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Conditions that Trigger Guanosine Tetraphosphate Accumulation in *Caulobacter crescentus*

TERESA A. CHIAVEROTTI,[1] GARY PARKER,[1] JONATHAN GALLANT,[2] AND NINA AGABIAN[1]*

Departments of Biochemistry[1] and Genetics,[2] University of Washington, Seattle, Washington 98195

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Caulobacter crescentus accumulated guanosine tetraphosphate in response to nitrogen starvation but not in response to amino acid starvation. Nitrogen starvation also acted specifically to inhibit certain transitions in the *C. crescentus* life cycle, and guanosine tetraphosphate may act as an intracellular regulator of cell cycle events.

Cells have developed complex regulatory mechanisms which ensure that intracellular metabolic and biochemical processes respond rapidly and appropriately to external environmental changes. The nucleotides guanosine-5'-diphosphate-3'-diphosphate (ppGpp) and guanosine-5'-triphosphate-3'-diphosphate (pppGpp) are two intracellular pleiotropic regulators in procaryotes which act to inhibit the synthesis of stable RNA, ribosomal proteins, and phospholipids (for reviews see references 1, 6, and 7).

In *Escherichia coli*, ppGpp accumulates in response to many conditions including starvation for an energy source or certain inorganic ions, nitrogen source starvation (3, 8), or reduction of aminoacylated-tRNA through starvation for essential amino acids. We report here that the dimorphic bacterium *Caulobacter crescentus* does not accumulate ppGpp in response to a reduced supply of aminoacylated-tRNA but does accumulate ppGpp when starved for an assimilable nitrogen source. Furthermore, we suggest that ppGpp may act as a regulator of specific cell cycle events in addition to its general pleiotropic regulatory effects.

C. crescentus CB15 (ATCC 19089) was grown at 30°C with rotary shaking in minimal glucose medium (12). Strains SC146 and SC392 are spontaneously derived mutants of CB15 obtained from Bert Ely and require arginine and proline for growth, respectively. Strains SC146 and SC392 were grown in minimal glucose medium with 0.1 mg of arginine or proline added per ml, respectively. Cells were labeled for 10 min with 500 μCi of $^{32}PO_4$ (New England Nuclear Corp.; carrier-free) per ml. Nucleotides were extracted by adding formic acid to a final concentration of 0.1 N, and after 30 min at room temperature the extracts were neutralized by the addition of 0.25 volume of 1.0 M Tris (pH 8.0) and stored frozen at −20°C. Labeled nucleotides were resolved by two-dimensional thin-layer chromatography on polyethyleneimine-impregnated cellulose plates (Brinkman), using the solvent system described by Nishino et al. (11). Labeled nucleotides were visualized by autoradiography and quantitated by scraping the plates and counting the scrapings by liquid scintillation. To normalize for differences in the uptake and incorporation of ^{32}P under different conditions, all results are reported as the ratio of label in ppGpp to that in GTP.

The two-dimensional thin-layer chromatography system used in this study is capable of separating ppGpp and pppGpp from each other and from other nucleotides. Table 1 presents the results of experiments with the amino acid auxotrophs. In these experiments, strains SC146 and SC392 were starved for arginine and proline, respectively, and the amount of labeled ppGpp which had accumulated at the end of 10 min was determined. No significant labeling of ppGpp was found in samples taken at 3, 10, and 20 min of arginine starvation with arginine auxotroph SC146 (data not shown). In other experiments, we found that growth-inhibitory concentrations of *O*-methylthreonine, serine-hydroxamate, and allyl glycine (which block synthesis or activation of isoleucine, serine, and lysine, respectively) also failed to stimulate ppGpp synthesis. Additionally, these amino acids analogs failed to restrict net RNA synthesis. Thus, it appears that reduced aminoacylation of five different tRNA species does not elicit the stringent response (6) in *C. crescentus*, in contrast to *E. coli* and other bacterial species.

The results presented in Table 1, however, clearly indicate that conditions do exist which stimulate ppGpp production in *C. crescentus*. When arginine auxotroph SC146 was starved for arginine and ammonia simultaneously, ppGpp synthesis was stimulated, and the ratio of labeled ppGpp to GTP was 50 times greater than in cultures incubated with arginine or ammonia

TABLE 1. *ppGpp synthesis in strains CB15, SC146 (Arg⁻), and SC392 (Pro⁻)*[a]

Strain	Addition(s) to HG	10^3 cpm/ml		ppGpp/GTP
		ppGpp	GTP	
SC146	NH_4^+, Arg	2.6	166	0.015
SC146	NH_4^+	0.82	138	0.006
SC146	Arg	2.8	145	0.019
SC146	None	36.6	48.3	0.757
SC392	NH_4^+, Pro	2.4	193.3	0.012
SC392	NH_4^+	—[b]	159.4	<0.001
SC392	Pro	17.4	45.5	0.382
SC392	None	32.2	50.6	0.636
CB15	α-Methyl glucoside	2.2	125	0.017
CB15	NH_4^+	0.89	178	0.005
CB15	None	62.9	67	0.939

[a] Cells were incubated at 30°C in medium which contained Hutner trace metals, 0.2% glucose, and 2×10^{-4} M PO_4^{-3} (HG). NH_4Cl when added was 10 mM, and arginine or proline when added was 0.1 mg/ml. For ^{32}P labeling, cells were grown overnight to an absorbance at 660 nm of 0.4 to 0.5 and were then harvested by centrifugation at 9,000 × *g* for 10 min. The cells were washed twice at 4°C with the appropriately modified medium and then suspended at an absorbance of 0.4 to 0.5 in modified medium that had been prewarmed to 30°C. Cells were allowed to equilibrate in the appropriate medium for 5 min before labeling commenced. Cells were pulsed for 10 min with ^{32}P, and labeled nucleotides were determined as described in the text. The ppGpp/GTP ratio of incorporated ^{32}P increased during the first 10 min under the conditions used in these experiments.

[b] —, Not detectable.

as a nitrogen source. Since *C. crescentus* can use arginine as the sole nitrogen source, it is evidently the absence of a nitrogen source which stimulates ppGpp synthesis. Similarly, proline auxotroph SC392 exhibited a 50-fold increase in the ppGpp-to-GTP labeling ratio when starved for both proline and ammonia. In fact, this mutant exhibited significant ppGpp synthesis when starved for ammonia in the presence of proline. Unfortunately, very little is known about amino acid metabolism in *C. crescentus*, although both arginine and proline are able to act as sole nitrogen sources for SC146, SC392, and prototrophic strains, and both amino acids are also able to support growth of glutamate synthase mutants (4). Our data suggest that proline is a poorer nitrogen source than arginine.

Table 1 also presents the results of experiments in which wild-type *C. crescentus* cells were starved for components of minimal medium. The addition of α-methyl glucoside resulted in a condition equivalent to glucose deprivation and stimulated a threefold increase above control cultures in the amount of labeled ppGpp present. When nitrogen was withdrawn from the medium, there was approximately a 200-fold increase in the ppGpp-to-GTP ratio above that in control cultures.

Altogether our data indicate that nitrogen starvation stimulates ppGpp synthesis in *C. crescentus*. We have observed that when synchronous swarmer cells are suspended in a medium which lacks a nitrogen source, these cells are no longer able to differentiate to form stalked cells (T. A. Chiaverotti and N. Agabian, manuscript in preparation). Both swarmer and stalked cells synthesize ppGpp when starved for nitrogen, and the levels which accumulate do not differ significantly for the two cell types (G. Parker and L. Palmer, unpublished data). Only the stalked cells of *C. crescentus* are able to initiate DNA replication, and DNA replication is restricted to the stalked cell stage (2, 5). Manoil and Kaiser (9, 10) have suggested that ppGpp may play a role in inducing fruiting body formation in another developmental procaryote, *Myxococcus xanthus*. It is interesting to speculate that in *C. crescentus*, ppGpp may act to regulate the transition from a swarmer cell to a stalked cell and that this regulation may include the inhibition of DNA replication initiation.

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ADDENDUM IN PROOF

Further experiments have shown that the rate of growth of the prototrophic strain using proline as a nitrogen source is slower than when arginine is used and that the maximum density reached by the prototrophic strain using proline as a nitrogen source is less than when arginine or ammonia is used.

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