

Residual Glycogen Metabolism in *Escherichia coli* is Specific to the Limiting Macronutrient and Varies During Stationary Phase

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During prolonged periods of nutrient deprivation, many microorganisms accumulate energy storage molecules such as glycogen to cope with nutrient-limiting conditions. The stationary phase growth of microorganisms has recently been recognized as a period in which active (also termed “residual”) metabolism occurs. In order to determine whether residual metabolism varies between different forms of stationary phase induced by various macronutrient limitations, sequential enzymatic reactions were used to quantify cellular glycogen contents and characterize the profile of glycogen accumulation in *Escherichia coli* MG1655 cells grown in carbon- and nitrogen-limited conditions. Nitrogen-limited cultures exhibited the highest amount of glycogen accumulation, which steadily increased from exponential phase to 60 h after the onset of stationary phase, after which a decrease was observed. In carbon-limited cultures, exponential phase glycogen accumulation was comparable to the non-limited and nitrogen-limited cultures, while in stationary phase the accumulated glycogen persisted for a longer time than in the non-limited cultures, after which it decreased until it was negligible. The results of the study indicated that residual metabolism, examined through glycogen accumulation, was variable both between and within stationary phases induced by limitation of the macronutrients studied.

The stationary growth phase is now recognized as a period of ongoing metabolic activity, rather than a period of dormancy (1). Instead of an abrupt cessation of growth when insufficient amounts of nutrients are present, the bacterial physiology is altered in order to maintain cell viability and ensure prolonged survival during adverse conditions (2, 3). Depending on the specific macronutrient that limits growth, distinct physiological responses are observed (4). Nutrient-scarce conditions are common in natural environments, forcing bacteria to remain prevalently in stationary phase; it has been estimated that 60 % of Earth’s biomass consists of microorganisms in stationary phase (5). Due to its prevalence in nature, the study of stationary phase growth has gained attention in recent years, in hopes that the production of secondary metabolites and storage compounds during stationary phase growth may be exploited for biosynthetic, therapeutic, and antibiotic applications.

In *Escherichia coli*, the physiological changes in transition to stationary phase are brought about by a variety of regulatory mechanisms, such as the RpoS sigma factor and the stringent response, which actuate general stress responses to nutrient limitation (2). The response to the availability (or lack) of specific macronutrients is determined by separate systems, such as carbon, nitrogen and phosphorus sensing systems (4). Distinctly regulated transitions into stationary phase are possible, which may lead to distinct metabolic profiles once stationary phase has been reached.

One of the responses to nutrient limitation of many microorganisms is the accumulation of carbon and energy storage molecules, such as glycogen, in order to withstand nutrient limitation for prolonged periods of time. The accumulation of storage molecules enables continuation of

exponential growth once the limiting nutrient is replenished, allowing success in competition during cycles of growth and nutrient-limitation. Indeed, *E. coli* is capable of producing large amounts of glycogen (over 50 % of the dry cell weight) in response to nitrogen limitation, and the accumulated glycogen can increase the viability of the bacteria (6).

Due to the key role of glycogen in the survivability of cells during nutrient deprivation, its accumulation was chosen as an indicator of residual metabolism for the study. Although it has previously been found that nitrogen limitation induces glycogen accumulation in *E. coli*, the accumulation (and possible utilization) of glycogen along the stationary growth phase has yet to be characterized. Additionally, whether the accumulation and utilization vary within different stationary phases induced by limitation of different macronutrients also has yet to be addressed. To compare the differences in glycogen accumulation profiles between and within growth conditions, enzymatic quantification of glycogen using amyloglucosidase digestion and coupled hexokinase/glucose-6-phosphate dehydrogenase assay was performed on wild type *E. coli* MG1655 grown under nitrogen- and carbon-limited conditions, as compared to nutritionally adequate conditions, in order to characterize residual metabolism in the stationary growth phase.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *E. coli* MG1655 cells obtained from the MICB 421 Culture Collection, Department of Microbiology and Immunology, University of British Columbia, Vancouver were grown in 500 ml of modified M9 minimal salts media (0.5 g/L NaCl, 7.0 g/L Na₂HPO₄, 3.0 g/LKH₂PO₄, 0.2 g/L MgSO₄·7H₂O, lacking NH₄Cl) in 2 L Erlenmeyer flasks at 37 °C,

aerated by shaking at 200 rpm. The M9 salts base medium was supplemented with 0.4 % (v/v) glycerol and 10 mM NH₄Cl to obtain the non-limited medium, while the supplemented glycerol was reduced to 0.04 % for the carbon-limited medium, and NH₄Cl supplementation was reduced to 1 mM for the nitrogen-limited medium. Overnight cultures for initial inoculums were grown in non-limited M9 medium.

Sampling. Culture samples were placed on ice mid-exponential phase and along stationary growth phase based on turbidity, measured by an Evolution 60S spectrophotometer (Thermo Scientific). At each time point, 30 ml of culture was split into two conical centrifuge tubes on ice for glycogen quantification and cell dry weight determination. Samples were centrifuged at 2500 × g at 4 °C for 10 min, and resulting cell pellets were stored at -80 °C.

Determination of bacterial dry weight. The collected cell pellets were resuspended in 1 ml of water and vacuum filtered on pre-weighed 0.45 μm pore size nitrocellulose filters for 5 min. The filters were then dried in an oven at 95 °C for approximately 16 h until a constant weight was measured.

Enzymatic quantification of glycogen. The following protocol for enzymatic quantification of glycogen through digestion with amyloglucosidase followed by quantification of the resulting glucose was adapted from previously established methods (7). The collected cell pellets were washed by resuspension with 1 ml of 50 mM TAE buffer followed by centrifugation at 12 000 × g for 2 min and removal of the supernatant. The washed cell pellets were resuspended in 1.25 ml of 200 mM sodium acetate buffer (pH 4.5) and lysed through bead beating using a FastPrep-24 (MP Biomedicals) with 0.1 mm glass beads for two 45 s cycles at 6.0 m/s. The resulting lysates were then placed in an 80 °C water bath for 20 min for denaturation of endogenous enzymes. Twenty μl samples of the lysates were incubated at 50 °C with either 2 μl of 200 U/ml amyloglucosidase (Sigma-Aldrich; 10115) or 2 μl of water for 30 min, allowing differentiation between glycogen-derived glucose and glucose already present in the cells by subtracting the detected glucose content in the water-treated samples from the glucose content in the amyloglucosidase-treated samples. Using a modified protocol of the Glucose (HK) Assay Kit (Sigma-Aldrich; GAHK20), 100 μl of the HK reagent was added to the 22 μl samples and incubated at room temperature for 30 min. The absorbance of the solution was measured at 340 nm using an Evolution 60S spectrophotometer (Thermo Scientific). A standard curve was constructed for extrapolation of the absorbance data using serial dilutions of a glucose stock solution. The net amounts of glycogen-derived glucose calculated for each growth condition were divided by the respective cell dry weights to account for different population densities between samples.

Percentage of total available carbon sequestered as glycogen. The amount of carbon source (i.e. glycerol) added to each growth medium was converted to hexose (i.e. glucose) equivalents by dividing the total number of moles of added glycerol by two to determine the maximum possible moles of glucose (i.e. assuming all available carbon in the medium was dedicated towards glucose synthesis):

$$\text{Maximum possible moles of glucose in 500 ml culture} = \frac{\text{Moles of glycerol added to 500 ml of culture}}{2}$$

The amount of moles of glycogen-derived glucose was then divided by the maximum possible moles of glucose to obtain the percentage of total carbon detected as glycogen:

$$\% \text{ total carbon detected as glycogen} = \frac{\text{Moles of glycogen derived glucose detected in 20 } \mu\text{l sample}}{\text{Maximum moles of glucose in 500 ml culture}} \times \frac{500 \text{ ml culture}}{20 \mu\text{l sample}} \times 100\%$$

The percentages were then normalized against the respective cell dry weights for each growth condition to obtain the percentage of total available carbon detected as glycogen per unit of cell weight at the selected sampling time:

$$\% \text{ total carbon detected as glycogen per unit cell weight} = \frac{\% \text{ total carbon detected as glycogen}}{\text{Cell dry weight}}$$

RESULTS

Confirmation of growth limitation and differences in growth rates. A confirmation of culture growth limitation by the macronutrients first needed to be established. Thus, growth curves were determined in media with reduced concentrations of nutrients based on previous studies on nutrient limitation (6, 7). Carbon and nitrogen specifically were chosen for limitation because of their use in the previously cited studies.

Growth curves were observed to have different approaches to stationary phase and final culture densities under each growth condition, with the limited cultures reaching an OD₅₀₀ of ~0.5 and the non-limited culture reaching an OD₅₀₀ of ~1.4 (Fig. 1). The growth of the nitrogen- and carbon-limited cultures reached culture densities that were reduced threefold compared to the non-limited cultures, indicating that the cultures were indeed limited by the lower concentrations of the macronutrients provided. The nitrogen-limited cultures displayed a lower exponential growth rate in comparison to the cultures in the other growth conditions, and reached stationary phase about 30 h later than the carbon-limited cultures. In contrast, the carbon-limited cultures had more rapid exponential growth rates, comparable to those of the non-limited cultures. In addition, the cultures appeared to deplete the available nutrients, reaching turbidities above that of the stationary phase, after which a decrease in turbidity likely due to cell death occurred (Fig. 1).

Glycogen accumulation varied between and within stationary phases. In order to characterize the flux of glycogen accumulation over time and compare the relative amounts of glycogen between growth conditions, an enzymatic approach was chosen to quantify the glycogen levels. It was assumed that changes in glycogen accumulation between growth conditions supported the theory that characteristically different metabolism occurred in stationary phases induced by differential nutrient-deprivation.

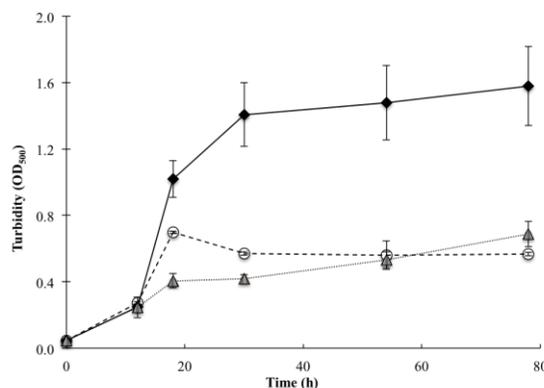


FIG 1 Effect of nutrient limitation on growth. Non-limited culture is indicated by (◆), Carbon-limited is indicated by (○) and nitrogen-limited is indicated by (▲). The error bars represent standard deviation for duplicate cultures.

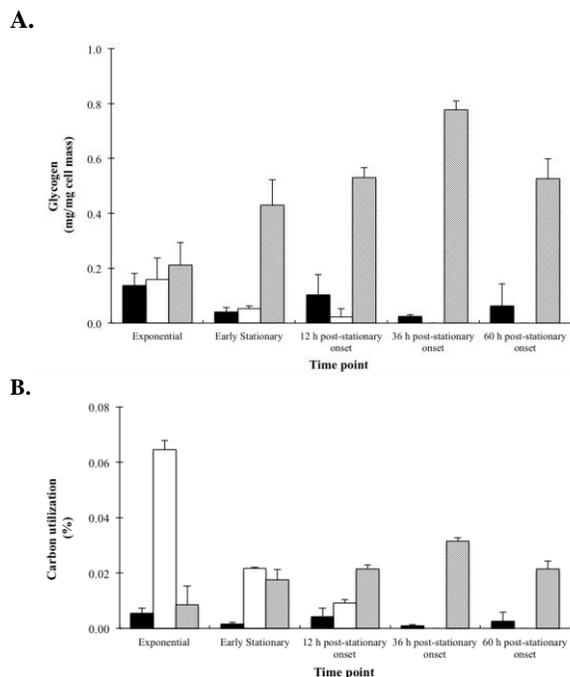


FIG 2 Effect of nutrient deprivation. (A) Glycogen accumulation. (B) Percentage of total available carbon stored as glycogen. The percentage of carbon utilization is denoting a percentage of total available carbon relative to mg of cell mass. The non-limited condition is indicated by (■), the carbon-limited condition is indicated by (□), and the nitrogen-limited condition is indicated by (▒). The error bars represent standard deviation for duplicate cultures.

The cultures in all three nutrient conditions displayed similar amounts of accumulated glycogen in the exponential phase at approximately 20 % of dry cell weight, indicating that the cultures in exponential growth maintained similar metabolism of glycogen (Fig. 2A). In the nitrogen-limited cultures, glycogen levels steadily increased following exponential phase, peaking at 36 h after the onset of stationary phase (80 % of the cell dry weight). Glycogen levels in nitrogen-limited cultures then decreased to 52 % of the dry cell weight at 60 h post-stationary phase, displaying a mobilization of the glycogen stores. In contrast, all glycogen in the carbon-limited cultures was completely mobilized by 36 h after the onset of stationary phase. Glycogen levels of the non-limited cultures varied in stationary phase from 2 to 20 % of the cell dry weight, perhaps indicating a cycle of accumulation and mobilization of glycogen.

During stationary phase, glycogen levels detected in nitrogen-limited cultures were manifold higher than the other growth conditions, with approximately 30 times greater accumulated glycogen at 36 h post-stationary phase onset, displaying massively upregulated synthesis of glycogen. It was observed though, that during filtration, the effluent fluid was slightly turbid, indicating that some cell mass had escaped the filter. The loss of cell mass during filtration would have resulted in a reported cell weight that was up to 25% lower than the actual cell

weight, and thus resulted in a higher reported value of glycogen content per unit of cell weight.

Proportion of glycogen sequestration indicated differential regulation. In order to elucidate the extent of glycogen accumulation within growth conditions, and to determine how glycogen levels fluctuated over time in response to a decreasing pool of total available carbon, comparisons of the proportions of total available carbon sequestered as glycogen in the different growth conditions were performed to provide further insight in the regulation of residual metabolism. The proportion of carbon detected as glycogen in the non-limited and nitrogen-limited cultures during exponential phase growth was approximately 90% lower than that of the carbon-limited cultures, showing that despite there being a tenfold lower concentration of the carbon source available in the medium, glycogen accumulation was unaffected (Fig. 2B).

During early stationary phase, the proportion of available carbon sequestered as glycogen was similar in both the carbon- and nitrogen-limited cultures (0.02 %), despite there being the same absolute amount of glycogen per mg of dry cell mass in the non-limited cultures and eightfold more glycogen in the nitrogen-limited cultures (0.40 mg/mg cells) as compared to the carbon-limited cultures (0.05 mg/mg cells). The carbon-limited cultures retained significant amounts of the available carbon as glycogen through the transition to and early stages of stationary phase, further demonstrating that glycogen synthesis was not suppressed, despite the lower amounts of available carbon in the medium. As there was no detectable presence of glycogen, the proportion of available carbon sequestered as glycogen was also regarded as negligible in the carbon-limited cultures as the cultures progressed into later stages of stationary phase.

DISCUSSION

The growth pattern observed in each condition demonstrated that selective deprivation of macronutrients changed the growth rate and the method that a bacterial population may use to transition from exponential to stationary phase. The reduced exponential growth rate of the nitrogen-limited culture and the resulting slower approach to stationary phase may have been due to the different ways in which the nutrient availability is sensed in *E. coli*. Specific carbon, nitrogen and phosphorus sensory systems have been described which actuate particular regulatory responses and the RpoS sigma factor regulon in unique ways (5). RpoS exerts control over hundreds of genes and many corresponding cell functions in the transition to stationary phase growth, thus shaping the characteristics of stationary phase growth. Clearly, depending on the limiting-nutrient, distinct large-scale regulatory changes in metabolic activity will occur, changing the manner by which a culture approaches stationary phase.

Glycogen accumulation remained at negligible levels in both the non-limited and carbon-limited conditions (Fig. 2A). In the non-limited growth condition, where no macronutrient was particularly limiting, glycogen

accumulation was not expected to occur on a substantial scale, as glycogen production for carbon storage is not required when carbon is present in excess. In carbon-limited cultures, carbon becomes critically limiting in the growth medium. Thus, cells need to continually utilize the available carbon to maintain basal metabolic processes, and lack the resources (i.e. excess carbon) to stockpile significant stores of glycogen. Nonetheless, exponential-phase glycogen levels in the carbon-limited cultures were comparable to those found in the non-limited cultures at the same time point, suggesting that there were no observable differences in the residual metabolisms between the two growth conditions prior to stationary phase onset. However, any stores of glycogen detected in the carbon-limited cultures rapidly diminished as stationary phase continued, until no more glycogen was detected after 12 h post-stationary phase onset, providing further evidence that residual metabolism was ongoing, as cells continued to mobilize glycogen as an energy source.

Assays of nitrogen-limited cultures revealed that glycogen accumulation peaked at approximately 80 % of the dry cell mass at 36 h post-stationary phase onset, an extremely large value. However, past studies have found that glycogen levels were significantly higher in nitrogen-deprived cells, and constituted up to 60 % of the cell dry weight (10). Thus, even accounting for the cells lost during vacuum filtration, the cells grown under nitrogen-deprivation accumulated significantly higher amounts of glycogen than the other growth conditions. The analysis of the proportions of total available carbon in the medium sequestered as glycogen revealed that although the transition to stationary phase is heavily regulated, the difference in regulation between the carbon- and non-limited culture growth conditions was not detectable until 12 h after the onset of stationary phase. However, the nitrogen-limited cultures already had a marked increase in glycogen accumulation by the onset of stationary phase, showing that differences exist in the immediacy of the regulation of residual metabolism among the cultures in the different growth conditions.

While the mobilization of glycogen observed in the nitrogen-limited cultures late in stationary phase can be partially attributed to the maintenance of basal metabolism during stationary phase, stationary growth phase cells can also convert glycogen to trehalose, both of which are viable energy sources for usage in basal metabolism (10). Carbon stores are known to be preferentially kept as trehalose in prolonged stationary phases due to its protective functions for osmotic stress and heat shock. Interestingly, when cells are brought out of stationary phase, trehalose is also metabolized before glycogen and its levels are quickly reduced to those comparable with exponential phase growth (11). Thus, the decrease in accumulated glycogen observed at 60 h after the onset of stationary phase may be at least partially due to conversion to trehalose, which could be measured by trehalase digestion and subsequent coupled hexokinase enzymatic reactions to determine the amount of trehalose-derived

glucose equivalents. The amount of additional glucose could be due to the conversion to trehalose.

Another implication to consider is the effect of quorum sensing on metabolic regulation, and subsequently, glycogen production and carbon utilization under different growth conditions. Bacteria have been known to utilize various signaling molecules to integrate cell population density and nutrient concentrations in order to construct a rudimentary snapshot of the ecological conditions within the growth medium (12). Quorum sensing molecules, such as homoserine lactone and autoinducer-2, are known to upregulate the expression of *rpoS* and *csrA*, respectively (13, 14). It is possible that the release of autoinducer-2 may have affected glycogen metabolism through the increase in expression of *csrA*, a negative regulator of glycogen biosynthesis (15), which may have accounted for the low levels of glycogen observed in carbon-limited conditions after 12 h post-stationary onset.

In conclusion, nitrogen-limited *E. coli* cultures accumulated higher levels of glycogen and sequestered the greatest proportion of total available carbon as glycogen during stationary phase, compared to non-limited and carbon-limited cultures, which displayed insubstantial levels of glycogen accumulation in stationary phase growth. Differences were implied in the timing of regulation of glycogen metabolism, and glycogen accumulation was observed to be variable along the course of stationary phase growth. The findings illustrated that there was an association between different macronutrient limitations and the profile of glycogen accumulation in stationary phases. Presuming that glycogen was a representative indicator, the results of the study showed that residual metabolism was variable between different growth conditions, and also along a temporal scale as growth progressed through stationary phase.

FUTURE DIRECTIONS

In order to further elucidate the effects of macronutrient deprivation on residual metabolism in bacterial populations, other macronutrients, such as phosphorus, an essential nutrient in biosynthesis of nucleotide and regulation of protein activity, could be subjected to similar analysis. Phosphorus limitation could induce changes in residual metabolic profile, such as the activation of glycogen synthesis genes (16). Furthermore, since the values obtained for accumulated glycogen in the study may have been inflated due to loss of cell mass during filtration, future experiments should focus on the reduction of the loss of cell mass for more accurate results. Expanding the sampling window (e.g. multiple days post-stationary phase onset) may also provide additional valuable insights into the metabolic differences between prolonged nutrient-deprived conditions over an extended time period. At these time scales, the 'growth advantage in stationary phase' phenomenon may take effect and consequently impact residual metabolism, whereby cells of older generations outcompete younger cells, producing a wave pattern of growth and death along a median in long-term stationary phase (17).

Since this study focused on one aspect of residual metabolism, namely the accumulation of glycogen, a more complete characterization of residual metabolism could be achieved by investigating the overall metabolism through transcriptomic analyses. Given the widespread transcriptional control of the transition into the stationary growth phase, an interesting investigation could identify aforementioned genes suspected to be responsible for the shaping of residual metabolism and follow their expression with reverse transcription quantitative PCR analysis. The results of transcriptomic analyses may provide a means to exploit the metabolic changes in stationary phase, such as the optimization of metabolite production or increasing efficiency of residual metabolism. Furthermore, metabolomic analyses of nutrient-limited cultures using gas chromatography-mass spectrometry may provide a more comprehensive understanding of the cell physiology and residual metabolism. The resulting metabolomes may prove crucial towards developing future applications in the pharmaceutical and biotechnological industries.

ACKNOWLEDGEMENTS

This project was supported by the Department of Microbiology and Immunology, University of British Columbia, Vancouver, BC. We would like to thank the department for their contribution of laboratory space, equipment, and materials. We would also like to thank Dr. William Ramey for his support and assistance throughout the duration of the project.

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