***relA* and Related Loci are Growth Rate Determinants for *Escherichia coli* in a Recycling Fermenter**

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Three sharply distinct phases were found in the growth of *Escherichia coli* in a continuous fermenter with 100% cell recycle. They were: an initial phase of batch-type growth reaching exponential rates that terminated abruptly when the glucose concentration in the fermenter fell below the cell's transport threshold; a second phase of linear growth whose rate was dependent on the rate of glucose provision to the fermenter, but whose length was fixed and independent of growth rate; and a third phase of slower linear growth, whose rate was also dependent on the glucose provision rate, characterized by a restriction in RNA accumulation, and indefinite in length. Guanosine 5'-diphosphate 3'-diphosphate (ppGpp) accumulation commenced at the start of phase 2 and rose throughout the phase. It reached a maximum at the start of phase 3, concurrent with the curbing of RNA accumulation; thereafter, the ppGpp concentration was constant unless the rate of glucose supply was increased, at which point it fell to a phase 2 level and the cells returned to a corresponding phase 2 growth rate. The length of growth phase 2 was changed in a precise manner by mutations in the *relA*, *relX* and *spoT* loci. Phase 2 was longer for *relA* and *relX* mutants (decreased capability for ppGpp synthesis) and shorter for *spoT* mutants (decreased capability for ppGpp degradation). It is concluded that the continuously falling energy supply per cell in a recycling fermenter invokes ppGpp accumulation by at least three biochemical mechanisms and that the third, final growth phase in the fermenter is an indefinitely extended manifestation of strong regulation by ppGpp.

INTRODUCTION

In a fermenter with 100% cell recycle the growing microbial population is completely retained in the fermentation vessel while fresh medium and spent broth are added to and removed from the vessel at equal rates. If the rate at which the carbon-energy substrate is supplied to the growing culture is kept constant, since the total cell mass in the fermenter is continuously increasing, then the rate at which energy is supplied per unit cell mass is continuously dwindling. For such circumstances, calculation of the effect of the maintenance energy requirement upon growth rate (Pirt, 1975; Schultz & Gerhardt, 1969) predicts a steady decrease toward zero for the growth rate, yielding a growth curve smoothly approaching an asymptote, whose value in turn should be predictable for any particular culture (Chesbro *et al.*, 1979) from its maintenance coefficient. When *Escherichia coli* was grown in a recycling fermenter under carbon-energy limitation the observed pattern of growth differed significantly from the pattern predicted by application of the maintenance energy concept (Chesbro *et al.*, 1979). Instead, a growth pattern with three phases that were sharply distinct both kinetically and biochemically was observed. The first phase was one of batch-type growth becoming exponential in rate. The exponential growth terminated abruptly when the glucose concentration in the fermentation vessel fell below the concentration limit

for transport by *E. coli*. In the immediately successive second growth phase, the growth rate was constant and a function of the rate at which glucose was supplied to the culture. The second phase terminated equally abruptly after an interval independent of the growth rate in either phases 1 or 2. For the strain of *E. coli* B used, this interval was 22–24 h. In the third phase of growth, the growth rate was still constant and a function of the glucose provision rate, but was a fraction of the phase 2 rate. Because the cell mass, X , increased continuously during phases 2 and 3 while the growth rate, dX/dt , was constant and positive within the two phases, the specific growth rate (μ), $(1/X) \cdot dX/dt$, decreased continuously within both phases, undergoing a change in its own rate of decline within phases 2 and 3. Further, because of the constancy of the growth rate within phases 2 and 3, the mass doubling time became the reciprocal of μ , or $X \cdot dX/dt$, so that the length of the doubling time continuously increased in phases 2 and 3, leading eventually in phase 3 to doubling times in excess of 100 h.

Thus, the recycling fermenter provides access to very slow specific growth rates and very long mass doubling times in a range that has not often been studied in bacteria. This range is reached in the recycling fermenter along highly reproducible growth rate transients which are of fixed length in the case of phase 2, and of indeterminate length in the case of phase 3.

The rates of RNA, DNA, protein and lipopolysaccharide synthesis remained in constant proportion during phase 2 growth, which thus resembled states of balanced growth in this respect. In phase 3, however, the rate of RNA synthesis was depressed more than the other rates of macromolecular syntheses, so that the pattern of macromolecular syntheses in phase 3 resembled that found in cultures with an elevated concentration of the regulatory nucleotide guanosine 5'-diphosphate 3'-diphosphate (ppGpp), the 'engine of stringency' (Fiil *et al.*, 1977).

We report here results of examining the concentrations of ppGpp in the three phases of an *E. coli* culture grown in the recycling fermenter, and the effects of mutations in the *relA*, *relX* and *spoT* loci upon the growth phase pattern.

METHODS

Bacteria and growth conditions. *Escherichia coli* B was the strain previously described (Chesbro *et al.*, 1979). *Escherichia coli* strains NF859, NF859X (*relA1*), NF162 (*relA1 spoT1*), NF161 (*spoT1*) and NF1035 (*relA2 relX*) were all gifts from J. Gallant, University of Washington, and *Escherichia coli* H10407 (Ent⁺), an enterotoxigenic strain, was a gift from S. Falkow, University of Washington. Cultures were maintained on Davis & Mingioli (1950) basal medium (DMM) agar slants containing 1.25 mg glucose ml⁻¹ with appropriate amino acids added at 20 µg ml⁻¹ for the growth of auxotrophic strains. Fresh cultures were transferred from frozen storage at intervals. The basal medium containing a known glucose concentration, 10 µg dithiothreitol ml⁻¹ (Chesbro *et al.*, 1979) and appropriate amino acid supplementation for auxotrophs was used in all experiments in the recycling fermenter, except those involving ³²P. The medium was prepared and autoclaved in 18 l batches.

Recycling fermenter operation. The fermenter system has been described previously (Chesbro *et al.*, 1979). Briefly, sterile medium, flushed with oxygen-free nitrogen, was added to the reaction vessel at a constant rate. The vessel contents (450 ml) were pumped continuously across a 0.2 µm polycarbonate filter (Nuclepore, Pleasanton, Calif., U.S.A.), spent medium was drawn off, and the microbial population was returned to the vessel. Since inflow and outflow rates were equal, the fluid level in the fermenter was constant. The glucose provision rate (GPR) was the product of the substrate concentration of the medium (S_R) multiplied by the rate at which medium flowed into the fermenter (F_R). To start, the fermenter was inoculated with approximately 30 ml of a 12 h anaerobic culture. The reaction vessel was maintained at 30 °C and the pH of the medium was kept at 7.0 by the addition of sterile 2 M-NaOH.

Growth, DNA, RNA and protein analysis. Analyses of microbial mass, culture turbidity, viable count, DNA, RNA and protein were performed as described previously (Chesbro *et al.*, 1979).

Determination of ppGpp. To 4 l of modified DMM [containing (g l⁻¹): K₂HPO₄, 0.087; (NH₄)₂SO₄, 1.0; sodium citrate, 0.5; bicine, 16.317; MgSO₄·7H₂O, 0.1 (autoclaved separately); glucose, 3.75 (autoclaved separately); dithiothreitol, 0.01; at pH 7.0] was added 25 mCi (925 MBq) of carrier-free [³²P]orthophosphate (New England Nuclear). After inoculation of the fermenter, 2 ml samples were withdrawn at various intervals and made 2 M in formic acid by adding 153 µl of concentrated formic acid. They were then frozen at -70 °C, thawed, centrifuged, and the supernates were dried and resuspended in 200 µl of 10% (v/v) isopropanol, thus concentrating the suspension 10×.

The ppGpp in these formic acid extracts was separated by polyethyleneimine (PEI)-cellulose thin-layer chromatography (J. T. Baker Chemical Co., Phillipsburg, N.J., U.S.A.). The sheets were prewashed once in deionized water and dried. One-dimensional separation of the nucleotides was achieved by spotting between 2.5 and 10 μ l of ^{32} P-labelled nucleotide extract and chromatographing in 1.5 M-potassium phosphate buffer, pH 3.4 (Cashel *et al.*, 1968). ^{32} P-labelled nucleotides were detected by autoradiography using Kodak XR-5 X-ray film exposed for 24–72 h. Spots whose mobility corresponded to authentic standards of ppGpp and GTP (PL Biochemicals, Milwaukee, Wis., U.S.A.) were cut out and counted in a scintillation counter.

For qualitative identification of ppGpp and other nucleotides, a two-dimensional system was employed (Cashel *et al.*, 1968). The formic acid extracts were chromatographed in the first dimension with 3.3 M-ammonium formate/4.2% (w/v) boric acid, pH adjusted to 7.0 with NH_4OH . Sheets were then soaked in methanol for 5 min, water for 15 min, and dried. The solvent for the second dimension was 0.85 M- KH_2PO_4 , pH 3.4.

Cultures were tested for the relative production of ppGpp and guanosine 5'-triphosphate 3'-diphosphate (pppGpp) by the procedure described by Engel *et al.* (1979). Cells grown overnight on broth agar plates were transferred with a toothpick to make turbid 200 μ l of a Tris minimal glucose medium (Kaempfer & Magasanik, 1967) containing 40 μ M-phosphate, 1 mg serine hydroxamate ml^{-1} (Sigma), 300 μ g L-valine ml^{-1} (Sigma) and 10 μ Ci (370 kBq) ^{32} P ml^{-1} . This suspension was incubated for 1 h at 37 °C, then 50 μ l of 13 M-formic acid was added, and the solution was frozen for 15 min. Samples (10 μ l) were spotted on PEI-cellulose chromatography plates, developed for 10 cm with 1.5 M- KH_2PO_4 , pH 3.4 and exposed to X-ray film overnight.

RESULTS

Behaviour of E. coli B and H10407 in the recycling fermenter

Growth of *E. coli* B in the recycling fermenter at a GPR of 387 $\mu\text{mol h}^{-1}$ is shown in Fig. 1. Phase 1 growth consisted of a lag period followed by exponential growth. When the glucose concentration in the fermenter fell below 0.1 $\mu\text{g ml}^{-1}$, the transport limit of *E. coli* (Chesbro *et al.*, 1979; Shehata & Marr, 1971), phase 1 ended and phase 2 began. In this second phase the growth rate was constant. For *E. coli* B, phase 2 lasted 22–24 h before phase 3 commenced (Chesbro *et al.*, 1979). The growth rate in phase 3 dropped to a new, lower value which then remained constant for at least another 150 h. Because the growth rate was constant in phases 2 and 3, while cell mass continuously increased, the specific growth rate (μ) fell throughout phase 2 and phase 3, and since mass doubling time in these phases of linear growth was $1/\mu$, the doubling time increased continuously.

Figure 1 shows changes in colony-forming units, biomass, DNA, RNA and protein in *E. coli* B before and after phase 3 cells were subjected to an upshift in GPR. The upshift was imposed by increasing fourfold the flow rate of medium into the fermenter. During phase 2, the concentrations of DNA, RNA and protein increased in constant proportion. At the onset of phase 3, the rates of synthesis of these parameters decreased, but not in proportion (Table 1), and the cells became impoverished in RNA as its synthesis dropped by 80% from its phase 2 rate. In comparison, protein synthesis dropped by 43% from its phase 2 rate.

For the first 6 h after the fourfold upshift, the rate of cell division was 13-fold, and the rate of biomass increase was 7-fold, greater than their respective rates immediately before the upshift. However, the rate of biomass increase was very nearly four times its phase 2 value. Protein synthesis increased 5-fold and the rate of RNA synthesis about 40-fold. The increase in the rate of DNA synthesis was relatively modest, about 2-fold. After 10 h, its rate of synthesis doubled fairly abruptly. At the same time, the rates of RNA synthesis, cell division and growth commenced falling, apparently toward asymptotes.

Figure 2 shows the growth pattern of strain H10407 in the recycling fermenter at a GPR of 412 $\mu\text{mol h}^{-1}$. The most obvious difference from the pattern observed with strain B is in the length of phase 2 – in this case close to 50 h. When H10407 was grown at various GPR, the growth rates in phases 2 and 3 were dependent on GPR, but the length of phase 2 remained at 50 h, as it had remained at 22–24 h for *E. coli* B despite changes in GPR and growth rate (Chesbro *et al.*, 1979).

Fig. 1. Growth of *E. coli* B in a recycling fermenter at pH 7.0, 30 °C, and a GPR of 387 $\mu\text{mol glucose h}^{-1}$. At 69 h, the GPR was shifted to 1552 $\mu\text{mol glucose h}^{-1}$. RNA ($\square$), DNA ($\triangle$), protein ($\blacksquare$), biomass ($\circ$), colony-forming units (c.f.u.) ($\bullet$).

Table 1. Change in culture parameters when a phase 3 culture of *E. coli* B was exposed to a fourfold increase in glucose provision rate

The GPR was 387 $\mu\text{mol h}^{-1}$ at an F_R of 55.8 ml h^{-1} during phase 2 and the first 45 h of phase 3; F_R was then increased to 216 ml h^{-1} . The phases are defined in the text.

Parameter	Percentage increase 6 h after upshift	Rate of change ($\mu\text{g ml}^{-1} \text{h}^{-1}$)		
		Phase 2	Phase 3	After upshift*
Dry weight	43	14.5	3.8	59.0
Protein	38	13.5	7.8	39.0
RNA	87	1.6	0.3	13.0
DNA	5	1.2	0.3	0.7
Bacterial numbers	30	—	—	—

* Calculated for the first 6 h.

Accumulation of ppGpp

The changes in RNA and protein synthetic rates at the entry of the culture into phase 3, their relative proportions during phase 3, and their changes after the upshift resembled similar changes in many strains of *E. coli* during carbon-energy shifts (Gallant, 1979; Nierlich, 1978). Such changes have been shown to be closely associated with cellular concentrations of ppGpp (Gallant, 1979; Nierlich, 1978).

Figure 3 shows the concentration of ppGpp in *E. coli* B at different times during the three phases and after a fourfold upshift in GPR. The concentration of ppGpp in phase 2 was

Fig. 2

Fig. 3

Fig. 4

Fig. 2. Growth of *E. coli* H10407 in a recycling fermenter at pH 7.0, 30 °C, and a GPR of 412 $\mu\text{mol glucose h}^{-1}$.

Fig. 3. ppGpp content of *E. coli* B growing in a recycling fermenter at a GPR of 1250 $\mu\text{mol glucose h}^{-1}$ until shifted to 4800 $\mu\text{mol glucose h}^{-1}$ at 45 h. Other conditions were as in Fig. 1. Klett units (○), ppGpp (bars).

Fig. 4. Growth of a wild-type and *rel* system mutants of *E. coli* in a recycling fermenter at pH 7.0, 30 °C. The cultures and respective GPR ($\mu\text{mol glucose h}^{-1}$) were: (a) NF161 (*spoT*), 416; (b) NF162 (*relA1 spoT*), 408; (c) NF859 (wild-type), 433; (d) NF859X (*relA1*), 416; (e) NF1035 (*relA2 relX*), 421.

double that in phase 1 and seemed to increase slightly throughout the phase. Its concentration nearly doubled again in phase 3 cells, then returned close to its early phase 2 concentration immediately after the glucose upshift.

Growth pattern of relA, relX and spoT strains of E. coli in the recycling fermenter

Two routes for the synthesis of ppGpp are believed to exist. The first involves the *relA* gene product, a ribosome-bound ATP:GTP pyrophosphate transferase whose activity in the synthesis of pppGpp and ppGpp requires the presence of mRNA and uncharged, codon-specific tRNA. These requirements couple this route to the cell's amino acid supply.

Fig. 5. Accumulation of RNA by *E. coli* strains NF161 (●) and NF1035 (○) in the experiments shown in Fig. 4.

(Because carbon source depletion also depletes the cell's amino acid supply, the *relA* gene product is also coupled indirectly to the carbon source supply.) The second system involves the *relX* gene product, and possibly the *relS* locus (Engel *et al.*, 1979) whose mode of action is less well defined, but which seems to couple ppGpp synthesis to energy downshifts. In this route, pppGpp is not an intermediate.

Regardless of the route by which ppGpp is made, it is subject to degradation by the *spoT* gene product, guanosine 3',5'-bis(diphosphate) 3'-pyrophosphohydrolase (ppGppase), an enzyme that also is ribosome-associated (Fiil *et al.*, 1977; Richter *et al.*, 1979), but which is inhibited by uncharged tRNA (Richter, 1980a) and possibly requires the existence of a transmembrane proton gradient (DeBoer *et al.*, 1975; Tetu *et al.*, 1980) for its activity. Glucose exhaustion (which presumably collapses the proton gradient) causes a rapid decrease in ppGpp degradation (DeBoer *et al.*, 1975; Tetu *et al.*, 1980), and it has been argued that inhibition of ppGppase plays an important role in elevating ppGpp concentrations during conditions of energy limitation as well as in amino acid starvation.

Mutations in the *relA* and *relX* loci produce a decreased ability to accumulate ppGpp and therefore should cause a delay in the appearance of phase 3, and a longer phase 2, if ppGpp is the determinant of the phase 2 to phase 3 transition. Conversely, in *spoT* strains, the diminished ppGpp degrading capability should bring about a faster accumulation of ppGpp, thus accelerating the appearance of phase 3 and shortening phase 2. Figure 4 shows that these loci affected the growth-phase pattern in the recycling fermenter by lengthening or shortening phase 2 in a manner consistent with their known effects upon ppGpp synthesis and the supposition that it is the accumulation of ppGpp that brings about phase 3 growth.

Strain NF1035 (*relA2 relX*), although retaining some capability for ppGpp synthesis (Pao & Gallant, 1978), has the most severely restricted capability for such synthesis among the strains studied, which, coupled with its possession of an active ppGppase, led to the expectation that it would either have the longest phase 2 of any strain studied, or would not enter phase 3 at all. In fact, it did enter phase 3 growth after the longest phase 2 yet observed, suggesting that ppGppase activity was ultimately depressed enough by either energy deficiency, or accumulation of uncharged tRNA, or both, to permit accumulation of ppGpp to the concentration necessary for phase 3 growth behaviour to begin.

Figure 5 shows the changes in RNA synthesis during the growth of strains NF161 and NF1035, which had the shortest and longest phase 2 growth intervals, respectively. The abrupt decrease observed in RNA synthesis at the phase 2/phase 3 transition was consistent with the expected effect of an increase in ppGpp concentration at that time. The other strains showed similar changes in RNA synthesis.

The effect of the individual loci upon the length of phase 2 is shown in Table 2. A change in the *spoT1* locus had about three times greater effect on the length of phase 2 than a change in

Table 2. Apparent growth yields and lengths of the phase 2 growth interval for *E. coli* strains of known *rel* system genotype shown in Fig. 4

<i>E. coli</i> strain	Apparent growth yield (Y_a) [mg biomass ($\mu\text{mol glucose}$) ⁻¹]		Length of phase 2 (h)	Increase or decrease in length (h) of phase 2 associated with mutant allele		
	Phase 2	Phase 3		<i>relA1</i>	<i>spoT1</i>	<i>relX</i>
NF161 (<i>spoT</i>)	0.017	0.007	13	↑ 11	↑	
NF162 (<i>relA1 spoT</i>)	0.014	0.007	24	↓	↓ 44	
NF859 (wild-type)	0.016	0.006	57		↓ 45	
NF859X (<i>relA1</i>)	0.014	0.005	69	↑ 12	↓	
NF1035 (<i>relA2 relX</i>)	0.016	0.005	84			↑ 15

the *relX* locus, and about four times greater than a change in the *relA* locus. However, changes in the loci had no obvious effect on the apparent growth yield (Y_a) in phase 2 growth, which averaged 0.016 mg cell dry wt ($\mu\text{mol glucose}$)⁻¹. There did seem to be a trend in Y_a values for phase 3 growth that was inversely correlated with the length of phase 2: Y_a for phase 3 decreased from 0.007 mg cell dry wt ($\mu\text{mol glucose}$)⁻¹ for strain NF161 to 0.005 mg cell dry wt ($\mu\text{mol glucose}$)⁻¹ for strain NF1035. The meaning of this correlation, assuming it is significant, is not yet clear to us.

Comparative synthesis of pppGpp and ppGpp in different *E. coli* strains

Although autoradiograms of *E. coli* B extracts showed the presence of ppGpp in this strain, pppGpp was absent. The latter nucleotide is believed to be the precursor of ppGpp *in vivo* when ppGpp is synthesized by the *relA*⁺ gene product (stringent factor) during amino acid starvation. Tight mutants of the *relA* locus, such as *relA1*, show no increase in the concentration of either nucleotide above basal levels when subjected to amino acid starvation (Gallant, 1979). However, both parent and *relA* mutant strains show elevated ppGpp, but not pppGpp, concentrations when subjected to a carbon-energy downshift (Gallant, 1979). This synthesis seems to involve the *relX* and *relS* loci (Engel *et al.*, 1979) but can be independent of both the *relA* and *relX* loci (Atherly, 1979). The *spoT*⁺ gene product, ppGpp pyrophosphatase, affects the relative accumulations of the two nucleotides in a complex way dependent on the genetic background of a particular strain (Fiil *et al.*, 1977).

A carbon-energy downshift of the sort imposed by the recycling fermenter necessarily subjects the cells to both an energy shortage and amino acid starvation as the supply of glucose-derived intermediates per cell dwindles. The resemblance of the growth pattern of *E. coli* B in the fermenter to that of strain NF162 (*relA1 spoT1*), and of strain H10407 to strain NF859 (*relA*⁺ *relX*⁺ *spoT*⁺), led to the expectation that each pair of strains should show corresponding behaviour in the accumulation of pppGpp and ppGpp when tested by the procedure of Engel *et al.* (1979), which subjects cells in batch growth to a simultaneous energy downshift and amino acid starvation. When tested in this way, *E. coli* H10407 and NF859 produced both nucleotides, whereas *E. coli* B produced only ppGpp, as did NF162 and NF859X (*relA1*). Strain NF161 (*relA*⁺ *spoT*), which is isogenic with NF162, produced a small but detectable amount of pppGpp in addition to ppGpp.

The close resemblance between the growth of strains B and NF162 in the recycling fermenter and in their production of nucleotides suggested that strain B, like NF162, might also show a reduced capacity for ppGpp degradation. The stability of ppGpp in *E. coli* B cells was tested by treating phase 3 cells with arsenate. Following exposure to arsenate, the half-life of ppGpp was greater than 16 min, while that of GTP, for comparison, was of the order of 3 min. Since ppGpp has a half-life of less than 1 min in *spoT*⁺ cells (Fiil *et al.*, 1977), *E. coli* B is either deficient in ppGpp pyrophosphatase activity, in addition to being deficient in stringent factor activity, or arsenate inhibited the pyrophosphatase in some way, perhaps by lowering the transmembrane proton gradient which has been suggested (Tetu *et al.*, 1980) as important to the *in vivo* activity of the enzyme.

DISCUSSION

In phase 3, the ultimate growth stage we have been able to produce in the recycling fermenter, the cellular concentration of ppGpp is comparable with those produced in populations of *E. coli* by carbon-energy source downshifts (Johnsen *et al.*, 1977; Pao & Gallant, 1978; Winslow, 1971), and protein and RNA syntheses behave in ways typical of ppGpp restriction of rRNA synthesis.

Energy-deficient cells have frequently been produced as a means of studying ppGpp synthesis, its effects, and more generally, the regulation and co-ordination of macromolecular syntheses. Strategies for producing such cells have included their being subjected to a shift from a more preferred substrate, usually glucose, to a less preferred one, such as succinate (Lazzarini *et al.*, 1971); permitting energy substrate exhaustion to occur (Boquet *et al.*, 1973; Jacobson & Jacobson, 1980); starvation by temperature shifts that inhibit thermally sensitive steps of energy pathways (Cashel, 1975; Lund & Kjeldgaard, 1972); exposure of anaerobes to oxygen (Glass *et al.*, 1978) or imposition of anaerobiosis on aerobic cultures (Lund & Kjeldgaard, 1972); use of analogues competitive with energy substrates (Hansen *et al.*, 1975; Johnsen *et al.*, 1977); and employment of drugs that uncouple oxidative phosphorylation or inhibit substrate level phosphorylation (Cashel, 1975; Richter, 1980*b*). It has been argued (Jacobson & Jacobson, 1980) that categorizing all these strategies as 'energy-source shift-downs' is suspect since the different treatments affect regulatory behaviour in demonstrably different ways and information about their effects on intermediary metabolism and cellular energy balance is usually absent.

At least some of this criticism is avoided in the production of energy-deficient cells in a recycling fermenter, since dissimilatory metabolism as reflected in fermentation balances is constant throughout the three phases, biomass yield per unit glucose utilized is constant within each phase, and the composition of the milieu is constant throughout the phases unless experimentally altered (Chesbro *et al.*, 1979).

An important feature of the recycling fermenter, as a strategy for calling into play processes that regulate and co-ordinate biosynthetic events at the genetic/metabolic level during energy shortage, is the considerable expansion of the periods of time over which these processes are evoked and exert their influence: the rise time between basal and maximum observed concentrations of ppGpp in cells growing exponentially with doubling times of 1–2 h is less than 1 h, while the comparable time for *E. coli* B in the recycling fermenter is the length of phase 2, i.e. 22–24 h.

The length of phase 2 is constant within rather close limits for a given strain of *E. coli* and independent of growth rate (Chesbro *et al.*, 1979), that is, its length seems to be determined by a cellular 'clockwork'. Since the termination of phase 2 is concurrent with the accumulation of ppGpp to a concentration sufficient to curb RNA accumulation, the 'clockwork' that determines the length of phase 2 is the linkage of ppGpp synthesis and degradation to the rate of energy supply per cell.

Because the rates of macromolecular synthesis in phase 3 resemble in their relative proportions those characteristic of cultures in the stringent response state, it was not surprising to find ppGpp elevated in these cells. Since phase 2 cells resemble cells in steady state growth in the constant proportions of their macromolecular syntheses, however, and therefore are not overtly undergoing the stringent response, it was surprising to find the concentration of ppGpp rising as soon as the glucose concentration in the culture broth fell below the limits of the phosphotransferase system (the condition initiating phase 2) and remaining at a concentration between the basal level of phase 1 and the ultimate level of phase 3. Lagosky & Chang (1980) have recently re-evaluated the relation between basal pool concentrations of ppGpp and RNA synthesis in *E. coli* (Hansen *et al.*, 1975) using an analytical procedure that finds the cellular concentration of ppGpp to be three to five times that shown by acid extraction procedures. They found that RNA synthesis responded relatively sensitively to changes in the concentration of ppGpp in *relA1 spoT1* cultures undergoing amino acid starvation and resupplementation. It is not clear how this relates to the apparent absence of such an effect on RNA synthesis in our phase 2 cultures. The experimental conditions varied considerably between their study and ours: the culture in the recycling fermenter is undergoing no abrupt, exogenously imposed transition in growth rate due to either amino acid or energy deficiency during phase 2, and the observation interval, 22–24 h, is considerably longer than the 3 h maximum interval used by Lagosky & Chang (1980), or, indeed, the observation interval of any studies on cell responses to changing ppGpp concentrations of which we are aware.

However, phase 2 cells may be showing a response to the increased ppGpp concentration in a different area of synthesis since in *E. coli* B the division rate is slowing and cells are becoming progressively larger through an increase in cell diameter (Chesbro *et al.*, 1979). Thus, while the concentration of ppGpp in phase 2 cells may not be high enough to affect the activity of RNA polymerase and the transcription of rRNA, it may be high enough to affect the activity of the enzymes of phospholipid and peptidoglycan synthesis which are susceptible to ppGpp regulation (Gallant, 1979; Nierlich, 1978), and thus the synthesis of membrane and septum and the elongation of the cell. It therefore seems likely that the different concentrations of ppGpp in phase 2 and phase 3 cells are responsible for the temporal separation between the alteration in rates of processes determining cell geometry and volume that takes place in phase 2 and the alteration in rates of processes determining cell mass that takes place in phase 3.

Different questions arise in connection with the observed changes in the rates of macromolecular syntheses and cell division that accompanied a fourfold upshift in glucose provision rate in phase 3. The global growth rate became four times the phase 2 rate rather than the phase 3 rate, while the ppGpp concentration dropped to about that of phase 2. The rates of RNA and protein synthesis and of cell division rose immediately after the upshift while the major increase in the rate of DNA synthesis lagged behind the other rates by about 10 h. This is the reverse of the situation usually observed in cells growing exponentially (Brunschede *et al.*, 1977) when they are subjected to an energy or temperature upshift. In that case, the change in cell division rate lags behind the change in DNA synthesis rate. This has been interpreted to mean that rounds of DNA synthesis underway when the shift was made had to be completed before the rate of cell division could increase (Loeb *et al.*, 1978), and that the rate of cell elongation was fixed for the duration of any division cycle.

In our experiments, the elevated DNA content per colony-forming unit in phase 3 just prior to upshift was very close to that reported by Tang & Helmstetter (1980) for a temperature-sensitive, DNA-initiation mutant of *E. coli* B/r shifted to a non-permissive temperature. They accounted for DNA enrichment in their culture as being due to the presence of multiple chromosome forks. Whether the DNA enrichment in phase 3 *E. coli* B cells was due to multiple forks on finished chromosomes, or to multiple copies of finished chromosomes, either explanation would fit the possibility that, during phase 3, the interdiction

of elongation and septum formation by ppGpp caused the accumulation of 'primed sites' (Sloan & Urban, 1976) in zones of cell wall addition and incipient septation. These 'primed sites' would result in cells poised for completion of cell elongation, septum formation and cell division whose potential for these actions was realized immediately after the upshift reduced the ppGpp concentration and provided carbon-energy at a higher rate.

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