Cloning, characterization and disruption of a (p)ppGpp synthetase gene (relA) of Streptomyces coelicolor A3(2)

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Summary
An internal segment of the (p)ppGpp synthetase gene, relA, of Streptomyces coelicolor A3(2) was amplified from genomic DNA using the polymerase chain reaction and used as a hybridization probe to isolate the complete gene from a cosmid library. relA lies downstream of a gene (apt) that apparently encodes adenosine phosphoribosyltransferase and is transcribed from two promoters, relAp1 and relAp2, and by transcriptional readthrough from apt. While the level of relAp2 transcripts remained relatively constant, relAp1 activity apparently peaked during transition phase, following a decline in readthrough transcription from apt. Disruption of relA using an att derivative of the temperate phage fiC31 abolished ppGpp synthesis on amino acid depletion. When grown on agar, the disruptants grew more slowly than a control lysogen or the cessation of growth, in determining the onset of secondary metabolism (Chater and Bibb, 1996).

In Escherichia coli, the highly phosphorylated guanine nucleotides ppGpp and pppGpp have been implicated in growth-rate control of gene expression (Sarubbi et al., 1988; Hernandez and Bremer, 1990; 1993; Schreiber et al., 1991), and in regulating stationary-phase gene expression, mediating at least some of their effects through activation of the stationary-phase sigma factor, σ5 (Gentry et al., 1993). Does (p)ppGpp play a role in growth-phase-dependent gene expression in streptomycetes and, more specifically, in triggering the onset of antibiotic production? Positive correlations were first observed between (p)ppGpp and antibiotic biosynthesis in Streptomyces aureofaciens (Simuth et al., 1979) and Streptomyces gali- laeus (Hamagishi et al., 1980), producers of chlorotetra-cycline and aclacinomycin, respectively. Later, Ochi (1986) observed an eightfold increase in formycin production by Streptomyces lavendulae after provoking (p)ppGpp accumulation by amino acid depletion. Moreover, a relC mutant of S. lavendulae defective in ribosomal protein L11, and consequently deficient in (p)ppGpp synthesis on amino acid starvation, was impaired in formycin production (Ochi 1986; 1990a). Similar relC mutants were isolated from Streptomyces antibioticus (Ochi, 1987), Strepto- myces griseoflavus (Ochi, 1988), Streptomyces griseus (Ochi, 1990a), and Streptomyces coelicolor (Ochi, 1990b).

The mutants accumulated only low levels of (p)ppGpp after nutritional shiftdown (on average c. 15% of wild type) and were deficient in antibiotic production, leading to the suggestion that (p)ppGpp plays a central role in triggering antibiotic biosynthesis (Ochi, 1990b). Consistent with this idea, reduced levels of actinomycin biosynthetic enzymes and mRNA were found in a relC mutant of S. antibioticus (Kelly et al., 1991), and (p)ppGpp accumulation was noted to coincide with transcription of the pathway-specific activator gene, bpaA, for bialaphos production by Streptomyces hygroscopicus (Holt et al., 1992). However, Bascaran et al. (1991) concluded that (p)ppGpp did not play a role in antibiotic production in Streptomyces clavuligerus. Production of cephalosporins (mainly cephamycin C) occurred during a phase of slow exponential growth (doubling times of c. 7h and 18h in rich and minimal media, respectively), and increased in stationary phase, while ppGpp levels remained constant from the beginning of growth. relC-like mutants accumulated lower amounts of ppGpp than the wild-type strain after nutritional shiftdown.

Introduction
Streptomycetes are Gram-positive mycelial soil bacteria that undergo a complex process of morphological differentiation leading to sporulation (Chater and Hopwood, 1993). They also produce a plethora of secondary metabolites, many with important applications as antibiotics in human medicine and in agriculture. Antibiotic production in streptomycetes is generally confined to stationary phase in liquid cultures, and usually coincides with the onset of morphological differentiation in agar-grown cultures. While little is understood of the mechanisms that regulate antibiotic production (Champness and Chater, 1994), most of the published data are consistent with a role for growth rate.

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(varying between 8% and 85% of the wild-type levels), but there was no simple correlation between the levels of ppGpp and cephalosporin production (some produced more, and others less, cephalosporin than the wild-type strain).

We have examined the relationship between (p)pGpp synthesis and the production of undecylprodigiosin and actinorhodin in S. coelicolor (Strauch et al., 1991; Takano et al., 1992; Takano and Bibb, 1994). Transcription of pathway-specific activator genes for each antibiotic (redD for undecylprodigiosin and actII-ORF4 for actinorhodin) conspicuously increased as the culture entered stationary phase, coinciding with a peak in the level of (p)pGpp; this was followed by transcription of representative biosynthetic structural genes, and production of the antibiotics. In contrast, transcription of the rrnD mRNA gene set decreased markedly (E. Takano and M. J. Bibb, unpublished). (p)pGpp synthesis was also induced by amino acid depletion during rapid exponential growth, and was followed by an immediate and marked decrease in rrnD transcription (Strauch et al., 1991). Transcription of actII-ORF4 was detected within 30 min of the nutritional shift-down and transcription of the act biosynthetic genes after a further 30 min (Takano and Bibb, 1994).

Although correlations between (p)pGpp synthesis and the onset of antibiotic production clearly exist, they do not establish a role for the highly phosphorylated nucleotides in triggering antibiotic biosynthesis. Although the relC mutants isolated by Ochi (1986; 1987; 1988; 1990a,b) show a marked reduction in antibiotic production, they grow at about half the maximal rate of the parent strain, presumably reflecting impaired protein synthesis. Thus it is difficult to assess whether the effect on antibiotic production is a direct consequence of reduced levels of (p)pGpp, or an indirect effect of the L11 mutation on protein synthesis.

In E. coli, (p)pGpp is synthesized predominantly by the ribosome-bound RelA, which is activated when an uncharged tRNA binds to the acceptor site of translating ribosomes (Cashel and Rudd, 1987). Therefore (p)pGpp synthesis can be invoked by amino acid depletion or by addition of tRNA synthetase inhibitors, such as serine hydroxamate (SHX) (Tosa and Pizer, 1971). Interestingly, the enzyme responsible for (p)pGpp degradation in E. coli, SpoT, is a homologue of RelA, the two proteins showing 31% amino acid sequence identity (Metzger et al., 1989). Although the mechanism is not understood, starvation for carbon and energy reduces SpoT activity and results in elevated levels of (p)pGpp (Cashel and Rudd, 1987). Furthermore, the residual level of (p)pGpp synthetic activity observed in relA null mutants probably reflects a synthetic role for SpoT in addition to its degradative function (Hernandez and Bremer, 1991; Xiao et al., 1991), although this has not been demonstrated in vitro. A relA homologue from Vibrio sp. strain S14 has been cloned, sequenced and disrupted (Flärdh et al., 1994); the mutant exhibited relaxed control of RNA synthesis, and failed to accumulate (p)pGpp upon addition of SHX. relA-dependent (p)pGpp synthesis was also invoked by carbon-source depletion, which is not observed in E. coli; presumably glucose deprivation in the vibrio results in a rapid decrease in the level of aminoacylation of at least some tRNAs. relA homologues have also been identified in Streptococcus equisimilis H46A (Mechold et al., 1993), Mycobacterium leprae (EMBL Accession Number U00011) and Mycoplasma genitalium (Peterson et al., 1995); genetic and/or biochemical studies that might differentiate between synthetic and degradative roles for these genes have not been published. The isolation of relC mutants of S. coelicolor (Ochi, 1990b), and the ability of the wild-type strain to produce (p)pGpp on amino acid depletion or on addition of SHX (Strauch et al., 1991), suggests that S. coelicolor possesses a ribosome-dependent (p)pGpp synthetase. In an attempt to establish if (p)pGpp plays an essential role in triggering antibiotic production in S. coelicolor, we set out to isolate and disrupt its relA homologue.

Results
Isolation of a relA homologue from S. coelicolor using the polymerase chain reaction
Alignment of the amino acid sequences of RelA homologues from E. coli (RelA (Metzger et al., 1988) and SpoT (Metzger et al., 1989)), M. leprae (EMBL U00011), S. equisimilis H46A (Mechold et al., 1993) and Vibrio sp. strain S14 (Flärdh et al., 1994) revealed several blocks of conserved amino acids (Fig. 1). Those corresponding to amino acid residues 317–324 and 426–433 of E. coli RelA were chosen for the design of 16-fold and 64-fold degenerate oligonucleotides for use as primers in the polymerase chain reaction (PCR), using the preferred codons from 64 streptomyctenes (Wright and Bibb, 1992); BamHI sites were incorporated at the 5' end of each primer to facilitate subsequent cloning. An amplified product of approximately 400 bp was obtained, consistent with the size expected for a relA homologue. The PCR product was digested with BamHI and cloned in BamHI-cleaved pBluescript SK+ in E. coli DH5α, yielding pJ6034. Sequencing from both ends of the pJ6034 insert using universal and reverse primers revealed the expected internal segment of a relA homologue.

The pJ6034 insert was isolated as a BamHI fragment, labelled with 32P by random oligonucleotide priming, and used as a probe to isolate six hybridizing cosmids from a library of S. coelicolor M145 DNA. Digestion of each of the six cosmids DNAs with BglII, Pvull, SacI and

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EcoRI + BamHI revealed common restriction fragments, and Southern analysis using the same probe identified hybridizing PstI fragments of approximately 4 kb and 6 kb. Cloning of these gel-purified PstI fragments in PstI-cleaved pBluescript SK+ yielded pIJ6031 (4 kb insert) and pIJ6037 (6 kb insert). A restriction map of the contiguous 10 kb region is shown in Fig. 2. Hybridization of the probe to cosmids L2 and L7 (H. M. Kieser, personal communication) localized the relA homologue on Asel fragment L and, more precisely, to a position between bidD and argA in the 12 o’clock region of the combined physical and genetic map of the S. coelicolor chromosome (Kieser et al., 1992).

RelA of S. coelicolor has an N-terminal extension that is absent from all known homologues

The sequence of a 4515-nucleotide (nt) region that extends 2058 nt upstream and 2457 nt downstream of the PstI site within relA was determined (EMBL X87267). Analysis of the sequence using the frame program (Bibb et al., 1983) revealed three open reading frames (ORFs) with protein-coding character (Fig. 2). The largest of these (relA; nt positions 796–3336) would encode a protein of 647 amino acids (94.2 kDa) with a high degree of similarity to all of the known RelA homologues (Fig. 1). S. coelicolor RelA is most similar to the homologue from M. leprae (66% amino acid sequence identity), and least similar to RelA of Vibrio sp. strain 14 (38% identity), and has an N-terminal extension of approximately 90 amino acids that is absent from all of the other homologues. Usually, translation of the relA transcript apparently initiates at a UUG codon (Fig. 3). Five nucleotides upstream of the UUG codon there is a sequence (AGGAG) that is complementary to a region close to the 3’ end of the 16S rRNA of S. coelicolor (Baylis and Bibb, 1987) that is likely to serve as a ribosome-binding site.

A second ORF of 546 nt (nt positions 65 to 610) occurs upstream of, and reading in the same direction as, relA and is preceded 7 nt upstream by a potential ribosome-binding site (GGA). The ORF would encode a protein of 182 amino acids (18.6 kDa) with a high degree of identity to adenine phosphoribosyltransferases (APTs) from a variety of organisms. The most similar homologue found in searches of the databases was the APT of E. coli (51% amino acid sequence identity; Hershey and Taylor, 1986). APTs catalyse the conversion of adenine and 5-phosphoribosyl pyrophosphate to AMP in a salvage reaction that is energetically less costly than de novo synthesis.

The third ORF of >1095 nt is located 3’ of relA and is oriented in the opposite direction (nt positions 3421 to 4515; Fig. 2). It terminates 86 nt from the end of the relA coding region; its N-terminus appears to occur outside of the sequenced region. Searches of the databases revealed similarity (46% amino acid sequence identity in an overlap of 175 amino acids) to an ORF of unknown function from M. leprae (EMBL U00019) that is not located adjacent to the mycobacterial relA homologue. A 22 nt inverted repeat occurs in the non-coding region between relA and the convergent ORF (nt positions 3358–3404), and may function as a factor-independent bidirectional transcriptional terminator (reviewed in Lewin, 1994).

Transcription of relA occurs from multiple promoters in a growth-phase-dependent manner

To map the transcriptional start site of relA, S1 nuclease protection experiments were carried out using RNA from S. coelicolor strain M145 at different stages of growth. A 458 bp SacI–SalI fragment (nt positions 474–931) that includes the non-coding region between apt and relA (Fig. 2) was cloned in pBluescript SK+ that had been cleaved with Smal and SalI, yielding pIJ6068. The 498 bp SalI–SalI fragment of pIJ6068, uniquely labelled at the 5’ end of the SalI site (which lies 136 nt downstream of the UUG start codon) was used as probe; the presence of 40 nt of poly linker at the unlabelled end of the probe served to distinguish between probe reannealing and transcriptional readthrough from apt. Putative transcriptional start sites were identified at nt positions 682 and 751–752 (relAp1 and relAp2, respectively, in Figs 3 and 4b); a protected fragment corresponding in size to the Stul–SalI segment of the probe (Fig. 4b; FLP) indicated additional transcription of relA from a promoter upstream of, or within, apt. While the level of transcripts initiated at relAp2 remained constant, transcription from relAp1 appeared to peak during transition phase, following a marked decrease in the level of transcriptional readthrough from apt (Fig. 4b). An inverted repeat of nine nucleotides occurs between the apt and relA coding regions (nt positions 716–738), and overlaps the –35 region of relAp2 (Fig. 3).

Since the changes in the levels of the relAp1 and readthrough transcripts coincided with elevated levels of ppGpp during transition phase, the effect of nutritional shiftdown on relA transcription was assessed (Fig. 5). While there was a clear decrease in the level of the readthrough transcript 30 min after shiftdown, there was a marked increase in the amount of relAp1 transcript.

To confirm that the RNA-protected fragments assigned to relAp1 and relAp2 represented transcriptional start sites, rather than artefacts of S1 nuclease digestion, the 498 bp SacI–SalI and 466 bp BamHI–SalI fragments of pIJ6068 were used as templates for in vitro transcription assays using RNA polymerase isolated from S. coelicolor strain M145. Both fragments gave run-off transcripts of approximately 250 nt and 180 nt (Fig. 6) that corresponded to the expected sizes predicted from the S1
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Fig. 1. Alignment of the amino acid sequence of *S. coelicolor* RelA (Screla) with *E. coli* ReiA (Ecrela), *E. coli* SpoT (Ecspot), *Vibrio* sp. strain 14 RelA (Vsrela), and RelA homologues from *M. leprae* (Mlrel) and *S. equisimilis* (Serel). The sequences were aligned and displayed using the PILEUP and PRETTYBOX programs, respectively, of the UWGCG package (Devereux et al., 1984). The locations of the conserved amino acid sequences that were used to design the PCR primers are indicated by arrows under the alignment. Black boxes indicate amino acids present in at least four of the six sequences, and shaded boxes represent conservative substitutions at these positions.


Disruption of relA results in abolition of ppGpp synthesis on amino acid depletion, but has no marked effect on actinorhodin production.

To assess the role of relA in (p)ppGpp synthesis and antibiotic production in *S. coelicolor*, the gene was disrupted using an att derivative of the temperate *Streptomyces* phage φC31 (Bruton and Chater, 1983). An internal 934 bp segment of the relA coding region (Fig. 2) that extended from a BamHI site (nt position 1290) located 494 nt downstream of the translational start codon to a PvuII site (nt position 2227) located 1108 nt upstream of the translation stop codon was cloned in the *E. coli* vector pMT3000 (Page et al., 1994) that had been cleaved with BamHI and SmaI, yielding pJ6070. The internal relA fragment was excised from pJ6070 as a 942 bp BamHI-SstI fragment, and ligated with the att-c+ φC31 phage vector KC516 (Rodicio et al., 1985) that had been cleaved with BglII and SstI; KC516 carries the tsr gene that confers resistance to the antibiotic thiostrepton (Thio). The ligated mixture was used to transfect protoplasts of *S. lividans* strain 1326, and likely recombinant clones were identified by plaque hybridization using the 934 bp BamHI-relA-PvuII fragment labelled with 32P by random oligonucleotide priming as a probe. Restriction analysis of phage DNA derived from eight positive plaques revealed that six contained the required construct, KC936. After single-plaque purification, high-titre phage stocks of KC936, and of the att+c+ phage vector KC301 (Hopwood et al., 1985), were prepared from *S. lividans* 1326 top layers and spotted onto lawns of *S. coelicolor* strain J1929, a Pgl- derivative of *S. coelicolor* M145 (Bedford et al., 1995). After sporulation, the plates were replicated to the rich medium R5 supplemented with 5 µg ml−1 Thio to obtain lysogens. After two rounds of single-colony purification on the same selective medium, seven independent ThioR isolates derived from KC936 and one from the control phage KC301 were replicated to lawns of *S. lividans* 1326 spores and shown to release phages. Southern analysis of BamHI-digested total DNA isolated from each of the KC936 lysogens using the purified BamHI-PvuII fragment as a 32P-labelled probe confirmed disruption of relA (Fig. 7). The 3.2 kb BamHI fragment in J1929 was
replaced by fragments of 5.1 kb and > 40 kb, as predicted from the restriction maps of the relA region and of KC516. Southern analysis of the same DNA samples digested with PstI again gave the pattern expected from disruption of relA.

All seven independent J1929::KC936 relA disruptants were streaked out for single colonies on R5 and minimal medium, each supplemented with 5 µg ml⁻¹ Thio, together with the control lysogen J1929::KC301. All of the disruptant colonies grew more slowly than those containing KC301. All of the J1929::KC936 derivatives appeared to sporulate normally and produced the purple-pigmented antibiotic actinorhodin, but their mature colonies were significantly smaller than those of J1929::KC301. The difference in growth rate and in the size of mature colonies was also observed on R2, SFM and SMMS agar media, both in the presence and absence of 5 µg ml⁻¹ Thio.

To assess the effect of disrupting relA on (p)pGpp synthesis, J1929::KC936 and J1929::KC301 were grown in SMM liquid medium containing 5 µg ml⁻¹ Thio until mid-exponential phase (OD₄₅₀ = 0.5) and subjected to amino acid depletion by rapidly transferring the cultures to SMM lacking amino acids. Nucleotides were extracted with 1 M formic acid 0,7.5 and 15 min after nutritional shift-down and analysed by high-performance liquid chromatography (HPLC) (Fig. 8). While the levels of ppGpp in J1929::KC301 7.5 min after amino acid depletion (542 and 292 pmol mg⁻¹ dry weight in duplicate experiments) were comparable to earlier estimates for S. coelicolor strain M145 (200 pmol mg⁻¹ dry weight; Strauch et al., 1991), there was no increase in the level of ppGpp in the relA disruptant J1929::KC936 (7.3 and 7.8 pmol mg⁻¹ dry weight, respectively). While the level of GTP in the relA⁻ J1929::KC301 dropped markedly on shift-down, there was no reduction in J1929::KC936 (Fig. 8).

Growth of S. coelicolor in SMM is limited by the availability
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of nitrogen (E. Takano and M. J. Bibb, unpublished), and (p)ppGpp synthesis occurs in S. coelicolor strain M145 during the transition between exponential growth and stationary phase (Strauch et al., 1991). To assess the effect of the relA mutation on ppGpp synthesis during this transition period, ppGpp levels were monitored by HPLC. While the level of ppGpp peaked at 14.3 pmol mg\(^{-1}\) dry weight in J1929::KC301 (similar to the 16 pmol mg\(^{-1}\) dry weight observed by Strauch et al., 1991), it remained at a constant level in J1929::KC936.

Disruption of relA appeared to have no deleterious effect on actinorhodin production in liquid culture, either after nutritional shiftdown or upon entry into stationary phase. On the contrary, in both cases actinorhodin production by J1929::KC936 preceded that by J1929::KC301 by several hours. Surprisingly, in contrast to the agar-grown cultures, J1929::KC936 did not show a reduction in growth rate in liquid SMM when compared with J1929::KC301. With agar-grown cultures, although actinorhodin production in the mutant was delayed by a few hours on SMMS, there was no perceptible difference on minimal medium, R2 and R5, and on SFM it occurred several hours earlier than in the relA\(^+\) strain. The same results were obtained both in the presence and absence of Thio.

Discussion

Earlier attempts to isolate a (p)ppGpp synthetase gene from S. coelicolor using cloned relA homologues from E. coli (54 mol% G+C), Serratia marcescens (59%) and M. leprae (59%) as hybridization probes failed (Takano, 1993; R. Chakraburtty, unpublished), presumably reflecting the considerable differences in base composition between genera (the GC content of the S. coelicolor relA homologue is 70.4%). In contrast, the PCR-based strategy reported here readily allowed for the isolation of a relA homologue from S. coelicolor; comparison of the degenerate primer sequences with the sequence of relA revealed 20/23 identities for the 5' primer and 23/23 for the 3' primer. Indeed, the same primers and amplification conditions gave PCR products of a similar size from Mycobacterium smegmatis and Myxococcus xanthus genomic DNAs (with GC contents deduced from the nucleotide sequence databases of 65% and 68%, respectively; R. Chakraburtty, unpublished), suggesting that they may be useful for the isolation of relA homologues from other bacterial species with a relatively high genomic GC content.

Although the PCR product might have represented a
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Fig. 7. Southern analysis of relA disruptants of S. coelicolor J1929. One microgram of BamHI-digested genomic DNA from seven independent J1929::KC936 isolates, and from the parental strain J1929 was probed with the purified BamHI–relA–PvuII fragment that had been labelled with 32P by random oligonucleotide priming. SM, size markers derived from phage λ DNA that had been digested with PstI (the filter was also probed with phage λ DNA that had been labelled by random oligonucleotide priming).

functional homologue of spoT involved in (p)ppGpp degradation rather than synthesis, disruption using the attC31 derivative KC516 established that the S. coelicolor gene encodes a (p)ppGpp synthetase. ppGpp synthesis invoked by amino acid depletion in mid-exponential phase, and during the transition between exponential growth and stationary phase under conditions of nitrogen limitation, was abolished in the relA mutant. While ATP levels after shiftdown were similar in both J1929::KC936 and J1929::KC301 (perhaps reflecting the higher intracellular concentrations and wide-ranging metabolic role of ATP), the concentrations of CTP and UTP were higher in J1929::KC301 than in the mutant, presumably indicating stringent control of stable RNA synthesis in the relA− strain. While GTP levels in the relA mutant after shiftdown were similar to those of the other NTPs, they fell markedly in the relA+ strain (as previously noted by Strauch et al., 1991), presumably reflecting, at least in part, ppGpp synthesis.

Since the level of ppGpp observed in the relA mutant (on average 1.7% of that in the relA+ strain after shiftdown and close to the limit of detection of the HPLC assay) did not increase upon amino acid depletion, it seems unlikely that it reflects reconstitution of a functional relA gene by excision of KC936 (Southern analysis failed to reveal any prophage excision in cultures grown in the presence of Thio; Fig. 7). It may reflect relA-independent ppGpp synthesis mediated either by a functional homologue of spoT or by a homologue of a novel (p)ppGpp synthetase activity recently identified in S. antibioticus (Jones, 1994a,b; G. H. Jones, personal communication). Alternatively, the N-terminal segment of RelA that remains after disruption may possess synthetic activity that does not require ribosome activation; consequently, the level of ppGpp would not be expected to increase upon amino acid depletion, consistent with the experimental observations.

The results presented here suggest that elevated levels of (p)ppGpp are not essential for triggering actinorhodin production in S. coelicolor. However, since differences in the timing of actinorhodin synthesis were observed between J1929::KC936 and J1929::KC301 with some media, it is possible that variations in the level of ppGpp can influence the onset of actinorhodin production. Moreover, since the relA mutant still makes ppGpp, we cannot exclude the possibility that the phosphorylated nucleotide is required for transcription of antibiotic biosynthetic genes. In this context, it may be pertinent to note the effect of disrupting relA of S. coelicolor J1501 with KC936 (data not shown). J1501 (hisA1 uraA1 strA1 pgf− SCP1− SCP2−) is a commonly used strain, but for reasons that are not understood it produces much less actinorhodin and undecylprodigiosin than prototrophic plasmid-free strains. It also differs from the wild-type strain in the regulation of carbon-source utilization (S. Angell, personal communication) and its genome has undergone several large deletions and duplications (Kieser et al., 1992). Disruption of relA with KC936 reproducibly gave J1501::KC936 lysogens that were either unaffected in antibiotic production or that produced undecylprodigiosin but not actinorhodin; the two phenotypes occurred in approximately equal numbers. While the basis for this heterogeneity and for the difference from J1929 is not clear, it is possible that the metabolism of ppGpp in J1501 differs from the wild-type

Fig. 8. HPLC analysis of ppGpp and NTP levels after amino acid depletion of J1929::KC301 (relA+) and J1929::KC936 (relA). The results are the average values from two independent experiments. NTP levels were standardized to those observed immediately before amino acid depletion in each culture (100%); ppGpp levels were standardized to the levels observed 7.5 min after shiftdown in the J1929::KC301 cultures. Nucleotide concentrations (in pmol mg−1 dry weight) immediately before shiftdown were: J1929::KC301 - ATP, 5668 and 5495; CTP, 490 and 647; UTP, 696 and 968; GTP, 1063 and 2056; ppGpp, >1 and 1.7. J1929::KC936 - ATP, 6347 and 4641; CTP, 520 and 752; UTP, 1468 and 1685; GTP, 1800 and 1022; ppGpp, 8.3 and 10.6.© 1996 Blackwell Science Ltd, Molecular Microbiology, 19, 357–368
strain, and that the deficiency in actinorhodin production reflects either lower basal levels or the complete absence of ppGpp in J1501::KC936 (disruptants unaffected in actinorhodin production might have undergone secondary mutations that compensate for the loss of (p)ppGpp synthesis). The isolation of further mutants of J1929 that are completely devoid of (p)ppGpp should determine whether the highly phosphorylated nucleotides play an obligate role in actinorhodin production in the wild-type strain. If they do not, then the frequently observed correlation between ppGpp synthesis and the onset of antibiotic production might simply reflect the coincidental growth-phase-dependent initiation of antibiotic synthesis and the role of ppGpp in the growth-rate control of primary metabolic genes.

Of the six fully sequenced relA homologues, only for spoT of E. coli is there convincing evidence for ppGpp degradative activity. Comparison of all six amino acid sequences (Fig. 1) reveals several conserved regions, but fails to identify motifs that might be used to discriminate readily between enzymes with synthetic or degradative activities, and there seems to be little conservation of gene organization around the relA homologues studied so far that might give a clue to function. The apt and relA genes of S. coelicolor are preceded by ORFs (EMBL X85969) that are homologous to secDF of E. coli; SecD and SecF appear to act to improve the efficiency of protein export, but are not absolutely required (Pogliano and Beckwith, 1994). Both relA and spoT of E. coli are contained within polycistronic transcription units, and neither maps close to apt or secDF (Rudd and Caskey, 1987); the relA homologue of Vibrio sp. strain S14 is preceded by a homologue of the gene found immediately upstream of E. coli relA (Flärdh et al., 1994). The relA homologue of M. leprae is preceded by an ORF reading in the same direction (EMBL U00011), and is followed by a converging ORF, but neither encodes a protein similar to those flanking S. coelicolor relA. In S. equiscilis, the relA homologue is followed by a small ORF of unknown function that is transcribed in the same orientation as the putative (p)ppGpp synthetase, and then by the converging streptokinase gene, spc (Mechold et al., 1993). Only in M. genitalium is there a resemblance of the gene organization found in S. coelicolor; the relA homologue of M. genitalium (designated spoT by Peterson et al., 1995) is preceded by a small ORF of unknown function, and then by a homologue of apt.

S. coelicolor relA is transcribed from multiple promoters in a growth-phase-dependent manner. The start site for the relAp2 transcript, which persists at a constant level throughout growth, is preceded by a 10-base pair sequence (TACCAT) that is similar to the consensus sequence (TAGYYT, where Y = G or A) deduced by Strohl (1992) for streptomycete promoters that are probably transcribed by the major RNA polymerase holoenzyme (Brown et al., 1992), but lacks a recognizable -35 region. Perhaps the inverted repeat (Fig. 3) extending from -13 to -33 binds a transcriptional activator that would obviate the need for a conventional -35 sequence. However, since transcription from relAp2 occurred in vitro with purified RNA polymerase, the requirement for any such activator is presumably not absolute. Interestingly, transcription from relAp1 peaked during transition phase, and after nutritional shiftdown, raising the possibility that relAp1 is itself subject to positive stringent control. Although the level of the RNA-protected fragment corresponding to relAp1 was noticeably lower than that derived from relAp2, the longer transcript may be translated more efficiently, and while differential transcription of relA in E. coli appears to play no role in regulating ppGpp synthesis, it is possible that increased levels of relA transcription contribute, at least in part, to the elevated levels of ppGpp in S. coelicolor. Alternatively, increased levels of transcription from relAp1 may simply compensate for the decrease in readthrough transcription from apt, which is presumably subject to negative stringent control, and may serve to maintain constant levels of RelA. While the sequences upstream of the relAp1 transcription start site do not resemble any other known streptomycete promoter (Strohl, 1992), the sequence TTGCA, which starts 10–11 bases upstream of the start site, matches perfectly the consensus -12 sequence of promoters recognized by σ54 (Gross et al., 1992); further analysis will be required to identify the RNA polymerase holoenzyme responsible for initiating transcription at relAp1. The significance of the transcriptional readthrough from apt, and its growth-phase dependence, remains to be determined.

Experimental procedures

Bacterial strains, and culture and transformation conditions

S. coelicolor strains J1929 (Bedford et al., 1995) and M145 (Hopwood et al., 1985), and S. lividans strain 1326 (Hopwood et al., 1985), were manipulated as previously described (Hopwood et al., 1985). SMM is the modified minimal medium of Takano et al. (1992); SMMS is a solidified version of SMM containing 1.5% (w/v) agar, but lacks antifoam and PEG 6000. SFM contains 2% (w/v) mannitol, 2% (w/v) soya flour and 2% (w/v) agar in tap water, and was autoclaved twice. E. coli K-12 DH5α (Hanahan, 1983) was grown and transformed according to Sambrook et al. (1989), and transformants were selected with carbenicillin at a final concentration of 200 µg ml⁻¹.

Isolation of plasmid DNA from E. coli

Plasmid DNA was isolated from E. coli strains using the following procedure (C. Thomas, personal communication), which reproducibly gave DNA of high purity that could be used directly for sequencing. A 10 ml culture grown overnight at 37° C in L broth containing 200 µg ml⁻¹ carbenicillin was harvested by centrifugation, resuspended in 1 ml of TEG (25 mM Tris pH 8, 10 mM EDTA, 50 mM glucose), and left at room
temperature for 5 min; 2 ml of 0.2 M NaOH/1% (w/v) SDS was added and the mixture was left at room temperature for 5 min. Sodium acetate (pH 4.8) (1.5 ml of 3 M) was added and the mixture was vortexed, left on ice for 10 min, and microcentrifuged for 10 min. The supernatant was harvested by centrifugation, washed with 70% (v/v) ethanol, and redisolved in 1 ml of water. LiCl (333 µl of 6 M) was added and the solution was left at -20°C for at least 20 min. After centrifugation, the supernatant was transferred to a clean tube, and 70 µl of 3 M sodium acetate (pH 6) and 3.5 ml of ethanol were added. After storage at -20°C for at least 20 min, the DNA was harvested by centrifugation, washed with 70% (v/v) ethanol, and the pellet resuspended in 200 µl.

**PCR**

The synthetic oligonucleotides 5'-CATGGATCCAACGG-CG-TACGAGAT(C)O(C)AC(CT)(GCTGGCA) and 5'-CAT-GGATCCTGTTG(C)C(A)(G)TGTA(G)C(G)GCGTA(G)CGGGAGTC (bracketed nt indicate positions of degeneracy) were used in the DNA PCR (Erlch, 1989) to amplify an internal segment of the relA homologue from S. coelicolor M145 DNA. The reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.1% (w/v) gelatin, 200 µM of each of the four dNTPs, 2.5 U of Taq polymerase, 40 pmol of each primer, and 50 ng of chromosomal DNA in a final volume of 100 µl. After denaturation at 95°C for 5 min, the samples were subjected to 25 cycles of denaturation (94°C, 1 min), annealing (55°C, 40 s), and extension (72°C, 50 s) and then incubated for 10 min at 72°C. PCR products were analysed by agarose (1.2% w/v) gel electrophoresis.

**Nucleotide sequencing studies**

The nucleotide sequence of the relA region was determined by dye-deoxy-sequencing (Sanger et al., 1977) using the Promega TaqTrack sequencing kit and a mixture of double- and single-stranded DNA templates derived by subcloning from pIJ6031 and pIJ6037. Synthetic oligonucleotides were used to close gaps in the sequence. The region encoding relA and apt submitted to the databases (EMBL X82672) was sequenced on both strands; parts of the sequence downstream of relA (nt positions 3351 to 4515) were determined on only one strand and the data are available from the authors on request.

**S1 nuclease protection studies**

pIJ6068, pBluescript SK+ with the 458 bp SstI–relA–SalI fragment (nt positions 474–931) cloned between the SmaI and SalI sites of the vector, was digested with SalI and calf-intestinal alkaline phosphatase, and the DNA extracted with phenol:chloroform (1:1, v/v), ethanol-purified and digested with SstI. The gel-purified 498 bp SstI–SalI fragment (10 pmol) was labelled using polynucleotide kinase and 30 pmol of [γ-³²P]ATP (3000 Ci mmol⁻¹, Amersham) (Sambrook et al., 1989). Unincorporated label was removed from the probe by passage through a Pharmacia Nick column. Fifty micrograms of RNA, isolated from cultures of S. coelicolor M145 grown in SMM liquid medium (Takano et al., 1992), were used in each S1 nuclease protection experiment with about 0.2 pmol (approximately 10⁰ Cerenkov c.p.m.) of the probe. Hybridizations were carried out in NaTCA buffer (Murray, 1986) at 45°C overnight after denaturation at 65°C for 15 min. S1 nuclease digestions and analysis of RNA-protected fragments were carried out as previously described (Janesbery et al., 1989). The endpoints of the RNA-protected fragments were determined by sequencing a sample of the same end-labelled fragment that was used as probe using the method of Maxam and Gilbert (1980).

In *vitro* transcription

RNA polymerase was isolated from a transition-phase culture of S. coelicolor M145, as described by Buttner and Brown (1985), and *in vitro* run-off transcription assays were performed according to Buttner et al. (1987). The 498 bp Sst–SalI and 466 bp BamHI–SalI fragments of pIJ6068 were purified by gel electrophoresis and used as templates. Transcripts were analysed on denaturing 6% polyacrylamide gels using the Gibco BRL 100 bp ladder that had been end-labelled with ³²P as size marker.

**Plaque lifts and Southern hybridization**

Plaque lifts and Southern blots (Southern, 1975) were carried out as described by Hopwood et al. (1985), using gel-purified DNA fragments containing internal segments of relA labelled with ³²P by random oligonucleotide priming (Sambrook et al., 1989).

**Amino acid depletion and (p)ppGpp assays**

Exponentially growing cultures (OD_{540nm} values of 0.5–0.6) were harvested by filtration (Whatman nitrocellulose filters, 0.45 µm pore size) from SMM medium containing 5 µg ml⁻¹ Thio, rapidly washed with either SMM or SMM lacking casamino acids, resuspended in the same medium that was used for washing, and quickly returned to the shaking incubator; the 15 mM (NH₄)₂SO₄ present in SMM provides a source of inorganic nitrogen for the continued growth of cultures depleted of amino acids. (p)ppGpp assays were carried out as described by Strauch et al. (1991).

**Note added in proof**

We have made a relA null mutant of the prototrophic S. coelicolor strain M600 in which most of the relA coding region (corresponding to amino acid residues 168–686) has been deleted. On SMMS medium the mutant, which must lack relA-dependent ppGpp synthetase activity, does not produce β-actinorhodin (the blue diffusible lactone form of actinorhodin); L. Bystrykh, personal communication) or actinorhodin (the purple mycelial form); on the richer R5 medium, β-actinorhodin production is abolished, although some actinorhodin is made, while on R2 medium (R5 lacking yeast extract) there is no apparent effect on antibiotic production. We believe that the N-terminal segment of RelA that remains after the disruption reported in this paper possesses ribosome-independent
ppGpp synthetase activity, and that the elevated basal level of ppGpp observed in the disruptant (8.5 and 10.6 pmol mg\(^{-1}\) dry weight compared with >1 and 1.7 pmol mg\(^{-1}\) dry weight for the relA congenic strain) represents the constitutive synthesis of ppGpp, and accounts for the reduction in growth rate observed on solid media. These recent results also suggest that under some nutritional conditions ppGpp plays an essential role in antibiotic production in 

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