ISOLATION AND CHARACTERIZATION OF AN RNA RELAXED MUTANT OF B. SUBTILIS

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An RNA relaxed strain of B. subtilis was isolated. This strain, auxotrophic for lysine, synthesizes ribosomal RNA when starved for lysine. The parental stringent strain stops stable RNA synthesis when starved for lysine. Upon starvation, the stringent strain synthesizes two unusual nucleotides which appear to be the same as those synthesized in amino acid starved stringent E. coli strains. The lysine starved relaxed B. subtilis mutant does not synthesize these nucleotides to a significant extent.

Wild-type E. coli strains manifest a stringent response to amino acid starvation i. e., net RNA synthesis stops along with net protein synthesis (1). Relaxed mutants which have lost the normal control i. e., which continue to synthesize ribosomal and transfer RNA in the absence of protein synthesis have been isolated in E. coli and S. typhimurium strains (2, 3). Although the mechanism for this RNA regulation is still obscure, two unusual nucleotides are synthesized in amino acid starved stringent strains but not in amino acid starved relaxed strains (4). These nucleotides were identified as guanosine 5' diphosphate 2' (or 3') diphosphate (ppGpp) and with less surety, guanosine 5' triphosphate 2' (or 3') diphosphate (pppGpp) by Cashel (5). A number of studies have indicated that these nucleotides may be involved in the regulation of ribosomal RNA synthesis in E. coli (6, 7). This paper describes the isolation of a relaxed RNA mutant of the gram positive bacterium B. subtilis. The relaxed mutant accumulates ribosomal and transfer RNA during lysine starvation. The mutant also fails to synthesize ppGpp or pppGpp during

lysine starvation whereas the parent strain synthesizes appreciable amounts of both nucleotides.

Materials and Methods.

B. subtilis strain BR16 lys^- was obtained from R. Doi.

SM medium: $(NH_4)_2SO_4$, 2g; K_2HPO_4, 14g; KH_2PO_4, 6g; $Na_3C_6H_5O_7$, 1g; $MgSO_4$, 0.2g per liter H_2O.

SC medium: NH_4Cl, 2g; NaCl, 5g; KCl, .037g; Tris, 6g; $MgSO_4$, 2 X 10^{-4}M; $FeCl_3$, 10^{-4}Mg; $Na_3C_6H_5O_7$, 0.1g. Potassium phosphate (pH 7) was added to a final concentration of 10^{-3}M for P^{32} labeling experiments. Glucose (0.4%) and glutamate (40 µg/ml) were added to the basal medium. Lysine was added to 40 µg/ml where indicated.

Selection: A B. subtilis culture was mutagenized with ethyl methane sulfonate (8). The mutagenized cells were grown overnight in SM medium plus lysine. Relaxed mutants were sought according to the procedure of Martin (3). The mutagenized bacteria were diluted and collected on 0.45 µ millipore filters such that 100-200 cells were trapped on the filter. The filter pads were then placed on SM agar plates plus lysine and incubated at 37°C until microcolonies appeared. Then the filter pads were transfered to SM agar plates lacking lysine and supplemented with C^{14}-uracil (0.05 µc/ml, 1 µg/ml). The incubation was continued for 30 minutes and the filter pads were exposed to autoradiography. Dark spots on the film corresponding to microcolonies indicated potential relaxed mutants. A number of such microcolonies was picked, purified and tested for the stringent-relaxed phenotype.

Results.

One of the colonies thus selected manifested the relaxed phenotype. Figure 1 shows the incorporation of C^{14}-uracil in the presence and absence of lysine for both the mutant and parent strains. The wild-type parent strain shows no measurable stable RNA synthesis during lysine starvation while the mutant accumulates RNA at a rate characteristic of exponential growth for almost a generation. Although the data is not shown, protein synthesis is reduced in both the wild-type and mutant strains to the same degree during

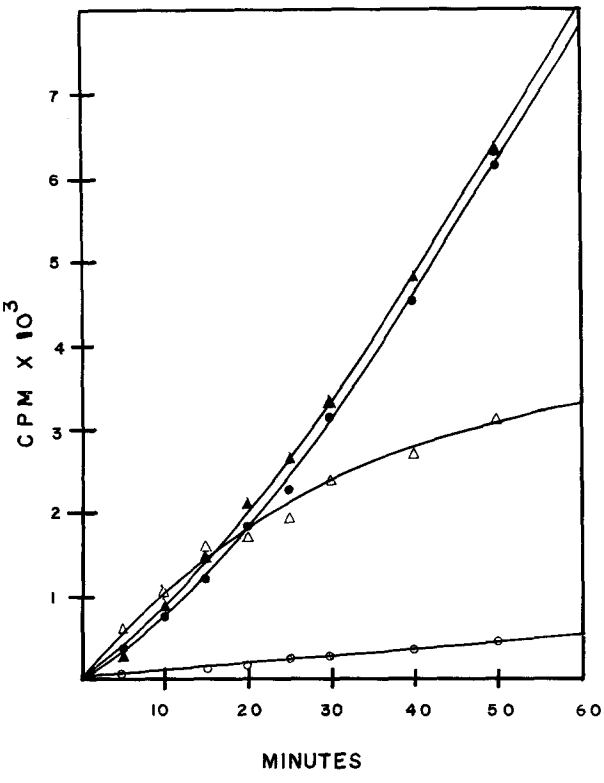

Figure 1. Incorporation of C^{14}-uracil in RNA. B. subtilis cultures were grown to mid logarithmic phase in SM medium plus lysine and tryptophane. (This strain is also a leaky tryptophane auxotroph.) The bacteria were filtered and resuspended in medium plus C^{14}-uracil (0.05 µc/ml, 5 µg/ml).

O------O RC^{str} - lysine
●------● RC^{str} + lysine
Δ------Δ RC^{rel} - lysine
▲------▲ RC^{rel} + lysine

lysine starvation and is about five percent of the exponential rate. In conformity with the nomenclature in E. coli we propose that the mutant allele be called rel and the phenotypes be referred to as RC^{str} for the parent and RC^{rel} for the mutant.

The RNA synthesized during lysine starvation of the RC^{rel} mutant was characterized further by sucrose density gradient analysis. Figure 2 shows the profile of radioactive RNA extracted from an exponential culture as well as from lysine starved RC^{str} and RC^{rel} strains. The data show that 23s, 16s, and 4s RNA accumulate in the RC^{rel} strain while no stable RNA species accu-

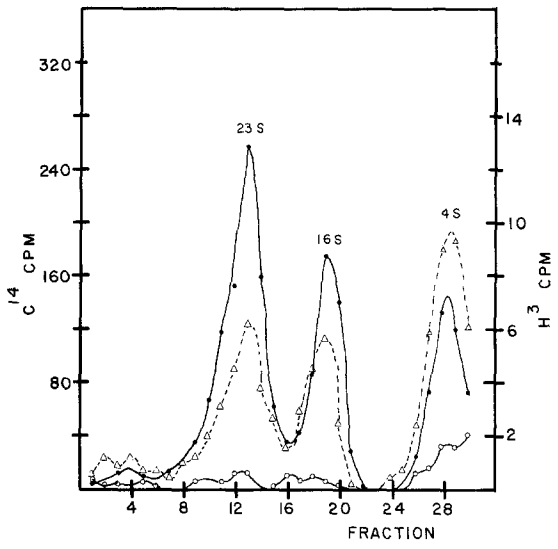

Figure 2. Distribution of RNA synthesized in RC^{str} and RC^{rel} strains starved for lysine. After 5 minutes starvation, cultures were labeled with C^{14}-uracil for 20 minutes. RNA was extracted with Tris saturated phenol (pH 7.4), precipitated with ethanol and redissolved in Tris buffer pH 7.4. The RNA was separated on a 5-20% sucrose gradient centrifuged at 37,000 RPM for 5 hours.

O------O C^{14} labeled RNA, RC^{str} strain
Δ------Δ C^{14} labeled RNA, RC^{rel} strain
●------● H^3 RNA from exponential culture

mulate in the lysine-starved RC^{str} strain.

The synthesis of one or more unusual nucleotides, ppGpp and pppGpp have been demonstrated in amino acid starved RC^{str} E. coli strains (4). Furthermore, it has been proposed that ribosomal RNA synthesis is regulated in amino acid starved bacteria by the presence of these nucleotides (6, 7). Table I shows the levels of ppGpp (and presumably pppGpp) during exponential growth and lysine starvation for both the RC^{str} and RC^{rel} B. subtilis strains. Neither strain synthesizes measurable amounts of the two nucleotides during exponential growth. The RC^{str} strain shows a marked increase in both nucleotide levels on starvation. We are reasonably confident of the identification of ppGpp as it co-chromatographs precisely with purified ppGpp isolated from E. coli (generously supplied by M. Cashel). Positive identification, however, requires further chemical analysis. The data in Table I show also that the

Table I

Strain	Min. after starvation	ppGpp (picomoles/OD)	pppGpp (picomoles/OD)
B. subtilis	Exponential	1.1	1.9
	2	7.6	13.
RC^{str}	5	36.	32.
	10	44.	49.
	20	26.	27.
B. subtilis	Exponential	2.2	4.1
	2	3.2	5.7
RC^{rel}	5	3.0	1.9
	10	1.8	4.7
	20	1.1	2.0
E. coli (CP78)	10	102	26
RC^{str}			

Table I. Bacteria were grown in SC medium with P^{32} (50 μc/μMole) and lysine. Cultures were starved by filtration and resuspension in identical medium without lysine. Samples were taken by filtering 4 ml and quickly immersing the filter in 1 ml cold 0.3 M PCA. After 30 minutes the PCA was neutralized with KOH and the samples centrifuged. A portion of the supernatant was chromatographed on polyethyleneimine thin-layer plates according to Cashel (6). The nucleotides were localized by autoradiography, cut out and counted in a gas flow counter.

level of ppGpp is 2-3 times greater in an amino acid starved E. coli strain while the level of pppGpp is half that of the B. subtilis strain. The RC^{rel} strain fails to show any significant increase in ppGpp or pppGpp on starvation.

Discussion.

A rel mutant of B. subtilis has been isolated whose properties are almost identical with the rel mutants of E. coli. The finding of such a mutant among gram positive bacteria emphasizes the significance of the normal amino acid regulation of RNA synthesis among prokaryotes. The synthesis of two guanine nucleotides is correlated with the cessation of RNA synthesis in the lysine starved RC^{str} strain. The level of these guanine nucleotides does not increase in the RC^{rel} strain where net RNA synthesis continues at an undiminished rate.

Further properties of the mutant are being investigated including its ability to sporulate normally.

ACKNOWLEDGMENTS

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