Materials and methods

Microorganisms and culture conditions

S. coelicolor A3(2) M145 (SCP-1, SCP-2, prototroph) and cosmid SC6E10 were kindly donated by Luis Servín-González, Instituto de Investigaciones Biomédicas, UNAM, México D. F. The *S. coelicolor sco2127* null mutant (Δ*sco2127*) was constructed by Forero et al. (in preparation) using the PCR targeting procedure [12]. For seed cultures, approximately 10^6 spores (maintained in 20% glycerol) were used to inoculate 250-ml Erlenmeyer flasks containing 50 ml minimal medium (NMMP) [13] supplemented with 50 mM mannitol. The seed cultures were incubated 48 h at 29°C under agitation (180 rpm). The cells were collected by centrifugation ($10,400 \times g$ for 10 min), washed two times and resuspended in 5 ml saline solution. For studies on SCO2127 synthesis, 250-ml baffled Erlenmeyer flasks containing 50 ml NMMP were inoculated with 1 ml of the washed seed cultures and incubated under similar conditions. At desired times, cells were collected by centrifugation ($10,400 \times g$ for 10 min) and disrupted by sonication (eight 30-s 60-W pulses, leaving 1 min between

each pulse), centrifuged at $5,900 \times g$ for 10 min and the sample supernatants (20 μg total protein) used for western blot analyses. Growth was monitored by following the absorbance at 600 nm. Remaining glucose in the culture medium was enzymatically determined, as previously described [6].

Escherichia coli M-15 (containing the pREP4 plasmid that encodes for the *lac* repressor in *trans*, ensuring a tightly regulated expression) was obtained from a commercial kit (Qiagen). Cells were grown in LB broth (Sigma) supplemented with ampicillin and kanamycin (100 and 25 μg/ml, respectively) at 37°C and 200 rpm. *E. coli* strain DH5α (containing the cosmid SC6E10) was grown in the same LB, supplemented with ampicillin (100 μg/ml).

Expression and purification of SCO2127-His₆

The *sco2127* gene was amplified by PCR using as template the *S. coelicolor* cosmid SC6E10 containing this region (Fig. 1). The oligonucleotides were designed based on the reported *sco2127* gene sequence [5] covering both the gene transcriptional start and terminator. The primers used for *sco2127* amplification were: fwd (5′-AGGAGTCCGT<u>CTA GA</u>GCGAAG), rev (5′-GGC<u>AAGCTT</u>ACCCGAGGC). Restriction sites for *Xba*I and *Hind*III were included in the amplified fragment. The PCR conditions for *sco2127* amplification were previously described [8]. The PCR product (694 bp) was cloned into pGEM-T Easy (Promega) and the *sco2127* gene verified by sequencing (Laragen, Inc.). For *sco2127* overexpression, the fragment was restricted with *Sac*I-*Hind*III, subcloned into pQE30 (Qiagen) (SCO2127-His₆) and used to transform by electroporation *E. coli* M-15, following the manufacturer′s instructions. Transformed cells were plated on LB agar

Fig. 1 Diagram showing the followed steps for amplification and cloning of *sco2127* into the overexpression plasmid pQE-30. *PT5* T5 promoter, *lac O* lac operator, *RBS* ribosome-binding site, *ATG* start codon, *6xHis* 6xHis tag sequence, *MCS* multiple cloning site, *Col E1* Col E1 origin of replication
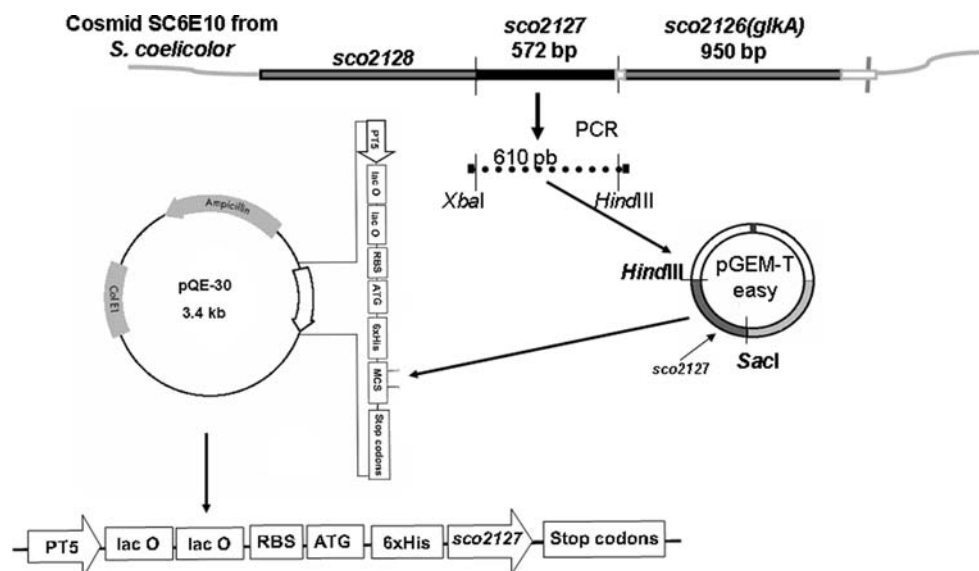
(Sigma), supplemented with ampicillin and kanamycin (100 and 25 µg/ml, respectively) and cultivated for 18 h at 37°C. One resistant colony was isolated from the plates and cultivated in LB (10 ml) with the same antibiotics. After 18 h incubation, the culture was transferred to a 2-L Fernbach flask containing 500 ml LB and cultivated for 2–5 h (until cells reached an OD of 0.5–0.8 at 600 nm). At this time, 0.4 mM IPTG was added to the culture medium and the cells incubated for at least 1 h, under similar conditions. Fifty-milliliter samples of the induced cells were harvested by centrifugation ($4,060 \times g$ for 5 min) and the pellet resuspended in 300 µl of a lysis solution [5 µl of protease inhibitor cocktail (Sigma) and 995 µl of lysis buffer (20 mM KH_2PO_4; 0.5 M NaCl; 50 mM imidazol and 20 mM β-mercaptoethanol)]. The above cell suspension was disrupted by sonication (three 10-s pulses of 60 W, leaving 1 min between each pulse) and centrifuged in cold at $5,900 \times g$ for 10 min. The supernatant was saved and the pellet resuspended in 200 µl of the same lysis solution, sonicated again (three 20-s pulses) and centrifuged under same conditions. Both supernatants were mixed, and 0.5 ml of 20% Triton X-100 (Sigma) was added and adjusted to get approximately a 1% v/v final concentration (since the presence of detergent has no effect on the protein binding to the sepharose matrix and reduces non-specific binding). The mixture was gently shaken for 30 min and centrifuged in cold at $4,500 \times g$ for 15 min. After centrifugation, the supernatant (750 µl) was added to a 1.5-ml Eppendorf tube containing 250 µl Ni-sepharose (GE Healthcare Bio-Sciences AB; catalog no. 17-5268-01), incubated 8 min at room temperature with agitation and centrifuged at $500 \times g$ for 5 min. The supernatant (containing the non-adsorbed proteins) was then recovered and stored at 4°C before SDS-PAGE analysis. The Ni-sepharose was washed with five volumes of the washing buffer (20 mM KH_2PO_4; 0.5 M NaCl; 50 mM imidazol), shaken for 5 min and centrifuged at $500 \times g$ for 3 min. This procedure was repeated nine more times. Finally, the Ni-sepharose was treated with two volumes (1.0 ml each) of elution buffer (20 mM KH_2PO_4; 0.5 M NaCl; 300 mM imidazol), shaken for 5 min, centrifuged at $500 \times g$ for 3 min and the supernatant (containing the purified SCO2127-His$_6$) recovered. The elution procedure was repeated at least four more times. The final supernatant (5 ml) was stored at 4°C before being analyzed by SDS-PAGE and used as antigen for anti-SCO2127 antibody production. The SCO2127-His$_6$ band was visualized by western blot assay, with a primary anti-histidine monoclonal antibody (Roche Molecular Biochemicals) and a secondary anti-mouse immunoglobulin antibody conjugated with alkaline phosphatase (Zymed), according to the manufacturer's instructions.

Protein electrophoresis

Standard procedures were used to carry out electrophoresis [14]. The supernatant samples containing 15 µg protein were subjected to SDS-PAGE analyses.

Anti-SCO2127 antibody production

For anti-SCO2127 antibody production, 2 ml of an emulsion containing 50 mg pure SCO2127 in Freund's complete adjuvant (1:1) was injected intradermally into a healthy male New Zealand white rabbit aged 3–4 months (body weight ~2.5 kg) following the protocol described by Hu et al. [15].

Immunoblotting

After SDS-PAGE, the proteins were electrotransferred using an electrophoretic transfer cell (Mini Trans-Blot®, Biorad-170-3930) to a PVDF membrane (Immobilon P, Millipore) previously activated in pure methanol for 20 s. The transference was carried out at 60 V for 90 min in 1X transfer buffer, which contained (l^{-1}) 14.4 g glycine, 3.0 g Tris and 100 ml methanol in distilled water. The membranes (with transferred proteins) were incubated at room temperature for 30 min in membrane-washing buffer [0.05% (v/v) Tween 20 (Sigma) and 3% (w/v) Difco skim milk in PBS]. Then, the anti-SCO2127 antibodies (1:5,000) were added and incubated 1 h under the same conditions. The solution was discarded and the membrane gently washed three times (1 min each) with the same buffer and incubated again under similar conditions in washing buffer with 3% (w/v) skim milk containing goat anti-rabbit IgG (H+L) HRP conjugate (1:10,000; Zymed). The membranes were washed two more times (1 min each) with membrane-washing buffer and finally developed with 0.05% (w/v) 3,3′-diaminobenzidine tetrahydrochloride (Sigma), 0.02% (w/v) nickel chloride hexahydrate and 0.1% (v/v) of 30% hydrogen peroxide. The developing procedure was stopped with distilled water.

Results

Construction of sco2127-His$_6$

The *sco2127* gene was amplified by PCR using as template the cosmid SC6E10 containing this region. The amplified region was cloned into the pGEM-T Easy vector for PCR products, and then the *sco2127* gene was subcloned into the expression plasmid pQE30 and the recombinant plasmid used to transform *E. coli* M-15 cells (Fig. 1). Transformed colonies were isolated by their ability to grow on LB agar
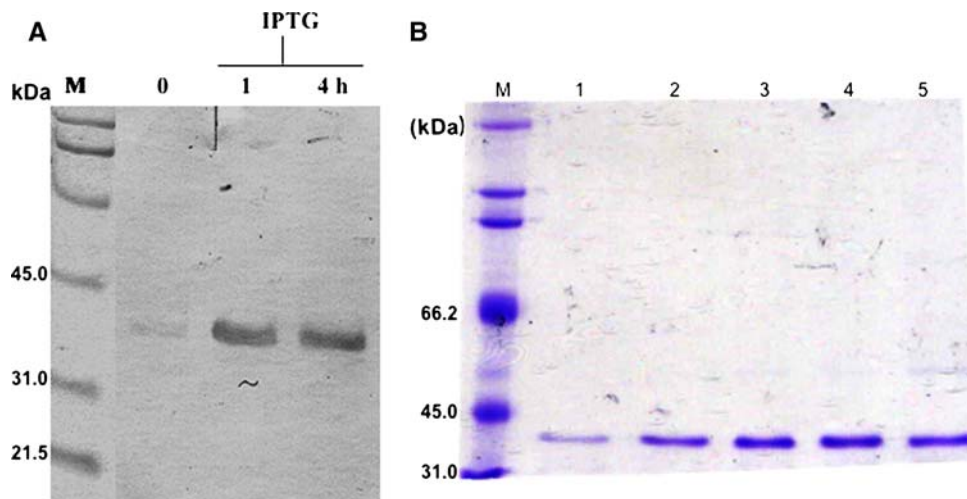
Fig. 2 **a** Western blot analyses of SCO2127-His$_6$. SDS-PAGE (8%) from *E. coli* M-15 transformed with pQE30-*sco2127*. The crude extracts were obtained from cultures grown during 1 and 4 h in the presence of 0.4 mM IPTG. Extracts from 0 h were not induced. The first track shows the molecular weight marker. **b** SDS-PAGE (8%) showing the serial elution of SCO2127-His$_6$ protein (*lanes 1–5*) from extracts of *E. coli* M-15 transformed with the plasmid pQE30-*sco2127*. The protein (SCO2127-His$_6$) was eluted with 300 mM imidazol from a Ni-sepharose column. The gel was stained with Coomassie blue

with ampicillin. In order to verify the correct orientation of the gene fusion, the resulting plasmid was sequenced.

Expression and purification of sco2127-His$_6$

In order to establish the conditions for protein expression, the transformed *E. coli* M-15 cells were incubated with 0.4 mM IPTG and assayed after 1 and 4 h. A western blot assay, using anti-histidine monoclonal antibodies (Fig. 2a), revealed a single band of 34 kDa. As shown in the same figure, there were no significant differences between the two incubation times on SCO2127-His$_6$ expression, and only a faint band was detected when the cells were not exposed to the inducer.

In order to obtain a purified sample of the SCO2127-His$_6$ product, the transformed *E. coli* M-15 strain containing the plasmid pQE30-sco2127 was grown in the presence of IPTG and its crude extracts passed through a Ni-sepharose column. Figure 2b shows the purified samples of SCO2127-His$_6$ after elution with 300 mM imidazol.

Polyclonal antibodies production

Polyclonal antibodies against the purified SCO2127-His$_6$ were produced in rabbits, following the procedure of Hu et al. [15]. As shown in Fig. 3, the antibodies gave a positive reaction against both *S. coelicolor* and *S. peucetius* var. *caesius* cell extracts grown with 100 mM glucose. The reaction appeared as a single band in the western blot membrane. On the other hand, no reaction was obtained using extracts from a *S. coelicolor* mutant lacking the *sco2127* gene (Δ*sco2127*).

Time course of SCO2127 synthesis

In order to establish the expression pattern of SCO2127 during the growth cycle of *S. coelicolor* grown in minimal medium supplemented with 50 and 100 mM glucose, protein extracts were analyzed by western blot at different fermentation times. As can be seen in Fig. 4a, when 50 mM glucose was utilized for growth, the culture reached the stationary phase after 72 h, coinciding with most of the carbohydrate consumption. In addition, SCO2127 was clearly detected in crude extracts from the beginning of the fermentation and during the logarithmic growth phase, but decreased at later stages of the microbial growth (Fig. 4c). On the other hand, when the cells were grown in the presence of 100 mM glucose (Fig. 4b), the cultures did not reach the stationary growth phase after 72 h fermentation. In addition, at this incubation time, approximately 40% of the carbohydrate still remained in the culture medium, and SCO2127 was clearly visualized (Fig. 4d).

Discussion

During the last years, our research group has been interested in the study of the *sco2127* gene and its role in CCR [8, 9]. In *S. coelicolor*, this region (576 pb) is located upstream of the *glkA* gene, and no evident function has been conferred to its possible expression product. We have obtained experimental evidence in *S. peucetius* var. *caesius* supporting that in the presence of glucose, SCO2127 plays a key role in the CCR process. Its effect seems to take place through the activation of glucose uptake and *glk* expression
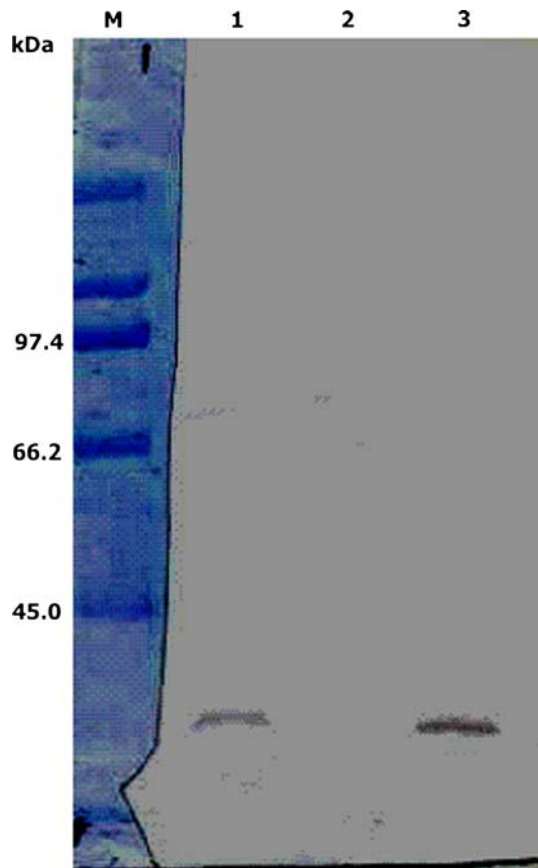
Fig. 3 Western blot using rabbit anti-SCO2127 polyclonal antibodies against extracts obtained from different *Streptomyces* strains grown in 100 mM glucose. *S. coelicolor* (*lane 1*), *S. coelicolor* Δ*sco2127* mutant (*lane 2*) and *S. peucetius var. caesius* (*lane 3*). The extracts were electrophoresed on 8% SDS-PAGE

[8, 9], allowing formation of the glucose catabolites responsible for this regulatory process [3]. However, its mechanism of action is not clear since *sco2127* lacks DNA-binding motifs that would indicate direct regulation of the *glk* promoter. As the predicted function of SCO2127 does not imply a simple and measurable reaction, it has been difficult to follow its effect during fermentation. Therefore, in order to get a better understanding of the SCO2127 mechanism of action, first of all it was essential to produce, purify and characterize this protein. In the present work, *sco2127* was subcloned into pQE30 to attach a histidine tag (SCO2127-His_6) and was overexpressed in *E. coli* M-15. This procedure allowed purification of enough SCO2127 using Ni-sepharose to be able to produce polyclonal antibodies. The antibodies were able to detect SCO2127 from crude extracts of both *S. coelicolor* and *S. peucetius* var. *caesius*, giving a single protein band of 34 kDa in western blot analyses. These results suggested some similarities of SCO2127 in these *Streptomyces* species. In agreement with this observation, SCO2127 and its corresponding ortholog from *S. peucetius var. caesius* show a 61% identity in their amino acid composition (J.K. Sohng, personal communication). When the protein was monitored in *S. coelicolor* cultures grown in the presence of 50 mM glucose, SCO2127 was detected at the beginning of the fermentation and during the logarithmic growth phase, but decreased at later stages of the microbial growth. Its main production at the logarithmic growth phase agrees with its predicted involvement in CCR as SCO2127 was expressed closely linked to the glucose concentration present in the culture medium. Detection of SCO2127 at the beginning of the fermentation

Fig. 4 Growth (*open circle*) and residual glucose concentration (*open triangle*) of *S. coelicolor* cells grown in 50 (**a**) and 100 mM glucose (**b**). Western blot assays showing the reaction of rabbit anti-SCO2127 polyclonal antibodies against crude extracts obtained from the above cultures grown in 50 (**c**) and 100 mM glucose (**d**), electrophoresed on 10% SDS-PAGE
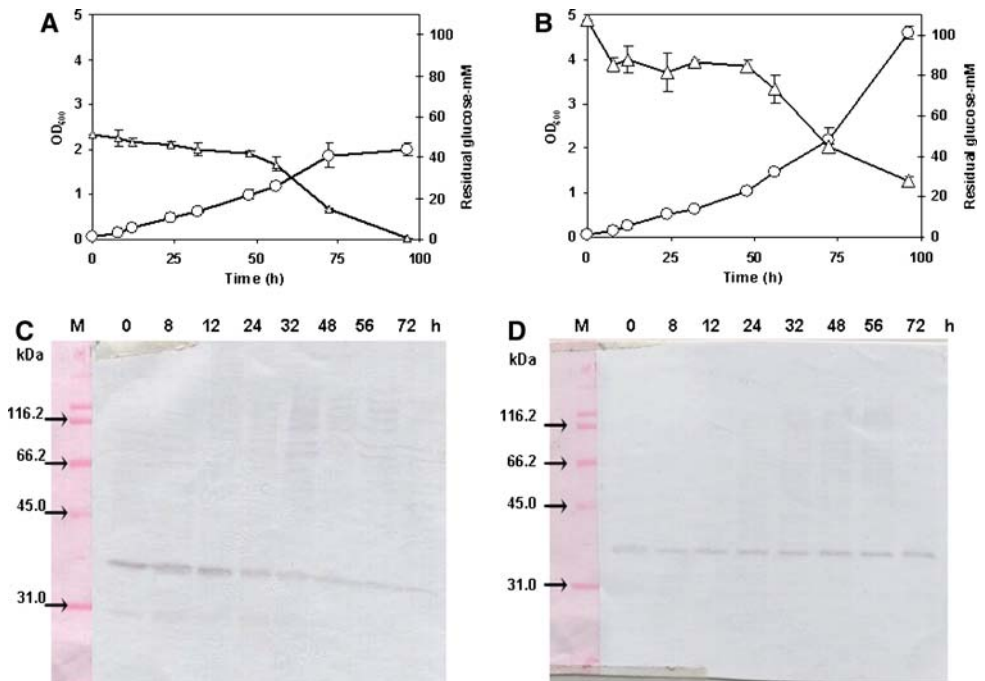
may be related to its possible role in the transport of carbohydrates [9] as the seeds cultures were grown in the presence of 50 mM mannitol. On the other hand, cultures grown in 100 mM glucose showed SCO2127 even at later stages of the microbial growth, coinciding with a 40% of available glucose. We have previously reported that SCO2127 may stimulate transcription of *glkA*, and probably that of the glucose permease gene as well [8]. It seems reasonable to think that stimulation of both activities allow efficient glucose consumption with the concomitant production of glucose catabolites, compounds presumably involved in eliciting CCR [3]. However, a direct effect of SCO2127 on the activation of Glk or GlcP1 cannot be discarded. In this regard, the apparent binding of Glk to the major glucose transport system of *S. coelicolor* (GlcP1) has been reported [16]. In connection to this finding, and in addition to its effect on *glk* expression, SCO2127 might also stimulate the binding between Glk and GlcP1, increasing the efficiency of glucose metabolism.

References

1. Hodgson D (1982) Glucose repression of carbon source uptake and metabolism in *Streptomyces coelicolor* and its perturbation in mutants resistant to 2-deoxyglucose. J Gen Microbiol 128:2417–2430

2. Angell S, Lewis CG, Buttner M, Bibb JM (1994) Glucose repression in *Streptomyces coelicolor* A3(2): a likely regulatory role for glucose kinase. Mol Gen Genet 244:135–143. doi:10.1007/BF00283514

3. Ramos I, Guzmán S, Escalante L, Imriskova I, Rodríguez-Sanoja R, Sanchez S, Langley E (2004) The glucose kinase alone cannot be responsible for carbon source regulation in *Streptomyces peucetius* var. caesius. Res Microbiol 155:267–274. doi:10.1016/j.resmic.2004.01.004

4. Ikeda H, Seno ET, Bruton CJ, Chater KF (1984) Genetic mapping, cloning and physiological aspects of the glucose kinase gene of *Streptomyces coelicolor*. Mol Gen Genet 196:501–507. doi:10.1007/BF00436199

5. Angell S, Schwartz E, Bibb JM (1992) The glucose kinase gene of *Streptomyces coelicolor* A3(2): its nucleotide sequence, transcriptional analysis and role in glucose repression. Mol Microbiol 6:2833–2844. doi:10.1111/j.1365-2958.1992.tb01463.x

6. Escalante L, Ramos I, Imriskova I, Langley E, Sanchez S (1999) Glucose repression of anthracycline formation in *Streptomyces peucetius* var. caesius. Appl Microbiol Biotechnol 52:572–578. doi:10.1007/s002530051562

7. Segura D, Gonzalez R, Rodriguez-Sanoja R, Sandoval T, Escalante L, Sanchez S (1996) *Streptomyces* mutants insensitive to glucose repression showed deregulation of primary and secondary metabolism. Asia Pac J Mol Biol Biotechnol 4:30–36

8. Guzmán S, Carmona A, López R, Escalante L, Ruiz B, Rodríguez-Sanoja R, Sanchez S, Langley E (2005) Pleiotropic effect of the *SCO2127* gene on the glucose uptake, glucose kinase activity and carbon catabolite repression in *Streptomyces peucetius* var. caesius. Microbiology 151:1717–1723. doi:10.1099/mic.0.27557-0

9. Guzmán S, Ramos I, Moreno E, Ruiz B, Rodríguez-Sanoja R, Escalante L, Langley E, Sanchez S (2005) Sugar uptake and sensitivity to carbon catabolite repression in *Streptomyces peucetius* var. caesius. Appl Microbiol Biotechnol 69:200–206. doi:10.1007/s00253-005-1965-7

10. Bertram R, Schlicht M, Mahr K, Nothaft H, Saier MH Jr, Titgemeyer F (2004) In silico and transcriptional analysis of carbohydrate uptake systems of *Streptomyces coelicolor* A3(2). J Bacteriol 186:1362–1373. doi:10.1128/JB.186.5.1362-1373.2004

11. Gullo VP, McAlpine Lam KS, Baker D, Petersen F (2006) Drug discovery from natural products. J Ind Microbiol Biotechnol 33:523–531. doi:10.1007/s10295-006-0107-2

12. Gust B, Kieser T, Chater KF (2002) REDIRECT© Technology: PCR-Targeting System in *Streptomyces coelicolor*. John Innes Centre, Norwich

13. Kieser T, Bibb MJ, Buttner MJ, Chater KM, Hopwood D (2000) Practical *Streptomyces* Genetics. John Innes Foundation, Norwich

14. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685. doi:10.1038/227680a0

15. Hu YX, Guo JY, Shen L, Chen Y, Zhang ZC, Zhang YL (2002) Get effective polyclonal antisera in one month. Cell Res 12:157–160. doi:10.1038/sj.cr.7290122

16. van Wezel GP, König M, Mahr K, Nothaft H, Thomae AW, Bibb MJ, Titgemeyer F (2007) A new piece of an old jigsaw: glucose kinase is activated posttranslationally in a glucose transport-dependent manner in *Streptomyces coelicolor* A3(2). J Mol Microbiol Biotechnol 12:67–74. doi:10.1159/000096461