sp2563 nor its Orthologue *sco2127* Seem to be the Only Factors Eliciting Carbon Catabolite Repression in two *Streptomyces* strains.

Alejandro Jiménez, Brenda Cabrera, Fanny C. Cordero, Beatriz Ruiz, Romina Rodríguez-Sanoja, Víctor Tierrafría and Sergio Sánchez.

Departamento de Biología Molecular y Biotecnología. Instituto de Investigaciones Biomédicas. Universidad Nacional Autónoma de México. México D.F. 04510.
E-mail: sersan@biomedicas.unam.mx

ABSTRACT

In *Streptomyces peucetius* var. *caesius* and *Streptomyces coelicolor*, secondary metabolism is strongly regulated by different sugars in a process known as carbon catabolic repression (CCR). We have previously reported on the isolation and characterization of a spontaneous *S. peucetius* var. *caesius* mutant resistant to 2-deoxyglucose (Dog^R), insensitive to CCR. This phenotype was reverted to the original one by transformation with the *S. coelicolor sco2127* gene, suggesting a role of this gene in CCR. Here we sequenced, analyzed, and compared the gene sequences between the *sco2127* orthologue gene from the original *S. peucetius* var. *caesius* strain (*sp2563*) and its Dog^R mutant derivative. No differences were found in the *sp2563* gene base sequences between these strains. In addition, when the effect of 2-deoxyglucose (2-DOG) was tested on the growth of a wild type *S. coelicolor* strain and its Δ*sco2127* mutant derivative on L-arabinose, both strains exhibited growth suppression by 2-DOG. Under this condition, two types of 2-DOG resistant mutants were isolated and in one of them actinorhodin production resulted insensitive to CCR. These results strongly suggested that *sco2127* and its orthologue *sp2563* gene products are not the only factors responsible for CCR in these streptomycetes.

INTRODUCTION

Streptomyces peucetius var. *caesius* is an industrially, clinically relevant Gram-positive filamentous bacteria with a complex differentiation process associated with the production of secondary metabolites. *S. peucetius* var. *caesius* produces doxorubicin, an anthracycline antibiotic widely used in chemotherapeutic regimens for cancer

treatment (Arcamone *et al*., 1969). Biosynthesis of anthracyclines and other secondary metabolites are tightly regulated by carbon catabolic repression (CCR) mediated by glucose (Demain, 1989; Segura et al., 1996). However, the regulatory mechanism of this effect in streptomycetes is not completely understood.

The first attempt to understand the CCR mechanism in the genus *Streptomyces* was reported by Hodgson (1982) in *Streptomyces coelicolor*. This strain is unable to grow in L-arabinose or glycerol when a non-metabolized glucose analogue, 2-deoxyglucose (2-DOG) is present in the culture medium. He found that mutants isolated as resistant to the growth inhibition by 2-DOG showed low glucose kinase (Glk) activity and decreased sensitivity to sugar repression of carbon source utilization and agarase production (Hodgson, 1982). Angell *et al*. (1994) demonstrated that transformation of these mutants with *sco2126* (*glkA*) and *sco2127* genes restores Glk activity and CCR sensitivity. However, the mutant transformed with *glkA* alone does not recover the wild type phenotype. Further studies on *sco217* have been conducted in a null mutant of this gene constructed from the *S. coelicolor* wild type strain (Chavez et al., 2011). This Δ*sco2127* mutant showed insensitivity of morphological differentiation to CCR.

Similarly, it has been demonstrated that *S. peucetius* var. *caesius* is unable to grow in lactose when 2-DOG is present in the culture medium (Segura *et al*., 1996). Mutants of this microorganism isolated as resistant to the growth inhibition by 2-DOG (Dog^R) exhibit low glucose kinase activity, reduced glucose incorporation and insensitivity of primary (β-galactosidase, D-xylose isomerase) and secondary metabolism (anthracyclines production) to CCR (Dog^R phenotype) (Segura *et al*., 1996; Escalante et al., 1999). When Dog^R mutants are transformed with *sco2127*, Glk activity as well as glucose uptake increase to levels higher than those of the original strain and anthracyclines production become supersensitive to the presence of glucose (Guzmán *et al*., 2005). These observations strongly suggest that *sco2127* some how participates in the regulation of glucose-mediated catabolic repression (Angell *et al*., 1994; Guzmán *et al*., 2005).

sco2127 has an orthologue gene in *S. peucetius* var. *caesius* named *sp2563.* The products of *sco2127* and *sp2563* are putative proteins with a molecular weight of 34 kDa, whose function is still unknown (Chávez *et al*., 2009). In the present work we look for the presence of mutations in the *sp2563* gene of a *S. peucetius* var. *caesius* Dog^R mutant that might explain its Dog^R phenotype. In addition, we examined sensitivity of the *S. coelicolor* Δ*sco2127* null mutant to 2-DOG, and its potential to produce a Dog^R phenotype. We expected from both approaches to obtain relevant information about *sp2563* and *sco2127* participation in the CCR phenomenon of these *Streptomyces* strains.

MATERIALS AND METHODS

Microorganisms and culture conditions

S. coelicolor A3(2) M145 (SCP-1, SCP-2, prototroph) was obtained from John Innes Institute, UK. The *S. coelicolor* null mutant (Δ*sco2127*) was constructed using the PCR targeting procedure (Gust *et al*., 2002). *S. coelicolor* and Δ*sco2127* spores were maintained in 20% glycerol. For seed cultures 10^6 spores were inoculated into 250-mL baffled Erlenmeyer flasks containing 50 mL minimal medium (NMMP) (Kieser *et al*., 2000), supplemented with 50 mM mannitol.

S. peucetius var. *caesius* NRRL B-5337 was obtained from the ARS Culture Collection, U.S. Department of Agriculture, Peoria, IL, USA. Spontaneous mutants (Dog^R) were isolated from the *S. peucetius* var. *caesius* original strain cultured in a medium containing 2-DOG and lactose as described elsewhere (Segura *et al*., 1996). The mutant and the original strains were grown on Petri dishes containing solid YMG medium containing 0.4 g Yeast Extract, 1.0 g Malt Extract, 0.4 g glucose and 1.5% Agar per 100 mL distilled water. Cells (0.25 mg/mL of dry weight) of each strain were used to inoculate 250-mL baffled Erlenmeyer flasks containing 50 mL YMG medium.

All the cultures were incubated at 29°C, under agitation (180 rpm) for 48 h.

sp2563 *sequence amplification and cloning*

Total DNA was isolated and purified as previously described (Kieser *et al*., 2000). For *sp2563* amplification, two sets of oligonucleotides were designed based on the consensus of six *sp2563* orthologous genes (*S. coelicolor,*

Streptomyces avermitilis, Streptomyces griseus, Streptomyces scabies, Streptomyces venezuelae and *Streptomyces clavuligerus*). The *sp2563*-fwd (5'-GTTCGGGTAACGTC GGAGGTACG AACCGTAGGC-3') and *sp2563*-rev (5'-CGAGCCCCGCTAAG CCCAACCGTACCCGAG-3') were used to amplify a 650 bp fragment correspondent to the *sp2563* sequence from *S. peucetius* var. *caesius*. The *sp2563*Dog^R-fwd (5'-CCTGCCGTCCG CCCTGCGCCGCTGCACCGT-3'), and *sp2563*Dog^R-rev (5'-AGTCCCATGCA TCCCTCAGTTCCGGTCGA-3') primers were designed to amplify the *sp2563* from the *S. peucetius* var. *caesius* Dog^R mutant. They amplified a 654 bp fragment located between *sp2562* (*glk*) and *sp2564* genes.

For PCR amplification the DNA was denatured at 94°C for 5 min, followed by 25 cycles of 94°C for 30 s, 50°C (*sp2563*-fwd/*sp2563*-rev) or 55°C (*sp2563*Dog^R-fwd/*sp2563*Dog^R-rev), and 72°C for 1 min. A final elongation at 72°C for 7 min was performed. The PCR products were analysed in 1% agarose gels with ethidium bromide.

The amplified sequences were purified using the QIAEX KIT (QIAGEN), according to manufacturer's specifications. The sequences were cloned into the pGEM®T Easy Vector (Promega) to generate pGEM-*sp2563* and pGEM-*sp2563*Dog^R plasmids which were transformed into *Escherichia coli* DH5α.

sp2563 *sequencing and analysis*

To analyse the sequence of *sp2563*, the pGEM-*sp2563* and pGEM-*sp2563*Dog^R plasmids was sequenced by LARAGEN Sequencing & Genotyping Inc. (USA). The obtained sequence was analyzed using data basis from the Basic Local Alignment Tool (BLAST). To corroborate the *sp2563* gene location, the 870 bp amplicon was also sequenced in the same way. Afterwards both, wild type and Dog^R sequences were compared to each other using BLAST.

Generation of Δsco2127 *derived Dog^R mutants*

Δ*sco2127* cells were grown in NMMP with 10 mM L-arabinose supplemented with 50 mM 2-DOG. After 10 days incubation, spontaneous mutants were detected and isolated for further analysis.

RESULTS

Sequencing of sp2563 *and* sp2563 Dog^R *genes*

Using the *sp-2563*Dog^R-fwd and *sp-2563*Dog^R-rev primers, the *sp2563* Dog^R mutant sequence was amplified, sequenced and compared to that of the original *S. peucetius* var. *caesius* strain, amplified with *sp2563*-fwd and sp2563-rev primers. As can be seen in Table 1 both sequences were 100% identical, indicating that no mutations were present in the *sp2563* Dog^R gene (Figure 1).

The amplified 654 bp *sp2563* sequence was also compared with six orthologous streptomycetes genes reported in "The StrepDB – Streptomyces Annotation Server". As seen in Table 2, the sequence showed 100% identity in 61% of the analyzed *sco2127* gene from *S. coelicolor* and a gradient of similarity to other orthologous genes.

Table 1 Comparison of the *spvc2563* gene sequences between the original *S. peucetius* var. *caesius* strain and its Dog^R mutant derivative.

Gene	Length (bp)	GC (%)	Max score	Total score	Query coverage	E value	Max identity
spvc2563	654	74	1201	1201	100%	0.0	100%
spvc2563 Dog^R	654	74	1201	1201	100%	0.0	99%

Parameters of sequence comparison are shown in the table.

```
Spvc 11  CATGCTNCGGNGCCATGGCGGCCGCGGGAATTCGATTAGTCCCATGCATCCCTCAGTTCC  70
         |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
DogR 10  CATGCTCCGGNGCCATGGCGGCCGCGGGAATTCGATTAGTCCCATGCATCCCTCAGTTCC  69

Spvc 71  GGTCGAGCCCCGCTACGGCCAACCGTACCCGAGCGGCTTCAGTCAAGGTCGATGTGTTCC 130
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
DogR 70  GGTCGAGCCCCGCTACGGCCAACCGTACCCGAGCGGCTTCAGTCAAGGTCGATGTGTTCC 129

Spvc 131 CCGGTACCGGTGCCCTCGTCGCGGGGGCGCGCCGCGGTGTCGCGCGTGGTCCAGCGCCGC 190
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
DogR 130 CCGGTACCGGTGCCCTCGTCGCGGGGGCGCGCCGCGGTGTCGCGCGTGGTCCAGCGCCGC 189

Spvc 191 TCCTGCGCCTCGACGGCGGAGCGGTACGCGGCGAGCAGTGCGGAACCGGCGCTCGCCAGG 250
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
DogR 190 TCCTGCGCCTCGACGGCGGAGCGGTACGCGGCGAGCAGTGCGGAACCGGCGCTCGCCAGG 249
```

```
Spvc 251 TGGTCGAAGACGTCGGGGTTGCGCTCGATGACGGGCTCGACGGCGGCCTTGGCCTGCCGC 310
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
DogR 250 TGGTCGAAGACGTCGGGGTTGCGCTCGATGACGGGCTCGACGGCGGCCTTGGCCTGCCGC 309

Spvc 311 ACGACCTGGTTGACCACCTGCTGGGCGGCGCCCCCCGCGACCGCGCCGAGCAGCGGTGAC 370
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
DogR 310 ACGACCTGGTTGACCACCTGCTGGGCGGCGCCCCCCGCGACCGCGCCGAGCAGCGGTGAC 369

Spvc 371 TGCAGTCCGGACAGCTTGTCGGCGACGGCGTCGACGAGTTTGCGCAGTTCCTCGGCGGCC 430
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
DogR 370 TGCAGTCCGGACAGCTTGTCGGCGACGGCGTCGACGAGTTTGCGCAGTTCCTCGGCGGCC 429

Spvc 431 GAGCCGGGCGGCGGGCCGTGCTGGGCGCGGCGGCGGGCCTGCTCCGCCGCCAGGTCCTCG 490
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
DogR 430 GAGCCGGGCGGCGGGCCGTGCTGGGCGCGGCGGCGGGCCTGCTCCGCCGCCAGGTCCTCG 489

Spvc 491 GCGCACGCCTGCGCCCAGGCGTCGTCGTCGGTCGCCCGGACCTCGTCACGCTCCTCGTCG 550
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
DogR 490 GCGCACGCCTGCGCCCAGGCGTCGTCGTCGGTCGCCCGGACCTCGTCACGCTCCTCGTCG 549

Spvc 551 CGCACAGTGCTGCCGCGCTCCGTACTGCCGCGCGTCGTGCTCTCACGCGCCGTGCCGTCG 610
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
DogR 550 CGCACAGTGCTGCCGCGCTCCGTACTGCCGCGCGTCGTGCTCTCACGCGCCGTGCCGTCG 609

Spvc 611 TGCACCGGTCCGAGCGCGTCCTCCCCTGCTTCGGACGTAGGGCGCTCATCGCTC        664
         |||||||||||||||||||||||||||||||||||||||||||||||||||
DogR 610 TGCACCGGTCCGAGCGCGTCCTCCCCTGCTTCGGACGTAGGGCGCTCATCGCTC       663
```

Fig. 1 Sequence alignments between the original *S. peucetius* var. *caesius* *spvc2563* gene with the *spvc2563* gene from a Dog^R mutant derivative. The sequence alignment was carried out using BLAST.

Evaluation of the Δsco2127 *mutant ability to grow in 2-DOG*

In order to confirm that Dog^R phenotype is independent of sco2127, we examined the sensitivity of a *S. coelicolor* Δ*sco2127* null mutant to 2-DOG, and its potential to produce a Dog^R phenotype. Similar to *S.*

Table 2 Comparison of the *spvc2563* gene sequence with six orthologous genes.

Genes	Length (bp)	GC (%) (approx)	Max score	Total score	Query coverage	E value	Max identity
spvc2563	654	74	1173	1215	100%	0.0	100%
sco2127	576	76	394	449	61%	7e-114	100%
sav6073	516	74	421	421	63%	4e-122	83%
sclav1341	582	76	233	260	58%	1e-65	74%
sgr5376	480	76	297	382	52%	1e-84	93%
scab67541	480	75	405	405	56%	3e-117	84%
sven1787	486	74	250	340	57%	2e-70	93%

spvc.- *S. peucetius* var. *caesius*; sco.- *S. coelicolor*; sav.- *S. avermitilis*; sclav.- *S. clavuligerus*; sgr.- *S. griseus*; scab.- *S. scabies* ; sven.- *S. venezuelae*.

coelicolor M145 wild type strain, Δ*sco2127* was unable to grow in arabinose with 2-DOG (not shown). Under this condition, spontaneous mutants were detected after 10 days incubation and isolated for further analysis. Two different phenotypes were observed for these Δ-sco2127 Dog^R mutants. As shown in Table 3, in type 1 resistant mutants, even though morphological differentiation was insensitive to glucose, actinorhodin production was sensitive to the carbohydrate, forming a red pigment, similar to that observed for undecylprodigiosin formation. On the other hand, in type 2 resistant mutants both, morphological differentiation and antibiotic production exhibited resistance to the presence of glucose, resembling in this way the Dog^R phenotype. The blue pigment produced by this mutant was characteristic of actinorhodin production.

DISCUSSION

It has been reported that *S. peucetius* var. *caesius* isolated as resistant to 2-DOG exhibited a Dog^R phenotype and Guzmán *et al.* (2005) reported that complementation of these mutants with *sco2127* recovers the original phenotype. These findings

Table 3 Effect of D-glucose on morphological differentiation and actinorhodin production in the *S. coelicolor* Δ-*sco2127* strain and two Dog^R mutants derived from it[a].

S. coelicolor strains	Morphological differentiation[b]	Actinorhodin production
M145 wild type strain	Sensitive	Sensitive
Δ-sco2127	Insensitive	Sensitive
Δ-sco2127 Dog^R 1	Insensitive	Sensitive (red)
Δ-sco2127/ Dog^R 2	Insensitive	Insensitive (blue)

[a] Strains grown in solid NMMP medium supplemented with 100 mM glucose.

[b] MF: Morphological differentiation. Formation of aerial mycelium formation and sporulation

suggest that both *S. coelicolor sco2127* and its orthologue *S. peucetius* var. *caesius sp2563* gene products may be involved in CCR in these organisms. However, sequence alignment of the *sp2563* gene between the original *S. peucetius* var. *caesius* strain and its derivative Dog^R mutants showed 100% identity, indicating that no mutations of this gene were present in the CCR resistant mutants. This in these Dog^R mutants. Furthermore, between the two types of Δ*sco2127* mutants resistant to 2-DOG, one resulted insensitive to the glucose effect for actinorhodin production and resembled a Dog^R phenotype, result discarded a mutation of *sp2563* as the possible cause of Dog^R phenotype. It is not worthy to mention that sp2562 encoding for Glk also lacks of any mutations in this strain.

In agreement with this result, it was found that a Δ*sco2127* null mutant was unable to grow in the presence of 2-DOG, supporting again that the *sco2127* and likely *sp2563* gene products are not responsible for CCR excluding the expression product of *sco2127* as the only cause of CCR.

However, we cannot explain why morphological differentiation in the Δ*sco2127* mutants is insensitive to CCR nor that one of the Δ*sco2127* resistant to 2-DOG mutants phenotype

resulted sensitive to the glucose effect and produced only the characteristic red pigment undecylprodigiosin. One possibility is that transcription of *sco2127* is necessary for optimum *sco2126* (*glkA*) expression and that both expression products are required for CCR (Ramos *et al*., 2004). This possibility may explain also the recovery of CCR responsiveness when the *S. peucetius* var. *caesius* Dog^R mutant is complemented with *sco2127*.

Finally, we consider that more research about Dog^R mutants is needed in order to understand the key modifications responsible for resistance to CCR.

ACKNOWLEDGEMENTS

We are indebted to M.A. Ortiz for strain preservation studies. The financial support of DGAPA (PAPIIT IN209210) and CONACYT (CB2008-100564-IIBO) are greatly appreciated.

REFERENCES

Angell S, Lewis CG, Buttner MJ & Bibb JM (1994) Glucose repression in *Streptomyces coelicolor* A3(2): a likely regulatory role for glucose kinase. *Mol. Gen. Genet.* 244:135-143.

Arcamone F, Cassinelli G, Fantini G, Grein A, Orezzi P, Pol C & Spalla C (1969) Adriamycin, 14-hydroxydaunomycin, a new antitumor antibiotic from *Streptomyces peucetius* var. *caesius*. *Biotechnol. Bioeng.* 11:1101-1110.

Chávez A, García-Huante Y, Ruiz B, Langley E, Rodríguez-Sanoja R & Sanchez S (2009) Clonning and expression of the *sco2127* gene from *Streptomyces coelicolor* M145. *J. Ind. Microbiol. Biotechnol.* 36:649-654.

Chávez A, Forero A, Sanchez M, Rodríguez-Sanoja R, Mendoza G, Servin-González L, Sánchez B, García-Huante Y, Rocha D, Langley E, Ruiz B and Sanchez S (2011) Interaction of SCO2127 with BldKB and its possible connection to carbon catabolite regulation of morphological differentiation in *Streptomyces coelicolor*. *Appl. Microbiol. Biotechnol*. 89:799–806.

Demain AL (1989) Carbon source regulation of idiolite biosynthesis in actinomycetes. In *Regulation of Secondary Metabolism in Actinomycetes*, pp. 127 – 131. Ed. S. Shapiro. Boca Ratón, FL. CRC Press.

Escalante L, Ramos I, Imriskova I, Langley E & Sanchez S (1999) Glucose repression of

anthracyclines formation in *Streptomyces peucetius* var. *caesius*. *Appl. Microbiol. Biotechnol*. 52:572-578.

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

Guzmán S, Carmona A, Escalante L, Imriskova I, López R, Rodríguez-Sanoja R, Ruiz B, Servín-Gonzalez L, Sánchez 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.

Hodgson D (1982) Glucose repression of carbon source uptake and metabolism in *Streptomyces coelicolor* A3(2) and its perturbation in mutants resistant to 2-deoxyglucose. *J. Gen. Microbiol*. 128:2417-2430.

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

Ramos I, Guzmán S, Escalante L, Imriskova I, Rodríguez-Sanoja R, Sanchez S & Langley E (2004) Glucose kinase alone cannot be responsible for carbon source regulation in *Streptomyces peucetius* var. *caesius*. *Res. Microbiol.* 155:267-274.

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