CARBON SOURCE-MEDIATED SHIFTS IN BACTERIAL COMPOSITION OF MIXED-SPECIES BIOINOCULANT AND METABOLISM OF BACILLUS MEGATERIUM QM B1551

A Thesis
Presented to the Faulty of the Graduate School
Of Cornell University
In Partial Fulfillment of the Requirements for the Degree of
Master of Science

by
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August 2017

ABSTRACT

I investigated the effects of carbon sources on both the bacterial composition of an agricultural bioinoculant product and the metabolism of a plant growth-promoting rhizobacterium, *Bacillus megaterium*. The first part of the study focused on evaluating an indigenous microorganism (IMO) bioinoculant that is mixed with water and a carbon source, such as maize bran or sugar, and incubated before applying to crops as an alternative to chemical fertilizers. Using Illumina 16S rRNA gene sequencing, I evaluated shifts in bacterial communities that resulted from incubating IMO with different carbon sources (maltose, glucose, sucrose, glucose with fructose, and maize bran) for varying lengths of time (48 and 72 hours) in limited aeration conditions. The following results were found: i) unaltered IMO paste consisted of primarily fermenting organisms, ii) incubating IMO with sterile growth media shifted the population towards dominance by *Bacillus and Paenibacillus* genera*,* iii) increased incubation time increased relative abundance of *Clostridium* (an anaerobic organism) and iv) non-sterile substrates encouraged growth of bacteria present on the substrate itself. These results indicate that variations in how the product is prepared can have a large impact on the final product that is ultimately applied to crops. For the second part of the study, I applied a ^{13}C-assisted metabolomics profiling technique to *Bacillus megaterium* QM B1551, an organism similar to one identified in the IMO, to investigate the co-metabolism of glucose and three common disaccharides: sucrose, maltose, or cellobiose. Growth experiments indicated that each disaccharide can serve as a sole growth substrate, in accordance with genetic analysis that predicted diverse metabolic capabilities. However, intracellular metabolite labeling following growth on labeled and unlabeled mixed substrates revealed a hierarchy in disaccharide catabolism in the presence of glucose: (i) complete inhibition of cellobiose catabolism, (ii) minimal catabolism of maltose, and (iii) unbiased catabolism of sucrose. Our findings provide insights on selective carbon turnover by soil *Bacillus* species. Overall, this work contributes to

the understanding of both mixed-species and single-species bioinoculants which are increasingly being looked towards as chemical fertilizer replacements.

BIOGRAPHICAL SKETCH

Tracy Youngster earned her Bachelor of Arts degree in Biological Sciences from Rutgers University in 2011. From 2011-2014, she worked at the Marine Biological Laboratory in Woods Hole, MA on a sustainable aquaculture project. In 2014, she joined the Soil and Crop Sciences graduate program under the supervision of Dr. Ludmilla Aristilde. She was also mentored by Dr. James Shapleigh, who served as a minor committee member for her MS thesis.

ACKNOWLEDGEMENTS

I sincerely thank Dr. Ludmilla Aristilde for her continued scientific guidance and support throughout my MS project. I also acknowledge the mentorship from Dr. James Shapleigh, who greatly helped with my understanding of bacterial physiology. I thank current and past members of the Aristilde lab for their camaraderie. I especially thank Julie Wushensky for her significant role in the *Bacillus megaterium* research and for her good spirit which helped enormously during long days and nights in the lab. I am grateful that Jenny Kao-Kniffin allowed me to use her lab space to conduct my sequencing prep work. In the Kao-Kniffin lab, I am very thankful for Terry Bell who took the time to teach me the sequencing protocol and who was readily available to help me process the enormous amounts of data generated. I also thank Maria Gannett who helped me troubleshoot computer problems when Terry was not around! The Cornell Statistical Consulting Unit, specifically Erika Mudrak, was instrumental and helped me understand how to best display and understand my data. Additional thanks to the Genomics Genomics Facility at Cornell University for their assistance with the 16S Illumina sequencing. I also thank Randy Ruth and Maka for making sure I had a life outside of work, and my parents and sister for sharing their own graduate school experiences which helped give me perspective. Finally, this work would have not been possible without the financial support from Schooner Capital and Vin Ryan, and I deeply thank them for their investment in this project.

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LIST OF ABBREVIATIONS

IMO Indigenous microorganism

PGPR Plant growth promoting rhizobacteria

QA/QC Quality assurance/quality control

PCR Polymerase chain reaction

OD Optical density

OTU Operational taxonomic unit

PTS Phosphotransferase system

ABC ATP-binding cassette

LC-MS Liquid chromatography-mass spectrometry

PP Pentose phosphate

AA Amino acid

LB Lysogeny broth

G6P Glucose-6-phosphate

F6P Fructose-6-phosphate

F1P Frutose-1-phosphate

FBP Fructose-1,6-bisphosphate

C6P Cellobiose-6-phosphate

M6P Maltose-6-phosphate

S6P Sucrose-6-phosphate

DHAP Dihydroxyacetone phosphate

GAP Glyceraldehyde-3-phosphate

DHAP Dihydroxyacetone phosphate

PEP Phosphoenolpyruvate

R5P Ribose-5-phosphate

Xu5P Xylulose-5-phosphate

3-PG 3-phosphoglycerate

PEP Phosphoenolpyruvate

A-CoA Acetyl-CoA

α-KG α-Ketoglutarate

Tyr Tyrosine

3P-Ser 3-phosphoserine

Val Valine

Asp Aspartate

Glu Glutamate

IMP Inosine monophosphate

UMP Uridine monophosphate

CHAPTER 1: BACTERIAL COMMUNITY SHIFTS IN AN INDIGENOUS BIOINOCULANT IN RESPONSE TO NUTRIENT CONDITIONS

1.1 Introduction

Bioinoculants, or biofertilizers, are products containing living microorganisms that enhance plant growth when applied to seed, plant surfaces, or soil (Vessey, 2003). These products are increasingly being considered as an alternative to chemical-based agricultural amendments due to high cost and potential negative environmental impacts of chemical fertilizers (Adesemoye et al., 2009; Vessey, 2003). Biofertilizer commercialization can be traced back to 1896 in the United States and United Kingdom with the inoculation of legumes with rhizobia (Smith, 1992). In addition to rhizobium inoculants, production of biofertilizers containing other plant growth-promoting rhizobacteria has increased over recent years (Vessey, 2003). Plant growth-promoting rhizobacteria (PGPR) is a group of bacteria that can colonize plant root systems and promote plant growth directly or indirectly via various mechanisms including synthesis of plant-growth promoting compounds (e.g. phytohormones), biological nitrogen fixation, or limiting harmful effects of phytopathogenic organisms (Gray et al., 2004). Amongst the important genera of PGPR, which have been identified and extensively studied, are *Bacillus*, *Pseudomonas*, and *Rhizobium*. These bacteria are often used in bioinoculant formulations for their demonstrated abilities to stimulate plant growth (Hermann and Lesueur, 2013; Compant et al., 2010). However, there is often inadequate governmental regulation of bioinoculant products and therefore little incentive for manufacturers to implement rigorous quality control procedures (Lupwayi et al., 2000; Schenck zu Schweinsberg-Mickan, 2009). Bioinoculant evaluation must be performed to determine if the products contain the promoted PGPR and to identify culturing conditions that will maintain these populations.

Implementation of quality assurance and quality control (QA/QC) practices is necessary for limiting bioinoculant variability (Hermann and Lesueur, 2013). Important quality assurance

parameters that are often overlooked include growth medium, aeration, and sterility of the carrier. For quality control, it is important to maintain the population of PGPR as well as preventing growth of opportunistic pathogens (Hermann and Lesueur, 2013). Failing to address these important factors has led to the presence of many ineffective and highly criticized biofertilizers (Herrmann and Lesueur, 2013). Additional QA/QC challenges exist for bioinoculants that contain a diverse assemblage of bacteria due to strain- and species-specific growth rates and requirements (Stephens and Rask, 2000). One category of such mixed-species bioinoculants, termed “indigenous microorganisms” (IMO), is obtained by a low-cost method of cultivating soil microorganisms in a region local to where the product will be used (Park and DuPonte, 2008). The general practice involves burying a carbohydrate-rich substrate, such as rice or potato, in vegetated soil for several days to allow rhizospheric bacteria to colonize the substrate. This substrate, which is populated by the indigenous rhizospheric bacteria, is then removed from the soil, mixed with sugar or molasses, and allowed to ferment for about one week. The fermentation process is aimed at both increasing the populations of beneficial bacteria and reducing the populations of pathogenic bacteria. This fermented product is then diluted with water or directly mixed with soil to be applied as an agricultural amendment (Park and DuPonte, 2008). Purported benefits of IMO-associated products include increased soil fertility, increased crop biomass, and reduction of piggery odors. Variations of IMO products produced on small-scale farms worldwide make use of carbon sources and soils available in their communities (Kumar and Gopal, 2015; Park and DuPonte, 2008). This low-resource and local approach to bioinoculant formulation is compelling, yet there is little scientific evidence that it successfully cultivates PGPR and that the intended organisms are present in the final product.

This study aimed at evaluating the effects of various nutrient and growth conditions on the starting bacterial population in a Ugandan IMO product. The product is a sweet potato-based bioinoculant paste cultured with bacteria from bamboo roots; this product is incubated with water and a carbon source, such as maize bran or sugar, prior to application. The

objectives of this study were to: (1) determine the starting bacterial population of the IMO product; (2) characterize the bacterial populations in response to incubation time and growth substrates; and (3) determine the impact of non-sterile growth substrates on the final bacterial population. To test these objectives, IMO paste was incubated in growth medium containing various nutrient concentrations and carbon source types for 48 and 72 hours. After incubations were complete, the bacterial communities were analyzed using Illumina 16S rRNA gene sequencing. Results indicated that the IMO paste itself primarily contained bacteria that are typically found in fermented food products. After IMO was incubated with sterile growth media, the resulting bacterial communities were dominated by *Bacillus* and *Paenibacillus* species and the relative abundance of *Clostridium* species increased in many samples as incubation time increased. The non-sterile maize bran promoted growth of several Gram-negative genera of bacteria that were not dominant in any of the sterile incubations or the initial IMO paste. These findings show that variations in preparation of an IMO bioinoculant product can drastically alter the resulting bacterial community ultimately applied to crops. This variability, which can lead to an ineffectual product that does not promote plant growth as intended, has important implications for the successful application of IMO bioinoculants.

1.2 Materials and Methods

1.2.1 IMO bioinoculant

The IMO bioinoculant, which was produced in Uganda, was made from sweet potatoes that were buried in a bamboo forest soil for 3-5 days, removed from the soil, mixed with molasses, and allowed to ferment for one week. The resulting fermented paste was packaged in plastic containers. A container of IMO was obtained in January 2016 and stored in 4°C after opening. Instructions for preparing IMO are to mix two tablespoons of IMO paste with 55 gallons of water and two kilograms of a carbon source, such as maize bran or sugar. This mixture is then

covered and allowed to incubate for 48 hours before applying to crops. Variations in the culturing conditions (described below) are derived from these instructions.

1.2.2 Culturing conditions

A sterile spatula was used to mix 10 mg of IMO paste with 25 mL growth medium in 50 mL sterile conical centrifuge tubes. Except for when noted otherwise, all growth media were adjusted to pH 7.0 and were filter-sterilized (0.22 µm nylon; Waters Corporation, Massachusetts). Major salts were added in the following concentrations: 89 mM K_2HPO_4, 56 mM NaH_2PO_4, 0.80 mM $MgSO_4 \cdot 7H_2O$, 19 mM NH_4Cl, 34 µM $CaCl_2 \cdot 2H_2O$, and 8.5mM NaCl. For media containing minor salts, the following concentrations were used: 1.9 µM H_3BO_3, 0.86 µM $CuSO_4 \bullet 5H_2O$, 7.7 µM $ZnSO_4 \bullet 7H_2O$, 0.75 µM $MnSO_4 \bullet H_2O$, 0.26 µM $NiCl_2 \bullet 6H_2O$, 0.31 µM $Na_2MoO_4 \bullet 2H_2O$, 30 µM $FeSO_4 \bullet 7H_2O$. For growth media containing a carbon source, the carbon-equivalent substrate concentration of 55 mM C was added as glucose alone, fructose alone, 1:1 glucose:fructose, sucrose alone, or maltose alone. A complex carbon source, maize bran (Honeyville Inc., Brigham City, Utah), was added at 5 g/L. The maize bran could not be filter-sterilized and was instead autoclaved at 121°C and 15 psi for 20 mins and subsequently added to filter-sterilized minimal medium. Non-autoclaved maize bran was also added to sterilized minimal medium at 5 g/L. The following solutions were prepared without carbon sources as additional controls including: sterile water, minimal medium with major salts in the absence of minor salts, and minimal medium with both major and minor salts.

Batch cultures with the IMO paste in growth media containing a sugar (n=3) were incubated for two periods of time: 48 and 72 hours. Incubations without carbon along with those containing maize bran were only incubated for 48 hours. Test tubes were placed in a 30°C stationary incubator with non-permeable screw top caps. Biomass growth at the end of the incubation time was measured by optical density at 600 nm (OD_{600}) using an Agilent Cary UV-visible spectrophotometer (Santa Clara, CA).

1.2.3 DNA extraction, PCR amplification, and Illumina MiSeq sequencing

After incubations were complete, the samples were centrifuged at 5478 g for five minutes. In samples where a dense cell pellet formed, the supernatant was poured off and DNA was isolated from 250 mg of the cell pellet. In samples where there was less visible growth and a smaller, more fragile pellet formed, the supernatant was carefully pipetted off and discarded until approximately 1mL was left. This smaller volume was subsequently homogenized and 250 µL was pipetted out and used for DNA extractions. To analyze the unaltered IMO bioinoculant, DNA was isolated from 250 mg of the product straight from the original container. In addition, DNA was isolated from 250 mg of non-autoclaved maize bran. MoBio PowerSoil DNA Isolation Kits (MoBio Laboratories Inc., Carslbad, CA, USA) were used according to manufacturer's instructions for all DNA isolations.

The universal bacteria primers 341F (5' – 195 CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') were used for 16S rRNA gene polymerase chain reaction (PCR) mixes and DNA was diluted 1:10 with PCR grade water. The PCR mixes and cycling conditions were based on the Howard et al. (2017) methods. Amplicons were cleaned using MagBio HighPrep PCR beads (MagBio Genomics, Gaithersburg, MD, USA) in 96-well plates. Barcode indexes were attached using the method described in Howard et al. (2017). Barcoded amplicons were normalized using the SequalPrep Normalization Kit (Thermo Fisher Scientific, Waltham, MA, USA). The 16S rRNA samples were pooled, concentrated, and processed using methods outlined in Howard et al. (2017) and pools were sequenced on the Illumina MiSeq at the Cornell Genomics Facility (Ithaca, NY) using a 600-cycle MiSeq Reagent Kit v.3.

1.2.4 Sequence processing

Sequences were processed based on the Brazilian Microbiome Project Pipeline (Pylro et al., 2014) and Mothur v.1.36.1 (Schloss et al., 2009) was used to trim primers, remove singleton sequences, and merge paired-end sequences as described in Howard et al. (2017). Clustering of 97% OTUs and chimera removal were performed using USEARCH v.7 (Edgar, 2010) and OTU sequences were classified in MOTHUR using the GreenGenes v. 13.8 16S database. R v. 3.2.1 was used for further statistical analyses.

1.2.5 Statistical Analyses

Statistical analysis was performed in R v. 3.2.1. Statistical differences between bacterial communities between the various treatments were determined by performing a PERMANOVA (method=bray, perm=999). The phyloseq package was used for creating all barplots (McMurdie and Holmes, 2013).

1.3 Results and Discussion

1.3.1 *Growth phenotypes of IMO incubations*

The overall growth of organisms in the various incubations, as determined by OD_{600} measurements, was dependent on the medium used for incubation (Fig. 1). The three conditions containing growth media without a carbon source (sterile water, medium containing only major salts, and medium containing both major and minor salts) all resulted in minimal growth measurements at both 48 and 72 hours (0.12 ± 0.01 and 0.14 ± .01, 0.13 ± .01 and .14 ± .01, 0.14 ± .01 and 0.15 ± .01, respectively) (Fig. 1). Growth was markedly increased when a carbohydrate substrate was added (Fig. 1). The growth measurements for glucose, maltose, glucose with fructose, and sucrose at 48 and 72 hours were 0.32 ± 0.01 and 2.0 ± 0.04, 0.34 ± 0.02 and 2.3 ± 0.06, 0.32 ± 0.02 and 2.4 ± 0.05, and 0.39 ± 0.03 and 2.5 ± .06, respectively (Fig. 1). A visible film also formed on the top of all incubations containing medium with sugar that did not disperse with vortexing prior to taking an OD_{600} measurement. This biofilm was

absent in incubations containing media without a carbon source. It should also be noted that the optical density could not be taken for the condition containing media with maize bran due to interference of large particles of maize in the growth medium. Comparing these growth measurements between samples with and without a carbon source reveals that the carbon present in the IMO paste (residual from the sweet potato fermentation process) was not sufficient for supporting substantial growth in these incubations.

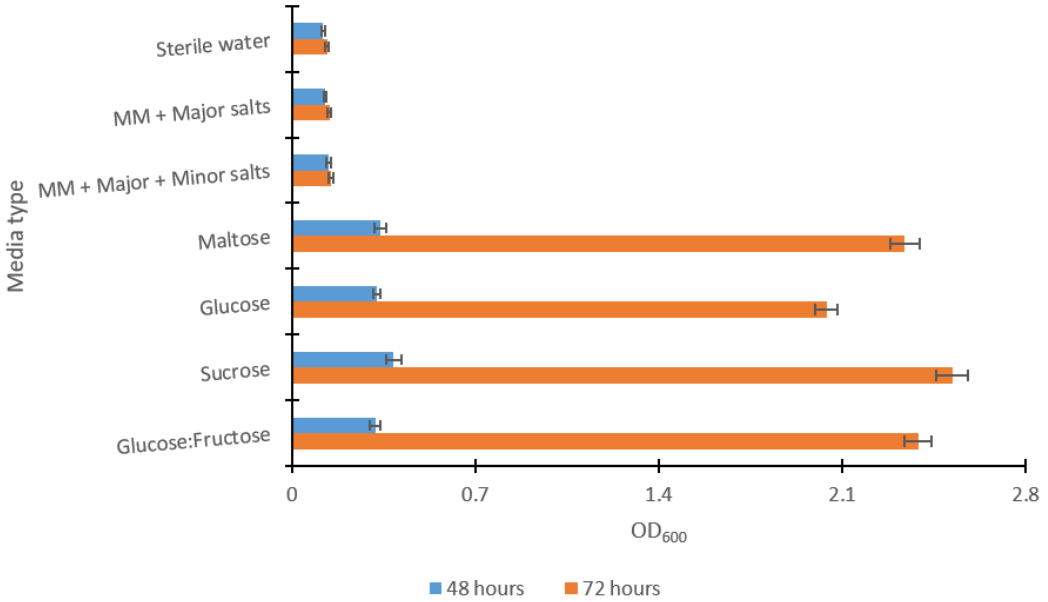

Figure 1: Optical density measurements at 600 nm (OD_{600}) of the IMO product cultured with different growth media. Measurements were obtained after 48 and 72 hours of incubation. Sterile water, minimal media (MM) with only major salts, and MM with both major and minor salts did not contain a carbon source. Maltose, sucrose, 1:1 glucose:fructose, and glucose all contained 55 mM carbon equivalents of the sugar along with both major and minor salts. All conditions were done in three biological replications (n=3) and error bars represent ± standard deviation.

1.3.2 *Bacterial composition of IMO paste*

The bacterial community of the unaltered IMO paste was evaluated to determine the initial community present prior to mixing the paste with growth media. This community was found to

contain *Lactobacillus*, *Clostridium*, and *Acetobacter* genera in about 75%, 12.5%, and 12.5% relative abundances, respectively (Fig. 2). *Lactobacillus* is a genus consisting of microaerophilic bacteria that ferment sugars to produce lactic acid and are commonly found in fermented food products (Makarova et al., 2006). *Acetobacter* is another genus commonly found in fermented food and produces acetic acid, a main component of vinegar (Yamada, 2000). The *Clostridium* genus consists of obligate anaerobes and a dominant species in the paste has high similarity to *Clostridium pasteurianum,* an organism capable of fermenting carbohydrates to both acids and solvents (Dabrock et al., 1992). These results demonstrate that the fermentation process used for producing IMO promoted growth of fermenting organisms, which are not organisms typically associated with plant growth promoting capabilities (Lugtenberg and Kamilova, 2009).

1.3.3 *Effect of metal nutrients on community divergence in the absence of carbon sources*

The following conditions all contained IMO incubated with an inorganic nutrient solution that did not contain a carbon source. Samples containing only sterile water as the growth medium did not exhibit any growth (Fig. 1); we were also unable to isolate DNA from these conditions. Growth was minimal in incubations containing media with only salts and no carbon source (Fig. 1) but DNA isolation from these samples was possible. These samples were found to contain about 10% *Paenibacillus* and 90% *Bacillus* genera (Fig. 2). The samples incubated with growth media containing both major salts and minor salts consisted of about 60% *Bacillus,* 25% *Ralstonia*, and 10% *Paenibacillus* (Fig. 2). These genera of bacteria were not dominant organisms in the unaltered IMO paste and were initially present at relative abundances of <0.01%. Interestingly, although there was no significant increase in growth in these samples compared to the sterile water control, the dominant organisms identified differed greatly from the dominant organisms present in the IMO paste. It is possible that the transfer into a neutral pH medium with nutrients allowed for growth of these initially less dominant organisms but growth could not continue without an additional carbon input. *Bacillus* and *Paenibacillus* are closely

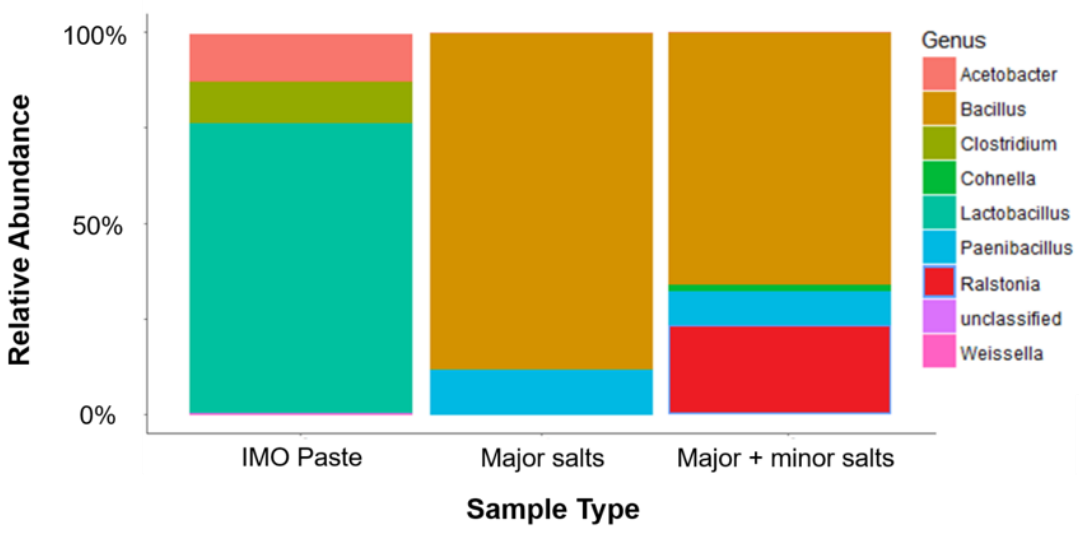

Figure 2: 100% plots of the mean relative abundance of dominant genera of bacteria based on 16S rRNA gene sequencing in the unadulterated IMO paste, a mixture of IMO paste in medium containing major salts, and a mixture of IMO paste in a medium containing both major and minor salts. Three biological replicates (n=3) were done for each condition.

related genera of aerobic endospore-forming bacteria that are commonly found in bulk and rhizosphere soils (Gardener 2004). *Bacillus* species have been extensively studied and several species are classified as PGPR, including *B. subtilis* and *B. megaterium* (Lugtenberg and Kamilova, 2009; Ortíz-Castro et al., 2008). Species of *Paenibacillus* have been reported to suppress plant pathogens, which is another important mechanism for promoting plant growth (Son et al., 2009). We note that species of *Ralstonia,* a genus of non-spore forming Gram-negative bacteria, have been classified as plant and human pathogens (Ryan and Adley, 2013; Satou et al., 2006). The species-level classification was unknown for many identified organisms and we cannot confirm whether these bacteria are related to those identified as either beneficial or pathogenic.

1.3.4 *Effect of monosaccharide and disaccharide substrates on community divergence*

The IMO paste was incubated with maltose, glucose, sucrose, or equimolar glucose:fructose for 48 or 72 hours to evaluate how carbon source and incubation time would impact the resulting bacterial community. These results show that while the overall growth in incubations containing a sugar were much higher than those lacking a carbon source, the relative abundances of genera were very similar and consisted primarily of *Bacillus* and *Paenibacillus* species (Fig. 1, 2, 3). Community composition in the incubation containing maltose consisted of about 70% *Bacillus* and 30% *Paenibacillus* genera after 48 hours of incubations (Fig. 3). However, after 72 hours of incubation, the community composition contained about 75% *Bacillus*, 15% *Paenibacillus*, and 10% *Ralstonia* (Fig. 3). At 48 hours, the glucose incubations consisted of about 90% *Bacillus* and 10% *Clostridium* genera and after 72 hours this shifted to 85% *Bacillus* and 15% *Clostridium* genera (Fig. 3). Sucrose incubations at 48 hours were almost 100% *Bacillus* and, after 72 hours, contained 60% *Bacillus* and 40% *Clostridium* (Fig. 3). The growth media containing the fructose:glucose mixture consisted of about 80% *Bacillus* and 20%

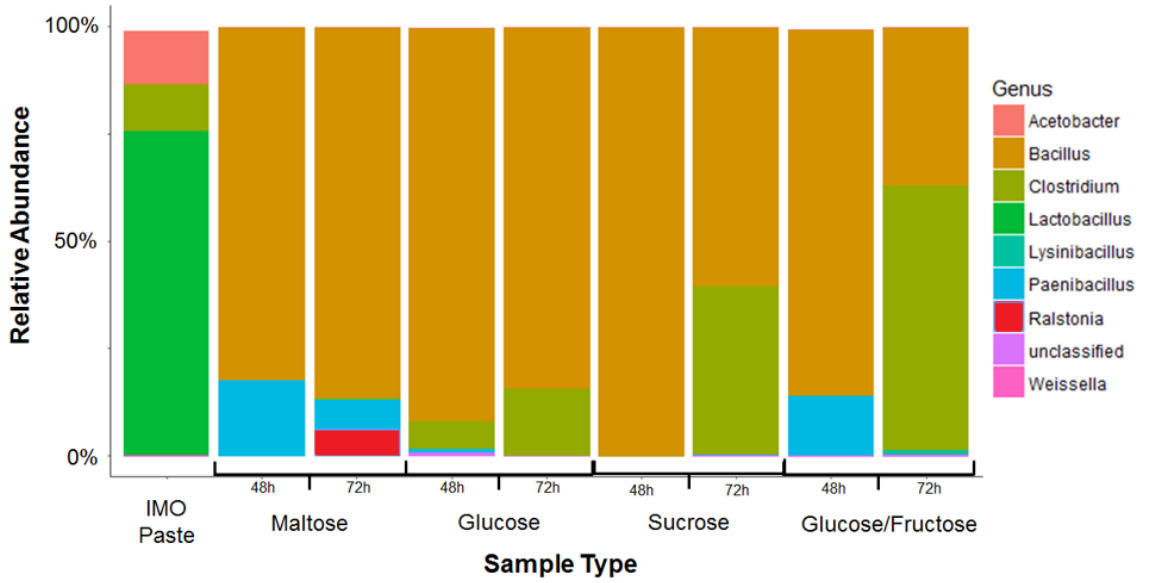

Figure 3: 100% plots of the mean relative abundance of dominant genera of bacteria based on 16S rRNA gene sequencing in unaltered IMO paste and following incubations with maltose, glucose, sucrose, or an equimolar glucose:fructose mixture for 48 and 72 hours of incubation. Data are averages from three biological replicates (n=3).

Paenibacillus genera after 48 hours and 40% *Bacillus* and 60% *Clostridium* after 72 hours (Fig. 3).

An increase in the relative abundance of *Clostridium* species was observed in samples containing glucose, sucrose, and glucose:fructose as incubation time increased from 48 and 72 hours (Fig. 3). The *Clostridium* growing in the incubations was identified as the same *Clostridium* dominant in the IMO paste and is highly similar to *Clostridium pasteurianum,* an obligate anaerobe (Dabrock et al., 1992; Kolek et al., 2015). These incubations were performed in sealed test tubes in a stationary incubator so no oxygen was introduced into the system after initially mixing the IMO in growth medium. As growth of aerobic organisms, such as *Bacillus* and *Paenibacillus,* increased (Fig. 1, 3), oxygen likely decreased due to respiration. A decrease in oxygen over time could have led to more suitable conditions for *Clostridium*. These results indicate that the bacterial community compositions were influenced more by time of incubation than specific type of sugar. This variability demonstrates that appropriate incubation times and aeration conditions need to be determined for improving consistency of the final IMO product.

1.3.5 *Effect of complex carbon source on community divergence*

Maize bran was used as a complex carbon source in the IMO growth media as it is the substrate that consumers are instructed to use for mixing with IMO and water before applying as a bioinoculant. The incubation containing autoclaved maize bran promoted dominance of *Bacillus* species, similar to results seen in incubations containing only salts or salts with sterile sugar as a carbon source (Fig. 4). Non-sterile maize bran was also used as a carbon source to determine the effects of the native bacterial population present on the substrate itself. These results show a striking difference compared with all other incubations and the non-sterile maize bran resulted in a community dominated by about 33% *Enterobacter* and 33% *Pseudomonas* species, the remainder unclassified at the genus level of classification (Fig. 4). The order level of classification showed that Pseudomonadales and Enterobacteriales dominated these

samples, compared with incubations containing sterile maize bran which was predominantly composed of the order Bacillales (Fig. 5). Isolated DNA from the non-sterile maize bran itself but the number of bacterial rRNA gene sequences was extremely low (57 ± 9) compared with the minimum cutoff set of 3,000 and the data is not shown here. The dominant sequences identified in the incubation containing non-sterile maize bran were not identified in the original IMO paste and, therefore, the dominant organisms must have come from bacteria present on the non-sterile substrate. These results show that the microbial community on the substrate used for incubating IMO can have a larger impact on the resulting bacterial community than the IMO itself, even when the bacteria on the non-sterile substrate is present at very low levels. This type of bioinoculant product is primarily mixed in an agricultural setting with non-sterile water and substrates so the implications of this finding are great. The process of incubating IMO with a carbon source is to promote proliferation of bacteria present in the IMO, and if bacteria present

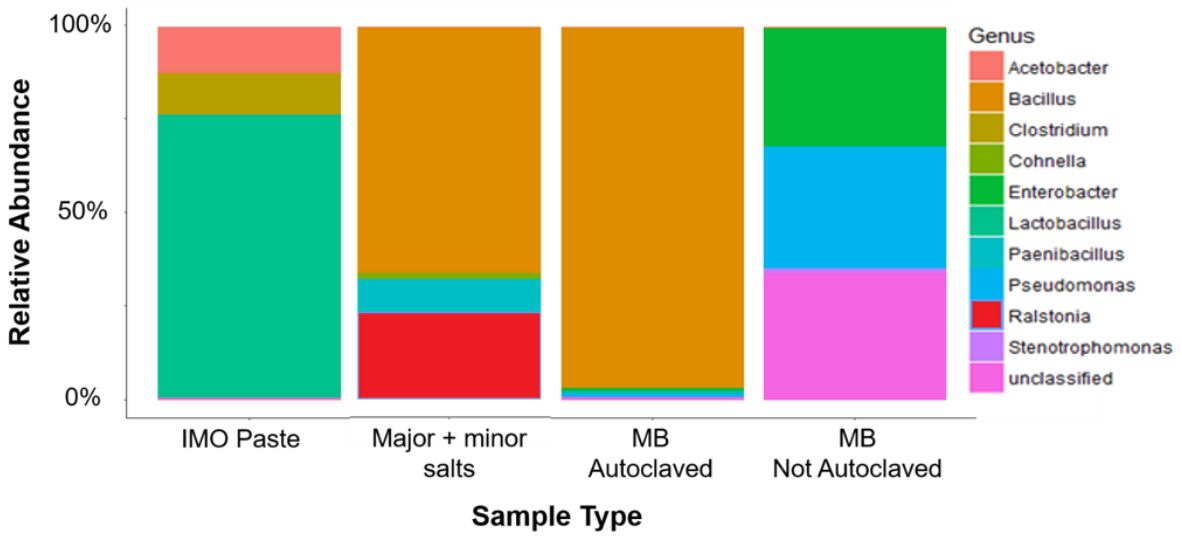

Figure 4: 100% plots of the mean relative abundance of dominant genera of bacteria based on 16S rRNA gene sequencing in the unadulterated IMO paste and following incubations with medium containing both major and minor salts, autoclaved maize bran (MB) with both major and minor salts, and non-sterile MB with both major and minor salts. Data are averages from three biological replicates (n=3).

on the substrate or in the water outcompetes the bacteria initially present in the IMO then this introduces an enormous amount of variability.

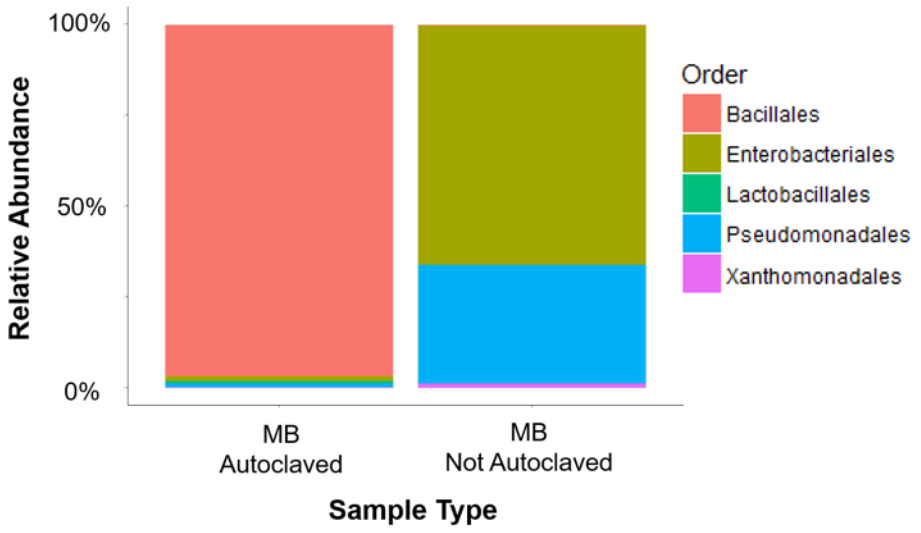

Figure 5: 100% plots of the mean relative abundance of dominant orders of bacteria based on 16S rRNA gene sequencing in IMO incubated with autoclaved maize bran (MB) with both major and minor salts and non-sterile MB with both major and minor salts. These are the same MB samples represented in Figure 3 but showing the order level of classification. Data are averages from three biological replicates (n=3).

1.4 *Conclusions*

Bioinoculants are becoming increasingly popular as an alternative to chemical-based fertilizers yet there is little external regulatory oversight into bioinoculant production (Adesemoye et al., 2009). The QA/QC protocols established by the manufacturers are often insufficient, resulting in inconsistent products with unproven microbial populations (Hermann and Lesueur, 2013). We evaluated IMO bioinoculants as this category is produced worldwide yet has received little scientific attention. Our primary research objectives were to: (1) determine the starting bacterial

population of the IMO product; (2) assess changes, if any, of bacterial populations in response to growth substrates; (3) assess the effects of incubation time on the resulting bacterial population; and (4) to determine the impact of non-sterile growth substrates on the final bacterial population. Results showed that the composition of the IMO paste consisted primarily of fermenting bacteria including *Lactobacillus, Acetobacter,* and *Clostridium* species. This is not surprising considering the product is made from fermented sweet potato, although these species are not typically utilized in PGPR bioinoculants (Lugtenberg and Kamilova, 2009). All incubations containing IMO and sterile media, with or without a carbon source, shifted the populations towards dominance by *Bacillus* and *Paenibacillus* species. The addition of a sterile carbon source did not drastically change the community composition, although lack of carbon did significantly repress overall growth. Organisms were transitioned from a complex and nutrient-rich paste to a dilute growth medium which could have allowed *Bacillus* and *Paenibacillus* species to outcompete the fermenting organisms dominant in the paste. *Clostridium* species increased in many of the incubations containing sugar after 72 hours of incubation, likely indicating depleted oxygen conditions which encouraged growth of anaerobic bacteria. The incubations containing non-sterile maize bran produced the most surprising results and these communities were dominated by *Enterobacter* and *Pseudomonas.* These organisms are thought to have proliferated from a low concentration of bacteria present on the non-sterile maize bran and were able to outcompete the IMO bacteria. This has large implications for bioinoculant products that instruct consumers to mix the product with non-sterile water and substrates as the bacteria being introduced from the environment can have a greater effect on the resulting composition than the bacteria from the bioinoculant itself. Overall, these results show that the IMO paste consists of a dynamic community of bacteria including fermenting organisms, rhizospheric bacteria, and many unknown organisms and the relative abundances of these organisms can shift depending on specific culturing conditions. This makes developing an effective QA/QC protocol that guarantees consistency in the initial product

and the incubated product that is used in the field particular challenging. Further research is needed to determine a method for production and preparation that does promote growth of target PGPR organisms. If that can be established, then the product would need to be evaluated for success in the field. While challenging, the development of a consistent and efficacious bioinoculant product that can be produced using limited resources would be advantageous for many small-scale farmers.

CHAPTER 2: GLUCOSE-INDUCED SELECTIVE INHIBITION OF DISACCHARIDE CATABOLISM IN A SOIL BACILLUS SPECIES: BACILLUS MEGATERIUM QM B1551

2.1 Introduction

Bacillus species are an important genus of Gram-positive bacteria that are found in diverse environmental matrices and can utilize a wide range of carbon sources (Stülke and Hillen, 2000). Several of the species, including *Bacillus megaterium* and *Bacillus subtilis,* have been used in bioinoculant products due to their reported plant growth-promoting abilities (Vary, 1994; Rodríguez and Fraga, 1999; Ortíz-Castro et al., 2008). These products are used to increase plant growth and are introduced into the rhizosphere where breakdown products of plant materials provide a major source of carbon for bacteria in soil environments. Notable amongst these compounds are the sugar dimers or disaccharides (cellobiose, maltose, and sucrose) and their respective monomers (glucose and fructose) (Graaff et al., 2010; Kogel-Knabner, 2017). The disaccharides mentioned have an identical carbon formula but differing glycosidic linkages (Fig. 6A). Sucrose, a dimer containing glucose and fructose linked via an α-1,2 glycosidic bond, is a transport sugar in plants and is the most abundant disaccharide in soils (Lunn, 2008; Reid and Abratt, 2005). Maltose is a glucose dimer with α-1,4 glycosidic bonds and is formed via amylase breakdown of starch. Cellobiose is a glucose dimer with β-1,4 glycosidic bonds and is produced via microbial hydrolysis of cellulose in soils (Schellenberger et al., 2011). These disaccharides are also commonly used as an enrichment material for bioinoculants (Omer 2010). Understanding how *Bacillus* species catabolize these disaccharides, which are used in commercial product formulations along with being present in the rhizosphere environments, can help optimize growth of these organisms for use in bioinoculant products.

Sugar uptake in *Bacillus* species occurs via phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) and ATP-binding cassette (ABC) transporters (Stülke and Hillen, 2000; Quentin et al., 1999). Involvement of ABC transporters has not been reported for

sugars used in this study despite presence of relevant genes in *B. subtilis* and *B. megaterium* (Quentin et al., 1999; Schönert et al., 2006). Transcriptional analysis has revealed that *B. subtilis* grown on either glucose, fructose, cellobiose, maltose, or sucrose as the sole carbon source expresses the relevant PTS transporters (Schönert et al., 2006; Steinmetz et al., 1989; Tangney et al., 1992; Tobisch et al., 1997; Stülke and Hillen, 2000). Transcriptional elucidation of disaccharide PTS enzymes have not yet been conducted in *B. megaterium.* However, as illustrated in Fig. 6C (Aristilde, 2017; Sasnow et al., 2016), similar PTS uptake proteins were proposed from the genome of *B. megaterium* (Kanehisa et al., 2017; Kanehisa et al., 2016; Kanehisa et al., 2000). In both *B. megaterium* and *B. subtilis,* cellobiose enters the cell, becomes phosphorylated to cellobiose-6-phosphate which is then converted into glucose and G6P via 6-phospho-β-glucosidase (Fig. 6C) (Kanehisa et al., 2017; Kanehisa et al., 2016; Kanehisa et al., 2000). In *B.* megaterium, maltose is converted into maltose-6-phosphate which is then dephosphorylated back to intracellular maltose via the maltose 6'phosphate phosphatase enzyme (Fig. 6C) (Kanehisa et al., 2017; Kanehisa et al., 2016; Kanehisa et al., 2000). The intracellular maltose is then converted into glucose via an α-glucosidase (Fig. 6C) (Kanehisa et al., 2017; Kanehisa et al., 2016; Kanehisa et al., 2000). This differs from the pathway used in *B. subtilis* which uses a maltose-6'-phosphate glucosidase enzymes to produce G6P and glucose from maltose-6-phosphate (Mokhtari et al., 2013; Kanehisa et al., 2017; Kanehisa et al., 2016; Kanehisa et al., 2000). In both *B. megaterium* and *B. subtilis,* sucrose enters the cell via a PTS transport protein, is phosphorylated into sucrose-6P which is then hydrolyzed to produce G6P and fructose (Fig. 6C). Intracellular fructose is subsequently phosphorylated to F6P (Fig. 1) (Kanehisa et al., 2017; Kanehisa et al., 2016; Kanehisa et al., 2000). In addition to the PTS proteins, *B. subtilis* has been shown to produce an intracellular sucrase and an extracellular levansucrase which can cleave sucrose into its monomers (fructose and glucose) (Steinmetz et al., 1989; Crutz and Steinmetz, 1992). *B. megaterium* has been found to produce an extracellular levansucrase enzyme, similar to the one found in *B.*

subtilis (Chambert et al., 1973; Meng and Futterer, 2003; Biedendieck et al., 2007; Crutz et al., 1990). Genomics studies also have also shown that *B. megaterium* contains an intracellular sucrase similar to the one identified in *B. subtilis* (Kanehisa et al., 2017; Kanehisa et al., 2016; Kanehisa et al., 2000).

Inhibition of disaccharide uptake has been reported in *B. subtilis* in the presence of glucose, a preferred carbon source in many bacteria (Stülke and Hillen, 2000). In *B. subtilis* grown on a mixture of glucose and cellobiose, repression of the *licBCAH* operon was observed; this operon consists of genes encoding for the PTS enzymes responsible for the uptake of β-linked glycosides, including cellobiose (Tobisch et al., 1996). *Bacillus subtilis* grown on glucose with maltose has been shown to inhibit maltose uptake and decreased α-glucosidase activity has been reported (Tangney et al., 1992; Stülke and Hillen, 2000; Schönert et al., 2006; Deutscher et al., 1994). Repression of sucrose uptake has also been observed in *B. subtilis* grown on a mixture of glucose with sucrose (Débarbouillé et al., 1991; Rutberg, 1997; Stülke et al., 1997). Sucrose regulation is controlled by antiterminator proteins although the mechanism for regulation of sucrose uptake has been shown to differ from the regulation of cellobiose and maltose uptake (Kanehisa et al., 2017; Kanehisa et al., 2016; Kanehisa et al., 2000; Stülke and Hillen, 2000; Rutberg, 1997; Stülke et al., 1997). Repression of these disaccharides has not been studied in *B. megaterium* although expression of amylase, an enzyme that cleaves starch into maltose, has been shown to be repressed by presence of glucose (Lee et al., 2001).

In the present study, we employed a metabolomics approach to investigate the hierarchy of glucose catabolism with respect to disaccharide catabolism in *B. megaterium* QM B1551. Based on prior findings with *B. subtilis* as well as previous genetic analysis of *B. megaterium,* we put forth the following three hypotheses regarding the co-catabolism of glucose and a disaccharide*:* (1) cellobiose uptake will be completely repressed in media containing glucose with cellobiose, (2) maltose uptake will be completely repressed in media containing glucose

with maltose, and (3) sucrose uptake will be repressed in media containing glucose with sucrose, but to a lesser extent than cellobiose or maltose. High-resolution liquid chromatography-mass spectrometry (LC-MS) was applied to track the simultaneous incorporation of stable isotope-labeled and unlabeled substrates from mixed-sugar mixtures into intracellular metabolites in glycolysis, the PP pathway, AA biosynthesis and ribonucleotide biosynthesis. Our results show that in the presence of glucose, *B. megaterium* completely represses cellobiose catabolism. Maltose utilization was greatly repressed in the presence of glucose, although small fractions of maltose were incorporated into metabolites produced throughout various pathways in central carbon metabolism. In contrast, *B. megaterium* did not repress sucrose catabolism in the presence of glucose and instead incorporated sucrose into metabolites to a similar extent as cells grown on glucose. Results indicate that even though maltose and cellobiose are repressed in the presence of glucose, there is a hierarchy and cellobiose is almost completely inhibited in the presence of glucose while small fractions maltose is able to be metabolized. Future studies are needed to elucidate the mechanisms that create this hierarchy of sugar utilization in mixed-substrate media in *B. megaterium* and to determine if these hierarchies could have implications for plant growth-promoting properties of *Bacillus* species.

2.2 Methods

2.2.1 *Culturing conditions*

Bacillus megaterium QM B1551 was obtained from the Bacillus Genetic Stock Center (Vary collection, Bacillus Genetic Stock Center, Ohio State University; http://www.bgsc.org/) as freeze-dried cultures on filter disks. According to BGSC instructions, a filter disk was placed on Luria-Bertani (LB) agar plates, several drops of liquid LB were placed on the disk, and the plate was incubated at 30°C until colonies formed. After initial growth, plates were placed in 4°C conditions for storage. For growth experiments, liquid cultures of *B. megaterium* QM B1551

were grown in 250mL baffled flasks at 30°C in a G24 environmental incubator shaker (New Brunswick Scientific, Edison, NJ) at 220rpm covered by permeable silicone sponge closures to allow gas exchange (Sasnow et al., 2016). All media was filter-sterilized (0.22µm nylon; Waters Corporation, MA) and adjusted to pH 7.0. Growth medium contained: 89 mM K_2HPO_4, 56 mM NaH_2PO_4, 0.8 mM $MgSO_4 \cdot 7H_2O$, 18.7 mM NH_4Cl, 34 µM $CaCl_2 \cdot 2H_2O$, 8.5mM NaCl, 1.9 µM H_3BO_3, 0.86 µM $CuSO_4 \bullet 5H_2O$, 7.7 µM $ZnSO_4 \bullet 7H_2O$, 0.75 µM $MnSO_4 \bullet H_2O$, 0.26 µM $NiCl_2 \bullet 6H_2O$, 0.31 µM $Na_2MoO_4 \bullet 2H_2O$, and 30 µM $FeSO_4 \cdot 7H_2O$.

Carbon-equivalent substrate concentrations were 55mM C and added as glucose alone, 50:50 glucose:maltose, 50:50 glucose:sucrose, or 50:50 glucose:cellobiose. Cultures were started in nutrient-rich LB broth and conditioned to the minimal growth medium by transferring cells twice into the fresh medium (Sasnow et al., 2016). Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}) using an Agilent Cary UV-visible spectrophotometer (Santa Clara, CA). The initial OD_{600} was between 0.05 and 0.06 when cells were transferred from LB to minimal media, and then again to minimal media. Cells were transferred during mid-expontential phase, which for *B. megaterium* was between OD_{600} of 3.0-5.0.

2.2.2 *Intracellular metabolite labeling*

For intracellular labeling, isotopically labeled substrates from Cambridge Isotopes (Tewksbury, MA) and Omicron Biochemicals (South Bend, IN) were used as growth media substrates. Labeled substrate schemes for steady state experiments included: [U-$^{13}C_6$]-glucose, 50:50 [U-$^{13}C_6$]-glucose:unlabeled sucrose, 50:50 [U-$^{13}C_6$]-glucose:unlabeled cellobiose, and 50:50 [U-$^{13}C_6$]-glucose:unlabeled maltose. Cultures were grown in their respective media in conditions described above (two biological replicates) and 3mL of cells were filtered (0.22 µm pore size nylon filters; Fisher Scientific, Pittsburgh, Pennsylvania) at two times points in mid-exponential phase (OD_{600} of 0.90-1.1 and 2.0-2.3). Filtered cells were quenched in 2mL of 4°C quenching

solution containing 40:40:20 acetonitrile:methanol:water in order to lyse cells and this quenching solution containing lysed cells was then centrifuged at 1062 g (five minutes). Two 100-µL aliquots of the supernatants were taken of each sample for technical replicates and these aliquots were dried under nitrogen gas and re-suspended in 100-µL of LC-MS water (Fisher Scientific, Pittsburg, PA) for LC-MS analysis.

2.2.3 *Metabolomics analysis via LC-MS*

Extracted metabolites were analyzed by reversed-phase ion-pairing liquid chromatography using ultra-high performance liquid chromatography (UHPLC; Thermo Scientific DionexUltiMate 3000) coupled with high resolution/accurate-mass mass spectrometer (MS) (Thermo Scientific Q Exactive quadrupole-Orbitrap hybrid MS) with electrospray ionization (ESI) operated in negative mode. LC-MS methods outlined in Aristilde et al. (2017) were used for our analysis. Solvent A contained 97:3 (v/v) LC-MS grade H2O: methanol with acetic acid (15 mM) and tributylamine (10 mM) and Solvent B contained 100% methanol. The solvent gradient with respect to solvent A was the following: 0 min, 100%; 2.5 min, 100%; 5 min, 80%; 7.5 min, 80%; 10 min, 45%; 12 min, 45%; 14 min, 5%; 17 min, 5%; 18 min, 0%; 25 min, 0%. The sample run lasted 25 minutes and the flow rate remained 180 µL min-1 for the entire run. The column temperature was set at 25°C and 10 µL of sample was injected (Lu et al., 2010). A Waters Acquity UPLC BEH C18 column (1.7µm with column size 2.1 x 100mm) (Waters Corporation, Massachusetts, USA) was used. Metabolites from key metabolic pathways were analyzed and included: gluconate, glucose-6-phoshpate, fructose-6-phosphate, dihydroxyacetone phosphate, 3-phosphoglycerate, phosphoenolpyruvate, pyruvate, citrate, α-ketoglutarate, succinate, fumarate, malate, aspartate, ribose-5-phosphate, xylulose-5-phosphate, tyrosine, 3-phosphoserine, valine, aspartate, glutamate, inosine monophosphate, and uridine monophosphate. The Metabolomics Analysis and Visualization Engine (MAVEN) software package (Clasquin et al., 2012) was used for metabolite identification and isotope enrichment

and the ^{13}C-labeled fractions were corrected for natural ^{13}C abundance.

2.3 Results and Discussion

2.3.1 *Growth phenotypes*

We confirmed the ability of *B. megaterium* to catabolize each substrate successfully, albeit the growth rates differed (Fig. 6B). During growth on 55 mM C provided as glucose alone, the specific growth rate was 0.68 ± 0.08 h^{-1} (Fig. 6B), in agreement with previously reported growth rates ranging from 0.67 to 1.0 h^{-1} for several strains of glucose-grown *B. subtilis* (Tobisch et al., 1997; Tännler et al., 2008). When the cells were grown on carbon-equivalent maltose or sucrose alone, we obtained similar growth rates (respectively, 0.59 ± 0.03 h^{-1} and 0.71 ± .07 h^{-1}) as the glucose-grown cells (Fig. 6B). By contrast, cells grown on equimolar carbon of cellobiose exhibited a near 50% decrease in growth rate (Fig. 6B), indicating that cellobiose was a less favorable carbon source than sucrose, maltose and glucose. Interestingly, the growth rates for cells fed on equimolar carbon (27.5 mM C each) of glucose:cellobiose, glucose:maltose, and glucose:sucrose mixtures (respectively, 0.67 ± 0.08 h^{-1}, 0.68 ± 0.07 h^{-1}, and 0.68 ± 0.05 h^{-1}) were similar to the growth rate of the cells grown on glucose alone (Fig. 6B). It can be inferred from these data that the cells utilized carbons from both substrates to support the same biomass growth rate as was obtained with glucose alone. However, no change in growth rate with 2-fold or 4-fold less glucose concentration (respectively, 27.5 mM C and 12.75 mM C) (Fig. 6B) implied that the lower glucose concentration in the mixture would not exert a carbon starvation condition in the absence of disaccharide uptake from the mixture. Therefore, no conclusion could be made regarding the extent of the disaccharide assimilation based solely on the growth phenotype of the cells grown on the mixture.

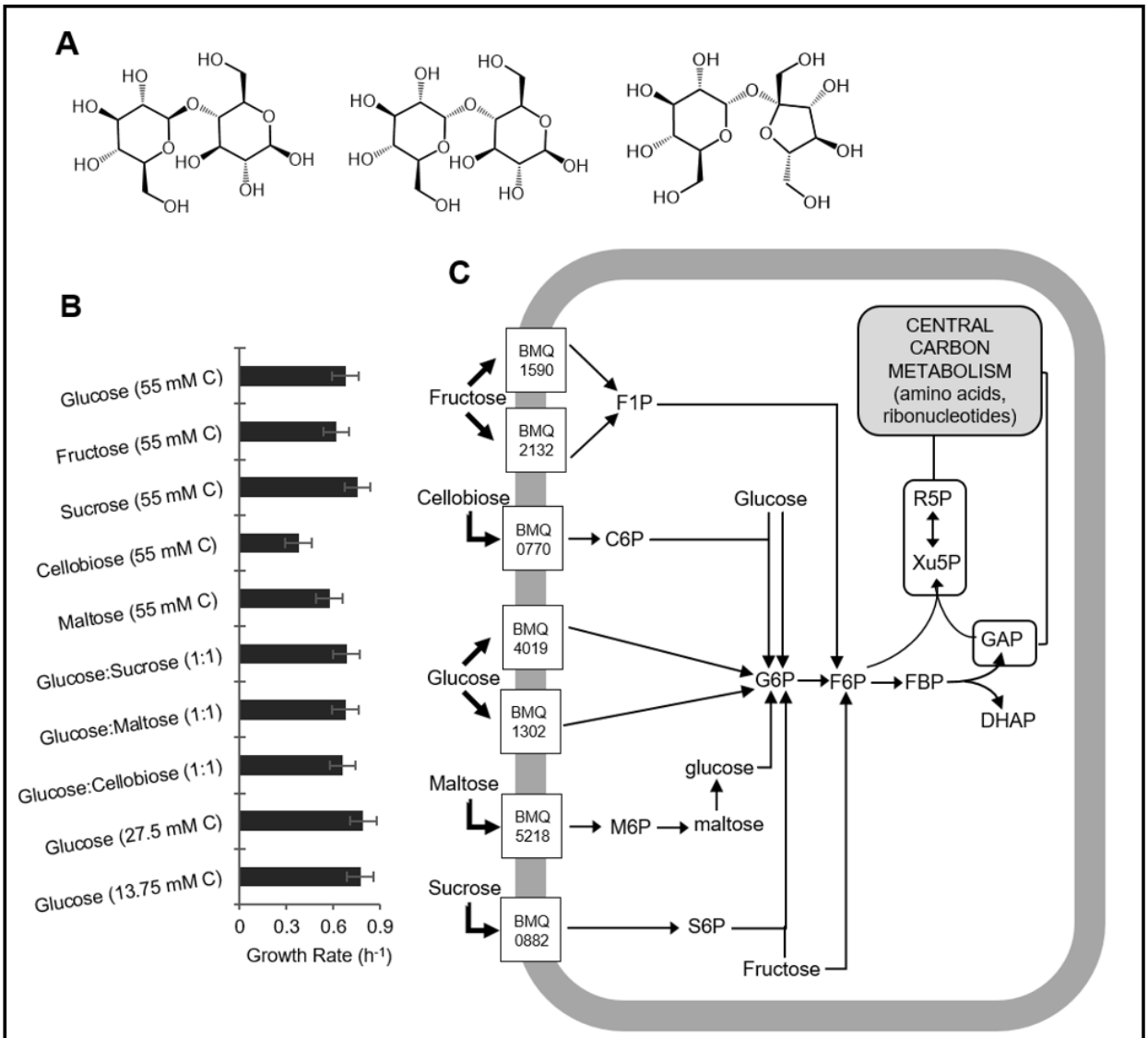

Figure 6: (A) Chemical structures of cellobiose (left), maltose (middle), and sucrose (right). (B) Growth rates (h^{-1}) of *Bacillus megaterium* QM B1551 grown on single and mixed substrate media; data (average ± standard deviation) were from biological replicates (n=3). (C) Schematic of sugar catabolic pathways in *Bacillus megaterium* QM B1551. Boxes represent relevant phosphotransferase sugar transport proteins (Kanehisa et al., 2017; Kanehisa et al., 2016; Kanehisa et al., 2000; Kanehisa et al., 2016; Kanehisa et al., 2000). Legend for metabolite names: glucose-6-phosphate, G6P; fructose-1-phosphate, F1P; fructose-6-phosphate, F6P; fructose 1,6-bisphosphate, FBP; cellobiose-6-phosphate, C6P; maltose-6-phosphate, M6P; sucrose-6-phosphate, S6P; glyceraldehyde-3-phosphate, GAP; dihydroxyacetone phosphate, DHAP; xyulose-5-phosphate, Xu5P; ribose-5-phosphate, R5P.

2.3.2 *Proof-of-concept labeling experiments*

To determine the co-utilization of glucose with disaccharides, labeling experiments were conducted with labeled and unlabeled glucose. Proof-of-concept labeling experiments were conducted with *B. megaterium* fed on [U-$^{13}C_6$]-glucose alone or with equimolar unlabeled glucose (Fig. 7). The ^{13}C-labeling data, which were obtained at two separate times during mid-exponential phase, confirmed that steady-state was achieved in the intracellular labeling (Buescher et al., 2015). When the cells were fed the labeled glucose alone, 100% of both G6P and F6P were fully ^{13}C-labeled (Fig. 7). During growth on equimolar labeled and unlabeled glucose, about 40-50% of these hexose-phosphate metabolites were fully-labelled in accordance with equal assimilation of labeled and unlabeled glucose (Fig. 7) (Aristilde, 2017). Compared with fully ^{13}C-labeled FBP in cells grown on ^{13}C-labeled glucose alone, the FBP in cells grown on the mixture of labeled glucose with unlabeled glucose was 35% nonlabeled, 17-19% triply ^{13}C-labeled and 35% fully ^{13}C-labeled (Fig. 7). Cells grown on fully ^{13}C-labeled glucose produced fully ^{13}C-labeled DHAP, but when the growth medium also contained unlabeled glucose, DHAP contained nearly equal amounts of nonlabeled (45%) and triply ^{13}C-labeled (46%) labeled fractions (Fig. 7). Therefore, the FBP labeling implied the involvement of gluconeogenic flux rather than only forward glycolytic flux (Fig. 7) (Aristilde, 2017). Glycolytic flux would primarily produce nonlabeled and fully ^{13}C-labeled FBP whereas the combination of labeled and nonlabeled triose phosphates would generate additionally triply ^{13}C-labeled FBP (Fig. 7). The pentose-phosphate metabolites in the PP pathway, Xu5P and R5P, also reflected the expected incorporation of labeled and unlabeled glucose (Fig. 7) Both PP metabolites were nearly 100% fully ^{13}C-labeled when cells were grown on fully ^{13}C-labeled glucose alone (Fig. 7). When cells were grown on the labeled glucose with unlabeled glucose mixture, Xu5P and R5P were found to be 32-37% nonlabeled, 9-14% doubly ^{13}C-labeled, 9-14% triply ^{13}C-labeled, and 34-38% fully ^{13}C-labeled (Fig. 7). The relatively smaller fractions of doubly and triply ^{13}C-labeled

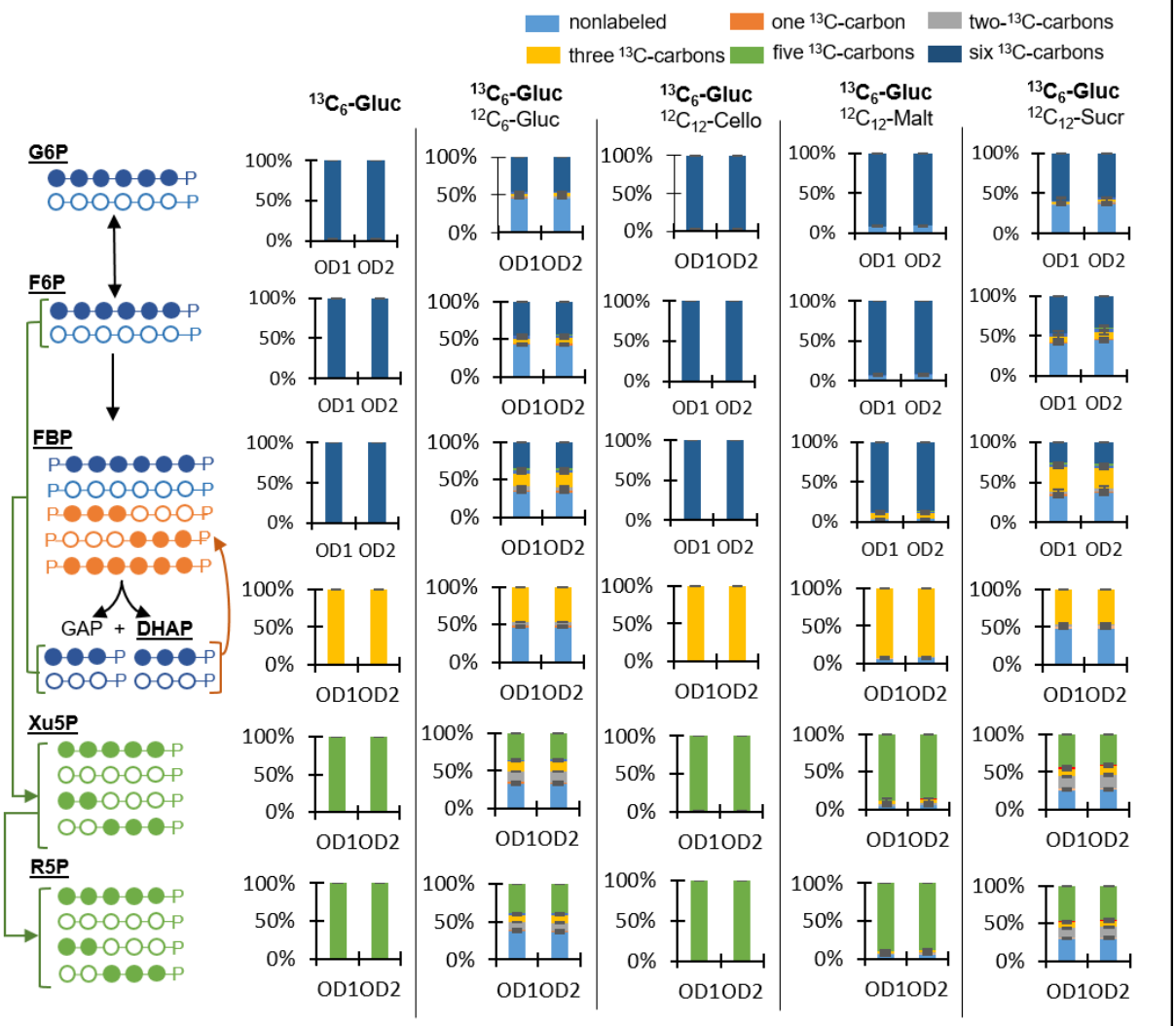

Figure 2: Mixed sugar catabolism of stable isotope-labelled glucose with unlabeled glucose, cellobiose, maltose, or sucrose. The carbon mapping on the left illustrates the different labelling forms of the metabolites based on isotopic enrichment. The filled circles and open circles represent, respectively, ^{13}C-labeled carbons and unlabeled carbons; black, red, and blue circles are assigned the labeled schemes in glycolytic, gluconeogenic, and pentose-phosphate pathways, respectively. The carbon mapping on the left illustrates various labeling patterns observed for key metabolites. Color codes for the labelling isotopologues: nonlabelled carbon (light blue), one ^{13}C-carbon (orange), two ^{13}C-carbons (gray), three ^{13}C-carbons (yellow), five ^{13}C-carbons (green), six ^{13}C-carbons (dark blue). Data from two separate time points in mid-exponential phase [optical densities (ODs) 1 and 2] are displayed to highlight that the cells were in pseudo-steady state. The measured data (average ± standard deviation) were from biological replicates (n=2). Non-noticeable error bars were in cases where standard deviation values were small.

PP pathway metabolites resulted from mixtures of nonlabeled and fully ^{13}C-labeled glycolytic metabolites.

These proof-of-concept results were used as a guide to determine the extent of co-catabolism of substrate mixtures composed of fully ^{13}C-labeled glucose and nonlabeled disaccharides (cellobiose, maltose, or sucrose). Labeling patterns that resembled those produced when cells were grown on fully ^{13}C-labeled glucose alone indicated that *B. megaterium* repressed the catabolism of the disaccharide in the presence of glucose. Labeling patterns that resembled those in cells grown on the labeled glucose with unlabeled glucose mixture indicated that *B. megaterium* can metabolize the disaccharides similarly to glucose (Aristilde, 2017).

2.3.3 *Glucose metabolism with respect to a* β*-linked disaccharide: cellobiose*

We confirmed that steady-state was achieved in intracellular labeling in the cells grown on the [U-$^{13}C_6$]-glucose with nonlabeled cellobiose (glucose:cellobiose) mixture (Fig. 7). We obtained metabolite labeling patterns in cells grown on the glucose:cellobiose mixture that were identical to those obtained in the cells grown on fully ^{13}C-labeled glucose alone (Fig. 7). Glycolytic metabolites (G6P, F6P, FBP, DHAP) and PP pathway metabolites (Xu5P, R5P) were exclusively fully ^{13}C-labeled, thus indicating complete repression of cellobiose catabolism in the presence of glucose (Fig. 7). Our results are consistent with complete repression of cellobiose PTS gene expression in the presence of glucose observed in *B. subtilis* (Tobisch et al., 1996).

2.3.4 *Glucose metabolism with respect to α-linked disaccharides: maltose and sucrose*

Cells grown on a mixture of [U-$^{13}C_6$]-glucose with either nonlabeled maltose (glucose:maltose) or nonlabeled sucrose (glucose:sucrose) were confirmed to exhibit steady state ^{13}C-labeling during exponential phase (Fig. 7). Metabolite labeling patterns in cells grown on the glucose:maltose mixture were primarily fully ^{13}C-labeled with a persistent presence of

small, nonlabeled fractions (<15%) (Fig. 7). Specifically, the hexose phosphates G6P and F6P were 6-9% nonlabeled and 91-94% fully ^{13}C-labeled whereas these metabolites were 100% fully ^{13}C-labeled during growth on the glucose:cellobiose mixture (Fig. 7). The FBP ^{13}C-labeling was in agreement with F6P labeling with 90% fully ^{13}C-labeled carbon, but FBP contained 8% triply ^{13}C-labeled carbon whereas F6P contained 5% nonlabeled carbon (Fig. 7). The PP metabolites also showed small fractions of nonlabeled carbon along with 88% fully ^{13}C-labeled carbon (Fig. 3). The persistence of the small fraction of nonlabeled and partially-labeled glycolytic and PP pathway metabolites was consistent with uptake and incorporation of small amounts of maltose-derived carbon (Fig. 7). In contrast to cellobiose, the presence of glucose did not result in complete repression of maltose utilization. These results are consistent with transcriptional studies in *B. subtilis* that revealed repression of maltose catabolism enzymes in the presence of glucose and provides additional information on the extent of this repression in *B. megaterium* (Fisher and Sonenshein, 1991; Schönert et al., 1999; Schönert et al., 2006; Tangney et al., 1992).

By contrast to the significant inhibition of maltose catabolism in the presence of glucose, we found that sucrose metabolism was not compromised by the presence of glucose. The metabolite labeling patterns during growth on equimolar labeled glucose with unlabeled sucrose (glucose:sucrose) resembled those obtained with cells fed on the mixture with equimolar labeled glucose with unlabeled glucose. In cells grown on glucose:sucrose, G6P was 35-37% nonlabeled and 55-59% fully ^{13}C-labeled and F6P was 40-44% nonlabeled and 40-46% fully ^{13}C-labeled. The remaining fractions were triply ^{13}C-labeled (Fig. 7). The FBP in cells grown on glucose:sucrose contained about 23-26% nonlabeled, 24-30% triply ^{13}C-labeled, and 26% fully ^{13}C-labeled fractions (Fig. 7). The labeling patterns for DHAP were about 48% nonlabeled and 46% fully ^{13}C-labeled, the remainder present as small fractions of singly and doubly ^{13}C- labeled carbon (Fig. 7). In the PP pathway, Xu5P and R5P from cells grown on glucose:sucrose were

25-29% nonlabeled, 12-18% doubly ^{13}C-labeled, 7-11% triply ^{13}C-labeled, and 40-47% fully ^{13}C-labeled (Fig. 7). This labeling is similar to the labeling seen in pentose-phosphates in cells grown on the labeled glucose with unlabeled glucose mixture. The results of this study show that *B. megaterium* can catabolize sucrose in the presence of glucose which indicates that at least one mechanism of sucrose metabolism is not repressed in the presence of glucose.

2.3.5 *Contribution of disaccharides to biomass precursors*

The central role of cellular metabolism is to channel nutrients towards biosynthetic building blocks for biomass growth. Important biomass precursors that are produced in central carbon metabolism include amino acids and the nucleoside monophosphates, inosine monophosphate (IMP) and uridine monophosphate (UMP). Amino acids are important for *de novo* protein synthesis while IMP and UMP are used in *de novo* ribonucleotide biosynthesis (Aristilde et al., 2017). Amino acids are produced throughout central carbon metabolism in glycolysis, the PP pathway, and the TCA cycle (Fig. 5). We evaluated the carbon labeling of tyrosine, 3-phosphoserine, valine, aspartate, and glutamate as they are all produced from different precursors and can represent amino acid synthesis in various pathways of central carbon metabolism (Fig. 5). Biosynthesis of these nucleoside precursors involves metabolites from glycolysis, the PP pathway, and the TCA cycle (Aristilde, 2017).

Our results show that when *B. megaterium* was grown on the glucose:cellobiose mixture, the labeling pattern of all amino acids resembled the labeling pattern of AAs grown on fully ^{13}C-labeled glucose (Fig. 8). This further emphasized that complete repression of cellobiose occurred in the presence of glucose (Fig. 8). Cells grown on the glucose:maltose mixture produced AAs that were primarily fully ^{13}C-labeled but had a higher percentage of nonlabeled carbon than the AAs from cells grown on fully ^{13}C-labeled glucose (Fig. 8). Tyrosine was about 69% fully ^{13}C-labeled with the remainder consisting of small fractions of nonlabeled

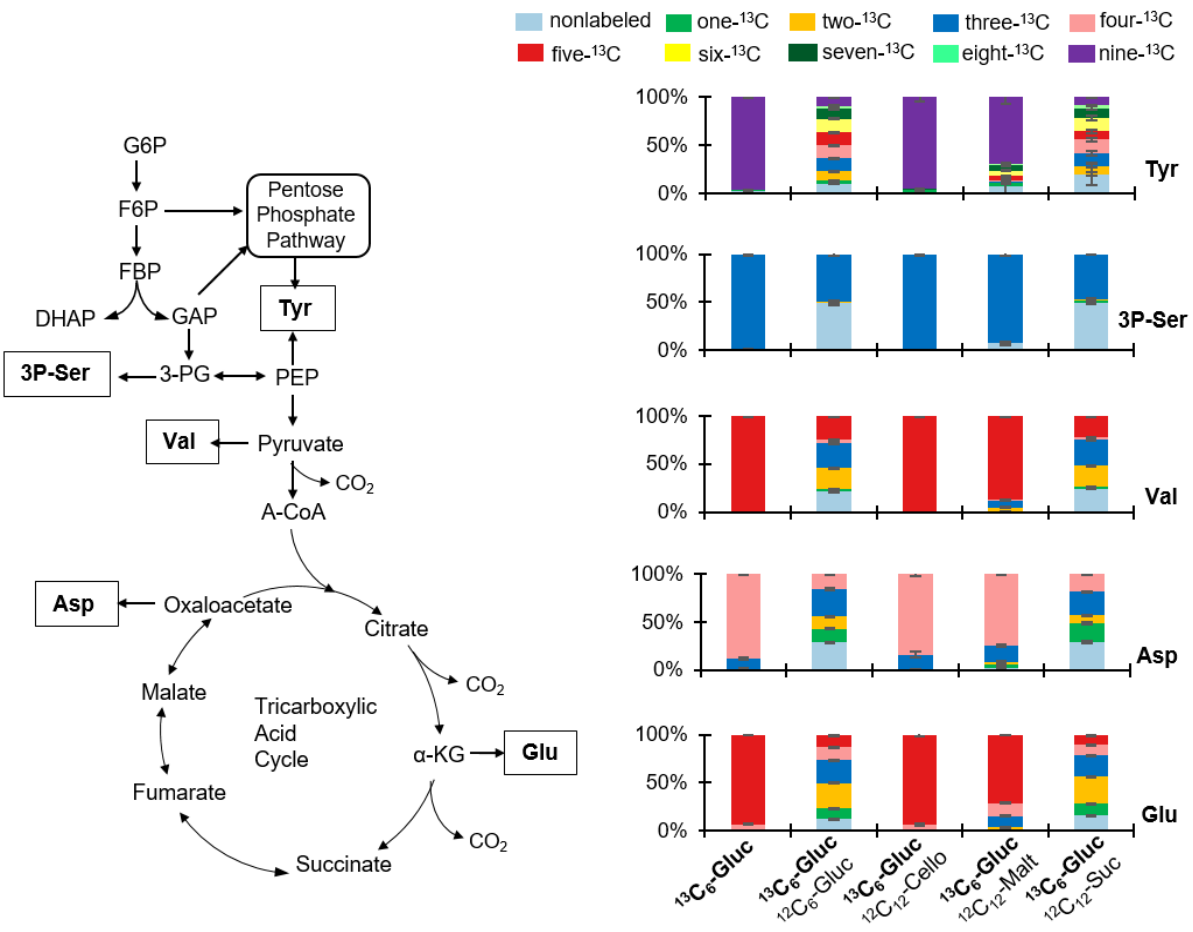

Figure 8: Incorporation of assimilated substrates into five amino acids: tyrosine (Tyr), 3-phosphoserine (3P-Ser), valine (Val), aspartate (Asp), and glutamate (Glu). On the left is a schematic overview of the central carbon metabolism that indicates the metabolite precursors to the different amino acids (shown in bordered text boxes). Legend for metabolite names: glucose-6-phosphate, G6P; fructose-6-phosphate, F6P; fructose 1,6-bisphosphate, FBP; glyceraldehyde-3-phosphate, GAP; dihydroxyacetone phosphate, DHAP; 3-phosphoglycerate, 3-PG; phosphoenolpyruvate, PEP; acetyl-CoA, A-CoA; α-ketoglutarate, α-KG. Color codes for the labeling isotopologues: nonlabeled carbon (light blue), one ^{13}C-carbon (green), two ^{13}C-carbons (orange), three ^{13}C-carbons (dark blue), four ^{13}C-carbons (pink), five ^{13}C-carbons (red), six ^{13}C-carbons (yellow), seven ^{13}C-carbons (eight), nine ^{13}C-carbons (purple). The measured data (averaged ± standard deviation) were from two independent biological replicates (n=2) and two technical replicates taken at mid-exponential phase when optical densities (measured at 600 nm) were ~0.9-1.1. Non-noticeable error bars were in cases where standard deviation values were small.

through octuply ^{13}C-labeled carbon (Fig. 8). The 3-phosphoserine was found to be about 7% nonlabeled and 92% fully ^{13}C-labeled (Fig. 8). Valine was about 87% fully ^{13}C-labeled with the remainder consisting of nonlabeled through quadrply ^{13}C-labeled fractions (Fig. 8). Aspartate was found to be about 17% triply ^{13}C-labeled, 74% fully ^{13}C-labeled, and the remainder as small fractions of nonlabeled through doubly ^{13}C-labeled carbon (Fig. 8). Glutamate was 12% triply ^{13}C-labeled, 13% quadruply ^{13}C-labeled, and 71% fully ^{13}C-labeled with a small fraction of doubly ^{13}C-labeled carbon (Fig. 8). This indicates that the small fraction of maltose observed entering glycolysis contributed to AA biosynthesis in *B. megaterium* (Fig. 7 and 8). Cells grown on the glucose:sucrose mixture have AA labeling that matches the patterns seen in the mixture of fully ^{13}C-labeled and unlabeled glucose indicating that *B. megaterium* can incorporate sucrose into AAs produced in all central carbon metabolism pathways in the presence of glucose to a similar extent as glucose alone (Fig. 8).

In accordance with complete inhibition of cellobiose catabolism in the presence of glucose, cells grown on the labeled glucose with cellobiose exhibited IMP and UMP labeling patterns similar to cells grown on labeled glucose alone (Fig. 9). This reflects the labeling patterns seen in glycolysis and the PP pathway metabolites which indicates cellobiose is not being metabolized in the presence of glucose. Cells grown on the labeled glucose with maltose mixture produced IMP and UMP with greater fractions of nonlabeled carbon as compared with the IMP and UMP produced from cells grown on fully ^{13}C-labeled glucose alone (Fig.9). The IMP in cells grown on glucose:maltose was 59% fully ^{13}C-labeled with the remainder being octuply and nonuply ^{13}C-labeled (Fig. 9). The UMP in these cells was 61% fully ^{13}C-labeled with the remainder being septuply and octuply labeled (Fig. 9). Cells grown on the glucose:sucrose mixture produced IMP and UMP with similar labeling patterns as the cells grown on the labeled and unlabeled glucose mixture indicating that sucrose was incorporated into these ribonucleotide precursors to a similar extent as glucose (Fig. 9).

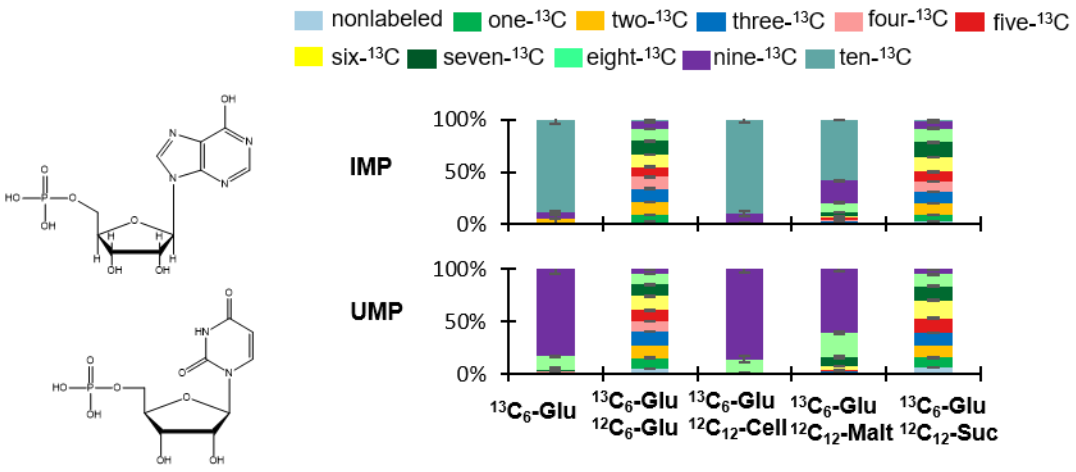

Figure 9: The structures on the left are inosine monophosphate (top) and uridine monophosphate (bottom). Color codes for the labelling isotopologues: non-labeled carbon (light blue), one ^{13}C-carbon (green), two ^{13}C-carbons (orange), three ^{13}C-carbons (dark blue), four ^{13}C-carbons (pink), five ^{13}C-carbons (red), six ^{13}C-carbons (yellow), seven ^{13}C-carbons (eight), nine ^{13}C-carbons (purple), ten ^{13}C-carbons (teal). The measured data (averaged ±standard deviation) were from biological replicates (n=2). Non-noticeable error bars were in cases where standard deviation values were small.

2.4 Conclusions

Bacillus species are important in soil environments and have been cultivated and utilized in bioinoculant products for their plant growth-promoting abilities, which include phosphate solubilization and production of secondary metabolites that suppress soil-borne plant pathogens (Stülke and Hillen, 2000; Chen et al., 2007). There is great diversity within the *Bacillus* genus yet much of the work on disaccharide catabolism has focused on transcriptional studies in the model organism, *Bacillus subtilis*. In pursuance of understanding an environmentally prevalent and commercially important species that is genetically distinct from *B. subtilis*, we evaluated disaccharide catabolism in *B. megaterium* (Vary, 1994). This study focused on the disaccharide metabolism of *B. megaterium* QM B1551 and the following three hypotheses were evaluated

here: (1) cellobiose uptake will be completely repressed in media containing glucose with cellobiose, (2) maltose uptake will be completely repressed in media containing glucose with maltose, and (3) sucrose uptake will be repressed in media containing glucose with sucrose, but to a lesser extent than cellobiose or maltose. Using ^{13}C tracer experiments, intracellular metabolite labeling was monitored to evaluate these hypotheses.

Cellobiose incorporation into intracellular metabolism was not observed indicating complete repression of cellobiose PTS genes in the presence of glucose. Maltose incorporation into intracellular metabolism was also inhibited in the presence of glucose, although to a lesser extent. Transcriptional studies have indicated repression of both cellobiose and maltose PTS genes in the presence of glucose in *B. subtilis* and the reason for the difference seen in the extent of repression in our study cannot be determined from our study or previous work (Tobisch et al., 1996; Tangney et al., 1992; Stülke and Hillen, 2000; Schönert et al., 2006). Subsequent studies are needed to determine what controls the extent of disaccharide repression in the presence of glucose. In contrast with cellobiose and maltose catabolism, repression of sucrose uptake was not observed in *B. megaterium*. This conflicts with transcriptional studies in *B. subtilis* that have shown sucrose uptake via a PTS protein is repressed in the presence of glucose (Kanehisa et al., 2017; Kanehisa et al., 2016; Kanehisa et al., 2000; Stülke and Hillen, 2000). It is not understood if this discrepancy is because of differences in metabolic machinery between the two species, or if the intracellular sucrase and extracellular levansucrase are not repressed and contribute significantly to sucrose catabolism. Transcriptional studies on *B. megaterium* for the various conditions tested in our study are needed to determine the cause of continued sucrose uptake in the presence of glucose. Better understanding disaccharide metabolism of *B. megaterium* helps to both optimize bioinoculant formulations that include *B. megaterium* and gives us a better general understanding of the metabolic diversity within the *Bacillus* genus.

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