Morphological differentiation and clavulanic acid formation are affected in a *Streptomyces clavuligerus* *adpA*-deleted mutant

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The TTA codon-containing *adpA* gene of *Streptomyces clavuligerus*, located upstream of *ornA*, is in a DNA region syntenous with the homologous region of other *Streptomyces* genomes. Deletion of *adpA* results in a medium-dependent sparse aerial mycelium formation and lack of sporulation. Clavulanic acid formation in this mutant decreases to about 10 % of the wild-type level depending on the medium, whereas its production is strongly stimulated by increasing the *adpA* copy number. Quantitative transcriptional analysis indicates that expression of the clavulanic acid regulatory genes *ccaR* and *claR* decreases seven- and fourfold, respectively, in the $\Delta adpA$ mutant, resulting in a large decrease in expression of genes encoding biosynthesis enzymes for the early steps of clavulanic acid formation and a smaller decrease in the expression of genes for the late steps of the pathway. An ARE box, 5'-TCTCATGGAGACATAGCGGGGCATGC-3', is present upstream of *adpA* and efficiently binds *S. clavuligerus* Brp protein, as shown by electrophoretic mobility shift assay (EMSA) analysis. The transcription level of *adpA* is higher in the absence of Brp, as shown in *S. clavuligerus* Δbrp , suggesting a connection between *adpA* expression and the γ -butyrolactone system in *S. clavuligerus*.

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INTRODUCTION

Streptomycetes are of particular interest as producers of a variety of well-known enzymes and secondary metabolites of commercial value, such as antibiotics, anti-tumour agents, immunosuppressors and enzyme inhibitors. Secondary metabolite production is specifically regulated at several levels of control, involving a regulatory network with different degrees of complexity. In some cases the regulatory network affects the production of one of the antibiotics produced by the strain, while in other cases the production of several antibiotics along with morphological development is affected.

A well-characterized regulatory cascade of *Streptomyces griseus* controls morphological and biochemical differentiation to secondary metabolism (Ohnishi *et al.*, 2005). A-factor, a microbial hormone active at about 100 nM concentration, binds the cytoplasmic receptor protein ArpA and releases it from specific DNA sequences named ARE boxes. An ARE box, located upstream of the pleiotropic regulator gene *adpA*, is responsible for the negative control exerted by ArpA on *adpA* expression. Derepression of *adpA* results in the formation of AdpA, a

member of the AraC/XylS-type regulatory protein subfamily, which activates a gene regulon. This includes genes (*adsA*, *ssgA*) for morphogenesis and spore formation (Yamazaki *et al.*, 2000, 2003), and for chymotrypsin, trypsin, serine proteases and metalloendopeptidases (*sprA*, *B*, *D*, *T*, *U*; *sgmA*) (Kato *et al.*, 2002, 2005; Tomono *et al.*, 2005b) with multiple functions in cell development. In addition, in *S. griseus*, AdpA activates expression of pathway-specific regulatory genes (*strR*, *griR*), triggering streptomycin and grizaxone biosynthesis and resistance (Higashi *et al.*, 2007; Tomono *et al.*, 2005a). Genes belonging to the AdpA regulon possess, upstream of their promoters, specific AdpA-binding sequences, such as 5'-TGCGSNGWWY-3' in *S. griseus*, where S=G/C, W=A/T, Y=T/C and N is any nucleotide (Ohnishi *et al.*, 2005; Yamazaki *et al.*, 2004).

In the model micro-organism *Streptomyces coelicolor*, *adpA_c* (previously *bldH*) is not essential for undecylprodigiosin production but is required for actinorhodin formation (Takano *et al.*, 2003). In addition, *adpA_c* is normally expressed in butyrolactone non-producing *scbA*- and *scbR*-disrupted mutants (*ScbR* being orthologous to ArpA and Brp in *S. griseus* and *Streptomyces clavuligerus*, respectively). Therefore, ArpA control over *adpA* expression appears to be different in *S. coelicolor*. The lack of

Abbreviation: EMSA, electrophoretic mobility shift assay.

antibiotic production and sporulation by *adpA*-negative mutants of *Streptomyces ansochromogenes* has been related to the presence of multiple AdpA-binding sites in the upstream region of *sanG*, a gene encoding the specific activator protein for nikkomycin production (Pan *et al.*, 2009).

All *adpA* genes contain a TTA codon translated in *Streptomyces* by the rare *bldA*-encoded tRNA^{leu} (Chater & Chandra, 2008; Lawlor *et al.*, 1987). TTA codons are present in many antibiotic-specific regulators, which explains the non-producing phenotype of *S. coelicolor bldA* mutants (Fernández-Moreno *et al.*, 1991; White & Bibb, 1997). The inability to translate *adpA* also explains the bald phenotype of the *S. coelicolor bldA* mutant (Nguyen *et al.*, 2003; Takano *et al.*, 2003), in which aerial mycelium formation is restored by complementation with a TTA-free *adpA* gene (Nguyen *et al.*, 2003).

In *S. clavuligerus*, cephamycin C and clavulanic acid production are activated by the CcaR regulator (Pérez-Llarena *et al.*, 1997). CcaR formation is modulated by regulatory proteins such as Brp, a butyrolactone receptor protein, and AreB, an IclR-like protein that connects primary and secondary metabolism (Santamarta *et al.*,

2005, 2007). In addition, clavulanic acid production is specifically activated by ClaR, a regulator under CcaR control (Paradkar & Jensen, 1998; Pérez-Redondo *et al.*, 1999).

In this paper we report that *S. clavuligerus* AdpA is part of a regulatory cascade that controls antibiotic production and study whether its involvement in biochemical and morphological differentiation is similar to that found in *S. griseus*.

METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strain DH5 α was maintained on Luria broth agar plates (Sambrook *et al.*, 1989) and grown in Luria broth liquid medium at 37 °C. Cultures of plasmid-bearing cells were supplemented with ampicillin (50 μ g ml⁻¹), chloramphenicol (25 μ g ml⁻¹), kanamycin (25 μ g ml⁻¹) or apramycin (50 μ g ml⁻¹) as appropriate. *E. coli* Ess22-35 and *Klebsiella pneumoniae* ATCC 29665 were used in cephamycin C and clavulanic acid bioassays, respectively (Liras & Martín, 2005).

S. clavuligerus ATCC 27064 and *S. clavuligerus* mutant strains were maintained on 2% agar TSB medium (30 g l⁻¹ tryptic casein soy

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant features*	Reference or source
Strains		
<i>S. clavuligerus</i> ATCC 27064	Wild-type; cephamycin C and clavulanic acid producer	ATCC†
<i>S. clavuligerus</i> Δ <i>adpA</i>	<i>adpA</i> -deleted mutant	This study
<i>S. clavuligerus</i> Δ <i>bldA</i>	<i>bldA</i> -deleted mutant	Trepanier <i>et al.</i> (2002)
<i>S. clavuligerus</i> Δ <i>adpA</i> [pCPA2]	<i>adpA</i> -deleted mutant complemented with <i>adpA</i> and its own promoter region	This study
<i>S. clavuligerus</i> pMS83	Wild-type strain containing the integrative vector pMS83	This study
<i>S. clavuligerus</i> pIJ699	Wild-type strain transformed with the multi-copy vector pIJ699	This study
<i>S. clavuligerus</i> pIJ <i>adpA</i>	Wild-type strain transformed with pIJ <i>adpA</i>	This study
<i>E. coli</i> ET12567	Methylation-deficient	Kieser <i>et al.</i> (2000)
<i>E. coli</i> ET12567(pUZ8002)	Methylation-deficient; transfer functions from pUZ8002	Kieser <i>et al.</i> (2000)
<i>E. coli</i> Ess22-31	β -Lactam antibiotic-supersensitive	Romero <i>et al.</i> (1984)
<i>E. coli</i> DH5 α	General cloning host	Stratagene
<i>E. coli</i> pGEX2T- <i>brp</i>	Brp protein heterologous expression host	Santamarta <i>et al.</i> (2005)
<i>K. pneumoniae</i> ATCC 29665	Indicator strain for clavulanic acid bioassay	Romero <i>et al.</i> (1984)
Plasmids		
pBluescript II KS(+)	<i>E. coli</i> general cloning vector; Amp ^r	Stratagene
pTC192-Km	pUC19-derived vector containing Kan-resistance gene (<i>aphII</i>) from Tn5 transposon	Rodríguez-García <i>et al.</i> (2006)
pIJ773	Apr ^r cassette in pIJ699	Gust <i>et al.</i> (2003)
pIJ699	Multi-copy <i>Streptomyces</i> vector containing Thio-resistance gene	Kieser & Melton (1988)
pMS83	Integrative vector used for <i>adpA</i> -deleted mutant complementation.	M. Smith, University of Aberdeen
pIJ <i>adpA</i>	<i>adpA</i> and its promoter region in pIJ699	This study
p <i>ΔadpA</i>	<i>adpA</i> :: <i>acc</i> -inactivation construct	This study
p <i>PadpA</i>	<i>adpA</i> and its promoter region (448 bp) in pBluescript II KS(+)	This study
pCPA2	<i>adpA</i> genetic complementation vector	This study

*Amp, ampicillin; Apr, apramycin; Kan, kanamycin; Thio, thiostrepton.

†ATCC, American Type Culture Collection.

broth) at 28 °C. ME medium, containing (in g l⁻¹) MOPS (21), glucose (5), yeast extract (0.5), meat extract (0.5), caseine peptone (1), agar (20), pH 7.0 (Sánchez & Braña, 1996), or TBO medium, containing (in g l⁻¹) tomato paste (20), oat flakes (20), agar (20), pH 6.5 (Higgins *et al.*, 1974), were used to test the morphological differentiation and spore formation ability of *Streptomyces* mutant strains when compared with the wild-type strain. For antibiotic production studies and transcriptional analysis, SA defined or TSB complex medium was used (Lorenzana *et al.*, 2004; Paradkar & Jensen, 1998). Strains were grown in 500 ml baffled flasks containing 100 ml TSB medium at 28 °C and 220 r.p.m. for 24 h. Five millilitres of the culture were harvested, and the mycelium was washed with 0.9% NaCl and used to inoculate SA medium. Triplicate cultures were incubated at 28 °C and 250 r.p.m. The growth rate was determined by measuring the DNA concentration using the diphenylamine reaction (Burton, 1968).

Nucleic acid manipulations. General DNA manipulations were performed using standard techniques (Sambrook *et al.*, 1989). *Streptomyces* genomic and plasmid DNA preparations, *S. clavuligerus* conjugation with *E. coli* ET1257/pUZ8002 as donor strain, and *Streptomyces* protoplast transformation were done following standard methods (Kieser *et al.*, 2000). Nucleic acid hybridizations were performed following the DIG system protocol (Roche), and colorimetric detection was carried out with nitro blue tetrazolium (NBT) and 5-bromo-1-chloro-3-indolyl phosphate (BCIP).

RNA samples from *S. clavuligerus* strains were prepared using RNeasy Mini spin columns (Qiagen), as previously described by Santamarta *et al.* (2005), and treated with DNase I (Qiagen) and Turbo DNase (Ambion) to eliminate chromosomal DNA contamination. PCR and RT-PCR were performed in a T-gradient (Biometra) thermocycler. Plasmids and oligonucleotides used in this work are shown in Tables 1 and 2, respectively.

Plasmid construction

Construction of p Δ adpA for *adpA* deletion. DNA fragments upstream (UP-*adpA*, 1983 bp) and downstream (D-*adpA*, 2267 bp) of *adpA* were amplified by PCR using oligonucleotides UpadpA-O1 and UpadpA-O2, and DWadpA-O1 and DWadpA-O1, respectively. Once sequenced, fragment UP-*adpA* was subcloned into the *EcoRV* site of pBluescriptII KS to form pUP-*adpA*. The apramycin-resistance cassette from pIJ773, containing the origin for conjugation (oriT) and the apramycin-resistance gene, was ligated into a filled blunt *HindIII* site of pUP-*adpA*, to obtain the vector pU : *aac*; the PCR-amplified D-*adpA* fragment was subcloned into a filled blunt *ClaI* site of vector pU : *aac* to give plasmid pU : *acc:D* in such a way that the apramycin-resistance gene was expressed divergently from the *ornA* gene. After *SpeI* linearization, pU : *acc:D* was ligated to the 1.4 kb *XbaI* DNA fragment containing the *aphII* gene for kanamycin resistance, isolated from plasmid pTC192-Km, leading to plasmid p Δ adpA. This plasmid was conjugated into *S. clavuligerus*, and apramycin-resistant transconjugants were subjected to sporulation on solid soy-mannitol medium in the absence of antibiotic and then plated onto antibiotic-supplemented medium. Apramycin-resistant kanamycin-sensitive gene *adpA*-replacement mutants were confirmed by Southern hybridization.

Construction of pCPA2 to complement *S. clavuligerus* Δ adpA. The *adpA* gene with its own promoter (1580 bp) was PCR-amplified using oligonucleotides PadpA-O1 and PadpA-O2. The amplified fragment was subcloned into the *EcoRV* site of pBluescriptII KS, giving pPadpA. *PvuII* digestion of pPadpA produced a 2034 bp fragment that was ligated into the *EcoRV* site of pMS83, leading to pCPA2. Plasmid pMS83 derives from pMS82 and uses the integration site for *Streptomyces* phage Φ B1 (Gregory *et al.*, 2003).

Construction of pJadpA to amplify the copy number of *adpA*.

The *adpA* gene with its own promoter was amplified using oligonucleotides PadpA-O1 and PadpA-O2. The amplified and sequenced fragment was subcloned in the *DraI* site of the high-copy-number plasmid pIJ699, producing plasmid pIJadpA, used to overexpress *adpA* in *S. clavuligerus*.

Mobility shift assays. The ARE_{adpA}-containing probe was isolated as a 448 bp *AvaI* fragment from plasmid pIJadpA. Once labelled with DIG-11-dUTP (DIG Gel Shift kit, 2nd generation, Roche) for chemiluminescence detection it was applied to DNA-binding assays using pure rBrp protein (0.5 µg) (Santamarta *et al.*, 2005). Once electrophoretic mobility shift assays (EMSAs) had been performed (Santamarta *et al.*, 2007), gels were transferred in 0.5 × TBE buffer to Hybond-N+ membranes (GE Healthcare) and developed for detection of DIG-11-dUTP-labelled fragments.

Immunodetection of AdpA

Generation of antibodies. A peptide corresponding to amino acids 385–399 of the AdpA sequence (NH₂-CAGHGRPSLPQRSAP-COOH) was commercially synthesized (NeoMPS, Strasbourg, France). To raise polyclonal antibodies, the peptide (3 mg) was resuspended in 1 ml PBS (pH 7.5) and thoroughly mixed with Freund's complete adjuvant. The solution was injected subcutaneously at multiple sites in New Zealand rabbits. The injections were repeated after 2 and 4 weeks using 1 mg peptide only. A blood sample was taken 1 week after the final injection. After allowing the blood to clot at room temperature, the serum was collected by centrifugation and stored at –20 °C until further use. To purify antibodies, the immunizing peptide was coupled to CNBr-activated Sepharose 4B according to the supplier's instructions. The serum was passed through the affinity column and washed with PBS (pH 7.5). Antibodies were eluted by washing the column with 0.1 M glycine (pH 3.0) and collected fractions (1 ml) were immediately neutralized by the addition of 1 M Tris and stored at –20 °C until use.

Western-blotting assays. AdpA was immunodetected in *S. clavuligerus* cell-free protein extracts as follows. Mycelium from a 36 h culture was washed with and resuspended in lysis buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.5). After disruption by sonication, cell debris was removed by centrifugation at 4 °C and 14 000 r.p.m. for 30 min. Samples (5 µg protein) were electrophoretically separated by 12% SDS-PAGE and blotted to a PVDF membrane (Immobilon-P, Millipore) for immunodetection using an alkaline phosphatase-conjugated anti-rabbit secondary antibody.

PCR, RT-PCR analysis and real-time RT-PCR. Total DNA of *S. clavuligerus* was used to amplify: (i) the complete *adpA* ORF (1206 bp) using oligonucleotides *adpA*-O1/*adpA*-O2; (ii) *adpA* downstream (2267 bp) and upstream (1983 bp) regions using DWadpA-O1/DWadpA-O2 and UpadpA-O1/UpadpA-O2, respectively; (iii) a fragment containing the *adpA* promoter region and ORF using the oligonucleotides PadpA-O1/*adpA*-O2. Every PCR (20 µl) was performed as described by Kieser *et al.* (2000) and contained 300 ng DNA template, 0.5 mM each oligonucleotide, 28 mM each dGTP and dCTP, 12 mM each dATP and dTTP, 1 mM MgCl₂, 5% DMSO and 0.8 U Platinum Pfx DNA Polymerase (Invitrogen). With small variations of annealing temperature, the PCR program was as follows: after the first step at 95 °C for 30 s, the annealing temperature was reduced in a touch-down of 1 °C from 65 to 58 °C in one cycle, and an annealing temperature of 58 °C was used in the next 25 cycles with an extension step of 2 min at 72 °C. The PCR products were confirmed for size and purity by agarose gel electrophoresis, isolated from the gel using the Qiagen II DNA Cleanup System (Qiagen) and sequenced.

Table 2. Oligonucleotide pairs used in this study

Oligonucleotide	Sequence (5'→3')	Description
<i>adpA</i> -O1	GGATCCATGAGTCAGGACTCCGC	Forward for <i>adpA</i> cloning
<i>adpA</i> -O2	TTATGGCGCGCTCCGCTG	Reverse for <i>adpA</i> cloning
DW <i>adpA</i> -O1	GTGCTCGGCGAAGGGGTGGACA	Forward for <i>adpA</i> downstream region
DW <i>adpA</i> -O2	CGGCAGTGCTCCGCTCCAGTG	Reverse for <i>adpA</i> downstream region
Up <i>adpA</i> -O1	GTACTCCCGGCCGACTTCTCT	Forward for <i>adpA</i> upstream region
Up <i>adpA</i> -O2	ATGCCCCGCTATGTCTCCA	Reverse for <i>adpA</i> upstream region
<i>adpA</i> -O3	GGCGGCCCCATTTTTGAGAGTTC	Forward for <i>adpA</i> RT-PCR
<i>adpA</i> -O4	CGTGCCGCGCCGAGGTGAGC	Reverse for <i>adpA</i> RT-PCR
<i>adpA-ornA</i> -O1	GGCCCTCGTCCGTCCTCCTG	Forward for intergenic region <i>adpA-ornA</i> RT-PCR
<i>adpA-ornA</i> -O2	AGCCTTCCCCGGTTCCCTCACAT	Reverse for intergenic region <i>adpA-ornA</i> RT-PCR
<i>bls2</i> -O1	GAGATCTACAACCGGGACGA	Forward for <i>bls2</i> RT-PCR and quantitative RT-PCR
<i>bls2</i> -O2	AGGTCATAGCGTTCCAGCAG	Reverse for <i>bls2</i> RT-PCR and quantitative RT-PCR
<i>brp</i> -O1	GGCGCTCTACTTCCACTTCCGT	Forward for <i>brp</i> RT-PCR
<i>brp</i> -O2	CCAGCGCGGGCATCAGA	Reverse for <i>brp</i> RT-PCR
<i>brp</i> -O3	AGGGGGCGCTCTACTTCCACTTC	Forward for <i>brp</i> quantitative RT-PCR
<i>brp</i> -O4	TCGCCTCATCGATCGCCTCCT	Reverse for <i>brp</i> quantitative RT-PCR
<i>car</i> -O1	GCCGGGGCGAAGGTCCAT	Forward for <i>car</i> RT-PCR
<i>car</i> -O2	ATCCGCTGCTCGTACATCTCCTT	Reverse for <i>car</i> RT-PCR and quantitative RT-PCR
<i>car</i> -O3	GGTGTCGATCATCCGGGTCCAGT	Forward for <i>car</i> quantitative RT-PCR
<i>ccaR</i> -O1	CCGGGCCAGGTCATCTCC	Forward for <i>ccaR</i> RT-PCR
<i>ccaR</i> -O2	CCGCGTAGTAGGCCTTCATCAG	Reverse for <i>ccaR</i> RT-PCR
<i>ccaR</i> -O3	TCGCGGACTCCATCGACCTCTT	Forward for <i>ccaR</i> quantitative RT-PCR
<i>ccaR</i> -O4	GGCGGGCCCTTCCACAG	Reverse for <i>ccaR</i> quantitative RT-PCR
<i>ceaS2</i> -O1	TGGGGAAGGTGTTTGGGGTTGT	Forward for <i>ceaS2</i> RT-PCR
<i>ceaS2</i> -O2	GGTTTCGCGGGGTGTTTCG	Reverse for <i>ceaS2</i> RT-PCR
<i>ceaS2</i> -O3	GCCGAGCGCCTGAACATCC	Forward for <i>ceaS2</i> quantitative RT-PCR
<i>ceaS2</i> -O4	GCGGTCCACCGGGGCAACAT	Reverse for <i>ceaS2</i> quantitative RT-PCR
<i>claR</i> -O1	GCCGGGCGGCGGTTCTTC	Forward for <i>claR</i> RT-PCR
<i>claR</i> -O2	GCCCCGCCAGTGGAAGACAC	Reverse for <i>claR</i> RT-PCR
<i>claR</i> -O3	CGGGCGGCGGTTCTT	Forward for <i>claR</i> quantitative RT-PCR
<i>claR</i> -O4	TCGTCGAGCAGGGGTTCC	Reverse for <i>claR</i> quantitative RT-PCR
<i>cas2</i> -O1	CGCAAGCGGCTGGTGATGGAG	Forward for <i>cas2</i> RT-PCR
<i>cas2</i> -O2	GGTCGTTTCGCTCCCCGTAGAGC	Reverse for <i>cas2</i> RT-PCR
<i>cas2</i> -O3	GCAAGCGGCTGGTGATGG	Forward for <i>cas2</i> quantitative RT-PCR
<i>cas2</i> -O4	GGTCTCCGAGGACAGGTAGTGC	Reverse for <i>cas2</i> quantitative RT-PCR
<i>cyp-fd</i> -O1	GCTGTGCGGCGGGCAACC	Forward for <i>cyp</i> and <i>fd</i> RT-PCR
<i>cyp-fd</i> -O1	CGGGCACAGCTCGGCACAG	Reverse for <i>cyp</i> and <i>fd</i> RT-PCR
<i>cyp</i> -O3	ACGAACTCGACGGCTATCTG	Forward for <i>cyp</i> quantitative RT-PCR
<i>cyp</i> -O4	ACATCGGGACCATCTCCTC	Reverse for <i>cyp</i> quantitative RT-PCR
<i>gcaS</i> -O1	GCCGGCCGCTTCTCTATG	Forward for <i>gcaS</i> RT-PCR
<i>gcaS</i> -O2	GCAGCCGGTCCTTCTCGTTC	Reverse for <i>gcaS</i> RT-PCR
<i>gcaS</i> -O3	GGTCAACTGGAGCCTGTGTA	Forward for <i>gcaS</i> quantitative RT-PCR
<i>gcaS</i> -O4	CCGCGAACTTGGCATAGTC	Reverse for <i>gcaS</i> quantitative RT-PCR
<i>hrdB</i> -O1	CGCGGCATGCTCTTCT	Forward for <i>hrdB</i> quantitative RT-PCR
<i>hrdB</i> -O2	AGGTGGCGTACGTGGAGAAC	Reverse for <i>hrdB</i> quantitative RT-PCR
<i>oat2</i> -O1	GACGCCCCGGGGATTCTGTGT	Forward for <i>oat2</i> RT-PCR
<i>oat2</i> -O2	TCGCCCCGCCGACGCTGA	Reverse for <i>oat2</i> RT-PCR
<i>oat2</i> -O3	CACCGTCTCTCGCCTCCAC	Forward for <i>oat2</i> quantitative RT-PCR
<i>oat2</i> -O4	CGTTCTCTCTCGCCCTCCAG	Reverse for <i>oat2</i> quantitative RT-PCR
<i>oppA1</i> -O1	CGGGGTACGGGGAGTGG	Forward for <i>oppA1</i> RT-PCR and quantitative RT-PCR
<i>oppA1</i> -O2	CGGAGGAAGTTCCAGGTGTA	Reverse for <i>oppA1</i> RT-PCR and quantitative RT-PCR
<i>oppA2</i> -O1	CCCACGGGTTGCGGAAGT	Forward for <i>oppA2</i> RT-PCR
<i>oppA2</i> -O2	CACCCAGCGGGGCAAGTT	Reverse for <i>oppA2</i> RT-PCR
<i>oppA2</i> -O3	GCAAGCGGCTGGTGATGG	Forward for <i>oppA2</i> quantitative RT-PCR
<i>oppA2</i> -O4	GCAGTACGCGGCGGACAAGAT	Reverse for <i>oppA2</i> quantitative RT-PCR
<i>orf12</i> -O1	GGCGATGGGGCTGCTGAC	Forward for <i>orf12</i> RT-PCR and quantitative RT-PCR

Table 2. cont.

Oligonucleotide	Sequence (5'→3')	Description
<i>orf12</i> -O1	GTGCGCGACGGGTGGTA	Reverse for <i>orf12</i> RT-PCR and quantitative RT-PCR
<i>orf13</i> -O1	CTGCGCTGGCTGCTGGTGTA	Forward for <i>orf13</i> RT-PCR and quantitative RT-PCR
<i>orf13</i> -O1	CTGCCGCCGGGAGATGC	Reverse for <i>orf13</i> RT-PCR and quantitative RT-PCR
<i>orf14</i> -O1	CGAACGACGACGAAACG	Forward for <i>orf14</i> RT-PCR and quantitative RT-PCR
<i>orf14</i> -O2	CGAGCGAGCCGACCATGT	Reverse for <i>orf14</i> RT-PCR and quantitative RT-PCR
<i>ornA</i> -O1	GATCGACTGGAGATGACC	Forward for <i>ornA</i> RT-PCR and quantitative RT-PCR
<i>ornA</i> -O2	CACGATGTCCACCCCTTC	Reverse for <i>ornA</i> RT-PCR and quantitative RT-PCR
<i>pah2</i> -O1	TCGACGCCGGGGACATCAAT	Forward for <i>pah2</i> RT-PCR
<i>pah2</i> -O2	CCGCTGGCCGACCTTCTC	Reverse for <i>pah2</i> RT-PCR
<i>pah2</i> -O1	CCTACGACGGGGCACCAG	Forward for <i>pah2</i> quantitative RT-PCR
<i>pah2</i> -O2	TCATGTGCAACGGCGTCAGATTG	Reverse for <i>pah2</i> quantitative RT-PCR
<i>PadpA</i> -O1	CCCATTGCGACGCTCGCAC	Forward for <i>adpA</i> and promoter region cloning

Gene expression analysis by RT-PCR and real-time RT-PCR was performed as previously described by Santamarta *et al.* (2007). Negative controls to confirm the absence of contaminating DNA on RT-PCR amplification were carried out with each set of primers and Platinum *Taq* DNA polymerase (Invitrogen). When real-time RT-PCR was performed, controls were included using RNA to preclude the amplification of chromosomal DNA. Relative quantification of gene expression was performed by the $2^{-\Delta\Delta C_t}$ method.

cDNAs for real-time RT-PCR analysis were synthesized using SuperScript III reverse transcriptase (Invitrogen). In total, 1 µg RNA was annealed at 70 °C for 5 min with 250 pmol random primers (Invitrogen) and 1 µl 10 mM dNTPs in a final volume of 14 µl. The mix was then supplemented with 4 µl 5× First-Strand buffer, 1 µl 0.1 M DTT and 1 µl SuperScript III reverse transcriptase, and kept at 25 °C for 5 min and 55 °C for 1 h. The retrotranscription reaction was stopped by heating at 70 °C for 15 min. Real-time RT-PCRs were carried out on a StepOnePlus thermocycler (Applied Biosystems). Reactions contained 2 µl cDNA reaction mixture diluted 1:3, 10 µl SYBR Green PCR Master Mix (Applied Biosystems) and 300 nM specific primers in a volume of 20 µl, and were performed in triplicate. The *hrdB*-like gene, encoding the major sigma factor in *S. coelicolor* A3(2) (Aigle *et al.*, 2000; Buttner *et al.*, 1990), was used as an

internal control to quantify the relative expression of the target genes. PCR conditions were as follows: 2 min at 50 °C, 10 min at 90 °C, 30 cycles of 15 s at 95 °C, and 1 min at 60–64 °C, depending on the primer pair. Specific product amplification was checked by the melting curve and agarose gel electrophoresis. In parallel, control PCRs were performed using RNA as template to preclude amplification of chromosomal DNA. Two biological replicates were employed for each strain; the efficiencies of the primers were measured by serial dilutions of genomic DNA as template.

RESULTS

Organization of the *S. clavuligerus* *adpA*-containing DNA region

A 5 kb DNA sequence containing the *adpA* gene was provided by DSM (Delft, The Netherlands). The DNA sequence and ORFs present in the fragment (Fig. 1a) totally coincide with those later published by the Broad Institute and will be named with the published nomenclature.

Fig. 1. Organization of the *adpA*-carrying region in *S. clavuligerus*. (a) Gene organization of a 5 kb DNA fragment of *S. clavuligerus* carrying the *adpA* gene. (b) Organization of the same region in *S. clavuligerus* $\Delta adpA$ mutants. (c) Pattern of hybridization of *Accl*-, *Sall*- and *SmaI*-digested DNA from *S. clavuligerus* 27064 (1), *S. clavuligerus* $\Delta adpA1$ (2) and *S. clavuligerus* $\Delta adpA2$ (3).

S. clavuligerus adpA encodes a 399 amino acid protein with 84–88 % identity to those of *S. griseus*, *Streptomyces avermitilis* and *S. coelicolor*. Like other AdpA orthologous proteins, it possesses two helix–turn–helix motifs (amino acids 238–280 and 286–330), characteristic of the AraC/XylS family of proteins, a domain pfpI/DJ-1 (amino acids 55–193) for dimerization, and a UUA codon-translated leucine residue (Leu²²³).

Upstream, divergent and separated from *adpA* by a 1.7 kb non-coding region is SSCG_05478, which encodes a protein of the universal stress family; 83 nt downstream of SSCG_05478 and in the opposite orientation is located a gene encoding a glutamine–D-fructose-6-phosphate amidotransferase. Downstream of *adpA* and separated by 10 nt is SSCG_05473, encoding an oligoRNase with 88 % identity to OrnA from *S. coelicolor* and *S. griseus*. Next to it, SSCG_100047 encodes a tRNA^{His}, followed 823 nt downstream and in the opposite orientation by an incomplete ORF (SSCG_05472) for a histidine kinase (Fig. 1a). Thus, there is a considerable synteny between this *S. clavuligerus* DNA region and the homologous ones in *S. coelicolor* and *S. griseus*.

To understand the effect of AdpA on morphological differentiation and antibiotic production in *S. clavuligerus*, we proceeded to disrupt the *adpA* gene by using plasmid p Δ *adpA*. The plasmid was constructed to delete 151 bp of the promoter region and 812 bp of the 5' end of *adpA* (Fig. 1b). Plasmid p Δ *adpA* was transferred to *S. clavuligerus* by conjugation and two of 28 recombinant colonies were apramycin-resistant and kanamycin-sensitive. These exconjugants were analysed by Southern hybridization using a 1.2 kb DNA probe containing the whole *adpA* gene. The hybridization pattern obtained (Fig. 1c) is consistent with the deletion of the expected 963 bp region in both exconjugants, which were named *S. clavuligerus* Δ *adpA*1 and Δ *adpA*2.

Morphological differentiation in *S. clavuligerus* Δ *adpA* mutants depends on culture media

Growth, aerial mycelium formation and sporulation of *S. clavuligerus* ATCC 27064 and the two Δ *adpA* mutants were studied. In TBO medium, *S. clavuligerus* ATCC 27064 produced aerial mycelium after 3–4 days of growth, and the characteristic grey-green colour of the spores was observed after 7 days. Only a sparse aerial mycelium was developed by the mutants after 10 days and no spores were formed even after longer incubation times (Fig. 2c). However, in ME medium, an excellent sporulation medium for *S. clavuligerus* ATCC 27064, the Δ *adpA* mutants were able to form aerial mycelium and to sporulate. Since the genetic characterization and morphological behaviour of both exconjugants were identical (data not shown), all the work was performed with exconjugant Δ *adpA*1, named *S. clavuligerus* Δ *adpA*.

Expression of *ornA* in *S. clavuligerus*

The *ornA* gene, located downstream of and in the same orientation as *adpA*, is not essential in *S. griseus* and *S.*

Fig. 2. Characterization of *S. clavuligerus* Δ *adpA*. (a) Amplification of the *S. clavuligerus* ATCC 27064 intergenic *adpA*–*ornA* region using oligonucleotides *adpA*–*ornA*-O1 and *adpA*–*ornA*-O2. Lanes: 1, positive PCR control with DNA as template; 2, RT-PCR amplification of the intergenic region; 3, RT-PCR negative control reaction lacking retrotranscriptase. (b) RT-PCR amplification of *ornA* using oligonucleotides *ornA*-O1 and *ornA*-O2 with *S. clavuligerus* ATCC 27064 (lane 4) and *S. clavuligerus* Δ *adpA* (lane 5). M, molecular mass standard. In the scheme below is shown the location of oligonucleotides *adpA*–*ornA*-O1 and *adpA*–*ornA*-O2 (marked 'a' and 'b') and *ornA*-O1 and *ornA*-O2 (marked 'c' and 'd'). (c) Growth, aerial mycelium formation and sporulation in TBO medium of *S. clavuligerus* ATCC 27064 (1), *S. clavuligerus* [pMS83] (2), *S. clavuligerus* Δ *adpA* (3) and *S. clavuligerus* Δ *adpA* [pCPA2] (4).

coelicolor, although its deletion partially affects growth and aerial mycelium formation (Ohnishi *et al.*, 2000; Sello & Buttner, 2008). The small (10 bp) *adpA*–*ornA* intergenic region present in *S. clavuligerus* suggests that both genes are transcriptionally coupled in this strain. To assess whether this was the case, oligonucleotides *adpA*–*ornA*-O1 and *adpA*–*ornA*-O2 were designed to amplify by RT-PCR a 407 bp fragment corresponding to the intergenic region. In addition, oligonucleotides *ornA*-O1 and *ornA*-O2 were used to detect *ornA* expression in *S. clavuligerus* Δ *adpA*, in which the promoter and 5' end of *adpA* are deleted. RNA samples were isolated from 24 h (TSB medium) and 40 h (SA medium) cultures. The amplification fragment obtained (Fig. 2a, lanes 1 and 2) with oligonucleotides *adpA*–*ornA*-O1 and *adpA*–*ornA*-O2 confirmed that the two genes are transcriptionally coupled in the wild-type strain. In addition, an amplified fragment corresponding to an *ornA* transcript was detected in the wild-type and the Δ *adpA* mutant using oligonucleotides *ornA*-O1 and *ornA*-O2 (Fig. 2b, lanes 4 and 5); this result confirms the presence of an additional monocistronic *ornA* mRNA. Real-time RT-PCR, using *ornA*-O1 and *ornA*-O2, was performed to quantify the *ornA* expression level in the wild-type strain and the Δ *adpA* mutant. The relative expression value obtained for *ornA* in *S. clavuligerus* Δ *adpA* SA cultures grown for 40 h was 0.148, which indicates a decrease in expression of 6.7-fold in the mutant compared with the wild-type strain (relative expression value of 1).

This decrease in *ornA* expression can be explained through the loss of *ornA* transcripts initiated from the *adpA* promoter.

Brp specifically binds the *adpA* promoter region

Brp is a butyrolactone receptor protein, homologous to *S. griseus* ArpA, and acts as a negative modulator of antibiotic biosynthesis in *S. clavuligerus*. It recognizes and binds ARE boxes present in the *ccaR* and *brp* promoter regions (Santamarta *et al.*, 2005). Bioinformatic analysis and comparison of the *adpA* promoter region with the ARE_{ccaR} and ARE_{brp} boxes indicated the presence 149 bp upstream of the *adpA* start codon of a putative ARE sequence, 5'-TCTCATGGAGACATAGCGGGGCATGC-3'. This sequence possesses stretches of identity with *S. clavuligerus* ARE_{ccaR} and ARE_{brp} and with the ARE boxes of regulatory gene promoters of other *Streptomyces* species, including the ArpA-binding sequence in the *S. griseus* *adpA* promoter (Fig. 3a).

To determine Brp binding to the *adpA* promoter region, the electrophoretic mobility of a 448 bp ARE_{adpA}-containing fragment in the presence of *S. clavuligerus* r-Brp was tested by EMSAs. In parallel, the binding to the ARE_{brp} and

Fig. 3. An ARE box is present upstream of *adpA*. (a) Location of the ARE box upstream of the *adpA* gene in *S. clavuligerus*. Sequence of the ARE_{adpA} box and comparison with ARE_{ccaR} and ARE_{brp} boxes of *S. clavuligerus*, as well as with ARE sequences present upstream of *S. griseus* *adpA*, *Streptomyces pristinaespiralis* *papR* and *Streptomyces virginiae* *barA*. (b) Gel shift electrophoresis of a 448 bp DNA fragment carrying the ARE_{adpA} box using pure recombinant *S. clavuligerus* r-Brp protein (0.5 µg). Lanes: 1, free probe; 2 and 3, 0.5–4 µg r-Brp; 4 and 5, sequence-specificity assay using one- and 10-fold amounts of unlabelled probe; 6, sequence-specificity assay using 10-fold amounts of a heterologous unlabelled 445 bp PvuII DNA fragment isolated from pBSKSII.

ARE_{ccaR} boxes was tested. A clear mobility shift of the ARE_{adpA}-containing probe was observed using increasing amounts of r-Brp protein, as shown in Fig. 3(b), lanes 2 and 3. The binding specificity of Brp for this sequence was tested through direct-competition reactions by increasing the amounts of competitor probe, which resulted in a progressively reduced signal of the shifted labelled ARE_{adpA}-containing probe (Fig. 3b, lanes 4 and 5), and by using a heterologous competitor probe that did not disturb the specific binding (Fig. 3b, lane 6). Therefore, the ARE_{adpA} sequence is functional and specifically binds Brp.

Real-time RT-PCR quantification of *adpA* expression was performed in the wild-type strain and the *S. clavuligerus* Δ *brp* mutant. A consistent slight increase of 2.65-fold in *adpA* expression was observed in *S. clavuligerus* Δ *brp* cultures grown for 24 h in TSB medium. This suggests that Brp acts as a negative modulator of *adpA* expression in *S. clavuligerus*, as occurs in *S. griseus*.

The translation of *adpA* is regulated by *bldA*

The TTA codon-containing *adpA* gene of *S. coelicolor* is not translated in the *S. coelicolor* Δ *bldA* mutant (Nguyen *et al.*, 2003; Takano *et al.*, 2003). To test whether the same occurs with the TTA codon located in *S. clavuligerus* *adpA*, the presence of the AdpA protein was analysed in *S. clavuligerus* ATCC 27064, *S. clavuligerus* Δ *adpA* and *S. clavuligerus* Δ *bldA* cell extracts through immunodetection assays using anti-AdpA antibodies. Repeatedly, an AdpA immunodetection signal was observed in 36 h TSB cell-free extracts of *S. clavuligerus* ATCC 27064 (Fig. 4a, lane 1), while this band was not present in *S. clavuligerus* Δ *adpA* or in *S. clavuligerus* Δ *bldA* cell extracts (Fig. 4a, lanes 2 and 3).

Fig. 4. Immunodetection of AdpA in *S. clavuligerus* ATCC 27064 and derived mutants. (a) Western blotting with anti-AdpA antibodies of cell extracts (5 µg protein each) of *S. clavuligerus* ATCC 27064 (1), *S. clavuligerus* Δ *adpA* (2) and *S. clavuligerus* Δ *bldA* (3). (b) RT-PCR amplification of *adpA* (left) and PCR to confirm the absence of contaminating DNA (right). Oligonucleotides *adpA*-O3 and *adpA*-O4 were used on mRNA from 24 h TSB cultures of *S. clavuligerus* ATCC 27064 (1) or *S. clavuligerus* Δ *bldA* (2).

To confirm that this lack of AdpA protein in the $\Delta bldA$ mutant was not due to lack of expression, amplification analysis of *adpA* was performed by RT-PCR. RNA from 24 h TSB cultures of *S. clavuligerus* ATCC 27064 and the $\Delta bldA$ mutant was retrotranscribed using primers *adpA*-O3 and *adpA*-O4. The amplification of a DNA fragment corresponding to the *adpA* transcript in *S. clavuligerus* $\Delta bldA$ (Fig. 4b, lanes 1 and 2), confirmed that the absence of AdpA is due to lack of mRNA translation.

Clavulanic acid production is especially affected in *S. clavuligerus* $\Delta adpA$

Growth and antibiotic production by *S. clavuligerus* ATCC 27064 and *S. clavuligerus* $\Delta adpA$ were analysed in defined SA (Fig. 5, upper panels) and in complex TSB media (Fig. 5, lower panels). Inactivation of *adpA* did not have a significant effect on growth in either medium (Fig. 5, left panels). Clavulanic acid production was strongly reduced in *S. clavuligerus* $\Delta adpA$ to 14% of the wild-type level in TSB medium at 36 h and to 5% at 60 h in SA medium. Production of cephamycin C by the $\Delta adpA$ mutant was almost at wild-type level in complex TSB medium but decreased in SA medium after 36 h of growth (Fig. 5, right panels). Both cephamycin C and clavulanic acid were restored to control levels in *S. clavuligerus* $\Delta adpA$ [pCPA2] (Fig. 6a), which carries the *adpA* gene in the integrative plasmid pMS83. Introduction of pCPA2 in the *S. clavuligerus* $\Delta adpA$ mutant also restored aerial mycelium formation and sporulation in TBO medium (Fig. 2c).

Multiple copies of *adpA* increase antibiotic production levels in *S. clavuligerus*

To determine whether antibiotic production was affected by increasing the *adpA* gene dosage, a DNA fragment containing *adpA* with its own promoter was subcloned into the multi-copy plasmid pIJ699, giving plasmid pIJ*adpA* (Table 1). Growth, cephamycin C and clavulanic acid production of the transformant *S. clavuligerus* [pIJ*adpA*] and its control, *S. clavuligerus* [pIJ699], were analysed in SA-grown cultures. The growth of both transformants was reduced when compared with *S. clavuligerus* ATCC 27064, probably due to the antibiotic added for plasmid selection. However, production of cephamycin C and clavulanic acid was clearly enhanced in the strain carrying multiple copies of *adpA* (Fig. 6b). After 60 h of culture, clavulanic acid production was of the order of 204 and 218 %, respectively, compared with the control *S. clavuligerus* pIJ699.

Transcriptional analysis of genes involved in clavulanic acid biosynthesis in an *S. clavuligerus* $\Delta adpA$ mutant

Deletion of *adpA* in *S. clavuligerus* strongly affects clavulanic acid production. To assess whether the pathway-specific regulators of clavulanic acid biosynthesis are under AdpA control, *ccaR* and *claR* transcription was analysed by RT-PCR in *S. clavuligerus* ATCC 27064 and in *S. clavuligerus* $\Delta adpA$ (data not shown). Transcriptional studies were performed using as template RNA samples isolated after growth in SA medium for 40 h, at which point the most drastic decrease in clavulanic acid

Fig. 5. Cephamycin and clavulanic acid production by *S. clavuligerus* $\Delta adpA$. Growth (left panels), and production of clavulanic acid (centre panels) and cephamycin C (right panels) by *S. clavuligerus* ATCC 27064 (open circles) and *S. clavuligerus* $\Delta adpA$ (closed circles) grown in SA (upper panels) and TSB (lower panels) media.

Fig. 6. Complementation of the $\Delta adpA$ mutant and effect of additional copies of *adpA* in the control strain. (a) Complementation of *S. clavuligerus* $\Delta adpA$ by the *adpA*-carrying pPCA2 integrative plasmid. Growth, and cephamycin C and clavulanic acid production in SA medium of *S. clavuligerus* 27064 (open squares), *S. clavuligerus* $\Delta adpA$ (closed squares), *S. clavuligerus* [pMS83] (open circles) and *S. clavuligerus* $\Delta adpA$ [pCPA2] (closed circles). (b) Effect of multiple copies of *adpA* on growth, and cephamycin C and clavulanic acid production of *S. clavuligerus* ATCC 27064 (open squares), *S. clavuligerus* [pIJ699] (open circles) and *S. clavuligerus* [pIJadpA] (closed circles) grown in SA medium.

production by the $\Delta adpA$ mutant was observed. No significant differences in amplification of *ccaR* or *claR* transcripts were observed between the analysed strains (data not shown); therefore, the transcriptional analysis was extended to the whole clavulanic acid cluster. The results indicate that all the genes under study are expressed in the mutant strain, in which clavulanic acid production is not totally abolished. Only the amplification of *ceaS2*, *bls2*, *pah2*, *cas2*, *claR*, *car* and *oppA2* transcripts decreased slightly in *S. clavuligerus* $\Delta adpA$ compared with the wild-type strain. To confirm these differences, a relative quantification by real-time RT-PCR was performed. The expression levels obtained for the different genes in *S. clavuligerus* *adpA* in relation to those of the wild-type strain (assigned a relative value of 1) are shown in Fig. 7. Transcription levels of 0.14 and 0.24 were found for *ccaR* and *claR*, encoding positive regulators for clavulanic acid biosynthesis (seven- and fourfold less expression than in the wild-type strain, respectively), while expression of *brp* was barely affected, with a relative value of 0.67 (not shown).

All biosynthetic genes analysed appeared to be down-regulated in the $\Delta adpA$ strain. The most dramatic decreases

in expression levels were observed in the early biosynthetic genes (*ceaS2*, *bls2*, *pah2* and *cas2*), with relative values ranging from 0.0098 (*cas2*) to 0.052 (*pah2*). This group of four genes are co-transcribed from the *ceaS2* promoter, although *cas2* has been described as also expressed in a monocistronic transcript (Paradkar & Jensen, 1995). This means a strong decrease in expression of the early genes, of the order of 50-fold lower for *ceaS2* and *bls2*.

The expression level of genes encoding late steps of the pathway (*car*, *gcaS2*) in *S. clavuligerus* $\Delta adpA$ was variable, since the relative value for *car* was 0.113 and that for *gcaS2* was 0.379 (i.e. about eight- and threefold less than the wild-type strain, respectively). Other essential genes of unknown function in clavulanic acid biosynthesis, such as *cyp-fd*, *orf12* and *orf13*, showed relative values (0.132, 0.130 and 0.153, respectively) similar to those of *car*. Expression of the oligopeptide permease-encoding gene *oppA2* (0.080) was strongly affected, while expression of *oppA1*, *oat2* and especially *orf14* was less affected (0.215, 0.356 and 0.569, respectively, i.e. about 4.6-, 3- and 1.7-fold lower). These results allow us to explain the decrease in clavulanic acid production observed in the $\Delta adpA$ mutant strain and

Fig. 7. Expression of clavulanic acid biosynthesis genes. The organization of the *S. clavuligerus* ATCC 27064 clavulanic acid biosynthesis gene cluster is shown above. Transcriptional units are indicated with arrows. The quantitative RT-PCR of the different genes using the oligonucleotides indicated in Table 2 is shown below. The relative values are referred to 1, the assigned relative value for the expression of each gene in *S. clavuligerus* ATCC 27064. Error bars were calculated by measuring the standard deviation among biological replicates of each sample. The mRNA templates were from 40 h cultures grown in SA medium.

suggests that AdpA acts as a positive regulatory modulator of clavulanic acid gene expression.

DISCUSSION

Depending on the culture medium, the AdpA-negative mutants of *S. clavuligerus* are blocked in sporulation and show sparse aerial mycelium formation. A similar medium-dependent, sporulation-negative phenotype has been described in *S. coelicolor adpA* mutants; however, in other *Streptomyces* species, *adpA* mutants display a fully bald phenotype (Nguyen *et al.*, 2003; Ohnishi *et al.*, 1999; Pan *et al.*, 2009; Takano *et al.*, 2003). Cephamycin C and especially clavulanic acid formation is impaired in *S. clavuligerus* $\Delta adpA$. Expression of *ornA*, encoding an oligoRNase involved in morphological differentiation (Ohnishi *et al.*, 2000; Sello & Buttner, 2008), is lower in *S. clavuligerus* $\Delta adpA$. However, the lack of sporulation observed in *S. clavuligerus* $\Delta adpA$ is not due to the low transcription of *ornA*, since the strain complemented *in trans*, *S. clavuligerus* $\Delta adpA$ (pCPA2), which still has a low expression of *ornA*, sporulates normally and produces wild-type levels of cephamycin C and clavulanic acid.

Since all *adpA* genes described so far contain a TTA codon, which is not translated in mutants blocked in the *bldA*

gene, it has been postulated that all the morphological differences observed in *S. coelicolor bldA* mutants are due to lack of *adpA* translation (Nguyen *et al.*, 2003; Takano *et al.*, 2003). *S. clavuligerus* $\Delta bldA$ displays a bald phenotype but produces both cephamycin and clavulanic acid; therefore, the *S. clavuligerus* $\Delta adpA$ and *S. clavuligerus* $\Delta bldA$ mutants exhibit different phenotypes in relation to aerial mycelium formation and antibiotic production. This might be due to the expression or lack of expression of other still-uncharacterized genes; furthermore, *S. clavuligerus* $\Delta bldA$ correctly translates the TTA codon-containing *ccaR* gene, for the clavulanic acid/cephamycin C-specific regulatory protein CcaR (Trepanier *et al.*, 2002; Santamarta, 2002).

The different behaviour of the *S. clavuligerus bldA* mutant with respect to *adpA* and *ccaR* translation might be due to the differences in the TTA 5' flanking nucleotides (Trepanier *et al.*, 2002). After comparison of all *Streptomyces bldA*-dependent TTA codons it has been suggested that TTAY sequences (where Y is C or T) are susceptible to be *bldA*-dependent as is the case for the TTA codon in *adpA* (TTAC), while TTAR sequences (where R is G or A), such as in the TTA codon of *ccaR* (TTAG), are not *bldA*-dependent. As shown above, the absence of AdpA detection by immunoassays of *S. clavuligerus* $\Delta bldA$ supports the theory of Trepanier and co-workers.

The decrease of cephamycin C and, especially, of clavulanic acid production in *S. clavuligerus* $\Delta adpA$ and their overproduction in transformants carrying multiple copies of *adpA* suggest that AdpA is a positive modulator in the antibiotic regulatory cascade of *S. clavuligerus*. However, the observed effect is more drastic in relation to clavulanic acid production, probably reflecting differences in the regulatory cascades for the two antibiotics (Paradkar & Jensen, 1998). The transcription in the $\Delta adpA$ mutant of clavulanic acid biosynthesis regulatory genes *ccaR* and *claR* was about seven- and fourfold lower than in the wild-type strain, which explains the strong decrease in expression of genes *ceaS2*, *bls2*, *pah2* and *cas2* for the early steps of the clavulanic acid pathway as well as the moderate decrease of the late biosynthesis genes.

Direct AdpA binding to sequences in the pathway-specific regulatory genes that control antibiotic production has been demonstrated in *S. griseus*, *S. ansochromogenes* and *S. coelicolor* (Higashi *et al.*, 2007; Pan *et al.*, 2009; Park *et al.*, 2009; Tomono *et al.*, 2005a). The consensus sequence in *S. griseus* is 5'-TGGCSNGWWY-3', and two types of AdpA binding have been described. In type I, the binding site contains two consensus sequences, while in type II, AdpA binds a single consensus sequence. In the intergenic *cmcH-ccaR* region, between the ARE box and the *tsp* points described for *ccaR*, two possible sequences for AdpA binding are located: (i) 406 bp from the ATG start codon there is a single (type II) sequence, 5'-TGGCCGGATT-3'; and (ii) 565 nt upstream from the ATG there are two direct sequences, 3'-TGGCCCTTTT-14-TGGCCGCTGT-5'. In both cases, the sequences are located in the DNA strand complementary to *ccaR*. Whether these sequences are true sites for AdpA binding has not yet been confirmed, since the purification of *S. clavuligerus* recombinant AdpA has been hampered by the instability in *E. coli* of all the expression vectors carrying *adpA* that have been constructed.

The butyrolactone receptor Brp binds to the ARE box-containing probe, as shown by EMSA; in addition, it has been reported that a Brp-disrupted strain produces 1.5- to threefold more clavulanic acid and cephamycin C than the wild-type strain (Santamarta *et al.*, 2005). This work demonstrates a connection between the butyrolactone and AdpA regulation systems: Brp binds an ARE box present upstream of *adpA*, which leads to repression of *adpA* in the wild-type strain and to a 2.5-fold increase of *adpA* transcript in the *S. clavuligerus* Brp-disrupted mutant. The AdpA regulation pattern shown by *S. clavuligerus* resembles that described for *S. griseus* (Ohnishi *et al.*, 1999, 2005). Thus, *ccaR* expression is controlled directly by Brp and indirectly through the Brp-dependent AdpA regulator.

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