Vol. 95, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 14, 1980 Pages 924-931

THE relA GENE IS NOT REQUIRED FOR GLYCOGEN ACCUMULATION DURING NH_4^+ STARVATION OF *Escherichia coli*

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Received June 18,1980

SUMMARY. When glucose is the carbon source, glycogen accumulates in partial (NH_4^+) or total (NH_4^+ and required amino acids) nitrogen starvation in *Escherichia coli* strains that are $relA^+$ or $relA^-$. But glycogen accumulates only in the $relA^+$ strain when required amino acids are depleted or isoleucine starvation is induced by valine addition. However, when glycerol is the carbon source, glycogen accumulates in both $relA^+$ and $relA^-$ strains after valine addition. We conclude that the relA gene is not required for glycogen accumulation when synthesis of all nitrogen-containing compounds of the cell is limited or abolished. We also conclude that, although the relA gene is needed for glycogen to accumulate in amino acid starvation, this requirement can be replaced by a high cellular concentration of 3',5'-cyclic AMP.

Bridger and Paranchych (1) have suggested that the relA gene is required for the accumulation of glycogen by *Escherichia coli* during nitrogen starvation in the presence of a source of carbon and energy. These investigators have further hypothesized that a relA gene product, ppGpp,[1] is the coordinator for the metabolic responses of *E. coli* to nitrogen starvation (2). We show here that the relA gene is not required for glycogen accumulation by *E. coli* during NH_4^+ or total nitrogen starvation. Thus, ppGpp is not the major coordinator of this metabolic response.

The other group of investigators showed that the accumulation of carbohydrate that occurs during amino acid starvation in the presence of a source of carbon and energy is dependent on the presence of the relA gene. Using a more specific, enzymatic assay we show here that the carbohydrate that accumulates in these conditions is glycogen. In addition, we present evidence

[1]Nonstandard abbreviations: ppGpp, guanosine 3'-diphosphate 5'-diphosphate; ppGp, guanosine 3'-monophosphate 5'-diphosphate.

0006-291X/80/150924-08$01.00/0

that a high cellular level of 3',5'-cyclic AMP can replace the relA gene requirement for glycogen accumulation during amino acid starvation.

METHODS. The organisms used in this study were derived from *E. coli* K12. The stringent strain used is CP78 (arg, his, thr, leu, thi). The relaxed strain, CP79, is the isogenic relA$^-$ derivative of CP78 (3). Bacteria were grown aerobically at 37°C with shaking in the minimal medium previously described (4). The medium was supplemented with thiamine and the required amino acids (100 µg/ml), and either glucose (2 g/liter) or glycerol (4 g/liter) as a carbon source. Ammonium ion (as NH_4Cl) was added either in large excess (600 mg/liter) or as the limiting nutrient (50 to 65 mg/liter). Cultures (1 liter) were inoculated with 1 ml of a suspension in Trypticase Soy Broth (Baltimore Biological Laboratories) on the day before each experiment and were grown overnight (16 to 24 hours).

Growth was followed by measuring the absorbance of the culture, suitably diluted with water, at 450 nm (1 cm light path) using a Beckman DU spectrophotometer. An absorbance of 1.0 in a fully supplemented, growing culture corresponds to approximately 140 mg of cellular protein per liter for CP78 and 120 mg/liter for CP79. (NH_4^+ starvation or addition of valine reduced the amount of protein synthesized per increase in the A_{450} by 15 to 40%, depending on the strain and the type of starvation. No increase in protein occured during starvation for the required amino acids.)

Samples for the determination of glycogen, NH_4^+, and protein were prepared in trichloracetic acid (TCA) as previously described (4). Cellular glycogen was measured by heating the washed TCA pellet in 2.5 N NaOH at 100°C for 30 min. to solubilize the sample and destroy free glucose, degrading the glycogen with amyloglucosidase (5-7), and measuring the liberated glucose with a fluorometric hexokinase procedure (5, 6). Protein was determined by the procedure of Lowry, *et al.* (8), using crystalline bovine serum albumin as the standard. The concentration of NH_4^+ in the neutralized TCA supernate (4) was determined using the phenolhypochlorite procedure (Urea Nitrogen, Technical Bulletin # 640, Sigma Chemical Co.).

RESULTS. Both the stringent (relA$^+$) and the relaxed (relA$^-$) *E. coli* strains accumulated large amounts of glycogen during starvation for NH_4^+ in the presence of an excess of glucose (Fig. 1). This accumulation was greater in the relA$^-$ strain than in the isogenic relA$^+$ strain. The exhaustion of NH_4^+ in these cultures produces only a partial nitrogen starvation. The amino acids that are required for growth of these amino acid auxotrophs can also serve as sources of nitrogen. (However, the large decrease in growth rate that followed the disappearance of NH_4^+ in the medium (Fig. 1) shows that these amino acids are a comparatively poor source of nitrogen.)

In order to evaluate the effect of the relA gene on glycogen accumulation during total nitrogen starvation, cultures of the stringent and relaxed strains growing on glucose were centrifuged, washed and resuspended in (otherwise complete) medium that contained neither NH_4^+ nor the required amino acids.

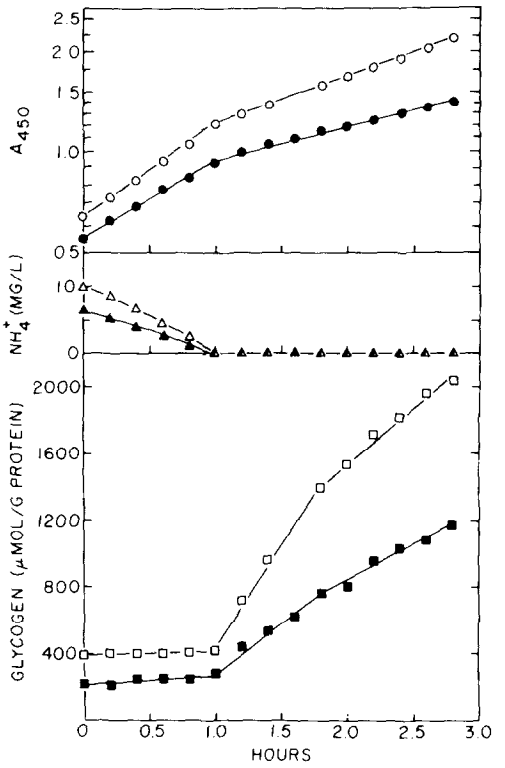

Fig. 1. The effect of a limiting amount of NH_4^+ in cultures of *E. coli* CP78, relA$^+$ (closed symbols) and CP79, relA$^-$ (open symbols): growth (●, ○), medium NH_4^+ concentration (▲, △), and glycogen accumulation (■, □). Cultures were grown and NH_4^+, glycogen and protein were determined as described in METHODS. The time at which NH_4^+ in the medium was exhausted was arbitrarily set at 1.0 hour.

During this total nitrogen starvation, both strains accumulated large amounts of glycogen (Fig. 2). Partial nitrogen starvation (no NH_4^+) of these resuspended cells produced an even greater accumulation of glycogen in both strains, but starvation for the required amino acids increased glycogen accumulation only in the relA$^+$ strain (Fig. 2). Control cultures, resuspended in complete medium, showed the same rapid growth and slow accumulation of glycogen as observed before centrifugation and resuspension.

The addition of valine to K12 strains of *E. coli* is well known to cause a prompt starvation for isoleucine (9). When we added valine to a culture of the relA$^-$ strain growing on glucose, only a brief increase in the rate of glycogen accumulation occured and then the rate returned essentially to that

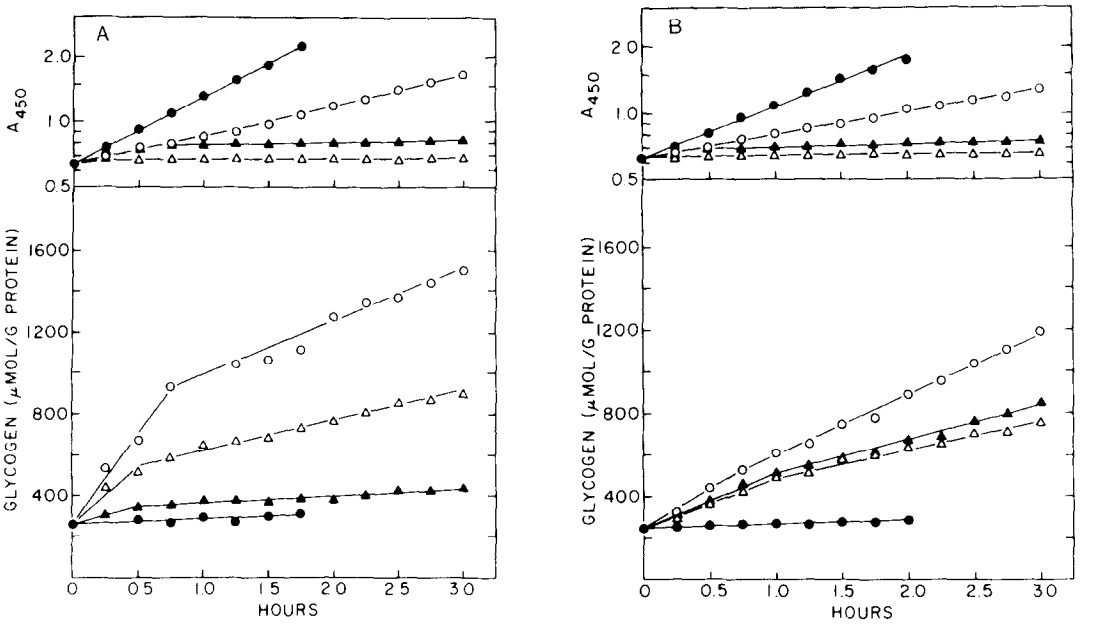

Fig. 2. Growth and glycogen accumulation in the absence of NH_4^+ or required amino acids or both. Cultures were grown in complete medium, collected, washed and then resuspended in complete medium (●), medium without amino acids (o), without NH_4^+ (▲), or without either amino acids or NH_4^+ (Δ).

A) CP78, $relA^+$
B) CP79, $relA^-$

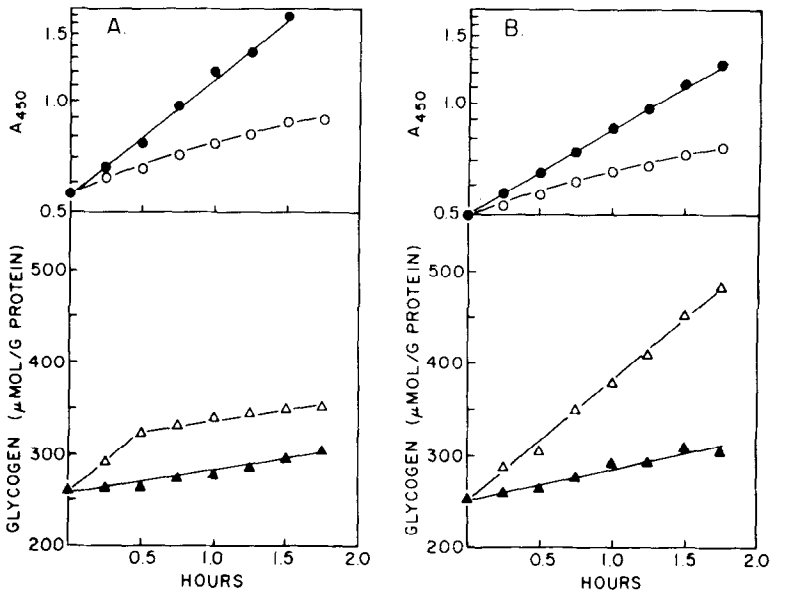

Fig. 3. Growth (●, o) and glycogen accumulation (▲, Δ) in control (closed symbols) and amino-acid-starved (open symbols) cultures of *E. coli* CP79, $relA^-$. Amino acid starvation was induced by adding L-valine, 500 μg/ml of culture, at 0 hours.

A) Growth with glucose as carbon source
B) Growth with glycerol as carbon source

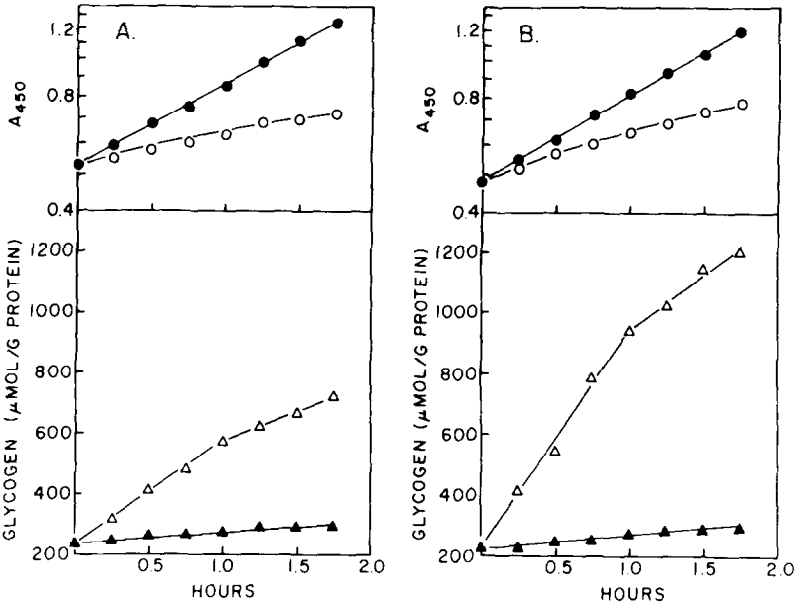

Fig. 4. Growth (●, ○) and glycogen accumulation (▲, △) in control (closed symbols) and amino-acid-starved (open symbols) cultures of *E. coli* CP78, relA$^+$. Amino acid starvation was induced by adding valine at 0 hours, as described in Fig. 3.

A) Growth with glucose as carbon source
B) Growth with glycerol as carbon source

observed in the control culture, so that only a small amount of glycogen had accumulated after about two hours (Fig. 3). However, when this organism was grown on glycerol, addition of valine produced a continuous increased rate of accumulation of glycogen causing a significant accumulation after about two hours (Fig. 3). Valine addition to cultures of the relA$^+$ strain resulted in a substantial accumulation of glycogen when either glucose or glycerol served as the source of carbon and energy (Fig. 4).

DISCUSSION. The accumulation of glycogen that follows both partial nitrogen starvation (NH_4^+ exhaustion) and total nitrogen starvation of the amino-acid-requiring auxotrophs used in this study is independent of the relA gene. Thus, the relA gene is not required for the glycogen accumulation that occurs when synthesis of all nitrogen-containing compounds slows drastically or stops.

In a relA$^+$ amino acid auxotroph using glucose as the source of carbon and energy, the cellular level of ppGpp, a product of the relA gene, increases

dramatically when NH_4^+ is exhausted from a minimal medium that contains the required amino acid supplements (10). However, during NH_4^+ starvation of the isogenic relA$^-$ strain, ppGpp increases only to the very low level that is present in the relA$^+$ strain prior to NH_4^+ depletion (10). In a relA$^+$ prototroph, the total nitrogen starvation that results from exhaustion of NH_4^+ from a minimal medium results in as much as a 20-fold increase in cellular ppGpp (11, 12). However, no increase in ppGpp was detected in an isogenic relA$^-$ strain (12). These observations, that there is little or no increase in ppGpp during NH_4^+ starvation in relaxed strains, coupled with our observation that a relaxed strain accumulates as much or more glycogen than the isogenic stringent strain does during NH_4^+ starvation, indicate that ppGpp does not play a role in the increase in glycogen accumulation that follows either total or partial starvation for a source of nitrogen.

Our data clearly indicate that in order for glycogen to accumulate during an amino acid starvation in the presence of glucose, the relA gene must be intact. When the relA gene is functional, amino acid starvation results in an increased level of two nucleotides with regulatory properties, ppGpp (13) and ppGp (14). (The nucleotide ppGp is apparently a product of ppGpp (14).) Presumably, an increase in one or both of these nucleotides is necessary for glycogen to accumulate during amino acid starvation in the presence of glucose.

However, we also show here that an *E. coli* culture growing on glycerol does not require the relA gene to accumulate a significant amount of glycogen during amino acid starvation induced by valine addition. During growth on glycerol, synthesis of cyclic AMP occurs at a high rate, but cyclic AMP synthesis is almost totally inhibited in a culture growing on glucose (15). This difference apparently contributes to the much higher cellular level of cyclic AMP seen in cells growing on glycerol than in cells growing on glucose (16). The inhibition of growth by valine would thus be expected to result in a greater accumulation of cyclic AMP in the cells growing on glycerol than in

the cells growing on glucose. We have previously presented evidence that an increase in the cellular level of cyclic AMP causes an increase in the rate of glycogen accumulation (17). Thus, the significant accumulation of glycogen that followed valine addition to the relA$^-$ strain growing on glycerol can apparently be accounted for by an increase in the cellular level of cyclic AMP.

The results presented here indicate that high cellular levels of cyclic AMP can compensate for the lack of an increase in relA gene products following amino acid starvation and allow an increase in glycogen accumulation to occur. From the data presented here it is not possible to determine if the relA gene product - cyclic AMP interaction is sequential (i.e., relA gene product → cyclic AMP → glycogen accumulation) or parallel (i.e., relA gene product → glycogen accumulation ← cyclic AMP). Based on a parallel mechanism, one could propose that cyclic AMP is the factor that causes the increase in glycogen accumulation following NH_4^+ or NH_4^+ plus amino acid starvation that we observed. However, the cellular level of cyclic AMP does not increase in NH_4^+-limited *E. coli* (18). Both sequential (19) and parallel (20) mechanisms have been proposed for the interaction of cyclic AMP and the relA gene product ppGpp in the regulation of other cellular processes. Work is in progress to determine whether the interaction involved in the regulation of glycogen synthesis is sequential or parallel.

Finally, it should be noted that we have presented *in vivo* and *in vitro* evidence that cellular levels of ppGpp greater than those that usually result from amino acid starvation can inhibit, rather than stimulate, glycogen synthesis (21, 22). Thus it is possible that, depending on the cellular level, ppGpp can either stimulate or inhibit glycogen accumulation. Studies are in progress to determine how these possible dual effects contribute to the regulation of glycogen synthesis *in vivo*.

ACKNOWLEDGEMENTS. We thank Dr. Michael Cashel, Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, for kindly providing the *E. coli* strains used in this study. This research was supported by Public Health Service Grant AM18670.

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