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Limiting phosphorus may result in impaired growth and decreased glucose content in *Escherichia coli* MG1655

Niloufar Benam, Sahitya Hajirawala, Jasper Hoi Chun Luong, Jiayi Yang

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

SUMMARY Inorganic phosphate is an important source of phosphorus for *Escherichia coli* metabolism. In addition to carbon and nitrogen, the presence of these molecules within the bacterium's environment contributes to its survival and growth. As such, extended periods of nutrient starvation results in metabolic and physiological changes to allow the organism to survive and adapt. Such a change is the activation of the stationary phase stress response primed by RpoS expression that results in glycogen accumulation. We hypothesized that the response occurs when phosphorus is limited to conserve metabolic energy in the form of glycogen as essential nutrients are limited. To determine the effects of phosphorus and nitrogen limitation on cellular growth and stationary phase glycogen accumulation of *E. coli*, MG1655 wild-type cells were subjected to nutrient limitation, and subsequent cellular glycogen quantification was attempted using a previously-established enzymatic glucose assay. Bacterial growth curve analysis showed that phosphorus-limiting conditions significantly impaired growth across all growth phases compared to non-limiting conditions and nitrogen-limiting conditions. Likewise, cellular glucose levels quantified after amyloglucosidase/hexokinase treatment may indicate a reduction of cellular glucose levels when phosphorus was limited compared to non-limiting conditions. Similarly, a possible reduction in cellular glucose levels relative to non-limiting conditions was observed in the nitrogen-limiting conditions but only when treated with amyloglucosidase. Altogether, the results of the investigation did not implicate any conclusive findings between phosphorus or nitrogen limitation and changes in cellular glycogen levels, but rather suggested that limiting phosphorus may lead to a reduction in bacterial growth and cellular glucose levels.

INTRODUCTION

Bacterial growth is facilitated by the presence of essential nutrients that are required for ongoing metabolic activity and synthesis of the molecules required for bacterial growth and survival (1). Macromolecules including carbon, nitrogen, and phosphorus are acquired from the environment and contribute to cellular metabolism, structural maintenance, as well as other cellular processes such as signal transduction and cell division (1–3). These macromolecules are also the building blocks for bacterial growth as they are essential components for amino acids, nucleic acids, and bacterial cell wall components such as peptidoglycans and phospholipids. The absence of these essential nutrients often limits the metabolic activity of bacteria and drives them to constantly scavenge the environment for these molecules (1). However, as nutrient scarce conditions are common occurrences in nature, bacteria often have responses that prolong their survival by altering their physiology and metabolic patterns during adverse conditions (4).

According to Peterson *et al.*, *Escherichia coli* utilizes a "two-stage starvation" response composed of scavenging and foraging. This response is used to determine whether the environmental resources are becoming limiting in regards to glucose, inorganic phosphate, and ammonia (1). The scavenging response is hypothesized to occur first, where the scavenging regulon involving catabolite activator protein (CAP) and cyclic AMP (cAMP) receptor protein is used for alternate carbon source utilization when glucose is not present. On the other hand, NtrB/NtrC and PhoR/PhoB control for nitrogen (glutamine) and phosphate scavenging respectively (1). The scavenging response is then followed by the foraging response where an increased expression of foraging proteins allows for the uptake of essential nutrients from the environment. If the foraging response is unable to obtain adequate amounts

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Address correspondence to: https://jemi.microbiology.ubc.ca/

of macronutrients, the organism would enter stationary phase and initiate a stress response.

In *E. coli*, phosphate sensing is controlled by the phosphate transport system (Pst) in conjunction with the PhoR-PhoB two-component system (1). During starving conditions like phosphorus limitation, a signalling cascade phosphorylates and activates PhoB, which is involved in transcription of stress-response genes (1). A study by Marzan *et al.* proposes that active PhoB is also involved in the transcription of small non-coding RNAs (sRNA) that upregulate production of RpoS (2). This phenomenon of increased RpoS expression and activity is bolstered by other previous studies (5). RpoS is a stress-related sigma factor that functions in regulating gene expression during stationary phase and is expressed when cells are exposed to stress conditions like nutrient deprivation (6). Some studies use glycogen accumulation as a common indicator for increased RpoS expression (5); however, the scientific reasoning behind this is yet to be investigated.

According to Hengge-Aronis *et al.*, one possibility is that RpoS may positively regulate glycogen synthesis through upregulation of glycogen-associated gene *glgS* that was initially uncharacterized (7). However, it was later discovered by Rahimpour *et al.* that the GlgS protein is a surface composition regulator which controls nucleotide and flagellum synthesis, such that the response only indirectly reflects glycogen accumulation. They further elaborate that this glycogen accumulation response occurs through a signalling cascade. Under nutrient-limiting conditions, the upregulation of RpoS inhibits expression of CsrA (glycogen biosynthesis repressor) which allows for the expression of the *glgBXCAP* operon (8). The proteins transcribed from this operon are directly involved in glycogen synthesis.

Glycogen molecules are polysaccharides that exist as long chains of linked glucose units, acting as a carbon and energy storage molecule in *E. coli*. It is synthesized and regulated by the *glgBXCAP* operon consisting of glycogen synthase (*glgA*), ADP glucose pyrophosphorylase (*glgC*), glycogen branching enzyme (*glgB*), glycogen debranching enzyme (*glgX*) *and* catabolic glycogen phosphorylase (*glgP*) (9, 10). As a physiological adaptation from *E. coli*, the response to accumulate glycogen ensures that sufficient energy is available during metabolic transitions or long-term nutrient starvation (11).

As a follow-up to Fung *et al.*'s study on glycogen accumulation under nitrogen and carbon limitation, we decided to explore the effects of limiting phosphorus on glycogen accumulation in *E. coli*. As there have not been conclusive findings that confirm the link between phosphorus starvation, glycogen accumulation, PhoR/PhoB expression, RpoS expression, and *glgBXCAP* transcription, the investigation on the effects of limiting phosphorus during the growth of *E. coli* would provide a better understanding of the stress-induced survival mechanisms that the bacterium undergoes to enhance its survival. By understanding the underlying mechanisms behind nutrient starvation phenotypes like glycogen accumulation, researchers can exploit the production of such storage molecules and metabolites for biosynthetic and therapeutic use.

As there is an incomplete understanding between glycogen accumulation response and nutrient limitation during stationary phase, we wanted to investigate the role of phosphorus limitation on glycogen accumulation and growth. We hypothesized that stationary-phase *E. coli* cells grown in phosphorus-limiting media will accumulate more glycogen than cells grown in non-limiting media. This is because phosphorus starvation induces the expression of RpoS, which inhibits CsrA, allowing for the expression of the *glgBXCAP* operon and ultimately leading to glycogen synthesis mediated by *glg*-related proteins. However, due to experimental limitations we were unable to confirm or reject our hypothesis as we did not achieve any conclusive findings regarding glycogen accumulation specifically. Instead, we discovered that limiting phosphorus may lead to both the suppression of *E. coli* growth and relative fold decrease in NADH and glucose abundance.

METHODS AND MATERIALS

Bacterial strain, media and growth conditions. Wild-type *E. coli* strain MG1655 was obtained from the MICB 421 Laboratory Stock Collection, Department of Microbiology and Immunology, University of British Columbia, Vancouver, BC. The M9 minimal salts medium used for non-limiting conditions contained 0.5g NaCl, 7.0g $Na\text{-}_2HPO_4$, 3.0g KH_2PO_4, and 3mM $(NH_4)_2SO_4$ per liter, with both nitrogen (N)-limiting containing and phosphorus (P)-limiting media containing 10% of original nitrogen content (3mM $(NH_4)_2SO_4$ or 0.7g

Na-$_2HPO_4$ & 0.3g KH_2PO_4 respectively). All M9 salt base mediums were adjusted to pH 7.4, supplemented with 0.4% (w/v) glucose, 10 mM $MgSO_4$ and 10mM $CaCl_2$. Overnight cultures used for the inoculation of the various modified M9 media were cultured at 37$_o$C in Lysogeny Broth (LB) medium using a shaking incubator (200 rpm) for 16 hours prior to inoculation.

Growth curve analysis. To determine an approximate time point of when cells reach stationary phase in the various conditions, kinetic measurements of OD_{600} were performed in the EPOCH 2 Microplate Reader (Biotek) in triplicates. Raw OD_{600} values were adjusted using various associated minimal mediums without inoculums. Inoculations were performed such that final dilution of inoculum in each minimum media begins its kinetic OD_{600} measurements below 0.2.

Sampling and normalization of cell density. Stationary phase MG1655 culture in various mediums cultured in 500 mL Erlenmeyer flasks were extracted after 6 hours of incubation at 37$_o$C using a shaking incubator (200 rpm) as per the performed growth curve. Performed in duplicates, 20mL of collected cells were diluted to an OD_{600} of 0.5 to normalize cell amount across the different samples, and were subsequently centrifuged within conical Oakridge tubes to perform pelleting, centrifuged at 8,500 rpm at 4$_o$C for 10 minutes, and stored at -80$_o$C prior to extraction.

Cellular glucose extraction for subsequent enzymatic quantification. Collected cell pellets were resuspended in 1mL 50X TAE buffer followed by centrifugation at 12,000 x g for 2 minutes and decantation. Cell lysis by bead beating using the FastPrep-24 (MP Biomedicals) with 0.1mm glass beads (2 x 45s cycles at 6m/s) was performed after resuspension of cells in 1.25mL 0.2M Potassium Acetate buffer (0.14M CH_3COOK, 0.06M CH_3COOH; pH 6.0). Lysates were placed in 80$_o$C water blocks for 20 minutes to denature endogenous enzymes and proteins.

Quantification of glycogen after amyloglucosidase treatment. Cellular glycogen levels were enzymatically quantified as recommended from the previous established method to convert glycogen into glucose to perform glucose quantification (4). 20µL of cell lysates samples were incubated on a 50$_o$C heat block for 30 minutes with either 2µL of 200 U/mL amyloglucosidase (Sigma Aldrich; ROAMYGL) or 2µL dH_2O, such that differentiation from glycogen derived glucose and pre-existing glucose could be differentiated during the assay. With a modified Glucose (HK) Assay Kit (Sigma-Aldrich; GAHK20), the 22µL samples were incubated with 100µL of the HK reagent for 30 minutes at room temperature, endpoint absorbance was measured at 340nm using an EPOCH microplate reader (Biotek) for enzyme converted glucose quantification.

RESULTS

Limiting phosphorus impaired *E. coli* growth over time. As phosphorus is an essential nutrient for bacteria (1), we wanted to determine how long it takes cells to reach stationary phase and whether limiting phosphorus has an effect on bacterial growth. Fig 1 shows the growth curve generated under three conditions, where the plotted OD_{600} is normalized with the corresponding blank media at each time point. Growth in P-limiting media appeared to be lower than the growth in non-limiting media. In addition, under P-limiting conditions, as shown in Fig 1, the bacteria reached stationary phase and death phase after 3 and 14 hours of incubation, respectively. In contrast, bacteria grown in non-limiting media reached stationary phase and death phase at later times (6 and 17 hours, respectively). Although our growth curve does not follow the trend of a traditional sigmoidal growth curve as observed in Fung *et al.* (4), our curve does show a clear death phase, which is likely preceded by a stationary phase. From our growth curve, we decided that 6-8 hours is a sufficient time frame for *E. coli* to reach a stationary phase under all M9 minimal media conditions. According to the growth curve (Fig 1), N-limiting media did not seem to suppress bacterial growth, as the trend closely follows that of non-limiting media. The growth curves for both N-limiting and non-limiting conditions appeared to reach a possible stationary phase and death phase around the same time point. Under all three conditions, the growth curves illustrated (Fig 1) contain

irregularities. There appears to be a notable increasing trend followed by a decreasing trend once the assumed stationary phases are reached. For both non-limiting and N-limiting conditions, an unusual increase is observed at around 8 hours, whereas this similar increase is observed at around 12 hours in the P-limiting condition. Despite the growth irregularities, the growth curve clearly shows that phosphorus limitation significantly hinders *E. coli* growth, as suggested by Fung *et al.* (4).

Limiting phosphorus may reduce intracellular NADH and glucose levels. To determine whether the effects of phosphorus limitation on glycogen accumulation are similar to those of carbon and nitrogen starvation as shown in the previous study (4), we measured the relative abundance of NADH using a glucose assay kit. Fig 2 shows a significant fold change decrease of NADH in phosphorus-limiting condition relative to the non-limiting condition, which also indicates a significant fold decrease of intracellular glucose levels. This finding is consistent for phosphorus-limiting conditions both with and without amyloglucosidase treatment, where the glucose content decreased to an amount less than half of the levels corresponding to non-limiting control. It was also found that there is a significant fold change decrease of NADH (and thus relative glucose) in the N-limiting condition relative to the non-limiting control but only in the presence of amyloglucosidase. The N-limiting treatment absent of amyloglucosidase was difficult to compare to the non-limiting control due to the large variation within the technical replicates. Furthermore, under both N- and P-limiting conditions, NADH levels appeared to be lower with amyloglucosidase treatment than without (when compared to the non-limiting conditions). This implies that a lower level of glucose was present after treatment with amyloglucosidase when compared to without. Our data altogether demonstrates that limiting phosphorus could result in the decrease of NADH and cellular glucose content relative to a non-limiting control.

DISCUSSION

In this study, we investigated whether limiting phosphorus has an effect on glycogen accumulation as a stress response in stationary-phase *E. coli* as a follow-up to a previous study exploring similar trends in nitrogen and carbon limitation (4). First, we determined the incubation time for *E. coli* MG1655 to reach stationary phase under non-limiting, N-limiting, and P-limiting conditions using growth curve assays. After establishing that 6-8 hours allowed cells to reach a stationary phase, glycogen content was sought to be determined by a glucose assay. In short, our results show that phosphorus limitation may lead to both the impairment of *E. coli* growth and fold decrease of NADH and glucose relative to non-limiting conditions. These findings partly align with our biological model, as phosphorus is a required nutrient for many cell components and growth, such that limiting it would inhibit growth. However, our hypothesis cannot be fully accepted nor rejected as we were unable to confidently find a

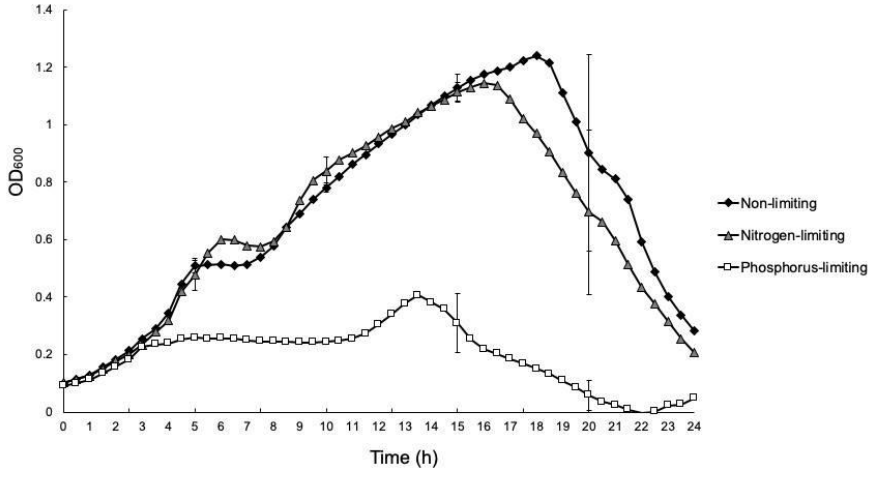

FIG. 1 Phosphorus-limiting conditions inhibit growth relative to non-limiting and nitrogen-limiting conditions. Cells were grown in M9 minimal media under non-limiting, nitrogen (N)-limiting, and phosphorus (P)-limiting conditions. The optical density (OD) was measured at 600 nm every 30 minutes for a 24-hour period. The error bars represent ± range of standard deviation for 3 replicates (n = 3) every 5 hours. OD_{600} values are relative to blanking with sterile M9 minimal

relationship between phosphorus limitation and glycogen.

The growth curve (Fig 1) revealed that bacteria grown under P-limiting conditions reached an earlier death phase when compared to bacteria grown under non-limiting conditions and N-limiting conditions. A possible explanation for this observation is that phosphorus is an essential element for prolonged *E. coli* survival (12). Juhna *et al.*'s study showed that with a higher supply of phosphate, *E. coli* cells died at a slower rate and reached death phase 2 days later than the cells which were only supplied with minimal phosphate. Thus, limiting phosphorus could contribute to an earlier death phase as seen in Fig 1. This might implicate that phosphorus limitation under our growth conditions impacted MG1655's general survival to a greater extent compared to the other two conditions, illustrating that it requires inorganic phosphates at larger amounts than what was provided to maintain its physiological and metabolic functions under stationary phase in a possible stress response.

Under N-limiting conditions, the bacterial growth followed similar trends to that of non-limiting condition as shown in Fig 1, suggesting that nitrogen limitation possibly had no impact on growth. This trend contrasts the growth pattern presented by Fung *et al.* as they showed that limiting nitrogen also suppresses growth (4) similar to our findings under phosphorus-limiting conditions. From our findings, phosphorus limitation impairs bacterial growth whereas nitrogen does not. These findings are inconclusive about the biological significance of nitrogen in bacterial growth as our growth trends are irregular and do not correspond to those presented by Fung *et al*. This discrepancy might be due to the nitrogen source we used was in the form of ammonium sulfate ($(NH_4)_2SO_4$), whereas they used ammonium chloride (NH_4Cl).

As shown in Fig 1, the stationary phase was not easily resolved as abnormal increasing and decreasing trends were observed in the growth curve. When looking at the wells inoculated with bacteria, we noticed small clumps in the media which we theorized to be bacteria aggregates as the clots were absent in the wells with blank media. Research conducted by Langmuir *et al.* has indicated that there are 50% more stationary-phase *E. coli* cells aggregating in the LB media (0% glucose) compared to the LB media supplied with 0.5% (w/v) glucose (13). In limiting conditions, cells tend to use up their energy source (14). This could imply that although the M9 minimal media contained 0.4% (w/v) glucose initially, the actual glucose concentrations might have dropped below 0.4%, possibly causing the cells to aggregate and form noticeable clumps. Therefore, most *E. coli* cells in limiting conditions may aggregate during the stationary phase due to low glucose levels. Cells may gradually lose this ability to aggregate and disperse once the death phase is reached. Therefore, cell aggregation may have affected absorbance (OD_{600}) readings, contributing to the abnormal increasing and decreasing trends displayed in our growth curve. However, if this aggregation effect is the result of limiting other nutrients (i.e. phosphorus or nitrogen), then this effect is unavoidable within the limitations of our project.

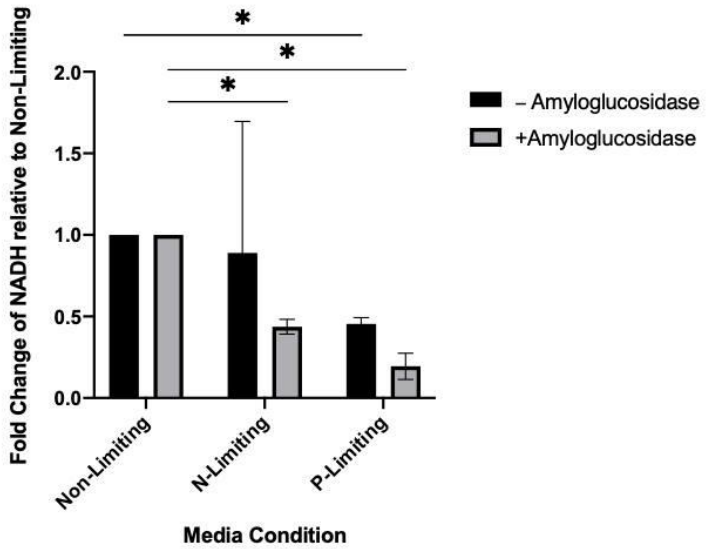

FIG. 2 Fold change decrease of NADH in phosphorus-limiting condition relative to non-limiting condition. MG1655 *E. coli* cells were treated with or without amyloglucosidase under non-limiting, nitrogen (N)-limiting, and phosphorus (P)-limiting conditions in M9 minimal media. Fold changes of NADH were relative to the non-limiting control and determined by measuring absorbance at 340nm using a glucose HK assay kit. As per manufacturing guidelines, levels of NADH correspond to equivalent glucose levels. Each treatment was performed in triplicate (n=3) and statistical significance was determined through a paired t-test where $\alpha = 0.05$. Asterisks (*) represent p-values < 0.05.

After acquiring the growth curve and establishing an incubation time that allows cells to possibly reach stationary phase, we then proceeded to analyze relative glycogen levels using amyloglucosidase and Glucose HK Assay Kit. Amyloglucosidase is an enzyme that breaks down glycogen into glucose subunits. Hexokinase (HK) uses ATP to phosphorylate glucose, producing glucose-6-phosphate (G6P). G6P dehydrogenase then oxidizes G6P and thereby reduces NAD+ to NADH (15). The resulting NADH emits fluorescence at 340 nm and the corresponding absorbance was measured (15). As only phosphorylated glucose will ultimately produce NADH, the measured NADH level is an indirect representation of the actual glucose content. Although our data remains inconclusive regarding *E. coli*'s glycogen accumulation as a stress response mediated by phosphorus deprivation, we did find that limiting phosphorus may result in a significant fold decrease of NADH (and therefore glucose) relative to non-limiting conditions as shown in Fig 2. Since NADH is an indirect indicator for relative glucose content, the core finding is that of glucose. A study by Gerard *et al.* provides corresponding evidence that when wildtype *E. coli* strains ENZ361 and MG1655 are starved for inorganic phosphate (P_i), glucose concentrations gradually decrease over time and are associated with an increase in acetate concentrations (16). For cells to maintain active aerobic metabolism, they continuously degrade glucose into acetate, allowing the cells to harness energy and survive under phosphate deprivation (16). These results correlate with our data (Fig 2) where a fold decrease in relative glucose (NADH) content is observed under phosphorus-limiting conditions. Gerard *et al.*'s study also indicates that this phenotype may be a result of the protective role of RpoS during stress response, which evidently increases in activity during P_i starvation (16). These findings partly align with our hypothesis, where RpoS expression plays a critical role during phosphorus limitation. In this case, not only might it impact glycogen accumulation, but it may also reflect glucose concentrations in phosphorus-deprived stationary cells. However, our data does not fully confirm nor deny our hypothesis as we did not determine the impact of phosphorus limitation on glycogen specifically.

As for nitrogen limitation, we did not observe any conclusive trends or findings that illustrate how limiting nitrogen may have affected cellular glucose content. Data shown in Fig 2 indicates that after amyloglucosidase treatment, there is a significant fold decrease in NADH and glucose in N-limiting conditions relative to the non-limiting control. In contrast, in the equivalent treatment without amyloglucosidase, there is no significant difference likely due to the large variance observed in this treatment group. Our results may suggest that limiting phosphorus has a more robust effect than limiting nitrogen; however, this is not conclusive as the media conditions were adjusted differently to limit each nutrient. Therefore, any differences between the importance of nitrogen and phosphorus as nutrient sources for bacterial growth and survival cannot be inferred through our results.

Furthermore, the results obtained for the fold changes in NADH levels under phosphorus limiting and nitrogen limiting conditions (Fig 2) are rather unusual. As previously mentioned, the NADH levels appear to be lower with amyloglucosidase treatment when compared to without. However, this trend is unexpected as upon treatment with functional amyloglucosidase, conversion of glycogen into glucose occurs, and more glucose should be available for reducing NAD+ into NADH. Hence, amyloglucosidase treatment should likely result in a higher level of NADH being produced. However, we observed an opposite trend. Some possible reasons behind these results could be the inactivity of the enzyme or technical issues within the glucose assay. As an example, the heating step within the glucose assay used for inactivation of cellular proteases could have possibly led to the inactivity of the amyloglucosidase enzyme. We could not further examine these possibilities due to not being able to repeat and optimize the glucose assay. Our inability to test the activity of the amyloglucosidase enzyme and generate a glycogen standard curve were additional limiting factors for further understanding the unexpected trends observed and correlating changes in cellular glucose to changes in cellular glycogen. Not having definite results regarding changes in glycogen limits our ability to address our research question.

Conclusions Based on the experimental results obtained, it is not possible to reach a definite conclusion regarding the effects of phosphorus limitation on the accumulation of glycogen within *E. coli* MG1655 cells. Based on our results, a significant decrease in the cellular glucose levels and suppression of growth was observed under phosphorus-limiting conditions.

However, such results cannot be correlated to changes in glycogen accumulation. This is due to the unavailability of a glycogen standard curve for translating the changes observed in cellular glucose levels to changes in glycogen storage levels. The experiment is therefore inconclusive regarding changes in glycogen levels and will not be able to clearly provide an answer to the original experimental question. Hence, the stated hypothesis regarding an increase in cellular glycogen levels under phosphorus-limiting conditions cannot be confirmed nor refuted. However, we did observe that phosphorus-limitation had a significant effect on impeding growth compared to nitrogen-limitation and reduced intracellular glucose levels by about 50% compared to non-limiting conditions.

Future Directions The initial goal of our project was to determine whether glycogen accumulates in *E. coli* cells when they were grown in phosphorus-limiting M9 media. We discovered that limiting phosphorus suppressed bacterial growth, and potentially decreased cellular glucose relative to the non-limiting control. To elaborate on our study, a standardized glycogen curve is needed to associate the absorbance values at 340 nm to the amount of glycogen accumulated in the nutrient-limiting media. We would likely achieve a linear or curve-like trend as increasing the amount of glycogen will increase the associated absorbance. This would allow for the correlation of glucose levels as measured using the HK glucose assay kit to actual glycogen levels after glucose to glycogen conversion using amyloglucosidase.

The standardized glycogen curve can also confirm the functionality of amyloglucosidase enzyme because if the enzyme is properly functional, adding the same volume of enzyme into different concentrations of glycogen should result in different levels of glucose conversion. Furthermore, to determine the optimal level of enzyme, we would also test varying amounts of amyloglucosidase with the same glycogen amount; this procedure ensures maximal glycogen to glucose conversion. In addition, to maintain the enzyme activity of amyloglucosidase, glucose assay can be optimized by using protein inhibitors instead of heat inactivation, since enzyme activity is affected by temperature (17). If the enzyme activity is maintained with the presence of protein inhibitors, we might expect to see an increase in glucose concentration compared to the results from the procedure that uses heat inactivation. Doing this would ensure that the enzyme is functional throughout the assay.

For future analysis, an RNA extraction and qPCR of the *rpoS* gene which is involved in stress response (6), *phoR* and *phoB* which function in phosphate sensing (1), and *glg*-related genes in the *glgBXCAP* operon involved in glycogen synthesis (8) under each limiting media condition, would be useful to determine the underlying biological mechanism of glycogen metabolism during nutrient starvation. Theoretically, if these genes are involved in stress response and glycogen accumulation, they would be upregulated. This would indicate whether or not these genes may be involved in glycogen accumulation during phosphorus-limitation. Altogether, performing these future experiments would help bolster our biological understanding regarding the underlying mechanisms of glycogen production under phosphorus limitation and validate our hypothesis.

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