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Streptomyces relC Mutants with an Altered Ribosomal Protein ST-L11 and Genetic Analysis of a *Streptomyces griseus relC* Mutant†

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Several relaxed (*rel*) mutants have been obtained from *Streptomyces* species by selecting colonies resistant to thiopeptin, an analogue of thiostrepton. Using two-dimensional gel electrophoresis, I compared the ribosomal proteins from *rel* and *rel*$^+$ pairs of *S. antibioticus*, *S. lavendulae*, *S. griseoflavus*, and *S. griseus*. It was found that all of the *Streptomyces rel* mutants thus examined had an altered or missing ribosomal protein, designated tentatively ST-L11. These *rel* mutants therefore could be classified as *relC* mutants and were highly sensitive to erythromycin or high temperature. A *relC* mutant of *S. griseus* was defective in streptomycin production, but phenotypic reversion of this defect to normal productivity was found at high incidence among progeny of the *relC* mutant. This phenotypic reversion did not accompany a reappearance of ribosomal protein ST-L11, and furthermore the ability of accumulating ppGpp still remained at a low level, thus suggesting existence of a mutation (named *sup*) which suppresses the streptomycin deficiency phenotype exhibited by the *relC* mutant. Genetic analysis revealed that there is a correlation between the *rel* mutation and the inability to produce streptomycin or aerial mycelia. The *sup* mutation was found to lie at a chromosomal locus distinct from that of the *relC* mutation. It was therefore concluded that the dependence of streptomycin production on the normal function of the *relC* gene could be entirely bypassed by a mutation at the suppressor locus (*sup*). The suppressing effect of the *sup* mutation on the *relC* mutation was blocked when the *afs* mutation (defective in A-factor synthesis) was introduced into a *relC sup* double mutant. It is proposed that the *sup* gene or its product can be a direct or indirect target for ppGpp.

The study of differentiation in *Streptomyces* species is of interest because they produce numerous antibiotics (physiological differentiation), usually in the process of aerial mycelium formation (morphological differentiation). The coupling of morphological and physiological differentiation suggests a mechanistic connection, clarification of which has only been attempted (for reviews, see references 3 and 14). Especially, it is frequently observed and widely accepted that morphological and physiological differentiation start concomitantly in response to nutrient limitation. One of the significant bacterial regulatory systems, coupled to nutrient limitation, is "stringent response," which causes immediate cessation of RNA synthesis and of other cellular reactions (for reviews, see references 2, 7, and 32). The guanine nucleotides ppGpp (guanosine 5'-diphosphate-3'-diphosphate) and pppGpp (guanosine 5'-triphosphate-3'-diphosphate) are believed to be responsible for this stringent response (9): when uncharged tRNA, a fraction of which would increase under amino acid limitation, enters the A site on a ribosome, *relA* gene product (stringent factor) catalyzes the synthesis of ppGpp and pppGpp. Transcription of numerous genes, including those for rRNA and tRNA, is immediately blocked by these nucleotides. A functional 50S ribosomal protein (designated L11 for *Escherichia coli*, BS-L11 for *Bacillus subtilis*, and BM-L11 for *Bacillus megaterium*) is required for synthesis of ppGpp and pppGpp upon amino acid limitation. Mutants with mutated L11, BS-L11, or BM-L11 protein fail to synthesize ppGpp and pppGpp, and thus they are called relaxed (*rel*) mutants (6, 34, 36). The ribosomal proteins L11, BS-L11, and BM-L11 are known to be homologous with each other (4, 35, 38) and are coded by the *relC* gene (= *rplK* gene in *Escherichia coli*) (29, 33). The mechanism of stringent response, briefly noted above, is probably oversimplified. Recent investigations suggest that in *E. coli* a "relaxed response" is not simply the absence of a stringent response; instead, in a *relA* relaxed strain, ppGpp levels drop precipitously rather than simply fail to accumulate (see reference 31 for a recent reference). In the present study, the relaxed phenotype is however defined for convenience as being a failure to suppress RNA synthesis coupled with a failure to accumulate ppGpp under amino acid deprivation.

The results of isolating and analyzing the relaxed mutants of several *Streptomyces* spp. led me to propose that morphological differentiation results from a decrease in the GTP pool, whereas physiological differentiation results from a more direct function of the *rel* gene product, ppGpp (18–23). In *B. subtilis* and *B. megaterium* (but not in *E. coli*), *relC* mutants were originally selected by resistance to thiostrepton (also known as bryamycin), an antibiotic that interferes with several ribosomal functions, eventually leading to inhibition of protein synthesis (4, 10, 30). Studies with *E. coli* (11), *B. subtilis* (38), and *B. megaterium* (4) have confirmed that *relC* proteins L11, BS-L11, and BM-L11 present in 50S subunits are principally involved in either creating or completing a binding site for thiostrepton: these proteins alone do not bind thiostrepton. The *rel* mutants of *Streptomyces* spp. used in the present study have been selected as spontaneously arising colonies resistant to thiopeptin (19–21, 23). Since thiopeptin is analogous to thiostrepton in both structure and mode of action (5), it is reasonable to assume that the lesion is expressed at the level of the ribosomal protein. The study presented here was undertaken on this assumption. Genetic analysis of the *rel* mutation of *S. griseus* was also attempted by using protoplast fusion; this analysis presents evidence for the existence of a mutation which suppresses the defects exhibited by the *relC* mutant.

† This paper is dedicated to the late Ernst Freese to thank him for his past courtesy.

MATERIALS AND METHODS

Strains and preparation of mutants. The strains used were *S. antibioticus* 3720 (= ATCC 14888), *S. lavendulae* MA406-A-1 (previously called *Streptomyces* sp. strain MA406-A-1), *S. griseoflavus* FERM 1805, and *S. griseus* IFO 13189. These are prototrophic wild-type strains. The *rel* mutants 49 (*S. antibioticus*), 32 and 123 (*S. lavendulae*), 6 (*S. griseoflavus*), and 3-3 (*S. griseus*) were selected from each parental strain as spontaneously arising colonies resistant to thiopeptin, as previously described (19–21, 23). The *rel* mutants 6 and 3-3 were used after purification by single-colony isolation just before use for experiments, because these two *rel* mutants (but not other *rel* mutants) were found to be unstable genetically, with highly frequent changes in colony morphology during storage. *B. subtilis* 60015 (*metC7 trpC2 rel*$^+$) and 61953 (*thr-5 trpC2 relC*) were provided by E. Freese, National Institutes of Health, Bethesda, Md. The *relC* (= *tsp-6 bry-2*) mutation in strain 61953 was originally selected by resistance to thiostrepton (34).

Mutagenesis for obtaining auxotrophic mutants of *S. griseus* was carried out by treating the spores with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (1.2 mg/ml, final concentration) for 1 h, as previously described (26). Double or triple auxotrophic strains were derived by sequential protoplast fusion of single auxotrophic mutants (see below). Although the original single auxotrophic mutants had partially impaired ability (decreased by 20 to 50%) for producing streptomycin or aerial mycelium probably because of alterations of DNA by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, it was easy, by using a protoplast fusion technique, to construct double or triple auxotrophic recombinants with normal productivity. The spontaneous reversion rates of each single requirement to prototrophy were between 10^{-8} and 10^{-9}. The medium used for determination of auxotrophy (synthetic medium I) was the same as previously described (21). Mutants (*afs*) defective in A-factor synthesis were obtained as described earlier (21), except that the cells were grown at 34°C instead of 36°C; the incidence of the *afs* mutants was about 20%. The amazingly high frequency of *afs* mutants after cultivation of *S. griseus* 13189 at high temperature has been taken as circumstantial evidence of plasmid involvement in A-factor synthesis in this strain (11).

Standard cross (matings). The method described by Hopwood (13) was used with slight modifications. A spore suspension of each auxotrophic strain was inoculated onto plates of sporulation agar (21) supplemented with appropriate requirements and then incubated at 30°C for 7 days. Frequency of recombinants was about 10^{-7}.

Fusion of protoplasts. The procedures for fusion and regeneration of protoplasts were essentially the same as that described previously (26). Auxotrophic strains were grown in SPY medium (21) at 30°C for 20 h (late exponential phase). The mycelia were harvested and protoplasts were prepared by incubating the mycelia in protoplast buffer (medium P [28]) containing lysozyme (2.5 mg/ml) but omitting $CuCl_2$. Fusion of protoplasts was performed by treating with 40% (wt/vol) polyethylene glycol 4000 (in protoplast buffer) at 30°C for 5 min. Fused protoplasts were then plated on regeneration medium (medium R1 [28]) omitting Casamino Acids and $CuCl_2$, with or without 2 mM (but 4 mM for glutamate) requirements and incubated at 25°C for 20 days until no new colonies were formed. (Long-term incubation was important since the recombinants carrying the *relC* mutation grew slowly on the regeneration medium). Frequency of regeneration from protoplast to mycelial form was about 10^{-3}, as determined with the parental strain. Frequency of recombinants, given as the ratio of prototrophic colonies growing on minimal regeneration medium to the total number of viable colonies on supplemented regeneration medium, was between 2×10^{-3} and 1×10^{-2}. The recombinants thus selected directly on the minimum regeneration medium were purified by single-colony isolation and then tested for their auxotrophy by replica plating on synthetic medium II with or without appropriate requirements. Synthetic medium II contained (per liter): soluble starch, 20 g; glycine, 5 g; sodium citrate (dibasic), 10 g; ammonium sulfate, 3 g; $MgSO_4$ (dehydrated), 10 g; NaCl, 5 g; KH_2PO_4, 0.5 g; $CaCl_2$, 50 mg; $ZnSO_4 \cdot 5H_2O$, 30 mg; $FeSO_4 \cdot 5H_2O$, 25 mg; $CuSO_4 \cdot 5H_2O$, 16 mg; and $MnSO_4 \cdot 4H_2O$, 12 mg (adjusted to pH 7.2 with NaOH). Both the growth rate and final biomass on medium II were much higher than on medium I. The *rel* genotype was determined on the basis of thiopeptin resistance after the recombinants were incubated on GYM agar (21) containing 4 μg of thiopeptin per ml; the *rel* but not the *rel*$^+$ recombinants grew on the medium 2 to 3 days after incubation at 30°C. Streptomycin productivity was determined by cultivating the recombinants in SPY medium (50 ml/250-ml flask) supplemented with requirements at 25°C for 3 days.

Assay for streptomycin, A-factor, and ppGpp. The procedures for each assay have been described earlier (19, 21).

Determination of RNA synthesis. RNA synthesis was determined by measuring the incorporation of [2-^{14}C]uracil into an acid-precipitable portion as previously described (19, 21).

Two-dimensional polyacrylamide gel electrophoresis. All of the strains were grown to mid-exponential phase in SPY medium. Preparations of ribosomes and ribosomal proteins and the procedures for two-dimensional polyacrylamide gel electrophoresis were described in a previous paper (25). The gels were run two to three times for different ribosomal protein preparations to confirm the reproducibility of the results obtained.

RESULTS

Phenotypic characterization of *rel* mutants. The *rel* mutants of *Streptomyces* spp. used here have been characterized as to their ability to produce antibiotic and aerial mycelia and also to accumulate ppGpp or RNA upon amino acid deprivation (19–21, 23). These characteristics are summarized in Table 1. The *relC* mutation of *B. subtilis* resulted in an increased sensitivity to erythromycin (10) and high temperature (34). Likewise, most of the *rel* mutants, with the exception of *S. antibioticus rel* mutant 49, were more sensitive to erythromycin and high temperature (Table 2). *S. griseoflavus rel* mutant 6 gave rise to colonies at high frequency (10^{-3}) which formed aerial mycelia normally as did its parental strain 1805 (23). They were of *rel*$^+$ type, as determined by their capability to accumulate ppGpp or to suppress RNA synthesis upon amino acid deprivation (23; Table 1). These *rel*$^+$ revertants, including the representative 6-R1, acquired normal sensitivity to both erythromycin and high temperature (Table 2). No reversion to *rel*$^+$ phenotype was detected in either *S. antibioticus* 49 or *S. lavendulae* 32 and 123, indicating genetic stability of these *rel* mutants. The *S. griseus rel* mutant 3-3 gave rise, at very high frequency (5 to 30%), to reversion of streptomycin deficiency to normal productivity when the mutant was stored for a long term (e.g., 6 months) on a slant in a cold room or when cultivated continuously for 2 weeks by inoculating the cells into fresh medium every 2 days. These phenotypic revertants pro-

TABLE 1. Summary of strain characteristics (19–21, 23)

Organism (antibiotic, produced) and strain	Source of strain	Amt (μg/ml) of thiopeptin needed for selection	*rel* genotype[a]	ppGpp accumulated[b] (pmol/mg [dry wt])	Antibiotic produced, (μg/ml)	Aerial mycelia[c]
S. antibioticus (actinomycin)						
3720			*rel*$^+$	82	110	+++
49	3720	3	*rel*	12	<1	+
S. lavendulae (formycin)						
MA406-A-1			*rel*$^+$	275	60	+++
32	MA406-A-1	3	*rel*	44	2	+
123	MA406-A-1	3	*rel*	53	2	+
S. griseoflavus (bicozamycin)						
1805			*rel*$^+$	337	72	+++
6	1805	40	*rel*	47	0.5	+
6-R1	6	NA[d]	*rel*$^+$	348	69	+++
S. griseus (streptomycin)						
13189			*rel*$^+$	815	120	+++
3-3	13189	3	*rel*	96	3	+
3-3-C	3-3	NA	*rel*	88	110–230	+++

[a] Determined by measuring RNA synthesis during Casamino Acid deprivation.
[b] Maximal ppGpp accumulated during Casamino Acid deprivation.
[c] +++, Abundant; +, sparse.
[d] NA, Not applicable.

duced normally not only streptomycin but also aerial mycelia; a representative strain, 3-3-C, is presented in Table 1. Unlike the case of *S. griseoflavus*, these phenotypic revertants did not accompany the restoration of the capability of accumulating ppGpp but retained their resistance to thiopeptin (Tables 1 and 2). These results raised the possibility of the existence of a mutation which suppresses the streptomycin deficiency phenotype resulting from the *rel* mutation. Since in *E. coli* RNA polymerase is a known target for ppGpp (9), I examined whether the strain 3-3-C carrying the putative suppressor mutation exhibited a relaxed response on RNA synthesis. Although the wild-type *S. griseus*, despite the accumulation of large amounts of ppGpp, exhibited incomplete stringency (i.e., incomplete suppression of RNA synthesis; Fig. 1A) under amino acid deprivation, strain 3-3-C clearly exhibited a relaxed response as did the *rel* mutant 3-3 (Fig. 1B and C). In order to analyze the *rel* and suppressor mutations genetically, a molecular analysis of the *rel* mutation was needed.

TABLE 2. Drug or temperature sensitivity of *Streptomyces rel* mutants

Strain	Resistance (up to μg/ml) of[a]:		Growth at high temperature[b]
	Thiopeptin	Erythromycin	
S. antibioticus			
3720 (*rel*$^+$)	1	0.1	+
49 (*rel*)	10	0.1	+
S. lavendulae			
MA406-A-1 (*rel*$^+$)	0.5	0.3	+
32 (*rel*)	6	0.05	−
123 (*rel*)	6	0.05	−
S. griseoflavus			
1805 (*rel*$^+$)	7	8	+
6 (*rel*)	200	1	−
6-R1 (*rel*$^+$)	7	8	+
S. griseus			
13189 (*rel*$^+$)	0.5	20	+
3-3 (*rel*)	7	2	−
3-3-C (*rel*)	30	4	−

[a] Mycelia were spread on GYM agar containing various amounts of drug and then incubated at 30°C. Resistance to the drug, defined as 50% growth of the control (no drug), was determined after 4 days of incubation, except that erythromycin resistance of *S. griseus* was determined after 1 day of incubation.
[b] Mycelia were spread on GYM agar. Growth was determined after 2 days of incubation at 40°C, except for that of *S. griseus*, which was incubated at 37°C. +, Growth more than 50% of control (30°C); −, growth less than 5% of control.

Two-dimensional gel electrophoresis of ribosomal proteins. I attempted two-dimensional polyacrylamide gel electrophoresis, using ribosomal proteins from *B. subtilis relC* and *rel*$^+$ strains (as a control) since their ribosomal proteins are well characterized. As shown in Fig. 2B, the *relC* strain 61953 lacked BS-L11 (marked by arrow), a result reported previously (34), and the patterns of protein spots in the gel were similar to those reported earlier (8, 38).

Accordingly, the ribosomal proteins of the *Streptomyces rel* mutants were then compared with those of the wild type. In *S. antibioticus*, the patterns of spots in the gels were essentially the same with one distinguishing difference: the spot in the wild type (arrow in Fig. 2C) was missing in the *rel* mutant 49 (Fig. 2D), and instead there was, though not convincingly, an extra spot (as indicated by the double-shafted arrow). This suggested the existence of a mutationally altered protein migrating differently during electrophoresis. No other ribosomal proteins were missing or altered in their electrophoretic mobility. (Several faint spots observed in Fig. A and C [indicated by arrowheads] are not observed in corresponding Fig. B and D, but these spots can be seen in the original slab gels.)

In *S. lavendulae*, two spots in the wild type were missing from the *rel* mutant 32 (Fig. 2E and F). It must, however, be noted that the spot indicated by the arrow titled at +60° in Fig. 2F is not convincingly missing since there was a reproducible faint spot in this region after several gels were run, and thus it might be a low-abundance protein. In contrast, there was no observable difference in a gel pattern of ribosomal proteins between the *rel* mutant 123 and parental strain (Fig. 2G). Apparently, strains 32 and 123 are different mutants, as expected from their distinctive colony structures (19).

Similarly, a spot in wild-type *S. griseoflavus* was absent

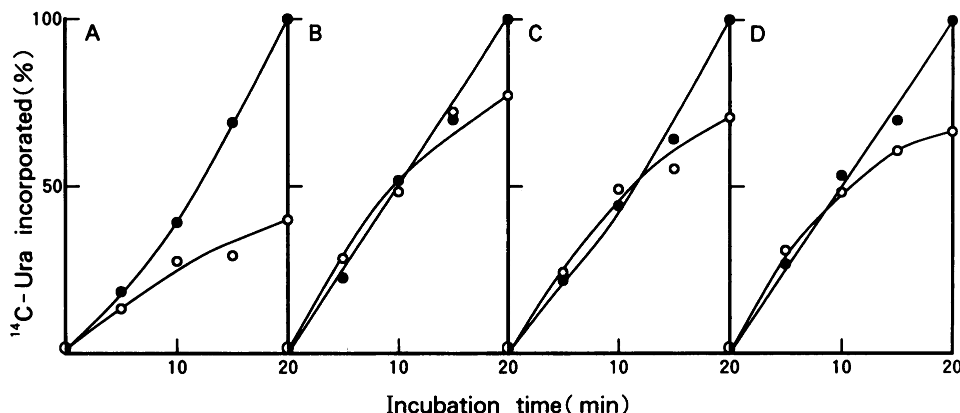


FIG. 1. RNA synthesis by cells of the *S. griseus* parental (13189) and mutant strains after deprivation of Casamino Acids. Cells, grown in synthetic medium containing 1% Casamino Acids to mid-exponential phase, were collected and transferred in synthetic medium containing [2-^{14}C]uracil with (●) or without (○) Casamino Acids and then incubated with shaking as previously described (21), except that synthetic medium contained 0.1% instead of 1% magnesium sulfate before and after transfer. The counts (in disintegrations per minute) measured after 20 min of incubation with Casamino Acids were designated as 100%. (A) Parental strain. (B) Strain 3-3. (C)Strain 3-3-C. (D) Strain 106.

from the *rel* mutant 6 (Fig. 2H and I). A revertant (6-R1) from the *rel* mutant to stringency was also analyzed: a protein which was absent in the *rel* mutant reappeared in the revertant (Fig. 2J). Thus, the change in ribosomal protein of *S. griseoflavus* was apparently reversed in such a way that the protein in the revertant migrated to the same position as the original protein of the wild-type strain.

In *S. griseus*, two spots in the wild type were clearly absent from the *rel* mutant 3-3, and there was no extra spot (Fig. 2K and L). The two proteins which were absent in mutant 3-3 were, as expected, still absent in strain 3-3-C carrying the putative suppressor mutation (Fig. 2M), thus accounting for its retention of the reduced ability to accumulate ppGpp (Table 1). Other ribosomal proteins from strain 3-3-C were also found not to vary from strain 3-3, suggesting that the suppressor mutation was not mediated through a third ribosomal protein. Accordingly, the ribosomal protein, altered or missing in *S. antibioticus* and *S. griseoflavus* (and also one of the two missing ribosomal proteins in *S. lavendulae* and *S. griseus*), was designated tentatively ST-L11.

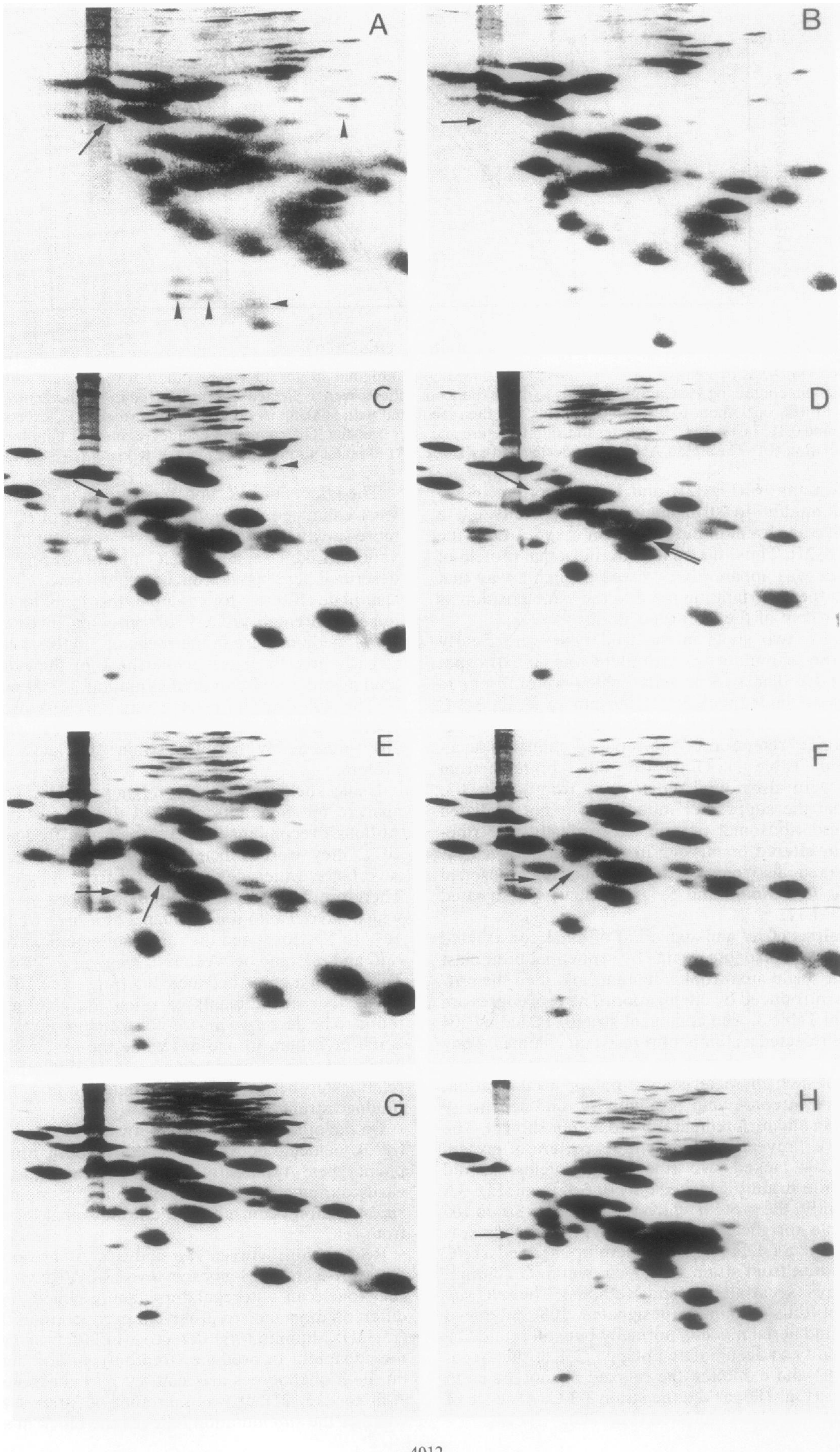
Genetic analysis of *rel* and *sup*. First of all, I constructed double or triple auxotrophic strains by sequential protoplast fusion of each single auxotrophic mutant, and then the *relC* mutation was introduced by conjugation. The procedures are summarized in Table 3. The conjugant strains, including 104 and 105, were selected as thiopeptin-resistant colonies. They exhibited the relaxed phenotype as in mutant 3-3 with respect to all of such characteristics as ppGpp accumulation, RNA synthesis, streptomycin productivity, and sensitivity to erythromycin and high temperature (data not shown). The strains, however, revealed a different gel pattern of protein spots; strain 104 lacked two ribosomal proteins, as did mutant 3-3, while strain 105 lacked only one of them (Fig. 3A and B). Evidently, the protein which was absent in strain 105 was responsible for the relaxed phenotype and thus was assumed to be the ST-L11 protein. I therefore derived a *relC sup* double mutant from strain 105 by cultivating it continuously for 14 days (see Materials and Methods). The *relC sup* double mutant thus obtained (designated 106) produced streptomycin and aerial mycelia normally but still retained a reduced capability to accumulate ppGpp (12% of that accumulated by 103) and exhibited the relaxed phenotype as to RNA synthesis (Fig. 1D), as did the strain 3-3-C. Absence of protein ST-L11 was also confirmed (Fig. 3C).

The effects of *relC* and *sup* mutations on the growth rate were examined. The *relC* mutant 61953 of *B. subtilis* grows more slowly than its isogenic *rel*$^+$ strain (unpublished observation). Likewise, all the *relC* mutants of *Streptomyces* spp. described here had a reduced growth rate in both liquid and agar plate cultures; for example, the doubling times of the *S. griseus* parental strain 13189 growing in SPY medium or GYM medium were in the range of 3.0 to 3.2 h as measured at early growth phase, while those of the *relC* mutant 3-3 (and also the *relC sup* double mutant 3-3-C) were 4.5 to 5.2 h. The difference in growth rate was less pronounced between strains 103 and 105 but not between strains 102 and 104, presumably because strain 105 lacks only ST-L11 protein.

Using such auxotrophic *rel-sup* strains, I attempted to analyze recombinants obtained during a standard mating. Although recombinants developed at a frequency of about 10^{-7}, they were difficult to distinguish from spontaneous revertants, which developed at a frequency of about 10^{-8}. Therefore, I next employed the protoplast fusion technique, which gave rise to recombinants at a high frequency of 2×10^{-3} to 1×10^{-2}, and the results of genetic crosses between *relC* and *rel*$^+$ and between *relC sup* and *rel*$^+$ are presented in Table 4. In a cross between 105 (*relC*) and 102 (*rel*$^+$), all of the tested recombinants carrying the *relC* mutation were found to be defective in streptomycin production (and also in aerial mycelium formation) while the *rel*$^+$ recombinants all exhibited normal productivity, suggesting a possible causal relationship between the *relC* mutation and the inability to produce streptomycin.

On the other hand, a cross between 106 (*relC sup*) and 102 (*rel*$^+$) yielded *relC* recombinants of both Stm$^-$ and Stm$^+$ phenotypes. Apparently, *relC* and *sup* mutations segregated easily during genetic recombination, thus indicating that the *sup* mutation occurred on a chromosomal locus distinctive from *relC*.

Relationship between *sup* and *afs*. *S. griseus* 13189 produces A-factor (2S-isocapryloyl-3S-hydroxymethyl-γ-butyrolactone), an autoregulatory factor which regulates both differentiation and streptomycin production in this organism (15, 21). Mutants (*afs*) defective in A-factor synthesis lose the capability to produce streptomycin and aerial mycelia, but both phenotypes are restored by exogenous addition of A-factor (15, 21). It was therefore of interest to determine whether the *sup* mutation relieves the block in streptomycin


A
B
C
D
E
F
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H

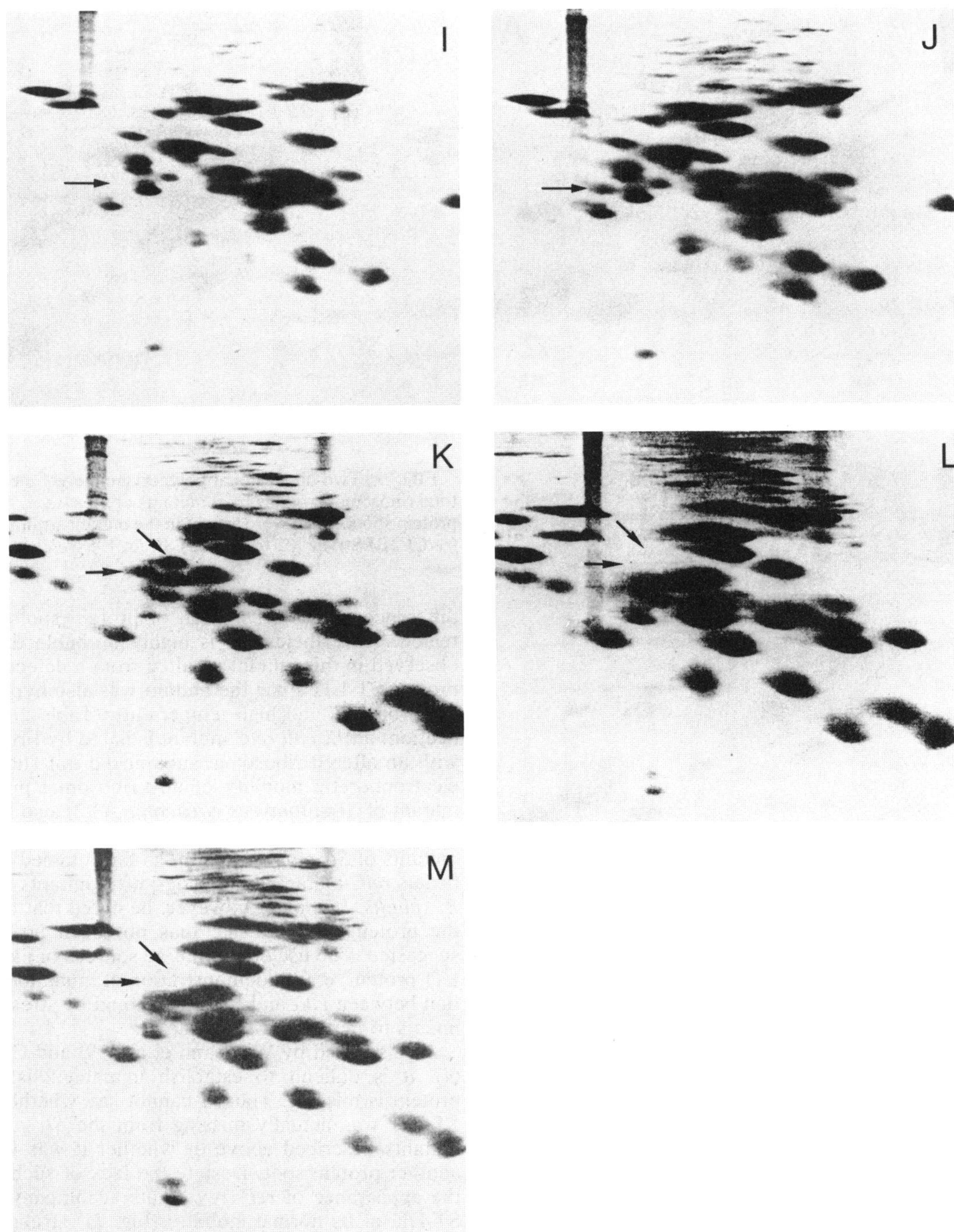

FIG. 2. Two-dimensional polyacrylamide gel electrophoresis of total ribosomal proteins. The gel system was based upon that of Kaltschmidt and Wittmann (16). The organisms analyzed were as follows: *Bacillus subtilis* 20015 (A) and 61953 (B); *Streptomyces antibioticus* 3720 (C) and 49 (D); *Streptomyces lavendulae* MA406-A-1 (E), 32 (F), and 123 (G); *Streptomyces griseoflavus* 1805 (H), 6 (I), and 6-R1 (J); *Streptomyces griseus* 13189 (K), 3-3 (L), and 3-3-C (M). Arrows indicate protein spots which were absent in the mutants.

production caused by the *afs* mutation. I therefore constructed triple mutants (*relC sup afs*) by introducing the *afs* mutation into strain 3-3-C or 106 (see Materials and Methods). The triple mutants tested (five strains for each) were all again blocked for production of both streptomycin and aerial mycelia. Clearly, the ability of the *sup* mutation to restore these processes was not exerted in the genetic background of *afs* mutants. Exogenous addition of A-factor (0.5 μg/ml, final concentration) restored both phenotypes of these triple mutants.

DISCUSSION

The *relC* mutants of *B. subtilis* and *B. megaterium* have a missing ribosomal protein (4, 34, 38). Mutants of *E. coli* lacking protein L11 are also found among revertants from

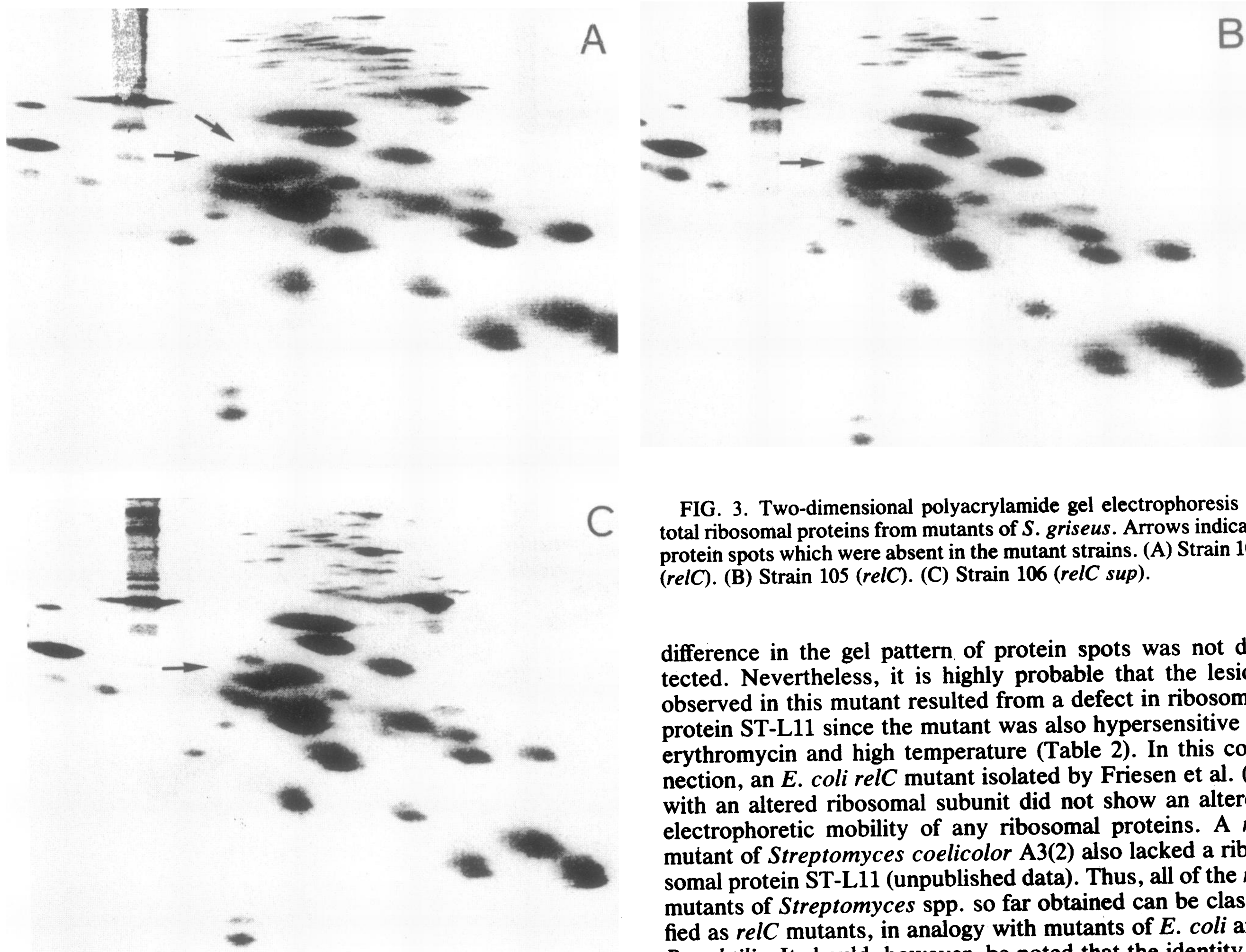

FIG. 3. Two-dimensional polyacrylamide gel electrophoresis of total ribosomal proteins from mutants of *S. griseus*. Arrows indicate protein spots which were absent in the mutant strains. (A) Strain 104 (*relC*). (B) Strain 105 (*relC*). (C) Strain 106 (*relC sup*).

erythromycin dependence to independence (37). Likewise, thiopeptin-resistant *Streptomyces rel* mutants tested had an altered or missing ribosomal protein, designated ST-L11. This is the first reported evidence of mutationally altered ribosomal proteins in streptomycetes. The only exception was in *S. lavendulae rel* mutant 123, wherein an observable

TABLE 3. Mutant strains of *S. griseus* used

Strain	Genotype	Streptomycin productivity[a]	Source
3-3	*relC1* (prototroph)	−	21
3-3-C	*relC1 sup-1* (prototroph)	+	Spontaneous mutation of 3-3
101	*met relC1*	−	NTG of 3-3
102	*glu[b] ura trp*	+	This study
103	*arg ilv[c]*	+	This study
104	*glu ura trp relC1*	−	Conjugation of 101×102
105	*arg ilv relC1*	−	Conjugation of 101×103
106	*arg ilv relC1 sup-2*	+	Spontaneous mutation of 105

[a] + and −, Capability to produce streptomycin of 100 to 200 μg/ml or 0 to 3 μg/ml, respectively.
[b] Glutamate can be replaced by aspartate or ammonium sulfate.
[c] Requirement to isoleucine and valine is not strick.

difference in the gel pattern of protein spots was not detected. Nevertheless, it is highly probable that the lesion observed in this mutant resulted from a defect in ribosomal protein ST-L11 since the mutant was also hypersensitive to erythromycin and high temperature (Table 2). In this connection, an *E. coli relC* mutant isolated by Friesen et al. (6) with an altered ribosomal subunit did not show an altered electrophoretic mobility of any ribosomal proteins. A *rel* mutant of *Streptomyces coelicolor* A3(2) also lacked a ribosomal protein ST-L11 (unpublished data). Thus, all of the *rel* mutants of *Streptomyces* spp. so far obtained can be classified as *relC* mutants, in analogy with mutants of *E. coli* and *B. subtilis*. It should, however, be noted that the identity of the proteins as 'L11-like' has not been proven but only suggested. The use of alternative sources of identification of L11 protein, e.g., a demonstration of immunochemical relation between L11 and ST-L11, should be stressed as experiments to perform in the future.

As discussed by Wittmann et al. (39) and Cundliffe et al. (4), it is difficult to establish unambiguously whether a protein is missing. Thus, I cannot say whether the protein ST-L11 was actually missing from the *Streptomyces relC* mutants described above or whether it was hidden within another protein spot. Despite the lack of such information, the appearance of rel^+ revertants accompanying a protein ST-L11 at its normal mobility (Fig. 2J) strongly implicates ST-L11 as being a participant in the synthesis of (p)ppGpp and therefore presumably in the regulation of RNA synthesis.

While all of the thiostrepton-resistant mutants of *B. subtilis* tested were hypersensitive to erythromycin and appeared to be of *relC* (34), the *Streptomyces relC* mutants have been detected among thiopeptin-resistant isolates at a frequency of 2 to 10% (19–21, 23). Also, reversion from erythromycin dependence to independence in *E. coli* is accompanied, but not uniquely, by loss of the L11 protein (37). The *Streptomyces relC* mutants, including that of *S. coelicolor* A3(2), had higher sensitivity to erythromycin and high temperature. The only exception was found in a *S. antibioticus relC* mutant (Table 2). Conceivably, the changes in ribosomal conformation of this mutant, which presumably

TABLE 4. Genetic analysis of recombinants derived from protoplast fusion between *rel* and *rel*$^+$ or between *rel sup* and *rel*$^+$

Cross[a]	Genotype of recombinants tested[b]	No. of recombinants tested	Streptomycin productivity[c]	
			+	−
105 (*arg relC1*) × 102 (*ura trp*)	*arg*$^+$ *ura trp*$^+$ *relC1*	75	0	75
	arg$^+$ *ura*$^+$ *trp relC1*	33	0	33
	arg$^+$ *ura trp*$^+$ *rel*$^+$	52	52	0
	arg$^+$ *ura*$^+$ *trp rel*$^+$	37	37	0
106 (*arg relC1 sup2*) × 102 (*ura trp*)	*arg*$^+$ *ura trp*$^+$ *relC1*	64	54	10
	arg$^+$ *ura*$^+$ *trp relC1*	38	31	7

[a] Two auxotrophic markers, *ilv* and *glu*, were not employed in these crosses since *ilv* was leaky and *glu* was not expressed on synthetic medium II because of the presence of ammonium sulfate.

[b] The *relC* genotype was determined on the basis of resistance to thiopeptin (4 μg/ml).

[c] + and −, Capability to produce streptomycin of more than 90 μg/ml or 0 to 3 μg/ml, respectively.

harbors the possible counterpart of ST-L11 protein in its ribosome, still permitted normal function in protein synthesis under the stress of high temperature or erythromycin binding. Its conformational alteration was, however, apparently too extensive to maintain the ability to produce ppGpp.

In *E. coli*, *B. subtilis*, and *B. megaterium*, the ribosome plays a central role in sensing nutrient limitation, eventually leading to the synthesis of ppGpp (2, 7, 36). The present study, together with earlier ones (19–24), suggests that the mechanism of synthesis of ppGpp (and perhaps that of stringent response) in *Streptomyces* spp. upon nutrient limitation is analogous to that in *E. coli* and *B. subtilis*. All the *Streptomyces relC* mutants, including that of *S. coelicolor* A3 (2) (unpublished data), were impaired in their ability to produce a corresponding antibiotic (Table 1), and thus it is possible to envisage a model in which ppGpp would play a pivotal role in triggering antibiotic production, a typical physiological differentiation process (19–21, 23). In this paper, using protoplast fusion, I have demonstrated a correlation between the *rel* mutation and the inability to produce streptomycin (Table 4). It is, however, also possible that the observed changes in ribosomal proteins (and thus ribosomal conformation) per se resulting from the *relC* mutation, rather than from ppGpp, were responsible for the inability to produce antibiotics. This appeared to be the case in chloramphenicol acetyltransferase formation by *B. subtilis*; the structural alteration of the ribosome per se due to the *relC* mutation was presumed to be responsible for the failure in inducing the enzyme (1). This possibility is, however, unlikely in antibiotic production by *Streptomyces* spp., because *S. lavendulae relC* mutant 32 regained the ability to produce formycin, accumulating ppGpp under glucose deprivation (19). In support of the significance of ppGpp per se is the fact that a high-bicozamycin-producing strain of *S. griseoflavus*, used at industrial level, maintained ppGpp levels two- to threefold greater than those of its ancestral strain 1805 during growth in production medium (27).

It is now important to clarify what the target(s) of ppGpp is in initiating the antibiotic production. In *B. subtilis*, mutations that block the sporulation process at the earliest stage are defective in the sporulation-associated events. One of the typical mutations, *spo0A*, results in deficiency of antibiotic production. The dependence of antibiotic production on the *spo0A* gene product, however, can be bypassed by mutations at a suppressor locus called *abrB* (17, 40). The determination of target(s) for ppGpp responsible for initiating antibiotic production, however, may be difficult since ppGpp results in typical pleiotropic effects by regulating the activity of numerous target molecules (2, 7). From this point of view, a discovery of a suppressor mutation (*sup*) which can circumvent the effect of the *relC* mutation on streptomycin production is of interest. This suppressor mutation made the *S. griseus relC* cells capable of producing streptomycin without accumulating ppGpp. Some of the *S. griseus relC* mutants described in this paper grew slowly, indicating the pleiotropic nature of these mutants. The growth rate of the *relC sup* double mutant, however, was not restored to that of the wild-type strain but was similar to that of the *relC* mutant. These observations can be taken as an evidence that the restoration of streptomycin production and aerial mycelium formation by the *sup* mutation should not result simply from an effect on growth. Although the facts described above might in turn be taken as an indication that ppGpp is not involved directly in triggering antibiotic production, the *sup* gene or its product can be a candidate for the target for ppGpp, which may operate at the onset of secondary metabolism at late growth phase. Since *relC sup* double mutants still showed relaxed response with respect to RNA synthesis, suppression of RNA synthesis per se under nutrient limitation is apparently not essential for initiating secondary metabolism, at least in the genetic background of *sup* mutants. These observations, however, do not rule out the possibility that RNA polymerase is a ppGpp target responsible for initiating secondary metabolism. Since aerial mycelium formation by *S. griseus* is shown, though not yet established in a causal relationship, to be induced by decrease in intracellular pool size of GTP (but not by a direct effect of ppGpp) (21), it is rather surprising that the impaired ability of aerial mycelium formation in *relC* mutants was also overcome by the *sup* mutation. The *sup* mutation described here promises to be a very useful contribution to the understanding of the sensory pathway that connects physiology with the differential expression of genes.

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