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A New Relaxed Mutant of *Bacillus subtilis*

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A new relaxed mutant of *Bacillus subtilis* was isolated by screening Rif^r clones for alterations in stringent control. The Rif^r relaxed mutant which is described was found to contain a second-site mutation conferring a relaxed response to an energy source downshift and was partially relaxed after amino acid starvation. The new *rel* locus, called *relG*, was distinct from the two other known *rel* loci in *B. subtilis*, *relA*, and *relC*.

The accumulation of guanosine 5'-diphosphate 3'-diphosphate (ppGpp) and guanosine 5'-triphosphate 3'-diphosphate (pppGpp) in bacteria after amino acid deprivation and the ensuing stringent response have been well reviewed (6, 10). The mechanism by which (p)ppGpp is synthesized on the ribosome in the presence of any uncharged tRNA species has been demonstrated in vitro in *Escherichia coli* and has been shown to be similar in *Bacillus subtilis* (14, 17). Thus far, only two loci have been identified which govern stringent control in *B. subtilis*: *relA* is the gene coding for stringent factor, which is the enzyme responsible for synthesizing (p)ppGpp in response to amino acid starvation, and *relC*, which codes for ribosome protein L11, a protein also needed for (p)ppGpp synthesis on the ribosome (15).

Guanosine tetraphosphate also accumulates in bacteria after a carbon or energy source downshift. In *E. coli* this occurs in strains carrying nonsense mutations in *relA* (5) or a complete deletion of *relA* (2, 3). Thus, the response to a carbon or energy source downshift must be independent of *relA* in *E. coli*. Genetic studies have implicated two other genes in this circuit: *relX*, which maps near *relA* (6, 12), and *relS*, which maps in the vicinity of *lac* (4).

The *relX* gene was detected through the identification of two wild-type isoalleles in different lines of *E. coli*; it affects the basal level of ppGpp and nucleotide accumulation during a carbon or energy source downshift synergistically together with *relA* (12). The *relS* gene was detected through isolation of a mutant allele with a drastically diminished basal level of ppGpp; its interaction with *relA* has not been established because of the seeming inviability of the *relS relA*$^+$ genotype (4).

In this communication, we describe the isolation and characterization of a new mutant of *B. subtilis* in which both ppGpp accumulation and stable RNA snythesis are relaxed with respect to a carbon or energy source downshift. The initial goal of our selection scheme was not, in fact, centered on the downshift response. Previous observations have suggested that RNA polymerase itself may be involved in (p)ppGpp metabolism in *B. subtilis* (13). These observations consisted of the demonstration that certain RNA polymerase inhibitors induced transient accumulation of (p)ppGpp and that certain mutations in genes coding for RNA polymerase subunits in turn modify this effect (13). We therefore screened spontaneous rifampicin-resistant (Rif^r) mutants (presumably altered in *rpoB*) (8) for aberration of (p)ppGpp metabolism. Two of these mutants turned out to be relaxed with respect to downshift, and one was characterized in detail. The gene defined by the mutational lesion, which we term *relG*, maps in the *strA-spcA* region of the *B. subtilis* chromosome, far from the location of *relA*.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains are described in Table 1.

Media used for radioactive labeling of cells was the Tris-glucose media described by Nishino et al. (11). Lysine starvation was achieved by centrifuging a small volume of cells in a Beckman microfuge and then suspending the cell pellet in the same volume of minimal medium without lysine.

For the selection of Rif^r strains, Penassay broth antibiotic medium no. 3 from Difco Laboratories was used. Solid rich medium for maintaining stocks and selection of antibiotic-resistant strains was tryptose blood agar base (TBAB) (Difco Laboratories). Solid minimal medium for amino acid analog sensitivity testing was the Tris-glucose minimal medium of Nishino et al. (11), containing 1.5% agar.

Selection of Rif^r strains and isolation of a relaxed mutant. Cultures of strain BR16 were grown to 10^9 cells per ml, centrifuged, and resuspended in 10% volume Penassay broth. Approximately 1 to 2 $\times$ 10^9 cells were plated on TBAB containing 10 μg of rifampicin (Sigma Chemical Co.) per ml. Several starting

TABLE 1. Bacterial strains

Strain	Description	Source	Parent
168	Prototroph, *rel*$^+$		
BR16	*trpC2 lys rel*$^+$	17	168
BR17	*trpC2 lys relA*	17	168
R-4	*trpC2 lys* Rif^r *relG*	Spontaneous mutant, this work	BR16
R-6	*trpC2 lys* Rif^r *rel*$^+$	Spontaneous mutant, this work	BR16
W-R4	Rif^r *rel*$^+$	Constructed, this work	168
G4-s	*trpC2 lys strA relG*	Constructed, this work	BR16

cultures were used to ensure that mutants of independent origin were obtained.

Rif^r strains were patched onto TBAB plates containing 10 μg of rifampicin and were replica plated onto minimal plates containing 0.1 mg each of *O*-methylthreonine (*O*-MT) and α-amino B-hydroxyvaleric acid. This concentration of amino acid analogs allows growth of *rel*$^+$ strains but not *rel* strains (V. L. Price, unpublished data). Rif^r clones which did not grow on plates containing the amino acid analogs were retested for sensitivity to several different amino acid analogs by the disk test: cells were grown to mid-log stage in minimal medium, and 0.2 to 0.3 ml of culture was added to 2.5 ml of soft agar. This was overlayed onto Tris-minimal medium plates, and filter paper disks impregnated with 20 μl of amino acid analog (50 mg/ml) were placed on top. The radius of the zone of inhibition was measured after overnight growth.

Extraction and quantitation of nucleotides. Cells were grown in the presence of 300 μCi of ^{32}P (New England Nuclear Corp., carrier free) per ml for one generation to allow equilibration with the phosphate pools. Growth was followed with a Beckman spectrophotometer at 690 nm. Samples (0.1 ml) were removed at appropriate times for nucleotide extraction with 0.1 N formic acid by the method of Gallant et al. (7). After the samples were cooled for 20 min on ice, the pH was adjusted with 25% volume of 1 M Tris (pH 8.0), and samples were centrifuged and frozen until analysis.

Polyphosphorylated nucleotides were resolved by thin-layer chromatography on polyethyleneimine cellulose (Brinkman Instruments, Inc.), using 1.5 M KH_2PO_4.

Identification of nucleotides was done by testing for comigration with the authentic compound in the chromatographic separation system described above. Nucleotide triphosphates were obtained from Sigma Chemical Co., and ppGpp and pppGpp were obtained from PL Labs. Radioactivity labeled nucleotides were located by autoradiography, and the corresponding spots were cut from the thin-layer chromatographic plate. Radioactivity in each spot was determined in a toluene-based scintillant, and the adjustment for background and trailing was made by cutting out an adjacent equal-sized spot from the chromatogram. Specific activity of the medium was determined by counting small samples of the extract directly along with each set of experimental samples.

RNA synthesis. Net RNA synthesis was assayed by labeling cultures at mid-log stage with 10 μCi of [5-^{3}H]uridine per ml (New England Nuclear Corp.). Unlabeled uridine was also present at 10 μg/ml. Samples (20 μl) were removed at appropriate times and spotted onto cellulose chromatographic sheets (Kodak Co.) presoaked with 10% (wt/vol) trichloroacetic acid. The method of quantitation of RNA by separating trichloroacetic acid-precipitable material by descending paper chromatography described by Weinstein et al. (19) was followed.

To test a large number of transformants for the relaxed response to α-methylglucoside (α-MG) with respect to RNA synthesis, a modification of the above procedure was used. Clones to be tested were patched onto TBAB plates and, after overnight growth, were transferred with sterile toothpicks to small serotubes containing 1 ml of minimal medium. When the cultures had grown to visible turbidity, 40 μl was transferred to duplicate wells of a microtiter dish containing 10 μCi of [5-^{3}H]uridine per ml and 10 μg of unlabeled uridine per ml. One of the duplicate wells also contained 1% α-MG. The microtiter plates were incubated at 37°C for 30 to 40 min, then 20-μl samples from each well were trichloroacetic acid precipitated, and the precipitated RNA was separated chromatographically as described above. Strains subject to downshift control of net RNA synthesis (*relG*$^+$) showed a reduction in labeling by 70 to 80% in the α-MG wells; *relG* strains showed no significant reduction.

Preparation of transforming DNA. DNA for transformations was prepared by the method of Haworth and Brown (9), except that the lysed cell suspension was phenol extracted and ethanol precipitated twice, with an RNase treatment (70 μg of RNase per ml incubated 30 min at 37°C) after the first ethanol precipitation.

Bacterial transformations. The medium used for transformations was that described by Anagnostopoulos and Spizizen (1), and the transformation procedure used was that of Haworth and Brown (9) with the following modifications. After 4.5 h of growth in transformation medium, 0.1 ml of competent cells was mixed with 0.9 ml of transformation medium and 0.1 ml of transforming DNA at 1 μg/ml (final DNA concentration, 0.1 μg/ml). The reaction was incubated at 37°C for 90 min and then plated on selective medium or, to allow expression time, plated on a nitrocellulose filter on TBAB for 3 h before the filter was transferred to selective medium (TBAB plus antibiotic). The following antibiotic concentrations were used for selection: rifampicin, 10 μg/ml; streptomycin, 200 μg/ml; and spectinomycin, 100 μg/ml. It was observed that many antibiotic-resistant transformants were obtained without allowing for expression; therefore, transformation mixtures were plated directly on selective medium for strain constructions.

RESULTS

Isolation of the relaxed mutant. Independent RNA polymerase mutants were obtained by selecting for spontaneous Rif^r clones as described above. Approximately 220 Rif^r strains were tested for their inability to grow in the presence of low levels of certain amino acid analogs, an indirect test for the relaxed phenotype (16, 18). Among the Rif^r strains tested for growth inhibition in the presence of 0.1 mg of *O*-MT (an isoleucine analog) per ml and 0.1 mg of α-amino B-hydroxyvaleric acid (a methionine analog) per ml, five did not grow. These were then retested for sensitivity to four different amino acid analogs by the disk test. Three showed increased sensitivity to the analogs tested. The zones of inhibition surrounding filter-paper disks containing the various amino acid analogs are reported in Table 2. The mutant selected for this study, R-4, was inhibited to a greater extent than was its *rel*$^+$ parent and to a similar degree as was isogenic *relA* strain BR17.

The mutants showing increased sensitivity to the amino acid analogs were tested directly for the relaxed phenotype by monitoring stable RNA synthesis and the accumulation of (p)ppGpp. Two of the mutants had a relaxed phenotype after an energy source downshift (glucose to succinate downshift or the addition of 1% α-MG), but not in response to amino acid starvation. One mutant, R-4, was chosen for further analysis.

Characterization of the relaxed mutant. The mutant strain R-4 exhibited a slower growth rate in minimal medium than the *rel*$^+$ parent, BR16. The doubling time for the mutant in the Tris-minimal medium used was 75 min, compared with 48 min for BR16 and 60 min for *relA* strain BR17.

Figures 1 and 2 show the behavior of R-4 compared with its two counterpart strains under conditions which elicited the stringent response. In the presence of *O*-MT, an inhibitor of isoleucyl-tRNA synthetase, R-4 exhibited a normal stringent response: net RNA synthesis was sharply reduced, as in *rel*$^+$ parent strain BR16 (Fig. 1). During lysine limitation, on the other hand, R-4 was partially relaxed, exhibiting a much smaller reduction in net RNA synthesis than BR16, whereas *relA* mutant BR17 showed no reduction at all (Fig. 2).

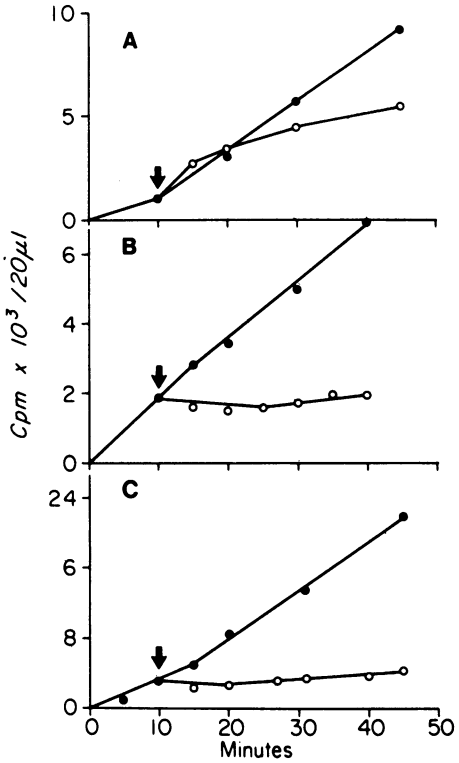

FIG. 1. Net RNA synthesis in the presence of *O*-MT. Net RNA synthesis was followed as described in Materials and Methods. *O*-MT (2 mg/ml) was added to logarithmically growing cultures at the time indicated by the arrow. Symbols: ●, control values; and ○, incorporation of label in the presence of *O*-MT. (A) BR17 (*relA*), (B) R-4 (*rif relG*), and (C) BR16 *rel*$^+$).

TABLE 2. Inhibition of growth by amino acid analogs[a]

Strain	Analog			
	O-MT	Norvaline	α-Amino B-hydroxy-valeric acid	Serine
BR16	4	5	0	5
BR17	15	9	16	11
R-4	12	6	15	10

[a] Numbers represent the radius in millimeters of the inhibition zone. All disks contained 20 μl of analog (50 mg/ml).

Figure 3 shows the response of the three strains to a carbon or energy source downshift, elicited by blocking glucose transport with the analog α-MG. It can be seen that net RNA synthesis was shut off entirely in the *rel*$^+$ parent strain BR16, restricted quite severely in the *relA* strain, but scarcely affected in R-4. Similar results were obtained when a downshift was produced by transferring cells from glucose to succinate medium (not shown). As an additional control, we show the behavior of one of the majority Rif^r mutants (R-6) which was not relaxed with respect to a downshift (Fig. 3, triangles), which was identical to that of the *rel*$^+$ parent strain.

The accumulation of (p)ppGpp under these various conditions is summarized in Table 3. In

TABLE 3. Accumulation of (p)ppGpp

Strain, condition	Nucleotide produced (nmol/A_{690})[a]	
	ppGpp	pppGpp
BR16 (*rel*$^+$)		
Basal	0.05	0.06
O-MT	0.50	0.64
Lysine starvation	0.15	0.30
α-MG	0.30	0.08
R-4 (Rif^r *relG*)		
Basal	0.04	0.02
O-MT	0.23	0.18
Lysine starvation	0.07	0.10
α-MG	0.08	0.02
G4-s (*strA relG*)		
Basal	0.02	0.015
O-MT	0.25	0.18
Lysine starvation	0.08	0.09
α-MG	0.04	0.014
W-R4 (Rif^r *relG*$^+$)		
Basal	0.03	0.02
O-MT	0.56	0.40
α-MG	0.15	0.03
BR17 (*relA*)		
Basal	—[b]	—
O-MT	—	—
Lysine starvation	—	—
α-MG	—	—

[a] Values (nanomoles per absorbance unit at 690 nm [A_{690}]) represent maximum amounts of nucleotide accumulated under various conditions and are an average of at least three trials.

[b] —, No detectable nucleotide was present.

each case, the results for R-4 corresponded to the behavior of net RNA synthesis: R-4 exhibited stringent accumulation of the nucleotides in response to both *O*-MT inhibition and lysine starvation. However, only about one-half of the ppGpp and one-third of the pppGpp levels obtained by the *rel*$^+$ parent strain were reached. Quantitatively, this resulted in little (p)ppGpp accumulation after lysine starvation. During an energy source downshift by α-MG, little or no accumulation over basal levels could be detected.

In the *relA* mutant (BR17), no accumulation of (p)ppGpp occurred after an energy source downshift (Table 3) despite the fact that net RNA synthesis was severely restricted (Fig. 3).

Genetic analysis of the mutation. Rif^r mutants have been found to contain an altered β subunit of RNA polymerase, coded for by the *rpoB* gene. To verify that the Rif^r allele of R-4 was also in *rpoB*, we mapped it by bacterial transformation. The Rif^r locus was found to be closely linked to *cysA* (35% cotransformation) and *strA* (41% cotransformation), agreeing well with the position of the *rpo*B gene in *B. subtilis*.

To determine whether the Rif^r locus of the mutant was also responsible for the relaxed phenotype for glucose downshift, DNA was isolated from R-4 and used to transform wild-type *B. subtilis* strain 168 to rifampicin resistance. (This strain is the parent of the lysine auxotroph BR16. It is more transformable than BR16; hence it was used in this cross.) The Rif^r transformants were then tested for the relaxed phenotype by measuring net RNA synthesis in the presence of α-MG as described above. Surprisingly, the Rif^r locus was separable from the mutation responsible for the relaxed phenotype. Among 55 Rif^r transformants tested, only 4 were also *relG*. The *relG* marker also contransformed weakly with *strA* (6% cotransformation) and *spcA* (8% cotransformation), but did not cotransform with *cysA*, as does *relC*. Therefore, the tentative map location would be in the *strA*-

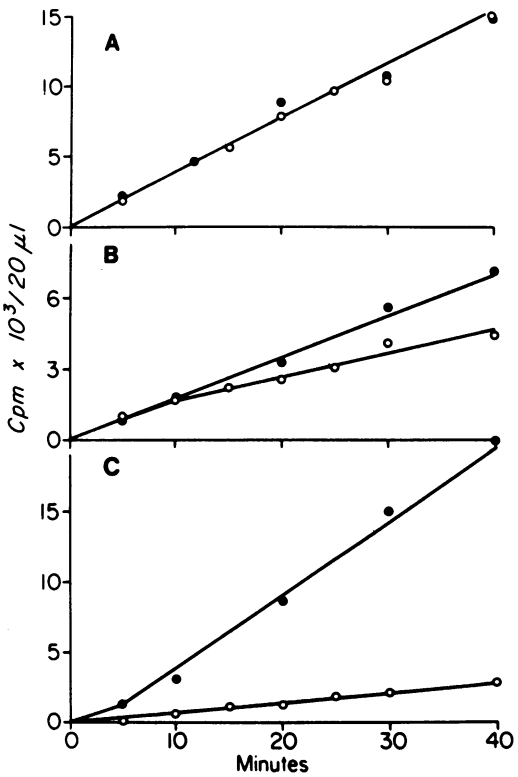

FIG. 2. Net RNA synthesis after lysine starvation. Cultures were centrifuged and suspended in an equal volume of Tris-minimal medium with lysine present (●) or without lysine (○). Net RNA synthesis was determined as described in Materials and Methods. (A) BR17 (*relA*), (B) R-4 (*rif relG*), and (C) BR16 (*rel*$^+$).

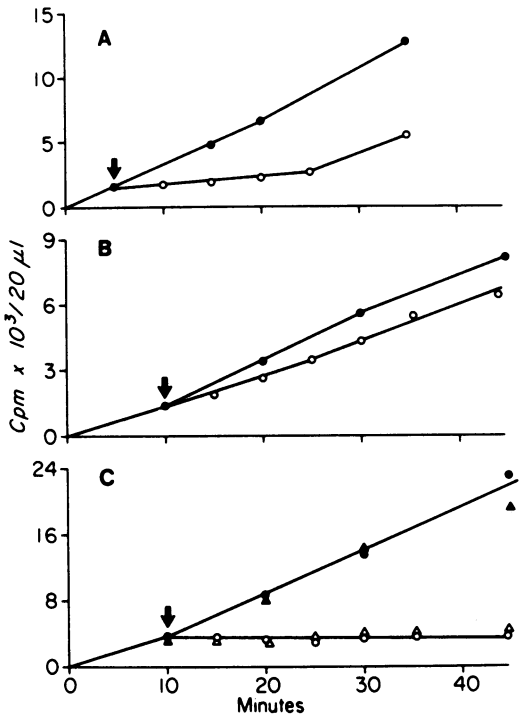

FIG. 3. Net RNA synthesis in the presence of α-MG. α-MG (1%) was added to logarithmically growing cultures at the time indicated by the arrow, and net RNA synthesis was determined as described in Materials and Methods. Closed symbols represent control values, and open symbols represent cultures containing α-MG. (A) BR17 (*relA*), (B) R-4 (*rif relG*), and (C) BR16 (*rel*$^+$), circles and R-6 (*rif rel*$^+$), triangles.

spcA region of the *B. subtillis* chromosome. This appears to be distinct from the *relC* locus, which maps in the order *cysA*, *relC*, *rif*, *strA* (15). Thus, the characteristics described above for the mutant strain R-4 are those of a double mutant of the genotype Rif^r *relG*. To determine whether the Rif^r mutation influences the relaxed phenotype, we isolated two strains carrying each mutant allele individually. W-R4 is one of the Rif^r transformants which was no longer relaxed with respect to downshift, and is thus Rif^r *relG*$^+$. Isolation of the converse single mutant was complicated by the fact that no direct selection was available for *relG*. To circumvent this difficulty, we made use of the close linkage in *strA* (streptomycin resistance) to the Rif^r locus. R-4 was transformed with DNA from a *strA* derivative of 168, which is rifampicin sensitive and *relG*$^+$. Streptomycin-resistant transformants were selected and then tested for rifampicin resistance and the *relG* phenotype of relaxed RNA synthesis in the presence of α-MG. An *strA* segregant was obtained which was rifampicin sensitive but had retained *relG*. This segregant, strain G4-s, resembled R-4 both in the relaxed response to downshift and its stringent response to O-MT. Its doubling time, at 120 min, is considerably slower than that of R-4.

Figure 4 shows the response of the Rif^r *relG*$^+$ and Rif^s *strA relG* strains to downshift. It was evident that relaxation of RNA synthesis with respect to downshift depended only on *relG*.

G4-S (*strA relG*) behaved similarly to R-4 (Rif^r *relG*) with respect to (p)ppGpp accumulation after an energy downshift and O-MT inhibition (Table 3). The strain containing the Rif^r locus alone (W-R4) accumulated nearly normal levels of (p)ppGpp after amino acid limitation. After energy downshift by α-MG addition however, slightly less (p)ppGpp accumulates than in the *rel*$^+$ Rif^s strain BR16.

DISCUSSION

We have described the isolation and characterization of what appears to be a new relaxed mutant of *B. subtilis*. Unlike the other *rel* strains so far described in *B. subtilis*, the *relG* mutation is specific for conferring a relaxed response to

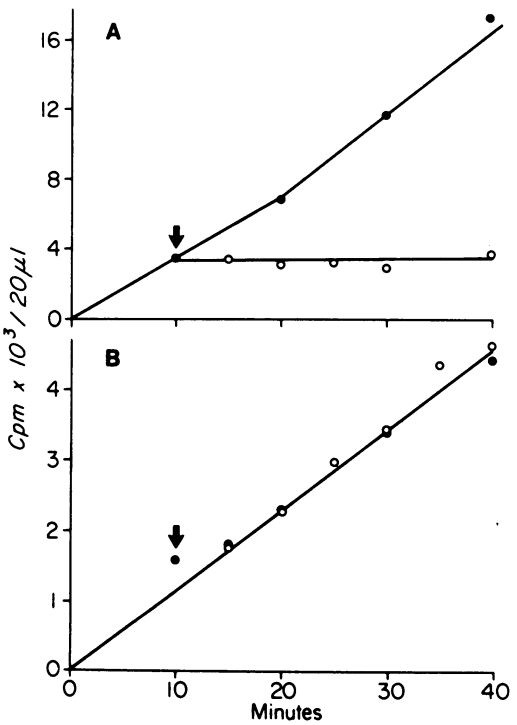

FIG. 4. Net RNA synthesis in the presence of α-MG. (A) Net RNA synthesis in strain W-R4 (*rif rel*$^+$); and (B) strain G4-s (*strA relG*) after the addition to 1% α-MG, indicated by the arrow. Experiments were performed as described in Fig. 2 legend and in Materials and Methods. ●, Control values; and ○, cultures containing α-MG.

glucose downshift rather than to amino acid starvation. The mutant that was isolated, R-4, was shown to be a double mutant containing both a Rif^r and *relG* mutation. Subsequent studies on strains that were constructed containing either the Rif^r marker alone or the *relG* mutation with a *strA* mutation showed that *relG* seemed to be responsible for the relaxed response to glucose deprivation: W-R4, containing just the Rif^r locus of R-4, had the stringent phenotype of a *rel*$^+$ strain, as did R-6, one of the spontaneously Rif^r strains which did not show increased sensitivity to amino acid analogs. The Rif^r mutation alone, therefore, does not suffice to give the relaxed phenotype. G4-s, which is *strA relG*, retained the relaxed characteristics of R-4. However, possible influence of the *strA* mutation cannot be eliminated.

The fact that this mutant can accumulate (p)ppGpp in response to amino acid starvation (albeit to a lesser extent than its *rel*$^+$ parent), but not after glucose deprivation, is evidence that two separate mechanisms for accumulating (p)ppGpp exist in *B. subtilis*. However, results here indicate that the two mechanisms are not mutually exclusive: the slightly relaxed phenotype of the *relG* strains toward amino acid limitation (reduced [p]ppGpp) and the absence of (p)ppGpp in the *relA* strain after an energy source downshift by α-MG (Table 3) indicate some form of interaction between *relA* and *relG*. In addition, both the *relA* mutant and a *relC* strain (not shown), which show relaxed RNA synthesis after amino acid starvation, show significant restriction (65 to 70%) of net RNA synthesis in the presence of α-MG without increasing (p)ppGpp. It follows that downshift control of net RNA synthesis depends on factors other than the level of (p)ppGpp and that these factors are affected by the mutational lesion in *relG* strains.

Detailed mapping of the *relG* locus has not yet been finished, although it appears to be in the *strA-spcA* region of the chromosome. The *relG* mutation appears to lie in a gene separate from the L11 (*relC*) gene as judged by its tentative map location and significant differences in the phenotype for the two strains with regards to stringent control: *relC* strains of *B. subtilis* behave similarly to *relA* after amino acid limitation or an energy source downshift, a response that was shown to be quite different from the *relG* phenotype as described above. *relG* is also sensitive to thiostrepton unlike *relC* strains, although this alone would not exclude them from being mutations in the same gene. It is likely that *relG* could be a ribosome protein or elongation factor, since the known *B. subtilis* genes for these translation components lie in this region.

How an energy source downshift serves to trigger the stringent response is not understood. It seems unlikely that an energy source downshift simply increases the level of uncharged tRNA, or the mechanism of (p)ppGpp synthesis would be the same as for amino acid starvation, which has been shown not to be the case. The exact nature of any interaction between the stringent response to amino acid limitation and to an energy source downshift is also not clear, but elements common to both (perhaps ribosome proteins or other translation factors) seem to be at work.

Further analysis of the nature of the *relG* mutation should answer questions regarding stringent control of energy metabolism and the possible role of RNA polymerase or ribosome proteins, or both, in this form of regulation.

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