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Stringency in the Absence of ppGpp Accumulation in *Rhodobacter sphaeroides*

RUTH ACOSTA AND DONALD R. LUEKING*

Department of Biological Sciences, Michigan Technological University, Houghton, Michigan 49931

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Leucine deprivation of either phototrophically or chemotrophically growing cells of *Rhodobacter sphaeroides* resulted in a restriction in the continued accumulations of cellular RNA, phospholipids, and protein. Phototrophically growing cells also displayed restrictions in the accumulations of cellular carotenoids and bacteriochlorophyll. Leucine deprivation, however, did not provoke the accumulation of cellular ppGpp or alter the steady-state levels of ppGpp, ATP, or GTP in cells of *R. sphaeroides*.

Numerous studies have implicated ppGpp as a pleiotrophic effector molecule for the coordination of metabolic events in procaryotic organisms (9, 18, 25). For example, metabolic adjustments associated with amino acid starvation and carbon or energy source downshift (18, 25), temperature shifts (21, 26), and nitrogen deprivation (1) are all thought to be mediated, in part, by the effector properties of ppGpp. However, the exact mechanisms by which ppGpp exerts its regulatory effects are, in most cases, unknown. Similarly, phototrophically grown cells of *Rhodobacter sphaeroides* (20) have been shown to rapidly accumulate ppGpp in response to an abrupt downshift in incident light intensity (7, 16), and Campbell and Lueking (7) have proposed that the coincident restrictions in biosynthetic activity observed following such a light transition (7, 11, 16, 23) are mediated by ppGpp. This proposal is especially compelling with regard to cellular phospholipid synthesis, because both the percent inhibition of the rate of phospholipid synthesis and the extent of accumulation of cellular ppGpp were found to be directly related to the magnitude of the light transition. However, a direct involvement of ppGpp in the control of phospholipid synthesis or other cellular biosynthetic activities was not established, and the mechanism responsible for the light-mediated formation of ppGpp was not identified.

To evaluate the potential effector role of ppGpp in the coordination of metabolic processes in *R. sphaeroides*, we found it of interest to determine whether *R. sphaeroides* possessed a typical ribosome-dependent, Rel^+ mechanism for the production of ppGpp and, if present, whether this mechanism was involved in the light-mediated formation of ppGpp. Cells of *R. sphaeroides* M29-5 (Leu^- Met^-) were grown in a succinic acid minimal medium as described by Lueking et al. (24) and were deprived of leucine by being washed and suspended in medium lacking this amino acid. Cells used for nucleotide measurements were adapted and grown in the low-phosphate minimal medium described by Campbell and Lueking (8). Phototrophic growth was conducted under an atmosphere of 95% N_2–5% CO_2 at 31°C and with saturating illumination (5,380 lx). Chemotrophic growth was conducted aerobically in the dark with constant sparging with a mixture of nitrogen-oxygen-carbon dioxide (74:25:1) (14, 15). Culture growth was monitored turbidimetrically with a Klett-Summerson colorimeter equipped with a red filter. RNA determinations were conducted with cells adapted (five mass doublings) and grown in medium containing [^{3}H]uracil (0.18 mM; specific activity, 10 μCi/μmol). Culture samples (0.1 ml) for RNA determinations were transferred to Whatman GLC filter disk and placed in ice-cold 10% trichloroacetic acid. The disks were then washed in 5% trichloroacetic acid–95% ethanol and dried, and the radioactivity was determined by scintillation counting. Treatment of the disk with 0.3 N NaOH (2 h, 60°C) removed ≥95% of the precipitable radioactivity. Phospholipids were extracted from whole cells by the method of Bligh and Dyer (5) as described by Ames (2). Samples were digested as described by Goldfine et al. (19), and lipid phosphorus was determined by the method of Bartlett (3). Lipid phosphorus values were multiplied by 25 to obtain micrograms of phospholipids. Protein was determined by the method of Lowry et al. (22). Cellular bacteriochlorophyll and carotenoids were quantitated in acetone-methanol (7:2 vol/vol) extracts (12, 13) as described by Wraight et al. (28). Nucleotide determinations were conducted exactly as described by Campbell and Lueking (7) with cells adapted and grown in low-phosphate minimal medium containing 100 μCi of carrier-free ^{32}P (New England Nuclear Corp.) per ml. Nucleotide identification was accomplished by both one- and two-dimensional thin-layer chromatography (7, 10).

Leucine deprivation of either phototrophically (Fig. 1) or chemotrophically (Fig. 2) growing cells of *R. sphaeroides* resulted in relatively immediate restrictions in the continued accumulations of cellular protein, phospholipid, and stable RNA (Fig. 1d to f; Fig. 2b to d). Phototrophically growing cells also displayed restrictions in the accumulations of cellular bacteriochlorophyll and carotenoids (Fig. 1b and c). Control studies showed that the washing procedure used for leucine starvation had no measurable effect upon cell growth or other parameters examined.

Since the restrictions of biosynthetic activities shown in Fig. 1 and 2 were less abrupt than those previously observed following a high-to-low light transition in phototrophically growing cells (7), it was concluded that the accumulation of ppGpp, if an accumulation occurred following amino acid starvation, was probably less rapid than that observed following a light transition. To examine this possibility, we tested the effect of leucine deprivation upon the cellular levels of ATP, GTP, and ppGpp in phototrophically and

* Corresponding author.

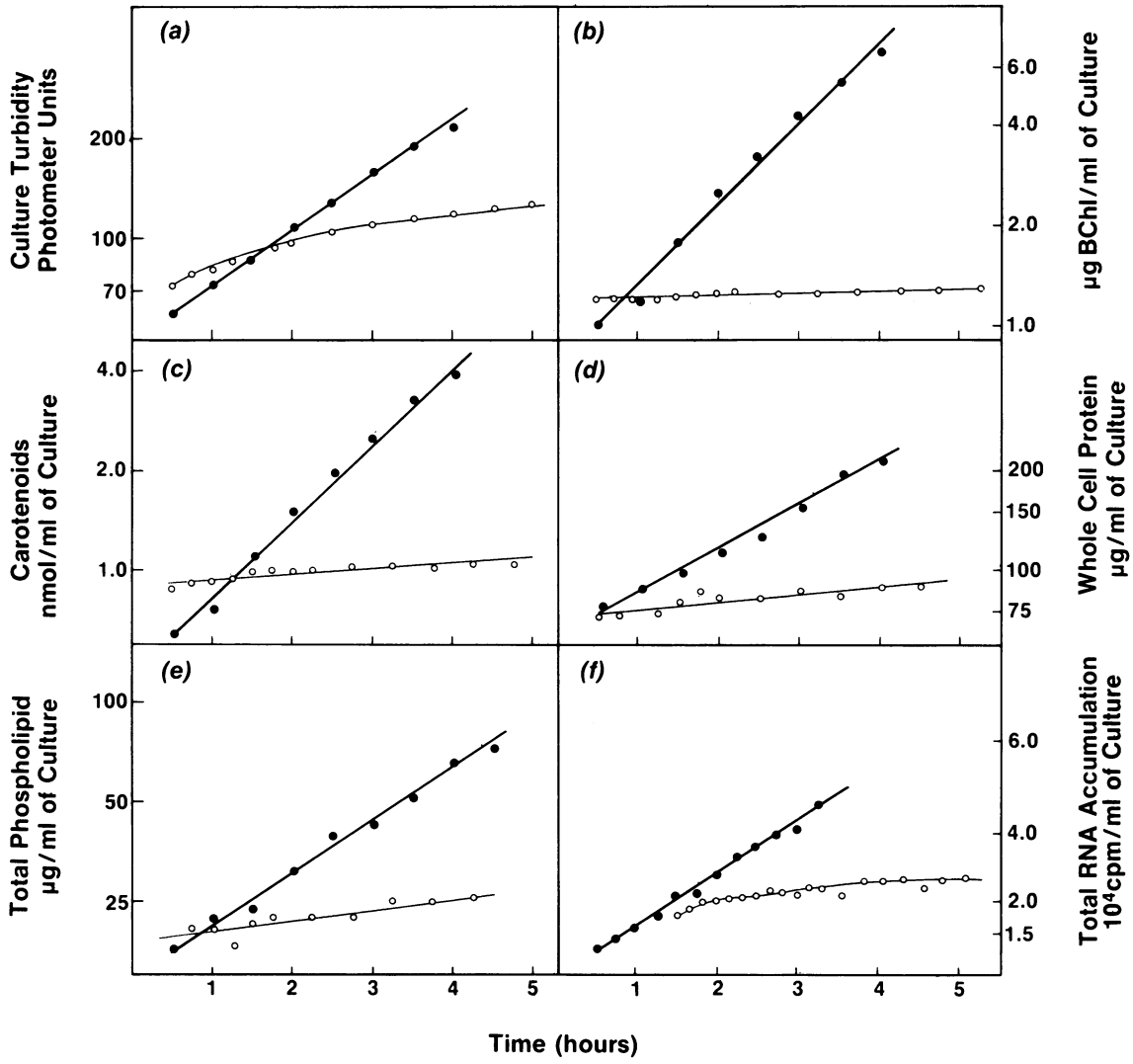

FIG. 1. Response of a high-light-adapted phototrophically growing culture of *R. sphaeroides* to leucine starvation. Cells adapted to logarithmic-phase growth (8×10^8 cells per ml) at a high light intensity (5,380 lx) were washed (twice) with succinic acid minimal medium and suspended in medium supplemented with 50 µg each of leucine and methionine per ml (●) or in medium lacking leucine (○). Cells used for RNA determinations were adapted and grown in medium containing [5-^{3}H]uracil as described in Materials and Methods. BChl, bacteriochlorophyll.

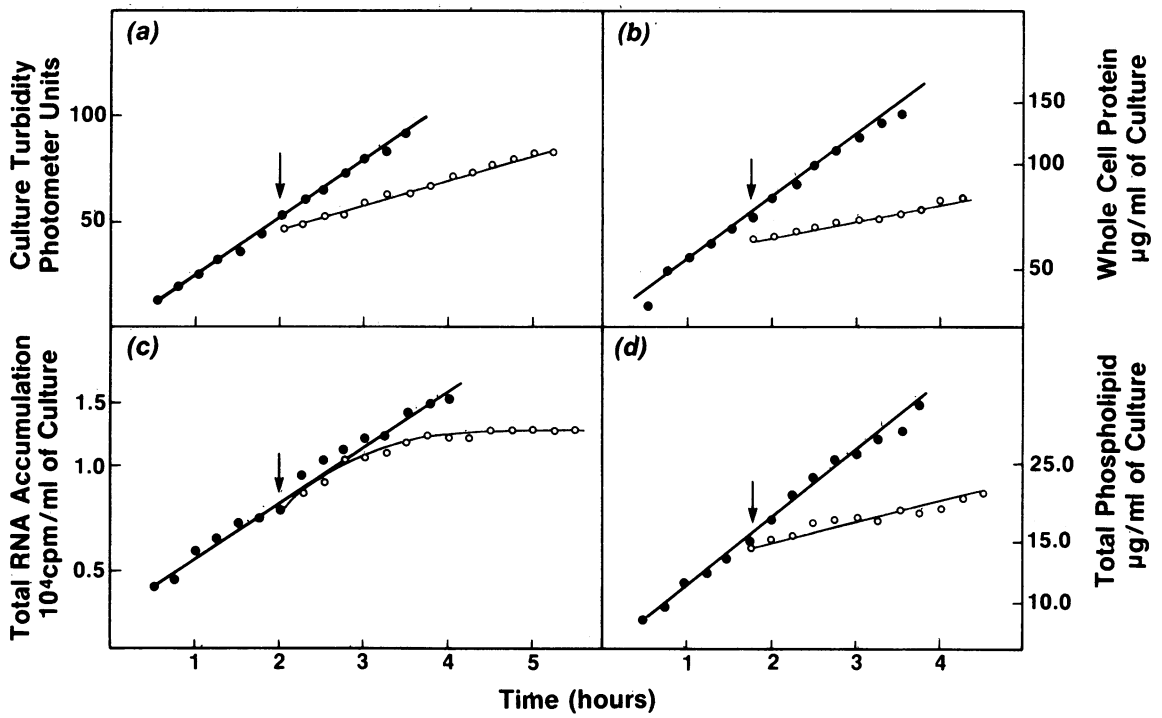

FIG. 2. Response of chemotrophically growing cells of *R. sphaeroides* to leucine starvation. Cells undergoing logarithmic-phase growth (5×10^8 cells per ml) were washed twice (at the time indicated by the arrows) with succinic acid minimal medium and suspended in medium either containing (●) or lacking (○) leucine (50 µg/ml).

chemotrophically growing cells. The steady-state levels of ATP (1.9 mM), GTP (0.48 mM), and ppGpp (0.15 mM) were the same in cells growing phototrophically and chemotrophically (Fig. 3), and these levels were in good agreement with the levels previously reported by Campbell and Lueking (7). Surprisingly, however, leucine deprivation (arrows in Fig. 3) had no effect upon the steady-state levels of the three nucleotides. Although the expected restrictions in biosynthetic activities were observed following leucine deprivation (Fig. 1 and 2), these restrictions occurred in the absence of ppGpp accumulation in both chemotrophically and phototrophically growing cells. Further, since the steady-state levels of ATP and GTP were unaffected by leucine deprivation (Fig. 3), the possibility that the observed stringent response to amino acid starvation is attributable to a condition of generalized energy stress seems unlikely.

Among procaryotes, reports of stringent responses to amino acid starvation without concurrent ppGpp accumulation are rare. *Rhizobium meliloti* does not accumulate ppGpp in response to amino acid starvation (4), although a significant accumulation of ppGpp occurs in this organism in response to carbon source or ammonium starvation. However, restrictions in cellular RNA accumulation in *R. meliloti* occur under all of these conditions (4). Similarly, Spadero et al. (27) reported that a histidine auxotroph of *Salmonella typhimurium* displayed a stringent response to histidine deprivation without concurrent ppGpp accumulation. Serine starvation of this strain, however, provoked the accumulation of ppGpp. Thus, these organisms, in addition to *R. sphaeroides*, are able to differentially accumulate ppGpp in response to specific environmental stimuli, and all possess ppGpp-independent regulatory mechanisms that restrict biosynthetic processes in response to amino acid starvation. Although the nature of these mechanisms is unknown, it was found that the inhibition of RNA accumulation in the *S. typhimurium* histidine auxotroph was dependent upon the presence of a functional *relA* locus (27). This finding prompted Spadaro et al. (27) to suggest that a secondary *relA*-dependent regulatory mechanism exists in *S. typhimurium* and becomes responsible for the coordination of biosynthetic processes in the event that the ppGpp-dependent mechanism fails.

The results of the present study do not preclude an effector role for ppGpp in the light-mediated control of metabolic activities in *R. sphaeroides*. They do indicate that ppGpp has no causal role in the stringent response to amino acid starvation displayed by *R. sphaeroides* and, regardless of whether ppGpp functions as a negative effector during a light transition, this organism must possess a ppGpp-independent regulatory mechanism that functions to coordinate metabolic activities in response to amino acid starvation. At present, information pertaining to this ppGpp-independent mechanism and to its relationship, if any, to the status of the *relA* locus is unavailable.

Finally, although the potential involvement of additional polyphosphorylated nucleotides cannot be eliminated, attempts to demonstrate species of polyphosphorylated guanosine and adenosine nucleotides (6) or adenylated nucleotides in ^{32}P-labeled amino acid-starved cells of *R. sphaeroides* were unsuccessful (data not shown). In particular, sustained attempts to demonstrate the presence of ppGp (phantom spot) (17) provided no evidence for the presence of this nucleotide in either chemotrophically or phototrophically growing cells.

(A preliminary report of this work was presented at the 85th Annual Meeting of the American Society for Microbi-

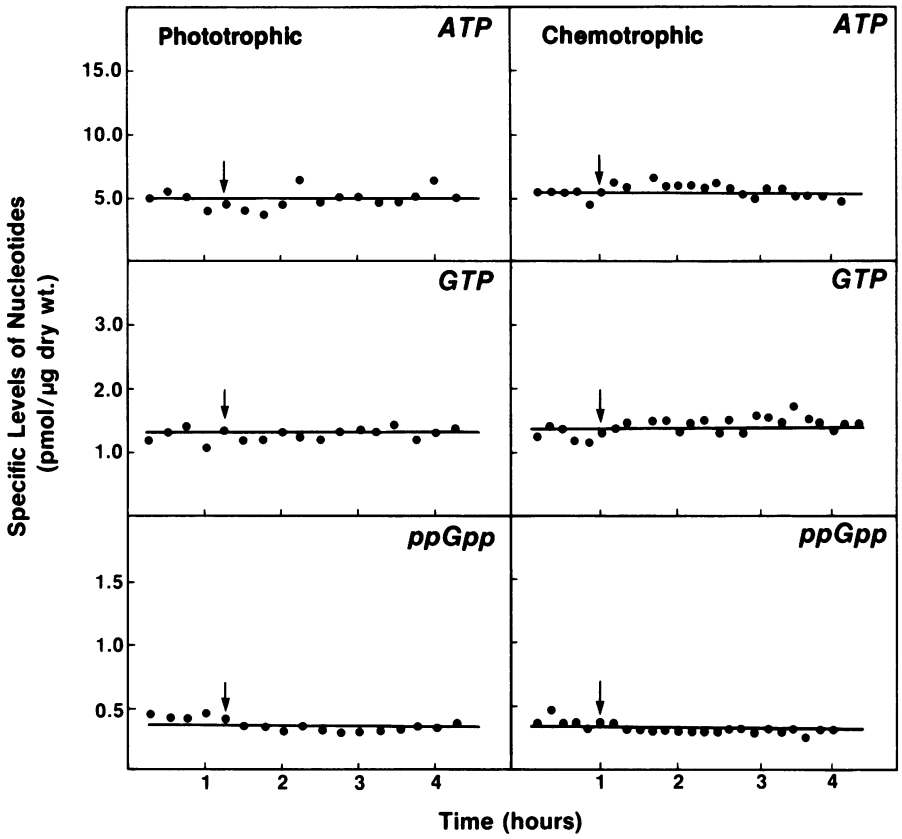

FIG. 3. Effect of leucine starvation on the cellular levels of ATP, GTP, and ppGpp in chemotrophically (5×10^8 cells per ml) and phototrophically (8×10^8 cells per ml) growing cells of *R. sphaeroides*. Cells were uniformly labeled by growth in low-phosphate (2 mM) minimal medium containing 100 μCi of ^{32}P per ml. Arrows indicate the start of leucine starvation. Nucleotide extraction and analysis were conducted as described by Campbell and Lueking (7).

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