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A 1.8-Mb-reduced *Streptomyces clavuligerus* genome: relevance for secondary metabolism and differentiation

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Abstract A large part (21 %) of the wild-type *Streptomyces clavuligerus* genome is located in a 1.8-Mb megaplasmid that greatly influences secondary metabolites biosynthesis even if the secondary metabolites are chromosomally encoded. The megaplasmid copy number may change depending on the nutritional and environmental conditions. The S. *clavuligerus oppA2::aph* mutant described by Lorenzana et al. (2004) does not form aerial mycelium, spores, and clavulanic acid, but overproduces holomycin. Transcriptomic studies, polymerase chain reactions (PCR), qPCR, and RT-qPCR analysis showed that *S. clavuligerus oppA2::aph* has a drastically reduced number of copies (about 25,000-fold lower than the parental strain) of plasmids pSCL1 (10.5 kb), pSCL2 (149.4 kb), and the megaplasmid pSCL4 (1.8 Mb). To clarify the role of the linear plasmids and the function of OppA2 in *S. clavuligerus oppA2::aph* we constructed *oppA2* mutants which contained: (1) a normal copy number of the linear plasmids, (2) completely lack of the linear plasmids, and (3) a *parA-parB*$_{pSCL4}$ mutant that resulted in lack of pSCL4. In addition, a strain with a functional *oppA2* gene was constructed lacking the megaplasmid pSCL4. The results confirmed that the *oppA2* gene is essential for clavulanic acid production, independently of the presence or absence of linear plasmids, but *oppA2* has little relevance on differentiation. We demonstrated that the lack of sporulation of S. *clavuligerus oppA2::aph* is due to the absence of linear plasmids (particularly pSCL4) and the holomycin overproduction is largely due to the lack of pSCL4 and is stimulated by the *oppA2* mutation.

Keywords *Streptomyces clavuligerus* · Clavulanic acid · Holomycin · Linear plasmids · pSCL4

Introduction

Streptomyces clavuligerus ATCC 27064 is the producer organism of two important compounds used in clinic, the β-lactam antibiotic cephamycin C and the β-lactamase inhibitor clavulanic acid (CA) (Baggaley et al. 1997; Liras et al. 2011). Genes for the biosynthesis of both compounds are located side by side in the 6.76 Mb *S. clavuligerus* chromosome (Ward and Hodgson 1993) and share a common SARP-type regulatory gene (*ccaR*) (Pérez-Llarena et al. 1997). Also located in the chromosome is the gene cluster for holomycin (Li and Walsh 2010; Robles-Reglero et al. 2013), a dithiolopyrrolone compound, described as RNA polymerase inhibitor (Oliva et al. 2001), with antitumoral properties (Webster et al. 2000; Li et al. 2007). In addition, *S. clavuligerus* has the potential to produce up to 48 putative secondary metabolites (Medema et al. 2010) including polyketides (PK), non-ribosomal peptides (NRP), mixed PK-NRP compounds, and terpenes.

The *S. clavuligerus* genome appears to be dynamic containing several small and one very large linear plasmid that might recombine with the chromosome. Three small linear plasmids, pSCL1 to pSCL3 of 10.5, 149.4, and 444.2 kb, have been described in *S. clavuligerus* ATCC 27064 (Wu and Roy 1993; Netolitzky et al. 1995). However, only the replicons for pSCL1 and pSCL2 have been identified in *S. clavuligerus*

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genome sequence (Medema et al. 2010; Song et al. 2010) while pSCL3 has been only detected by pulse field electrophoresis and might have been integrated in the chromosome. Sequencing of *S. clavuligerus* genome revealed a new large linear plasmid of 1.8 Mb, named pSCL4, packed with 25 gene clusters for secondary metabolites. The pSCL4 megaplasmid does not seem to have genes essential for primary metabolism, it contains a replication origin different from that of the chromosome and is supposed to be dispensable (Medema et al. 2010). However, no studies are available on the effect of complete removal of pSCL4 on growth, morphology, or the production of secondary metabolites encoded by chromosomally located gene clusters.

In previous studies on clavulanic acid biosynthesis we constructed by gene disruption the mutant *S. clavuligerus oppA2::aph* (Lorenzana et al. 2004). The *oppA2* gene is located in the clavulanic acid gene cluster and encodes an oligopeptide permease (Mellado et al. 2002). The OppA2 protein of *S. clavuligerus* has been crystallized and found to bind in vitro arginine and, probably, arginine containing intermediates of the clavulanic acid pathway (Mackenzie et al. 2009). Disruption of *oppA2*, results in lack of production of clavulanic acid but, interestingly, the original mutant overproduces large amounts of holomycin, a compound undetectable in the wild-type strain cultures (de la Fuente et al. 2002); this report was one of the first examples of the awakening of expression of a silent gene cluster in *Streptomyces.* The original *oppA2* mutant shows a bald phenotype (Lorenzana et al. 2004). In the present study we compare the *S. clavuligerus oppA2::aph* mutant with the wild-type strain using transcriptomic and quantitative polymerase chain reactions (qPCR) techniques and we prove that only trace amounts of the plasmids pSCL1, pSCL2, and pSCL4 are present in this mutant. The wild-type *S. clavuligerus* ATCC 27064 and the original mutant *S. clavuligerus oppA2::aph* have been used to get strains completely devoid of pSCL4 or devoid of the three plasmids. Particularly, *S. clavuligerus* mutants deleted in the pSCL4 *parA-parB* genes (SCLAV_p0885 and SCLAV_p0884) led to the complete loss of this plasmid. The behavior of these linear plasmid-free strains indicates that the linear plasmids have an important role on secondary metabolites biosynthesis and differentiation.

Materials and methods

Culture conditions and strains The origin and characteristics of the *Streptomyces* strains used in this work is shown in Table 1. To start *Streptomyces* seed liquid cultures the strains were grown in 100 ml of tryptic soy broth (TSB) medium at 28 °C and 220 rpm for 24 h. These seed cultures were used to inoculate (5 % *v*/*v*) 500 ml baffled flasks containing 100 ml of defined SA medium (starch-asparagine) (Aidoo et al. 1994) and the culture was grown for 84 h under the same conditions. ME medium (Lorenzana et al. 2004) was used to test sporulation and aerial mycelium formation. The strains used in this work are listed in Table 1.

Antibiotics assays Clavulanic acid and cephamycin C were quantified as indicated by Pérez-Redondo et al. (1998). Holomycin was determined by bioassay against *Micrococcus luteus* ATCC 9341 and by HPLC as described by de la Fuente et al. (2002).

S. clavuligerus oppA2::aph protoplasts formation and regeneration S. clavuligerus oppA2::aph was grown for 36 h in 100 ml TSB medium at 28 °C and 220 rpm to an optical density (OD_{600nm}) of 7 to 10. Ten milliliters of this

Table 1 Characteristic of the *Streptomyces* strains used in this work

Strain	Origin	Characteristics of the strains
S. clavuligerus ATCC 27064	ATCC	Wild-type strain
S. clavuligerus oppA2::aph (renamed *S. clavuligerus oppA2::aph* pSCL^{low})	Lorenzana et al. 2004	Clavulanic acid non-producer, holomycin overproducer strain. Kanamycin resistant. Non-sporulating strain. It carries low copy number of pSCL1, pSCL2 and pSCL4
S. clavuligerus oppA2::aph pSCL$^-$	This work	Clavulanic acid non-producer, holomycin overproducer strain. Kanamycin resistant. Non-sporulating strain. It lacks pSCL1, pSCL2, and pSCL4
S. clavuligerus Δ*oppA2::acc* pSCL$^+$	This work	Clavulanic acid non-producer, holomycin low producer strain. Apramycin resistant. It carries normal copy number of pSCL1, pSCL2, and pSCL4
S. clavuligerus pSCL4$^-$	This work	Holomycin producer strain. Non sporulating strain
S. clavuligerus Δ*oppA2::acc* pSCL4$^-$	This work	Clavulanic acid non-producer, holomycin overproducer strain. Apramycin resistant. Non-sporulating. It carries normal copy number of pSCL1 and pSCL2. It lacks pSCL4
S. clavuligerus Δ*ccaR::tsr*	Wang et al. 2004	Clavulanic acid, cephamycin C non-producer. Thiostrepton resistant

culture were used to inoculate 50 ml of YEMEG medium (García-Domínguez et al. 1989) supplemented with glycine (8 mg/ml). After 48 h the cells were washed with sucrose (10.3 %), resuspended in 2 ml of "P buffer" containing lysozime (1 mg) and kept at 30 °C for 30 min. The protoplasts obtained were washed three times with "P buffer", adequately diluted in "P buffer", and plated on R2YEG plates (Kieser et al. 2000) in which the glucose was substituted by glycerol, since *S. clavuligerus* is unable to utilize glucose (García-Domínguez et al. 1989).

Construction of plasmid-free and *oppA2* mutants

S. clavuligerus oppA2::aph pSCL^{-} This plasmid-free strain (lacking pSCL1, pSCL2, and pSCL4) derives from the original *S. clavuligerus oppA2::aph* pSCL^{low} and was obtained by protoplasts regeneration. The colonies obtained after protoplasts regeneration were grown in TSA and ME plates. DNA from 24 clones was separately purified and tested for PCR amplification of SCLAV_p1328, SCLAV_p0126, and SCLAV_p1452 genes, located at each end of pSCL4. None of the 24 clones analyzed gave amplification bands after 35 PCR cycles whereas using DNA from the wild-type strain the expected 413, 430, and 421 bp DNA bands corresponding to each gene amplification were observed. The strain was deposited in the University of León culture collection under the number ULCC-701.

S. clavuligerus ΔoppA2::acc pSCL^{+} A SuperCos1 *S. clavuligerus* ATCC 27064 cosmid library was used to locate cosmids containing the clavulanic acid gene cluster using the PCR amplified *orf14-oppA2-orf16* genes as probe. Additional mapping with restriction endonucleases and hybridization with other CA gene internal probes demonstrated that cosmid D11-7 contained the whole CA cluster. Gene deletion of *oppA2* was performed on the wild-type *S. clavuligerus* ATCC 27064 using the Redirect Method (Gust et al. 2002) and the oligonucleotides shown in Table S1. Four exconjugants designed *S. clavuligerus ΔoppA2::acc* pSCL^{+} were obtained. Each of them was tested for the adequate antibiotic resistance and confirmed by PCR and Southern hybridization; in addition, all the exconjugants were grown separately in liquid cultures and antibiotic production was studied to prove that they were identical.

S. clavuligerus pSCL4^{-}*, devoid of the megaplasmid* The proteins encoded by the *parA-parB* genes of pSCL4 are only 30–40 % identical to those encoded by the *parA-parB* genes of the chromosome suggesting that they are not able to complement each other. Using cosmid D10-10, in which the pSCL4 *parA-parB* genes are centrally located, oligonucleotides parAB-D and parAB-R and the Redirect method we obtained three *S. clavuligerus* exconjugants apramycin resistant, kanamycin sensitive. The clones (previously tested by PCR to confirm the substitution of the *parA-parB* genes in pSCL4 by the apramycin cassette), were grown in ME medium to allow sporulation. Spores were isolated, and individual clones were tested for growth in the presence and absence of apramycin. Most of the colonies apramycin sensitive showed a smaller size and were candidates as pSCL4-free clones. The lack of pSCL4 was tested as indicated in "Results". The strain was deposited in the University of León culture collection under the number ULCC-702.

S. clavuligerus ΔoppA2::acc pSCL4^{-} This double deletion strain was obtained as indicated before for S. *clavuligerus ΔoppA2::acc* pSCL^{+} but using *S. clavuligerus* pSCL4^{-}, devoid of pSCL4, instead of the wild-type strain as starter strain.

RNA isolation and purification Samples from the *Streptomyces* cultures were stabilized with two volumes of RNA Protect Bacteria Reagent (Qiagen) for 5 min, then 1 % β-mercaptoethanol was added. After 10 min the samples, in a cryogenic bath, were sonicated with six pulses of 30 s in a XL2020 Sonifier, set at 3.5 volts. The preparation was extracted with one volume of phenol/chloroform-isoamylic alcohol and the aqueous-phase was applied to RNeasy Mini Kit Columns (Qiagen) according to the manufacturer's instructions. RNA preparations were incubated with DNaseI (Qiagen) to eliminate DNA contamination. Sample quantification was done with a NanoDrop ND-1000 UV–vis spectrophotometer and the integrity of the RNA was determined in a Bioanalyzer 2100 (Agilent Technologies). Only RNAs with an RNA integrity number above 7.0 were used.

Polymerase chain reactions and RT-PCR analysis Oligonucleotide primers used in this work are shown in Table S1. All PCR reactions were performed in a T-gradient (Biometra) thermocycler using total DNA as template. The PCR reaction, performed as described by Kieser et al. (2000), contained in 30 μl volume: 30 nM DNA template, 0.2 mM dNTP each, 1 mM $MgCl_2$, 4 % dimethyl sulfoxide (DMSO), and 0.1 unit Go Taq polymerase (Promega). The amplification program was as follows: after a step of 95 °C for 30 s, the annealing temperature was reduced in a one cycle touch-down of 1 °C from 68 to 60 °C; an annealing temperature of 72 °C was used next (between cycles 20 and 35) with an extension step of 10 min at 72 °C. Quantification and purity analysis of all PCR products was determined using a NanoDrop ND-1000 190 Spectrophotometer (Thermo Scientific) and the fidelity of the amplification was confirmed by sequencing. Gene expression was studied by RT-PCR (Santamarta et al. 2007; López-García et al. 2010) using gene-specific primers (Table S1). Negative controls to confirm the absence of contaminating DNA were carried out with each set of primers.

cDNA was prepared using 200 ng RNA as template, oligonucleotides at 4 μg each, Superscript™ One-Step RT-PCR mixed Platinum® Taq (Invitrogen) 0.3 μl and 0.05 % DMSO 1 μl. The retrotranscription reaction was performed at 55 °C for 30 min. The samples were denaturalized at 94 °C for 2 min and then were amplified using 30 PCR cycles as follows: denaturation at 94 °C for 30 s, hybridization for 30 s at the optimal temperature according to the primers, extension 15–30 s at 72 °C, and a final extension of 10 min at 72 °C. Negative controls contained Platinum® Taq (0.18 μl) instead of the Superscript™ One-Step RT-PCR/Platinum® Taq mixture.

Quantitative RT-PCR and qPCR Gene expression analysis by quantitative RT-PCR (qRT-PCR) was performed as previously described (López-García et al. 2010) using RNA obtained as described above. Detection of plasmids pSCL1, pSCL2, and pSCL4 in the strains was performed by qPCR (Lee et al. 2006) using 2 ng of gDNA template.

Labeling and microarray hybridizations *S. clavuligerus* microarrays were obtained from Agilent Technologies, in the format of Agilent 8×15K. They include quadruple probes for about 800 genes and intergenic regions of some clusters involved in secondary metabolism. In addition, they included duplicated probes for 7,728 chromosomal genes (out of 7,825) of *Streptomyces coelicolor* genome. Culture conditions were optimized using SA medium and RNA was extracted at the exponential and early stationary growth phase of the cultures of *S. clavuligerus* ATCC 27064, *S. clavuligerus oppA2::aph*, and *S. clavuligerus* Δ*ccaR::tsr*. Four biological replicates were made for each condition. Labeling reactions were performed according to the recommendations described by BioPrime® Array CGH Genomic Labeling Systems (Life Technologies). Total RNA was labeled with Cy3-dCTP (Amersham) using random primers and SuperScript™ II reverse transcriptase (Invitrogen). gDNA was labeled with Cy5-dCTP (Amersham) from random primers extended with the Klenow fragment of DNA polymerase (Roche). The final products were purified with MinElute columns (Qiagen) and labeling efficiencies were quantified spectrophotometrically. Cy3-cDNA (300 ng) and Cy5-labeled gDNA (10 pmol) were mixed, vacuum dried, resuspended in 32 μl of hybridization solution (Agilent), and applied on the microarray surface. Hybridizations were carried out at 55 °C and extended to 60 h to improve the quality of the results (Sartor et al. 2004). Washing, scanning with an Agilent DNA Microarray Scanner G2565BA, and image quantification were carried out as reported previously (Rodríguez-García et al. 2007).

Identification of differentially transcribed genes and transcription profile classification Microarray data were normalized with the Bioconductor package limma (Smyth and Speed 2003; Smyth 2004). Weighted median was applied within arrays. Weights were assigned as follows: 1, probes corresponding to *S. coelicolor* genes showing a raw Cy3 intensity value higher than 2,000; 0.25, probes corresponding to *S. clavuligerus* genes; 0, the rest of probes. The normalized log2 of Cy3/Cy5 intensities is referred as the Mg value, which is proportional to the abundance of transcripts for a particular gene (Mehra et al. 2006). The information from within-array spot duplicates (Smyth et al. 2005) and empirical array weights (Ritchie et al. 2006) were taken into account in the linear models (Smyth 2004).

The transcription results of the six experimental conditions were compared using four contrasts. For each contrast *p* values and M*c* values (log measure of the differential transcription between a specific mutant and the wild strain) were calculated. False-discovery rate (FDR) correction for multiple testing was applied. For each contrast or comparison between two experimental conditions, a result was considered as statistically significant if the FDR-corrected *p* value was <0.05. M*c* positive values indicates a downregulation, and M*c* negative values indicates up regulation. The microarrays data have been deposited in NCBI-GEO Database with accession number GSE51435.

Results

Transcriptomic studies in *S. clavuligerus oppA2::aph* revealed that the pSCL4- and pSCL2-located genes are poorly expressed in this strain

A transcriptomic study was made to compare the original mutant *S. clavuligerus oppA2::aph* with the wild type strain, *S. clavuligerus* ATCC 27064. The microarrays used contained 800 genes for secondary metabolites biosynthesis, nitrogen and carbon utilization, arginine biosynthesis and all those genes known previously to the *S. clavuligerus* genome sequencing project.

Two culture times were chosen for the transcriptomic studies, i.e. exponential phase (t1) and stationary phase (t2) of growth in SA medium. No significant differences in the transcription of most of the genes for clavulanic acid biosynthesis were found between the wild type strain and the original *oppA2*-disrupted mutant (not shown). Forty seven of the 800 *S. clavuligerus* genes studied in the microarray (Table 2) are located in plasmid pSCL2, and forty genes belong to the megaplasmid pSCL4 (Table 3) but it lacks genes of pSCL1. Strikingly, all the genes located in either of these linear plasmids showed significant changes and 99 % of them were underexpressed. The average Mc values for genes located in pSCL2 was −2.35 and −2.34 at the exponential and stationary phase of growth, and those located in pSCL4 showed average Mc values of −2.57 and −3.17 at these two times. These results indicate a decrease in expression of the plasmid-

Table 2 Expression of genes located in pSCL2 in mutants *S. clavuligerus oppA2::aph* pSCL^{low} and *S. clavuligerus* Δ*ccaR::tsr* in relation to the control strain

Gene	Product	Exponential phase				Stationary phase			
		S. clavuligerus oppA2::aph pSCL^{low}		*S. clavuligerus* Δ*ccaR::tsr*		*S. clavuligerus oppA2::aph* pSCL^{low}		*S. clavuligerus* Δ*ccaR::tsr*	
		Mc	FDR	Mc	FDR	Mc	FDR	Mc	FDR
SclaA2_010100027605	Helicase	**−1.40**	1.68E-05	**1.02**	3.06E-03	**−1.70**	1.03E-07	**−0.11**	7.59E-01
SclaA2_010100027610	Hypothetical protein	**−1.33**	7.21E-08	**0.91**	3.35E-04	**−1.71**	8.21E-12	**0.14**	6.04E-01
SclaA2_010100027615	Hypothetical protein	**−1.87**	1.51E-06	**0.53**	2.99E-01	**−2.29**	3.43E-09	**0.32**	4.27E-01
SclaA2_010100027620	Hypothetical protein	**−2.23**	9.63E-03	**0.97**	3.92E-01	**−1.85**	2.28E-02	**1.24**	1.29E-01
SclaA2_010100027625	Hypothetical protein	**−2.55**	9.15E-28	**0.91**	4.42E-06	**−2.00**	1.59E-20	**1.27**	1.14E-10
SclaA2_010100027675	Secreted protein	**−3.08**	6.21E-31	**−0.11**	7.72E-01	**−2.75**	2.76E-27	**−0.18**	4.09E-01
SclaA2_010100027680	Hypothetical protein	**−1.07**	1.71E-03	**0.28**	5.91E-01	**−1.61**	9.01E-07	**−0.82**	1.07E-02
SclaA2_010100027690	Hypothetical protein	**−3.88**	6.37E-33	**0.57**	4.89E-02	**−2.91**	1.80E-23	**1.50**	3.23E-09
SclaA2_010100027700	Hypothetical protein	**−2.13**	1.84E-04	**0.39**	6.71E-01	**−2.03**	2.07E-04	**−0.54**	3.52E-01
SclaA2_010100027705	Ribonuclease H	**−0.28**	8.55E-01	**0.29**	8.59E-01	**−1.28**	1.57E-01	**−1.09**	2.32E-01
SclaA2_010100027710	Hypothetical protein	**−1.99**	3.31E-06	**0.24**	7.40E-01	**−1.65**	6.25E-05	**0.57**	1.77E-01
SclaA2_010100027715	Partitioning protein	**−3.21**	8.68E-19	**0.66**	8.42E-02	**−2.89**	2.66E-16	**1.19**	1.71E-04
SclaA2_010100027720	parB-like partition protein	**−3.88**	3.87E-15	**0.61**	3.15E-01	**−3.61**	7.97E-14	**0.96**	3.12E-02
SclaA2_010100027905	Hypothetical protein	**−0.75**	1.27E-01	**0.69**	2.07E-01	**−1.61**	1.30E-04	**−0.71**	9.72E-02
SclaA2_010100027910	Hypothetical protein	**−2.93**	1.55E-06	**1.30**	6.38E-02	**−2.81**	2.21E-06	**1.27**	3.05E-02
SclaA2_010100027915	ATP/GTP binding protein	**−3.01**	1.66E-08	**1.02**	1.01E-01	**−2.79**	8.41E-08	**1.19**	1.89E-02
SclaA2_010100027920	Transposase	**−1.40**	8.60E-07	**1.00**	7.48E-04	**−1.75**	8.16E-10	**0.11**	7.16E-01
SclaA2_010100027925	Acetyltransferase	**−1.31**	3.99E-02	**1.16**	9.58E-02	**−1.73**	2.65E-03	**0.06**	9.29E-01
SclaA2_010100027930	Hypothetical protein	**−2.80**	8.73E-18	**2.15**	5.01E-12	**−2.98**	1.56E-19	**1.27**	1.21E-05
SclaA2_010100027935	Hypothetical protein	**−2.51**	1.12E-11	**1.07**	4.80E-03	**−2.77**	9.81E-14	**0.86**	1.27E-02
SclaA2_010100027950	Hypothetical protein	**−2.27**	1.58E-06	**0.98**	7.36E-02	**−2.43**	1.67E-07	**0.68**	1.42E-01
SclaA2_010100027955	GntR-family regulatory protein	**−2.06**	2.80E-08	**0.85**	3.89E-02	**−2.25**	1.12E-09	**0.69**	5.42E-02
SclaA2_010100027965	Hypothetical protein	**−1.96**	1.38E-07	**0.69**	1.10E-01	**−2.12**	7.09E-09	**0.03**	9.40E-01
SclaA2_010100027970	Hypothetical protein	**−2.32**	8.94E-06	**0.50**	5.07E-01	**−2.48**	1.13E-06	**0.54**	3.03E-01
SclaA2_010100027975	Hypothetical protein	**−3.10**	1.08E-33	**0.50**	2.02E-02	**−3.25**	1.43E-35	**0.96**	7.27E-07
SclaA2_010100027980	Serine/threonine-protein Kinase	**−3.58**	7.02E-47	**0.20**	3.55E-01	**−3.15**	1.19E-41	**1.52**	1.21E-17
SclaA2_010100027980	Hypothetical protein	**−3.49**	2.80E-11	**0.38**	6.53E-01	**−2.90**	9.86E-09	**1.14**	2.05E-02
SclaA2_010100027985	Hypothetical protein	**−2.34**	2.14E-10	**0.21**	7.40E-01	**−2.08**	7.09E-09	**−0.18**	6.39E-01
SclaA2_010100027990	Hypothetical protein	**−1.90**	7.48E-14	**0.70**	6.76E-03	**−2.04**	1.57E-15	**0.11**	6.74E-01
SclaA2_010100027995	Hypothetical protein	**−1.70**	4.74E-04	**0.73**	2.35E-01	**−1.76**	1.51E-04	**0.11**	8.23E-01
SclaA2_010100028005	Regulatory protein	**−1.35**	1.71E-04	**0.60**	1.65E-01	**−1.48**	2.01E-05	**−0.22**	5.71E-01
SclaA2_010100028010	Hypothetical protein	**−1.23**	2.79E-01	**0.57**	7.19E-01	**−1.40**	1.28E-01	**−0.37**	7.07E-01
SclaA2_010100028015	Phosphatase	**−1.85**	5.27E-09	**0.88**	8.96E-03	**−1.98**	3.30E-10	**−0.16**	6.20E-01
SclaA2_010100028020	Hypothetical protein	**−2.65**	2.77E-16	**1.64**	8.91E-08	**−2.50**	3.59E-15	**1.18**	5.15E-05
SclaA2_010100028030	Hypothetical protein	**−1.46**	2.82E-03	**0.47**	4.98E-01	**−1.79**	1.17E-04	**−0.48**	3.27E-01
SclaA2_010100028035	RNA polymerase sigma factor	**−1.91**	1.44E-06	**0.58**	2.51E-01	**−2.14**	3.96E-08	**−0.23**	5.84E-01
SclaA2_010100028185	Hypothetical protein	**−3.40**	3.47E-10	**0.94**	1.38E-01	**−3.51**	6.45E-11	**0.75**	1.53E-01
SclaA2_010100028210	Hypothetical protein	**−2.35**	1.82E-10	**0.33**	5.33E-01	**−2.66**	7.77E-13	**0.42**	2.42E-01
SclaA2_010100028325	Hypothetical protein	**−2.42**	3.90E-14	**1.85**	2.91E-09	**−2.19**	2.04E-12	**0.42**	1.64E-01
SclaA2_010100028330	Hypothetical protein	**−1.56**	2.06E-03	**2.71**	8.91E-08	**−1.74**	2.78E-04	**0.98**	4.07E-02
SclaA2_010100028335	Transferase	**−1.99**	1.12E-05	**2.97**	1.84E-10	**−2.03**	3.91E-06	**1.82**	3.19E-05
SclaA2_010100028340	Putative regulatory protein	**−2.89**	2.17E-12	**1.15**	6.58E-03	**−3.15**	2.55E-14	**0.27**	5.12E-01
SclaA2_010100028340	Telomere-associated protein	**−2.75**	3.33E-17	**0.79**	1.61E-02	**−3.33**	2.26E-22	**0.02**	9.56E-01
SclaA2_010100028345	Terminal protein	**−1.84**	2.33E-02	**0.57**	6.53E-01	**−2.42**	1.11E-03	**−0.14**	8.67E-01

Table 2 (continued)

Gene	Product	Exponential phase				Stationary phase			
		S. clavuligerus oppA2::aph pSCL^{low}		*S. clavuligerus* Δ*ccaR::tsr*		*S. clavuligerus oppA2::aph* pSCL^{low}		*S. clavuligerus* Δ*ccaR::tsr*	
		Mc	FDR	Mc	FDR	Mc	FDR	Mc	FDR
SclaA2_010100028350	Hypothetical protein	**−1.41**	2.19E-08	**0.73**	6.58E-03	**−2.08**	2.98E-15	**−0.29**	2.42E-01
SclaA2_010100028355	Hypothetical protein	**−2.44**	4.82E-02	**0.72**	7.11E-01	**−2.13**	5.97E-02	**0.78**	5.19E-01
SclaA2_010100028360	Hypothetical protein	**−1.23**	1.04E-04	**0.75**	3.28E-02	**−1.60**	2.43E-07	**0.10**	7.66E-01

encoded genes in *S. clavuligerus oppA2::aph* ranging from 5.06-fold (Mc −2.34) to 9-fold (Mc −3.17).

To validate the transcriptomic results 11 genes strategically separated in the megaplasmid pSCL4 were tested by RT-PCR (Fig. 1a) using RNA extracted at the exponential phase of growth. The genes chosen have diverse functions including regulators, ferredoxin-related proteins, sigma factors, a GlnT-like protein or a SAM-dependent methyltransferase (Fig. 1b). As shown in Fig. 1a all these genes, with the exception of SCLAV_p1328 showed a good amplification in the wild type strain. In contrast none of the RNAs obtained from *S. clavuligerus oppA2::aph* at both culture times gave amplification. These results confirmed the previous transcriptomic analysis and demonstrated that in the *oppA2* mutant the megaplasmid pSCL4-encoded genes are expressed at very low levels or not expressed at all.

These lower values of expression of the plasmid genes in the original *oppA2* mutant may be explained by an unknown general mechanism of regulation of all the genes in the plasmids or, alternatively, might be due to lower plasmids copy number and therefore, reduced levels of the mRNA′s encoded in the *oppA2::aph* disrupted strain.

Plasmids pSCL1, pSCL2 and pSCL4 are present in very low copy number in *S. clavuligerus oppA2::aph*

The presence of the plasmids pSCL4 and pSCL2 in the *oppA2* mutant was also tested by PCR (Figs. 1c and 2a) with the oligonucleotides used for the qRT-PCR experiment. No amplification of the 11 genes located in pSCL4 was observed after 20 cycles of PCR amplification in *S. clavuligerus oppA2::aph*, while clear bands were detected in the wild type strain (Fig. 2a) and also in the control strain *S. clavuligerus* Δ*ccaR::tsr* (not shown). However, when the number of PCR cycles was increased to 35 cycles, clear amplification bands were found also in the *oppA2*-disrupted mutant (Fig. 2a). Three of the bands were sequenced and found to correspond to the expected genes. This amplification after a large number of cycles suggests that the multicellular hyphae of *S. clavuligerus oppA2::aph* contained a very low copy number of the megaplasmid pSCL4, but is not completely devoid of it. To confirm this hypothesis, qPCR analysis of genes located in these plasmids was made as described by Lee et al. (2006). qPCR quantification efficiency curves were obtained for genes of pSCL2 (*parA*$_{pSCL2}$, SclaA2_010100027930, SclaA2_010100028210, SclaA2_010100027690), genes of pSCL4 involved in plasmid stability (*parB*$_{pSCL4}$, *traA*) and in regulation of antibiotic production (*brp*; Fig. 2b and c) and for two genes located in pSCL1 (SclaA2_010100027590 and SclaA2_010100027570). No genes have been ascribed so far to plasmid pSCL3 which has only been detected by pulse field electrophoresis (Netolitzky et al. 1995) and whose identity is unclear. As control for the qPCR the chromosomal genes *adpA* and *hrdB* (not shown) were used. Amplification of *parA*$_{pSCL2}$, located in pSCL2, was 25,000-fold lower in *S. clavuligerus oppA2::aph* than in the wild-type strain and other genes tested were in the order of 10,000-fold lower. Similarly, as shown by qPCR, *brp*, *traA*, and *parB*$_{pSCL4}$, located in the megaplasmid pSCL4, were present only in trace amounts (10^4 lower concentration than in the parental strain) as occurs with the genes located in pSCL1. In contrast, in the control strain *S. clavuligerus* Δ*ccaR::tsr* (lacking the specific SARP regulator CcaR), all the genes tested were present in a number of copies similar to those of the wild-type strain (Fig. 2c). Consequently, the strain *S. clavuligerus oppA2::aph* was renamed *S. clavuligerus oppA2::aph* pSCL^{low} because it contains a very low number of copies of all plasmids.

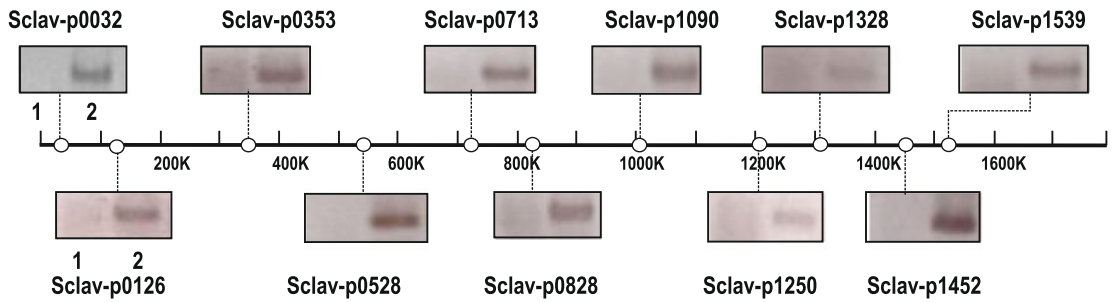
Characteristics of *S. clavuligerus*-derived strains constructed to discriminate the effects due to *oppA2* and to the lack of plasmids

In order to discriminate between the effects produced by the *oppA2* mutation and the lack of plasmids in *S. clavuligerus* we constructed the strains indicated in Table 1. The three strains indicated below knocked-out in *oppA2* were unable to produce clavulanic acid, although they are different in other characteristics as follows:

Table 3 Expression of genes located in pSCL4 in *S. clavuligerus oppA2::aph* pSCLLow and *S. clavuligerus* Δ*ccaR* in relation to the wild-type strain

Gene	Product	Exponential phase				Stationary phase			
		S.clavuligerus oppA2::aph pSCL^{low}		*S.clavuligerus* Δ*ccaR::tsr*		*S.clavuligerus oppA2::aph* pSCL^{low}		*S.clavuligerus* Δ*ccaR::tsr*	
		Mc	FDR	Mc	FDR	Mc	FDR	Mc	FDR
SCLAV_p0032	MMPL domain precursor	**0.08**	9.88E-01	**0.02**	9.90E-01	**−0.45**	8.02E-01	**−0.21**	9.11E-01
SCLAV_p0126	Hypothetical protein	**−3.55**	6.98E-26	**0.28**	4.96E-01	**−3.67**	5.12E-27	**−0.15**	6.20E-01
SCLAV_p0353	Acetyltransferase	**−0.81**	8.42E-05	**−0.26**	3.33E-01	**−0.83**	2.29E-02	**0.05**	9.07E-01
SCLAV_p0528	Putative lysR-family transcriptional regulator	**−1.68**	9.63E-03	**−0.15**	9.07E-01	**−2.26**	1.97E-04	**−1.22**	4.45E-02
SCLAV_p0713	Ferredoxin 2[4Fe-4S]-related protein	**−1.41**	2.96E-04	**0.86**	5.16E-02	**−2.14**	2.40E-08	**0.46**	2.36E-01
SCLAV_p0715	Putative membrane protein	**−3.71**	9.09E-16	**0.59**	2.90E-01	**−3.09**	2.76E-12	**0.31**	4.90E-01
SCLAV_p0763	Amidohydrolase:Amidohydrolase-like precursor	**−6.52**	4.90E-39	**−0.84**	3.63E-02	**−5.56**	2.48E-33	**−0.10**	7.94E-01
SCLAV_p0786	Hypothetical protein	**−3.23**	7.30E-14	**−0.70**	1.61E-01	**−5.02**	2.10E-25	**−1.91**	2.04E-06
SCLAV_p0787	Hypothetical protein	**−2.21**	5.20E-03	**−1.57**	8.02E-02	**−4.14**	5.75E-08	**−3.02**	5.27E-05
SCLAV_p0808	Putative transcriptional regulatory protein	**−1.49**	5.28E-03	**−0.43**	5.90E-01	**−1.80**	3.34E-04	**−0.70**	1.77E-01
SCLAV_p0826	Putative AraC-family transcriptional regulator	**−4.16**	1.40E-43	**0.88**	2.87E-05	**−4.59**	1.30E-47	**−0.05**	8.17E-01
SCLAV_p0828	Oxygenase-reductase PgaM	**−3.79**	9.56E-23	**−0.42**	3.42E-01	**−4.04**	8.13E-25	**−1.24**	1.36E-04
SCLAV_p0853	AfsR-like protein	**−1.05**	9.57E-04	**0.42**	3.15E-01	**−1.74**	2.43E-08	**−1.65**	1.13E-07
SCLAV_p0930	5-methyltetrahydropteroyltriglutamate–homocysteine methyltransferase	**−4.36**	9.11E-09	**0.02**	9.93E-01	**−4.29**	8.56E-09	**−0.75**	3.19E-01
SCLAV_p0935	Putative enoyl-CoA hydratase/isomerase	**−1.88**	1.51E-02	**−0.15**	9.19E-01	**−5.37**	2.09E-12	**−3.08**	2.02E-05
SCLAV_p0939	Putative methylmalonic acid semialdehyde DH	**−1.52**	2.32E-02	**−0.02**	9.93E-01	**−5.42**	9.26E-16	**−3.32**	1.19E-07
SCLAV_p0969	Putative 6-phosphogluconolactonase	**−2.35**	3.79E-19	**−1.35**	2.55E-08	**−2.38**	1.31E-19	**−1.13**	1.17E-06
SCLAV_p0975	Ribulose–phosphate 3-epimerase	**−1.77**	2.08E-10	**−1.25**	6.44E-06	**−2.01**	7.82E-13	**−1.36**	4.01E-07
SCLAV_p1007	CddY–*Rhodococcus ruber*	**−7.43**	2.42E-71	**−1.16**	1.46E-08	**−7.70**	4.92E-73	**−0.54**	4.59E-03
SCLAV_p1088	Alpha-L-fucosidase precursor	**−2.46**	6.35E-11	**−0.10**	8.82E-01	**−2.79**	2.18E-13	**−1.74**	1.33E-06
SCLAV_p1090	Putative RNA polymerase sigma factor	**−1.09**	9.20E-03	**0.22**	8.36E-01	**−1.28**	2.54E-02	**−1.16**	4.15E-02
SCLAV_p1122	Transcriptional activator	**−0.60**	4.44E-01	**1.11**	1.08E-01	**−1.58**	5.53E-03	**0.36**	5.68E-01
SCLAV_p1123	Putative methyltransferase	**−2.79**	8.86E-11	**3.26**	1.36E-13	**−2.55**	1.30E-09	**3.44**	7.86E-15
SCLAV_p1142	SclavP3 Predicted orf	**−1.11**	5.36E-07	**−1.01**	8.10E-06	**−1.88**	1.15E-15	**−1.77**	1.97E-14
SCLAV_p1152	ATP-binding region. ATPase-like:Histidine kinase	**−1.98**	2.27E-05	**−0.52**	4.12E-01	**−2.54**	3.85E-08	**−1.19**	8.19E-03
SCLAV_p1153	Transcriptional regulatory protein cutR	**−2.16**	5.10E-03	**−0.52**	6.71E-01	**−3.04**	2.91E-05	**−1.33**	6.92E-02
SCLAV_p1158	Hypothetical protein	**−2.00**	4.48E-03	**−0.31**	8.03E-01	**−3.09**	3.76E-06	**−1.26**	5.70E-02
SCLAV_p1203	Putative response regulator	**−2.47**	1.56E-09	**−0.76**	1.08E-01	**−3.19**	3.35E-14	**−1.83**	3.24E-06
SCLAV_p1217	Amino acid permease-associated region	**−2.90**	4.97E-11	**−0.94**	5.39E-02	**−3.63**	1.52E-15	**−1.34**	1.17E-03
SCLAV_p1235	Transcriptional regulator protein	**−1.74**	7.85E-05	**−0.89**	8.32E-02	**−2.44**	2.13E-08	**0**	1.00E-05
SCLAV_p1250	Regulatory protein	**−1.15**	5.35E-02	**−0.53**	5.04E-01	**−1.88**	4.13E-04	**−2.17**	4.95E-05
SCLAV_p1276	ABC transporter ATP-binding protein	**−2.85**	2.34E-09	**−0.77**	1.86E-01	**−2.35**	3.39E-07	**−0.89**	5.13E-02
SCLAV_p1292	Putative glutamate dehydrogenase	**−1.27**	3.70E-07	**−0.25**	4.81E-01	**−1.37**	2.41E-08	**−1.40**	1.60E-08
SCLAV_p1319	ThiJ/pfpI family protein	**−6.25**	8.76E-30	**−1.72**	1.75E-04	**−7.20**	5.66E-35	**−1.10**	1.03E-02
SCLAV_p1328	SAM-dependent *O*-methyltransferase	**−2.03**	3.43E-03	**−0.44**	7.79E-01	**−2.84**	1.07E-03	**−1.70**	5.13E-02
SCLAV_p1452	GlnT-like protein	**−3.39**	3.70E-28	**−0.60**	3.33E-02	**−4.50**	2.24E-38	**−1.54**	1.60E-09
SCLAV_p1461	Response regulator:transcriptional regulatory protein	**−1.06**	5.81E-04	**0.11**	8.41E-01	**−1.80**	3.23E-09	**−0.94**	1.32E-03
SCLAV_p1498	Gll2891 protein	**−0.62**	1.25E-02	**0.15**	7.06E-01	**−1.14**	1.07E-06	**−1.17**	6.50E-07
SCLAV_p1539	Related to folylpolyglutamate synthase	**−3.01**	1.25E-14	**−0.48**	3.17E-01	**−4.70**	8.86E-27	**−2.38**	1.76E-10
SCLAV_p1582	Putative DNA-binding protein	**−1.02**	6.22E-05	**−0.03**	9.62E-01	**−1.88**	7.88E-13	**−1.01**	4.12E-05

a



b

	Exponential phase				Stationary phase			
	S. clavuligerus oppA2::aph pSCL^{low}		*S. clavuligerus* Δ*ccaR*		*S. clavuligerus oppA2::aph* pSCL^{low}		*S. clavuligerus* Δ*ccaR*	
Gene (SCLAV_): PRODUCT	**Mc**	**FDR**	**Mc**	**FDR**	**Mc**	**FDR**	**Mc**	**FDR**
p0032. MMPL domain precursor	**0,08**	9,88E^{-01}	**0,02**	9,90E^{-01}	**-0,45**	8,02E^{-01}	**-0,21**	9,11E^{-01}
P0126: Hypothetical protein	**-3,55**	6,98E^{-26}	**0,28**	4,96E^{-01}	**-3,67**	5,12E^{-27}	**-0,15**	6,20E^{-01}
p0353: Acetyltransferase	**-0,81**	8,42E^{-05}	**-0,26**	3,33E^{-01}	**-0,83**	2,29E^{-02}	**0,05**	9,07E^{-01}
p0528:Putative lysR-family regulator	**-1,68**	9,63E^{-03}	**-0,15**	9,07E^{-01}	**-2,26**	1,97E^{-04}	**-1,22**	4,45E^{-02}
p0713:Ferredoxin 2[4Fe-4S] related protein	**-1,41**	2,96E^{-04}	**0,86**	5,16E^{-02}	**-2,14**	2,40E^{-08}	**0,46**	2,36E^{-01}
p0828:Oxygenase-reductase PgaM	**-3,79**	*9,56E^{-23}*	**-0,42**	3,42E^{-01}	**-4,04**	8,13E^{-25}	**-1,24**	1,36E^{-04}
p1090:Putative RNA polymerase sigma factor	**-1,09**	9,20E^{-03}	**0,22**	8,36E^{-01}	**-1,28**	2,54E^{-02}	**-1,16**	4,15E^{-02}
p1250:Regulatory protein	**-1,15**	5,35E^{-02}	**-0,53**	5,04E^{-01}	**-1,88**	4,13E^{-04}	**-2,17**	4,95E^{-05}
p1328:SAM-dependent O-methyltransferase	**-2,03**	3,43E^{-03}	**-0,44**	7,79E^{-01}	**-2,84**	1,07E^{-03}	**-1,70**	5,13E^{-02}
p1452:GlnT-like protein	**-3,39**	3,70E^{-28}	**-0,60**	3,33E^{-02}	**-4,50**	2,24E^{-38}	**-1,54**	1,60E^{-09}
p1539:Related to folylpolyglutamate synthase	**-3,01**	1,25E^{-14}	**-0,48**	3,17E^{-01}	**-4,70**	8,86E^{-27}	**-2,38**	1,76E^{-10}

c

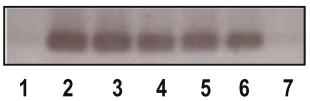


Fig. 1 Expression of genes located in the megaplasmid pSCL4 of *S. clavuligerus*. **a** Map of pSCL4. In each case the location and name of the gene studied by RT-PCR is indicated. The RT-PCR analysis corresponds to 24-h cultures in SA medium of *S. clavuligerus oppA2::aph* pSCL^{low} (2) and *S. clavuligerus* ATCC 27064 (3). **b** Microarrays expression values of the 11 genes tested in pSCL4 in *S. clavuligerus oppA2::aph* pSCL^{low} and *S. clavuligerus* Δ*ccaR*. **c** PCR amplification of the *parA*$_{pSCL2}$ gene in: (*1*) *S. clavuligerus oppA2::aph* pSCL^{low} (*1*), *S. clavuligerus* ATCC 27064 (*2*), *S. clavuligerus* Δ*oppa2::acc* pSCL^{+} (*3*, *4*), *S. clavuligerus* Δ*ccaR* (*5*, *6*). Negative control without DNA (*7*)

S. clavuligerus Δ*oppA2::acc pSCL*$^{+}$ obtained from the wild-type strain *S. clavuligerus* ATCC 27064 by deletion of *oppA2*, contains a copy number of plasmids pSCL1, pSCL2, and pSCL4 identical to that of the wild type strain (as determined by qPCR, not shown). It has a growth pattern similar to that of the wild type strain, and produced aerial mycelium and spores in ME plates. It has a growth pattern similar to that of the wild-type strain. This strain lacks a functional *oppA2* gene but has two important phenotypic differences with respect to the original *oppA2* mutant, i.e. the normal ability to sporulate (Fig. 3) and a weak yellow color (lower holomycin production) of the colonies in SA medium. These results indicate that the *oppA2* mutation of the original *S. clavuligerus oppA2::aph* pSCL^{low} is not responsible for the high production of holomycin.

S. clavuligerus oppA2::aph pSCL^{-} obtained by protoplast regeneration of the original *S. clavuligerus oppA2::aph* pSCL^{low}, it completely lacks pSCL1, pSCL2, and pSCL4 as determined by qRT-PCR of genes internal to these plasmids. This strain does not sporulate (Fig. 3).

S. clavuligerus pSCL4^{-} Colonies with reduced growth, obtained by deletion of the *parA-parB*$_{pSCL4}$ genes, as indicated in "Materials and methods", were shown to lack the megaplasmid pSCL4. Plasmid genes *brp*, *traA*, and *parB*$_{pSCL4}$ and the chromosomal *adpA* and *hrdB* genes, as control, were amplified. While PCR amplification of the chromosomal genes was in the same level than in the wild-type strain (Fig. 4), amplification of nine genes located in pSCL4 did not give amplification after 35 cycles of PCR. However,

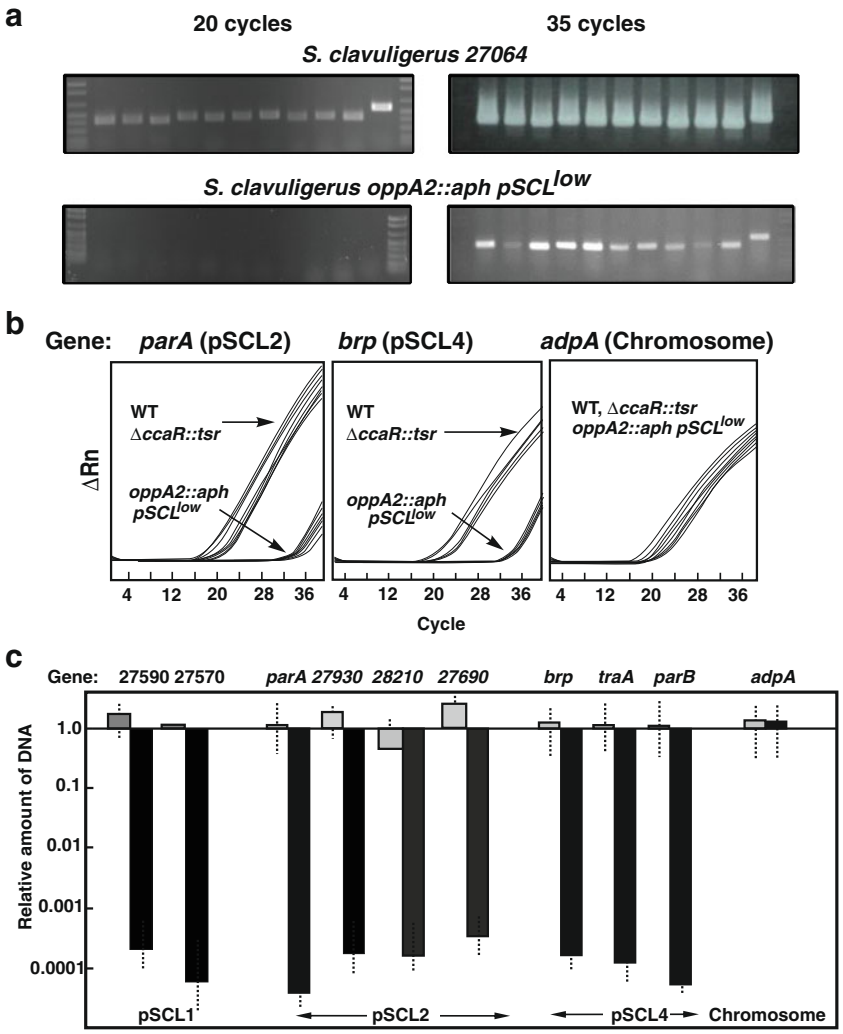


Fig. 2 Quantification of *S. clavuligerus* plasmids by PCR and qPCR. **a** PCR amplification of the 11 genes studied in Fig. 2 (in the same order) after 20 PCR cycles (*left panel*) and 35 cycles (*right panel*) of amplification using total DNA from *S. clavuligerus* ATCC 27064 (*upper panels*) or *S. clavuligerus oppA2::aph* pSCL^{low} (*lower panels*). **b** Amplification curves of the qPCR for the $parA_{pSCL2}$ gene, located in pSCL2, *brp* gene, located at pSCL4 and the chromosomal *adpA* gene in the three strains studied. **c** Relative amount of DNA for pSCL1 gene (SclaA2_010100027590 and SclaA2_010100027570), pSCL2 gene ($parA_{pSCL2}$, SclaA2_010100027930, SclaA2_010100028210, and SclaA2_010100027690), *brp*, *traA*, and *parB* (all in pSCL4) and the chromosomal *adpA* gene in *S. clavuligerus* Δ*ccaR* (*gray bars*), and *S. clavuligerus oppA2::aph* pSCL^{low} (*black bars*)

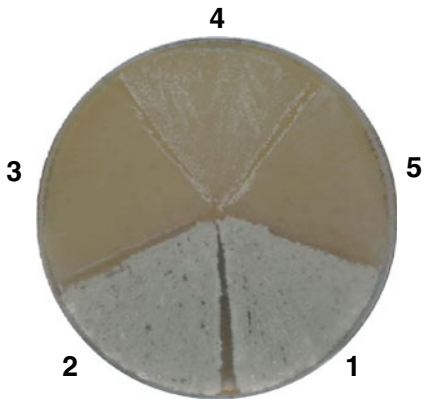


Fig. 3 Effect of the presence of plasmids on the morphologic differentiation of *S. clavuligerus* strains. The strains were grown in ME plates. *1) S. clavuligerus* ATCC 27064, *2) S. clavuligerus* Δ*oppA2::acc* pSCL^{+}, *3) S. clavuligerus* Δ*oppA2::acc* pSCL4^{-}, *4) S. clavuligerus* pSCL4^{-}, and *5) S. clavuligerus oppA2::aph* pSCL^{-}

plasmids pSCL1 and pSCL2 were present in this strain, as shown by the normal amplification of pSCL1 genes (SclaA2_010100027590 and SclaA2_010100027579) and pSCL2 genes ($parA_{pSCL2}$ and SclaA2_010100028210). Therefore, the strain obtained was named *S. clavuligerus* pSCL4^{-} (Fig. 4). These results indicates that the different linear plasmids have distinct *parA-parB* genes.

S. clavuligerus Δ*oppA2::acc pSCL4*$^{-}$ was obtained from *S. clavuligerus* pSCL4^{-} by deletion of the *oppA2* gene.

These two last strains, which lack the megaplasmid pSCL4, did not sporulate and showed a poor aerial mycelium formation ability (Fig. 3). Since the only common characteristic of the three strains, and the only difference with plasmid-containing *S. clavuligerus* Δ*oppA2::acc* pSCL^{+}, is the lack of pSCL4, we conclude that plasmid pSCL4 carries genes involved in the control of aerial mycelium formation and genes essential for sporulation.

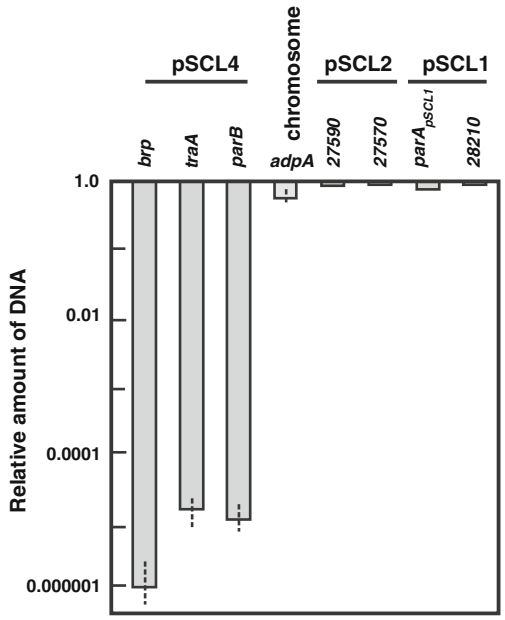


Fig. 4 Linear plasmids present in *S. clavuligerus* pSCL4$^-$. Study of genes located in pSCL4, pSCL1, pSCL2, and in the chromosome as shown by qPCR. The location of the tested genes is indicated above

The growth pattern in defined SA liquid medium was also different. Growth of *S. clavuligerus* Δ*oppA2::acc* pSCL$^+$ was slower than that of the wild-type strain and the DNA content of the mycelium (as a measure of the growth) was in the order of 80 % of that in the wild type. Growth of *S. clavuligerus* Δ*oppA2::acc* pSCL4$^-$ was even lower showing average DNA content in the order of 60 % of that in the wild-type strain. Therefore, the lack of plasmids is correlated with a slow growth of the strains. The plasmid carry genetic information required for optimal growth.

Antibiotic production by plasmid-less strains and *oppA2*-deleted mutants carrying a normal plasmid copy number

Production of cephamycin C and clavulanic acid Production of cephamycin and CA by the strains constructed above was tested in liquid defined SA medium. In general, cephamycin C production was not significantly affected by the absence of plasmids in the strains (Fig. 5, right panels).

Clavulanic acid production was slightly reduced (20–30 %) by the lack of the pSCL4 megaplasmid with respect to the wild-type strain (Fig. 5, upper left panel). However, the disruption or deletion of *oppA2* resulted in a complete lack of clavulanic acid production which is not restored by the presence of all the plasmids in *S. clavuligerus* Δ*oppA2::acc* pSCL$^+$ (Fig. 5, middle left panel). Clavulanic acid biosynthesis only depends on a functional *oppA2* gene as shown by comparing *S. clavuligerus* pSCL4$^-$ and *S. clavuligerus* Δ*oppA2::acc* pSCL4$^-$ (Fig. 5, lower left panel). This result confirms previous reports indicating that *oppA2* is essential for CA biosynthesis.

Production of holomycin Holomycin production (Fig. 5, central panels) by the wild-type strain is null; however, when plasmid pSCL4 is eliminated from the wild-type strain holomycin production reach 1,000 μg/mg DNA (185.4 μg/ml; Fig. 5, upper middle panel). The lack of pSCL4 is sufficient for holomycin production. The lack of pSCL4 in *S. clavuligerus* Δ*oppA2::acc* pSCL4$^-$ or the lack of all the plasmids in *S. clavuligerus oppA2::aph* pSCL$^-$ results in holomycin overproduction up to 1,600 μg/mg DNA (Fig. 5, central middle panel). The mutation of *oppA2* in *S. clavuligerus* Δ*oppA2::acc* pSCL$^+$, carrying all the plasmids, results in an holomycin production of 400 μg/mg DNA (Fig. 5, central middle panel) what indicates that OppA2 has some effect on holomycin production. No great differences in holomycin were observed by the lack of the small plasmids pSCL1 and pSCL2. However, the production values of *S. clavuligerus* Δ*oppA2::acc* pSCL4$^-$ and *S. clavuligerus oppA2::aph* pSCL$^-$ (which lacks all plasmids) are about 2.5-fold higher than those observed in the strain *S. clavuligerus* Δ*oppA2::acc* pSCL$^+$ that carries all the plasmids. Therefore, we can conclude that the mutation in *oppA2* results in production of holomycin but the additional loss of pSCL4 increases drastically the production of this antitumoral agent.

Discussion

Linear mini- and megaplasmids occur in *S. clavuligerus* ATCC 27064 (Medema et al. 2010) and in several other *Streptomyces* species (Kinashi 2011). Deletion of megaplasmids, if they are dispensable, is a valuable strategy to save energy and precursors of secondary metabolites encoded by genes located in the megaplasmid. Although three linear small plasmids pSCL1, pSCL2, and pSCL3 have been described using pulse field electrophoresis (Netolitzky et al. 1995) only pSCL1 and pSCL2 were clearly identified as separate replicons in genome sequencing (Medema et al. 2010; Song et al. 2010). Our results on the loss of pSCL1 and pSCL2 confirm that these plasmids are separate units and not chromosome integrated. The linear megaplasmid pSCL4 (1.8 Mb) is supposed to derive from the chromosome by double crossover with a smaller plasmid or by consecutive recombination events (Medema et al. 2010). The apparent lack of genes for primary metabolism suggested that pSCL4 is a dispensable plasmid and not a small chromosome.

Although the *whi* and *bld* genes, involved in aerial mycelium and spore formation are located in the *S. clavuligerus* chromosome, the pSCL$^-$ strains were unable to sporulate indicating that an additional, still unknown gene located in the plasmids is essential for these morphological changes.

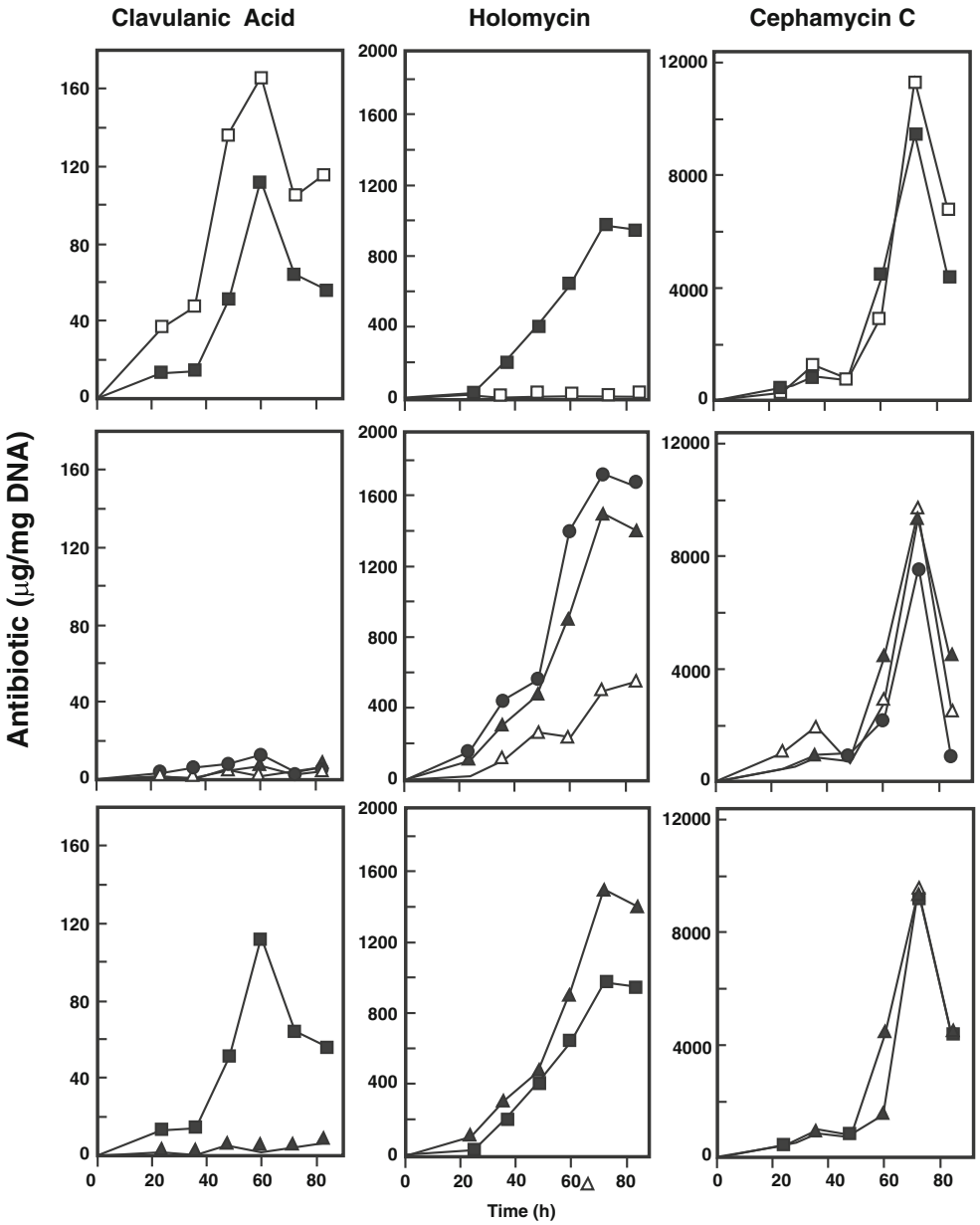


Fig. 5 Antibiotic production by *S. clavuligerus* strains with different genetic background. Cephamycin C production (*right panels*), clavulanic acid production (*left panels*), and holomycin production (*central panels*). Strains: *S. clavuligerus* ATCC 27064 (*open squares*), *S. clavuligerus* pSCL4^{-} (*closed squares*), *S. clavuligerus oppA2::aph* pSCL^{-} (*closed circles*), *S. clavuligerus* Δ*oppA2::acc* pSCL^{+} (*open triangles*), and *S. clavuligerus* Δ*oppA2::acc* pSCL4^{-} (*closed triangles*). The effect of the presence of plasmid pSCL4 in antibiotic production is shown in the *upper panels*. In the *middle panels* is shown the influence of plasmids pSCL1 and pSCL2 (*closed circles and triangles*) on antibiotic production in an *oppA2*-minus genetic background, a strain lacking only pSCL4 (*open triangles*) is included for comparison. The *lower panels* show the effect of the *oppA2* mutation (*closed triangles*) on antibiotic production by two strains lacking plasmid pSCL4

During the study of the original *S. clavuligerus oppA2::aph* mutant we observed that this strain (now designated *S. clavuligerus oppA2::aph* pSCL^{low}) has a greatly reduced number of copies of plasmids pSCL1, pSCL2, and pSCL4, what is responsible for some of the phenotypical alterations observed in this mutant. The original *oppA2* mutant strain shows very high production of holomycin (Lorenzana et al. 2004; Li and Walsh 2010), complete lack of clavulanic acid formation and was unable to produce aerial mycelium and spores.

The reduced copy number of linear plasmids suggested initially a mutation in the *par* genes or in other genes involved in plasmids replication or segregation. Each of *S. clavuligerus* plasmids has its own set of *par* genes. These genes are not very similar to each other and different from the *par* genes in the chromosome. The *parA-parB*$_{pSCL4}$ genes appear to be relatively stable and difficult to manipulate as shown by the lack of success of our initial trials to completely delete them. Therefore, the concomitant lower copy number of the three plasmids, pSCL1, pSCL2, and pSCL4, suggests that a gene controlling the copy number of all the plasmids (different from *parA-parB*) appears to be spontaneously altered in *S. clavuligerus oppA2::aph* pSCL^{low}.

In this work we constructed plasmid-free strains by protoplasts regeneration of *S. clavuligerus oppA2::aph* pSCL^{low} as shown by the lack of amplification of genes located along the whole length of pSCL4 megaplasmid after 35 cycles of PCR and by qPCR studies of genes located in the other plasmids. Strains carrying or lacking the plasmids, allowed to differentiate the role of the *oppA2*-mutation in plasmid-containing and plasmid-free backgrounds and highlighted the role of the lack of linear plasmids in the phenotype of *S. clavuligerus*

oppA2::aph pSCL^{low}. This work confirmed that: (1) the *oppA2* mutation has a role on activating the expression of the silent holomycin gene cluster. The loss of plasmids pSCL1, pSCL2, and pSCL4 causes a large increase of holomycin production by *S. clavuligerus oppA2::aph* pSCL^{-} in relation to *S. clavuligerus* Δ*oppA2::acc* pSCL^{+}. Similar results were observed with S. *clavuligerus* Δ*oppA2::acc* pSCL4^{-}, obtained after deletion of *parA-parB*$_{pSCL4}$. Therefore, the larger production of holomycin by these strains suggests that, (2) a repressor of the holomycin cluster is located in pSCL4. In fact, Kirby (1978) already associated the production of a yellow pigment (now known to be holomycin) by this strain with the presence of plasmids. The high production of holomycin by the plasmid-less strain is stimulated by the lack of OppA2 protein, although the mutation of the *oppA2* gene is not strictly required for holomycin production as shown by the holomycin production of *S. clavuligerus* pSCL4^{-} which contains a functional *oppA2* gene. Therefore, we can conclude that: (3) the effect of OppA2 and a holomycin putative repressor encoded by pSCL4 are additive.

The linear plasmids in the wild-type *S. clavuligerus* ATCC 27064 are quite stable in the absence of genetic manipulation. The pSCL4 deletion appears to be a random phenomenon; Charusanti et al. (2012) found that only one in 14 clones suffered the reduction in copy number or the loss of pSCL4 when challenged with pathogenic bacteria (Charusanti et al. 2012). As conclusion of our study it should be borne in mind that all the mutant strains obtained from *S. clavuligerus* ATCC 27064 should be checked for the presence of linear plasmids before a clear characterization of the effect of the mutations is done.

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