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.

INTRODUCTION

The stringent response, first described in enterobacteria as an inhibiton 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 (Bracken 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 thiopetin-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 (Martinez-Costa et al., 1996; Wendrich & Marahiel, 1997). In Streptomyces coelicolor A3(2), the relA/spoT gene (hereafter named relA; Chakraburty et al., 1996) encodes a 94 200 Da protein.
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 (Chakraburtty & 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 cephamycin 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. Bascaran 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 cephamycin C than the wild-type strain, suggesting that ppGpp is not essential for antibiotic biosynthesis in S. clavuligerus, other mutants produced reduced cephamycin 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 cephamycin 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 clavaminate 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 cephamycin 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 DH5α were used for cloning. E. coli Ess22-35 and Klebsiella pneumoniae ATCC 29665 were used to assay for cephamycin C and clavulanic acid production, respectively (Liras & Martin, 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 (Bascaran 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 (SMF) 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). dNTP 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 Swall 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/BglII-digested pBSKK+ (+) 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/HindII-digested pULGE211 to give pULGE212. Finally the neo gene, encoding kanamycin resistance, was isolated with KpnI from plasmid pTC192K (Rodriguez-Garcia et al., 2006) and inserted into the single KpnI site internal to relA.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 Norf–BamH1 fragment containing relA, a 2.6 kb Norf 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 Norf 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** truncated gene. The complete relA gene was amplified using oligonucleotides relA-O1 and relA-O2, and Pfu DNA polymerase, and confirmed by nucleotide sequencing. The conjugative, integrative plasmid pMS17 (Rodriguez-Garcia et al., 2005), which integrates
Table 1. Oligonucleotides used in this work

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>-mer</th>
<th>Use and comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>relA-O1</td>
<td>CCGTCTGAGCTCTGACCCGACCAC</td>
<td>22</td>
<td>To clone relA from S. clavuligerus genomic DNA, including the whole apt-relA intergenic region; relA-O1 and relA-O2 contain XhoI and NotI sites respectively (bold)</td>
</tr>
<tr>
<td>relA-O2</td>
<td>CCGTCTGAGCTCCGACTAAGGGGCCC</td>
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</tr>
<tr>
<td>relA-O3</td>
<td>GGGAGGCTCTAGAGGCGCTCCGACCTGATGG</td>
<td>30</td>
<td>For cloning the 1.9 kb internal fragment of relA, relA1.9; contain restriction sites for XhoI (3) and EcoRI (4) for subsequent cloning (bold)</td>
</tr>
<tr>
<td>relA-O4</td>
<td>CGGCCCCGAATTCACAGAGGGCGGACCCTGA</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>relA-O5</td>
<td>GTCGGATCCGATGAGCCAGGAC</td>
<td>21</td>
<td>To clone a ribosome-independent (p)ppGpp synthetase gene; include restriction sites for BamHI (5) and EcoRI (6) for subsequent cloning (bold)</td>
</tr>
<tr>
<td>relA-O6</td>
<td>GTCGAATTCCGATCTACTGCGG</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>claR-1</td>
<td>TATTCGAATTCGAGCTCCGACGGCCCGCACGACA</td>
<td>40</td>
<td>For high-resolution S1 mapping of claR (Paradkar &amp; Jensen, 1998); claR2 was 5'-end-labelled, the non-homologous tail is underlined</td>
</tr>
<tr>
<td>claR-2</td>
<td>TATTCGAATTCGAGCTCCGACGGCCCGCACGACA</td>
<td>36</td>
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<tr>
<td>cceS1</td>
<td>TGATCCTGCTGCCGAAATCCAGGAAAGGAGCCAGC</td>
<td>31</td>
<td>For high-resolution S1 mapping of the cceS2 transcript (Tahan et al., 2004); cceS2 was 5'-end-labelled, the non-homologous tail is underlined</td>
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<tr>
<td>bldG1</td>
<td>TCGATCTGACGACGGAGGTCTGCTGGGA</td>
<td>18</td>
<td>For high-resolution S1 mapping of the bldG transcript (Bignell et al., 2005); bldG2 was 5'-end-labelled, bldG1 has a 12 bp non-homologous tail</td>
</tr>
<tr>
<td>bldG2</td>
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<td>18</td>
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<tr>
<td>ccaR1</td>
<td>AGGTGTTTGGGGAGGATTTGCCGG</td>
<td>21</td>
<td>For high-resolution S1 mapping of two putative ccaR transcripts (Wang et al. 2004); ccaR2s was 5'-end-labelled</td>
</tr>
<tr>
<td>ccaR2</td>
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<td>21</td>
<td></td>
</tr>
<tr>
<td>cepD1</td>
<td>CTCTCTGAGGAGGAGGGACAAAA</td>
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<td>For high-resolution S1 mapping of the cepD transcript (Gomez-Escribano et al., 2006); cepDs2 was 5'-end-labelled</td>
</tr>
<tr>
<td>cepD2</td>
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<td></td>
</tr>
<tr>
<td>relAs1</td>
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<td>33</td>
<td>For high-resolution S1 mapping of the putative tsp upstream of relA; relAs2 was 5'-end-labelled</td>
</tr>
<tr>
<td>relAs2</td>
<td>GGCGGCTGACGACCCGATGTT</td>
<td>21</td>
<td></td>
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site-specifically into the phage dC31 attachment site of S. clavuligerus, was digested with BamHI and the 4.6 kb DNA fragment isolated. The amplified fragment carrying relA and its own promoter was ligated to the 4.6 kb BamHI DNA fragment to yield pULGE331, in which relA is in the opposite orientation to aec(3)/JV. This plasmid was used to complement the relA-null mutants. Religated BamHI-digested pMS17 (pMS17B) was used as vector control. The incomplete relA gene relA<sup>4</sup> 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<sup>4</sup> is expressed from the Streptomyces promoter tcp830 (Rodriguez-Garcia et al., 2005).

RNA extraction and purification. Streptomyces RNA extraction and purification were performed using the RNasey 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 Kissler et al. (2000) but using 1 × S1 digestion buffer in step 1 of the protocol. The oligonucleotides used to amplify the probes for high-resolution mapping were labelled with [γ-<sup>32</sup>P]ATP and polynucleotide kinase. Thirty micrograms of RNA was used in each reaction. A bld<sup>G</sup> probe was included as an internal control in every reaction; that for relA<sup>4</sup> was digested with BamHI and the 4.6 kb DNA fragment isolated. The incomplete relA<sup>4</sup> complement the whole transcript in the opposite orientation to the whole gene; include restriction sites for BamHI (5) and EcoRI (6) for subsequent cloning (bold)

Amino acid shift-down procedure. Amino acid shift-down experiments were carried out in MF medium (Bascarán 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 OD<sub>600</sub> 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 μm 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 × 250 mm 10 μm particle size Partisil SAX column on an Agilent 1100 HPLC system fitted with a photodiode array detector. The mobile phases (A) KH<sub>2</sub>PO<sub>4</sub>, 7 mM adjusted to pH 4.0 with phosphoric acid, and (B) KH<sub>2</sub>PO<sub>4</sub>, 0.5 M containing Na<sub>2</sub>SO<sub>4</sub>, 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; pppGpp, 25.2 min; ppppGpp, 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 *Pau*I (lanes 2, 4) with the relA1.9 probe or the neo probe. The very weak hybridization signal with the relA1.9 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 relA1.9 or the neo probes. Restriction sites for *Nco*I (N) and *Pau*I (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 Nofl sites in either the relA1.9 fragment or the inserted neo gene, the regions flanking relA were cloned by marker rescue using Nofl-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 BamHI and Ncol 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 (Martinez-Costa et al., 1998), we proceeded to construct a relA-deletion mutant of S. clavuligerus. The ∆relA mutant was confirmed by hybridization with the relA1.9 and neo probes (Fig. 1a, lane 5) and was named S. clavuligerus ∆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

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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 ∆relA::neo; 6, S. clavuligerus ∆relA::neo(pULGE261).
introduced by conjugation into *S. clavuligerus ΔrelA::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 ΔrelA::neo* (pULGE331), but not in derivatives containing pMS17B (data not shown). The integrative plasmid pULGE261, which carries an incomplete *relA* 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 ΔrelA::neo* (pULGE261) expressing the *relA* 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 ΔrelA::neo* and *S. clavuligerus relA::neo* to produce cephemycin 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 cephemycin C and clavulanic acid in both *relA* mutants was similar to that of the wild-type strain. However, the yield of clavulanic acid and cephemycin 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 ΔrelA::neo* grown in TSB medium was 3- to 4-fold higher than that from the wild-type strain, and cephemycin C production was 2.6-fold higher. In SA medium, the increases were even higher: 4-fold for clavulanic acid and 6-fold for cephemycin C at 72 h. The *S. clavuligerus relA::neo* mutant showed a similar pattern.

![Fig. 4](http://mic.sgmjournals.org) 749
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 ΔrelA::neo mutant

S. clavuligerus wild-type and ΔrelA::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 ΔrelA::neo mutant remained fairly steady throughout the fermentation. As previously described (Gomez-Escribano et al., 2006), ppGpp and (p)ppGpp peaked at 48 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 claR (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, claR 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

![Intracellular nucleotide levels in S. clavuligerus ATCC 27064 (filled symbols) and S. clavuligerus ΔrelA::neo (open symbols) grown in SA medium.](image)
and particularly of ccaR (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 ccaS2 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 ΔrelA::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* ΔrelA::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* ΔrelA::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,
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 \(D_{\text{relA}}\) 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 \(S. \text{clavuligerus relC} \) mutant (data not shown), in both SA and MF cultures, but gave a strong signal in \(S. \text{clavuligerus D}_{\text{relA}}::\text{neo} \) 30–60 min after shift-down. Thus this switch of tsp was observed only in the \(\Delta_{\text{relA}}\) mutant, suggesting that it is dependent on the absence of RelA.

**DISCUSSION**

Studies of relaxed mutants of different *Streptomyces* species impaired in (p)pGpp synthesis led to the suggestion that sporulation is elicited by a decrease in intracellular GTP content (Ochi, 1986). *S. \text{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. \text{clavuligerus D}_{\text{relA}}::\text{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 \(\Delta_{\text{relA}}\)-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
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 (Chakraburty & 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<sup>RI</sup> gene. The antibiotic phenotype of the 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 ΔrelA 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).
dependent negative regulation of antibiotic biosynthesis gene transcripts. This growth-
S. clavuligerus (p)ppGpp synthesis in SA-grown cultures of Consistent with our mutant analysis, we observed a peak in 1991).
relA proficient in cephamycin C production (Bascaran et al., 1991) when isolation of thiostrepton-resistant mutants that were our strain of S. clavuligerus, is responsible for the contrasting patterns of antibiotic production and the markedly different phenotypes of the isolation of thiostrepton-resistant mutants that were proficient in cephamycin C production (Bascaran 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 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.

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