

## Occurrence of the Stringent Response in *Streptomyces* sp. and its Significance for the Initiation of Morphological and Physiological Differentiation

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*Streptomyces* sp. MA406-A-1 produced formycin (a nucleoside antibiotic) in parallel with cell growth in a synthetic medium. When the synthetic medium was supplemented with 1% (w/v) Casamino acids, however, formycin was produced only after the end of exponential growth. The intracellular ppGpp pool increased gradually towards the end of exponential growth and was maximal at the beginning of formycin production. After shift down from Casamino acids medium to synthetic medium, the ppGpp pool increased immediately, while the GTP pool decreased; under such conditions, the ability to produce formycin increased eightfold. Relaxed (*rel*) mutants, the first isolated for a *Streptomyces* species, were found at high incidence (10%) among spontaneous thiopeptin-resistant isolates and had severely reduced abilities to accumulate ppGpp. These *rel* mutants also failed to produce formycin under the usual culture conditions and exhibited numerous pleiotropic effects such as an inability to produce melanin and an extended delay of aerial mycelium formation. Thus *Streptomyces* sp. exhibited a typical stringent response, and the response initiated (or was needed for) the induction of secondary metabolism. The response may have also participated in the initiation of aerial mycelium formation by decreasing the intracellular GTP pool.

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### INTRODUCTION

*Streptomyces* is a genus of the order *Actinomycetales*. A distinguishing characteristic of species of this genus is their ability to form aerial mycelium from substrate mycelium when cells encounter adverse environmental conditions such as nutrient limitation. The aerial mycelium subsequently can form spores (conidiospores), perhaps one of the most evolved differentiation processes in prokaryotic micro-organisms. Consequently, the formation of aerial mycelium and conidiospores by *Streptomyces* has been studied as a model for differentiation (Kalakoutskii & Agre, 1976; Ensign, 1978; Chater & Merrick, 1979; Chater, 1984).

Members of the genus *Streptomyces* also produce numerous antibiotics, which has been regarded as an example of 'secondary metabolism' as the cells do not need to produce antibiotics to grow normally. Although *Streptomyces* species produce antibiotics at about the same time that the mycelium undergoes differentiation, no causal links have been established between differentiation and formation of these secondary metabolites (Chater, 1984). Thus, *Streptomyces* are excellent organisms in which to study both differentiation and secondary metabolism.

In *Bacillus subtilis* and *Escherichia coli*, amino acid limitation produces the 'stringent response' whereby the rate of RNA synthesis and of numerous other cellular reactions, including GTP synthesis, is severely reduced. This effect is accompanied by an increase in the intracellular concentrations of ppGpp and pppGpp, compounds believed to be responsible for the stringent response (Cashel, 1975; Gallant, 1979). The stringent response can be avoided by the introduction of genes having the relaxed genotype. The best known is *relA* (Fiil & Friesen, 1968;

Swanton & Edlin, 1972), a mutation in the gene that codes for the factor needed for the synthesis of ppGpp and pppGpp (Block & Haseltine, 1973; Smith *et al.*, 1978). Another mutant gene is *relC* (Friesen *et al.*, 1974; Smith *et al.*, 1978), which codes for ribosomal protein L11 (Parker *et al.*, 1976; Smith *et al.*, 1980). During amino acid starvation in *E. coli* or *B. subtilis*, mutants carrying the relaxed genotype (*relA* and *relC*) accumulate neither ppGpp nor pppGpp (Cashel, 1975; Gallant, 1979). However, mutants of *E. coli* (but not of *B. subtilis*) bearing either the *relA* or the *relC* gene can still accumulate ppGpp during a shift down of energy source, e.g. glucose deprivation (Lazzarini *et al.*, 1971; Friesen *et al.*, 1974). The occurrence of the stringent response in *Streptomyces* sp. has been described by Riesenber *et al.* (1984).

The importance of the stringent response for differentiation has been demonstrated in *B. subtilis* (Freese, 1981; Freese & Heinze, 1984) and *Myxococcus xanthus* (Kaiser, 1984). In *B. subtilis* the decrease of intracellular GTP, caused by the stringent response (or more directly by the inhibitors of GMP synthesis), results in an initiation of sporulation (Lopez *et al.*, 1981; Ochi *et al.*, 1981, 1982). Aerial mycelium formation of *Streptomyces* has also been shown to be initiated by the addition of decoyinine (a specific inhibitor of GMP synthesis), which caused a decrease in the pool of GTP (Ochi, 1986). The results with *B. subtilis* showed that the stringent response initiates both sporulation and antibiotic production, but by different mechanisms (Ochi & Ohsawa, 1984; Ochi, 1985).

By isolating relaxed mutants of *Streptomyces* sp. MA406-A-1, I show in this paper that the stringent response (ppGpp) is needed to initiate formycin production in this organism.

#### METHODS

**Source of drugs.** ppGpp and pppGpp were purchased from Sanraku Co., Fujisawa, Kanagawa, Japan, DL-serine hydroxamate and D(-)-threo-chloramphenicol from Sigma and Casamino acids (vitamin-free) from Difco. [2-<sup>14</sup>C]Uracil and [2-<sup>14</sup>C]thymidine were from New England Nuclear. Formycin was a gift from Dr T. Shomura, Meiji Seika Co., Yokohama, Japan. Thiopeptin (an analogue of thiostrepton) was a commercial product of our company. All medium components were from commercial sources.

**Strains and preparation of mutants.** *Streptomyces* sp. MA406-A-1, a wild-type, prototrophic (formycin-producing) strain was provided by the Institute of Microbial Chemistry, Tokyo, Japan. The spontaneous thiopeptin-resistant mutants were obtained as resistant colonies, which developed 22 d after spreading spores on soluble starch/yeast extract/polypeptone agar (see below) containing 3 µg thiopeptin ml<sup>-1</sup>. [Growth of the parental strain was almost completely suppressed with 1 µg thiopeptin ml<sup>-1</sup>.] The resistant clones were purified by single colony isolation before subsequent study.

**Growth conditions.** Synthetic medium contained (per litre): glucose, 10 g; ammonium sulphate, 2 g; lysine.HCl, 2 g; glutamine, 2 g; NaCl, 5 g; KCl, 0.5 g; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.5 g; ZnSO<sub>4</sub>.7H<sub>2</sub>O, 10 mg; 1 M-sodium phosphate buffer (pH 7), 100 ml. [Lysine and glutamine were included because they are precursors of formycin biosynthesis and increase formycin production (Ochi *et al.*, 1979).] The medium was sterilized by autoclaving at 120 °C for 20 min; glucose solutions were autoclaved separately. Maltose/polypeptone/yeast extract medium contained (per litre): maltose, 30 g; polypeptone, 30 g; yeast extract, 10 g; NaCl, 5 g; KCl, 0.5 g; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.5 g; ZnSO<sub>4</sub>.7H<sub>2</sub>O, 10 mg; 1 M-sodium phosphate buffer (pH 7), 7 ml. Sporulation medium contained (per litre): soluble starch, 20 g; yeast extract, 4 g; agar, 20 g. Soluble starch/yeast extract/polypeptone agar (as agar medium for formycin production) contained 0.5% (w/v) polypeptone in addition to sporulation medium. All the media were adjusted to pH 7 with NaOH before autoclaving. Plates (diameter 8.5 cm) consisted of 25 ml medium solidified with 2% (w/v) agar.

A spore suspension for inoculation was prepared by scraping the spores from a slant of sporulation medium with an inoculating loop and transferring them to a sterile test tube containing approximately 100 glass beads (3 mm diameter) and 4 ml saline (0.9% NaCl). After continuous blending on a vortex mixer for 5 min, the spore suspension (3 ml) was inoculated into maltose/polypeptone/yeast extract medium (50 ml in a 250 ml flask). After culture for 2 d (for parent) or 3–4 d (for mutants) at 25 °C, cells were harvested by centrifugation (8000 g, 10 min), washed twice with 100 ml saline, and resuspended in the original volume of saline. Washed cells (2 ml) were inoculated into 50 ml synthetic medium, or synthetic medium supplemented with 1% (w/v) vitamin-free Casamino acids (in a 250 ml flask), and cultured at 30 °C on a rotary shaker (230 r.p.m.). To determine formycin production in nutrient medium, cultured broth (1 ml) was directly inoculated into fresh maltose/polypeptone/yeast extract medium (50 ml in a 250 ml flask), and cultured at 25 °C. [Formycin production with maltose/polypeptone/yeast extract medium decreased markedly if the culture was grown at 30 °C instead of 25 °C.]

**Curing of formycin production.** *Streptomyces* sp. MA406-A-1 was grown in maltose/polypeptone/yeast extract medium at 25 °C for 2 d. Portions (0.5 ml) were inoculated into 50 ml of the same medium but containing 5 µg

acriflavin ml<sup>-1</sup>, and cultured at 25 °C for 1 d. This procedure was repeated to increase curing efficiency. The cultured broth was homogenized with glass beads as described above and plated onto soluble starch/yeast extract/polypeptone agar after suitable dilution. Colonies that developed after 5 d incubation at 30 °C were examined for formycin production (see below) in maltose/polypeptone/yeast extract medium or on soluble starch/yeast extract/polypeptone agar.

**Assay of nucleotide pools.** Cells growing in 200 ml synthetic medium supplemented with 1% Casamino acids were collected on a filter paper (diameter 9 cm, no. 2, Toyo Roshi Co.) and washed with 50 ml synthetic medium, and the filters plus cells were quickly transferred to 200 ml synthetic medium. After incubation at 30 °C for the indicated time, the cells from 100 ml culture were rapidly collected as just described (without washing), and the filter was then immediately laid upside down onto 10 ml 1 M-formic acid in a Petri dish; the entire collection procedure was done within 15 s. A separate 100 ml sample was used to determine the dry cell mass. After 1 h incubation at 4 °C, the filter paper was removed by centrifugation (5000 g, 10 min). The supernatant fraction was filtered through a syringe (10 ml) fitted with a Millipore HAWP filter (0.45 µm pore size) to remove all remaining debris and then vacuum-evaporated to dryness for 10 h. The residue was dissolved in 300 µl deionized water. For the assay of nucleotides by HPLC, 50–100 µl of the dissolved residue was applied to a column of Particil PXS 10/25 SAX (Whatman), which had previously been washed with 0.05 M-H<sub>2</sub>SO<sub>4</sub> followed for 10 min with a high ionic strength buffer (see below) and then for 15 min with a low ionic strength buffer (see below). The nucleotides were eluted as described by Ochi *et al.* (1981) at a flow rate of 1.5 ml min<sup>-1</sup>, using a gradient of ionic strength made up by mixing a low ionic strength buffer (7 mM-KH<sub>2</sub>PO<sub>4</sub>, adjusted to pH 4 by H<sub>3</sub>PO<sub>4</sub>) and a high ionic strength buffer (0.5 M-KH<sub>2</sub>PO<sub>4</sub> + 0.5 M-Na<sub>2</sub>SO<sub>4</sub>, adjusted to pH 5.4 by KOH). The amount of each nucleotide was determined by comparison with the peak areas of materials eluted from HPLC columns and that of known amount of authentic nucleotides. The peak areas in absorbance profiles were automatically integrated by a computer (Hitachi 655-61, processor A). To normalize the amounts of nucleotides in different cultures, they were expressed relative to the cell dry weight measured at the time of harvesting, i.e. pmol (mg dry wt)<sup>-1</sup>.

**Determination of cell dry weight.** Cells from 100 ml culture were collected on filter paper (diameter 4 cm, no. 2, Toyo Roshi Co.) and washed with 200 ml deionized water. The filters with cells were then dried in an oven at 60 °C for 4 h and weighed after cooling in a desiccator.

**Assay for formycin.** Formycin was determined by a disc-plate method with *Xanthomonas oryzae* IFO 3312 as a test organism as described by Ochi (1986).

**Determination of RNA or DNA synthesis.** The cells, grown to mid-exponential phase, were harvested and resuspended to OD<sub>600</sub> = 2 in the specified medium containing 50 µM-[2-<sup>14</sup>C]uracil [0.05 µCi ml<sup>-1</sup> (1.85 kBq ml<sup>-1</sup>), for RNA synthesis] or 20 µM-[2-<sup>14</sup>C]thymidine [0.03 µCi ml<sup>-1</sup> (1.11 kBq ml<sup>-1</sup>), for DNA synthesis], and incubated at 30 °C with shaking. Samples (1 ml each) were removed at the indicated times and immediately added to 1 ml ice-cold 10% (w/v) TCA. After standing for 1–2 h at 0 °C, the suspensions were collected on membrane filters (Millipore HAWP, 0.45 µm), which were then washed with 5 ml ice-cold 5% (w/v) TCA. Radioactivity on the filters was estimated in a liquid scintillation counter with Aquasol (New England Nuclear) as scintillation fluid.

**Reproducibility.** The experiments shown in each Figure were repeated at least once to confirm reproducibility (except that in Fig. 1, which was not repeated); representative results are presented.

## RESULTS

### *Growth and formycin production*

In contrast to the growth of unicellular bacteria, the growth pattern of streptomycetes is one of hyphal elongation with branching and leads to the formation of clumps. The physiology within and outside these clumps may differ because of differences in nutrient transport. In this study clumping was largely avoided by inoculating relatively large amounts of mono-spore suspensions in making precultures (see Methods); consequently, the cell populations described below are expected to be more uniform than those in previously described studies.

When cells were inoculated into synthetic medium, they produced formycin during the exponential growth phase and ceased production when growth ended. In contrast, cells did not produce formycin during the exponential growth phase if the medium was supplemented with 1% (w/v) Casamino acids but began producing it after the end of exponential growth, showing a typical pattern of secondary metabolite synthesis.

The changes in intracellular nucleotide pool size during growth in synthetic medium supplemented with 1% (w/v) Casamino acids were measured (Fig. 1). The pool size of ppGpp during early exponential growth was very low [2–4 pmol (mg dry wt)<sup>-1</sup>] but it increased towards the end of exponential growth. A drastic increase was observed around 20 h, at which time cells had just entered stationary phase, apparently due to exhaustion of nutrient(s) which would

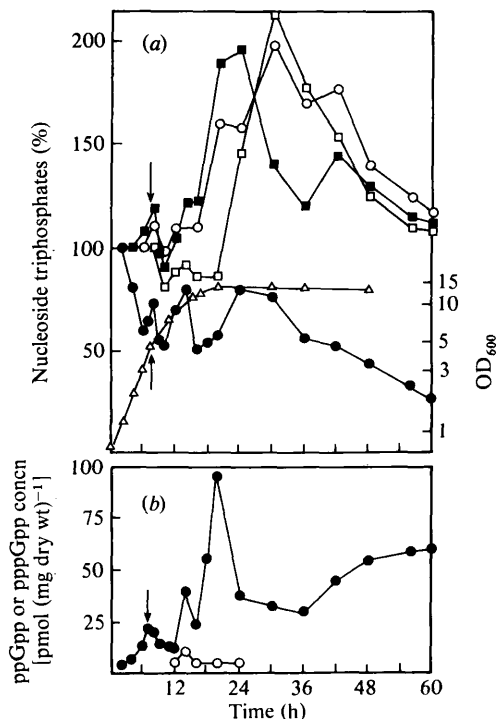


Fig. 1. Changes in the intracellular concentrations of nucleoside triphosphates, ppGpp and pppGpp during growth in synthetic medium supplemented with Casamino acids. *Streptomyces* sp. MA406-A-1 was grown in synthetic medium plus 1% (w/v) Casamino acids. At each of the indicated times, samples (20–100 ml) were withdrawn from the cultures, filtered and extracted as described in Methods. The pool size of each nucleotide at 2 h [pmol (mg dry wt)<sup>-1</sup>], designated as 100%, was: GTP, 1020; CTP, 403; ATP, 2060; UTP, 734. The arrows indicate the time at which exponential growth ceased. (a)  $\Delta$ , OD<sub>600</sub> of culture;  $\bullet$ , GTP;  $\circ$ , CTP;  $\blacksquare$ , ATP;  $\square$ , UTP. (b)  $\bullet$ , ppGpp;  $\circ$ , pppGpp.

increase the fraction of uncharged tRNA. At the same time, ATP, CTP and UTP all increased markedly, possibly due to arrest of RNA synthesis. When cells were grown in synthetic medium, relatively high levels of ppGpp [15–22 pmol (mg dry wt)<sup>-1</sup>] were detected throughout the exponential growth phase (results not shown).

A small amount of chloramphenicol (0.7  $\mu$ g ml<sup>-1</sup>, added just after inoculation) reduced formycin production by 65% without affecting growth. Since chloramphenicol inhibits ppGpp synthesis in *B. subtilis* (Ochi & Freese, 1983), these results raised the possibility that ppGpp is the physiological initiator of formycin production. To assess this possibility, I isolated relaxed (*rel*) mutants which might lack the ability to produce ppGpp.

#### Isolation of *rel* mutants

Relaxed (*relC*) mutants, lacking ribosomal protein BS-L11, have been previously recovered from *B. subtilis* cells as thiostrepton-resistant isolates (Smith *et al.*, 1978). In the present study I used thiopeptin [an analogue of thiostrepton; see Hensens & Albers-Schönberg (1983*a, b*) for its structure] to isolate drug-resistant mutants of *Streptomyces* sp. MA406-A-1. A number of thiopeptin-resistant colonies developed on the plates containing 3  $\mu$ g thiopeptin ml<sup>-1</sup> after 22 d incubation with a frequency of about  $2 \times 10^{-8}$ .

Among these, 20 strains selected randomly were tested for the ability to produce ppGpp during nutritional shift-down. Strains growing exponentially in synthetic medium supplemented with 1% Casamino acids were harvested and resuspended in synthetic medium (without Casamino acids). After a 10 min incubation, cells were collected and extracted and the extracts

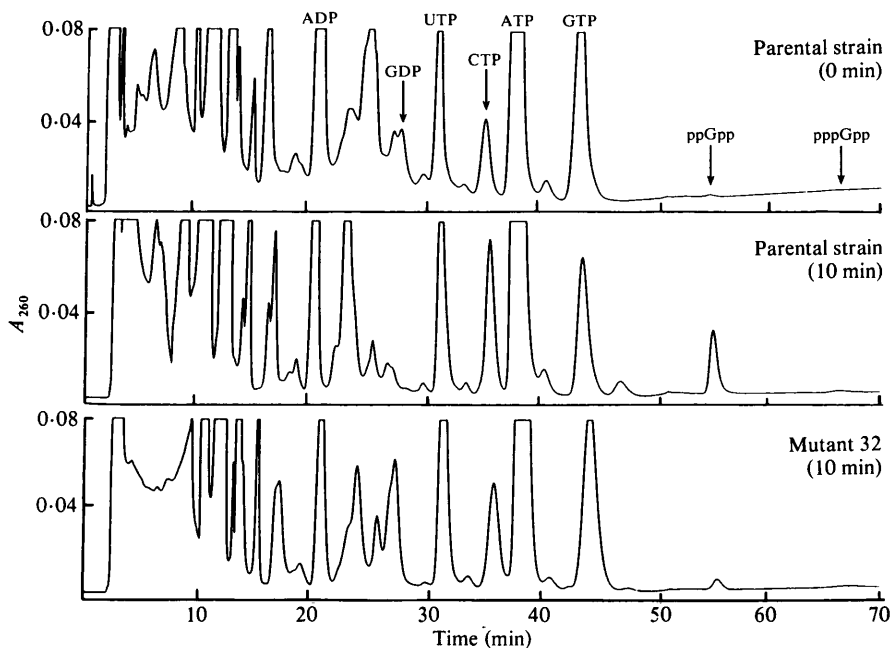


Fig. 2. Elution profile of nucleotides extracted from cells of the parental strain (MA406-A-1) or mutant 32 after deprivation of Casamino acids. The strains, grown to mid-exponential phase (for 4 h in parent, for 6 h in mutant) in synthetic medium plus 1% (w/v) Casamino acids, were harvested and transferred to fresh synthetic medium (without Casamino acids). After 10 min incubation with shaking, cells were collected and extracted and the extracts analysed by HPLC. Samples applied were equivalent to 15–17 mg cell dry wt in each profile.

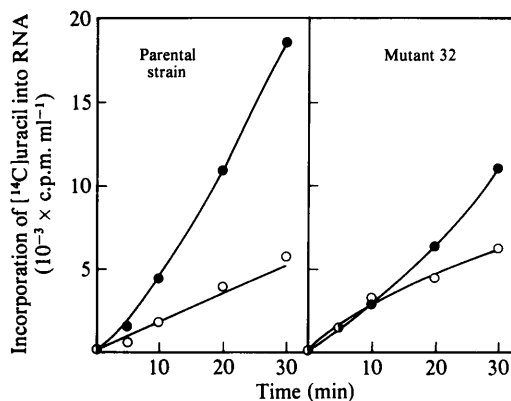


Fig. 3. Effect of deprivation of Casamino acids on RNA synthesis by cells of *Streptomyces* sp. MA406-A-1 and mutant 32. Cells, grown with 1% (w/v) Casamino acids as in Fig. 2, were transferred to synthetic medium containing  $[2\text{-}^{14}\text{C}]\text{uracil}$  with (●) or without (○) Casamino acids and incubated with shaking for 30 min (see Methods).

analysed by HPLC (see Methods). Two strains (32 and 123) accumulated severely reduced amounts of ppGpp, compared with the parental strain (Fig. 2). Strains 32 and 123 appear to be different mutants because their colony morphologies differ. The other 18 strains examined accumulated as much ppGpp as the parental strain (not shown). Mutant 32 was examined for its ability to synthesize RNA during amino acid starvation. As shown in Fig. 3, the RNA synthesis of the parental strain (MA406-A-1) was markedly reduced upon nutritional shift-down whereas

mutant 32 continued RNA synthesis for at least 20 min at a rate comparable with that in the presence of Casamino acids. Mutant 123 responded to deprivation of Casamino acids in a manner similar to that of mutant 32 (data not shown). Thus, both mutants exhibited the relaxed phenotype with respect to RNA synthesis. The *rel* mutations were apparently leaky in both strains, as both strains still produced measurable amounts of ppGpp.

#### *Characterization of rel mutants*

The *rel* mutation resulted in numerous pleiotropic effects: in particular, the *rel* mutants produced strikingly less formycin than the parental strain in both liquid and agar-plate cultures. This deficiency was more pronounced in complex than in synthetic medium: the parental strain produced 60  $\mu\text{g}$  formycin  $\text{ml}^{-1}$  in maltose/polypeptone/yeast extract medium, while the *rel* mutants (32 and 123) produced only 1–3  $\mu\text{g}$  formycin  $\text{ml}^{-1}$ . Those thiopeptin-resistant mutants which did produce as much ppGpp as the parent strain also formed 40–70  $\mu\text{g}$  formycin  $\text{ml}^{-1}$ . Thus, the ability to accumulate ppGpp was correlated with the ability to produce formycin. The decreased ability of *rel* mutants to accumulate formycin could not be attributed to an increased ability to inactivate formycin; formycin added exogenously to cultures of the mutant strains did not decrease in concentration upon incubation. In addition, the final pH values of the culture media of the parental strain and mutants were similar, suggesting that *rel* mutants did not accumulate abnormal amounts of acidic or alkaline metabolites.

If the sporulation medium was supplemented with 0.05% tyrosine, the parental strain but not the *rel* mutants produced a black pigment (melanin). Although the *rel* mutants retained their ability to form aerial mycelium and spores when cultured on sporulation medium, the amounts of aerial mycelium were less and the onset of formation of aerial mycelium was delayed considerably (5–7 d) compared with that of the parental strain (1–2 d). Furthermore, addition of a small amount of polypeptone (0.1–0.2%) to the medium suppressed completely the development of aerial mycelium in *rel* mutants but not in the parental strain. As in *E. coli* (Stephens *et al.*, 1975), the *rel* mutation rendered growth more sensitive towards an amino acid analogue, DL-serine hydroxamate.

#### *Changes in nucleotide pool after nutritional shift-down*

In *B. subtilis*, Sterlini & Mandelstam (1969) proposed a shift down for timely initiation of sporulation, where the cells were transferred from a glutamate + 1% Casamino acids medium to a replacement medium containing glutamate as the sole carbon source. Because the cells have to adapt to the absence of amino acids, the stringent response might be transiently effective; changes in nucleotide pools in *B. subtilis* cells after shift down suggested this was the case (Lopez *et al.*, 1981). I used a similar approach to study whether or not *Streptomyces* sp. exerts the stringent response (i.e. accumulation of ppGpp or pppGpp) during amino acid starvation.

The parental and one of the *rel* mutants (32) were grown to mid-exponential phase in synthetic medium supplemented with 1% Casamino acids, harvested by filtration and immediately resuspended in fresh synthetic medium (without Casamino acids). Cells of the parental strain accumulated ppGpp immediately after shift down, while cells of the *rel* mutant accumulated ppGpp to only 15% of that amount (Fig. 4).

In contrast, when the parental strain and *rel* mutant 32 were grown to exponential growth phase in synthetic medium, filtered and transferred to glucose-deficient synthetic medium, both strains accumulated 125–130 pmol ppGpp ( $\text{mg dry wt}^{-1}$ ) after 10–20 min (data not shown). Thus, strain 32 still retained the ability to accumulate ppGpp during glucose starvation, as is the case for *rel* mutants of *E. coli*.

#### *Formycin production after shift down*

Since the *rel* mutation resulted in a sharp decrease in the ability to produce formycin, it was possible that conditions causing ppGpp to accumulate might also increase the ability of cells to produce formycin. This was examined after deprivation of Casamino acids, wherein cells accumulated large amounts of ppGpp (see Fig. 4). To test this idea, cells of both the parental strain (*rel*<sup>+</sup>) and *rel* mutant were grown in synthetic medium supplemented with Casamino acids

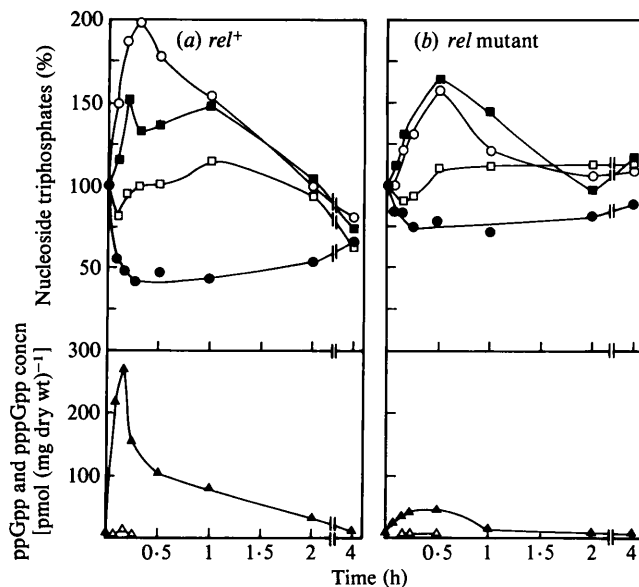


Fig. 4

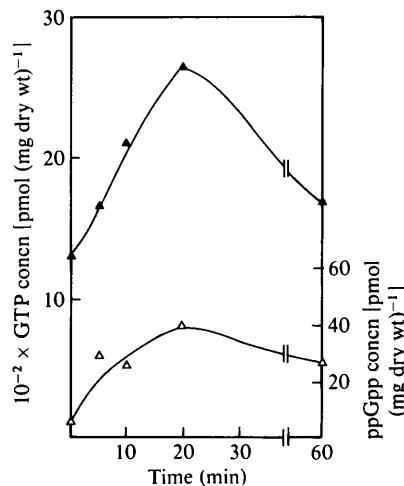


Fig. 5

Fig. 4. Changes in the intracellular concentrations of nucleoside triphosphates, ppGpp and pppGpp after shift down from synthetic medium containing 1% (w/v) Casamino acids to synthetic medium lacking Casamino acids. Cells of (a) parental strain or (b) mutant 32, grown to mid-exponential phase in synthetic medium plus 1% (w/v) Casamino acids as in Fig. 2, were harvested and transferred to fresh synthetic medium without Casamino acids, and then incubated at 30 °C with shaking. At the indicated times, cells were filtered and extracted and the nucleotides were separated and quantified by HPLC (see Methods). Just before the transfer of cells to conditions without Casamino acids, the concentrations of nucleotides [pmol (mg dry wt)<sup>-1</sup>; designated as 100%], measured in two separate experiments and averaged, were (parent) GTP, 1580; ATP, 2920; CTP, 722; UTP, 1040; (strain 32) GTP, 1680; ATP, 2790; CTP, 674; UTP, 1520. ●, GTP; ○, ATP; ■, CTP; □, UTP; ▲, ppGpp; △, pppGpp.

Fig. 5. Changes in the intracellular concentrations of ppGpp and GTP during deprivation of Casamino acids in the presence of chloramphenicol. Cells of the parental strain were grown in synthetic medium plus 1% (w/v) Casamino acids and transferred to synthetic medium containing chloramphenicol (2 µg ml<sup>-1</sup>) and growth was continued for 60 min. Other procedures were the same as in Fig. 4. ▲, GTP; △, ppGpp.

(as described above) and shifted down nutritionally by harvesting and transferring the cells to one-half and one-third of the original volume of fresh synthetic medium (cell density 2.2–2.5 mg dry wt ml<sup>-1</sup>). Cells of both strains were also grown, in parallel, in synthetic media without Casamino acids supplement but transferred to fresh medium as described above. These transferred cultures were all incubated with shaking for 8 h. Formycin production by *rel*<sup>+</sup> cells deprived of Casamino acids was drastically enhanced after a 2 h lag; increase in formycin titre was eightfold higher in cells shifted down than in those grown throughout in the synthetic medium. The *rel* cells did not produce any detectable amount of formycin under either of these experimental conditions.

Low levels of chloramphenicol inhibit the synthesis of both ppGpp and pppGpp without affecting cell growth of *B. subtilis* (Ochi & Freese, 1983). With *Streptomyces* sp., low levels of chloramphenicol (2 µg ml<sup>-1</sup>) similarly blocked accumulation of ppGpp induced by deprivation of Casamino acids (compare Fig. 5 with Fig. 4a). Virtually no change in growth rate was observed upon the addition of 2 µg chloramphenicol ml<sup>-1</sup> as determined after deprivation of Casamino acids, but 8 µg chloramphenicol ml<sup>-1</sup> caused 50% inhibition of the growth rate.

When cells of the parental strain were shifted down from medium containing Casamino acids, their increased ability to produce formycin could be blocked by the immediate re-addition of 1% Casamino acids or of chloramphenicol (2 µg ml<sup>-1</sup>); however, after 2–3 h, addition of Casamino

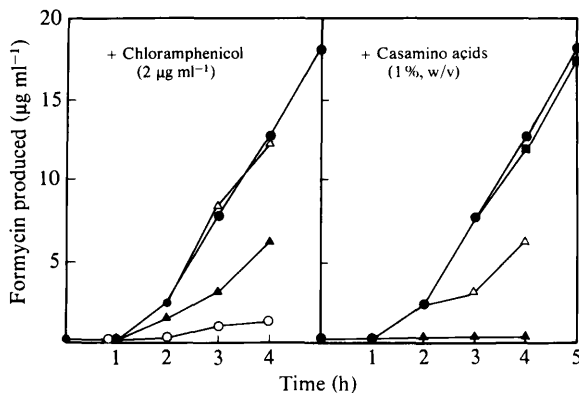


Fig. 6. Effect of chloramphenicol or Casamino acids on formycin production initiated by deprivation of Casamino acids. Cells of the parental strain were grown with 1% (w/v) Casamino acids as in Fig. 4 and then harvested and transferred to one-third of the original volume of fresh synthetic medium, giving 2.2 mg dry wt cells ml<sup>-1</sup>. Cultures were divided into five samples which were incubated at 30 °C. At various times, chloramphenicol (2 µg ml<sup>-1</sup>) or Casamino acids (1%, w/v) was added to one of the flasks and the formycin titre in the medium was measured at the times indicated. ●, No addition and addition at: ○, zero time; ▲, 1 h; △, 2 h; ■, 3 h.

Table 1. *Effect of glucose deprivation on formycin production in the presence or absence of chloramphenicol*

The results are means of four separate experiments and varied by  $\pm 20\%$  for formycin produced per cell and by  $\pm 15\%$  for ppGpp.

Strain	Transferred from synthetic medium to	Chloramphenicol (2 µg ml <sup>-1</sup> )	Formycin produced per cell* [µg (mg dry wt) <sup>-1</sup> ]	ppGpp† [pmol (mg dry wt) <sup>-1</sup> ]
Parent ( <i>rel</i> <sup>+</sup> )	Glucose-deficient	—	4.6	136
	synthetic medium	+	4.6	63
	Synthetic medium	—	1.5	22
		+	0.8	7
Mutant 32 ( <i>rel</i> )	Glucose-deficient	—	3.8	122
	synthetic medium	+	1.3	68
	Synthetic medium	—	0.3	14
		+	<0.2	12

\* Determined 10 h after transfer.

† Determined 20 min after transfer.

acids or chloramphenicol had no effect on formycin synthesis (Fig. 6), indicating that the stringent response has to function for 2–3 h to complete the initiation of formycin production.

Because the *rel* mutant could accumulate ppGpp during glucose starvation, it was worthwhile to examine formycin-producing ability under such conditions. Glucose deprivation after transfer from synthetic medium arrested growth completely during 10 h incubation (but DNA synthesis continued at half rate for at least 4 h), and during this period the *rel* mutant produced formycin to about the same extent as did the *rel*<sup>+</sup> parental strain (Table 1). Thus, the ability to produce formycin was tightly coupled with the accumulation of ppGpp.

#### *Curing of formycin production*

Culture of *Streptomyces* sp. MA406-A-1 with acridine dye resulted in a high frequency (about 50%) of colonies that had lost the ability to produce formycin (S. Yashima, personal communication), which is a result similar to that seen for antibiotic production by numerous



other *Streptomyces* species (Okanishi & Umezawa, 1978). After deprivation of Casamino acids these cured strains accumulated as much ppGpp as did the parental strain.

#### DISCUSSION

In bacteria, differentiation and induction of secondary metabolism start concomitantly in response to nutrient limitation. How do bacteria sense such adverse environmental conditions? The results of this study indicate that in *Streptomyces* sp. MA406-A-1 ppGpp may be an early signal, produced in response to nutrient limitation, which may trigger events such as formycin production. This suggestion is supported by the response of the parental strain both to nutritional shift-down and to chloramphenicol and by the behaviour of the *rel* mutants.

This is the first report of the isolation of *rel* mutants (32 and 123) in streptomycetes; they might be tentatively classed as *relC* mutants, in analogy with mutants of *E. coli* and *B. subtilis*, which lack ribosomal protein L11 (Smith *et al.*, 1978, 1980), but further work will be required to confirm this classification directly. Under glucose deprivation the cells of the *rel* mutants of *Streptomyces* sp. MA406-A-1 accumulated as much ppGpp as cells of the wild-type strain, a result consistent with what has been reported in *E. coli* (Lazzarini *et al.*, 1971; Friesen *et al.*, 1974).

The GTP pool size of *rel*<sup>+</sup> (but not *rel*) cells decreased extensively after deprivation of Casamino acids concomitant with the increased levels in ppGpp (Fig. 4). Since ppGpp (and pppGpp) inhibits IMP-dehydrogenase of *E. coli* and *B. subtilis* (Gallant *et al.*, 1971; Wu & Scrimgeour, 1973), which plays a role in GMP (and thus GTP) synthesis, the elevated levels of ppGpp may be responsible for the decline in the GTP of *Streptomyces* sp.

The ability to produce formycin was strongly correlated with the stringent response, suggesting that ppGpp may initiate (directly or indirectly) formycin production. In support of this suggestion is the fact that the *rel* mutation also abolished streptomycin production by *Streptomyces griseus* (unpublished observations). The only seeming disharmony was the lack of effect of chloramphenicol on formycin production by the *rel*<sup>+</sup> strain during glucose deprivation (see Table 1). Conceivably, *rel*<sup>+</sup> cells grown in synthetic medium had already been committed to produce formycin and this commitment was not abolished because of the absence of further growth, so that inhibition of ppGpp accumulation no longer affected formycin-producing ability. However, the results do not reveal anything about the molecular mechanism of ppGpp function, i.e. a role in transcription or translation. The decrease in GTP content *per se*, which presumably resulted from ppGpp accumulation, cannot be a signal for formycin production (Ochi, 1986). The sharp decrease in GTP pools associated with the observed stringent response may also play a role in the initiation of aerial mycelium formation. The delayed appearance of aerial mycelium in the *rel* mutants is consistent with this notion (Fig. 4; also Ochi, 1986). The relatively high ppGpp accumulation during growth in synthetic medium could be a reason for growth-associated formycin production. In fact, the addition of Casamino acids lowered the ppGpp pool size (Fig. 1), and suppressed formycin production throughout exponential growth. The occurrence of ppGpp in various *Streptomyces* species (Hamagishi *et al.*, 1981; Nishino & Murao, 1981) and its possible relationship to antibiotic production (An & Vining, 1978; Simuth *et al.*, 1979; Stastna & Mikulik, 1981) have been reported.

Antibiotic production has been most frequently described from a viewpoint of 'negative' regulatory features (Martin & Demain, 1980; Demain *et al.*, 1983), and it is frequently suppressed by high levels of carbon, nitrogen and phosphate. In *E. coli*, cAMP is involved in carbon catabolite repression. Although cAMP occurs in *Streptomyces* species (Gersch, 1979; Colombo *et al.*, 1982; Ensign, 1982; Surowitz & Pfister, 1985), it is uncertain whether it has an essential role in catabolite repression. In contrast with such negative regulations, compounds which 'positively' function have been reported recently. Mutants of *S. griseus* or *Streptomyces griseoflavus* lacking A-factor or defective in arginine synthesis, respectively, were deficient both in aerial mycelium formation and antibiotic production, but both processes could be restored by adding A-factor or citrulline (Kleiner *et al.*, 1976; Hara & Beppu, 1982; Ochi, *et al.*, 1984). At present, it is entirely unknown whether effects elicited by ppGpp could be superimposed on the positive and negative regulatory effects mentioned above. Such a possible interaction might be at least partially resolved by using the *rel* mutants described.

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