A relaxed (*rel*) mutant of *Streptomyces coelicolor* A3(2) with a missing ribosomal protein lacks the ability to accumulate ppGpp, A-factor and prodigiosin

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A relaxed (*rel*) mutant was found among 70 thiopeptin-resistant isolates of *Streptomyces coelicolor* A3(2) which arose spontaneously. The ability of the *rel* mutant to accumulate ppGpp during Casamino acid deprivation was reduced 10-fold compared to the wild-type. Analysis of the ribosomal proteins by two-dimensional PAGE revealed that the mutant lacked a ribosomal protein, tentatively designated ST-L11. It was therefore classified as a *relC* mutant. The mutant was defective in producing A-factor and the pigmented antibiotic prodigiosin, in both liquid and agar cultures, but produced agarase normally. Production of actinorhodin, another pigmented antibiotic, was also abnormal; it appeared suddenly in agar cultures after 10 d incubation. Although aerial mycelium still formed, its appearance was markedly delayed. Whereas liquid cultures of the parent strain accumulated ppGpp, agar cultures accumulated only trace amounts. Instead, a substance characterized only as an unidentified HPLC peak accumulated intracellularly in the late growth phase, just before aerial mycelium formation and antibiotic production. This substance did not accumulate in mutant cells. It was found in *S. lividans* 66 and *S. parvulus*, but not in seven other *Streptomyces* species tested. The significance of these observations, and the relationship of the mutant to earlier *rel* isolates of *Streptomyces* is discussed.

Introduction

The genus *Streptomyces* is of considerable interest because of its complex life cycle, which includes aerial mycelium formation and sporulation, and because of its ability to produce numerous antibiotics and extracellular enzymes (for reviews, see Chater, 1984, 1989; Hopwood, 1988; Ochi, 1990*b*). Within this genus, *Streptomyces coelicolor* A3(2) is genetically the best-characterized species, and presents a well-developed system for analysing the molecular genetics of individual phenomena (reviewed by Hopwood, 1973, 1988). Recent studies include the transcriptional control of agarase (Buttner *et al.*, 1988), rRNA synthesis (Baylis & Bibb, 1988), sporulation genes (Mendez & Chater, 1987; Guijarro *et al.*, 1988) and antibiotic-induced proteins (Murakami *et al.*, 1989). Since *S. coelicolor* A3(2) produces two pigmented antibiotics, actinorhodin and prodigiosin, in addition to two other antibiotics, this organism provides a useful system for studying the initiation of differentiation and secondary metabolism.

In micro-organisms, differentiation and secondary metabolism are usually simultaneous responses to nutrient limitation (for reviews, see Freese & Heinze, 1984;

Chater, 1984, 1989). Investigating the mechanisms by which adverse environments may alter gene expression in these responses, I have observed that morphological differentiation of *Bacillus subtilis* and *Streptomyces* spp. is accompanied by a decrease in the GTP pool, whereas antibiotic production is linked to a more direct function of the *rel* gene – ppGpp accumulation (Ochi & Ohsawa, 1984; Ochi, 1986*a, b*, 1987*a, b, c*, 1988). Recent genetic analyses of a *S. griseus* mutant demonstrated a link between the *rel* mutation and both inability to produce streptomycin and reduced ability to form aerial mycelium (Ochi, 1990*a*). In order to study the function of ppGpp using molecular genetics, I have isolated and characterized a *rel* mutant of *S. coelicolor* A3(2) which lacks the ability to accumulate ppGpp.

Methods

Chemicals. Thiopeptin (an analogue of thiostrepton) was a commercial product of my company. Guanosine 5'-monophosphate 3'-diphosphate (pGpp), adenosine 5'-monophosphate 3'-diphosphate (pApp) and adenosine 5'-diphosphate 3'-diphosphate (ppApp) were generous gifts from A. Yoshimoto, Sanraku Co., Fujisawa, Japan. Adenosine 5'-tetrphosphate (Apppp), *P*¹,*P*⁴-di(adenosine-5') tetraphosphate (AppppA), *P*¹,*P*⁵-di(adenosine-5') pentaphosphate

(ApppppA) and P^1, P^6 -di(adenosine-5') hexaphosphate (AppppppA) were purchased from Sigma.

Strains and preparation of mutants. *S. coelicolor* A3(2), a wild-type prototrophic strain, was provided by D. A. Hopwood, John Innes Institute, Norwich, UK. The spontaneous thiopeptin-resistant mutants were obtained as resistant colonies, which developed 20 d after spreading spores on ISP medium 2 (Difco) supplemented with 20 µg thiopeptin ml⁻¹. Growth of the parent strain was suppressed almost completely at 5 µg thiopeptin ml⁻¹. The resistant clones were purified twice by single-colony isolation. This procedure appeared to be necessary to obtain stable resistant clones.

Growth conditions. All incubations were carried out at 30 °C. CD medium contained (per l): glucose, 10 g; ammonium sulphate, 3 g; NaCl, 0.5 g; KCl, 50 mg; MgSO₄ · 7H₂O, 50 mg; ZnSO₄ · 7H₂O, 1 mg; 1 M-sodium/potassium phosphate buffer, pH 7, 50 ml. The medium was sterilized at 120 °C for 20 min; glucose solutions were autoclaved separately. Soluble starch/polypeptone/yeast extract medium (SPY medium; Ochi, 1987b) used in this study contained 0.1%, rather than 1%, MgSO₄. ISP medium 2 (Difco) contained (per l): Bacto-yeast extract, 4 g; Bacto-malt extract, 10 g; Bacto-dextrose, 4 g; Bacto-agar, 20 g (pH 7.3). ISP medium 3 (Difco) contained (per l) Bacto-oatmeal, 20 g; FeSO₄ · 7H₂O, 1 mg; MnCl₂ · 4H₂O, 1 mg; ZnSO₄ · 7H₂O, 1 mg; Bacto-agar, 18 g (pH 7.2). Plates (diameter 8.5 cm) contained 20 ml ISP medium 2 or ISP medium 3.

A monospore suspension, prepared as described previously (Ochi, 1987b), was used to inoculate SPY medium. After 2 d on a rotary shaker (230 r.p.m.), cells were washed twice with 100 ml saline and suspended in their original volume of saline. This suspension (2 ml) was then added to 50 ml CD medium (in a 250 ml flask) supplemented with 3% (w/v) vitamin-free Casamino acids (Difco) and cultured on a rotary shaker at 230 r.p.m. At the indicated times, cells were collected on a filter paper (Toyo Roshi no. 2, 9 cm diameter) and rapidly transferred to CD medium lacking Casamino acids, to cause amino acid depletion. Inoculation of the original medium with a monospore suspension was important, because pellet formation resulted in reduced ability (50–70%) to accumulate ppGpp during Casamino acid deprivation. This may be caused by heterogeneous ageing of mycelia in the pellet, or by residual Casamino acids trapped inside the pellet.

Assay of nucleotide pools. The intracellular concentrations of ppGpp, pppGpp and other nucleotides were assayed by HPLC on a 25 cm Partisil PXS 10 SAX column (Whatman; void volume 3.02 ml) after extraction with 1 M-formic acid as previously described (Ochi, 1986b). The maximum error in HPLC measurements was ±5%, as determined by multiple HPLC analysis of the same sample.

In plate cultures, the intracellular nucleotide concentrations were determined as follows. A 0.1 ml spore suspension (approx. 2×10^8 spores) was spread on cellophane (diameter 8 cm; UV-sterilized) previously placed on the agar medium. At various times, the cellophane sheet with mycelia was removed, quickly weighed, and laid upside-down in 1 ml methanol in a plastic Petri dish (diameter 8.5 cm); 10 ml 1 M-formic acid was added immediately afterwards. The entire sampling procedure was completed within 10 s. Due to the strong hydrophobicity of aerial mycelium, pre-treatment with methanol facilitated formic acid extraction; if it was omitted, extraction efficiency decreased by about 30%. After 1 h in a cold-room, the extract was analysed by HPLC. The pool size was expressed as pmol or nmol nucleotides per mg dry weight of cells, using 1 g wet weight equivalent to 134 mg dry weight, determined with cells grown on cellophane for 60 h.

Determination of RNA synthesis. RNA synthesis after Casamino acid deprivation was determined by measuring [2-¹⁴C]uracil incorporation into acid-precipitable cell material as described by Ochi (1986b).

Determination of [2-¹⁴C]uracil uptake. Cells were grown for 10 h in CD medium supplemented with 3% Casamino acids. Before and after Casamino acid deprivation, [2-¹⁴C]uracil was added to 20 µM final concentration (0.02 µCi ml⁻¹; 740 Bq ml⁻¹) and incubation continued with shaking. Samples (1 ml) were withdrawn at 2 min intervals for 6 min, and the cells were collected on a membrane filter (Millipore, pore size 0.45 µm, diameter 25 mm), washed with 5 ml 0.1 M-LiCl, and counted in a liquid scintillation counter, with Aquasol (New England Nuclear) as the scintillation fluid. Uptake was linear for at least 6 min.

Agarase determination. Agarase activity was determined by Gran's test as described by Hodgson & Chater (1981). Since agarase production is subject to catabolic repression (Bibb *et al.*, 1987), colonies were grown on CD medium in which glucose was replaced by 2% (w/v) peptone. After 3 or 5 d incubation, 0.05 M-iodine in 0.12 M-KI was applied to the agar plate. Agarase activity was indicated by a clear halo around a colony.

A-factor assay. A-factor was assayed with a *S. griseus* mutant defective in its synthesis, as previously described (Ochi, 1987b).

Two-dimensional (2-D) PAGE. The method for 2-D PAGE, originally described by Kaltschmidt & Wittman (1970), was as described in detail by Ochi (1989), as was the procedure for preparing ribosomes and total ribosomal protein. Electrophoretic separation was repeated two or three times for each preparation, to confirm the reproducibility of the results.

General reproducibility. The experiments shown in each Figure or Table were all repeated two or more times to confirm that the results were reproducible. Representative data are presented.

Results

Accumulation of ppGpp by S. coelicolor A3(2)

The ability of *S. antibioticus* to accumulate ppGpp is greatly affected by the culture conditions preceding nutritional shift-down (Ochi, 1987a). Therefore, initial experiments were done to define the conditions for maximal ppGpp formation by *S. coelicolor*. Cells were grown with varying amounts of Casamino acids for various times, and then transferred to Casamino-acid-free CD medium. The results are summarized in Table 1. Cells grown in nutritionally poor medium prior to Casamino acid deprivation accumulated less ppGpp. Further, maximum ppGpp synthesis was observed in cells grown for 10 h (mid-exponential phase) before transferral; accumulation was reduced to 50% or less in cells grown for 16 h (late exponential) or 24 h (post-exponential). The greatest accumulation (650 pmol ppGpp per mg dry weight) occurred with cultures initially grown in CD medium with 3% Casamino acids for 10 h.

Isolation of a rel mutant

Relaxed mutants with severely impaired ability to accumulate ppGpp have previously been isolated from several *Streptomyces* spp. as thiopeptin-resistant colonies

Table 1. Effect of growth conditions on the ability of cells to accumulate ppGpp after Casamino acid deprivation

Cells of *S. coelicolor* A3(2) grown in chemically defined (CD) medium supplemented with various amounts of Casamino acids were harvested at the times indicated, and transferred to CD medium without Casamino acids. After 15 min incubation with shaking, the cells were harvested and ppGpp was extracted. The ppGpp pool sizes just before the cells were deprived of Casamino acids were 15–45 pmol (mg dry wt)⁻¹.

Growth conditions before Casamino acid deprivation	ppGpp accumulated after 15 min Casamino acid deprivation [pmol (mg dry wt) ⁻¹]
Grown for 14 h in CD medium with Casamino acids (w/v):	
0.5%	65
1%	108
2%	257
3%	541
4%	477
Grown in CD medium with 3% (w/v) Casamino acids for:	
6 h	472
10 h	650
16 h	309
24 h	210

(Ochi, 1986*b*, 1987*a, b*, 1988). A similar method was used in this study to isolate a relaxed mutant of *S. coelicolor* A3(2).

The conditions developed for maximum ppGpp accumulation were used to test 70 strains, randomly selected from thiopeptin-resistant isolates, for their ability to accumulate ppGpp. Only strain 63 produced substantially (10-fold) less ppGpp than the parent strain after 15 min of Casamino acid deprivation.

In the parent strain, RNA synthesis, as measured by [¹⁴C]uracil incorporation into acid-precipitable cell material, was severely reduced on Casamino acid deprivation, but strain 63 continued RNA synthesis at an essentially unchanged rate (Fig. 1). Since in *Escherichia coli* and *B. subtilis* inhibition of uracil uptake can result not only from ppGpp accumulation (stringent response) but also from a decrease in GTP concentration, or both (Fast, 1978; Beaman *et al.*, 1983), uracil uptake by *S. coelicolor* was also measured. Uracil uptake by cells of the parent strain decreased by 15% after Casamino acid deprivation, and by 27% in cells of strain 63. Because there was no marked differential change in UTP pool size between the parent and mutant strains in the initial 15 min of Casamino acid deprivation (Fig. 2), there was no need to allow for differences in the specific radioactivity of intracellular UTP. It can be concluded that mutant 63 exhibits a typical relaxed (*rel*) phenotype with respect to RNA synthesis.

Fig. 1. RNA synthesis by (a) *S. coelicolor* A3(2) and (b) mutant 63 after deprivation of Casamino acids. Cells grown to mid-exponential phase (10 h for the parental strain and 16 h for the mutant) in CD medium plus 3% Casamino acids were collected and transferred, to give an OD₆₀₀ of 2, in CD medium containing [2-¹⁴C]uracil with (●) or without (○) Casamino acids, and incubated with shaking for 30 min.

Changes in the intracellular nucleotide pool on Casamino acid deprivation were examined. Cells of the parent strain accumulated high levels of ppGpp immediately (Fig. 2). Accumulation of pppGpp was maximal at 10 min, and was at most 20% of that of ppGpp (data not shown). The *rel* mutant accumulated far less ppGpp (and pppGpp, not shown) than the parent strain throughout 1 h incubation (Fig. 2). Unexpectedly, the decrease in the GTP pool of the parent strain was less extensive than that in the *rel* mutant. This result contrasts with observations on other *Streptomyces* spp. (Ochi, 1986*b*, 1987*a, c*) and may be attributed to the much sharper decline in RNA synthesis in *S. coelicolor* on Casamino acid deprivation (Fig. 1); under such circumstances, nucleoside triphosphate utilization might be much reduced. I therefore measured ppGpp and GTP pool sizes during growth in normal conditions. Wild-type cells grown to post-exponential phase (24–36 h) in SPY medium maintained ppGpp levels about fourfold higher, and GTP levels much lower, than those in *rel* cells (Table 2). This result is consistent with those obtained for other *Streptomyces* spp.

Characterization of the *rel* mutant

Ribosomal proteins. The *rel* mutant lacked a ribosomal protein, as determined by 2-D PAGE (Fig. 3). No other ribosomal protein was missing or altered in electrophoretic mobility. (Several faint spots are not visible in both photographs, but were, however, present on both the gels; these are indicated with arrowheads.) No new spot that might represent an altered form of the protein was

Fig. 2. Changes in the intracellular pools of nucleoside triphosphates and ppGpp after deprivation of Casamino acids. Cells of strain A3(2) (a) or mutant 63 (b), grown to mid-exponential phase in CD medium plus 3% Casamino acids as described in the legend to Fig. 1, were harvested and transferred to the same volume of fresh CD medium without Casamino acids and then incubated with shaking. Just before transfer, the nucleotide pool concentrations (mean of two samples) of GTP, ATP, UTP and CTP were 4.65, 12.0, 2.99 and 2.11 (strain A3(2)) and 5.06, 8.02, 2.70 and 2.05 (strain 63) nmol (mg dry wt)⁻¹, respectively. ●, GTP; ▲, ppGpp; ○, ATP; □, UTP; △, CTP.

seen on the gel, although it might have been hidden by another protein spot. Strain 63 can thus be classified as a *relC* mutant, and the missing protein was tentatively designated ST-L11 by analogy with *E. coli* and *B. subtilis* mutants which lack ribosomal proteins L11 and BS-L11, respectively (Friesen *et al.*, 1974; Smith *et al.*, 1978, 1980).

Fig. 3. 2-D PAGE of total ribosomal proteins. The gel system was based upon that of Kaltschmidt & Wittmann (1970). (a) *S. coelicolor* A3(2); (b) mutant 63. The arrows indicate a protein spot (ST-L11), which was absent in the mutant. Several faint spots, indicated by arrowheads, are not visible in both photographs, but were present in both the original gels.

Table 2. Intracellular nucleotide pools of cells grown in SPY medium

Cells of the parent strain or mutant 63 were grown in SPY medium with shaking. At the indicated times, the cells were harvested, extracted and assayed for intracellular nucleotides.

Strain	Time (h)	Nucleotide concentration [pmol (mg dry wt) ⁻¹]		
		ppGpp	GTP	ATP
Parent (<i>rel</i> ⁺)	24	103	945	4790
	36	46	820	4660
63 (<i>rel</i>)	24	28	1780	6440
	36	26	1470	4800

Antibiotic resistance. The *relC* mutant was resistant to 30 µg thiopeptin ml⁻¹ after 4 d incubation on ISP medium 2; the parent strain was resistant only to 2 µg ml⁻¹. However, although the parent strain grew normally in the presence of 10 µg erythromycin ml⁻¹, growth of the mutant was completely abolished by 0.1 µg ml⁻¹. Growth of mutant cells was also almost completely arrested at 40 °C, although the parent strain grew normally at this temperature.

Secondary metabolism. The *relC* mutant was strikingly deficient in production of the pigmented antibiotics prodigiosin and actinorhodin. The parent strain *S. coelicolor* A3(2) synthesized these compounds after 2–3 d incubation on ISP medium 2 or Hopwood's medium (Hopwood, 1967), but the mutant failed to produce either in 10 d incubation. Cultures were not examined for production of methylenomycin. However, after 10 d incubation, mutant cells suddenly produced a blue pigment identified by TLC as actinorhodin. The amount was about 20-fold greater than that formed by the parent strain, and no undecylprodigiosin was detected. Although intriguing, the mechanism of this abnormal production was not studied further.

A-factor (2*S*-isocapryloyl-3*S*-hydroxymethyl-γ-butyrolactone) is an autoregulating factor produced by *S. griseus*, controlling both differentiation and streptomycin production. Mutants unable to synthesize A-factor (*afs*) are defective in these processes (Horinouchi *et al.*, 1983; Ochi, 1987*b*). Previously isolated *relC* mutants of *S. griseus* were able to synthesize A-factor normally (Ochi, 1987*b*), but the *S. coelicolor relC* mutant was not. The parent strain produced A-factor at a lower level (5–10 ng ml⁻¹), detectable after 2 d incubation in liquid or solid ISP medium 2, with or without 100 mM-phosphate buffer (pH 7), but the mutant produced <1 ng ml⁻¹ under these conditions. Addition of exogenous A-factor to a final concentration of 0.01–1 µg ml⁻¹ did not restore antibiotic production in the mutant.

Aerial mycelium formation. The *relC* mutant retained the ability to form aerial mycelium on ISP medium 2, but the onset of formation was delayed by 5 d, and less was produced than in the parent strain. The addition of A-factor with or without 0.1–2 mM-decoyinine to cultures did not restore wild-type mycelium formation (data not shown).

Agarase production. *S. coelicolor* is distinctive in its ability to utilize agarose as a sole carbon source by excreting agarase into the medium (Hodgson & Chater, 1981; Bibb *et al.*, 1987). Both parent and mutant strains produced agarase normally, as determined by Gran's test, producing 3 mm diameter haloes on an agar plate.

Correlation between the relC mutation and the altered phenotype

Twenty isolates, varying in thiopeptin resistance from 15 to 80 µg ml⁻¹, were tested for pigmented antibiotic production, aerial mycelium formation, erythromycin sensitivity and temperature sensitivity. All exhibited the normal parental phenotype for most of these characteristics, although a few exhibited a slight reduction in antibiotic productivity, or a slight increase in temperature sensitivity, or both. The ribosomal proteins of five thiopeptin-resistant isolates were also analysed by 2D-PAGE; no difference was observed between the tested isolates and the parent strain, and the ST-L11 protein was at the normal location in each case. These results suggest that the abnormal behaviour of strain 63 results from the putative *relC* mutation.

No phenotypic reversion of the *relC* mutant was observed by examining for ability to form pigmented antibiotics or loss of erythromycin sensitivity.

Changes in the nucleotide pool during growth on agar media

Unlike *S. griseus*, *S. coelicolor* produces aerial mycelium and spores only on agar medium. Changes in the nucleotide pool during growth on ISP medium 2 were examined using the cellophane-sheet technique. The results may not accurately reflect the physiology of cells growing on the medium without the limitations in permeability due to the cellophane; however, the parent strain produced as much antibiotic and aerial mycelium with the cellophane as without it.

The intracellular pool size of GTP was high in early growth phase, but abruptly decreased at mid-growth phase in both parent and mutant strains (Fig. 4). This decrease was more pronounced, though not greatly so, in the parent strain; near the onset of mycelium formation (50–70 h), the lowest pool size measured was only one-fourth that observed in the mutant. The ppGpp pool size in the parent strain (5–10 pmol per mg dry weight) was well below that measured in liquid culture (about 100 pmol per mg dry weight; Table 2) throughout 90 h incubation. In the *relC* mutant, ppGpp pool size was 2–5 pmol per mg dry weight. pppGpp was undetectable (<5 pmol per mg dry weight) in both strains.

Analysis of nucleotide pools by HPLC (Fig. 5) indicated that the parent strain (but not the mutant) accumulated an unknown substance (US) intracellularly in the late growth phase of cultures on agar media (Fig. 4). Cultures were not tested for extracellular accumulation. The UV absorption spectrum of US in phosphate buffer at pH 5.4 (inset in Fig. 5) displayed a maximum at 255 nm and resembled that of ATP. US also accumu-

Fig. 4. Changes in the intracellular pools of GTP, ATP and an unknown substance in (a) strain A3(2) and (b) mutant 63 during growth on agar (ISP medium 2) covered with a cellophane sheet. The concentration of the unknown substance was calculated tentatively using the molar absorption coefficient of ATP. The arrow indicates the time at which antibiotic production and aerial mycelium formation began. ●, GTP; ○, ATP; ▲, unknown substance; △, dry cell wt per cellophane sheet.

lated intracellularly in the parent strain in liquid cultures, but was detected only during a period of about 6 h, between 24 and 30 h incubation.

In eukaryotic cells, some adenosine polyphosphate species may be signal nucleotides (reviewed by Jakubowski, 1986; Plateau *et al.*, 1989). However, US differed in HPLC retention time from known adenosine polyphosphates (AppppA, ApppppA, AppppppA, pApp, ppApp) (Fig. 5).

Finally, nine other *Streptomyces* spp. were tested for US accumulation. Since prolonged accumulation of US was only detected in agar cultures, cells were grown on agar plates of ISP medium 2 or ISP medium 3 for 4 d, and assayed daily for US, using HPLC. *S. lividans* 66 and *S. parvulus* ATCC 12434 accumulated as much US as *S. coelicolor*, but *S. antibioticus* 3720, *S. lavendulae* MA406-A-1, *S. griseus* IFO 13189, *S. griseoflavus* FERM 1805, *S. hygroscopicus* IFO 13472, *S. venezuelae* IFO 13096 and *S. violaceus* IFO 13103 did not accumulate detectable amounts.

Discussion

Thiopeptin-resistant *rel* mutants have been isolated previously from various *Streptomyces* spp., e.g. *S.*

antibioticus, *S. lavendulae*, *S. griseus* and *S. griseoflavus*. These were all demonstrated to have an altered or missing ribosomal protein, designated ST-L11. I have summarized their properties (Ochi, 1990a) with regard to ppGpp accumulation, RNA synthesis, antibiotic production, temperature sensitivity and drug sensitivity. More specifically, these properties comprise: (1) about 10-fold reduction in ppGpp accumulation during Casamino acid deprivation; (2) continued RNA synthesis during Casamino acid deprivation; (3) markedly reduced antibiotic production; (4) markedly late formation of aerial mycelium; (5) increased sensitivity to high temperatures (except *S. antibioticus relC* mutant 49); (6) increased sensitivity to erythromycin (except *S. antibioticus relC* mutant 49), but a 10- to 15-fold increase in thiopeptin resistance; (7) a somewhat reduced growth rate, especially in CD medium. *S. coelicolor* mutant 63 shares these properties. The 2-D PAGE gel positions of the ribosomal proteins which were altered or missing in these *relC* mutants were all similar, namely 7–11 mm from the origin in the first dimension, and 35–40 mm in the second (Ochi, 1990a; this study). Although considerable electrophoretic variability exists among the ribosomal protein patterns of different *Streptomyces* spp. (Ochi, 1989), the above evidence suggests that the

Fig. 5. Elution profile from HPLC of nucleotides extracted from cells of (a) strain A3(2) or (b) mutant 63 grown on agar covered with a cellophane sheet for 42 h (A3(2)) or 60 h (mutant 63). The samples injected were extracted from 6.5–7.7 mg cell dry wt. The unknown substance is labelled US. The numbered arrows indicate the retention times for: 1, AppppA; 2, ApppppA; 3, pApp; 4, AppppppA; 5, pGpp; 6, Apppp; 7, ppApp. The inset shows a UV spectrum of US in phosphate buffer at pH 5.4.

missing protein confers the observed phenotype. It is therefore referred to in all species as ST-L11; however, its identity as 'L11-like' has not been proven, but suggested by analogy with ribosomal protein functions in *E. coli* and *B. subtilis*. Confirmation requires demonstration of immunochemical or functional homology with the *E. coli* L11 protein.

One goal in studying the mechanism of morphological and physiological differentiation is to identify the biochemical effectors responsible. All *Streptomyces relC* mutants so far isolated are severely impaired in the production of an antibiotic (Ochi, 1990a; this study). In *E. coli*, ppGpp can affect gene expression by altering the selectivity of RNA polymerase *in vivo* and *in vitro* (Kajitani & Ishihama, 1984; Baracchini *et al.*, 1988). One

can envisage a model in which ppGpp could trigger antibiotic production in *Streptomyces* spp. in response to physiological conditions. However, although the phenotypic properties discussed have been tentatively related to the *relC* mutation, including the failure to produce prodigiosin, genetic analysis is required before the altered phenotype can be unambiguously attributed to the *relC* lesion. The availability of genetic markers and the high frequency of genetic recombination in *S. coelicolor* A3(2) (Hopwood, 1973) will be helpful in such a study. Further analysis at the molecular level is needed to establish a causal link between ppGpp accumulation and the onset of prodigiosin or actinorhodin production.

A striking difference between *relC* mutants of *S. griseus* and those of *S. coelicolor* is that the former produce A-factor normally, but the latter produce none. A-factor plays an important role in *S. griseus*, controlling differentiation and streptomycin production. In *S. coelicolor*, however, A-factor plays no such role; its synthesis and those of antibiotics are controlled at the same level in a hierarchical regulatory cascade (Horinouchi *et al.*, 1983; Hopwood, 1988), implying that it is a simple secondary metabolite in this organism. The non-production of both A-factor and antibiotics in the *S. coelicolor relC* mutant is consistent with this view.

In agar cultures, several unexpected results were obtained. Firstly, the *relC* mutant was delayed in the onset of aerial mycelium formation, despite a timely reduction in GTP pool size at the mid-growth phase (Fig. 4). Unless the different minimum pool sizes observed between the parent and mutant strains are significant, additional conditions may have to be met before the GTP decrease can trigger aerial mycelium formation. This possibility is supported by the different sporulation response of *S. coelicolor* A3(2) as compared with *S. griseus*, *S. griseoflavus* and *S. lavendulae* MA406-A-1. In contrast with observations of the latter three species (Ochi, 1986a, 1987b; Ochi *et al.*, 1988), in *S. coelicolor* the suppressive effect of 2–4% Casamino acids on aerial mycelium formation could not be reversed by decoyinine, a specific inhibitor of GMP synthetase, at any of the concentrations tested (unpublished results). Mutant 63 may be unable to achieve the required physiological conditions due to the *relC* lesion. Since A-factor did not restore normal mycelium formation, its presence appears not to play a determining role. It is also noteworthy that Granozzi *et al.* (1990) reported a transitory cessation of macromolecular synthesis (particularly of RNA) just before aerial mycelium was formed in *S. coelicolor* A3(2).

Secondly, although mutant 63 produced no prodigiosin, it produced large amounts of actinorhodin after 10 d incubation on agar. No explanation can be offered for this at present, but it implies that different mechanisms initiate production of the two antibiotics.

Thirdly, the parent strain accumulated only trace amounts of ppGpp in agar cultures, but accumulated an unidentified substance (US), whose UV spectrum resembled that of ATP, just before differentiation. The mechanism of US synthesis evidently differs from that of ppGpp, because US did not accumulate during Casamino acid deprivation. The *relC* mutant did not accumulate US, implying that US synthesis may be controlled by ppGpp. Whether US acts as a signal molecule, together with or instead of ppGpp, in initiating antibiotic production by some *Streptomyces* spp. merits further investigation.

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