Two relA/spoT homologous genes are involved in the morphological and physiological differentiation of Streptomyces clavuligerus

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This study is focused on the involvement of the unusual nucleotide (p)ppGpp during the morphological and physiological differentiation of Streptomyces clavuligerus. In particular, the functional and structural elements of two genes encoding the proteins RelA and Rsh were identified. The relA gene encodes an 843 aa protein (RelA), while the rsh gene encodes a 738 aa protein (Rsh). The relA and rsh genes were disrupted by the insertion of a hygromycin resistance gene and an apramycin resistance gene, respectively. The synthesis of ppGpp in the relA gene-disrupted mutant was completely eliminated under conditions of starvation for amino acids, whereas synthesis persisted, but was greatly reduced in the rsh gene-disrupted mutant. The relA gene-disrupted mutant had a bald appearance on agar plate cultures and retarded growth in submerged culture, while the rsh-disrupted mutant was unchanged in growth characteristics relative to the wild-type culture. The production of both clavulanic acid and cephamycin C were completely abolished in the relA-disrupted mutant. Thus, it is concluded that the relA gene rather than rsh is essential for morphological and physiological differentiation in S. clavuligerus and that RelA primarily governs the stringent response of S. clavuligerus to starvation for amino acids.

INTRODUCTION

Streptomycetes are Gram-positive bacteria that form filamentous mycelia and also produce a wide variety of antibiotics and other secondary metabolites. The production of those antibiotics and metabolites in submerged culture is generally confined to the stationary growth phase or conditions where nutrients essential for growth are limiting. In addition, growth under these conditions on solid culture normally coincides with the onset of morphological differentiation (Chater & Bibb, 1996). The accumulation of highly phosphorylated guanine nucleotides, (p)ppGpp, is one of the first responses to a nutritional downshift (Cashel et al., 1996). The synthesis of (p)ppGpp from ATP and GTP in Escherichia coli is accomplished by two enzymes, ppGpp synthetase and ppGpp synthetase/hydrolase (Metzger et al., 1989). The ppGpp synthetase (RelA) protein catalyses ppGpp synthesis under conditions of amino acid limitation, in association with ribosomes (Haseltine & Block, 1973). The ppGpp synthetase/hydrolase (SpoT) is a bifunctional enzyme which synthesizes (p)ppGpp under carbon limitation in a ribosome-independent mode and also degrades (p)ppGpp by means of a manganese-dependent (p)ppGpp pyrophosphohydrolase activity. However, the synthetic activity in vivo is normally obscured by its more abundant degrading activity (Gentry & Cashel, 1996). (p)ppGpp binds to the β-subunit of RNA-polymerase, resulting in the reduction of rRNA transcription, probably due to the instability of open promoter/RNA-polymerase complexes at rRNA promoters (Chatterji et al., 1998). (p)ppGpp acts as a positive regulator of some other promoters as well as a negative regulator of rRNA transcription (Barker et al., 2001; Choy, 2000). In addition, the levels of the stationary-phase-specific sigma factor (σ8)-dependent genes in E. coli are closely regulated by (p)ppGpp (Brown et al., 2002). (p)ppGpp is also thought to signal nutritional downshift to initiate fruiting body development in Myxococcus xanthus (Garza et al., 2000), to activate quorum sensing in Pseudomonas aeruginosa (Delden et al., 2001), to induce the colicin synthesis in E. coli (Kuhar et al., 2001) and to contribute to virulence in Legionella pneumophila (Hammer & Swanson, 1999) and Mycobacterium tuberculosis (Primm et al., 2000).

Abbreviations: ppGpp, guanosine 3',5'-bispyrophosphate; RelA, ppGpp synthetase; Rsh, RelA/SpoT homologue; SpoT, ppGpp synthetase/hydrolase.

The GenBank accession numbers for the sequences reported in this paper are AF421216 and AF421217.
It has also been reported that (p)pGpp plays an important role during secondary metabolism and morphological differentiation in *Streptomyces* spp. (Ochi, 1986, 1987; Strauch *et al.*, 1991; Bascaran *et al.*, 1991; Hoyt & Jones, 1999). However, when the (p)pGpp synthetase-encoding gene (*relA*) of *Streptomyces coelicolor* A3(2) was characterized (Chakraburty *et al.*, 1996), a null mutation in the *relA* gene failed to produce actinorhodin and undecylenicodisogin in conditions of nitrogen limitation (Chakraburty & Bibb, 1997). A second RelA homologous protein, RshA, was also identified from the genome sequence of *S. coelicolor* (Mittenhuber, 2001). However, no experimental evidence is available to indicate if RshA, like RelA, can act as a bifunctional enzyme, both synthesizing and degrading (p)pGpp (Sun *et al.*, 2001). It has been reported that ppGpp was detected at very low levels prior to the accumulation of cephapycin C in *S. clavuligerus* NRRL3585, and that another form of guanine nucleotide, ppGp, was also simultaneously detected (Jones *et al.*, 1996). The synthesis of ppGp was not overtly associated with any ppGpp synthetic activity but rather with the ribosome. Nevertheless, ppGp was shown to play a regulatory role in the production of cephapycin C. In the current study, we describe the identification of the *relA/spoT* homologous genes, *relA* and *rsh*, from *Streptomyces clavuligerus* ATCC 27064. The involvement of the genes in morphological differentiation and the biosynthesis of antibiotics was demonstrated using mutants in which the *relA* and *rsh* genes were disrupted.

**METHODS**

**Bacterial strains, plasmids and maintenance.** *S. clavuligerus* ATCC 27064 and *Streptomyces lividans* TK24 were used throughout the current studies. Wild-type and mutants obtained were cultured on a solid inorganic salts starch medium (ISP4; Difco Laboratories). Spores formed on the agar plate medium (ISP4) were suspended in 20% (v/v) glycerol to give about 10^11 spores ml^{-1} and the spore suspension was stored at −70°C. *E. coli* DH5α was used for the propagation of cosmids. *E. coli* ESS was used as an indicator organism for determining the concentration of cephapycin C. *E. coli* ET12567 (MacNeil *et al.*, 1992) was used to obtain unmethylated DNA. Strains of *E. coli* were maintained on Lennox broth (LB) solid medium (Difco Laboratories) which consisted of 1% (w/v) Bacto tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose and 1.5% Bacto agar, supplemented with appropriate antibiotics for plasmid or cosmid maintenance. pWE15 (Wahl *et al.*, 1987) was used for the construction of the genomic library of *S. clavuligerus*, pBluescript KS(−) (Stratagene) was used as a general purpose cloning vector in *E. coli*. A *Streptomyces* high-copy-number plasmid containing a thiopeptin resistance gene, pLJ702 (Kieser *et al.*, 2000), and an *E. coli*–*Streptomyces* shuttle vector, pUWL-KS (Wehmeier, 1995) were used to prepare gene disruption mutants by double crossover. For complementation of disrupted genes, pSET152 (Bierman *et al.*, 1992) containing an apramycin resistance gene was used.

**Cloning and characterization of relA/spoT homologous genes.** DNA manipulation in *E. coli* and *Streptomyces* spp. was carried out according to the methods reported elsewhere (Sambrook & Russell, 2001; Kieser *et al.*, 2000). Restriction and modifying enzymes were used according to the manufacturers’ recommendations (Poscochem and Boehringer-Mannheim). DNA fragments were purified from agarose gels using the EZNA Gel Extraction Kit (Omega).

**Disruption of relA and rsh and phenotypic complementation.** The *relA/spoT* homologous genes were disrupted by insertion of a hygromycin resistance gene (*hyg*) and an apramycin resistance gene (*apr*) in the middle of each gene, respectively. In the case of the *relA::hyg* mutant construction, the *BglI–Smal* fragment (1542 bp) of *relA* in pSMF4103, a recombinant cosmid containing the *relA* region, was replaced with the *hyg* gene (1410 bp) in pOE829. The DNA fragment (2400 bp) containing *relA::hyg* was cloned into pBluescript KS(−), creating pSMF384. A *BglI–XhoI* fragment (3500 bp) from pSMF384 (which includes the *hyg* gene together with the remainder of *relA* and downstream sequences) was inserted into pLJ702 digested with *BglI*, creating pSMF387 and introduced into *S. lividans*, reisolated and introduced into *S. clavuligerus*, creating pSMF387. A disruption mutant defective in the *relA* gene resulting from replacement of *relA* by homologous recombination (double crossover) was isolated (Paradkar & Jensen, 1995). The transformant harbouring pSMF387 was subcultured five times in a rich medium (MMY; Stuttard, 1982) containing only hygromycin (200 μg ml\(^{-1}\)) and then transformants resistant to hygromycin but sensitive to thiopeptin (50 μg ml\(^{-1}\)) were selected as putative *relA* gene disruption mutants (AreA). The insertion inactivation of *relA* was confirmed by Southern hybridization (Sambrook & Russell, 2001) using the 717 bp SacI fragment, containing part of *relA* and part of *hyg*, labelled with the ECL direct nucleic acid labelling system (Amersham Pharmacia Biotech) as a probe. For the phenotypic complementation of the disrupted *relA* gene, a *BamHI–EcoRI* fragment (4500 bp) containing the entire *relA* for the construction of the cosmid library of *S. clavuligerus*, 100 μg *S. clavuligerus* chromosomal DNA was partially digested with Sau3AI (Poscochem) to produce a mean fragment size of approximately 50 kb. The reaction was stopped by phenol extraction, and the mixture was applied to a 50 ml sucrose gradient ranging from 10 to 40% and then ultracentrifuged at 100,000 g for 20 h. Fractions (1 ml) containing DNA fragments of 40–60 kb were pooled and concentrated by ethanol precipitation. The size-fractionated DNA (500 ng) was then ligated in a total volume of 20 μl with 1·4 μg BamHI-digested pWE15 that had been treated with calf intestinal alkaline phosphatase (Poscochem) to prevent recircularization. After ligation at 16°C for 12 h, the ligated DNA was packaged in bacteriophage λ particles by using the Giga Pack III Gold kit (Stratagene) and was introduced into *E. coli* DH5α by transduction. Oligonucleotide primers for PCR were designed based on the conserved sequences of *relA/spoT* homologous genes from various organisms. The synthetic oligonucleotides relAPI (5′-CTSCAGACGATCAGGAGGACAC-3′), relAPI (5′-CTTTGGGSSHSGCCGATGTAGGCCC-3′), spoTP1 (5′-CCC-AAGTTCACTSTACAGTCGTCGACG-3′) and spoTP2 (5′-CTCS-GGSSGSSACGGGGGTGCCAGCA-3′) were used as PCR primers for the amplification of internal segments of the *relA/spoT* homologue from *S. clavuligerus*. The PCR-amplified fragments were used to verify their identities and then used as probes to screen a pWE15-based cosmid library containing fragments of *S. clavuligerus* genomic DNA for cosmids containing *relA/spoT* homologous genes. The nucleotide sequence of the *relA/spoT* homologous gene region was determined by dideoxy-sequencing (Sanger method) using an automatic sequencer (ABI3730; Applied Biosystems). The regions encoding the *relA/spoT* homologous genes were sequenced on both strands and the entire sequence was assembled from sequence information obtained from analysis of overlapping clones using the DNAsis and Winstar programs (Hitachi Software). Sequences were compared with the database using the BLAST program (Altschul *et al.*, 1990) and multiple alignments were done with GENEDOC and CLUSTAL W (Bibb *et al.*, 1994; Higgins *et al.*, 1992). ORF analysis and prediction were done with FRAME 2.3.2 (www.nih.gov/∼/jung/cgi-bin/frameplot.pl; Ishikawa & Hotta, 1999). Phylogenetic analysis of the *RelA/SpoT* family of proteins was displayed using the PHYLIP program (Felsenstein, 1993).
Stringent responses in Streptomyces clavuligerus

sequence in pSMF4013 was cloned into an expression vector (pSET152), creating pSMF389. Plasmid pSMF389 was transformed into the non-methylating strain of E. coli ET12567 and the plasmid reisolated from the transformant of E. coli ET12567 was introduced by transformation to the protoplasts of the relA-disrupted mutant (ΔrelA). Strains showing a hygromycin-sensitive (HygS) and thiostrepton-sensitive (TsrS) phenotype were designated relA-complemented strains (C_relA).

To construct an rsh::apr mutant, an Ncol fragment (3840 bp) in pSMF41025, a recombinant cosmid containing the rsh (2217 bp) gene was used where the EcoRI–Apol fragment (551 bp) was replaced with the apr gene (1920 bp) in pSET152. The Ncol fragment (5320 bp) containing rsh::apr was cloned into plBlueScript KS(−), creating pSMF3812. A KpnI–XbaI fragment (5800 bp) from pSMF3812 was ligated to KpnI/XbaI-digested pUWL-KS, creating pSMF3813 and then introduced into S. lividans. Plasmid pSMF3813, purified from S. lividans, was then transformed into S. clavuligerus. A transformant harbouring pSMF3813 was subcultured five times using a rich medium (trypticase soy broth, TSB) containing only apramycin (50 µg ml⁻¹) and then Apr⁺ Tsr⁻ colonies were selected as potential disruption mutants (Δrsh). The insertion inactivation of the rsh gene was confirmed by Southern hybridization using a 1.7 kb Ncol–EcoRI fragment, containing part of rsh and part of apr, labelled with the ECL direct nucleic acid labelling system as a probe. For phenotypic complementation of the disrupted rsh gene, a 3.8 kb BamHI–EcoRI fragment, containing the entire rsh sequence and putative promoter region, in pSMF41025 was cloned into pUWL-KS, yielding plasmid pSMF3814. pSMF3814 was introduced by transformation into the non-methylating E. coli strain ET12567 and then reisolated and used to transform protoplasts of the rsh-disrupted mutant (Δrsh). Strains showing an Apr⁺ Tsr⁻ phenotype were designated rsh-complemented strains (C_rsh).

Analysis of intracellular (p)ppGpp levels. The stored spores were inoculated into a rich medium containing 0-4% (w/v) glucose, 0-4% yeast extract, 1-0% malt extract, 0-1% NZ-amime, 0-2% NaCl and 0-1% (w/v) inorganic salts mixture. The inorganic salts mixture consisted of 0-0025% MgSO₄.7H₂O, 0-0008% CuSO₄.5H₂O, 0-0015% FeSO₄.7H₂O, 0-0008% MnSO₄.4H₂O, 0-003% CaCl₂.2H₂O and 0-002% ZnSO₄.7H₂O. Culture growth in the seed culture medium was inoculated into the rich medium and cultured at 30 °C on a rotary shaker (150 r.p.m.). Seed cultures (2-5 ml) were used to inoculate 100 ml chemically defined (CD) medium supplemented with 1% Casamino acids. The CD medium consisted of 2% (w/v) glucose, 0-3% (NH₄)₂SO₄, 0-52% KH₂PO₄, 0-24% KH₂PO₄, 0-05% NaCl, 0-005% KCl, 0-005% MgSO₄.7H₂O and 0-0001% ZnSO₄.7H₂O (pH was adjusted to 7-0 before steam sterilization). Cultures growing in CD medium containing 1% Casamino acids were harvested during exponential growth phase by filtration (Whatman GF/C filters), washed rapidly with CD medium, resuspended in CD medium without Casamino acids and quickly returned to the shaking incubator. After the downshift, the culture samples were harvested at 0, 5, 10, 15, 30 and 60 min, then filtered. The filter cakes were transferred to 10 ml formic acid, incubated at 4 °C for 1 h and centrifuged for 10 min at 6000 × g. The supernatants (formic acid-extractable fractions) were clarified by filtration using a 0-45 µm filter and freeze-dried. The amount of ppGpp, pppGpp and GTP was determined by HPLC using a Partisol-10 SAX (4.6 × 250 mm) column (Whatman) as described previously (Ochi, 1986).

Antibiotic production and cultural characteristics of mutant strains. Antibiotic production by the wild-type and mutant strains of S. clavuligerus was assessed in a jar fermenter (KF-5L; Ko-Biotec). One millilitre of spore suspension was inoculated into 100 ml seed culture medium in 500 ml culture flasks and cultured at 30 °C for 3 d on a rotary shaker (150 r.p.m.). Seed culture medium (TSB) consisted of 1-0% (w/v) maltose, 0-5% glucose, 1-7% Bacto tryptone, 0-3% Bacto soytone, 0-25% NaCl, 0-25% K₃HPO₄ (pH was adjusted to 7-0 before steam sterilization). The seed culture (300 ml) was used to inoculate 3 l production medium in the jar fermenter. Production medium consisted of 1-0% (w/v) glycerol, 0-6% arginine, 0-2% KH₂PO₄, 0-06% MgSO₄.7H₂O, 0-005% FeSO₄.7H₂O, 0-005% CaCl₂.2H₂O, 0-005% MnCl₂.4H₂O, 0-005% ZnCl₂. The culture temperature was maintained at 30 °C and pH was adjusted to 7-0 by automatic addition of 1 M HCl or 1 M NaOH. Agitation was fixed at 300 r.p.m. and aeration was controlled to 1 vol. air (vol. medium)⁻¹ min⁻¹. To measure cell growth, triplicate samples (10 ml) of cultures were collected on pre-weighed filters (Whatman GF/C) by vacuum filtration. The filter cakes were washed twice with 10 ml distilled water and dried at 80 °C for 24 h and reweighed. The concentration of cephamycin C was assayed using the agar-diffusion method with the indicator organism E. coli ESS, and the concentration of clavulanic acid was quantified by HPLC after derivatization with imidazole (Foulstone & Reading, 1982; Paradkar & Jensen, 1995).

RESULTS

Construction of a genomic library and identification of relA/spoT genetic homologues from S. clavuligerus

A genomic library of S. clavuligerus was constructed using the cosmid vector pWE15. Probes for relA and spoT were constructed, independently, based on the published sequences of the two genes from various bacterial species. As a result of colony hybridization and Southern analysis using these probes, two plasmids, pSMF4103 and pSMF41025, were found to contain two ORFs that hybridized with the probes specific for the relA and spoT genes, respectively. The sequences of DNA containing the orf1 region in pSMF4103 (3561 bp; accession no. AF421216) and the orf2 region in pSMF41025 (3360 bp; accession no. AF421217) were deposited with GenBank. A DNA sequence comparison revealed that orf1 showed 88% homology to the relA gene of S. coelicolor (Chakraburtty et al., 1996) and that orf2 showed 67% homology to the rshA gene of S. coelicolor (Sun et al., 2001). The organization of the genes in the orf1 and orf2 regions of the plasmids, and the domains in the proteins as deduced from their DNA sequences are compared in Fig. 1. The HD domain is a predicted metal-chelating region in a new superfamily of metal-dependent phosphohydrolases (Aravind & Koonin, 1998). All putative bifunctional enzymes (the RelA/SpoT homologues) possess the HD domain and the domain is sufficient for (p)ppGpp hydrolase activity (Gentry & Cashel, 1996). The TG5 and ACT domains are conserved ATP/GTP-binding and GTP-binding domains, respectively (Gentry & Cashel, 1996). The presence of these conserved motifs in RelA and Rsh is in agreement with their biochemical functions because ATP and GTP are substrates of the reaction catalysed by (p)ppGpp synthetase. RelA and Rsh possess three domains, a (p)ppGpp-degrading domain and a (p)ppGpp synthesising domain (Fig. 1c and d).

A phylogenetic tree showed that there was a clear separation of the RelA and SpoT families on one hand and of two Rel
families of Gram-positive origin (Bacillus/Clostridium group and an actinobacterial group) as reported by Mittenhuber (2001) on the other. The protein (RelA) deduced from the orf1 sequence was grouped with other RelA proteins identified from Streptomyces spp. The protein (Rsh) deduced from the orf2 sequence was grouped only with the RshA protein of S. coelicolor (Fig. 2). From these comparisons, orf1 was tentatively assigned as a relA gene encoding a RelA-like protein, and orf2 as a rsh gene encoding a RshA-like protein in S. clavuligerus.

Disruption of relA and rsh genes and complementation of the disrupted genes

ΔrelA and Δrsh mutants were constructed by gene replacement at the relA and rsh loci in wild-type S. clavuligerus, respectively. When pSMF387 containing the relA::hyg region was introduced into wild-type S. clavuligerus, seven putative ΔrelA mutants were obtained. When pSMF3813 containing the rsh::apr region was introduced into wild-type S. clavuligerus, six putative Δrsh mutants were obtained. Two putative C_relA strains were obtained by introducing pSMF389 harbouring the entire relA gene into ΔrelA mutants. Three putative C_rsh strains were obtained by introducing pSMF3814 harbouring the entire rsh gene into Δrsh mutants. Southern hybridization data with a SacII fragment probe containing part of relA gene showed that the size of the positive band in the wild-type, the ΔrelA mutant and the C_relA strain was 717, 648 and 717 bp, respectively (Fig. 3a). In Southern blot analysis with an NcoI/EcoRI fragment probe containing the rsh gene, the size of the positive band in the wild-type, the Δrsh mutant and the C_rsh strain was 3-8, 5-2 and 3-8 kb, respectively (Fig. 3b). These data confirmed that the disruption and complementation events had taken place in each gene disruption mutant and complemented strain, respectively.

The relA and rsh genes are involved in synthesis of ppGpp and pppGpp

The concentrations of ppGpp, pppGpp and GTP in cells of the wild-type and mutant strains after depletion of Casamino acids were determined (Fig. 4a, b and c). The synthesis of ppGpp and pppGpp in the wild-type began immediately after the depletion of Casamino acids. pppGpp concentrations had already reached a peak by 5 min after removal of Casamino acids and declined rapidly thereafter. ppGpp levels rose more slowly to peak at 10 min after removal of Casamino acids and also declined more slowly. Both compounds were below detectable levels after 1 h (data...
not shown). In contrast, the production of ppGpp and pppGpp was completely abolished in the ΔrelA mutant. The Δrsh mutant also showed a decrease in the levels of ppGpp and pppGpp relative to the wild-type, but still retained some ability to accumulate these metabolites. The data indicate that the relA gene has a more dramatic effect on the production of ppGpp and pppGpp than does rsh. The profiles of GTP in the different strains were inversely related to those of ppGpp and pppGpp. The ΔrelA mutant showed no decrease in GTP pool size after nutritional downshift (Fig. 4c).

The relA and rsh genes are involved in morphological and physiological differentiation

The morphological characteristics of the wild-type and the disruption mutants were compared in cultures grown on ISP4 solid medium for 28 days. In comparison with the wild-type, the ΔrelA mutant showed a significantly retarded growth rate and a bald appearance, resulting from the lack of aerial mycelia and spores (Fig. 5). The growth of the Δrsh mutant was not significantly retarded (Fig. 5). Mycelia cultured for 3 d on cover slips inserted into ISP4 medium were also observed by phase-contrast microscopy (Fig. 6). It was evident that the ΔrelA mutant grew to form short, branched and clumped mycelia, whereas the Δrsh mutant grew in a manner more similar to the wild-type, but with slightly straighter hyphae. The mycelial growth of the ΔrelA mutant in submerged culture was retarded compared to the wild-type, whereas the growth of the Δrsh mutant was not greatly altered (Fig. 7a). The relA and rsh genes were also shown to be involved in the production of secondary metabolites such as clavulanic acid and cephamycin C in S. clavuligerus. The levels of cephamycin C and clavulanic acid in ΔrelA and Δrsh mutants were very significantly reduced by the disruption of the relA and rsh genes, relative to those in wild-type of S. clavuligerus (Fig. 7b and c).

DISCUSSION

This paper is the first report of the identification of two relA/spoT homologue genes, relA and rsh, from S. clavuligerus, in conjunction with an assessment of their involvement in morphological differentiation and antibiotic production (physiological differentiation) in this species. The synthesis of ppGpp and pppGpp in the wild-type were apparent...
within 10 min following depletion of Casamino acids. However, production of these metabolites was entirely abolished in the ΔrelA mutant despite the presence of an intact rsh gene. Interestingly, the production of ppGpp and pppGpp in the Δrsh mutant was also lowered compared to the wild-type, again despite the presence of an intact relA gene. These data indicate that the relA gene plays an essential role in the production of (p)ppGpp upon depletion of amino acids, which is in agreement with the report of Haseltine & Block (1973). Since the role of the rsh gene in the production of (p)ppGpp may be normally obscured by its more abundant degradative activity as reported by Gentry & Cashel (1996), the rsh null mutant had little effect on (p)ppGpp production and mycelial morphology.

The morphological differentiation of S. clavuligerus was also very significantly affected by the disruption of the relA gene, but not greatly by the disruption of the rsh gene. The altered morphological characteristics were completely restored by the complementation of the corresponding disrupted genes.

Fig. 3. Southern blot analysis showing the disruption and complementation of relA and rsh genes. SacII-digested (a) and NcoI-digested total DNAs (b) from ATCC 27064 wild-type strain, disruption mutants (ΔrelA and Δrsh) and complemented strains (CrelA and Crsh) of S. clavuligerus were separated by agarose gel electrophoresis (left). Replica electropherograms were blotted onto a nylon membrane and hybridized with the 717 bp SacII fragment probe (a) and the 1.7 kb NcoI/EcoRI fragment probe (b). The marker DNA is the 1 kb Plus DNA Ladder (Gibco) (a) or HindIII-digested λ DNA (b).

Fig. 4. Time-course comparison of the amount of ppGpp (a), pppGpp (b) and GTP (c) under amino acid downshift conditions in wild-type (filled circles), ΔrelA (open squares) and Δrsh (open circles) mutant strains of S. clavuligerus. After the downshift from CD medium containing 1% Casamino acids to the medium without Casamino acids, culture samples at 0, 5, 10, 15, 30 and 60 min were analysed for the amount of intracellular ppGpp, pppGpp and GTP using HPLC.
Failure to produce aerial mycelium in the ΔrelA mutant accompanied by no decrease in GTP pool size after nutritional downshift (Fig. 4c) is in agreement with the proposal that morphological differentiation of *Streptomyces* spp. is directly controlled by intracellular GTP level (Okamoto & Ochi, 1998). Thus, it was apparent that mycelial morphology and antibiotic production are severely affected by the disruption of the *relA* gene and both of these phenotypes are presumably associated with the consequent inability of the mutants to accumulate ppGpp and pppGpp after amino acid starvation. These results were very similar to those observed for the *relA* null mutant of *S. coelicolor* (Chakraburtty & Bibb, 1997). However, mutation of the *rsh* gene gave only minor changes in ppGpp and pppGpp accumulation or mycelial morphology, but nonetheless resulted in a major defect in production of cephamycin C and clavulanic acid. The effects of the *rsh* gene on physiological differentiation in *S. clavuligerus* are very different from those observed for *S. coelicolor*, where deletion of *rshA* had no effect on the production of the pigmented antibiotics, actinorhodin and undecylprodigiosin (Sun et al., 2001). It is conceivable that production of cephamycin C and clavulanic acid in *S. clavuligerus* is more sensitive to the intracellular level of (p)ppGpp than is actinorhodin production in *S. coelicolor*. Thus, only minor changes in (p)ppGpp production resulted in a major defect in antibiotic production in *S. clavuligerus*. It seems plausible that the stringent response of *S. clavuligerus* to starvation for amino acids is governed mainly by RelA and that the (p)ppGpp synthesized immediately after depletion of amino acids triggers the initiation of pathways for both morphological and physiological differentiation in this species.

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**Fig. 5.** Morphological characteristics in wild-type (wild), the disruption mutants (ΔrelA and Δrsh) and the complemented strains (C<sub>relA</sub> and C<sub>rsh</sub>) of *S. clavuligerus*. The morphological comparison was carried out on ISP4 solid medium (28 days) for all strains.

**Fig. 6.** Micrographs of wild-type, ΔrelA mutant and Δrsh mutant strains of *S. clavuligerus*. The strains were grown on cover slips inserted into ISP4 solid medium at 30 °C for 3 days. Mycelia on cover slips were observed by phase-contrast microscopy (Leica DM IRF2 and IRB). Bars, 12.5 (a) and 1 μm (b).
REFERENCES


