Formation of ppGpp in a Relaxed and Stringent Strain of *Escherichia coli* during Diauxie Lag*

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ABSTRACT: It has previously been thought that the capacity to produce guanosine 5′-diphosphate 3′(or 2′)-diphosphate (ppGpp) is specifically dependent on the allelic state of the *rel* gene of *Escherichia coli*. The present paper, on the contrary, reports that a relaxed as well as a stringent strain produced similar amounts of ppGpp when entering the glucose–succinate diauxie lag, during which time the accumulation of RNA was temporarily arrested. It is concluded that the formation of ppGpp is not specific for the amino acid control of RNA synthesis, nor due to a specific property of a stringent strain.

When a stringent strain (*rel*$^+$) is deprived of a required amino acid or when its ability to aminoacylate tRNA is restricted, the rate of RNA accumulation (net synthesis) is severely restricted (see review by Edlin and Broda, 1968)—a phenomenon which will be referred to here as "stringent response." Relaxed mutants (*rel*) in which such stringent response is greatly diminished have been isolated (Borek *et al.*, 1955; Fiil and Friesen, 1968). Neither the mechanism which leads to the stringent response nor the way by which mutation can alter the amino acid control of RNA accumulation is fully understood. However, Cashel and Gallant (1969) observed that two unusual phosphorylated compounds, MS I and MS II, appear during amino acid starvation in a stringent strain but not in a relaxed strain, and that their appearance and disappearance are closely correlated with the stringent response (Cashel, 1969). Cashel and Kalbacher (1970) then determined that the structure of MS I is guanosine 5′-diphosphate 3′(or 2′)-diphosphate (ppGpp) while the structure of MS II is as yet undetermined. Failure to detect the MS compounds in a uracil-requiring stringent strain during uracil starvation suggests that their formation is not simply a response to blocked RNA synthesis in a stringent strain (Cashel and Gallant, 1969). Cashel (1969) also showed that both stringent and relaxed strains produce a similar basal level of these compounds whether the cells are growing rapidly in glucose medium or slowly in succinate medium. These observations have encouraged the notion that the formation of MS compounds is specific for the stringent response (Cashel, 1969). On the contrary, the present paper reports that in a relaxed strain as well as in a stringent strain, the formation of ppGpp (MS I) occurred concomitantly with a temporary cessation of RNA accumulation during glucose–succinate diauxie lag, thus indicating that the formation of ppGpp is not specific for the stringent response, nor is it due to a specific property of the stringent strain.

Materials and Methods

Bacteria and Culture Conditions. A pair of *Escherichia coli* K-12 strains, CP 78 (*rel*$^+$) and CP 79 (*rel*), which are isogenic except at the *rel* locus (Fiil and Friesen, 1968) were used throughout. For bacterial growth, Tris–maleate synthetic medium designated as basal TM medium (Paranchych, 1966) was supplemented in the following ways. TMG medium: the basal TM medium was supplemented with glucose (a final concentration of 4 mg/ml), the required amino acids plus isoleucine (50 μg each/ml) and thiamine (10 μg/ml). TMGS medium: the basal TM medium was supplemented with amino acids and thiamine as in TMG medium. In addition, a limited amount of glucose (0.1 mg/ml) and disodium succinate hexahydrate (2 mg/ml) were added. It was found in our laboratory that the addition of isoleucine to these media lengthens the log phase of growth of CP 78 and CP 79, which permitted us to establish a clear-cut diauxie situation before the stationary phase was reached. Bacteria were grown at 37° on a gyrotory water bath shaker, and cell density was measured at an absorbance of 500 nm by means of a Bausch & Lomb spectrophotometer.

Chemicals. Uniformly labeled L-[^{14}C]alanine and [^{14}C]-uracil were obtained from International Chemical and Nuclear Corp.; carrier-free [^{32}P]orthophosphoric acid (in 0.1 N HCl) from Atomic Energy of Canada, Ltd.; D,L-β-2-thienylalanine and amino acids, from Nutritional Biochemical Corp.; polyethylenimine-cellulose thin-layer plates (MN-polygram cel 300 PEI), from Brinkmann Instruments.

Assay of RNA and Protein Accumulation. In order to assay accumulation of RNA and protein, aliquots of the cultures were mixed with one-tenth the volume of the RNA-labeling or protein-labeling mixture. The RNA-labeling mixture contained per milliliter: 5 μCi of [^{14}C]uracil, 100 μg of unlabeled uracil, and 200 μg of cytosine (to minimize the labeling of DNA). The protein-labeling mixture contained per milliliter: 1 μCi of [^{14}C]alanine and 500 μg of unlabeled alanine. At intervals, 0.1-ml portions of the cultures were withdrawn and assayed for the labeled RNA (cold trichloroacetic acid insoluble radioactivity) or labeled protein (hot trichloroacetic acid insoluble radioactivity) by the filter paper disk method (Bollum, 1968).

Assay of ppGpp. Carrier-free [^{32}P]orthophosphate was added to the cultures to final concentrations of 50–200 μCi/ml at the times indicated in the figure legends. The concentration of phosphate ions in all the media used was 10^{-3} M. No attempt was made to increase the specific activity by lowering the phosphate concentration of the media since the diauxie experiments required balanced growth conditions. At intervals, 0.1-ml portions of the cultures were withdrawn and mixed with an equal volume of 2 M formic acid. After incubating in an ice bath for at least 15 min, the suspensions were centrifuged at 5000*g* for 5 min. Portions (5 μl) of the supernatant were immediately chromatographed one dimensionally with 1.5 M KH_2PO_4 (pH 3.4) on polyethylenimine-cellulose thin-layer plates as previously described (Cashel *et al.*, 1969). Auto-

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radiography of the chromatograms revealed a similar nucleotide distribution pattern to that reported by Cashel (1969). The regions of the chromatograms corresponding to ppGpp (MS I) were cut out, immersed into 15 ml of toluene-based scintillation liquid, and counted in a Packard liquid scintillation spectrometer. Then, using a known specific activity of [^{32}P]phosphate in the medium, the amount of ppGpp was calculated as millimicromoles of phosphate per milliliter of culture of A_{500} = 1.0.

Results

A carbon source shift down is known to cause a temporary cessation of RNA accumulation in both stringent and relaxed strains of *E. coli*, in which case stringent and relaxed strains are indistinguishable (Neidhardt, 1963). Generally, a carbon source shift down is achieved by collecting the cells by centrifugation or filtration followed by resuspension into a poorer carbon source. Such shift-down experiments are inconvenient with respect to prelabeling of cells with radioactive precursors and immediate sampling after the shift down. In the following experiment, we examined the cellular production of MS compounds during diauxie, which involves a transient change in carbon source. Both CP 78 (*rel*$^+$) and CP 79 (*rel*) were cultured in TMGS medium containing limiting glucose and sufficient succinate. *E. coli*, under this condition, preferentially utilizes only the glucose at first. When glucose supply is exhausted, the so-called diauxie lag occurs, during which time enzymes for the utilization of the succinate will presumably be induced. The diauxie lag is subsequently followed by resumed growth on succinate. During glucose–succinate diauxie, cell density, RNA and protein accumulation, and amounts of ppGpp were assayed. The results are shown in Figure 1. As expected, RNA accumulation in both the relaxed and stringent strain ceased simultaneously with the break in the growth curves at middle-log phase. A significantly longer cessation of RNA accumulation was observed in CP 79 as compared to CP 78. To our surprise, both relaxed and stringent strains produced similar quantities of ppGpp as soon as RNA accumulation ceased. The amount of ppGpp quickly rose to a level higher than the subsequently maintained plateau value. This overshoot has been previously observed during the stringent response (Cashel, 1969). However, the plateau value of ppGpp did not return to the basal level when the RNA accumulation resumed. A slower decline of ppGpp to the plateau value was observed in CP 79, which might be related to the longer cessation of RNA accumulation in this strain.

Since the autoradiograms revealed the presence of very little MS II, no attempt was made to quantitate MS II in the chromatograms. Small but definite breaks in the protein accumulation curves were observed both in CP 78 and CP 79 at approximately 5–10 min after their RNA accumulation ceased.

Similar formation of ppGpp was also observed in both strains when the glucose–succinate diauxie was created in the presence of Casamino Acids instead of the required amino acids plus isoleucine.

In order to compare the amount of ppGpp formed during the diauxie lag with that formed during amino acid starvation, the effect of β-2-thienylalanine was studied. β-2-Thienylalanine is known to cause intracellular starvation of phenylalanine by acting as a false feedback inhibitor of phenylalanine biosynthesis (Ezekiel, 1965), and this analog has been previously used to cause the formation of ppGpp in other stringent strains (Wong and Nazar, 1970). Figure 2 illustrates

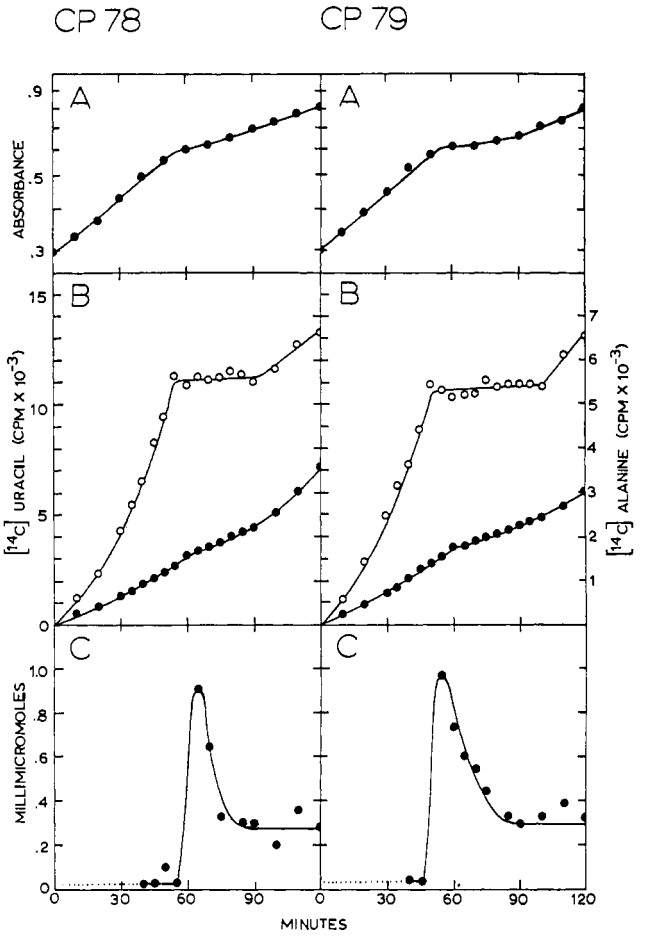

FIGURE 1: Formation of ppGpp during glucose–succinate diauxie lag in the presence of required amino acids and isoleucine. CP 78 (*rel*$^+$) and CP 79 (*rel*) were cultured at 37° in TMGS medium containing limiting glucose (0.1 mg/ml) and disodium succinate hexahydrate (2 mg/ml). When the culture reached an early-log phase (approximately 3 × 10^8 cells/ml), the culture was divided into four culture tubes of identical design. The RNA- and protein-labeling mixtures were added to the two separate tubes at zero time. [^{32}P]-Orthophosphate was added at 30 min to the third tube. The fourth portion was used for the determination of cell density. The periodic assays of cell density and accumulation of labeled RNA, protein, and ppGpp were performed as described in Materials and Methods. The left column indicates CP 78 and the right, CP 79. Part A indicates cell density. Part B indicates the incorporation of precursors into RNA (open circles) or protein (closed circles) in 0.1-ml portions of the cultures. In part C, the amounts of phosphate incorporated into ppGpp are expressed as millimicromoles of phosphate per milliliter of culture of A_{500} = 1.0.

the effects of β-2-thienylalanine on the accumulation of RNA and protein and the formation of ppGpp in CP 78 and CP 79. Upon addition of the analog, formation of ppGpp, with a concomitant inhibition of RNA accumulation, occurred in the stringent strain while the relaxed strain exhibited neither response. Protein accumulation in both strains was severely reduced indicating the effectiveness of phenylalanine starvation by the analog. It is noted that the maximum amount of ppGpp formed in CP 78 during phenylalanine starvation was approximately 2.5 times as large as that observed during diauxie lag.

Discussions

Both stringent and relaxed strains produced similar amounts of ppGpp during diauxie lag (created by a carbon shift down

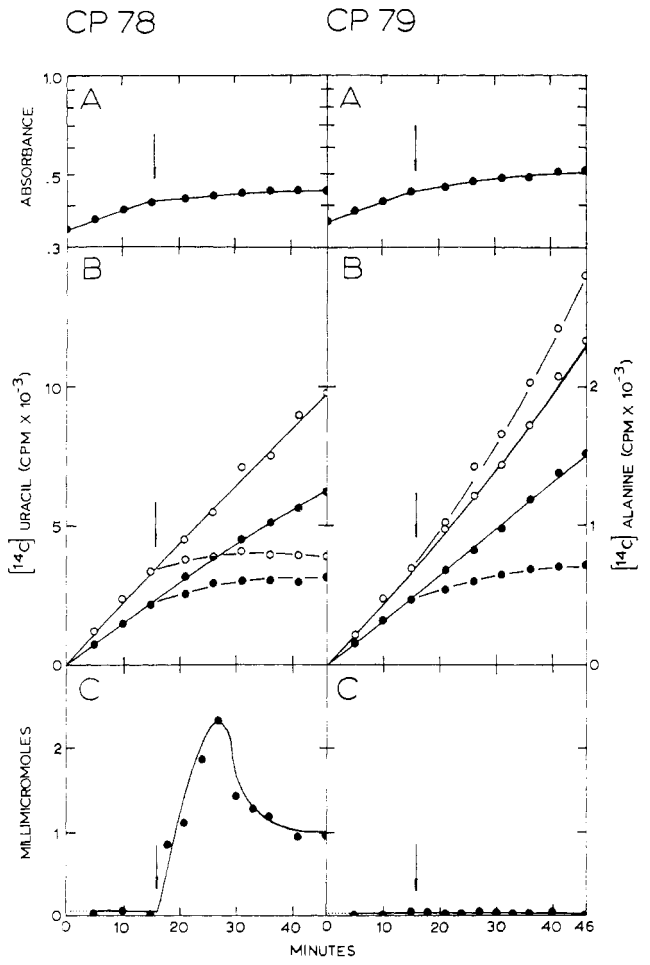

FIGURE 2: Effect of phenylalanine starvation on ppGpp formation. CP 78 (*rel*$^+$) and CP 79 (*rel*) were cultured in TMG medium containing normal amount of glucose (4 mg/ml). The RNA- and protein-labeling mixtures and [^{32}P]orthophosphate were added at zero time to three separate tubes. DL-β-2-Thienylalanine was added at 16 min (as indicated by arrows) to a final concentration of 500 μg/ml. The periodic assays of cell density and accumulation of labeled RNA, protein, and ppGpp were performed as described in Materials and Methods. The left column indicates CP 78 and the right, CP 79. Part A indicates cell density. Part B indicates the incorporation of precursors into RNA (open circles) or protein (closed circles) in 0.1-ml portions of the cultures, where the interrupted lines indicate the incorporation in the β-thienylalanine-treated cultures, while the continuous lines indicate the incorporation in the untreated controls. In part C, the amounts of phosphate incorporated into ppGpp are expressed as millimicromoles of phosphate per milliliter of culture of A_{500} = 1.0.

from glucose to succinate) under conditions where the rate of protein accumulation was only slightly affected (Figure 1). This fact eliminates the previous notion (Cashel, 1969) that the formation of ppGpp is specific for the stringent response. Furthermore, the capacity to produce ppGpp is not directly dependent of the allelic state of the *rel* gene. Therefore, the fact that the relaxed strain does not produce ppGpp during amino acid starvation is likely due to its continued RNA accumulation under this condition. On the other hand, the formation of ppGpp is clearly correlated with the inhibition of RNA accumulation during both diauxie lag and stringent response.

Upon amino acid deprivation the rate of RNA synthesis in a stringent strain rapidly falls to approximately 30% of the rate in the uninhibited control (Lazzarini and Dahlberg, 1971), while during glucose to lactate diauxie lag the rate of RNA synthesis is reduced only to approximately 90% of the rate during balanced growth on glucose (Lazzarini and Winslow, 1970). If the formation of ppGpp is correlated with the inhibition of RNA synthesis, the amount of ppGpp produced during stringent response is expected to be greater than that produced during diauxie lag, due to their inverse relationship. Comparison of Figure 1C and Figure 2C shows that the maximal amount of ppGpp formed in CP 78 during phenylalanine starvation was, in fact, greater by a factor of 2.5 than that formed during diauxie lag.

The present results provide little information as to the mechanism of ppGpp formation and its physiological function. However, the possibility suggested by Cashel and Gallant (1969) that MS compounds may arise from an "idling" of the translation machinery is unlikely, since ppGpp was formed while protein accumulation was only slightly affected (Figure 1). In view of the data in Figure 1C that the amount of ppGpp exhibited an only initial rise and subsequent decline to lower plateau values while RNA accumulation is still halted, there still exists a possibility that the formation of MS compounds are secondarily formed when RNA accumulation is reduced in some specific manner. On the other hand, inhibition of *in vitro* RNA synthesis by ppGpp (Cashel, 1970; Travers *et al.*, 1970) suggests its active role in the regulation of RNA synthesis. Wong and Nazar (1970) showed that MS compounds do not appear in the presence of rifampicin which is a known inhibitor of initiation of bacterial RNA synthesis (Hartmann *et al.*, 1967; Wehrli *et al.*, 1968). While their results may suggest that initiation of RNA synthesis is a prerequisite for MS production, it is entirely possible that ppGpp is formed by a mechanism which does not involve the rifampicin-sensitive initiation step and that such a mechanism may be common to the formation of ppGpp both during the stringent response and diauxie lag.

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