

Streptomyces clavuligerus *relA*-null mutants overproduce clavulanic acid and cephamycin C: negative regulation of secondary metabolism by (p)ppGpp

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The (p)ppGpp synthetase gene, *relA*, of *Streptomyces clavuligerus* was cloned, sequenced and shown to be located in a genomic region that is highly conserved in other *Streptomyces* species. *relA*-disrupted and *relA*-deleted mutants of *S. clavuligerus* were constructed, and both were unable to form aerial mycelium or to sporulate, but regained these abilities when complemented with wild-type *relA*. Neither ppGpp nor pppGpp was detected in the *S. clavuligerus relA*-deletion mutant. In contrast to another study, clavulanic acid and cephamycin C production increased markedly in the mutants compared to the wild-type strain; clavulanic acid production increased three- to fourfold, while that of cephamycin C increased about 2.5-fold. Complementation of the *relA*-null mutants with wild-type *relA* decreased antibiotic yields to approximately wild-type levels. Consistent with these observations, transcription of genes involved in clavulanic acid (*ceaS2*) or cephamycin C (*cefD*) production increased dramatically in the *relA*-deleted mutant when compared to the wild-type strain. These results are entirely consistent with the growth-associated production of both cephamycin C and clavulanic acid, and demonstrate, apparently for the first time, negative regulation of secondary metabolite biosynthesis by (p)ppGpp in a *Streptomyces* species of industrial interest.

Received 22 July 2007

Revised 16 November 2007

Accepted 29 November 2007

INTRODUCTION

The stringent response, first described in enterobacteria as an inhibition of stable RNA synthesis upon amino acid starvation, is mediated by the highly phosphorylated guanosine nucleotides ppGpp (Cashel & Kabalcher, 1970) and pppGpp (Haseltine *et al.*, 1972). In *Escherichia coli* and other enterobacteria the intracellular level of (p)ppGpp is controlled by RelA, a (p)ppGpp synthetase encoded by *relA*, and by the (p)ppGpp 3'-pyrophosphohydrolase activity encoded by *spoT* (Sy, 1977). RelA is ribosome-associated and is activated, presumably by conformational change, when uncharged tRNAs bind to the A site of the ribosome (Cashel *et al.*, 1996). Subsequent (p)ppGpp synthesis reduces the level of transcription of many genes that are required for rapid growth, while enhancing that of many others associated with stationary phase and other physiological stresses. Indeed, in *E. coli* ppGpp is now

firmly considered to be a global regulator of gene expression rather than simply a modulator of ribosome biosynthesis (Braeken *et al.*, 2006). Its mode of action has been studied extensively in *E. coli*, and involves reorienting gene transcription via binding to RNA polymerase (Magnusson *et al.*, 2005).

In actinomycetes, the accumulation of (p)ppGpp after amino acid starvation was first demonstrated in *Streptomyces hygroscopicus* (Riesenberg *et al.*, 1984). The isolation of thiopeptin-resistant mutants of several *Streptomyces* species, many of which were shown to be deficient in (p)ppGpp synthesis, subsequently revealed a general and positive correlation between (p)ppGpp synthesis, antibiotic production and morphological differentiation (Ochi, 1986, 1990). While in enterobacteria, *relA* and *spoT* encode two related proteins with different functions, actinomycetes and other Gram-positive bacteria possess a single bifunctional RelA/SpoT protein (Martínez-Costa *et al.*, 1996; Wendrich & Marahiel, 1997). In *Streptomyces coelicolor* A3(2), the *relA/spoT* gene (hereafter named *relA*; Chakraborty *et al.*, 1996) encodes a 94 200 Da protein

Abbreviation: tsp, transcriptional start point.

The GenBank/EMBL/DBJ accession number for the sequence of the 5.4 kb DNA fragment cloned in this work is AM408890.

which conferred (p)ppGpp hydrolysis activity on an *E. coli* *relA spoT* double mutant (Martínez-Costa *et al.*, 1998), thus behaving in a similar way to the bifunctional RelA/SpoT homologue of *Streptococcus equisimilis* (Mechold *et al.*, 1996). *relA*-null mutants of *S. coelicolor* are impaired in the stationary-phase production of two antibiotics, actinorhodin (Martínez-Costa *et al.*, 1996) and undecylprodigiosin, under conditions of nitrogen limitation (Chakraborty & Bibb, 1997).

Streptomyces clavuligerus is used for the production of the β -lactamase inhibitor clavulanic acid and consequently is of considerable industrial interest (Liras & Rodríguez-García, 2000); it also produces the β -lactam antibiotic cephamicin C (Liras, 1999). Prior to this study, and in contrast to other streptomycetes, the role of ppGpp and related highly phosphorylated guanosine nucleotides in the control of secondary metabolism in *S. clavuligerus* was unclear. Basarán *et al.* (1991) showed that a stringent response followed amino acid starvation in *S. clavuligerus* and resulted in increased ppGpp levels. While some mutants impaired in ppGpp synthesis produced higher levels of cephamicin C than the wild-type strain, suggesting that ppGpp is not essential for antibiotic biosynthesis in *S. clavuligerus*, other mutants produced reduced cephamicin C levels. The effect of these mutations on clavulanic acid biosynthesis was not studied. More recently we studied the effect of a well-characterized *rplK* (*relC*) mutation on clavulanic acid and cephamicin C production, finding that the mutation resulted in a reduction in (p)ppGpp synthesis and lower antibiotic production than in the parental strain (Gomez-Escribano *et al.*, 2006). While Jones *et al.* (1996, 1997) reported a burst of ppGpp synthesis prior to clavulanic acid production, they concluded that ppGpp was not required for transcription of the clavamate synthase (*cas*) gene involved in clavulanic acid biosynthesis. It was thus necessary to clarify the role of ppGpp in clavulanic acid biosynthesis using *relA*-null mutants unable to synthesize ppGpp. We therefore constructed two different *relA*-null mutants and show here that both surprisingly overproduce clavulanic acid and cephamicin C. This is in contrast to the findings of Jin *et al.* (2004), who reported that the production of both compounds required a functional *relA* gene.

METHODS

Strains and culture conditions. *E. coli* XL1-Blue and *E. coli* DH5 α were used for cloning. *E. coli* Ess22-35 and *Klebsiella pneumoniae* ATCC 29665 were used to assay for cephamicin C and clavulanic acid production, respectively (Liras & Martín, 2005). *S. clavuligerus* ATCC 27064 was used as the parental strain. *S. clavuligerus relC* (Gomez-Escribano *et al.*, 2006) was used for comparison purposes in S1 nuclease protection experiments. TSB complex medium or SA defined medium were used for studies of antibiotic production (Paradkar & Jensen, 1998; Lorenzana *et al.*, 2004). All *S. clavuligerus* cultures were carried out in triplicate in baffled flasks. Amino acid shift-down was carried out using defined MF medium (Basarán

et al., 1991). Growth of the cultures was determined by dry cell weight or by total DNA content, measured by the diphenylamine method (Burton, 1968). ME agar medium (Sánchez & Braña, 1996), as well as mannitol soya flour (SFM) medium (Kieser *et al.*, 2000), were used to assess morphological differentiation and sporulation.

DNA manipulations. Nucleic acid purification, DNA manipulation, *E. coli* and *Streptomyces* transformation and *E. coli*-*S. clavuligerus* conjugation were performed following standard methods (Sambrook *et al.*, 1989; Kieser *et al.*, 2000). Nucleic acid hybridizations were performed using the protocol given in the DIG-System kit (Roche) and colorimetric detection was achieved using nitro blue tetrazolium (NBT) and 5-bromo-1-chloro-3-indolyl phosphate (BCIP). PCR was performed using a Biometra TGradient Thermocycler and the conditions of Kieser *et al.* (2000). dTNP mixtures were prepared from individual nucleotides (Promega) using a ratio of 15A:15T:35G:35C to improve the amplification efficiency with high-G + C *Streptomyces* DNA. The oligonucleotides in Table 1 were used for subcloning, detection of the *relA*-null mutants and to obtain probes for S1 nuclease mapping. DNA sequencing was carried out using double-stranded DNA and the PCR method of Mullis & Faloona (1987). Nucleotide sequences were obtained on an ABI Prism Sequencer 310 (Perkin Elmer), and analysed using the following computer programs: Geneplot from DNASTAR, FASTA3 (EBI), CLUSTAL_X for multiple alignments (Higgins & Sharp, 1989) and the databases SWISS-PROtein, EMall (EMBL) and GenBank (USA). The DNA sequence of the 5.4 kb DNA fragment cloned in this work can be found in the EMBL database under accession number AM408890.

Disruption of *relA*. The 1.9 kb *relA* fragment was inserted into *EcoRI/XbaI*-digested pBSKS(+) to give pULGE211. A conjugation cassette carrying the *acc3(IV)* gene for apramycin resistance was isolated from plasmid pIJ733 (Gust *et al.*, 2003) and inserted into *EcoRI/HindIII*-digested pULGE211 to give pULGE212. Finally the *neo* gene, encoding kanamycin resistance, was isolated with *KpnI* from plasmid pTC192K (Rodríguez-García *et al.*, 2006) and inserted into the single *KpnI* site internal to *relA1.9* in pULGE212 to yield pULGE220. This plasmid was introduced into *S. clavuligerus* by conjugation and exconjugants were selected for kanamycin resistance and apramycin sensitivity. One, *S. clavuligerus relA::neo*, gave the pattern expected for a *relA*-disrupted mutant when hybridized with *neo* or *relA1.9* probes (Fig. 1, lanes 3 and 4).

Deletion of *relA*. Through a series of cloning steps carried out on the 16 kb *NotI*-*BamHI* fragment containing *relA*, a 2.6 kb *NcoI* fragment containing all of the *relA* coding region apart from 104 bp of N-terminal coding sequence (Fig. 2) was replaced by a neomycin/kanamycin-resistance cassette. The resulting plasmid, with *neo* in the same orientation as *relA*, was called pULGE240. The conjugation cassette with the apramycin-resistance marker from pIJ733 was inserted in the *NotI* site of pULGE240 to yield pULGE244. This plasmid was introduced into *S. clavuligerus* ATCC 27064 by conjugation and kanamycin-resistant exconjugants were screened for apramycin sensitivity, characteristic of double-crossover recombinants. DNA from kanamycin-resistant, apramycin-sensitive colonies was hybridized with *relA1.9* and *neo* probes. Lack of hybridization with the *relA* probe and hybridization with the *neo* probe (Fig. 1, lane 5), as well as the size of the hybridizing fragments, confirmed the construction of a *relA*-deleted mutant.

Construction of plasmids carrying the *relA* gene and the *relA*^{RI} truncated gene. The complete *relA* gene was amplified using oligonucleotides *relA*-O1 and *relA*-O2, and Pfx DNA polymerase, and confirmed by nucleotide sequencing. The conjugative, integrative plasmid pMS17 (Rodríguez-García *et al.*, 2005), which integrates

Table 1. Oligonucleotides used in this work

Name	Sequence	-mer	Use and comments
relA-O1	CCCTGCTCGAGCTCTGAACCAC	22	To clone <i>relA</i> from <i>S. clavuligerus</i> genomic DNA, including the whole <i>apt-relA</i> intergenic region; relA-O1 and relA-O2 contain <i>XhoI</i> and <i>NotI</i> sites respectively (bold)
relA-O2	CCGCTGCGGCCGCTAAGGGGCC	22	
relA-O3	GGGAGCTCTAGAGCGCCTCGACCTGGATGG	30	For cloning the 1.9 kb internal fragment of <i>relA</i> , <i>relA1.9</i> ; include restriction sites for <i>XbaI</i> (3) and <i>EcoRI</i> (4) for subsequent cloning (bold)
relA-O4	CGGCCGGAATTCCAAGAGCGGCGACCCGTA	30	
relA-O5	GTCGGATCCATGGCCAAGGAC	21	To clone a ribosome-independent (p)ppGpp synthetase gene; include restriction sites for <i>BamHI</i> (5) and <i>EcoRI</i> (6) for subsequent cloning (bold)
relA-O6	GTCGAATTCAGCTACTCCAGG	22	
claR-1	<u>TATTCGAATTCGGACGGCGCCCGGAGCGCGCCCGCCATCC</u>	40	For high-resolution S1 mapping of <i>claR</i> (Paradkar & Jensen, 1998); claR2 was 5'-end-labelled, the non-homologous tail is underlined
claR-2	CACGGTGCCGTGCCGGACACCCGTCCGCGGGGCCAC	36	
ceaS1	<u>TGGATCCGTCGCGAATCCAGGGAAGCCGAGC</u>	31	For high-resolution S1 mapping of the <i>ceaS2</i> transcript (Tahlan <i>et al.</i> , 2004); ceaS2 was 5'-end-labelled, the non-homologous tail is underlined
ceaS2	GGGCGGTGCATACACGGG	18	
bldGs1	<u>TCGATCGATCGACCGGGAGGTCGTCTGGGA</u>	30	For high-resolution S1 mapping of the <i>bldG</i> transcript (Bignell <i>et al.</i> , 2005); bldGs2 was 5'-end-labelled, bldGs1 has a 12 bp non-homologous tail
bldGs2	CCGACCTCGACGACCGTA	18	
ccaRs1	AGGTTTGCCGAGGATTTCCGG	21	For high-resolution S1 mapping of two putative <i>ccaR</i> transcripts (Wang <i>et al.</i> 2004); ccaRs2 was 5'-end-labelled
ccaRs2	ACATCATCCAGGTGTTTCATG	21	
cefDs1	CTCCTGTGGAGCGGGACAAAA	21	For high-resolution S1 mapping of the <i>cefD</i> transcript (Gomez-Escribano <i>et al.</i> 2006); cefDs2 was 5'-end-labelled.
cefDs2	TGTTGAGGTTGACGACGGTGG	21	
relAs1	<u>GCTAGCTAGCTACCTGATGGAGCTGGGCTTCCT</u>	33	For high-resolution S1 mapping of the putative tsp upstream of <i>relA</i> ; relAs2 was 5'-end-labelled
relAs2	GGGCTTGTCACCCGAGGTCTT	21	

site-specifically into the phage ϕ C31 attachment site of *S. clavuligerus*, was digested with *Bam*HI and the 4.6 kb DNA fragment isolated. The amplified fragment carrying *relA* and its own promoter was ligated to the 4.6 kb *Bam*HI DNA fragment to yield pULGE331, in which *relA* is in the opposite orientation to *aac(3)IV*. This plasmid was used to complement the *relA*-null mutants. Religated *Bam*HI-digested pMS17 (pMS17B) was used as vector control. The incomplete *relA* gene *relA^{RI}* was amplified from genomic DNA using oligonucleotides relA-O5 and relA-O6, confirmed by sequencing and inserted into *XbaI/EcoRV*-digested pMS17 to give pULGE261, in which *relA^{RI}* is expressed from the *Streptomyces* promoter tcp830 (Rodríguez-García *et al.*, 2005).

RNA extraction and purification. *Streptomyces* RNA extraction and purification was performed using the RNeasy kit (Qiagen) following the protocol at <http://www.surrey.ac.uk/SBMS/Fgenomics>. Phenol was obtained from BDH or Appligen-Oncor, and lysozyme from Sigma.

S1 endonuclease mapping. High-resolution S1 nuclease mapping was performed with sodium trichloroacetate buffer as described by Kieser *et al.* (2000) but using $1 \times$ S1 digestion buffer in step 1 of the protocol. The oligonucleotides used to amplify the probes for high-resolution mapping were labelled with [γ - 32 P]ATP and polynucleotide kinase. Thirty micrograms of RNA was used in each reaction. A *bldG* probe was included as an internal control in every reaction; that for *claR* (shown in Fig. 7c) was repeated without the *bldG* probe to allow visualization of the *claR*-tsp2 protected fragment, which differs from that of the *bldG*-tsp1 protected segment by only one nucleotide (Bignell *et al.*, 2005). Reactions for each strain were performed concomitantly, loaded on the same gel and exposed on the same film, allowing quantitative comparison of different time points and different strains for each probe.

Amino acid shift-down procedure. Amino acid shift-down experiments were carried out in MF medium (Basarán *et al.*, 1991) using 1 litre flasks with stainless steel springs for dispersed growth and containing 200 ml MF medium supplemented with 1% (w/v) Casamino acids (MFA). Flasks were inoculated with mycelium from a 24 h TSB culture to a final OD₆₀₀ of 0.25. During rapid growth (15–17 h after inoculation), 50 ml of each culture was quickly filtered (15 s maximum) through Whatman no. 1 filters (8.5 cm diameter) using a vacuum pump, washed with fresh MF medium and the mycelium resuspended in the same volume of MF or MFA (as control) medium.

Quantification of nucleotides. Nucleotides were extracted as described by Ochi (1986). Samples of the cultures taken from 0 to 60 min after shift-down were quickly filtered as above and the filters submerged upside-down in ice-cooled 1 M formic acid in a Petri dish. The extraction was kept at 4 °C for 60 min; the formic acid extract was then isolated by centrifugation, filtered through 0.45 μ m pore cellulose acetate filters, frozen in liquid nitrogen and freeze-dried. The dry samples were kept at –80 °C until analysed. Separation and quantification of the nucleotides were achieved using a 4.6 \times 250 mm 10 μ m particle size Partisil SAX column on an Agilent 1100 HPLC system fitted with a photodiode array detector. The mobile phases (A) KH₂PO₄ 7 mM adjusted to pH 4.0 with phosphoric acid, and (B) KH₂PO₄ 0.5 M containing Na₂SO₄ 0.5 M adjusted to pH 5.4 with NaOH, were used with the following gradient: time 0 min, 0% B; 3 min, 20% B; 15 min, 70% B; 25 min, 75% B; 35–45 min, 100% B. Under these conditions retention times were: ATP, 13.6 min; GTP, 15.4 min; ppGpp, 25.2 min; pppGpp, 37.5 min. Nucleotide standards were obtained from Sigma. ppGpp was kindly supplied by K. Ochi, National Food Research Institute, Tsukuba, Ibaraki, Japan. ppGpp and pppGpp were quantified using the absorption coefficient for GTP.

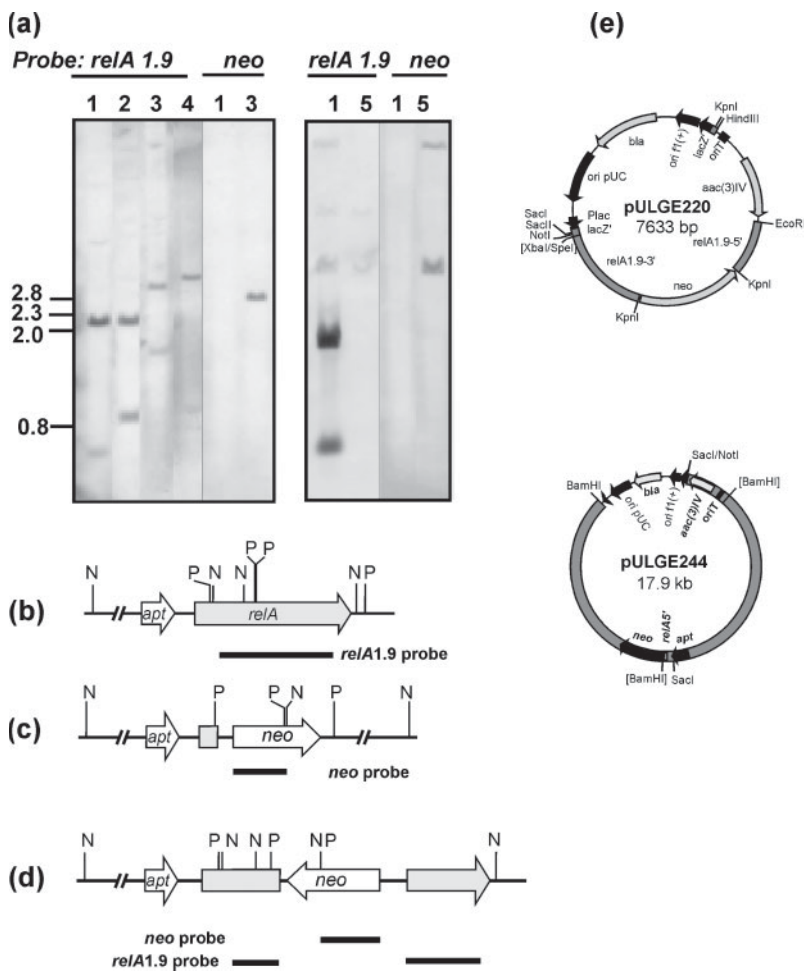


Fig. 1. Southern analysis of the *relA*-null mutants of *S. clavuligerus*. (a) Hybridization of total DNA from *S. clavuligerus* ATCC 27064 (lanes 1, 2), *S. clavuligerus relA::neo* (lanes 3, 4) and *S. clavuligerus ΔrelA::neo* (lane 5) digested with *Nco*I (lanes 1, 3, 5) or *Pvu*II (lanes 2, 4) with the *relA*1.9 probe or the *neo* probe. The very weak hybridization signal with the *relA*1.9 probe (about 4.5 kb) observed with *S. clavuligerus ΔrelA::neo* genomic DNA probably reflects hybridization with the related *rsh* gene of *S. clavuligerus* (Jin *et al.*, 2004). Restriction maps of the *relA* regions in *S. clavuligerus* ATCC 27064 (b), *S. clavuligerus ΔrelA::neo* (c) and *S. clavuligerus relA::neo* (d) are shown below the Southern analysis. (e) Plasmids pULGE220 and pULGE244, used respectively to disrupt and delete the *relA* gene. Bars indicate the regions hybridizing with the *relA*1.9 or the *neo* probes. Restriction sites for *Nco*I (N) and *Pvu*II (P) are indicated.

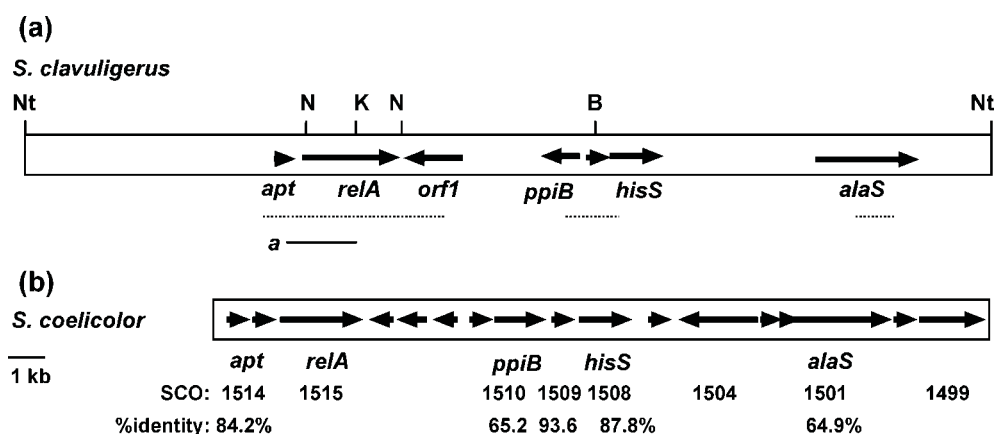


Fig. 2. Comparison of the *relA* loci of *S. clavuligerus* and *S. coelicolor*. (a) Restriction map of the *relA* region of *S. clavuligerus* ATCC 27064. Partially or completely sequenced ORFs are indicated by arrows. Restriction sites for *Bam*HI (B), *Nco*I (N; not all *Nco*I sites are shown) and *Not*I (Nt) are indicated. Solid bar a indicates the hybridization probe used to confirm the organization of the region upstream of *relA*. (b) Homologous regions in the *S. coelicolor* genome. The names of the genes and percentage amino acid sequence identities with proteins encoded by the regions sequenced in *S. clavuligerus* are indicated (see text for details). *orf1* corresponds to a hypothetical protein.

RESULTS

Cloning of *relA* of *S. clavuligerus*

Conserved sequences present in the *relA* genes of *S. coelicolor* and *S. antibioticus* were used to design oligonucleotides *relA*-O3 and *relA*-O4, which were used to PCR-amplify a 1.9 kb DNA fragment from total DNA of *S. clavuligerus* ATCC 27064. The nucleotide sequence of the amplified fragment (named *relA1.9*) is identical to the corresponding fragment published by Jin *et al.* (2004) and indicates that it encodes amino acids 140–771 of RelA. This fragment was then used to disrupt *relA* in *S. clavuligerus* (Fig. 1a, lanes 3 and 4). Since there are no *NotI* sites in either the *relA1.9* fragment or the inserted *neo* gene, the regions flanking *relA* were cloned by marker rescue using *NotI*-digested total DNA from *S. clavuligerus* *relA::neo*. A kanamycin-resistant *E. coli* transformant was found to carry a plasmid (pULGE221) with an insert of about 26 kb in which the *neo* gene was located between an 8 kb DNA fragment carrying the 5'-end of *relA* and its upstream region and a 16.6 kb DNA fragment carrying the 3'-end of *relA* and its downstream region. The complete sequence of *relA* was obtained by sequencing fragments of pULGE221. While the sequence of the *relA* protein-coding region was identical to that published by Jin *et al.* (2004), there were marked differences in the upstream region (see below). Upstream of *relA*, and separated by 176 nt, was a gene (*apt*) encoding a protein 84.2% identical to the adenine phosphoribosyltransferase of *S. coelicolor* (SCO1514), an enzyme involved in purine nucleotide biosynthesis (Fig. 2). Downstream of *relA*, in the opposite orientation and separated by 105 nt, was an ORF encoding a putative peroxidase with 52.2% identity to Bpro DRAFT_3308 from *Polaromonas* sp. The ends of several fragments obtained by *Bam*HI and *Nco*I digestion were also sequenced (Fig. 2). Analysis of the sequences revealed an ORF (*alaS*) encoding an alanyl-tRNA synthetase homologous to SCO1501, an ORF (*ppiB*) encoding a putative peptidyl-prolyl *cis/trans* isomerase homologous to SCO1510, and an ORF (*hisS*) encoding a histidyl-tRNA synthetase homologous to SCO1508. The arrangement of

the *S. clavuligerus* genes is similar to that found in *S. coelicolor* (Fig. 2) and in *S. avermitilis* (data not shown).

Interestingly, the sequence of the intergenic region of *S. clavuligerus* between *relA* and *apt* is markedly different from that published by Jin *et al.* (2004). We found an intergenic region of 176 nt between *apt* and *relA*, whereas only 29 nt were reported by Jin *et al.* (2004). A tandem duplication containing the probable *relA* ribosome-binding site in the sequence of Jin *et al.* (2004) was not present in our intergenic region. The nucleotide sequence found in our work is identical to that obtained independently by DSM, Delft, The Netherlands (M. van den Berg, personal communication) for the same wild-type strain. Thus the strain used by Jin *et al.* (2004) appears to have undergone deletion and rearrangement in the *relA* promoter region.

Construction of a *relA*-deleted mutant of *S. clavuligerus*

The *S. clavuligerus* *relA::neo* insertion mutant described above contains the whole *relA* gene in two fragments separated by *neo*. Since internal fragments of *relA* might still encode a functional ppGpp synthetase (Martínez-Costa *et al.*, 1998), we proceeded to construct a *relA*-deletion mutant of *S. clavuligerus*. The Δ *relA* mutant was confirmed by hybridization with the *relA1.9* and *neo* probes (Fig. 1a, lane 5) and was named *S. clavuligerus* Δ *relA::neo*. This mutant carries only 104 nt of the 5'-end of *relA*.

Characteristics of the *S. clavuligerus* *relA*-null mutants and of complemented transformants

Morphological differentiation. The *relA*-null mutants did not sporulate on ME agar (Fig. 3), the medium commonly used for *S. clavuligerus* sporulation, nor on SFM medium. In addition, the mutants failed to produce aerial mycelium and the brown pigment characteristic of *S. clavuligerus* when grown on ME agar (Fig. 3b, cultures 3 and 5) or on SFM medium (data not shown)

To complement the *relA* mutants, the integrative plasmid pULGE331 (carrying *relA* with its own promoter) was

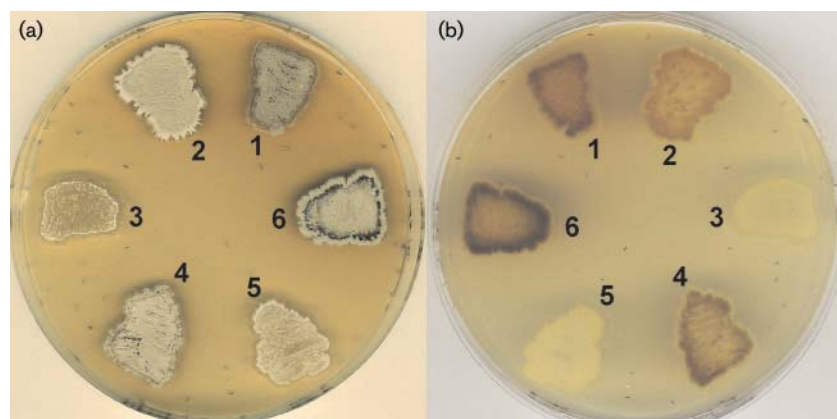


Fig. 3. Phenotypes of *S. clavuligerus* ATCC 27064 and the *S. clavuligerus* *relA*-null mutants grown on ME agar. (a) Top view of the plate. (b) Bottom view of the plate. 1, *S. clavuligerus* ATCC 27064; 2, *S. clavuligerus* 27064(pULGE261); 3, *S. clavuligerus* *relA::neo*; 4, *S. clavuligerus* *relA::neo*(pULGE261); 5, *S. clavuligerus* Δ *relA::neo*; 6, *S. clavuligerus* Δ *relA::neo*(pULGE261).

introduced by conjugation into *S. clavuligerus* $\Delta relA::neo$ and *S. clavuligerus relA::neo*. Exconjugants carrying the vector pMS17B were used as controls. Morphological differentiation was restored in *S. clavuligerus relA::neo*(pULGE331) and *S. clavuligerus* $\Delta relA::neo$ (pULGE331), but not in derivatives containing pMS17B (data not shown). The integrative plasmid pULGE261, which carries an incomplete *relA^{RI}* gene encoding a truncated RelA protein (amino acids 228–495) that is predicted to be able to form ppGpp in a ribosome-independent manner (Martínez-Costa *et al.*, 1998), was also introduced into the *relA*-null mutants by conjugation. Both *S. clavuligerus relA::neo*(pULGE261) and *S. clavuligerus* $\Delta relA::neo$ (pULGE261) expressing the *relA^{RI}* DNA fragment *in trans* recovered the ability to form aerial mycelium, spores and pigment (Fig. 3, cultures 4 and 6). Integration of the vector (pMS17) alone lacking the truncated *relA* failed to complement the two mutations.

Physiological differentiation. The ability of *S. clavuligerus* $\Delta relA::neo$ and *S. clavuligerus relA::neo* to produce cephamycin C and clavulanic acid was assessed in TSB and SA liquid media. Both mutants gave lower biomass yields (expressed as mg per mg DNA) in TSB at 36 h, and in SA at 72 h. The sequential pattern of production of cephamycin C and clavulanic acid in both *relA* mutants was similar to that of the wild-type strain. However, the yield of clavulanic acid and cephamycin C, expressed as μg per mg DNA, was consistently much higher in the *relA*-null mutants (Fig. 4). The yield of clavulanic acid from *S. clavuligerus* $\Delta relA::neo$ grown in TSB medium was 3- to 4-fold higher than that from the wild-type strain, and cephamycin C production was 2.6-fold higher. In SA medium, the increases were even higher: 4-fold for clavulanic acid and 6-fold for cephamycin C at 72 h. The *S. clavuligerus relA::neo* mutant showed a similar pattern

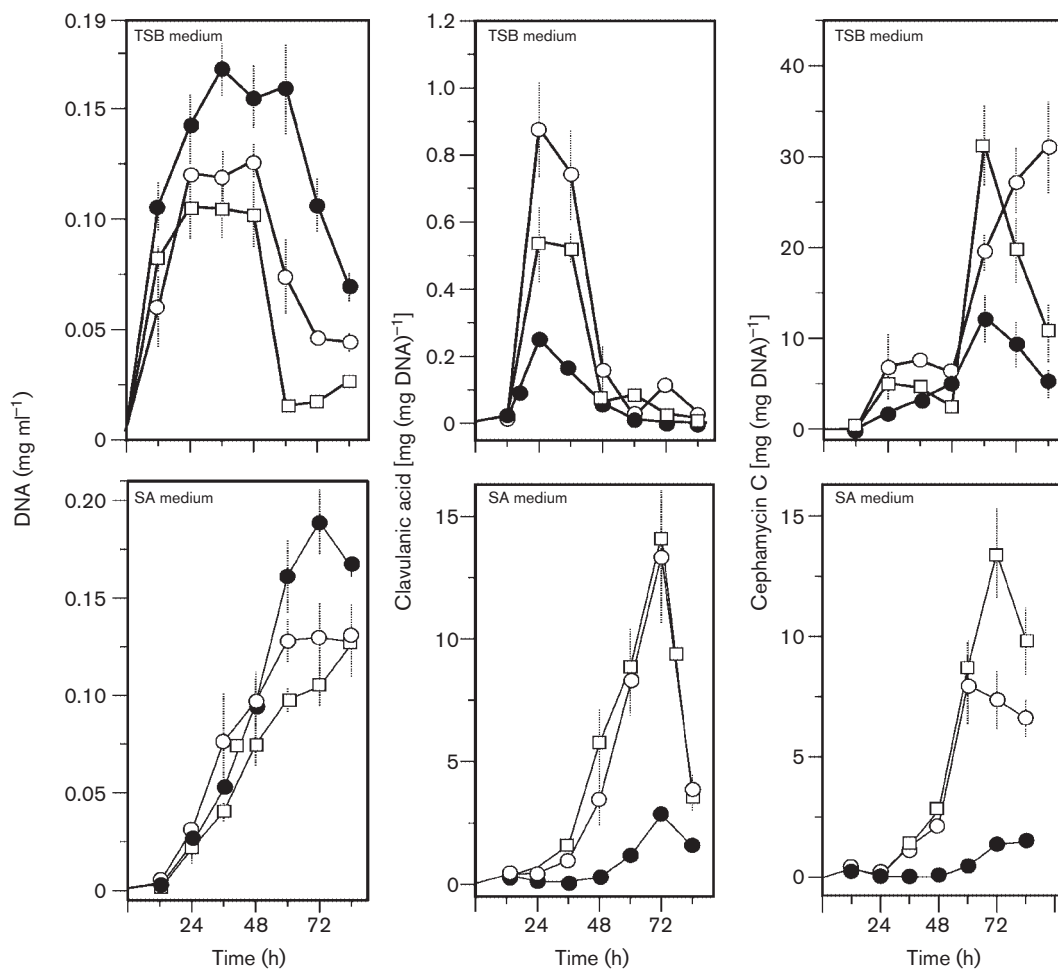


Fig. 4. Growth and clavulanic acid and cephamycin C production by *S. clavuligerus* ATCC 27064 and *relA*-null mutants grown in TSB medium (upper panels) and SA medium (lower panels). ●, *S. clavuligerus* ATCC 27064; □, *S. clavuligerus relA::neo*; ○, *S. clavuligerus* $\Delta relA::neo$. Data are means \pm SD of triplicate cultures.

of antibiotic production and higher yields of clavulanic acid and cephamycin C than the parental strain (Fig. 4).

When the *relA*-null mutants were complemented with the complete *relA*, production of clavulanic acid and cephamycin C decreased to almost wild-type levels. These results indicate that a functional *relA* gene exerts a negative effect on the production of both secondary metabolites in *S. clavuligerus*.

Nucleotide levels in *S. clavuligerus* ATCC 27064 and in the $\Delta relA::neo$ mutant

S. clavuligerus wild-type and $\Delta relA::neo$ were grown in SA medium and their ATP, GTP and polyphosphorylated nucleotide levels determined (Ochi, 1986). Samples were taken from the same cultures to measure clavulanic acid and cephamycin C production, and for S1 nuclease analysis of the expression of antibiotic biosynthesis genes (see later). Both cultures exhibited a similar increase in ATP levels (about 0.2 nmol per mg cell dry weight) as growth proceeded, with that in the mutant showing a delayed and more gradual rise (Fig. 5). A marked decrease in GTP level (about threefold) occurred in the wild-type strain during growth, but then levelled out upon entry into stationary phase; in contrast, the GTP content of the *S. clavuligerus*

$\Delta relA::neo$ mutant remained fairly steady throughout the fermentation. As previously described (Gomez-Escribano *et al.*, 2006), ppGpp and (p)ppGpp peaked at 48 h of growth in the wild-type. As expected, no polyphosphorylated guanine nucleotides were detected in the *relA*-deletion mutant.

Transcription of cephamycin C and clavulanic acid biosynthesis genes in cultures grown in SA medium

Transcription of *cefD*, encoding the isopenicillin N epimerase for cephamycin C biosynthesis (Kovacevic *et al.*, 1990), and of *ceaS2*, encoding the carboxyethylarginine synthase for clavulanic acid biosynthesis, was assessed by high-resolution S1 nuclease protection analysis. In parallel, transcription of the regulatory genes *ccaR* (Pérez-Llarena *et al.*, 1997) and *bldG* (Bignell *et al.*, 2005) (both involved in the regulation of cephamycin C and clavulanic acid production), and of *clpR* (Pérez-Redondo *et al.*, 1998, Paradkar & Jensen, 1998) (involved in regulating clavulanic acid biosynthesis), was determined, as was that of *relA*.

While the transcription profiles of *relA*, *bldG*, *clpR* and *ccaR* were broadly similar in both strains (although transcription of *ccaR* appeared somewhat higher and persisted for longer in the *relA*-deletion mutant), transcription of *cefD*

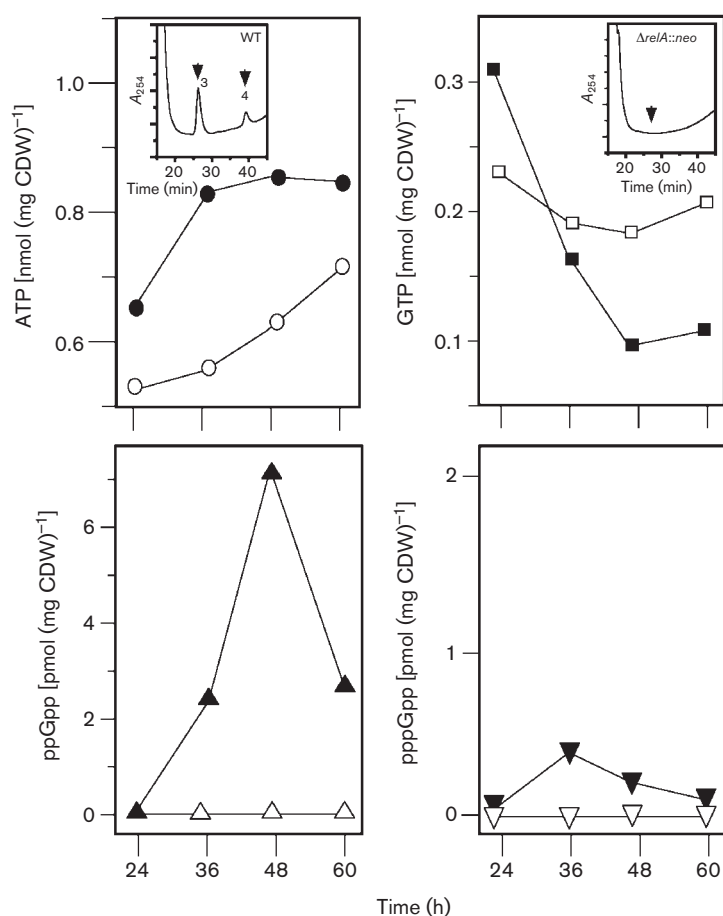


Fig. 5. Intracellular nucleotide levels in *S. clavuligerus* ATCC 27064 (filled symbols) and *S. clavuligerus* $\Delta relA::neo$ (open symbols) grown in SA medium. ●, ○, ATP; ■, □, GTP; ▲, △, ppGpp; ▼, ▽, pppGpp. CDW, cell dry weight. The insets show the location of the peaks of ppGpp (3) and pppGpp (4) in extracts of the wild-type strain as analysed by HPLC upon maximal synthesis after amino acid deprivation, and their absence in the similarly treated $\Delta relA::neo$ mutant.

and particularly of *ceaS2* (encoding the enzyme catalysing the first step in clavulanic acid biosynthesis) was much higher in the *relA*-deletion mutant. This was especially noticeable after 48 h, when expression dropped markedly in the wild-type strain. This difference in expression of *cefD* and *ceaS2* correlates well with the overproduction of both cephamycin C and clavulanic acid in the *relA*-deletion mutant (Fig. 6a, b).

Since 104 bp of the 5'-end of the *relA* gene was still present in $\Delta relA::neo$, the pattern of transcription of this gene could also be studied by S1 nuclease protection analysis. Transcription initiation of *relA* occurred at a thymine located 43 nt upstream of the triplet TTG proposed by Jin *et al.* (2004) as start codon (Fig. 7a). This thymine lies in the segment of the intergenic region that appears to be deleted in the strain used by Jin *et al.* (2004).

Nutritional shift-down switches expression of *claR* from *tsp1* to *tsp2*

The behaviour of *S. clavuligerus* ATCC 27064 and of *S. clavuligerus* $\Delta relA::neo$ after amino acid shift-down was determined by transferring cells from MFA medium to MF medium, lacking amino acids. Nucleotide contents after shift-down and transcription of the same set of antibiotic biosynthesis genes were analysed. As expected, (p)ppGpp production was higher in the wild-type strain 15 min after amino acid shift-down, concomitant with a reduction in the GTP level and an increase in ATP content. ppGpp and pppGpp were never detected in *S. clavuligerus* $\Delta relA::neo$, which showed a strong rise in both ATP and GTP levels after shift-down (Fig. 8).

Expression of *ccaR*, *relA* and *bldG* was similar in both strains after shift-down. Interestingly, transcription of *claR*,

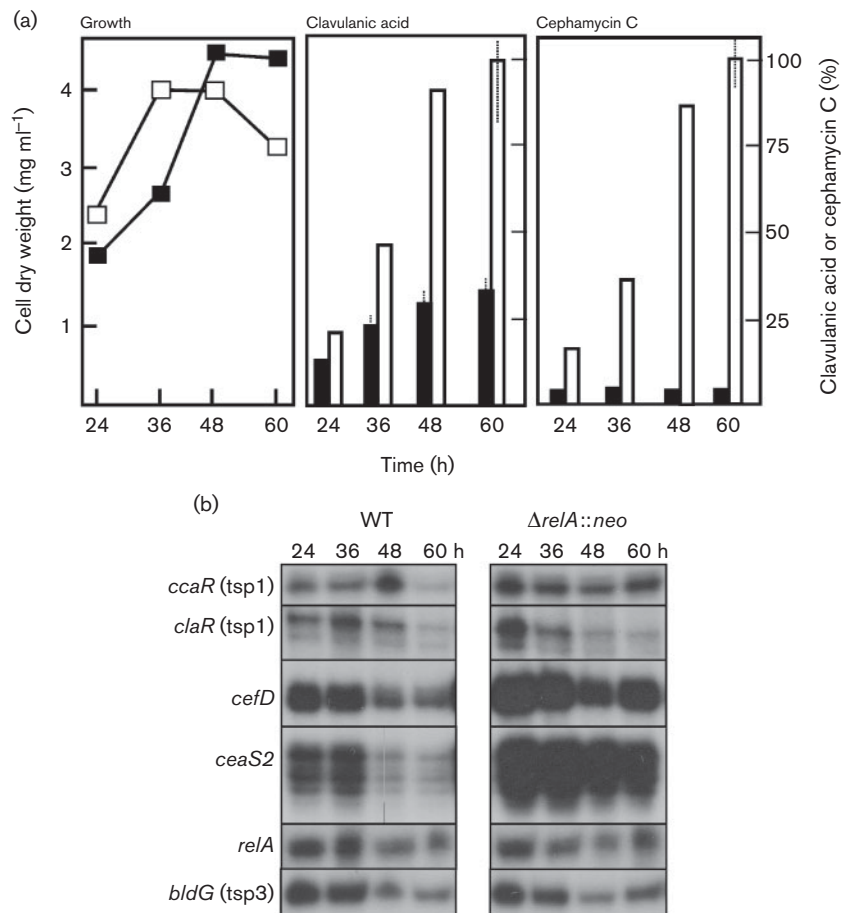


Fig. 6. Antibiotic production and expression of antibiotic biosynthesis genes. (a) Growth and production of clavulanic acid and cephamycin C by *S. clavuligerus* ATCC 27064 (black squares and bars) and *S. clavuligerus* $\Delta relA::neo$ (white squares and bars) grown in SA medium in the same experiment as used to extract RNA for expression analysis. The level of clavulanic acid and cephamycin C production corresponding to 100% is 8.42 and 25.16 μg per mg cell dry weight, respectively. (b) S1 nuclease protection analysis of expression of *ccaR*, *claR*, *cefD*, *ceaS2*, *relA* and *bldG* in the cultures shown in (a) using the probes obtained by PCR with the primers indicated in Table 1. Wild-type (left panel) and the $\Delta relA::neo$ mutant (right panel) at 24, 36, 48 and 60 h of culture.

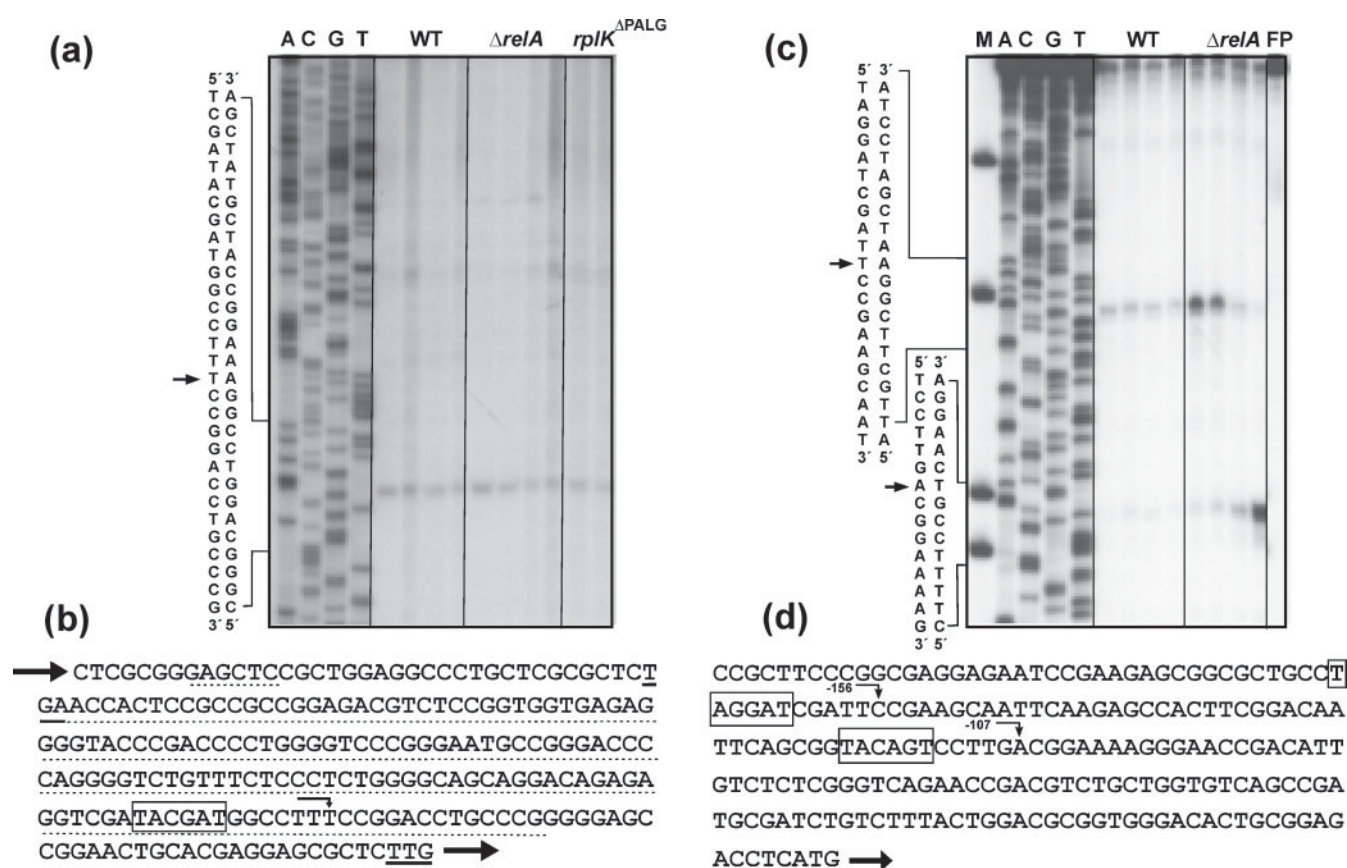


Fig. 7. Mapping of the *relA* and *claR* transcriptional start points (tsps). (a) S1 nuclease mapping of *relA* using RNA from *S. clavuligerus* ATCC 27064, *S. clavuligerus* $\Delta relA::neo$ and *S. clavuligerus* *relC* (Gomez-Escribano *et al.*, 2006) grown in SA medium for 24, 36, 48 and 60 h. (b) Intergenic region of *apt-relA*. The TGA codon of *apt* is underlined. The putative Pribnow (-10) sequence for *relA* is boxed. A bent arrow indicates the tsp. Dotted lines indicate the region which is not present in the sequence published by Jin *et al.* (2004). The *relA* TTG translation start codon is underlined. (c) S1 nuclease mapping of *claR* using RNA from *S. clavuligerus* ATCC 27064 and *S. clavuligerus* $\Delta relA::neo$ isolated 24, 36, 48 and 60 min after amino acid shift-down. FP, *claR* full-length probe (299 bp); M, molecular mass markers (140, 151, 200 and 249 bp). A, C, G and T correspond to the four lanes of a sequencing reaction of the full-length probe with ^{32}P -labelled reverse primer. (d) Intergenic region of *car-claR*. The putative Pribnow sequences are boxed. Bent arrows indicate tsps. The *claR* ATG translation start codon is underlined. In (a) and (c), arrows indicate sites of transcript initiation.

which in SA and MFA medium initiates at transcriptional start point (tsp) 1 (Gomez-Escribano *et al.*, 2006), corresponding to a cytosine located 155 nt upstream of the ATG start codon (Paradkar & Jensen, 1998), changed in the $\Delta relA$ mutant 30 min after shift-down to be transcribed predominantly from tsp2, an adenine located 107 nt upstream of the ATG start codon, a tsp not previously described (Figs 7c, d and 8). This adenine is located 5 nt downstream of a putative TACAGT Pribnow box. tsp2 was used poorly by the wild-type and the *S. clavuligerus* *relC*^{APALG} mutant (data not shown), in both SA and MF cultures, but gave a strong signal in *S. clavuligerus* $\Delta relA::neo$ 30–60 min after shift-down. Thus this switch of tsp was observed only in the $\Delta relA$ mutant, suggesting that it is dependent on the absence of RelA.

DISCUSSION

Studies of relaxed mutants of different *Streptomyces* species impaired in (p)ppGpp synthesis led to the suggestion that sporulation is elicited by a decrease in intracellular GTP content (Ochi, 1986). *S. clavuligerus* *relA*-null mutants are unable to form aerial mycelium and to sporulate (Jin *et al.*, 2004). In batch cultures in liquid SA medium, *S. clavuligerus* $\Delta relA::neo$ maintains a steady intracellular GTP content, which is twice that of the wild-type strain upon entry into stationary phase (Fig. 5); after amino acid shift-down, the GTP content in the $\Delta relA$ -null mutant increases threefold, in contrast to that in the wild-type, which remains relatively stable (Fig. 8). Thus our data are in agreement with the hypothesis of Ochi (1986) linking a

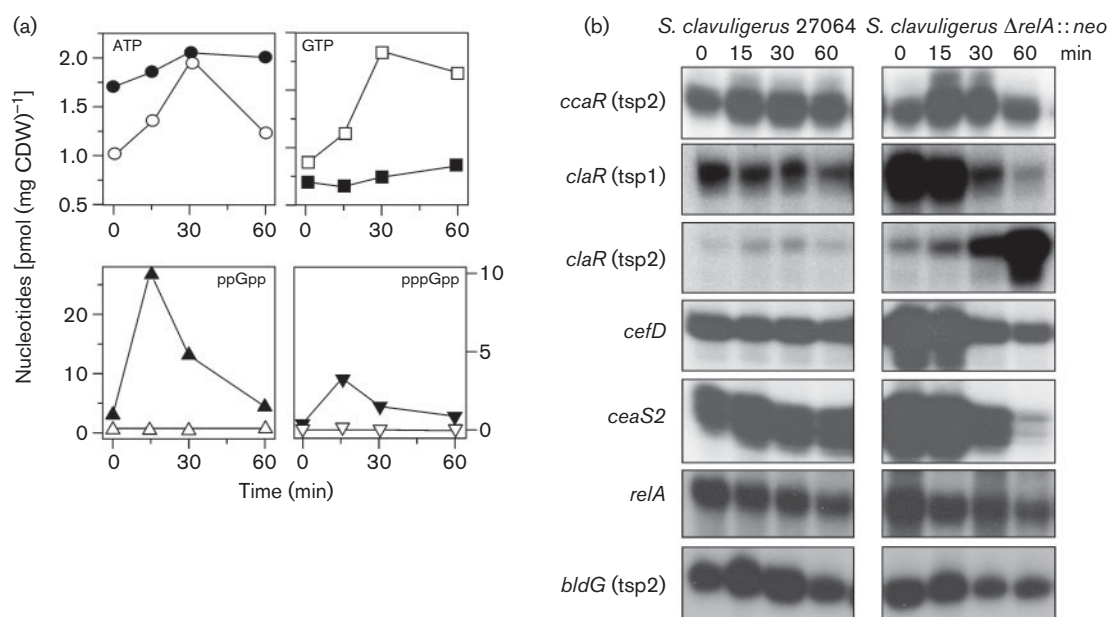


Fig. 8. Intracellular nucleotide contents and transcription of antibiotic biosynthesis genes after amino acid shift-down. (a) ATP, GTP, ppGpp and pppGpp levels in wild-type *S. clavuligerus* ATCC 27064 (filled symbols) and *S. clavuligerus* $\Delta relA::neo$ (open symbols) at different times after amino acid shift-down from MFA to MF medium. CDW, cell dry weight. (b) Expression of *ccaR*, *claR*, *cefD*, *ceaS2*, *relA* and *bldG* in *S. clavuligerus* ATCC 27064 (left panels) and *S. clavuligerus* $\Delta relA::neo$ (right panels) at 0, 15, 30 and 60 min after amino acid shift-down as determined by high-resolution S1 nuclease protection analysis.

decrease in GTP content with the initiation of sporulation in *Streptomyces*.

Ochi (1986) also proposed that (p)ppGpp synthesis is required for antibiotic production in streptomycetes. In support of this, a *relA*-null mutant of *S. antibioticus* was unable to produce actinomycin (Hoyt & Jones, 1999) and a *S. coelicolor* M600-derived *relA*-null mutant was impaired in antibiotic production under conditions of nitrogen, but not phosphate, limitation (Chakraborty & Bibb, 1997). However, a *S. coelicolor* J1501-derived *relA*-null mutant was impaired in actinorhodin but not in undecylprodigiosin or calcium-dependent antibiotic production (Martínez-Costa *et al.*, 1996), suggesting that the requirement for RelA is dependent upon background genotype and the growth medium used.

The work described here is believed to be the first report of a *Streptomyces relA*-null mutant that overproduces antibiotics. *S. clavuligerus relA*-null mutants do not synthesize detectable amounts of (p)ppGpp, yet they overproduce both clavulanic acid and cephamycin C when compared to the wild-type strain. This is also reflected in increased levels of transcription of antibiotic biosynthesis genes. Antibiotic production and morphological differentiation were restored to levels similar to those observed in the wild-type strain by complementation with a full-length *relA* or a truncated *relA^{RI}* gene. The antibiotic phenotype of the *S. clavuligerus relA*-null mutants (Fig. 4) contrasts with the results of Jin & *et al.* (2004). These authors constructed a *S.*

clavuligerus relA-null mutant unable to synthesize (p)ppGpp that was impaired in both sporulation and antibiotic production. In addition, they constructed a null mutant of a *relA*-homologous gene, *rshA*. The *rshA*-deleted mutant exhibited reduced (p)ppGpp synthesis (about 67% ppGpp and 42% pppGpp compared to wild-type), and was as severely impaired in antibiotic production as the $\Delta relA$ mutant, but showed almost normal sporulation. The authors concluded that just a slight decrease in (p)ppGpp can severely affect antibiotic production in *S. clavuligerus* without affecting morphological differentiation. The role of *rshA* is not clear since *relA*-null mutants completely lack (p)ppGpp formation (Jin *et al.*, 2004; this work) and an *rshA*-deleted mutant of *S. coelicolor* is unaffected in antibiotic production (Sun *et al.*, 2001).

The differing behaviour of our *S. clavuligerus relA*-null mutants and that published by Jin *et al.* (2004) appears to reflect differences in the parental strains used and potentially the growth media adopted in the respective studies. Sequencing of the region between *apt* and *relA*, and the 3'-end of *apt* (data not shown), revealed marked differences between the two strains. We found 147 additional nucleotides between *apt* and *relA*, and located the *relA* tsp at a thymine 43 nt upstream of the translation start codon, in a region not present in the strain used by Jin *et al.* (2004). The sequence we determined is identical to that obtained independently by DSM (Delft, The

Netherlands) for *S. clavuligerus* ATCC 27064 (M. van den Berg, personal communication), and it appears that the strain used by Jin *et al.* (2004), also described as ATCC 27064, has undergone a *relA* promoter deletion (note that we cannot exclude that the two isolates may differ further through the occurrence of additional DNA rearrangements and mutations acquired during separate subculturing). In addition, the culture media and growth conditions used in the two studies are different. While we used SA medium in baffled flasks (Paradkar & Jensen, 1998; Lorenzana *et al.*, 2004), Jin *et al.* (2004) used a different medium in a jar fermenter, conditions in which the antibiotic production occurred after entry into stationary phase in their wild-type strain. Either or both of these differences may be responsible for the contrasting patterns of antibiotic production and the markedly different phenotypes of the *relA*-null mutants. However, our results clearly indicate that (p)ppGpp is not required for antibiotic biosynthesis in our strain of *S. clavuligerus*, as previously implied from the isolation of thiostrepton-resistant mutants that were proficient in cephamycin C production (Bascarán *et al.*, 1991).

Consistent with our mutant analysis, we observed a peak in (p)ppGpp synthesis in SA-grown cultures of *S. clavuligerus* ATCC 27064 at the beginning of stationary phase that coincided with a clear decrease in the abundance of antibiotic biosynthesis gene transcripts. This growth-dependent negative regulation of antibiotic biosynthesis gene expression does not occur in the $\Delta relA$ mutant (see Figs 5 and 6), suggesting a negative role for (p)ppGpp in the expression of such genes in wild-type *S. clavuligerus*. While this may seem to disagree with earlier work in other streptomycetes, a striking difference is that in *S. clavuligerus* expression of the cephamycin C and clavulanic acid biosynthesis genes is growth-associated (Figs 4 and 6); i.e. it occurs during rapid growth and declines upon entry into stationary phase. Consequently, a priori, expression of the biosynthesis genes for both of these compounds would not be expected to be (p)ppGpp-dependent. Nevertheless, this is the first report of the negative regulation of secondary metabolite biosynthesis by (p)ppGpp. It will be interesting to see whether the expression of other secondary metabolic gene clusters that are transcribed during rapid growth are also negatively regulated by (p)ppGpp, and whether their levels of production increase in a *relA*-null mutant.

ACKNOWLEDGEMENTS

This work was supported by grants from the Junta de Castilla y León (LE21/00A) to J. F. M., from the Ministry of Science and Technology (Madrid) to P. L. (CICYT, BIO2000-0272), and by a grant to the John Innes Centre (M. J. B.) from the Biotechnology and Biological Sciences Research Council. J. P. G.-E. received a scholarship from the Ministry of Science, Education and Culture and a Marie Curie Training Fellowship from the EU. We gratefully acknowledge receipt of ppGpp from Dr K. Ochi. Valuable discussions with K. Chater and F. Malpartida are also acknowledged.

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Edited by: L. Heide