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Expression of the endogenous and heterologous clavulanic acid cluster in *Streptomyces flavogriseus*: why a silent cluster is sleeping

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Abstract Clusters for clavulanic acid (CA) biosynthesis are present in the actinomycetes *Streptomyces flavogriseus* ATCC 33331 and *Saccharomonospora viridis* DSM 43017. These clusters, which are silent, contain blocks of conserved genes in the same order as those of the *Streptomyces clavuligerus* CA cluster but assembled in a different organization. *S. flavogriseus* was grown in nine different media, but clavulanic acid production was undetectable using bioassays or by high-performance liquid chromatography analyses. Reverse-transcriptase polymerase chain reaction (RT-PCR) of *S. flavogriseus* CA biosynthesis genes showed that the regulatory genes *ccaR* and *claR* and some biosynthetic genes were expressed whereas expression of *cyp*, *orf12*, *orf13*, and *oppA2* was undetectable. The *ccaR* gene of *S. clavuligerus* was unable to switch on CA production in *S. flavogriseus*::[P_{fur}-$ccaR_C$], but insertion of a cosmid carrying the *S. clavuligerus* CA cluster (not including the *ccaR* gene) conferred clavulanic acid production on *S. flavogriseus*::[SCos-CA] particularly in TBO and YEME media; these results suggests that some of the *S. flavogriseus* CA genes are inactive. The known heptameric sequences recognized by CcaR in *S. clavuligerus* are poorly or not conserved in *S. flavogriseus*. Quantitative RT-PCR analysis of the CA gene clusters of *S. clavuligerus* and *S. flavogriseus* showed that the average expression value of the expressed genes in the former strain was in the order of 1.68-fold higher than in the later. The absence of CA production by *S. flavogriseus* can be traced to the lack of expression of the essential genes *cyp*, *orf12*, *orf13*, *orf14*, and *oppA2*. Heterologous expression of *S. clavuligerus* CA gene cluster in S. *flavogriseus*::[SCos-CA] was 11- to 14-fold lower than in the parental strain, suggesting that the genetic background of the host strain is important for optimal production of CA in *Streptomyces*.

Keywords Genomes · Clavulanic acid · Gene clusters · Heterologous expression · *Streptomyces clavuligerus*. *Streptomyces flavogriseus* · *Saccharomonospora viridis*

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Introduction

Clavulanic acid (CA) is a β-lactamase inhibitor with clavam structure produced industrially from cultures of *Streptomyces clavuligerus*. The CA molecule is formed by condensation of a five-carbon fragment of arginine (the ornithine moiety) with a three-carbon glyceraldehyde-3-phosphate unit (Khaleeli et al. 1999; Valentine et al. 1993) by the carboxyethylarginine (CEA) synthase encoded by *ceaS2* (Pérez-Redondo et al. 1999). The product of this condensation, carboxyethylarginine, is cyclized to proclavaminic acid by the β−lactam synthetase (*bls2*) (Bachmann et al. 1998) and modified in sequential steps by the clavaminate synthetase (*cas2*) and the proclavamic acid hydrolase (*pah2*) that removes the amidino group of arginine

to produce clavaminic acid (Marsh et al. 1992; Aidoo et al. 1994) (Fig. 1a). The known late steps of the CA pathway involve at least two enzymes, the glycyl clavaminic acid synthase (encoded by *gcaS*) forming the peptide-like intermediate N-glycyl-clavaminic acid. This compound is the precursor of clavaldehyde from which clavulanic acid is formed by the final enzyme of the pathway, clavaldehyde reductase (Car) (Pérez-Redondo et al. 1998; Nicholson et al. 1994).

Besides the biosynthetic genes encoding enzymes of the pathway, some additional genes located in the CA cluster are important for CA production. These include *oppA1* and *oppA2*, encoding two oligopeptide permeases; *cyp*, encoding a P450 cytochrome monooxygenase; and *orf12*, encoding a putative lipoprotein. Disruption of each of these genes led to lack of CA production (Bachmann et al. 1998; Mellado et al. 2002; Lorenzana et al. 2004). Other genes in the cluster (*orf13*, *orf14*, and *oat2*) are required for maximum CA production, but their disruption still allows some production of the β-lactamase inhibitor (for a review, see Liras et al. 2011).

The CA gene cluster of *S. clavuligerus* is adjacent to the cephamycin C gene cluster (including 17 genes from *bla* to *pbpR*). A SARP-type regulatory gene, *ccaR*, controlling clavulanic acid and cephamycin C biosynthesis (Pérez-Llarena et al. 1997a) is located within the cephamycin C gene cluster. A second regulatory gene, *claR*, of the LysR family, located in the CA cluster, controls the expression of genes for the late steps of the CA pathway (Pérez-Redondo et al. 1998; Paradkar et al.1998).

The ability to produce β-lactams and other secondary metabolites is usually restricted to a few species within different genera (Martín et al. 2000; Martín and Liras 2010), and this ability is due to (1) the ancestral assembling of the gene cluster or (2) to the acquisition of genes by horizontal transfer either in the same species (there are two sets of paralogous clavam genes in *S. clavuligerus*) or between different species (Liras et al. 1998). The availability of microbial genomes is revealing the existence of gene clusters for secondary metabolites which are not expressed. The awakening of these silent clusters is a

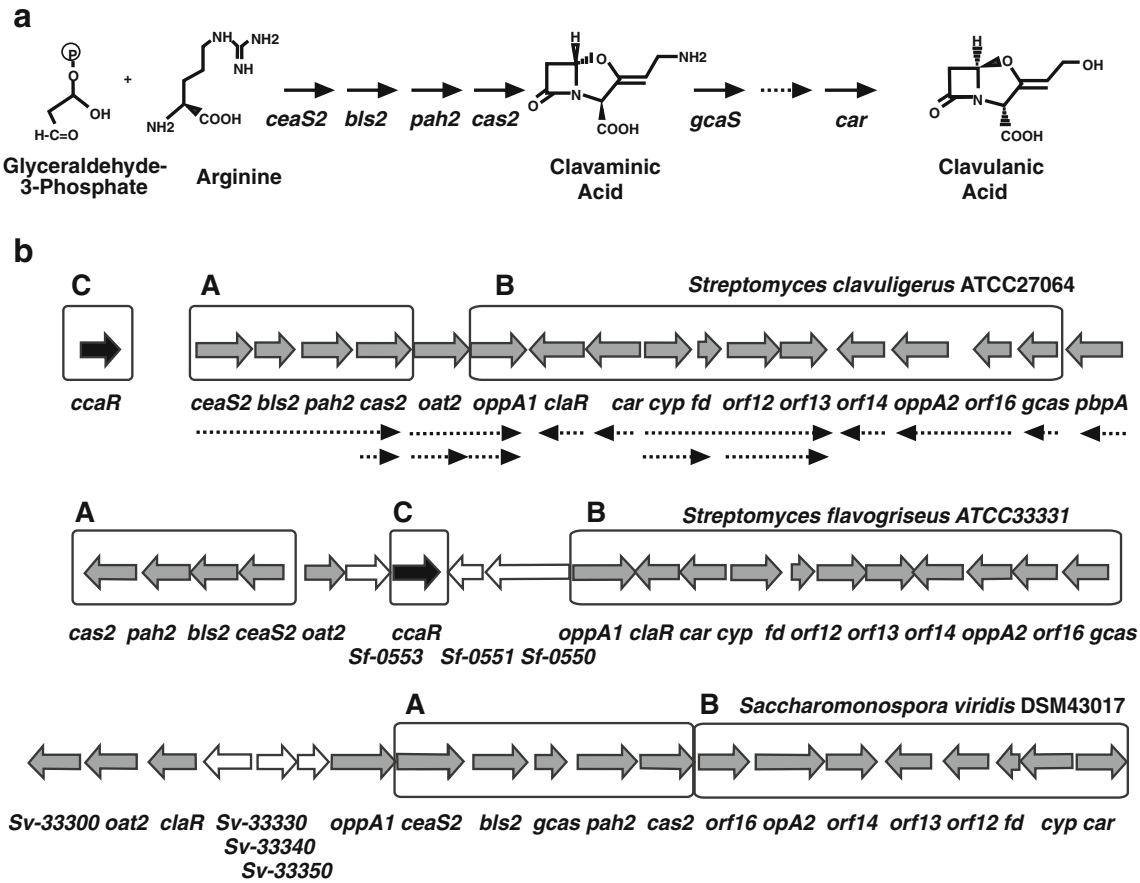

Fig. 1 Clavulanic acid biosynthesis pathway and gene clusters. **a** Scheme of the clavulanic acid pathway. The gene encoding enzymes involved in the different steps are indicated. **b** Clavulanic acid gene cluster in *S. clavuligerus*, *S. flavogriseus*, and *Sac. viridis*. The transcriptional pattern of the *S. clavuligerus* cluster is indicated with *broken arrows*. The grouped subclusters (blocks A, B, and C) referred in the text are enclosed in *square boxes*. The regulatory gene *ccaR* (block C) is indicated with a *black-filled arrow*

challenge for the pharmaceutical industry and requires understanding of the molecular basis for this lack of expression (Laureti et al. 2011).

Taking into account the increasing number of fully or partially sequenced microbial genomes (www.ncbi.nlm.nih.gov), we undertook the search for other putative CA gene clusters in different microorganisms that may contain (or lack) additional genes. Our aim was to test production of CA or related compounds in different media by high-performance liquid chromatography (HPLC) and biological assays to determine if those novel CA gene clusters were expressed.

In this work, the CA cluster of *Streptomyces flavogriseus* and *Saccharomonospora viridis* (hereafter abbreviated as *Sac. viridis*) was analyzed, and the production of CA by these strains was tested. In addition, the heterologous expression of the *S. clavuligerus* CA cluster in *S. flavogriseus* was allowed to identify non-expressed genes that explain the silent nature of the *S. flavogriseus* CA cluster.

Materials and methods

Strains and culture conditions

Streptomyces strains used in this work are described in Table 1. They were grown at 28 °C and 220 rpm in 500-ml baffled flasks containing 100 ml TSB medium for 24 h to an $OD_{600\ nm}$ of 6.5 and used to inoculate (5 % *v*/*v*) the final cultures. The following media were used to grow the *Streptomyces* species: defined SA and MG as described by Paradkar et al. (1998) and Doull and Vining (1989). Complex TSB, YEME, R5 media, MEY, or MS as described by Kieser et al. (2000), TBO as described by Higgens et al. (1974), and ISP4 (Difco ™). Kanamycin (50 μg/ml), thiostrepton (10 μg/ml), hygromycin (50 μg/ml), and nalidixic acid (25 μg/ml) were added to the cultures when required.

Clavulanic acid analysis

Clavulanic acid production was quantified using bioassay with *Klebsiella pneumoniae* ATCC 29665 as test strain as indicated by Romero et al. (1984). When required, the bioassays were repeated in plates lacking penicillin G. Broth of the cultures of *S. flavogriseus*::[SCos-AC] was derivatized with imidazole as described by Foulstone and Reading (1982). These samples were analyzed by HPLC in a SunFire column (5 μm, 4.6×250 mm; Waters, Milford, MA, USA), using an isocratic elution with acetonitrile and 0.1 % trifluoroacetic acid in water (10:90) at 1 ml/min. Peaks were detected at 318 nm. For HPLC-MS analysis, an Alliance chromatographic system coupled to a ZQ4000 mass spectrometer and an Atlantis T3 column (3 μm, 2.1×150 mm; Waters, Milford) was used. Samples (10 μl) were injected and eluted with 0.1 % formic acid in water during 4 min, followed by a linear gradient from 0 to 40 % acetonitrile over 16 min at 0.2 ml/min. MS analysis were done by electrospray ionization in the negative mode, with a capillary voltage of 3 kV and a cone voltage of 20 V. Clavulanic acid was detected by selected ion recording at *m*/*z* 198. Pure clavulanic acid (Antibióticos SA, León, Spain) was used as standard.

Table 1 *Streptomyces* strains used in this work

Strain	Origin	Characteristics of the strain
S. clavuligerus ATCC 27064	ATCC	Wild-type strain
S. clavuligerus Δ*ccaR*::*tsr*	Alexander and Jensen 1998	Clavulanic acid and cephamycin C non producer. Thiostrepton resistant
S. clavuligerus (Δ*ccaR*::*tsr*):: [P_{fur}-*ccaR*$_F$]	This work	Clavulanic acid and cephamycin C non producer. Thiostrepton resistant. It carries, integrated in the genome, the *ccaR*$_F$ gene expressed from the P_{fur} promoter
S. flavogriseus ATCC 33331	ATCC	Wild-type strain
S. flavogriseus:: [P_{fur}-*ccaR*$_C$]	This work	Clavulanic acid non producer. It carries the *ccaR*$_C$ gene expressed from the P_{fur} promoter of *S. coelicolor*, integrated in the genome. Hygromycin B resistant
S. flavogriseus::[SCos-CA]	This work	Clavulanic acid producer. It carries the [SCos-AC] containing the clavulanic acid gene cluster of *S. clavuligerus* integrated in the genome. Kanamycin and tetracycline resistant
S. flavogriseus::[P_{fur}-*ccaR*$_C$]:: [SCos-CA]	This work	Clavulanic acid producer. It carries integrated in the genome [SCos-AC] and the *ccaR*$_C$ gene expressed from the P_{fur} promoter. Kanamycin, tetracycline, and hygromycin B resistant
S. coelicolor M1146	Gomez-Escribano and Bibb 2011	Strain for expression of heterologous gene clusters

Nucleic acid manipulation

Oligonucleotides

Oligonucleotides used in this work (Laboratorios Conda, Madrid, Spain) are shown in Table S1. The oligonucleotides designed to discriminate orthologous genes of *S. clavuligerus* ATCC 27064 and *S. flavogriseus* ATCC 33331 were tested in both strains to ensure the specific amplification of the analyzed gene.

Isolation of nucleic acid

DNA was obtained by the modified Kirby method as described by Kieser et al. (2000). RNA was obtained using the RNeasy kit (Qiagen). Samples to extract RNA were taken at 24 and 48 h of culture. The RNA was quantified in a NanoDrop spectrophotometer, the integrity analyzed using a Bioanalyzer 2100 (Agilent Technologies), and the chips included in the RNA 6000 Nano LabChip® kit (Agilent).

Construction of plasmids [P_{fur}-$ccaR_F$] and [P_{fur}-$ccaR_C$]

Using the DNA of *S. flavogriseus* and the oligonucleotides Sfla_0552-D and Sfla_0552-R (Table S1), an 854-bp DNA fragment was amplified containing NcoI and HindIII digestion sizes at the ends. This DNA fragment, containing the $ccaR_F$ gene (Sfla_0552 gene, orthologous to $ccaR_C$) was ligated to plasmid pUFurReg digested with the same enzymes to introduce $ccaR_F$ in phase downstream of the constitutive promoter P_{fur} (Ortiz de Orue Lucana et al. 2003) to avoid the regulation by sigma–antisigma factors of *ccaR* (Bignell et al. 2005). The $ccaR_F$-containing fragment was isolated with BamHI-HindIII, filled with Klenow and subcloned in the EcoRV site of the integrative vector pMS82 (Matthew et al. 2003). The resulting plasmid [P_{fur}-$ccaR_F$] was transformed in *E. coli* ET12567[pUZ8002], which was later conjugated with *S. clavuligerus* Δ*ccaR*::*tsr* to give S. *clavuligerus* (Δ*ccaR*::*tsr*)::[P_{fur}-$ccaR_F$]. To construct [P_{fur}-ccaR$_C$], the *S. clavuligerus* $ccaR_C$ gene was amplified by PCR using oligonucleoties SCLAV_ccaR-D and SCLAV_ccaR-R which carry at the ends an NcoI and a HindIII site, respectively. The following steps were carried out in parallel with those described for [P_{fur}-$ccaR_F$].

Location and analysis of the [SCos-CA] cosmid

Two DNA fragments from the clavulanic acid gene cluster were amplified. They corresponded to (1) a 502-bp DNA fragment internal to *ceaS2* which was amplified with oligonucleotides ceaS2_D and ceaS2_R and (2) a 516-bp DNA fragment containing the intergenic region and part of the *orf18* and *gcaS* genes, which was amplified with oligonucleotides orf18-gcaS_D and orf18-gcaS_R. The amplified regions were used to scan a SuperCos gene library of *S. clavuligerus* DNA (Robles-Reglero and Liras, unpublished results). One cosmid giving positive hybridization with both probes, analyzed by restriction digestion, PCR of internal zones, and partial sequencing, was found to carry the whole CA gene cluster. This cosmid, which will be named [SCos-CA], carries an insert including genes from *ceaS2* to *gcaS* but not carry the *ccaR* gene.

Vector pFL1272, a pIJ787-derived plasmid (Eustáquio et al. 2005), was digested with XbaI, and the integration cassette (containing the *int* and tet^R genes, and the *att* site of ϕC31) was isolated and filled with Klenow in the presence of thymines and cytosines. Cosmid [SCos-CA] was digested at a single site with HindIII, the ends were filled with Klenow in the presence of adenines and guanines, and the linear fragment was ligated to the pFL1272 integration cassette. The resulting construction was transformed in *E. coli* DH5α and colonies resistant to both kanamycin and tetracycline were isolated. After the confirmation of the correct construction, the cosmid was introduced in *E. coli* ET12567[pUZ8002] which was conjugated with spores of *S. flavogriseus*. Exconjugants resistant to kanamycin were tested by PCR for the presence of the [SCos-CA] cosmid. Ninety percent of the exconjugants tested showed amplification by PCR of a 1,700-bp DNA fragment containing *oppA2* which was not amplified when DNA from *S. flavogriseus* ATCC 33331 was used.

RT-PCR

The one-step reverse-transcriptase polymerase chain reaction (RT-PCR) mixture contained in a 20-μl volume RNA template (200 ng), reaction mixture 1X, oligonucleotides 0.5 mM each, DMSO (5 %), *SuperScriptTM II* reverse transcriptase, and *Platinum® Taq* 2 U (Invitrogen, Carlsbad, CA, USA). Control reactions contained *Platinum® Taq* DNA polymerase but no reverse transcriptase. The reaction to synthesize cDNA was carried out at 55 °C for 30 min. Amplification by PCR of the cDNA (30 or 35 cycles) was as follows: 95 °C/30 s; 60 °C/30 s; 72 °C/40 s, and a final extension of 72 °C/10 min. Controls (not shown) to test the *S. clavuligerus* CA gene expression in the different media were always included using oligonucleotides already tested for *S. clavuligerus* CA gene amplification (Santamarta et al. 2011; López-García et al. 2010).

RT-qPCR

Gene expression analysis by reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) was performed as previously described (Kurt et al. 2013). The quantification of gene expression was performed by the $2^{-\Delta Ct}$ method (Livak and Schmittgen 2001; Schmittgen and Zakrajsek 2000), using the gene *hrdB* as internal control (Aigle et al. 2000).

Results

Genes for clavulanic acid biosynthesis occur in other actinomycetes different from *S. clavuligerus*

The complete clavulanic acid gene cluster occurs infrequently in the genome of actinomycetes. This cluster is located side by side to the cephamycin C gene cluster in *S. clavuligerus*, *Streptomyces jumonjinensis*, and *Streptomyces katsuharamanus* (Ward and Hodgson 1993), but no information on the CA gene cluster sequence of the last two species is available. *Streptomyces antibioticus* Tü1718, a clavam producer (Nobary and Jensen 2012; Janc et al. 1995), contains a complete *ceaS-bls-pah-cas* cluster for the early genes of CA biosynthesis but lacks the genes for the late steps of the CA pathway. A complete CA cluster is present in *S. flavogriseus* ATCC 33331 (Sfla_0539 to Sfla_0558) (Fig. 1B), previously unknown to be a clavulanic acid producer, and in *Sac. viridis* DSM 43017 (Svir_33300 to Svir_33490); this is an actinomycete, member of the *Pseudonocardiaceae* family, with Gram-negative staining properties (Pati et al. 2009) and a circular chromosome different to *Streptomyces* species which have a linear chromosome. In addition, some genes of the CA cluster are present in the Gram-positive bacterium *Anoxybacillus flavithermus* WK1.

The clusters in *S. flavogriseus* ATCC 33331 and *Sac. viridis* DSM 43017 contain all the CA genes and show a strong conservation of several blocks of genes of the known CA cluster but with important differences between them. All the CA proteins of *S. flavogriseus* show a similar length and higher percentage of amino acid identity with those of *S. clavuligerus* (ranging from 88 % for OppA1 to 61 % for ClaR) than with those of *Sac. viridis* (Table 2). In *Sac. viridis*, the *claR* gene, encoding the LysR-type regulator of the late steps of the pathway, has a poor identity (31 %) with respect to *S. clavuligerus claR* (as compared to the 61 % of *S. flavogriseus claR*), but the identity is spread throughout the whole gene what excludes frameshift mutations.

The GcaS protein encoded using *S. flavogriseus* and *Sac. viridis* supports the annotation of a short version for *S. clavuligerus* GcaS as suggested by Arulanantham et al. (2006). Also, the putative membrane protein encoded by *orf13* of *S. clavuligerus* has a stretch of 26 amino acids at the C-terminal end (amino acids 433 to 458) which are not present in the other orthologous proteins. However, the *orf12*-encoded putative lipoprotein of *S. clavuligerus* contains several internal amino acid stretches (amino acids 1–18, 49–55, 75–80, 146–155, 198–226, 346–358, 380–388, and 433–458) that are not present in the *S. flavogriseus* and *Sac. viridis* orthologous.

Organization of the clavulanic acid gene cluster in *S. flavogriseus* and *Sac. viridis*

All the genes described in the CA cluster of *S. clavuligerus* are present in *S. flavogriseus* with the exception of *pbpA*. This gene encodes a penicillin G binding protein (Ishida et al. 2006) but its involvement in CA biosynthesis is not clear (Liras et al. 2011). Genes with 79 and 39 % identity to the orthologous *S. clavuligerus pbpA* are 3.25 Mb and 3.2 kb away, respectively, from the CA cluster in *S. flavogriseus* and *Sac. viridis*.

Three blocks of genes (Fig. 1b) are almost identical in the CA cluster of the three strains and in the clavam producer *S. antibioticus*:

Block A

Block A (Fig. 1b) includes the genes for the early steps of CA biosynthesis (*ceaS2-bls2-pah2-cas2*) which are co-transcribed in *S. clavuligerus* (Santamarta et al. 2011), next to *oat2*, a non-essential gene related to arginine utilization, which is in the opposite orientation in *S. flavogriseus* and *Sac. viridis*. These genes are in the same order in *S. clavuligerus*, *S. flavogriseus*, and *S. antibioticus* (Nobary and Jensen 2012), but genes in block A are in the opposite orientation with respect to block B in *S. flavogriseus* (Fig. 1b).

Block B

A large set of genes (block B in Fig. 1b) in *S. clavuligerus* carries *oppA1-claR-car-cyp-fd-orf12-orf13-orf14-oppA2-orf16-gcaS* and includes late biosynthetic, regulatory, and signal transport genes. This block is fully conserved in *S. flavogriseus* and has the opposite orientation in relation to block A in *S. flavogriseus* and *Sac. viridis* as compared to *S. clavuligerus*. It is interesting that in *Sac. viridis*, block B lacks the *claR*, *oppA1*, and *gcaS* genes, which are located elsewhere in the cluster. In *Sac. viridis*, block A contains in addition, inserted between *bls2* and *pah2*, the *gcaS* gene which in *S. clavuligerus* and *S. flavogriseus* is located in block B.

The regulatory gene *ccaR* of *S. flavogriseus* is located inside the CA cluster at a different location than in *S. clavuligerus*

Block C

The third block (Fig. 1b) includes only the regulatory gene *ccaR*. This gene encodes the SARP-type regulatory protein CcaR that, in *S. clavuligerus*, binds heptameric sequences upstream of *ceaS2* and *claR* and activates expression of these genes and of those located downstream of *ceaS2* (Santamarta et al. 2011). The *ccaR* gene in *S. clavuligerus* is located at 21.5 kb away from the CA cluster, central to the cephamycin C gene cluster (Pérez-Llarena et al. 1997a). *S. flavogriseus* lacks a cephamycin C biosynthesis cluster, and in this species, *ccaR* is located downstream of Sfla _0553, in the middle of the CA cluster.

Table 2 Comparative analysis of clavulanic acid biosynthesis genes in *S. clavuligerus* ATCC 27064, *S. flavogriseus* ATCC 33331, and *Sac. viridis* DSM 43017

S. clavuligerus ATCC 27064	Gene	Product	Size (aa)[a]	*S. flavogriseus* ATCC 33331	Size (aa)[a]	Identity/ similarity (%)[b]	*Sac. viridis* DSM 43017	Size (aa)[a]	Identity/ similarity (%)[b]
SCLAV_4197	*ceaS2*	Carboxyethylarginine synthase 2	586	Sfla_0555	571	84/90	Svir_33370	556	68/79
SCLAV_4196	*bls2*	CEA beta-lactam-synthase 2	513	Sfla_0556	509	67/76	Svir_33380	512	51/63
SCLAV_4195	*pah2*	Proclavaminate amidinohydrolase 2	313	Sfla_0557	316	84/92	Svir_33400	343	68/78
SCLAV_4194	*cas2*	Clavaminate synthase 2	325	Sfla_0558	324	78/85	Svir_33410	323	63/75
SCLAV_4193	*oat2*	Glutamate N-acetyltransferase 2	399	Sfla_0554	392	80/86	Svir_33310	385	70/79
SCLAV_4192	*oppA1*	ABC-type dipeptide transport system	564	Sfla_0549	553	88/92	Svir_33360	549	83/90
SCLAV_4191	*claR*	Transcriptional activator	432	Sfla_0548	488	61/73	Svir_33320	468	31/48
SCLAV_4190	*car*	Clavaldehyde reductase	248	Sfla_0547	247	67/80	Svir_33490	247	68/79
SCLAV_4189	*cyp*	Cytochrome P450-SU2	408	Sfla_0546	410	77/87	Svir_33380	406	71/83
SCLAV_4188	*fd*	Ferredoxin	71	Sfla_0545	70	69/80	Svir_33470	68	60/69
SCLAV_4187	*orf12*	Beta-lactamase-like protein	458	Sfla_0544	432	61/67	Svir_33460	424	52/66
SCLAV_4186	*orf13*	Integral membrane protein	340	Sfla_0543	327	62/73	Svir_33450	314	63/75
SCLAV_4185	*orf14*	Acetyltransferase GNAT family protein	339	Sfla_0542	343	62/72	Svir_33440	331	62/72
SCLAV_4183	*oppA2*	ABC-type dipeptide transport system	562	Sfla_0541	567	81/88	Svir_33430	562	76/84
SCLAV_4182	*orf16*	DUF482 domain-containing protein	401	Sfla_0540	391	70/80	Svir_33420	384	68/78
SCLAV_4181	*gcas*	Biotin carboxylase	529	Sfla_0539	429	85/89	Svir_33390	428	76/85
SCLAV_4180	*pbpA*	Penicillin-binding protein	529	Sfla_3620	494	79/89	Svir_00380	488	39/56
SCLAV_4204	*ccaR*	SARP-type regulator	262	Sfla_0552	262	45/63	–	–	–
SCLAV_4202	*blp*	β-lactamase inhibitory protein	182	Sfla_0521	186	44/56	–	–	–

[a] Number of amino acids in the protein

[b] Amino acid identity/similarity to the orthologous *S. clavuligerus* protein

Surprisingly, no *ccaR* gene is present in the CA cluster of *Sac. viridis*; the gene with highest similarity to *ccaR* is Svir_28940 (32 % amino acid identity), located about 40 kb away from the CA cluster.

Heterogeneous genes inserted in the CA cluster of *S. flavogriseus* and *Sac. viridis*

Three genes, unrelated to the *S. clavuligerus* CA gene cluster, are inserted into the CA cluster of *S. flavogriseus*. One gene, Sfla_0550, encodes a protein with 53 % identity to NocE, a protein with a SGNH hydrolase-type esterase domain encoded by SCLAV_5162 in *S. clavuligerus* but outside the CA cluster. In *Nocardia uniformis*, NocE is a protein of unknown function encoded by a gene (*nocE*) located in the nocardicin cluster (Gunsinor et al. 2004). The second gene, Sfla_0551, encodes a small protein (93 amino acids) annotated as a transport-related protein but with no correspondence to any protein required for clavulanic acid biosynthesis. The third gene inserted in the cluster, Sfla_0553, encodes a 335 amino acid protein 51 % identical to SCLAV_4203, a protein of unknown function encoded by *orf11* which in *S. clavuligerus* is located in the cephamycin C gene cluster. It is unclear if the presence of these genes affects CA production in *S. flavogriseus* and the elucidation will require studies using deleted mutants.

Three CA biosynthesis-unrelated genes, Svir_ 33330, Svir_33340, and Svir_33350, are inserted in the CA cluster of *Sac. viridis* (Fig. 1b); they encode, respectively, a 625-amino acid penicillin-binding protein with putative transpeptidase activity, a 1,414-amino acid hypothetical protein with some similarity to NocE, and an 88-amino acid hypothetical protein.

Expression of the CA gene cluster and clavulanic acid production using cultures of *S. flavogriseus*

Due to the lack of a regulatory *ccaR* gene in *Sac. viridis* genome and since initial tests showed that *Sac. viridis* grows poorly in our standard media and growth conditions, we decided to focus in *S. flavogriseus* to study clavulanic gene expression.

Both *S. clavuligerus* and *S. flavogriseus* were grown in parallel cultures in nine different media, the complex YEME, MS, R5, MEY, TBO, ISP4, and TSB media and the defined MG and SA media under the normal growth conditions used for *S. clavuligerus*. While *S. clavuligerus* produced CA with different yields in all the media, no detectable production of CA by *S. flavogriseus* was observed at any time using bioassays or by HPLC analysis of broth of the liquid cultures. Supplementation of the *S. flavogriseus* cultures with the CA precursors glycerol 0 (2 % *v*/*v*), arginine (5 or 10 mM), or both did not result in CA production (data not shown).

To test whether the lack of CA production was due to the absence of transcription, RNA was purified from *S. flavogriseus* cultures after incubation in YEME, R5, TSB, and SA media for 24 and 48 h, and RT-PCR analyses were performed for the biosynthetic genes and some other genes essential for CA production using oligonucleotides specific for *S. flavogriseus* genes (see Table S1). Positive and negative PCR controls (not shown) confirmed that the sets of oligonucleotides used were effective in amplification and discriminate between *S. clavuligerus* and *S. flavogriseus* CA orthologous genes.

The results of the *S. flavogriseus* RT-PCR reactions are shown in Fig. 2. A clear expression of the *ccaR* and *claR* regulatory genes was observed, especially in YEME, R5, and SA media that suggested that the genes for the early and late steps of the pathway activated using these regulators might be also expressed. However, the expression of *orf12*, *orf14*, and *orf16* in *S. flavogriseus* was low or undetectable in all the tested media, and the expression of *cyp*, *car*, and *cas2* was low (data not shown), even after 35 amplification cycles. The low expression of some biosynthesis genes and the undetectable levels of others could explain the lack of CA production using *S. flavogriseus* in the culture conditions tested.

The $ccaR_F$ gene of *S. flavogriseus* and the $ccaR_C$ of *S. clavuligerus* are not functional in the heterologous strains

Although the endogenous *ccaR* gene (named $ccaR_F$ thereafter) was expressed in *S. flavogriseus* ATCC 33331, as shown by RT-PCR (see Fig. 2), the protein might be present at low levels or might not be functional what could explain the silent nature of *S. flavogriseus* CA cluster. To eliminate these hypotheses, we introduced the *S. clavuligerus ccaR* gene (that will be named $ccaR_C$) expressed from the P_{fur} promoter in *S. flavogriseus* ATCC 33331 by conjugation. The strain obtained, *S. flavogriseus*::[P_{fur}-$ccaR_C$], expressed $ccaR_C$ as detected by RT-PCR but did not produce clavulanic acid in any media (data not shown), suggesting that $ccaR_C$ is not functional in *S. flavogriseus*, perhaps due to the lack of conserved heptameric CcaR-binding sequences in the putative target promoters.

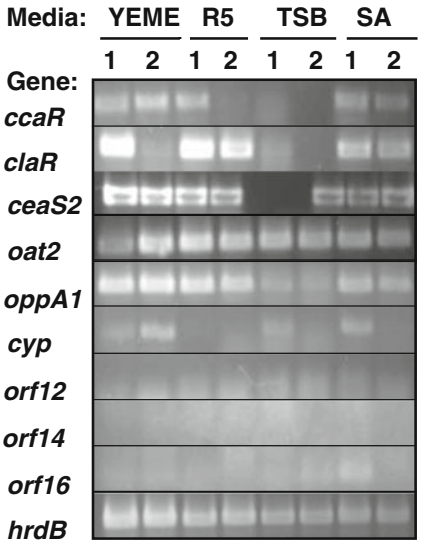

Fig. 2 Expression of the CA biosynthesis genes as detected by RT-PCR. Isolated RNA from *S. flavogriseus* cultures grown in YEME, R5, TSB, and SA media for 24 h (*1*) and 48 h (*2*) was submitted to 35 cycles of amplification for the genes indicated at the left side of the figure. The *hrdB* gene is used as external control

To determine whether the *S. flavogriseus* $ccaR_F$ was able to complement $ccaR_C$ in the disrupted mutant *S. clavuligerus* Δ*ccaR*::*tsr*, unable to produce clavulanic acid or cephamycin C, $ccaR_F$ (under the P_{fur} promoter) was introduced in *S. clavuligerus* Δ*ccaR*::*tsr*. Seven exconjugants of *S. clavuligerus* (Δ*ccaR*::*tsr*)::[P_{fur}-$ccaR_F$] were grown in solid and liquid TSB, MEY, and TBO media for up to 60 h. However, neither cephamycin C nor clavulanic acid was detected in any conditions in spite of the positive expression of $ccaR_F$, as detected by RT-PCR. This result suggests that the $CcaR_F$ protein is unable to complement the lack of $CcaR_C$ in the *S. clavuligerus* disrupted mutant, perhaps because $CcaR_F$ does not recognize promoters essential for clavulanic acid biosynthesis located in the CA gene cluster of *S. clavuligerus*. Positive controls complementing the Δ*ccaR S. clavuligerus* strain with $ccaR_C$ proved that the homologous regulatory protein complements the CA production as reported previously (Pérez-Llarena et al. 1997a, 1997b)

Heterologous expression of *S. clavuligerus* clavulanic acid cluster in *S. flavogriseus* ATCC 33331 and *S. coelicolor* M1146

Since the endogenous CA cluster of *S. flavogriseus* was not functional and $ccaR_C$ did not improve CA production, we decided to study the heterologous expression of the complete *S. clavuligerus* CA cluster (except the *ccaR* gene that is in the cephamycin C cluster) in *S. flavogriseus* ATCC 33331 and in the model organism *S. coelicolor* M1146 to analyze the influence of the genetic background of these strains on the CA cluster expression. The whole cluster for clavulanic acid, present in cosmid [SCos-CA], was introduced by conjugation in these strains and in derived strains carrying the $ccaR_C$ gene expressed from the P_{fur} promoter; the strains obtained were named *S. flavogriseus*::[SCos-CA], *S. flavogriseus*::[P_{fur}-$ccaR_C$]::[SCos-CA], and *S. coelicolor*::[P_{fur}-$ccaR_C$]::[SCos-CA].

These strains and their controls lacking [SCos-CA], were tested in the nine solid media previously described. No production of CA was ever detected in the *S. coelicolor* exconjugants (data not shown). However, a clear inhibition zone produced by CA was observed in plugs of *S. flavogriseus*::[SCos-CA] and *S. flavogriseus*::[P_{fur}-$ccaR_C$]::[SCos-CA] (Fig. 3 inset) but not in the control *S. flavogriseus* grown in MS, ISP4, and particularly in MEY and TBO media.

To validate these results, two exconjugants of *S. flavogriseus*::[SCos-CA] and *S. flavogriseus*::[P_{fur}-$ccaR_C$]::[SCos-

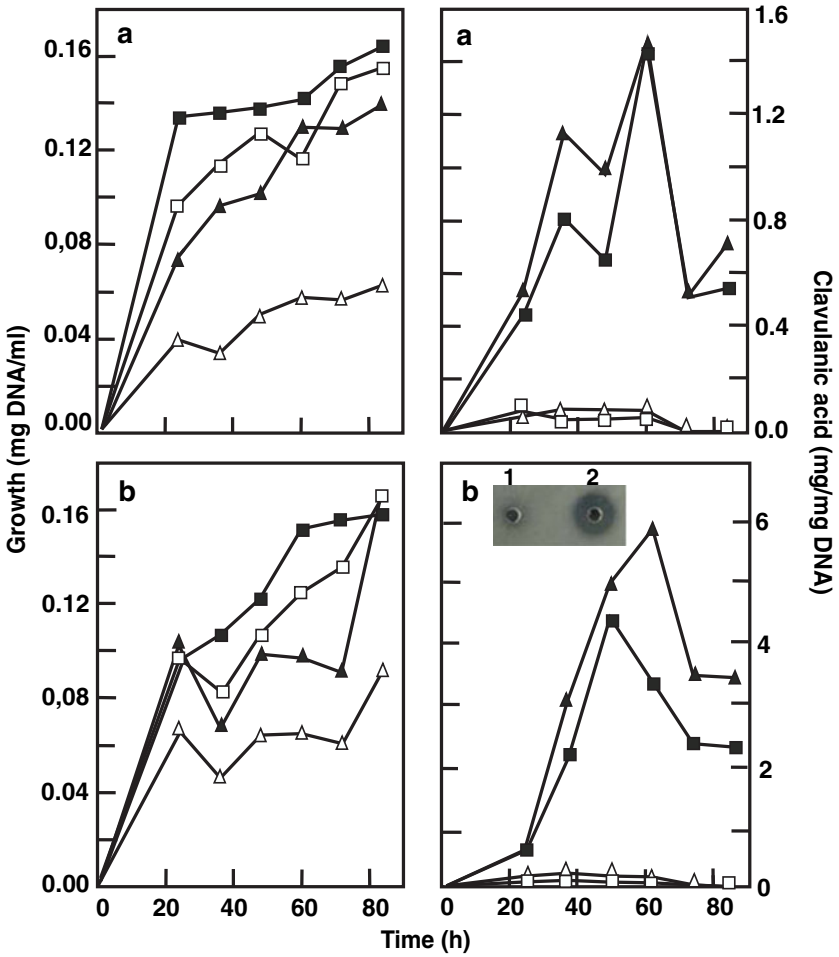

Fig. 3 Heterologous clavulanic acid production using exconjugants of *S. flavogriseus*. Growth (*left panels*) and clavulanic acid production (*right panels*) in MEY (**a**) and TBO medium (**b**) of *S. flavogriseus* ATCC 33331 (*open triangles*), S. *flavogriseus*::[P_{fur}-*ccaR*$_C$] (*open squares*), *S. flavogriseus*::[SCos-CA] (*black triangles*), and *S. flavogriseus* ::[P_{fur}-*ccaR*$_C$]::[SCos-CA] (*black squares*). *Inset in the lower-right panel*: bioassays of broth samples from *S. flavogriseus* ATCC 33331 (*1*) and *S. flavogriseus*::[SCos-CA] (*2*) cultures grown in TBO medium for 48 h

CA] were separately grown in MEY and TBO liquid cultures using cultures of *S. clavuligerus* ATCC 27064 and *S. flavogriseus* ATCC 33331 as controls (Fig. 3). The growth of the strains was relatively similar with the exception of S. *flavogriseus* ATCC 33331 that grew more slowly. The MEY medium supported a faster growth with top growth values at 45 h of cultivation, while in TBO, the maximum growth value was reached at 70 h. Production of clavulanic acid was particularly high in TBO medium with values of 4.5 to 6 μg CA/mg DNA (0.6 μg/ml) at 45 h, about four- to fivefold higher than in MEY medium. These values were, however, lower than those of *S. clavuligerus* ATCC 27064 grown in the same medium and conditions (not shown) which reached maximal values of 87 and 750 μg/mg DNA (28.5 and 164.5 μg/ml) in MEY and TBO media, respectively. The inhibition zone on penicillin-resistant *Klebisella* bioassays produced using broths of the cultures is shown in the inset of Fig. 3.

Taken together, these results indicate that the *S. clavuligerus* CA genes (except *ccaR*) are functional in *S. flavogriseus*. Therefore, the lack of CA production in *S. flavogriseus* is due to lack of CA biosynthesis enzymes.

Confirmation by HPLC-MS that the product is clavulanic acid

To further confirm that the product responsible for the inhibition zones was clavulanic acid, the broth of cultures of two *S. flavogriseus*::[SCos-CA] exconjugants was analyzed by HPLC. A small peak was detected with the same retention time as authentic clavulanic acid (not shown). These broths were also analyzed by HPLC-MS. By electrospray ionization in the negative mode and selected ion recording at *m*/*z* 198, a peak eluting at 14 min was detected in both the standard and the cultures of two exconjugants (Fig. 4A,B). In both cases, mass spectra of that peak showed a negative ion, $[M\text{-}H]^-$, with an *m*/*z* value of 198, as expected for clavulanic acid (Fig. 4C).

Expression of the endogenous and heterologous clavulanic acid gene clusters in *S. flavogriseus* as determined by RT-qPCR

To further investigate the lack of clavulanic acid formation in *S. flavogriseus* ATCC 33331, we compared by RT-qPCR the expression of the clavulanic acid genes of *S. flavogriseus* ATCC 33331 and *S. clavuligerus* ATCC 27064. In addition,

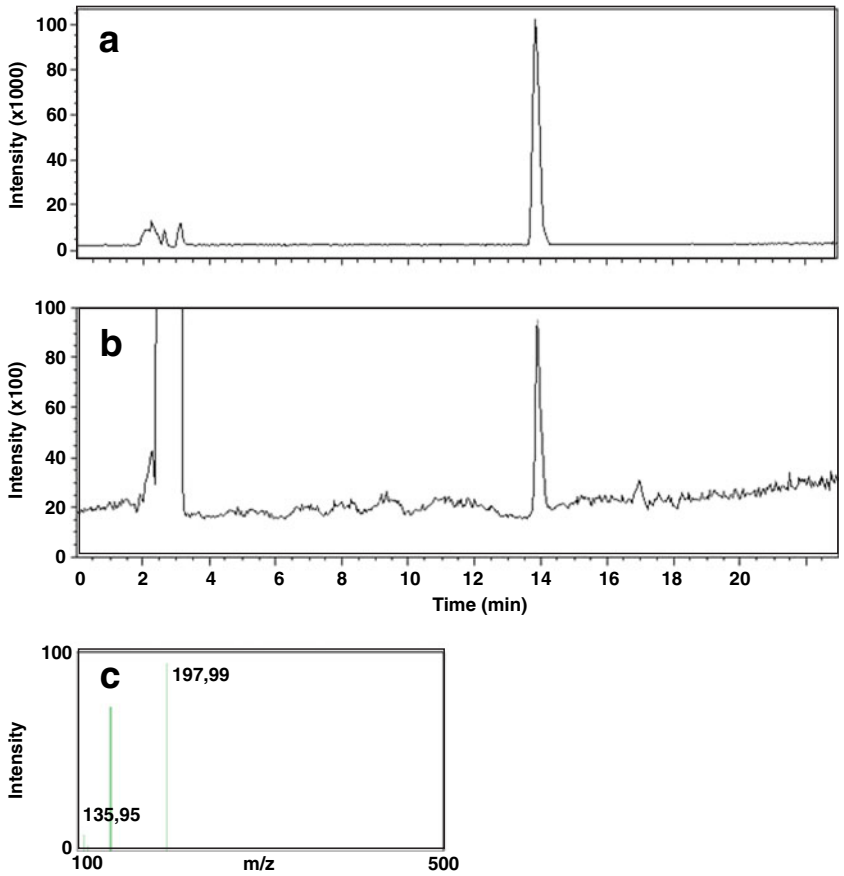

Fig. 4 Mass chromatography obtained by selected ion recording at *m/z* 198. **a** Clavulanic acid standards (0.1 μg); **b** broth (10 μl) from a 60-h culture of *S. flavogriseus*::[SCos-CA] grown in TBO medium. **c** Mass spectra of the clavulanic acid peak at chromatogram B detected in negative mode (M-H)

expression of both the endogenous and the heterologous CA gene clusters were studied (using specific sets of oligonucleotides) in *S. flavogriseus*::[SCos-CA] which carries the CA cluster of *S. clavuligerus*. The studies were done at 48 h of growth in liquid TBO medium which support high CA formation using all the strains.

All the CA genes were well expressed in control *S. clavuligerus* ATCC 27064 (Fig. 5A) with expression values between 4 (*ceaS*, *car*) and 6 (*oppA1*). Interestingly, several genes of the endogenous CA cluster of *S. flavogriseus* ATCC 33331 (not shown) and *S. flavogriseus*::[SCos-CA] (Fig. 5B, black bars) were expressed with a similar pattern but at lower intensities in both *S. flavogriseus* strains. All the endogenous genes for the early steps of the pathway (*ceaS2* to *cas2*) were expressed in *S. flavogriseus*::[SCos-CA] with expression values of about 2 to 2.3, while *oat2*, *oppA1*, and *gcaS* reached values of 4 to 4.6. These expression values, even though lower than those of *S. clavuligerus* (Fig.5A), should be high enough to support CA formation in *S. flavogriseus*. However, endogenous genes essential for CA formation as *cyp*, *orf12*, *orf13*, *orf14*, or *oppA2* were barely or not expressed (expression values around 0.01) in *S. flavogriseus* or *S. flavogriseus*::[SCos-CA]. The low expression of these genes, which might have an accumulative negative effect, is the most plausible explanation of the lack of clavulanic acid production in this strain. All *S. clavuligerus* original genes are poorly expressed in the recombinant *S. flavogriseus*::[SCos-CA] (without the *S. clavuligerus ccaR* gene) (Fig. 5B, white bars) with average values of expression of 0.3 to 0.4, which was about 11- to 14-fold lower than in their *S. clavuligerus* natural host; however, there was an expression of the exogenous *cyp*, *orf12*, *orf13*, or *oppA2* genes that although small, still was three- to fourfold higher than in *S. flavogriseus* ATCC 33331. This expression of the heterologous genes explains the formation of CA by the recombinant *S. flavogriseus*::[SCos-CA] which in TBO reaches 0.6 μg/ml compared to the 164.5 μg/ml produced by *S. clavuligerus* in the same medium. The absence of heterologous expression in *S. coelicolor* and the low expression in *S. flavogriseus* illustrate the important role of the rest of the genes (i.e., the genetic background) on the expression of the CA cluster.

Discussion

Streptomyces species are prolific producers of antibiotics and other related secondary metabolites (Martín et al. 2000; Bérdy 2012). The availability of an increasing number of genome sequences of species of *Streptomyces* (Pati et al. 2009; Ikeda

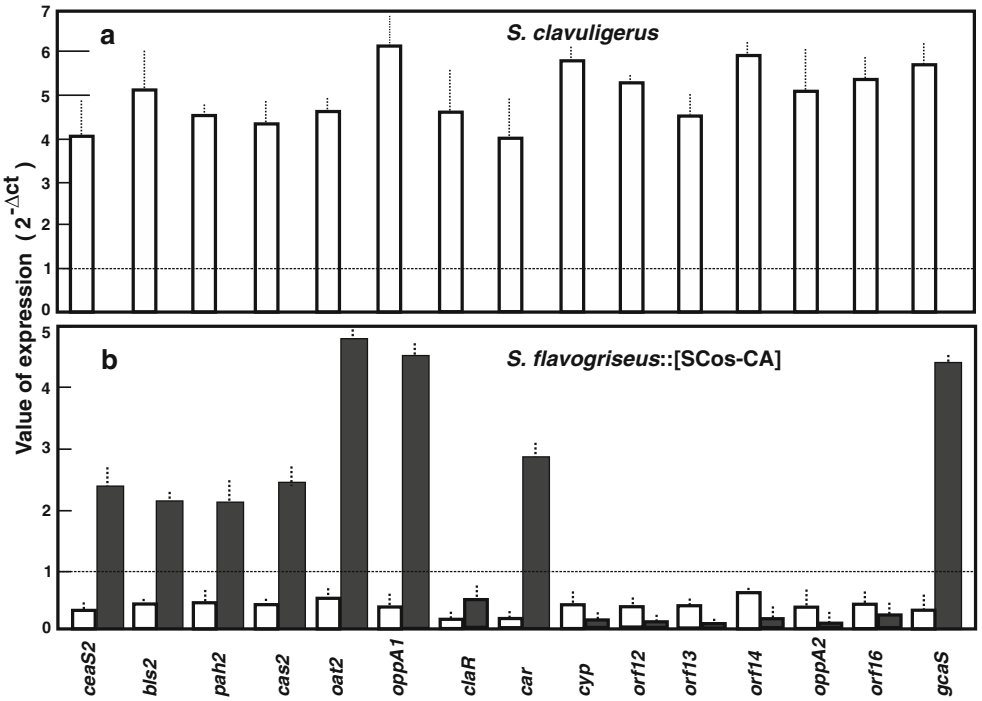

Fig. 5 Quantitative expression by RT-qPCR of the clavulanic acid biosynthesis genes. **A** *White bars* show the expression of the genes (indicated at the *bottom* of the figure) in *S. clavuligerus* grown in TBO medium after 48 h of culture. **B** Expression of the clavulanic acid biosynthesis genes in *S. flavogriseus*::[SCos-CA]. Expression of genes of the endogenous *S. flavogriseus* cluster is shown in *black bars*. Heterologous expression of genes of *S. clavuligerus* CA cluster in *S. flavogriseus*::[SCos-CA] is shown in *white bars*

et al. 2003; Bentley et al. 2002; Barreiro et al. 2012, among others) has provided evidence for the presence of about 20 to 30 gene clusters for secondary metabolites in each of the genomes (Bentley et al. 2002; Medema et al. 2010). However, the presence of these gene clusters does not mean that all of them are expressed (Laureti et al. 2011) Many secondary metabolites gene clusters may be silent or expressed only at very low levels under the culture conditions used in the laboratory (Brakhage and Schroeckh 2011), but they might be expressed in natural environment resulting in an ecological advantage for the strain. An important challenge is to understand why silent clusters are silent and to modify the genes to achieve their expression.

In this work, we report the presence of a complete clavulanic acid gene cluster in two actinomycetes, *S. flavogriseus* and *Sac. viridis*. The organization of CA genes in these genomes shows gene conservation in specific subclusters similar to those of *S. clavuligerus*; however, these subclusters are rearranged in a patchwork-like organization, indicating that reorganization of these blocks has occurred during evolution of the clusters probably associated with horizontal transfer phenomena. We found previously a similar patchwork-like arrangement of the cephamycin C gene clusters in *Amycolatopsis* (formerly *Nocardia*) *lactamdurans* and *S. clavuligerus* (Liras et al. 1998; Enguita et al. 1998). It is very interesting that the genes of block A for the early steps of CA biosynthesis are also required for clavam biosynthesis in *S. antibioticus*, a strain that does not produce clavulanic acid and lacks blocks B and C (Nobary and Jensen 2012). These observations suggest that the full CA pathway has been evolutively assembled by combining an early pathway (up to clavaminic acid formation, block A) common to the biosynthesis of antifungal clavams and the present "late" biosynthesis pathway of CA (block B).

An important difference between the CA cluster in *S. clavuligerus* and *S. flavogriseus* is the presence in the later of the *ccaR* gene, encoding a SARP-type regulator (block C in Fig. 1b) (Pérez-Llarena et al. 1997a). These differences suggest that intense reorganization processes have occurred within the genus *Streptomyces* that cannot be explained by simple rearrangements during vertical inheritance of the CA genes from a common *Streptomyces* ancestor.

S. flavogriseus did not produce CA in eight different culture media, in which *S. clavuligerus* produces high levels, and therefore, the *S. flavogriseus* CA cluster might be considered as silent. Several of the *S. flavogriseus* CA biosynthesis genes analyzed by RT-PCR were found to be expressed at different degrees depending on the media but always at low levels as reflected by the requirement of a high number of PCR cycles needed to detect their expression. RT-qPCR studies allowed to identify which genes of the CA cluster are really silent. They are *cyp*, *orf12*, *orf13*, *orf14*, and *oppA2*, all known to be essential for CA formation in *S. clavuligerus* (Mellado et al. 2002; Li et al. 2000; Lorenzana et al. 2004). Some of these genes are underexpressed in the absence of CcaR (Santamarta et al. 2011). The high expression of the genes for the early steps of the pathway and the poor expression of other genes suggest that *S. flavogriseus* is in an intermediate evolutive stage and might lose eventually the functionality of the genes for the late steps, leading to a strain similar to *S. antibioticus* (Nobary and Jensen 2012).

Heterologous expression of particular genes to increase antibiotic production is a common strategy. Moreover, the heterologous expression of complete gene clusters is a new approach for novel antibiotic production based on the analysis of gene clusters located in rare actinomycetes (Tong et al. 2013) or in *Streptomyces* with unstable antibiotic gene expression levels. Specific *S. coelicolor* host strains have been constructed with this purpose (Gomez-Escribano and Bibb 2011). In some cases, as that of aminocoumarins or trithiazolylpyridine-containing derived compounds, the heterologous expression in genetically modified strains of *S. coelicolor* and *S. lividans* is satisfactory (Eustáquio et al. 2005; Flinspach et al. 2010; Young and Walsh 2011) but other gene clusters, as that of holomycin, are poorly expressed (Huang et al. 2011; Robles-Reglero et al. 2013). When the *S. clavuligerus* CA cluster was introduced in *S. flavogriseus*, the genes were expressed although at very low levels, as detected by RT-qPCR, independently of the presence of the *S. clavuligerus* regulatory activator $CcaR_C$. Indeed, the introduction of the *S. clavuligerus* regulatory ccaRC gene, expressed from the P_{fur} promoter, does not improve significantly the low production of CA in *S. flavogriseus*.

An interesting question is why some genes of the *S. flavogriseus* CA gene cluster are not expressed since both the *ccaR* and *claR* regulatory activators are well expressed in this species (Fig. 2). The simplest explanation is that the promoter regions of those genes have evolved in *S. flavogriseus* being unable to make stable interactions with the RNA polymerase and the CcaR or ClaR positive regulators. A similar situation has been found with a 19-kb thienamycin-like cluster located 41 kb away from the CA cluster in *S. flavogriseus*. The thienamycin cluster, although apparently complete, is silent in several media (Blanco 2012) although there are no expression studies available.

S. flavogriseus is unable to use the heterologous $CcaR_C$ regulatory protein of *S. clavuligerus* for the expression of its own CA genes when introduced in plasmid pMS82. Moreover, the *S. flavogriseus* $CcaR_F$ protein activates expression of its own (homologous) CA genes in the wild-type *S. flavogriseus* to a lower degree than in *S. clavuligerus*. The lack of effect of $CcaR_C$ on the *S. flavogriseus* CA genes might be explained by the lack of detectable heptameric sequences for $CcaR_C$ binding (Santamarta et al. 2011) in the promoter regions of the *S. flavogriseus* CA biosynthesis genes.

The low degree of activation of the *S. clavuligerus* CA genes by $CcaR_C$, when the structural genes and the $CcaR_C$ regulator (both from *S. clavuligerus*) are introduced in *S. flavogriseus*, suggests that a molecule required for full CcaR-mediated induction of homologous CA genes is deficient (at least partially) in *S. flavogriseus*. This might be a still-unknown ligand required for full CcaR activity as occur in other *Streptomyces* species (Wang et al. 2009; Xu et al. 2010). Interestingly, the constructions carrying [P_{fur}-*ccaR*$_C$] and [SCos-CA] result in heterologous production of clavulanic acid in *S. flavogriseus* but not in *S. coelicolor*. This might reflect differences in the genetic background in both host microorganisms, with *S. coelicolor* phylogenetically much more distant in relation to *S. clavuligerus* than *S. flavogriseus* (Zhou et al. 2011). We propose that in many cases, heterologous expression may be limited by the lack of appropriate pools of precursors (as a result of different biosynthetic steps) or of unknown ligands or protein modifying systems that may decrease or even prevent the expression of an apparently complete gene cluster. Indeed, the availability of arginine, a well-known precursor of CA (Valentine et al. 1993; Romero et al. 1986), is probably affected by the very different organization of the *arg* gene clusters in *S. clavuligerus* and *S. coelicolor* (Rodríguez-García et al. 1997). Heterologous expression is easier if the host used has a very similar or at least a related pathway providing adequate precursors (e.g., rare amino acid biosynthetic pathways for heterologous expression of a non-ribosomal peptide compounds). This is the case of *S. flavogriseus* as host for the heterologous expression of CA genes when compared with *S. coelicolor*.

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References

Aidoo KA, Wong A, Alexander DC, Rittammer RA, Jensen SE (1994) Cloning, sequencing and disruption of a gene from *Streptomyces clavuligerus* involved in clavulanic acid biosynthesis. Gene 147:41–46

Aigle B, Wietzorrek A, Takano E, Bibb MJ (2000) A single amino acid substitution in region 1.2 of the principal sigma factor of *Streptomyces coelicolor* A3(2) results in pleiotropic loss of antibiotic production. Mol Microbiol 37:995–1004

Alexander DC, Jensen SE (1998) Investigation of the *Streptomyces clavuligerus* cephamycin C gene cluster and its regulation by the CcaR protein. J Bacteriol 180:4068–4079

Arulanantham H, Kershaw NJ, Hewitson KS, Hughes CE, Thirkettle JE, Schofield CJ (2006) ORF17 from the clavulanic acid biosynthesis gene cluster catalyzes the ATP-dependent formation of *N*-glycyl-clavaminic acid. J Biol Chem 6:279–287

Bachmann BO, Li R, Townsend CA (1998) Beta-Lactam synthetase: a new biosynthetic enzyme. Proc Natl Acad Sci USA 95:9082–9086

Barreiro C, Prieto C, Sola-Landa A, Solera E, Martínez-Castro M, Pérez-Redondo R, García-Estrada C, Aparicio JF, Fernández-Martínez LT, Santos-Aberturas J, Salehi-Najafabadi Z, Rodríguez-García A, Tauch A, Martín JF (2012) Draft genome of *Streptomyces tsukubaensis* NRRL 18488, the producer of the clinically important immunosuppressant Tacrolimus (FK506). J Bacteriol 174:3756–3757

Bentley SD, Chater KF, Cerdeño-Tárraga AM, Challis GL, Thomson NR, James KD, Harris DE, Quail MA, Kieser H, Harper D, Bateman A, Brown S, Chandra G, Chen CW, Collins M, Cronin A, Fraser A, Goble A, Hidalgo J, Hornsby T, Howarth S, Huang CH, Kieser T, Larke L, Murphy L, Oliver K, O'Neil S, Rabbinowitsch E, Rajandream MA,

Rutherford K, Rutter S, Seeger K, Saunders D, Sharp S, Squares R, Squares S, Taylor K, Warren T, Wietzorrek A, Woodward J, Barrell BG, Parkhill J, Hopwood DA (2002) Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). Nature 417:141–147

Bérdy J (2012) Thoughts and facts about antibiotics: where we are now and where we are. J Antibiot 65:385–395

Bignell DR, Tahlan K, Colvin KR, Jensen SE, Leskiw BK (2005) Expression of *ccaR*, encoding the positive activator of cephamycin C and clavulanic acid production in *Streptomyces clavuligerus*, is dependent on *bldG*. Antimicrob Agents Chemother 49:1529–1541

Blanco G (2012) Comparative analysis of a cryptic thienamycin-like gene cluster identified in *Streptomyces flavogriseus* by genome mining. Arch Microbiol 194:549–555

Brakhage AA, Schroeckh V (2011) Fungal secondary metabolites - strategies to activate silent gene clusters. Fungal Genet Biol 48:15–22

Doull JL, Vining LC (1989) Culture conditions promoting dispersed growth and biphasic production of actinorhodin in shaken cultures of *Streptomyces coelicolor* A3(2). FEMS Microbiol Lett 3:265–268

Enguita FJ, Coque JJ, Liras P, Martín JF (1998) The nine genes of the *Nocardia lactamdurans* cephamycin cluster are transcribed into large mRNAs from three promoters, two of them located in a bidirectional promoter region. J Bacteriol 180:5489–5494

Eustáquio AS, Gust B, Galm U, Li SM, Chater KF, Heide L (2005) Heterologous expression of novobiocin and clorobiocin biosynthetic gene clusters. Appl Environ Microbiol 71:2452–2459

Flinspach K, Westrich L, Kaysser L, Siebenberg S, Gomez-Escribano JP, Bibb M, Gust B, Heide L (2010) Heterologous expression of the biosynthetic gene clusters of coumermycin A(1), clorobiocin and caprazamycins in genetically modified *Streptomyces coelicolor* strains. Biopolymers 93:823–832

Foulstone M, Reading C (1982) Assay of amoxicillin and clavulanic acid, the components of Augmentin, in biological fluids with high-performance liquid chromatography. Antimicrob Agents Chemoter 22:753–762

Gomez-Escribano JP, Bibb MJ (2011) Engineering *Streptomyces coelicolor* for heterologous expression of secondary metabolite gene clusters. Microb Biotechnol 4:207–215

Gunsinor M, Breazeale SD, Lind AJ, Ravel J, Janc JW, Townsend CA (2004) The biosynthetic gene cluster for a monocyclic beta-lactam antibiotic, nocardicin A. Chem Biol 11:927–938

Higgens CE, Hamill RL, Sands TH, Hoehn MM, Davis NE, Najarahan R, Boeck LD (1974) The occurrence of desacetoxycephalosporin C in fungi and *Streptomyces*. J Antibiot 27:298–300

Huang S, Yudong Z, Zhiwei Q, Xiaoling W, Mayca O, Chen L, He J, Yu Y, Deng H (2011) Identification and heterologous expression of the biosynthetic gene cluster for holomycin produced by *Streptomyces clavuligerus*. Process Biochem 46:811–816

Ikeda H, Ishikawa J, Hanamoto A, Shinose M, Kikuchi H, Shiba T, Sakaki Y, Hattori M, Omura S (2003) Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*. Nat Biotechnol 21:526–531

Ishida K, Hung TV, Liou K, Lee HC, Shin CH, Sohng JK (2006) Characterization of *pbpA* and *pbp2* encoding penicillin-binding proteins located on the downstream of clavulanic acid gene cluster in *Streptomyces clavuligerus*. Biotechnol Lett 28:409–417

Janc JW, Egan LA, Townsend CA (1995) Purification and characterization of clavaminate synthase from *Streptomyces antibioticus*. A multifunctional enzyme of clavam biosynthesis. J Biol Chem 270:5399–5404

Khaleeli N, Li R, Townsend CA (1999) Origin of the β-lactam carbons in clavulanic acid from an usual thiamine pyrophosphate-mediated reaction. J Am Chem Soc 121:9223–9224

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

Kurt A, Álvarez-Alvarez R, Liras P, Özcengiz G (2013) Role of the *cmcH–ccaR* intergenic region and ccaR overexpression in cephamycin C biosynthesis in *Streptomyces clavuligerus*. Applied Microbiol Biotechnol. doi: 10.1007/s00253-013-4721-4724

Laureti L, Song L, Huang S, Corre C, Leblond P, Challis GL, Aigle B (2011) Identification of a bioactive 51-membered macrolide complex by activation of a silent polyketide synthase in *Streptomyces ambofaciens*. Proc Natl Acad Sci USA 108:6258–6263

Li R, Khaleeli N, Townsend CA (2000) Expansion of the clavulanic acid gene cluster: identification and in vivo functional analysis of three new genes required for biosynthesis of clavulanic acid by *Streptomyces clavuligerus*. J Bacteriol 182:4087–4095

Liras P, Rodríguez-García A, Martín JF (1998) Evolution of the clusters of genes for β-lactam antibiotics: a model for evolutive combinatorial assembly of new β-lactams. Internat Microbiol 1:271–278

Liras P, Santamarta I, Pérez-Redondo R (2011) Clavulanic acid and clavams biosynthesis and regulation. In: Dyson P (ed) *Streptomyces* Molecular Biology and Biotechnology. Caister Academic Press, Norfolk, UK, pp 167–178

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real time quantitative PCR and the 22DDCt Method. Methods 25:402–408

López-García MT, Santamarta I, Liras P (2010) Morphological differentiation and clavulanic acid formation are affected in a *Streptomyces clavuligerus adpA*-deleted mutant. Microbiology 156:2354–2365

Lorenzana LM, Pérez-Redondo R, Santamarta I, Martín JF, Liras P (2004) Two oligopeptide-permease-encoding genes in the clavulanic acid cluster of *Streptomyces clavuligerus* are essential for production of the beta-lactamase inhibitor. J Bacteriol 186:3431–3438

Marsh EN, Chang MD, Townsend CA (1992) Two isozymes of clavaminate synthase central to clavulanic acid formation: cloning and sequencing of both genes from *Streptomyces clavuligerus*. Biochemistry 31:12648–12657

Martín JF, Liras P (2010) Engineering of Regulatory Cascades and Networks Controlling Antibiotic Biosynthesis in *Streptomyces*. Current Op in Microbiol 13:263–273

Martín JF, Gutiérrez S, Aparicio JF (2000) Secondary metabolites. In: Lederberg J (ed) Encyclopedia of Microbiology, vol 4, 2nd edn. Academic Press, San Diego, pp 213–236

Matthew AG, Till R, Smith MCM (2003) Integration site for *Streptomyces* phage ΦBT1 and development of site-specific integrating vectors. J Bacteriol 185:5320–5323

Medema MH, Trefzer A, Kovalchuk A, van den Berg M, Müller U, Heijne W, Wu L, Alam MT, Ronning CM, Nierman WC, Bovenberg RA, Breitling R, Takano E (2010) The sequence of a 1.8-mb bacterial linear plasmid reveals a rich evolutionary reservoir of secondary metabolic pathways. Genome Biol Evol 2:212–224

Mellado E, Lorenzana LM, Rodríguez-Sáiz M, Díez B, Liras P, Barredo JL (2002) The clavulanic acid biosynthetic cluster of *Streptomyces clavuligerus*: genetic organization of the region upstream of the *car* gene. Microbiology 148:1427–1438

Nicholson NH, Baggaley KH, Cassels R, Davison M, Elson SW, Fulston M, Tyler JW, Woroniecki ST (1994) Evidence that the intermediate biosynthetic precursor of clavulanic acid is its N-aldehyde analogue. J Chem Soc Chem Commun 1994:1281–1282

Nobary GS, Jensen SE (2012) A comparison of the clavam biosynthetic gene clusters in *Streptomyces antibioticus* Tü1718 and *Streptomyces clavuligerus*. Can J Microbiol 58:413–425

Ortiz de Orue Lucana D, Tröller M, Schrempf H (2003) Amino acid residues involved in reversible thiol formation and zinc ion binding in the *Streptomyces reticuli* redox regulator FurS. Mol Genet Genomics 268:618–627

Paradkar AS, Aidoo KA, Jensen SE (1998) A pathway-specific transcriptional activator regulates late steps of clavulanic acid biosynthesis in *Streptomyces clavuligerus*. Mol Microbiol 27:831–843

Pati A, Sikorski J, Nolan M, Lapidus A, Copeland A, Glavina del Rio T, Lucas S, Chen F, Tice H, Pitluck S, Cheng JF, Chertkov O, Brettin T, Han C, Detter JC, Kuske C, Bruce D, Goodwin L, Chain P, D'haeseleer P, Chen A, Palaniappan K, Ivanova N, Mavromatis K, Mikhailova N, Rohde M, Tindall BJ, Göker M, Bristow J, Eisen JA, Markowitz V, Hugenholtz P, Kyrpides NC, Klenk HP (2009) Complete genome sequence of *Saccharomonospora viridis* type strain (P101). Stand Genomic Sci 1:141–149

Pérez-Llarena F, Martín JF, Galleni M, Coque JJ, Fuente JL, Frère JM, Liras P (1997a) The *bla* gene of the cephamycin cluster of *Streptomyces clavuligerus* encodes a class A beta-lactamase of low enzymatic activity. J Bacteriol 179:6035–6040

Pérez-Llarena FJ, Liras P, Rodríguez-García A, Martín JF (1997b) A regulatory gene (*cca*R) required for cephamycin and clavulanic acid production in *Streptomyces clavuligerus*: amplification results in overproduction of both ß-lactam compounds. J Bacteriol 179:2053–2059

Pérez-Redondo R, Rodríguez-García A, Martín JF, Liras P (1998) The *cla*R gene of *Streptomyces clavuligerus*, encoding a LysR-type regulatory protein controlling clavulanic acid biosynthesis, is linked to the clavulanate-9-aldehyde reductase (*car*) gene. Gene 211:311–321

Pérez-Redondo R, Rodríguez-García A, Martín JF, Liras P (1999) Deletion of the *pyc* gene blocks clavulanic acid biosynthesis except in glycerol-containing medium: evidence for two different genes in formation of the C3 unit. J Bacteriol 181:6922–6928

Robles-Reglero V, Santamarta I, Álvarez-Álvarez R, Martín JF, Liras P (2013) Transcriptional analysis and proteomics of the holomycin gene cluster in overproducer mutants of *Streptomyces clavuligerus*. J Biotechnol 163(1):69–76

Rodríguez-García A, Ludovice M, Martín JF, Liras P (1997) Arginine boxes and the *argR* gene in *Streptomyces clavuligerus*: evidence for a clear regulation of the arginine pathway. Mol Microbiol 25:219–28

Romero J, Liras P, Martín JF (1984) Dissociation of cephamycin and clavulanic acid biosynthesis in *Streptomyces clavuligerus*. Appl Microbiol Biotechnol 20:318–325

Romero J, Liras P, Martín JF (1986) Utilization of ornithine and arginine as specific precursors of clavulanic acid. Appl Environ Microbiol 52:892–897

Santamarta I, López-García MT, Kurt A, Nárdiz N, Pérez-Redondo R, Álvarez-Álvarez R, Martín JF, Liras P (2011) Characterization of DNA-binding sequences for CcaR in the cephamycin–clavulanic acid supercluster of S*treptomyces clavuligerus*. Mol Microbiol 81:968–981

Schmittgen TD, Zakrajsek BA (2000) Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophys Methods 46:69–81

Tong L, Yuanyuan D, Qiu C, Jingtao Z, Weiming Z, Kui H, Wenli L (2013) Cloning, characterization and heterologous expression of the indolocarbazole biosynthetic gene cluster from marine-derived *Streptomyces sanyensis* FMA. Mar Drugs 11:466–488

Valentine BP, Bailey CR, Doherty A, Morris J, Elson SW, Baggaley KH, Nicholson NH (1993) Evidence that arginine is a later metabolic intermediate than ornithine in the biosynthesis of clavulanic acid by *Streptomyces clavuligerus*. J Chem Soc Chem Commun 1993:1210–1211

Wang L, Tian X, Wang J, Yang H, Fan K, Xu G, Yang K, Tan H (2009) Autoregulation of antibiotic biosynthesis by binding of the end product to an atypical response regulator. Proc Natl Acad Sci U S A 106:8617–8622

Ward JM, Hodgson JE (1993) The biosynthetic genes for clavulanic acid and cephamycin production occur as a 'super-cluster' in three *Streptomyces*. FEMS Microbiol Lett 110:239–242

Xu G, Wang J, Wang L, Tian X, Yang H, Fan K, Yang K, Tan H (2010) "Pseudo" gamma-butyrolactone receptors respond to antibiotic signals to coordinate antibiotic biosynthesis. J Biol Chem 285:27440–27448

Young TS, Walsh CT (2011) Identification of the thiazolyl peptide GE37468 gene cluster from *Streptomyces* ATCC 55365 and heterologous expression in *Streptomyces lividans*. Proc Natl Acad Sci USA 108(32):13053–13058

Zhou Z, Gu J, Du YL, Li YQ, Wang Y (2011) The -omics Era- a toward a systems-level understanding of *Streptomyces*. Current Genomics 12:404–416