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Investigation of Media Effects

on *Sinorhizobium meliloti* Glucose Minus

University of Redlands Honors Research Thesis

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Submitted to the faculty of the University of Redlands in partial fulfillment of the requirements
for the degree of Bachelor of Science in the Department of Chemistry

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Abstract

Although nitrogen fertilizers have certain benefits, the over application of such compounds often results in damages to the ecosystem. In this project, we focus our study on *Sinorhizobiom meliloti*, a species that lives in symbiosis with alfalfa plants, and through its nitrogen fixation capabilities, restores nitrogen levels in the soil. In our study we aim to gain a better understanding of the carbon metabolism in *S. meliloti*, mainly by looking at growth patterns in the presence of different carbon sources. Our research picked up on Erik Arvey's discoveries which pointed out that sucrose inhibits growth of certain glucose minus mutants of *S. meliloti* on a lactate and aspartate medium.[13] Due to this rather odd mutant behavior, we began our experimentation by growing mutant strains on other similar disaccharides such as cellobiose and trehalose, the breakdown products of sucrose, fructose and glucose, and increasing fructose concentrations. We even generated new mutant strains via TN5 mutagenesis to investigate their behavior as well. Overall we found out that other disaccharides don't inhibit growth and fructose which caused the highest degree of inhibition managed to slow down the growth over a longer period of time. Sucrose, also appeared to inhibit growth on succinate and aspartate medium, and nitrogen and lactate medium, suggesting that this growth delayed in the mutants could be due to a metabolite accumulating in the cell, or that the cells waste energy in breaking down sucrose which cannot be further metabolized. We also grew *S. meliloti* mutant and wildtype strains on gluconolactone, which helped us pin-point our library of mutations in the Entner-Doudoroff pathway.

Introduction

Fertilizers are used to enrich the soil with nutrients that can enhance the growth of plants and play a crucial role in agriculture today. Worldwide in 2011 an estimate of 105.3 million tons of nitrogen have been used [1]. While there are some advantages of using fertilizers such as accessible price and high nutrient content, the over-application usually results in negative outcomes [2]. Oversupply of nitrogen leads to softening of plant tissue, which can lead to a plant that is susceptible to diseases and pests. Yet, the most devastating effect of excess fertilizer is the negative impact it has on the environment. This situation can lead to pollution of water resources, disturbances in soil pH, eradication of micro-organisms and friendly insects and increase in populations of harmful bacteria [2]. Fortunately, naturally occurring micro-organisms such as rhizobia can optimize the growth of many leguminous plants important in our diet and the diet of farm animals due to their nitrogen fixing properties. It is reported that rhizobia can fix 50-300 kg N/ha which can replace up to 80 - 90 million tons of N fertilizers [3]. Furthermore, nitrogen fixing bacteria have also proved to be far less expensive than fertilizers [3].

In the atmosphere, nitrogen is plentiful. However, plants cannot assimilate nitrogen in the form present in the atmosphere. As a way to cope with this dilemma, leguminous plants and the rhizobia bacteria form a symbiotic relationship. The symbiosis consists in the plant providing numerous carbon sources that the bacteria can grow on, and in return the bacteria converting nitrogen from the atmosphere into ammonia, a form of nitrogen that can be consumed by plants [3]. The rhizobia bacteria usually live in the soil where carbon resources are scarce. When encountering a plant that can form nodules, the bacteria and the plant exchange molecular signals with each other, resulting in changes in gene expression [3]. The rhizobia attach to the plant's root-hair causing the hair to curl. This mechanism entraps the rhizobia in the root hair, which

leads to the formation of the infection thread [3]. Around the infection thread plant cells start to divide and the new cells are then invaded by the bacteria. Within the cellular environment the bacteria start to fix nitrogen in exchange for access to an abundance of dicarboxylic acids produced by the plant. This ultimately results in a mutualistic symbiotic relationship between bacteria and the plant [3].

In the process of nodulation, the rhizobia encounter a variety of environments, each more or less plentiful in carbon sources. In the soil, the rhizobia have access to little carbon sources which can be used for energy needs. This nutrient-depleted environment is beneficial for the plant's ability to attract the nitrogen fixing bacteria. Leguminous plants attract nitrogen fixing bacteria by simply releasing a variety of sugars and peptides surrounding the roots that facilitate the growth of microorganisms. This area is known as the rhizosphere. After the bacteria enter the rhizosphere it is only a matter of time until they infect the plant and start the nodulation process. Overall, as compared to the soil, the rhizosphere and environment within the plant, offer the bacteria a more optimal place to grow due to the availability of various dicarboxylic acids, monosaccharaides and polysaccharides. [3]
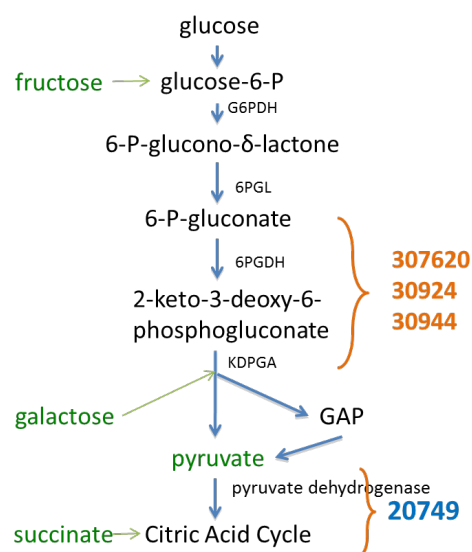

Figure 1 [5]. The Entner-Doudoroff Pathway and possible mutant strain locations.

In bacteria, the break-down of glucose occurs via the Entner-Doudoroff (ED) pathway. [12] This pathway represented in Figure 2 is a series of enzymatic reactions that convert glucose to pyruvate which enters the citric acid cycle in order to produce energy in form of ATP. Other monosaccharides such as fructose and galactose are also metabolized by the rhizobia. These sugars enter the ED-pathway at different steps as metabolites. Disaccharides, or polysaccharides are usually broken down into their monomers, which then also enter the ED-pathway. Therefore, the ED-pathway is highly significant in the metabolism of carbon compounds.

Since bacteria are constantly facing different environments with a variety of carbon sources available, catabolite repression (CR) evolved to be the mechanism which allowed bacteria to make the most efficient use of available resources. CR is a process that evolved on the principle that utilizing the best available carbon source first until it is consumed from the environment will account for the fastest growth of the colony.[10] Only when the best available sugar is expended will the next best carbon compound be considered for consumption. Sugars that would gain priority would be sugars located at lower entry points in the ED-pathway (Figure 1) mainly because those sugars are closest to the end-point and require the least amount of energy to be metabolized. For example, succinate which is a metabolite found in the citric acid cycle will induce CR on glucose in *S. meliloti*. [15] The process of CR varies from organism to organism,

and the exact mechanism of CR in *S. meliloti* is unknown.

Figure 2. A. Mechanism of glucose induced CR in *E. coli*. **B.** Proposed mechanism for succinate induced CR in *S. meliloti*. [4]

A study conducted by Pinedo and Gage (2009) investigated possible mechanisms of CR in
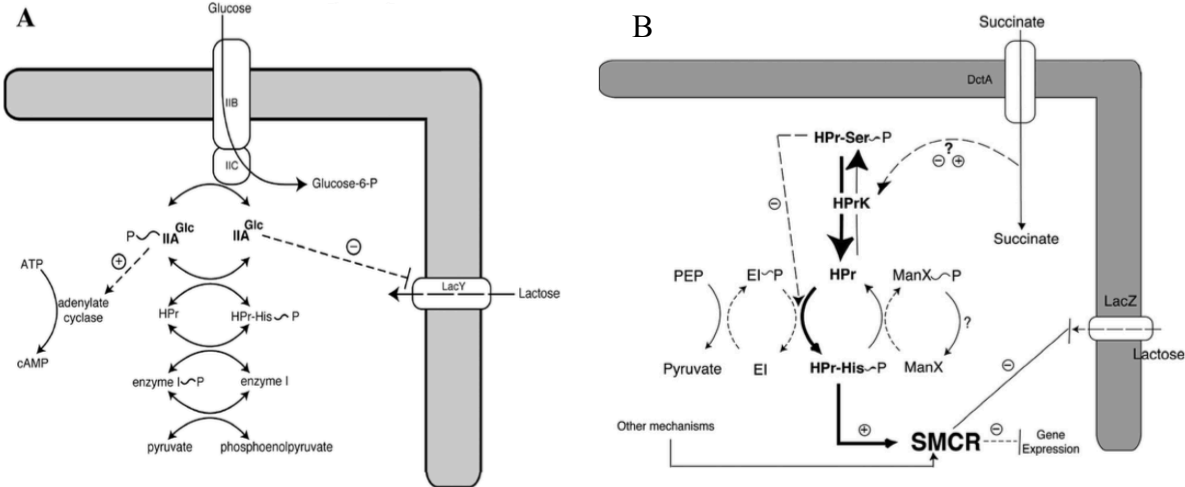
S. meliloti, a representation of their proposed mechanism is shown in figure 3. [4] In *E. coli*, glucose induced CR occurs as follows: glucose is taken into the cell and phosphorylated by the transporter protein (Figure 3A). When glucose gains a phosphate group, protein IIA becomes dephosphorylated, and loses a phosphate group. Dephosphorylating the IIA protein blocks lactose uptake via LacY transporter, a process known as inducer exclusion. However, when there is no glucose, IIA-protein is phosphorylated. This state of the protein activates adenylate cyclase, which in turn catalyzes the reaction that turns ATP into cAMP. Increasing concentrations of cAMP activate the lac operon which leads to a series of events that lead to the expression of enzymes able to metabolize lactose. In figure 3B, we can see that the mechanism proposed for succinate induced CR in *S. meliloti* is less clear and more complicated. Not only is succinate not phosphorylated, but it is also not sure how it affects the HPR kinase enzyme which seems to have a function in inducer exclusion CR on lactose. [4]

Velazquez et al (2004) on *Pseudomonas putida* looked at glucose induced CR. *P. putida* is

a strain that contains the TOL plasmid in its genome which offers it the ability to grow on

toluene, m-xylene or p-xylene[6]. In this organism, glucose induces CR by preventing the

transcription of genes located on the TOL plasmid. Also, because *P. putida* lacks certain

enzymes, glucose and gluconate are exclusively metabolized via the ED-pathway. In this study,

the mutants were grown on a minimum growth medium (M9), with casamino acids in the

presence and absence of glucose under various conditions: genetic modification that forced

fructose to be exclusively metabolized via the ED-pathway, a mutation that stimulated the

expression of ED-enzymes, and a mutation interrupting the ED-pathway. Their results indicated

that when fructose was exclusively metabolized via the ED-pathway, fructose induced CR and

hindered the growth of the bacteria. Furthermore, when the ED-enzymes were stimulated a

decrease in glucose induced CR was observed, and when the ED-pathway was hindered, glucose
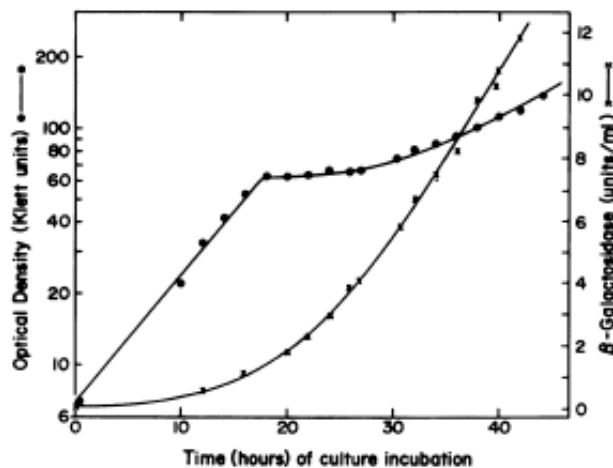
induced CR was increased[6].

Figure 3. Catabolite repression in *Sinorhizobium meliloti* growing on succinate and lactose.[7]

The experiment regarding CR in *S. meliloti* done by Ucker and Singer (1978) is depicted

in figure 3. Here it is observed that from 0 to about 20 hours the cells grow relatively fast but the

activity of β-galactosidase is at minimum. This indicates that the cells mainly consume succinate during this growth period. Soon after 20 hours, the growth halts and at the same time a drastic increase in β-galactosidase is observed. Soon after this increase, cells resume growth but at a slower rate. Due to the high activity of β-galactosidase it can be concluded that cells during this phase grow on lactose.[7] This phenomenon is an example of CR, mainly, the decrease in β-galactosidase activity such that cells can utilize succinate first.

Because in the presence of glucose, a decrease in β-galactosidase activity occurred, β-galactosidase assays are a useful tool to measure CR.[14] β-galactosidase is an enzyme that breaks down lactose by disrupting the glycosidic bond linking together the two monomers that form lactose, glucose and galactose. In the β-galactosidase assay, o-nitrophenyl-β-D-galactoside (ONPG) and the enzyme are added to a cuvette and time is allowed to pass. As the enzyme breaks down the glycosidic bond, O-nitrophenol is released which has a yellow color. A spectrometer will measure the absorbance of the solution at 420 nm. Knowing the absorbance and the time it took for the reaction to take place, using Miller's equation (1) we can calculate the β-galactosidase enzyme activity[8].

(1)
$$1000 * \frac{(Abs_{420} - (1.75 * Abs_{550}))}{(t * v * Abs_{600})} = 1 \text{ Miller Unit}$$

Where Abs_{420} is the absorbance of the yellow ONPG at 420 nm, Abs_{550} is the scatter from cell debris at 550 nm, Abs_{600} is the cell density at 600 nm, t is the reaction time in minutes, and v is the volume of cells in millimeters. Since the 420 nm accounts for the absorbance of both ONPG and the cells, in order to get a more accurate picture of the concentration of ONPG alone, we subtract the absorbance at 550 which accounts for cell debris.

Some carbon containing compounds are not only useful for generating energy but they also protect the bacteria against osmotic pressure. Osmotic pressure occurs when the environment of a cell contains a high concentration of certain electrolytes impenetrable to the cell membrane. Since water has a tendency to go from a lower chemical potential to a greater potential, water crosses the cell membrane to leave the cell and enter the environment. In order to prevent the water from leaving the cell, bacteria have evolved the mechanism of osmoprotection in which certain disaccharides accumulate in the cytoplasm creating a chemical potential within the cell equal to the chemical potential outside the cell[9]. This process is highly beneficial at counteracting osmosis.

Initially our research was focused on why sucrose, which is an osmoprotectant and a source of energy, inhibits growth of certain *Sinorhizobium meliloti* glucose minus mutants on a lactate and aspartate medium, as Erik Arvey's has observed.[13] The main question asked was whether the inhibition is due to CR, or due to a metabolite accumulating when the mutants used were unable to break down certain sucrose byproducts, or simply due to a waste of energy. To understand this phenomenon, we tried to see if other disaccharides also induce a growth inhibition on the mutants. We have also replaced the lactate and aspartate in the media with a other of carbon and nitrogen sources discussed later. Growth of the mutants was also analyzed when increasing concentrations of fructose.

Finally, we focused our research on the study of glucose CR on *Sinorhizobium meliloti*. Studying CR served as a means to understanding sucrose growth inhibition but also gave us a better picture of the unknown process by which CR occurs in *S. meliloti*, which is a model organism that belongs to the Rhizobiaceaea family. Specifically, we focused on the means by which glucose induces CR on other sugars such as lactose. Glucose is important because it is the

primary source of energy and the byproduct of many disaccharides and polysaccharides breakdown. Overall, understanding steps involved in glucose mediated CR would provide us with a better with a better understanding of glucose metabolism in *S. meliloti* as well as other rhizobium bacteria.

One key step in studying CR, was to generate mutants that are deficient in certain locations in the ED-pathway. We believed that we already had mutants located downstream from gluconate (Figure 2), our goal was now to add new mutants upstream from gluconate to our library. Research has shown that in *Escherichia coli* glucose induces CR by blocking the expression of lac operon as certain metabolites during the pathway act as lac operon gene suppressors [10]. Based on *P. putida* findings we tested and looked for metabolites that could cause glucose catabolite repression in *S. meliloti*. Since different metabolites are produced at different points in the ED pathway, we generated more mutants deficient at various locations in the ED pathway. Our aim was to generate mutations that would prevent the formation of metabolites upstream from 6-P-gluconate. These mutants were created using Tn5 mutagenesis. In Tn5 mutagenesis, *E. coli* carry a genetically engineered transposon attached to the plasmid found within the cell. A transposon is a gene that cause mutations due to its ability to randomly insert itself into different places in a genome. *E. coli* were then incubated with *S. meliloti.* During the incubation, *E. coli* plasmid was transferred to *S. meliloti* in a natural process known as conjugation. Since *S. meliloti* received a new plasmid which also contained a transposon, which after the incubation period, new mutations were generated in the *S. meliloti* genome [11].
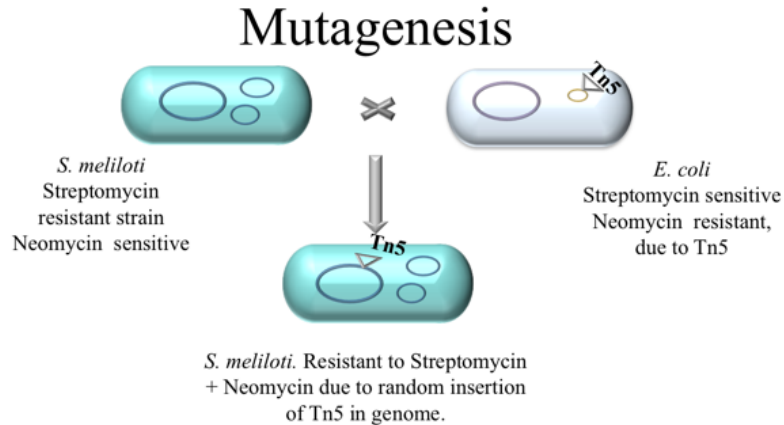
Figure 4. Mutagenesis summary.

Experimental

Bacterial strains, media, and growth conditions.

The bacterial strains used in this study were E. coli MM294 with the plasmid PRK602, and *S. meliloti* wildtype stain 1021 and SB112, and the mutants 20749, CT2141, 307620, 30924 and 30944. Strain SB112, unlike 1021, grows on gluconate. The bacterial growth was monitored with a Spectrophotometer 20 which measures optical densities of cells growing in liquid media at wavelength of 600 nm (OD_{600}).

Media

The LA9 liquid media was made using 6 g Na_2HPO_4, 3 g KH_2PO_4, and 0.5 g NaCl, all dissolved in 500 mL of deionized water, and autoclaved. After autoclaving, 1mL of 1.0 M $MgSO_4 \cdot 7H_2O$ (to give 1 mM), 0.25 mL of 1.0 M CaCl2 (to give 0.25 mM), 1 mL of 5 µg/mL CoCl2 (to give 5 ng/mL), 1 mL of 2.5% tryptone (autoclaved), and 10 mL of 0.1 mg/mL biotin (to give 1 µg/mL biotin) was added to the media. Biotin and tryptone were supplements added in order to help cells reach exponential phase faster. Unless otherwise specified, the carbon source we used was lactate and the nitrogen source we used was aspartate, both at 10 mM final concentration.

In LA9 media containing various disaccharides and monosaccharides, such as glucose, fructose, trehalose and cellobiose, were added to the media at a final concentration of 1 mM, unless otherwise indicated. The new volume addition for the sugar solution was subtracted from the total volume of sterile deionized water. In SA9 media, the lactate component was replaced with 10 mM succinate. In AA9 media, the lactate was removed and aspartate was used as the sole carbon and nitrogen source. To compensate for the missing carbon from the lactate, aspartate was used at 20 mM final concentration. In LN9 media, the aspartate was replaced with ammonium chloride, at 10 mM final concentration and lactate was used at 20 mM, to compensate for the loss of carbon from the aspartate molecule.

The M9T media had the same components as the LA9 media, except that it had no lactate or aspartate and instead only one carbon source at a final concentration of 0.2% and ammonium chloride as the nitrogen source. The agar M9 media contained 0.4 % of any single carbon source and no tryptone. Tryptone is known for accelerating bacterial growth, and was thus removed in order to observe a greater growth inhibition. The LB liquid media was made by dissolving 10 g of Bacto tryptone, 5 g of Bacto yeast extract, and 10 g of NaCl in 1 liter of deionized water and then autoclaved. The LB solid media was obtained by adding 15 g of agar to the LB liquid media prior to the autoclave step.

<u>Mutagenesis</u>

The mutagenesis was conducted in order to generate new mutant strains more upstream than 307620, 30924 and 30944. SB112 was inoculated in liquid LB media and *E. coli* MM294 PRK602 was inoculated in LB media containing neomycin 50 ug/ml, to make sure the plasmid PRK602 was maintained in the culture. Three LB plates, each containing a sterile filter were prepared. 1 mL of *E. coli* was placed in two different centrifuge tubes, 1 mL of *S. meliloti* was

also placed in two different centrifuge tubes. The four tubes were then centrifuged for 30 seconds, and the supernatant from each tube was removed. The tubes containing *E. coli* cells were then washed in 0.5 ml LB liquid media for two more times to remove any excess neomycin from the solution. To allow the mutagenesis to occur, the *S. meliloti* in one of the tubes was combined with *E. coli* cells and mixed well. The mixture was placed on a filter paper on the LB plates. For control, the contents in the tube containing *S. meliloti* were placed on a filter paper on the LB plates. The same procedure was repeated for the tube containing *E. coli* cells. The cells were then incubated at 30°C overnight.

The screen for our mutants consisted of diluting the mutant cells in deionized water to obtain about 50 cells per plate, and then spreading the cells on M9 plates containing 0.4% glucose, .04% gluconate, 200 µg/ml neomycin, and 400 µg/ml streptomycin. When the colonies grew, the colonies that were significantly smaller were picked and patched onto LB plates containing 400 µg/ml streptomycin and 200 µg/ml neomycin.
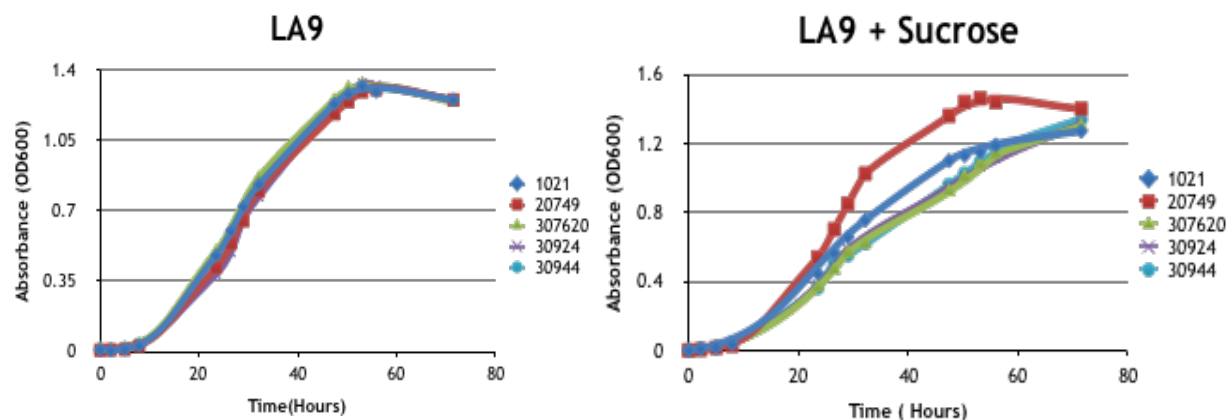
Results and Discussion

Figure 5. Growth on LA9 with and without sucrose.

Figure 5 shows the growth of each mutant and the wild-type strain on LA9 medium in the presence and absence of sucrose. Looking at the exponential growth we can see that when

sucrose is absent in the media, the mutants grow just the same as the wild-type. However, when sucrose is added, glucose-minus mutants (307620, 30924, and 30944) reach the stationary phase later as compared to the wild-type.
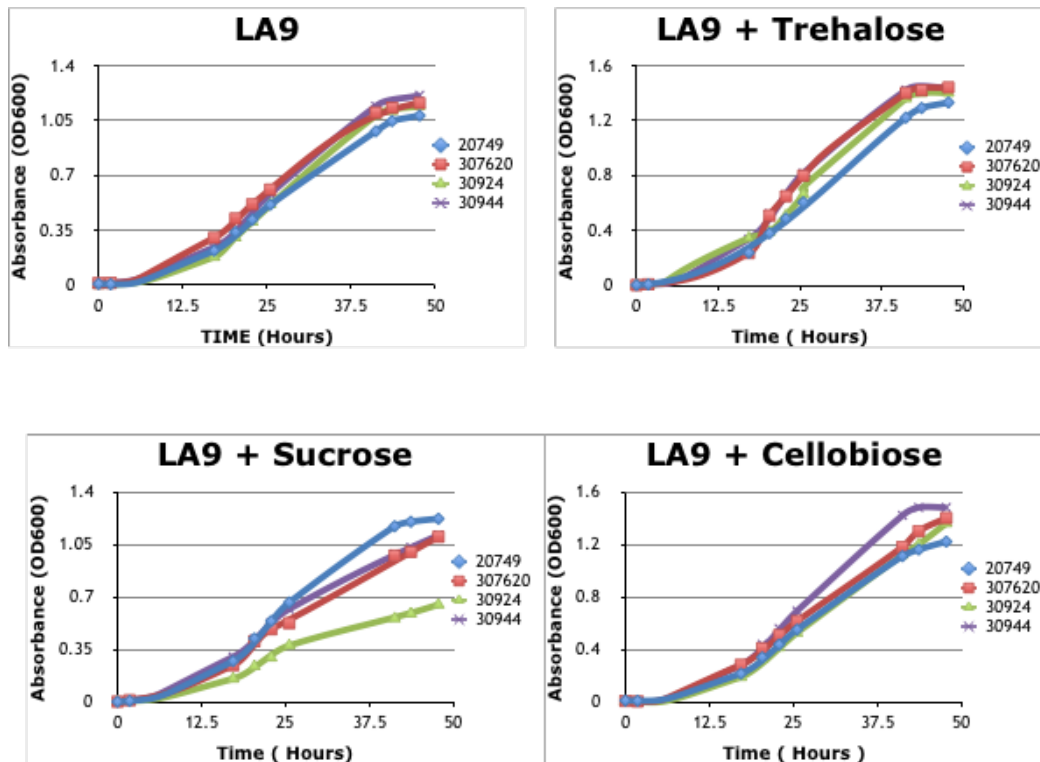

Figure 6. Growth on LA9 and other disaccharides.

Next, we checked the growth of the mutants on LA9 in the presence of other disaccharides. Cellobiose and trehalose are structurally different from sucrose because they are composed of two glucose monomers, as opposed to a glucose and a fructose monomer. Again, similar behavior was observed, on LA9 alone all mutants behaved the same. However, in the presence of other disaccharides, mutants 307620, 30924, and 30944 do not grow significantly slower. This phenomenon is seen especially on LA9 with sucrose. Due to an experimental issue, 20749 served as the wild-type in this experiment.
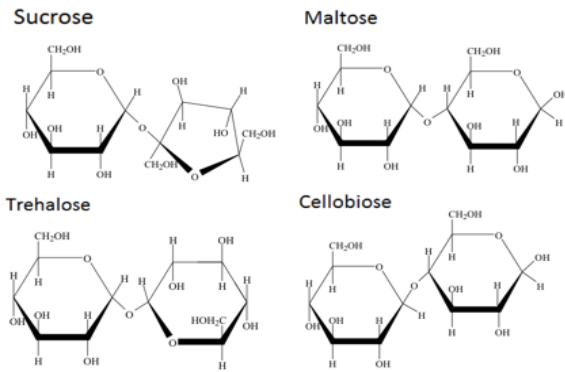
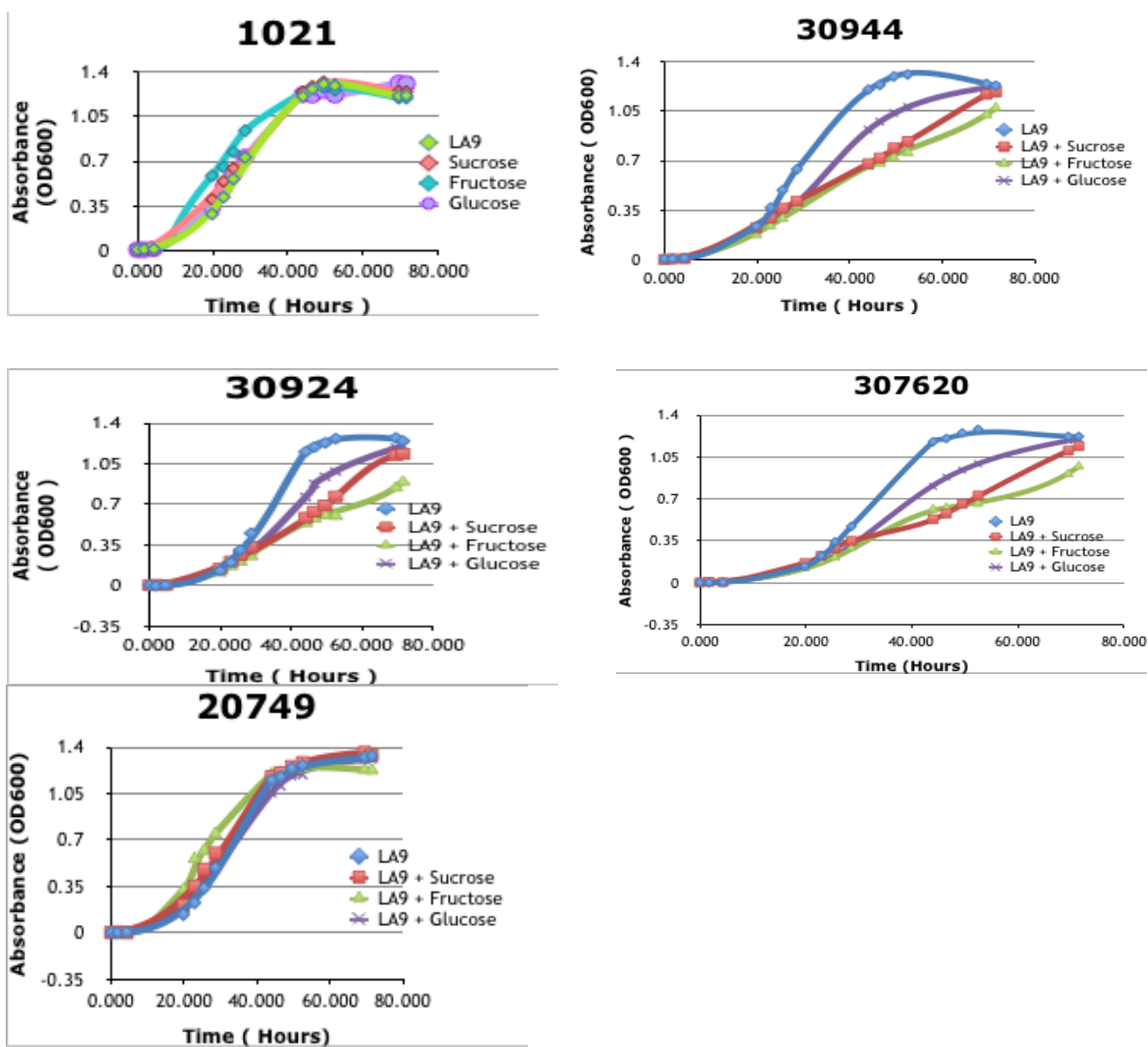**Figure 7.** Structure of sucrose, maltose, trehalose, and cellobiose

Figure 8. The wildtype strain and each mutant growing on LA9, and LA9 with sucrose, fructose

or glucose.

Sucrose was broken down into its components, fructose and glucose, and the wild-type and each of the mutants was inoculated in LA9 media and LA9 media with the monosaccharides (Figure 8). The wild-type strain and 20749 had the same growth curve regardless of the carbon source, however, the other mutants grew significantly slower when glucose or fructose were present in their media.
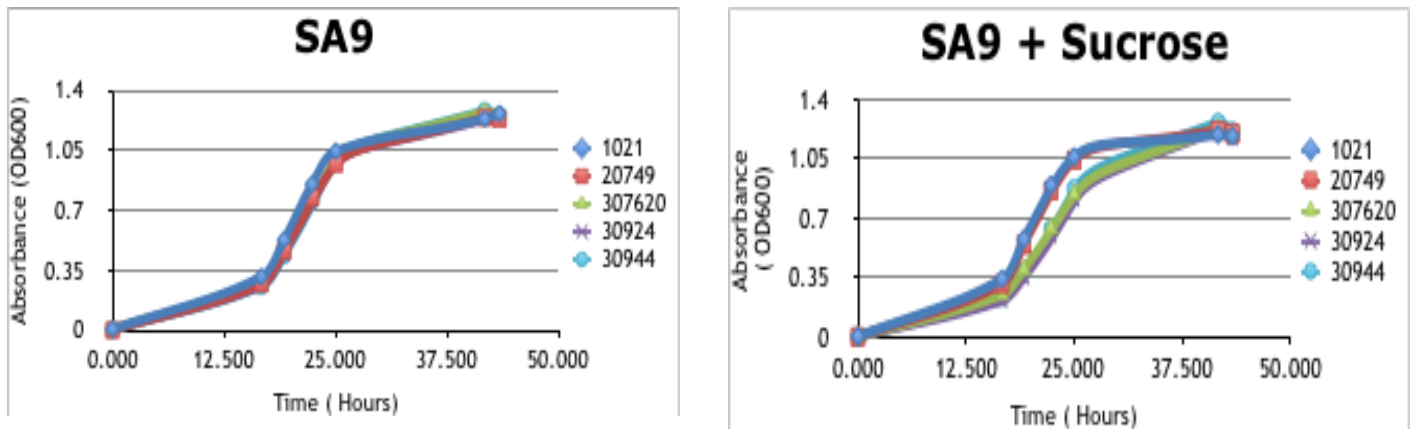

Figure 9. Growth on LA9 medium in which lactate was replaced with succinate, in the presence or absence of sucrose.

After conducting the experiments on the monosaccharides we suspected that the growth inhibition was due to CR. In order to check if CR occurs when mutants are growing on LA9 and sucrose, we replaced lactate which might be subject to CR by glucose with succinate (Figure 9). Since succinate is an intermediate in the citric acid cycle, we did not expect the mutants to show growth inhibition even if sucrose was present. Figure 9 shows that although the growth was not as delayed as previous experiments have shown, the growth curve of the mutants and the wild-type or 20749 were not similar when sucrose was added to the SA9 media. This hinted towards the idea that metabolites could be accumulating with the mutant cells, preventing them from utilizing energy in a most efficient manner.
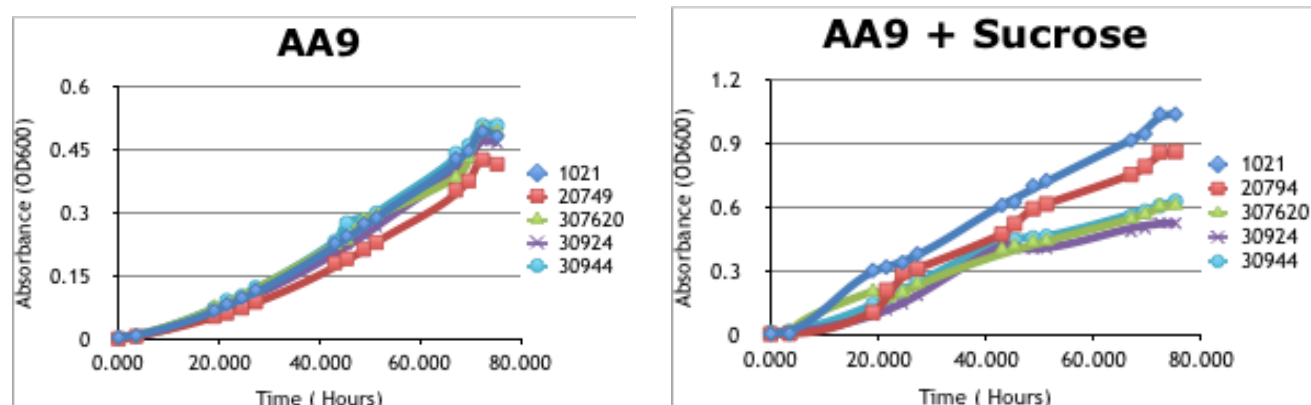
Figure 10. Growth on 20 mM aspartate and LA9 salts, in the presence or absence of sucrose.

We wanted to check that the reason we were seeing delayed growth on sucrose was not because glucose induced CR on lactate. Therefore, we made a new media in which lactate was completely removed and the bacteria was growing only on aspartate. In case of CR, sucrose should not have been able to inhibit growth on aspartate because aspartate is processed via a different pathway than ED-pathway, it may enter the citric acid cycle as oxaloacetate. Therefore, because growth inhibition was still present in bacteria growing on AA9 and sucrose (Figure 10), we hypothesized that this was not because CR but rather that this was further evidence that accumulation of metabolites produced while trying to break down sucrose in the mutant cells could be what is slowing down their growth.
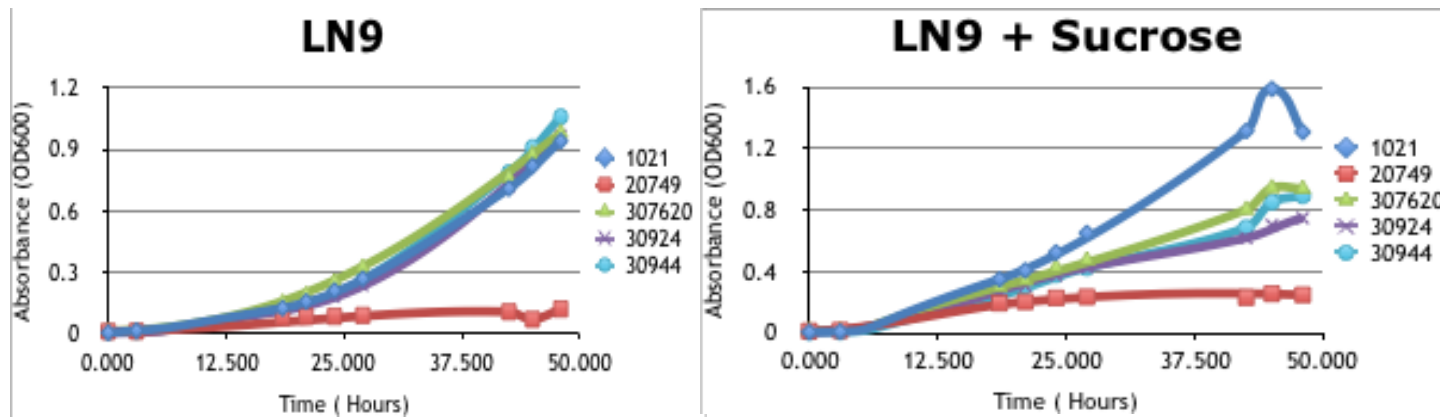
Figure 11. Growth on LA9 medium with aspartate replaced with ammonia, in the presence or absence of sucrose.

To make sure that there is no possibility for the carbon generated from metabolizing aspartate to interfere with the growth on the media in the presence of sucrose, we completely replaced aspartate with ammonia as the only nitrogen source. Growth inhibition still occurred in the mutants. 20749 was not able to grow at all due to its mutation after the ED-pathway and the fact that it can't use lactate as a carbon source.
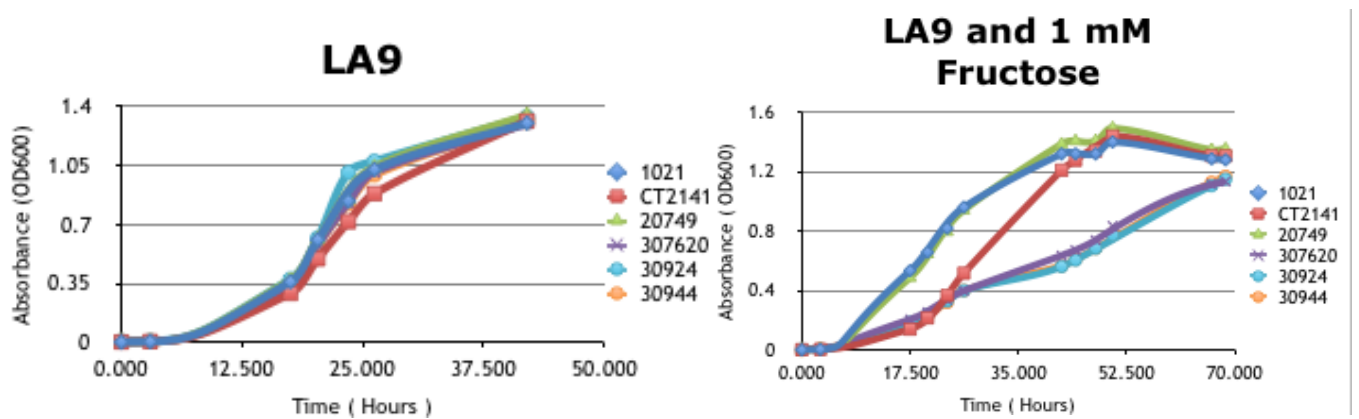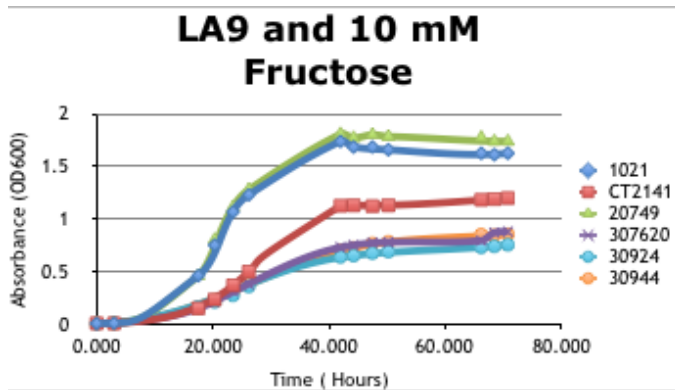
Figure 12. Growth on LA9 medium and LA9 medium with 1 mM fructose, and 10 mM fructose.

Since fructose was able to to induce the highest degree of growth inhibition as seen in figure 6, we decided to check and see what would happen if the mutants are grown on increasing concentrations of fructose. It can be seen from figure 12 that in the LA9 medium with no fructose present all mutants and wild-type grew at the same time. However, increasing fructose concentration also increased the amount of time it took for the mutants 307620, 30924, and 30944 to grow, or reach an absorbance as high as in the absence of fructose.
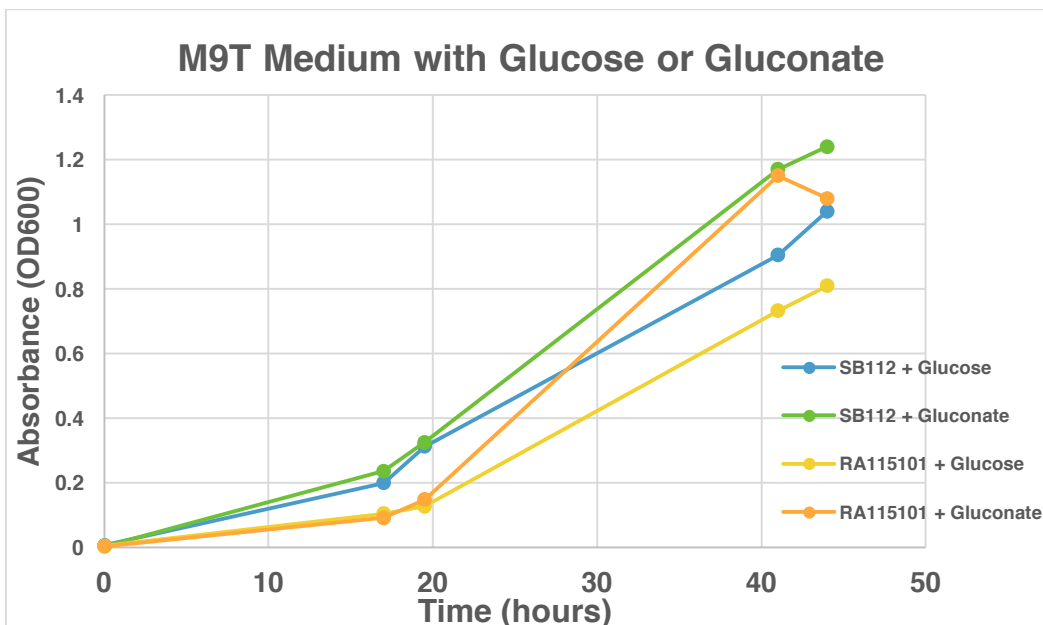

Figure 13. SB112 and mutant RA115101 growing on M9T medium in the presence of either glucose or gluconate.

A next step in our experiment was conducting mutagenesis, where we aimed to obtain mutants located upstream from gluconolactone. After we obtained the selection plates, one mutant that looked promising was RA115101. This mutant was inoculated in M9T with glucose or gluconate and it was observed that the mutant grew fine on both glucose and gluconate as compared to the wild-type SB112. Because the mutant was able to grow on gluconate, we could not conclude that the mutation was upstream from gluconate.

Furthermore, we were also interested in growing the bacteria on gluconolactone which is the metabolite just upstream from gluconate. This technique of growing mutants on gluconolactone would help us find mutants that are located upstream from gluconate.
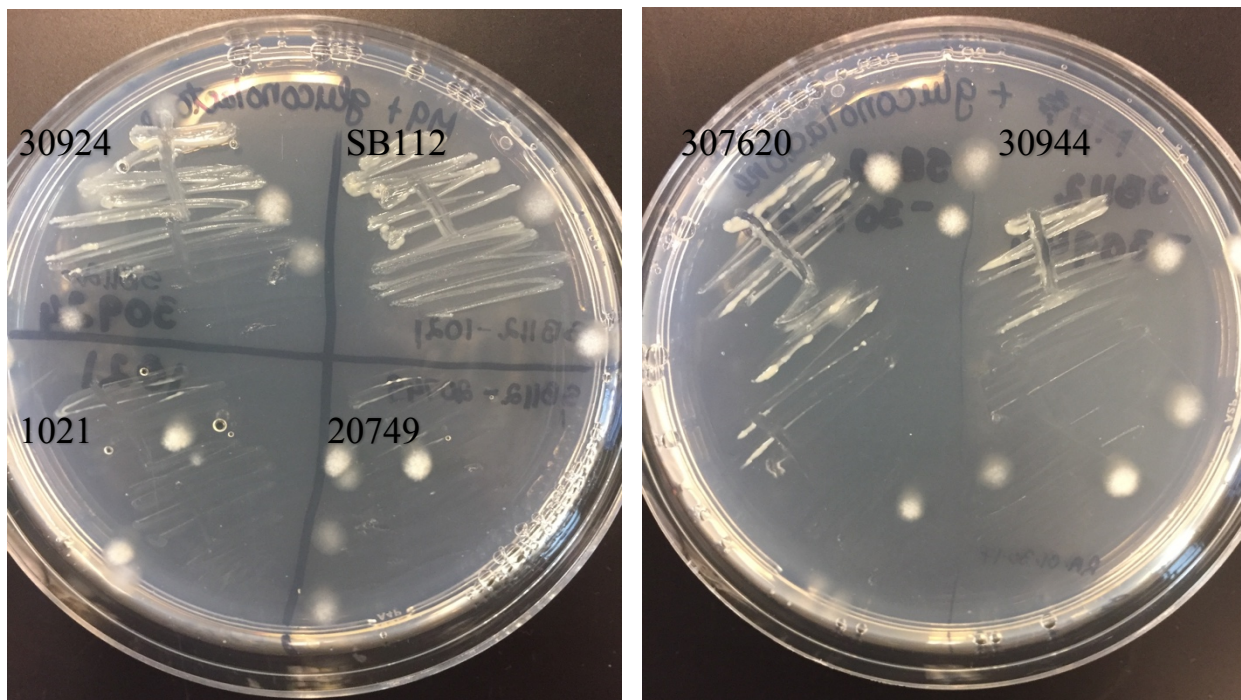

Figure 14. Growth on M9 medium and gluconolactone. The white colonies must be ignored as they represent contamination.

Figure 14 shows the growth of mutants and wild-type on gluconolactone. This experiment was conducted mainly to check and see if bacteria can grow a media containing

gluconolactone as the sole carbon source. We are able to see that indeed, the wild type SB112 and the mutants were able to grow on gluconolactone. This indicated that the mutant location could be anywhere upstream from gluconolactone and downstream from glucose-6-phosphate. However, one possible issue with gluconolactoe could be that it might spontaneously react to become gluconate. In figure 14, 20749 showed no growth because 20749 can only grow on pyruvate and other intermediates that are not processed down the ED-pathway.
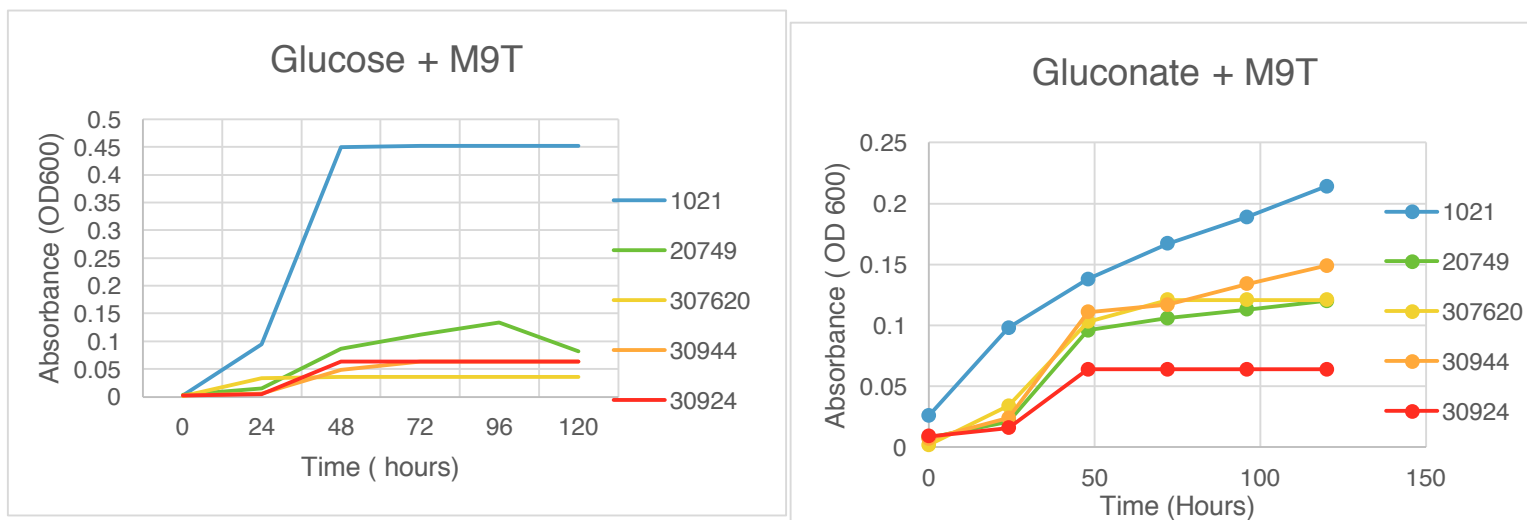

Figure 15. Mutant strains and wild type growth on M9T medium and glucose or gluconate.

In figure 15 we grew the mutants and the wild-type strains on M9T with glucose or gluconate. However, we did not have enough growth to be able to harvest cells to conduct an enzyme assay on the cells.

As future work, we would like to conduct more literature search on gluconolactone to see how stable it is and be able to trust that it does not react to become gluconate. We would also like to search the literature about ways in which gluconolactone is taken up in the cells and locations at which it enters the ED-pathway. Knowing more about this compound could help us develop our screening technique for mutants, and would allow for an easier process to find

mutants upstream from gluconate. It would also be helpful if we can repeat the growth on M9T with glucose or gluconate and obtain a substantial amount of cells that could allow us to conduct the B-galactosidase enzyme assay.

Summary of Results

After growing the mutant and wildtype strains on other disaccharides (cellobiose and trehalose) it was interesting to see that these sugars don't inhibit the growth of mutants. With sucrose remaining our main disaccharide inhibitor, we considered looking at how the mutants behave in the presence of sucrose break down products, fructose and glucose. Since fructose appeared to have a greater impact on growth, we further increased the fructose concentration to notice that the higher the fructose concentration, the slower mutants grew. Sucrose, also appeared to inhibit growth on succinate and aspartate medium, and nitrogen and lactate medium. This suggested that beside catabolite repression the growth delayed could also have been due to stress in the cell caused by metabolite accumulating, or possible wasted energy in breaking down sucrose which cannot be further metabolized. Furthermore, we were interested in pin-pointing the mutations that caused the deficiency in enzymes of the Entner–Doudoroff pathway. In order to do so we grew them on gluconolactone. After this experiment we noticed that except for the wildtype, 1021, the mutants and SB112 were able to thrive, while 20749 was unable to form significant colonies. This indicated that the mutant location in these strains could be anywhere upstream from gluconolactone and downstream from glucose-6-phosphate.

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