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Glucose-mediated catabolite repression in Sinorhizobium meliloti

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Abstract:

Alfalfa plants and the bacteria *Sinorhizobium meliloti* interact in a symbiotic relationship in which the alfalfa plant receives a useable form of nitrogen, ammonia, to use as a fertilizer, while the bacteria receive a carbon and energy source from the plant roots. During this exchange, there is formation of a root nodule, and these bacteria are implanted inside the alfalfa root cells. To convert the nitrogen by the process of nitrogen fixation for the alfalfa plant, Sinorhizobium meliloti requires a great deal of energy. To conserve the energy that it has, this bacterium performs what is known as catabolite repression. Catabolite repression is the process by which the bacterium uses the carbon source that is most energy efficient for it, and represses the genes responsible for all the other carbon sources. When observing succinate control over the production of B-galactosidase, catabolite repression was seen. (Ucker and Signer, 1978). Glucose-mediated catabolite repression and succinate-mediated catabolite repression were also observed with these bacteria, and an additive effect was observed when both were tested together, suggesting that glucose and succinate-mediated catabolite repression may occur by different mechanisms. (Jelesko and Leigh, 1994). Mutated strains 20749, 307620, 30944, and 30924 were used to identify genes responsible for glucose-mediated catabolite repression. Strains were grown in M9t media with various carbon sources (gluconate, glucose, lactose), and growth was observed by taking absorbance readings on a single-beam spectrophotometer. Growth curves constructed gave many conflicting results, and more growth experiments must be done to confirm growth of these strains on various carbon sources.

Introduction:

Legumes are a family of important crops that have a major impact on the agricultural world. They are grown primarily for their grain seed, called a pulse, for livestock forage, and as a soil enhancing green manure. Legumes are of interest to this project due to the prospect of them being a green manure.

Legumes and bacteria are in a symbiotic relationship with each other that provides better living conditions for both species involved. In this relationship between the plant species alfalfa and the bacterial species *Sinorhizobium meliloti*, the bacteria receive carbon sources from the plant, while the plant receives ammonium that can be used as a fertilizer. The plant is not capable of using atmospheric nitrogen directly, and it must be converted to the usable form of ammonia, which is done by the bacteria (Van Rhijn and Vanderleyden, 1995).

Across the country, many kinds of legume crops are produced, and so this relationship is one of interest for our society, and understanding how the relationship between these bacteria and the plant works can make it possible to generate better crops that are easier to produce. Furthermore, when a legume dies in a field, all its remaining nitrogen is released back into the soil. In the soil, the nitrogen is then available for future plants to use as fertilizer. A better understanding of how alfalfa and these bacteria work together could lead to production of crops that would require less artificial fertilizer, costing farmers less money, and providing a more natural product to consumers.

Sinorhizobium meliloti is found in a variety of environmental conditions around the plant that include the soil, the area surrounding the plant roots, and the actual root nodule. Nutrients vary from one area to another, and the root nodule contains the most concentrated nutrients

for the bacteria, while the soil itself contains the least amount of nutrients. In the bare soil, nutrients found can include ammonium, atmospheric nitrogen, organic matter, and nitrates (Donegan, et al., 1999). The area surrounding the roots as well as the root nodule are more nutrient rich. (Atlas and Bartha, 1987). The water concentration in the bare soil is very low, and the bacteria that are found in the bare soil struggle to survive due to the low nutrient and water conditions.

The area that surrounds the roots is called the rhizosphere. There are much higher concentrations of nutrients and water found here, as compared to the bare soil, so there are much higher concentrations of bacteria found in the rhizosphere. In the rhizosphere, the bacteria can encounter carbohydrates that are released by the plant roots. The plant also contributes other nutrients, which include amino acids, tannins, vitamins, keto acids, alkaloids, and phosphatides. In the rhizosphere, the plant releases higher levels of nutrients because there is more CO_2 in the rhizosphere, which induces the plant to release the nutrients (Atlas and Bartha, 1987). The plant, alfalfa in this case, in turn recognizes the bacteria in the soil, and wants to attract the *Sinorhizobium meliloti* to its roots to form the root nodules.

The root nodules are the area containing the highest level of nutrients for the bacteria. In the root nodules, the bacteria are inside the plant cells, which allow the bacteria to have access to all the nutrients that are held in the plant roots. The main nutrients they have access to are carbon sources. The bacteria have an abundance of carbon sources in the nodules, which in turn is an abundance of energy, which enables the bacteria to perform their metabolic processes. One of these metabolic processes is the process of nitrogen fixation, which allows

the bacteria to convert the unusable atmospheric nitrogen into the usable form of ammonia for the plant, thus completing the symbiotic relationship.

As the exchange of nutrients is happening, the bacteria also begin to move toward the root walls. The bacteria begin to build up around newly growing root hairs, which cause curling and branching of the hairs (Van Rhijn and Vanderleyden, 1995). As this happens, the bacteria are enveloped by the root hairs, and then the bacteria penetrate into the plant roots. Figure 1 displays the process of interacting with root hairs, and root nodulation. Once the bacteria enter the plant root, they spread and create an infection thread, where bacteria can then enter newly growing plant root cells. This is when the bacteria began the nitrogen fixation reaction.

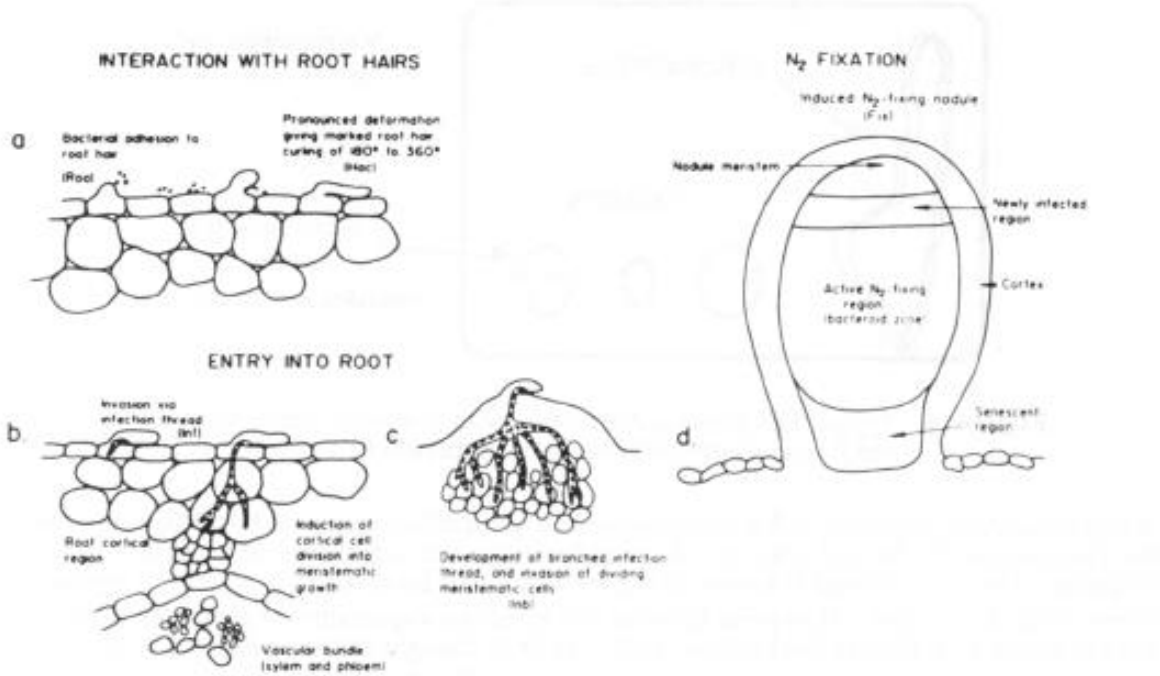

Figure 1- Nodulation
(Richardson and Rolfe, 1988)

To perform nitrogen fixation, the bacteria use the carbon sources that are found within the plant root nodules. Nitrogen fixation is the process of converting atmospheric nitrogen,

which the plant is not able to use, to the usable form of ammonia, by the reaction shown below in Scheme 1.

$$N_2 + 8e^- + 8H^+ + 16\ ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$

Scheme 1: Nitrogen Fixation (Dalton, 2007)

The supply of carbon from the plant root allows the bacteria to provide the plant with a constant supply of ammonia (O'Gara, et al., 1989).

Since the bacteria have multiple carbon sources available, the bacteria must choose which source to use to maximize efficiency. It has been shown in previous research that the primary carbon source the bacteria choose to use is succinate (Bringhurst and Gage, 2002) (Ucker and Signer, 1978). There has not been much research done into what other carbon sources the bacterium might also choose preferentially, while repressing the genes for the other sources.Because of this, the interest of this research was a different carbon source, glucose, which has not been studied extensively. The availability of glucose is high in the rhizosphere, where there is a high availability of nutrients in general.

S. meliloti catabolize glucose through a cycle known as the Entner-Doudoroff pathway (Geddes and Oresnik, 2014) shown in Figure 2.

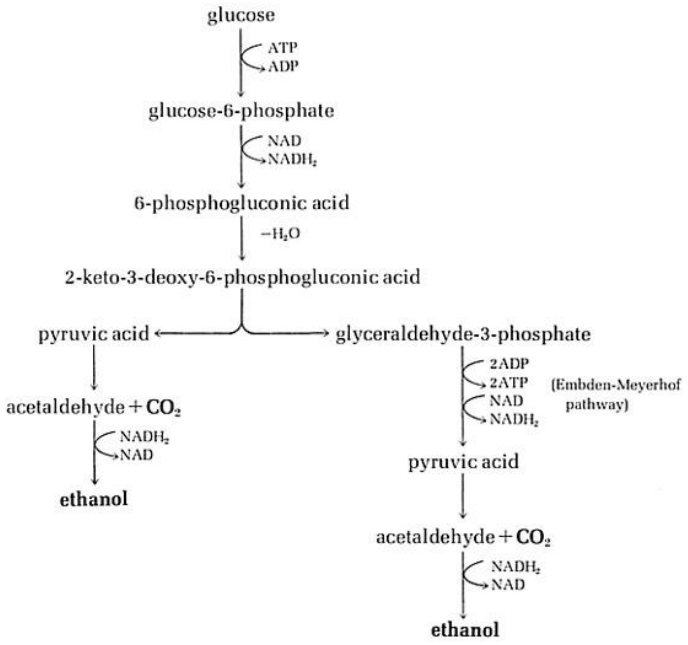

Figure 2- Entner-Doudoroff Pathway (Todar, 2004)

Pyruvate is converted to acetyl-CoA by the enzyme pyruvate dehydrogenase, and then acetyl-CoA goes into the citric acid cycle. Through the citric acid cycle, the acetyl-CoA is used to produce ATP for the cell to use as energy. Succinate is already a component in the citric acid cycle, and because of this, it is efficient for the cells to use it because it does not need to be converted into another form. Other carbon forms such as lactose require extra steps to be incorporated into the citric acid cycle. Lactose must be cleaved into glucose and galactose by β-galactosidase, as shown in Figure 3, to be brought into the Enter-Doudoroff pathway and then into the citric acid cycle, and these extra steps make lactose a less efficient source for the bacteria than succinate or glucose.

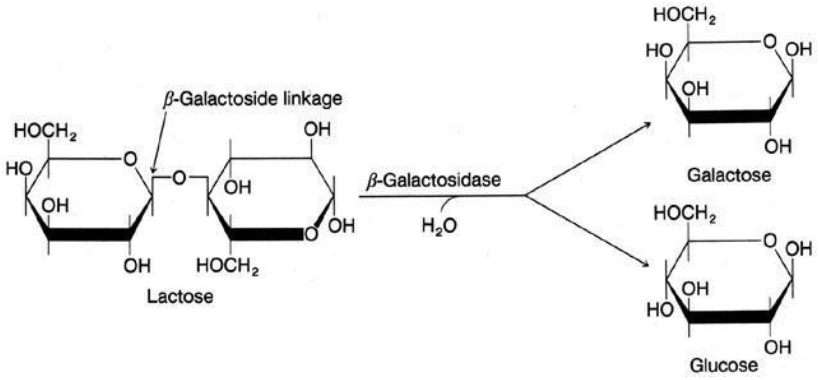

Figure 3- Lactose Metabolism (Pierce, 1993)

The process of the bacteria choosing one carbon source over another is called catabolite repression (Todar, 2004). The bacterium chooses which carbon source is most efficient for it to use energy-wise, and then prevents the production of enzymes that are needed to metabolize the other carbon sources.

In S. meliloti, the primary carbon source is succinate, while other carbon sources include glucose, lactose, fructose, pentose, and galactose (Bringhurst and Gage, 2002). Previous work done by Ucker and Signer found that succinate exhibited catabolite repression of lactose. They measured this catabolite repression by measuring β-galactosidase activity in the cells. Figure 4 shows a graph of their results, and plots the optical densities of the cells and the β-galactosidase activity against the time the cells were incubated. As can be seen in the figure, there was rapid cell growth and high optical densities early in the growth. At this point, the bacteria were metabolizing succinate. At approximately 18 hours, the growth levels off, because the bacteria had used up the supply of succinate and needed to grow on the lactose. At this point, as shown on the graph, the β-galactosidase activity greatly increases, as more β-

galactosidase is needed to metabolize the lactose that the cells are now growing on (Ucker and Signer, 1978). This shows an example of catabolite repression of succinate on lactose.

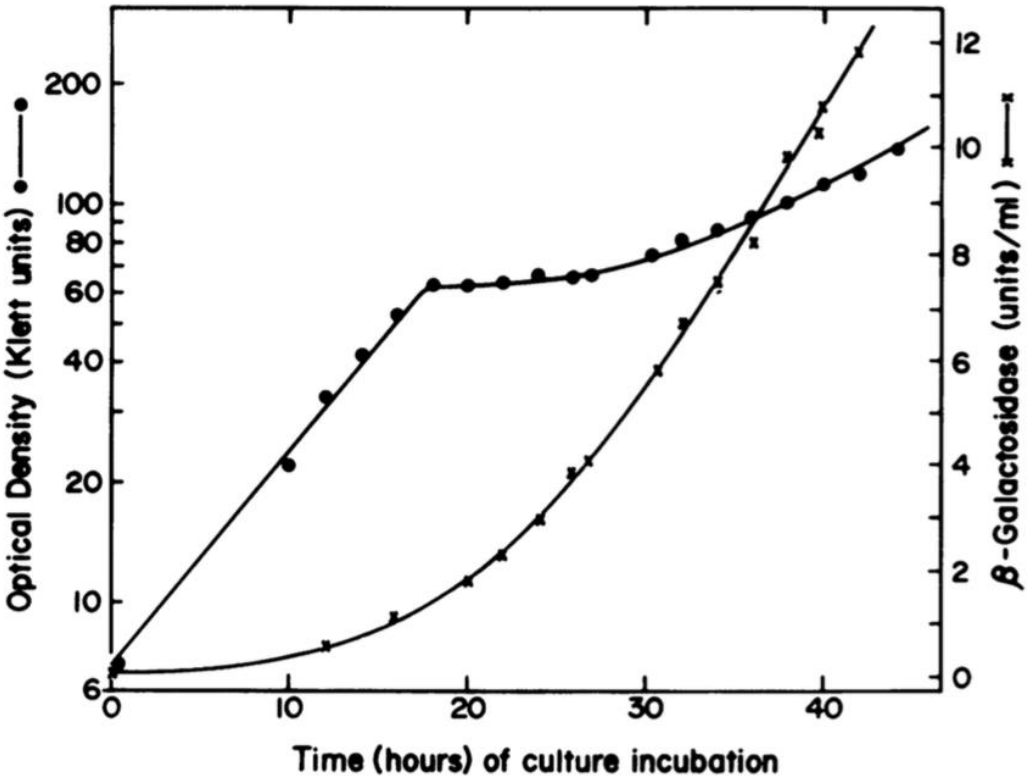

Figure 4- *S. meliloti* growth on Succinate and Lactose
(Ucker and Signer, 1978)

Other previous research has shown that the wild type *S. meliloti* also displays catabolite repression of lactose while in the presence of glucose (Jelesko and Leigh, 1994). When succinate and glucose are both present, there is an even greater repressive effect on lactose (Jelesko and Leigh, 1994). This suggests that there could be different mechanisms that control succinate and glucose in the production of β-galactosidase in the cell, and the pathways are independent from one another.

To find the mechanism for glucose-mediated catabolite repression, the genes involved in expressing this glucose effect needed to be analyzed. Mutated S. meliloti found not to exhibit catabolite repression in the presence of glucose and the presence of lactose may contain damaged catabolite repression genes. Mutants that might fit this description were found and characterized by Roxanna Apostol during previous research. These strains were all in the wild-type background (phenotype of the typical form as found in nature), and labeled as 1021 (wild type), 20749, 307620, 30944, and 30924. These strains are all glucose-minus mutants. These strains were also moved into the SB112 background (also conducted by Roxanna Apostol). The SB112 background mutants grow on gluconate, which likely enters the Enter-Doudoroff pathway farther along than glucose does.

This research is specifically focused on what happens to glucose in *S. meliloti.* To do this, we currently have, and are getting more, mutants that are blocked somewhere in the glucose metabolism pathway. This research is attempting to figure out whether these mutations still allow for catabolite repression, and also, where these mutants are blocked. Preliminary work suggests that these mutants might be blocked after 6-phosphogluconate in the Entner-Doudoroff pathway, however, recent work has shown that there might be questions regarding these results. Current work will focus on experiments regarding catabolite repression in these mutants.

It can be determined whether the cell is using glucose or lactose as a carbon source by looking at the activity of β-galactosidase. When comparing the β-galactosidase activity of cells that are just using lactose as their primary carbon source versus cells using glucose in the presence of lactose, the difference can be clearly seen. Previous research found that there is

about a one to three difference in the activity of β-galactosidase between cells grown on lactose alone compared to being grown in the presence of both glucose and lactose (Jelesko and Leigh, 1994).

The activity of the β-galactosidase can be measured using a UV-vis spectrophotometer. When β-galactosidase cleaves the β-D-galactosides, the colorless compound o-nitrophenol-β-D-galactosidase (ONPG, added to cells) is hydrolyzed into galactose and o-nitrophenol. The conjugate base, o-nitrophenolate, produces a yellow color, turning the cells from colorless to yellow. This yellow nitrophenol compound absorbs light at 420 nm (Miller, 1972). An OD_{600} value is measured at 600 nm, and this value determines the concentration of cells. ONPG is added in excess, which allows the assay to be linear, and the reaction is stopped by the addition of Na_2CO_3.

The addition of the sodium carbonate raises the pH to about 11, which is above the activity of the β-galactosidase (Miller, 1972). An OD_{420} reading is a mixture from the o-nitrophenol and light scattering from cell debris, and this interference is corrected for by a background reading at 550 nm (OD_{550}) (Miller, 1972).

$$\text{Light scattering} = 1.75 \times OD_{550}$$

The true absorbance can then be found by using the following equation (Miller, 1972):

$$\text{Units} = 1000 \times \frac{(OD_{420} - 1.75 \times OD_{550})}{t \times V \times OD_{600}}$$

OD_{600} is the cell density reading before the assay and OD_{420} and OD_{500} are recorded from the assay. T is the time in minutes, and v is the volume in mL of the culture used for the assay (Miller, 1972). The ratio of β-galactosidase activity for these cells grown on lactose alone versus

the cells grown on glucose in the presence of lactose can either verify or not verify the presence of catabolite repression.

The main goals of this research were to conduct a variety of growth experiments, both on solid and liquid media, to try and determine how the different strains were growing on different carbon sources, as well as to try and harvest cells for the assays. The cells were grown by taking previously frozen strains, streaking them onto plates, or swabbing into liquid media, and allowing them to incubate. The growing cells were checked continuously to produce growth curves, and determine patterns. Cells were harvested from these growth experiments, and will be used in the future for the assays. These experiments allowed us to begin to determine if catabolite repression was occurring, and what carbon sources were causing it to occur.

Methodology:

Strain Construction:

100 mL of LB was made by mixing 1g tryptone, 0.5 g yeast extract, and 1 g NaCl and 100 mL of DI water in a bottle. The bottle was autoclaved for an hour and a half, and allowed to cool and sit overnight. The following day, 250 µL $CaCl_2$, and 250 µL $MgSO_4$ were added to the LB to convert it to LBCM. 5 mL of the LBCM was measured out, and put into a sterile test tube. This was then inoculated with a previously made strain, wild type- 20749. This test tube was then placed into the shaking water bath overnight.

4.5 mL of fresh LBCM was measured out and added to the already 20749-inoculated LBCM. This was put back into the shaking water bath for two hours. After two hours, it was

removed and 100 µL of phage stock was added. This tube was then put back into the shaking water bath overnight.

The LBCM with 20749 from before was removed from the water bath. 150 µL of chloroform were added, and the tube was vortexed for 30 seconds to distribute the chloroform throughout the solution. This was then distributed into 4 sterile microfuge tubes (1mL in each), and the tubes were centrifuged for 10 minutes. 900 µL were taken from each tube and all added to one tube that was then labeled as a 20749-phage, and stored in the fridge.

In order to be able to do a phage transduction to get the 20749 strain into the SB112 background, LBCM needed to be inoculated with the SB112 strain. 4 mL of fresh LBCM was measured out and added to a sterile test tube. This was then inoculated with the SB112 and put into the shaking water bath overnight.

Four sterile microfuge tubes were prepared: tube 1 contained 300 µL of SB112 cells and 30 µL of 20749 phage; tube 2 contained 300 µL of SB112 cells and 3 µL of 20749 phage; a control tube 3 which contained 300 µL of SB112 cells and another control tube 4 that contained 30 µL of phage. All four tubes were incubated at room temperature for 15 minutes. For tubes 1, 2, and 3 the following was done: the tubes were centrifuged at 14000 rpm for 1 minute, the supernatant was poured off, the tubes were washed twice with 0.85% NaCl, and each tube was resuspended in 0.100 mL of 0.85% NaCl. Control tube 4 containing only the phage was simply left at room temperature until plating. When all four tubes were ready, they were plated onto streptomycin/neomycin plates.

Growth experiments:

Previously constructed mutant strains in both the wild-type (1021) and SB112 background were pulled from the freezer: 1021, 20749, 307620, 30944, 30924, SB112-1021, SB112-20749, SB112-307620, SB112-30944, and SB112-30924. All ten strains were streaked onto separate LB plates using sterile wooden toothpicks. They were left to incubate over a three-day period. Once grown, the plates were transferred to the fridge in order to preserve them for all of the growth experiments.

500 mL of 2x M9 was made using 11.33 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, and 1.0 g NH_4Cl. All four were measured out and put into a 1000 mL glass bottle; 500 mL of deionized water was added and the mixture was autoclaved for an hour and a half. Once removed from the autoclave, it was placed on the bench at room temperature.

24 mL of four different solutions was made up: M9t glycerol solution, M9t gluconate solution, M9t glucose, and M9t. M9t glycerol was made with 480 µL glycerol, 12000 mL 2xM9, 6 µL $CaCl_2$, 24 µL $MgSO_4$, 24 µL $CoCl_2$, 240 µL biotin, 24 µL tryptone, and 11.202 mL deionized water. The solution of M9t gluconate and M9t glucose were made identically, but with gluconate, and glucose as the carbon sources, respectively, instead of glycerol. The solution of M9t contained no carbon sources, and 11.682 mL deionized water, but was otherwise prepared identically to the other three.

Each of 5 sterile test tubes were filled with 4 mL of M9t glycerol solution. The five previously streaked plates with the wild-type background strains were removed from the fridge, allowed to warm to room temperature, and then swabbed with a sterile wooden stick.

The stick was used to inoculate each test tube with a different strain. The five test tubes were placed into a shaking water bath overnight.

5 sterile test tubes were filled with 4 mL of M9t gluconate solution, 5 sterile test tubes were filled with 4 mL of M9t glucose solution, and 5 sterile test tubes were filled with 4 mL M9t solution, for a total of 15 labeled test tubes. The previously inoculated test tubes were removed from the shaking water bath, and 2 µL of each strain were transferred to the newly prepared test tubes, using a micropipettor. This gave 1 tube of each strain in each different background. Absorbance readings were taken on a Spectronic 20, single-beam spectrophotometer with a set wavelength of 600 nm. The readings were taken once a day, starting on the day of inoculation, and continuing for five days afterwards. This growth experiment was repeated identically as above, but using the same mutant strains in the SB112 background, instead of the wild-type background.

24 mL of M9t glycerol was made using the same recipe as before. Each of 5 sterile test tubes were filled with 4 mL of M9t glycerol solution. Previously streaked plates containing all of the mutants in the wild type background) were removed from the fridge, allowed to warm to room temperature, and then swabbed with a sterile wooden stick. The stick was used to inoculate each test tube with a different strain. The five test tubes were placed into a shaking water bath overnight.

Solutions of M9t lactose, and M9t glucose + lactose (24 mL each) were made using the same recipes as before, but substituting lactose and glucose in as the carbon sources. 10 sterile test tubes were prepared, five containing 4 mL of M9t lactose solution each, and the other five containing 4 mL of M9t glucose + lactose solution each. The previously inoculated test tubes

were removed from the shaking water bath, and 2 µL of each strain were transferred to the newly prepared test tubes, using a micropipettor. This gave 1 tube of each strain in each different background. Absorbance readings were taken on a spectrophotometer with a set wavelength of 600 nm. The readings were taken once or twice a day, starting on the day of inoculation, and continuing for three days afterwards.

24 mL of M9t glycerol was made using the same recipe as before. Each of 5 sterile test tubes were filled with 4 mL of M9t glycerol solution. Previously streaked plates containing all of the mutants in the SB112 background were removed from the fridge, allowed to warm to room temperature, and then swabbed with a sterile wooden stick. The stick was used to inoculate each test tube with a different strain. The five test tubes were placed into a shaking water bath overnight.

Solutions of M9t lactose, and M9t gluconate + lactose (24 mL each) were made using the same recipes as before, but substituting lactose and gluconate in as the carbon sources. 10 sterile test tubes were prepared, five containing 4 mL of M9t lactose solution each, and the other five containing 4 mL of M9t gluconate + lactose solution each. The previously inoculated test tubes were removed from the shaking water bath, and 2 µL of each strain were transferred to the newly prepared test tubes, using a micropipettor. This gave 1 tube of each strain in each different background. Absorbance readings were taken on a spectrophotometer with a set wavelength of 600 nm. The readings were taken once or twice a day, starting on the day of inoculation, and continuing for three days afterwards.

The cells from the lactose and lactose plus glucose tubes containing the 1021 strain were harvested once the absorbance readings were at between 0.4-0.6. This ensured that the cell densities were high enough. All were harvested in the same way. For each tube: 1 mL at a time was taken from the tube, put into a sterile microfuge tube, and spun for 1 minute at 14000 rpm; the supernatant was poured out and this process was continued until the whole tube was done and a single pellet of cells remained. All four tubes were placed in a box and put into the -70^{O}C freezer.

Results:

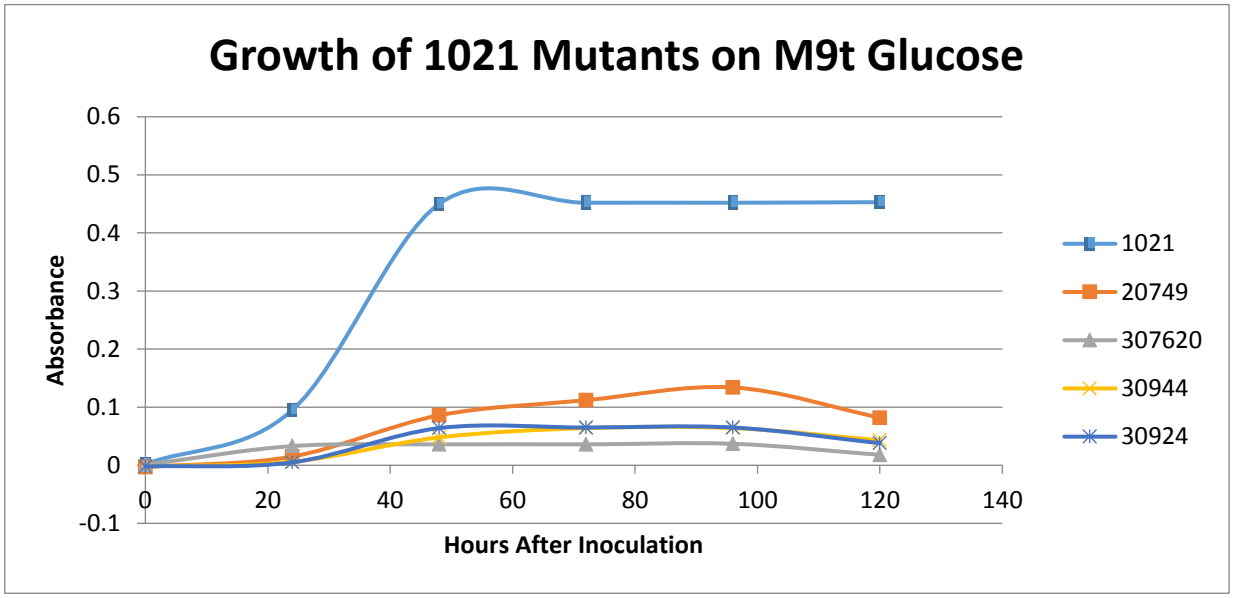

Figure 1: Growth of all five strains in the 1021 background in the M9t glucose solution over a period of 5 days; each color represents a different strain.

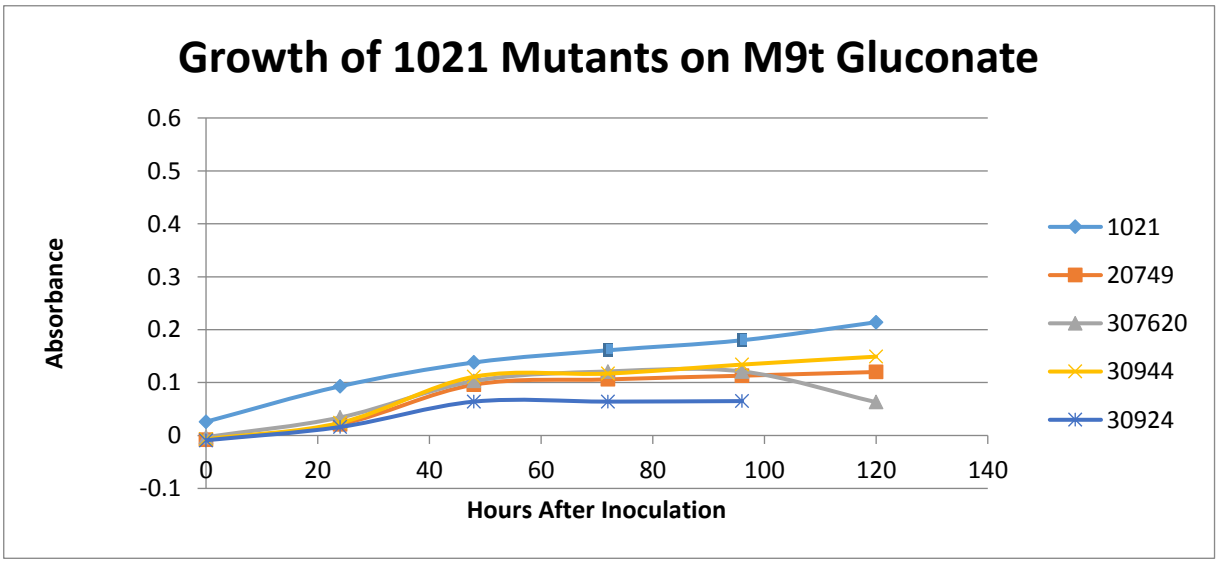

Figure 2: Growth of all five strains in the 1021 background in the M9t gluconate solution over a period of 5 days; each color represents a different strain.

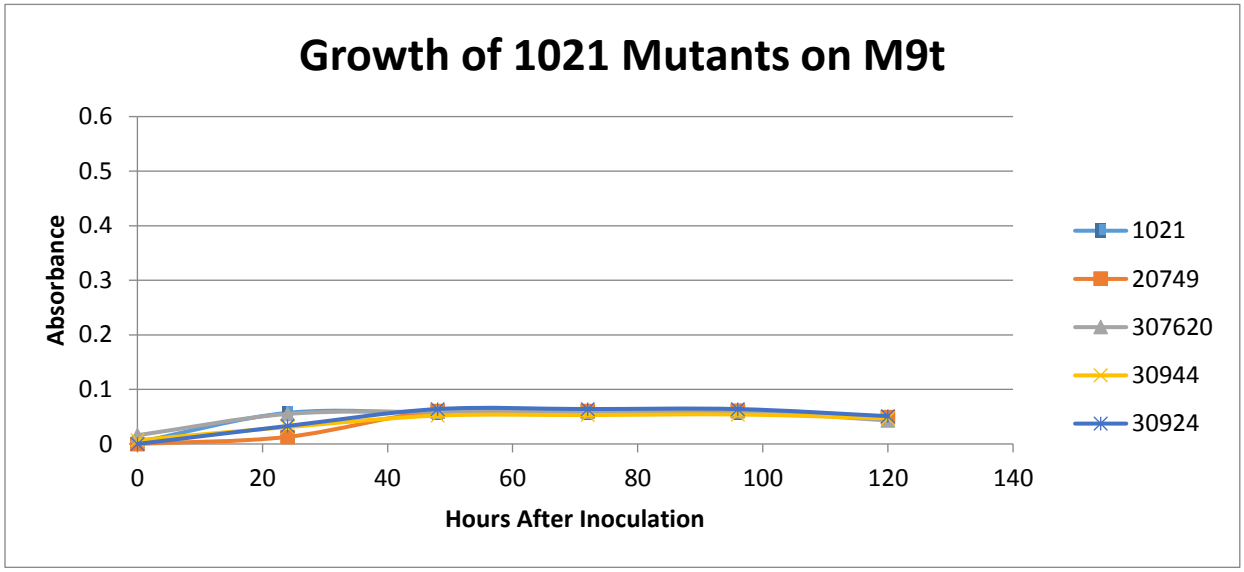

Figure 3: Growth of all five strains in the 1021 background in the M9t solution over a period of 5 days; each color represents a different strain.

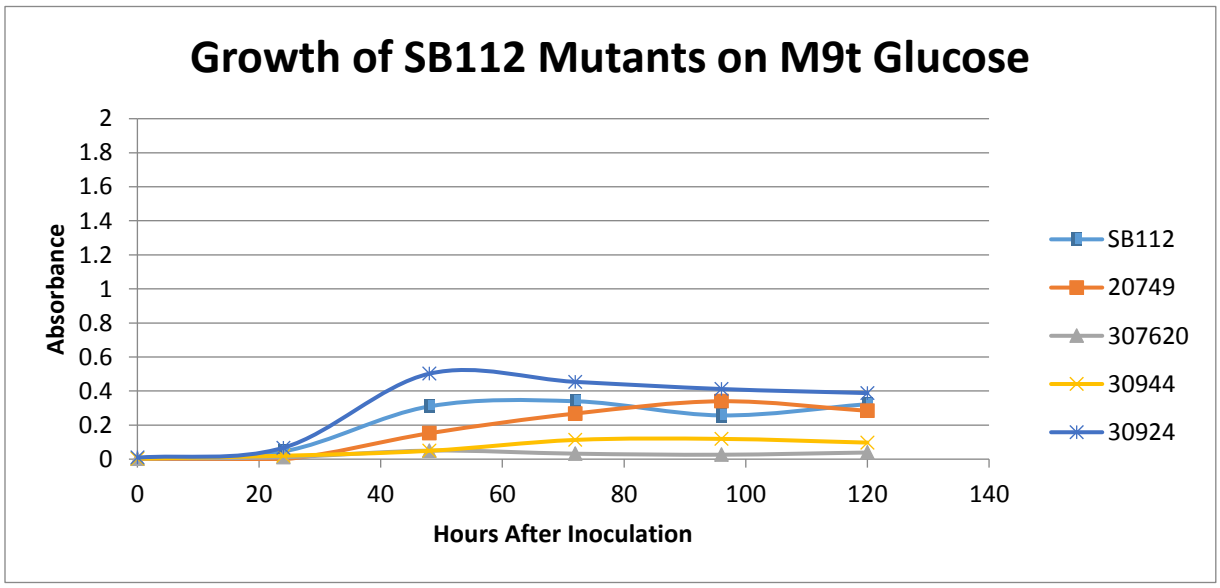

Figure 4: Growth of all five strains in the SB112 background in the M9t glucose solution over a period of 5 days; each color represents a different strain.

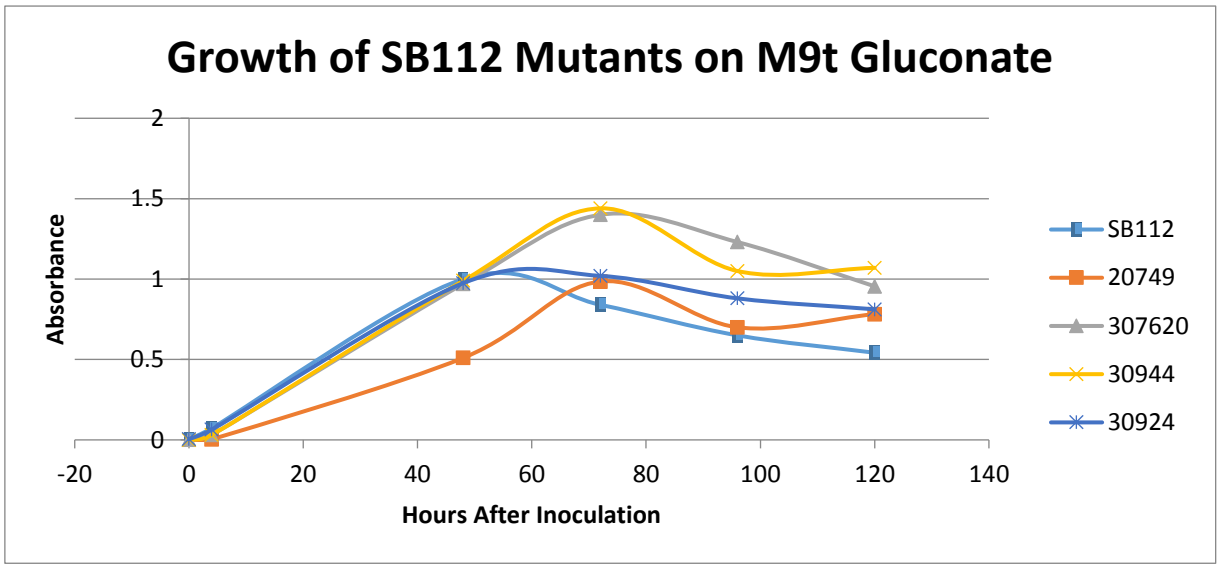

Figure 5: Growth of all five strains in the SB112 background in the M9t gluconate solution over a period of 5 days; each color represents a different strain.

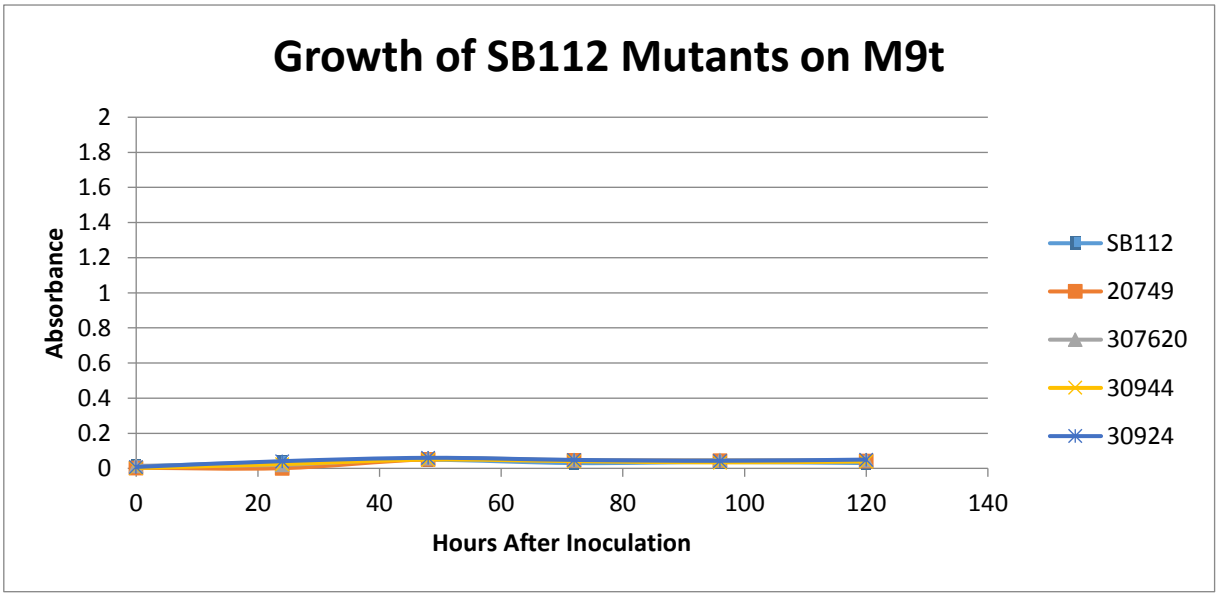

Figure 6: Growth of all five strains in the SB112 background in the M9t solution over a period of 5 days; each color represents a different strain.

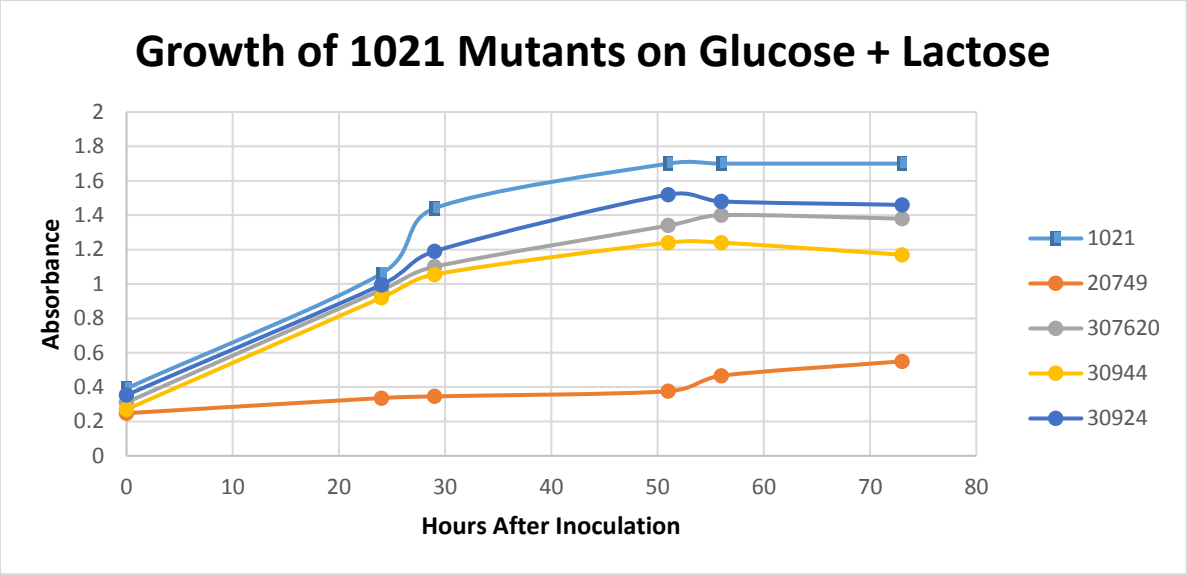

Figure 7: Growth of all five strains in the 1021 background on glucose and lactose; each color represents a different strain.

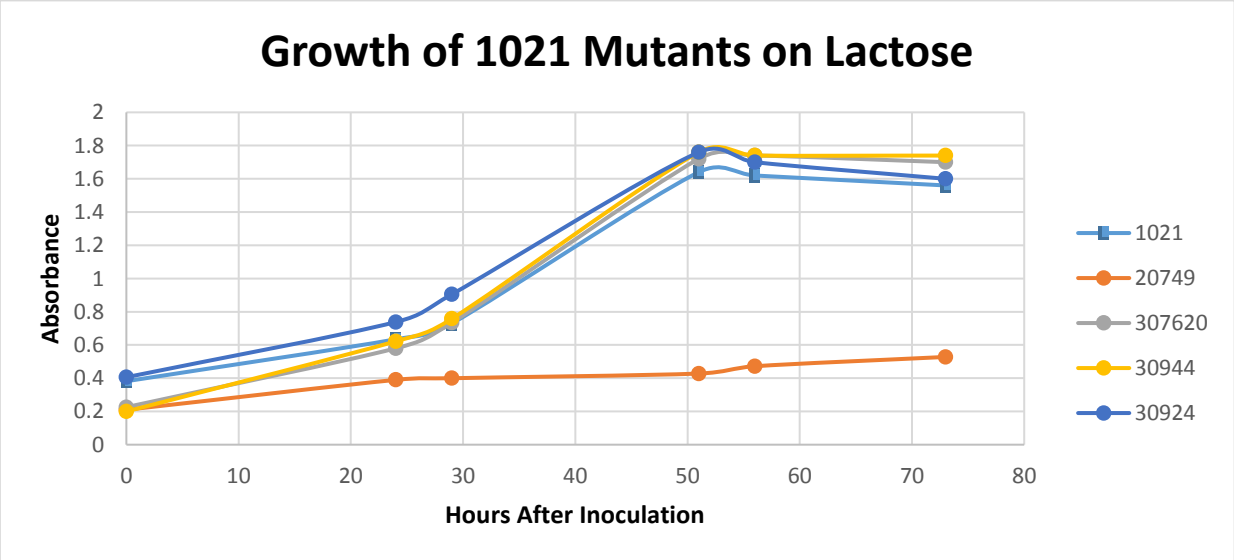

Figure 8: Growth of all five strains in the 1021 background on lactose; each color represents a different strain.

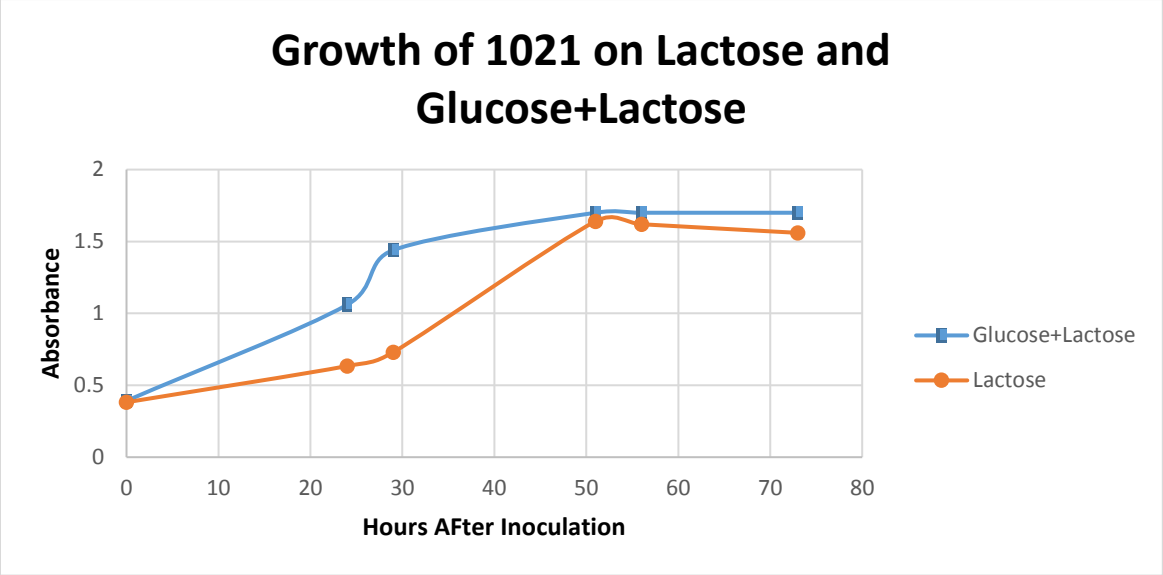

Figure 9: Growth of 1021 strain in glucose and glucose + lactose backgrounds

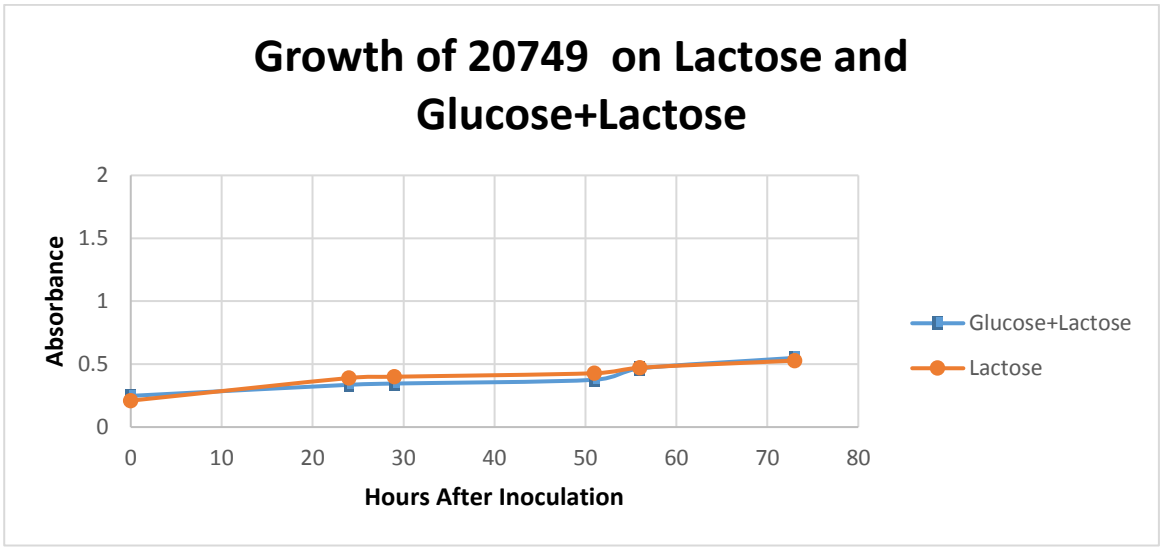

Figure 10: Growth of 20749 strain in glucose and glucose + lactose backgrounds

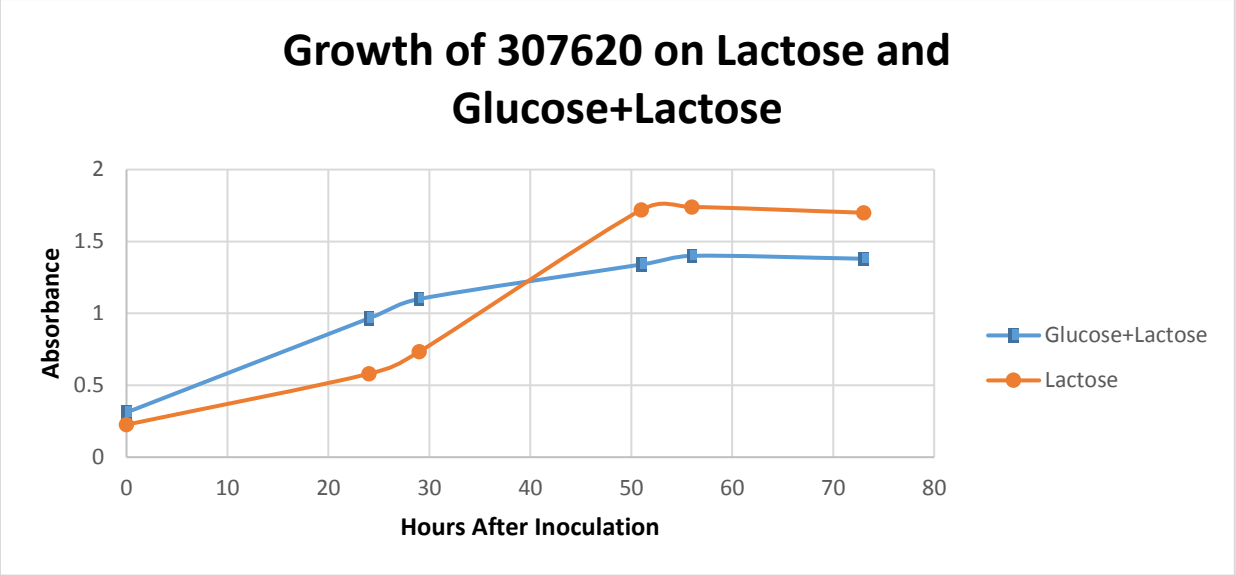

Figure 11: Growth of 307620 on lactose and glucose + lactose backgrounds

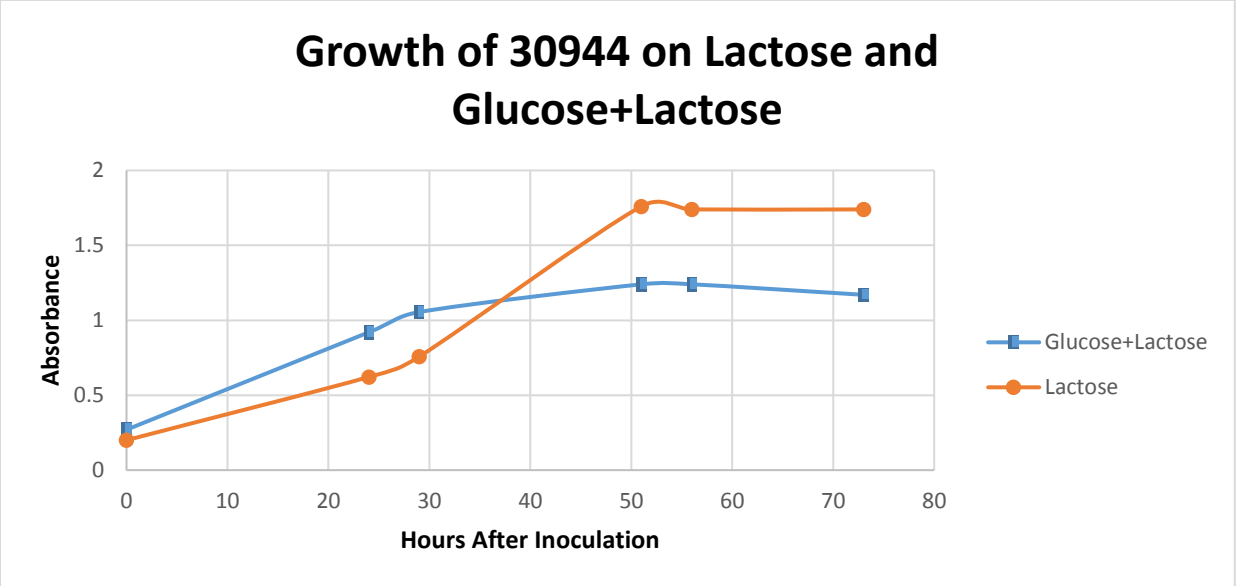

Figure 12: Growth of 30944 on lactose and glucose + lactose backgrounds

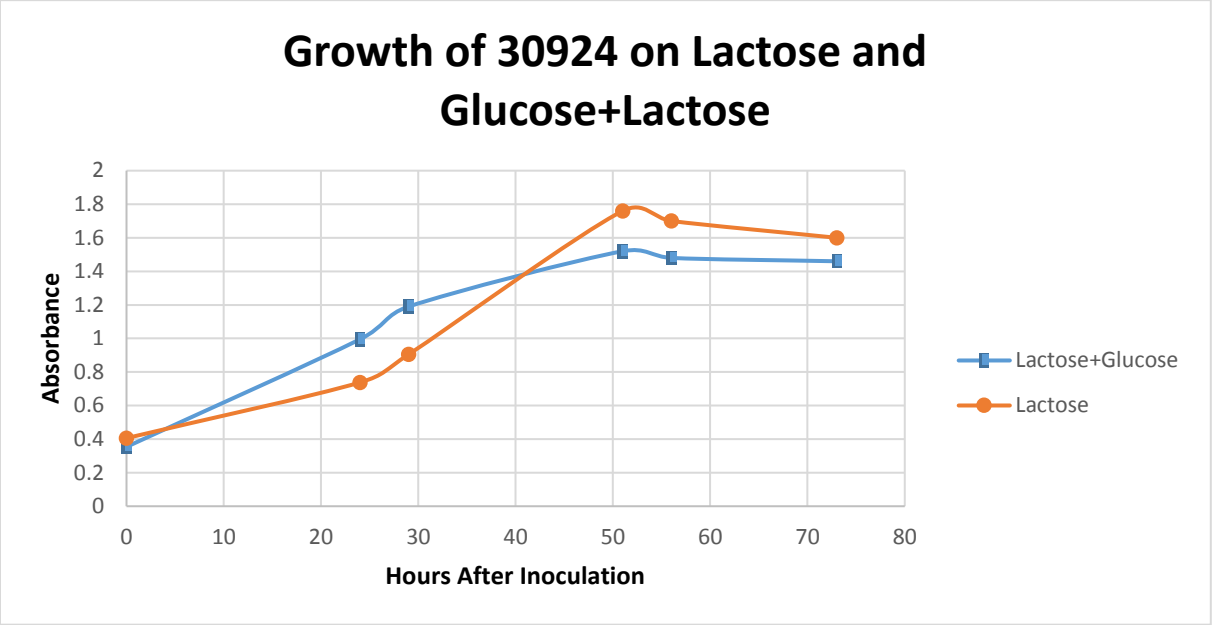

Figure 13: Growth of 30924 on lactose and glucose + lactose backgrounds

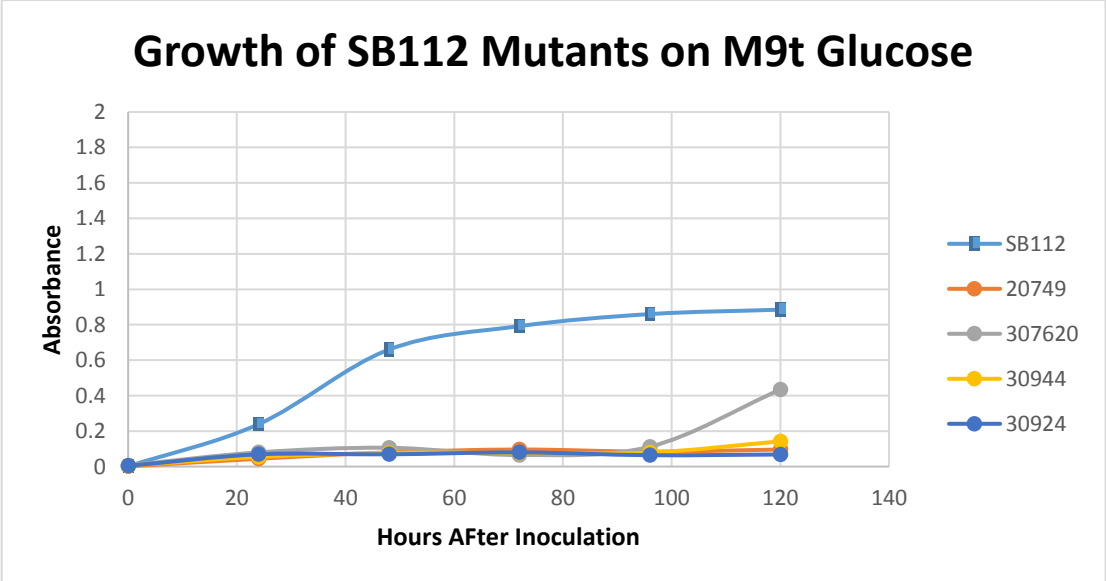

Figure 14: Growth of all five strains in the SB112 background in the M9t glucose solution over a period of 5 days; each color represents a different strain.

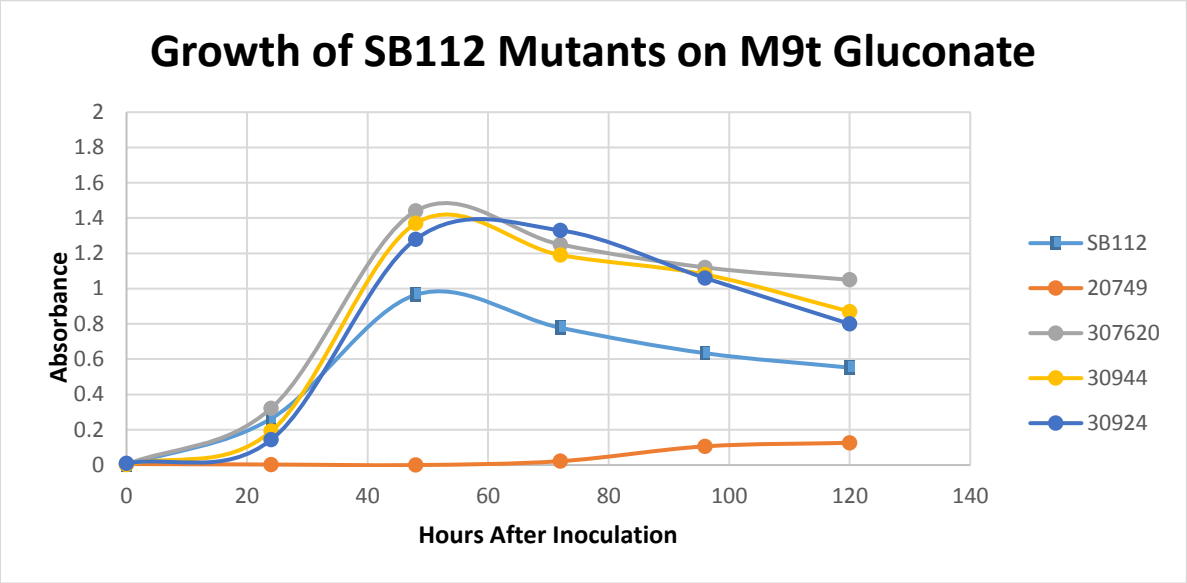

Figure 15: Growth of all five strains in the SB112 background in the M9t gluconate solution over a period of 5 days; each color represents a different strain.

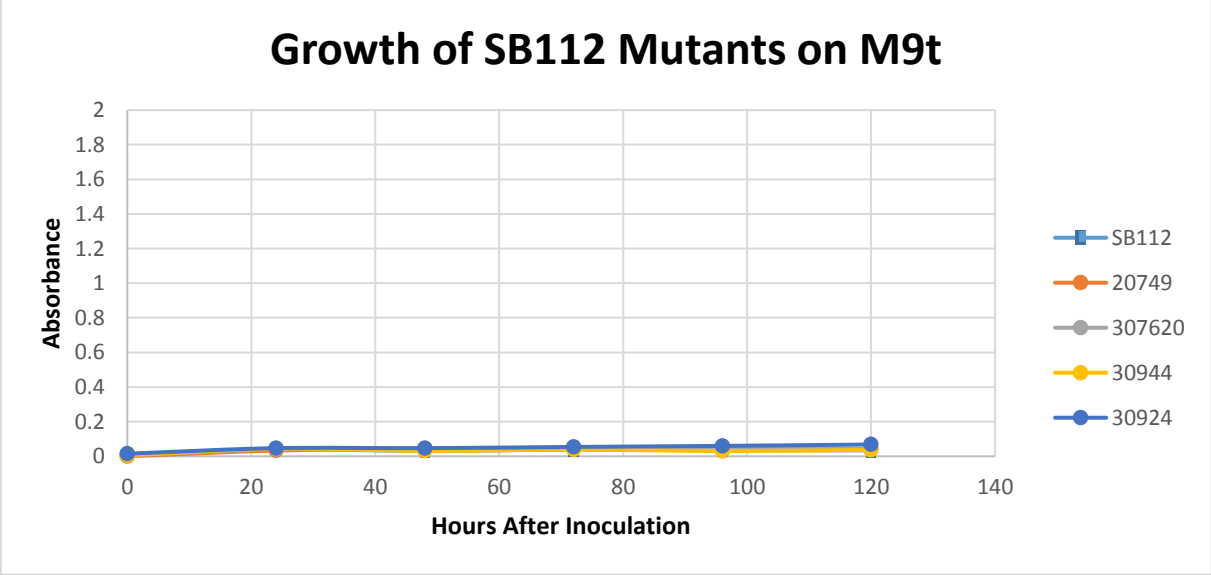

Figure 16: Growth of all five strains in the SB112 background in the M9t solution over a period of 5 days; each color represents a different strain.

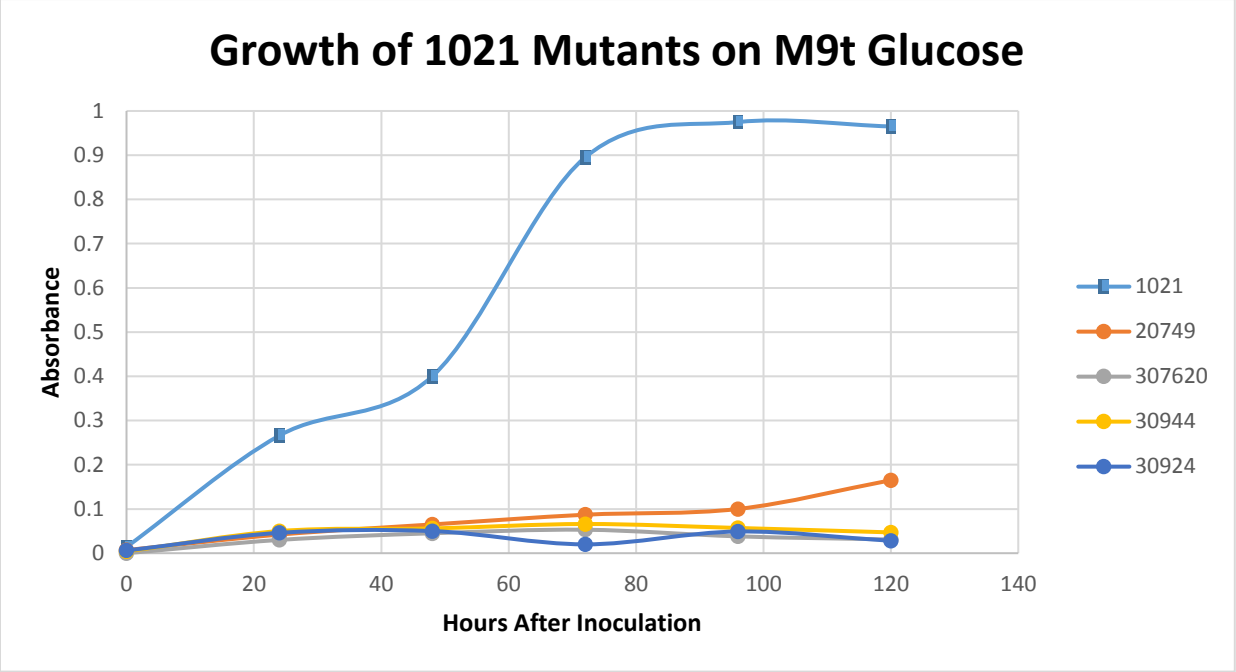

Figure 17: Growth of all five strains in the 1021 background in the M9t glucose solution over a period of 5 days; each color represents a different strain.

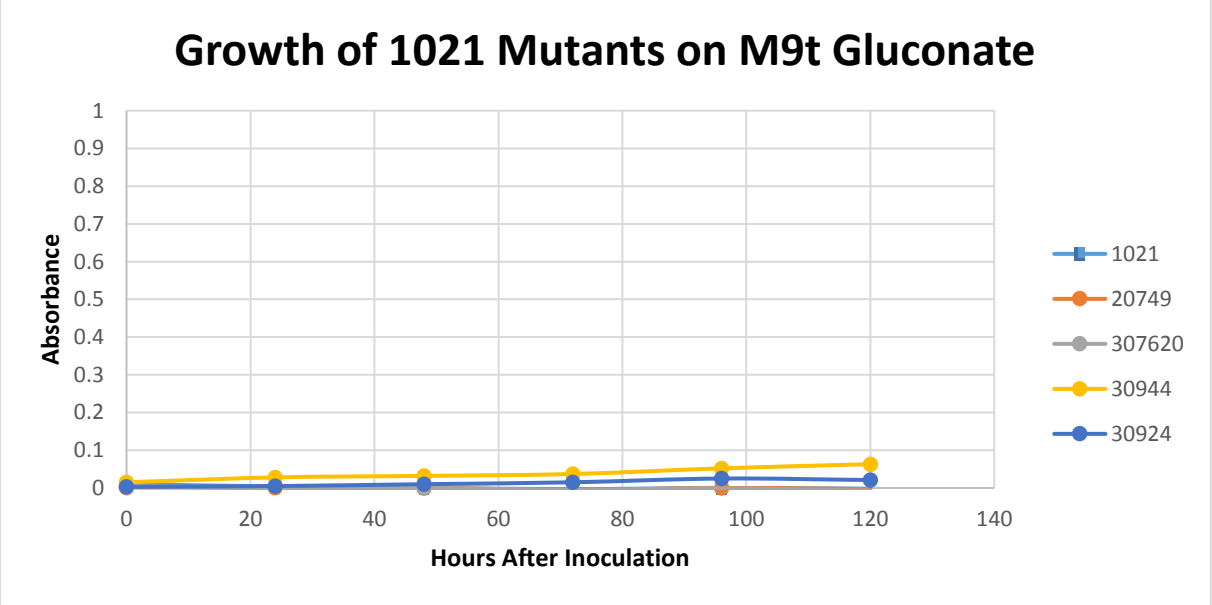

Figure 18: Growth of all five strains in the 1021 background in the M9t gluconate solution over a period of 5 days; each color represents a different strain.

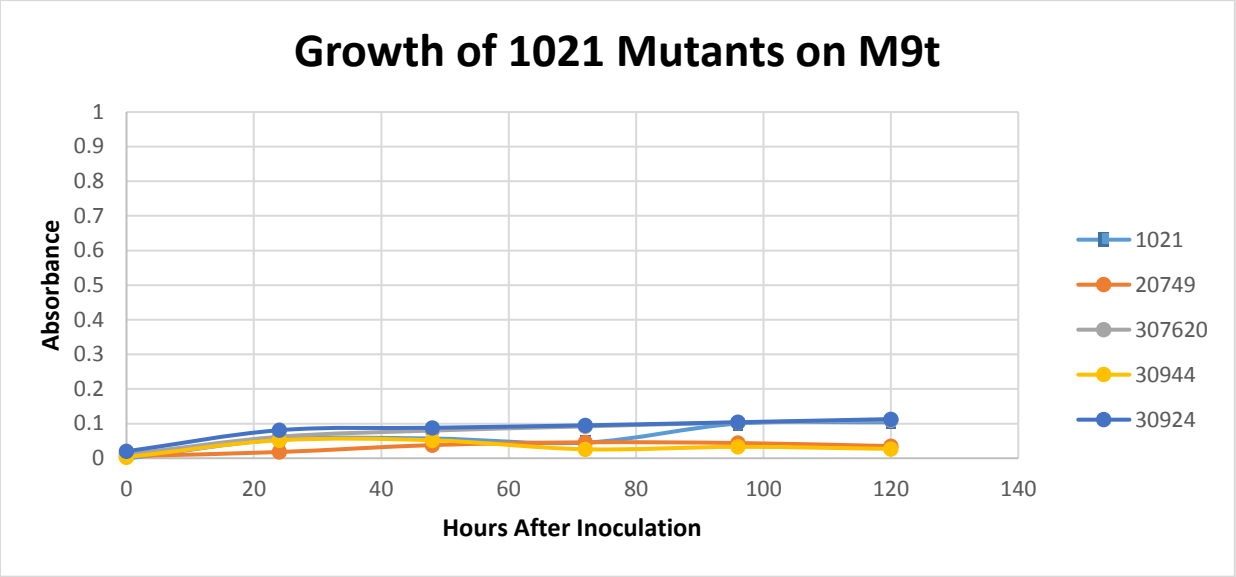

Figure 19: Growth of all five strains in the 1021 background in the M9t solution over a period of 5 days; each color represents a different strain.

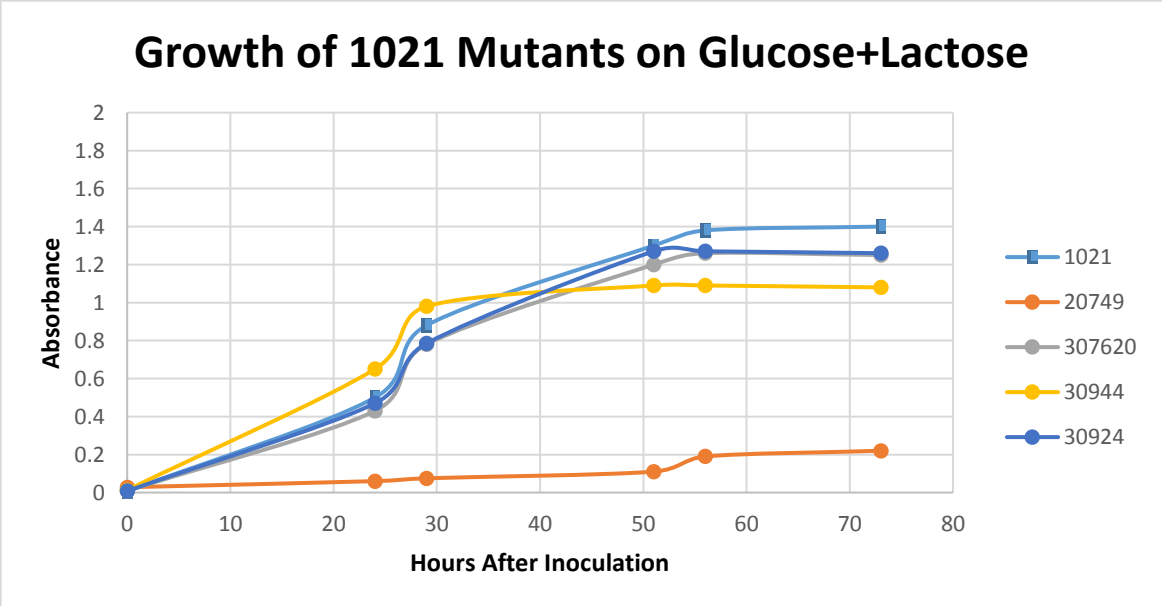

Figure 20: Growth of all five strains in the 1021 background on glucose and lactose; each color represents a different strain.

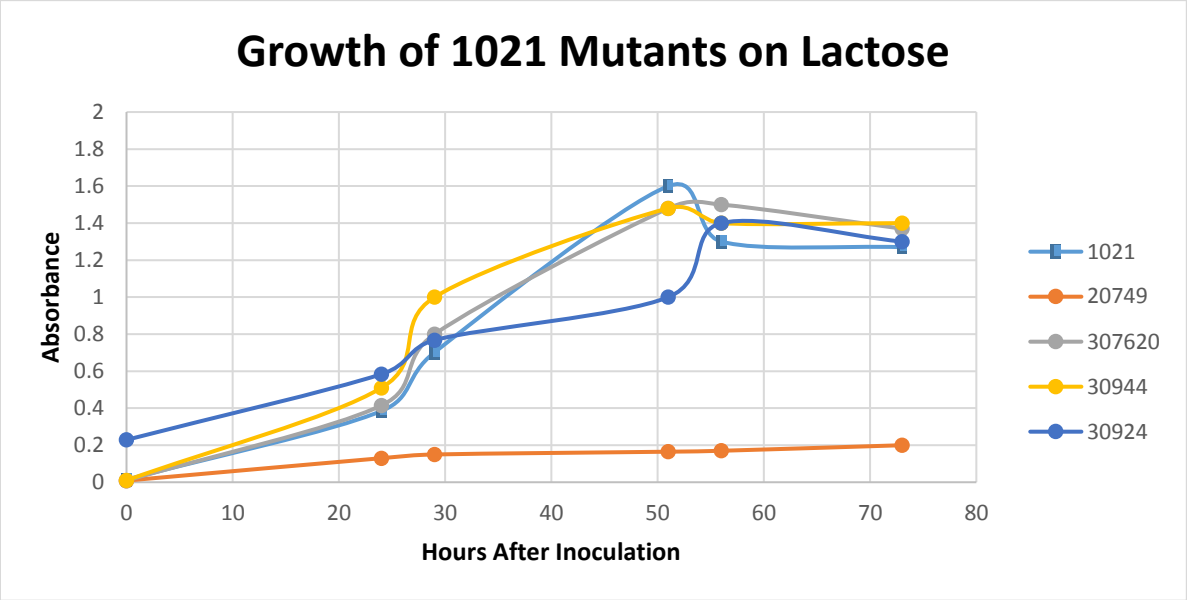

Figure 21: Growth of all five strains in the 1021 background on lactose; each color represents a different strain.

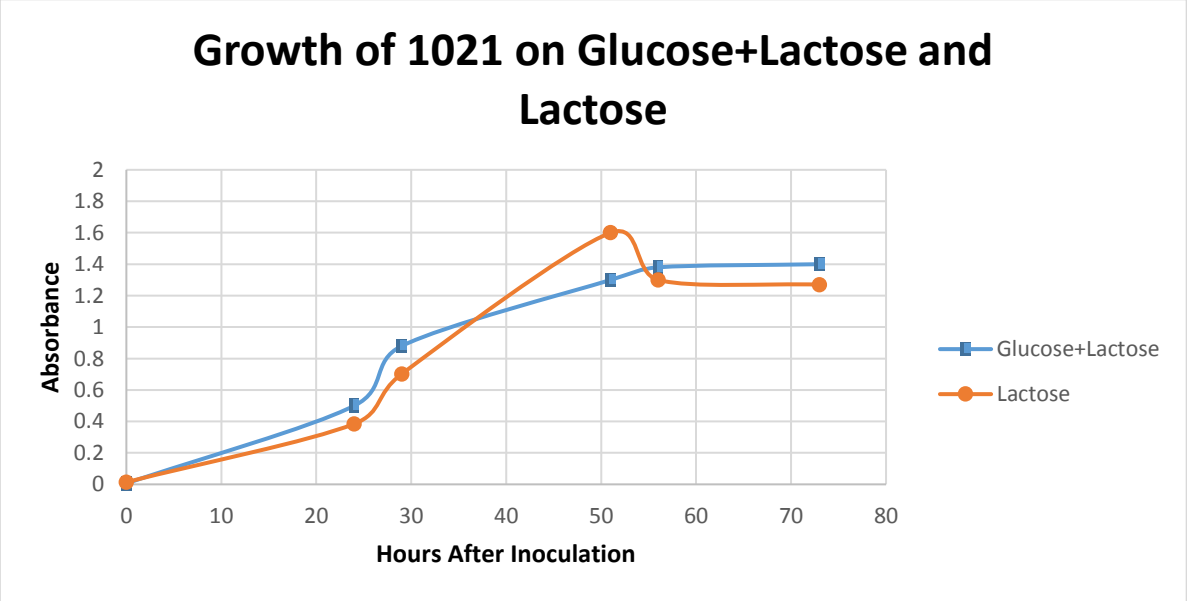

Figure 22: Growth of 1021 strain in glucose and glucose + lactose backgrounds

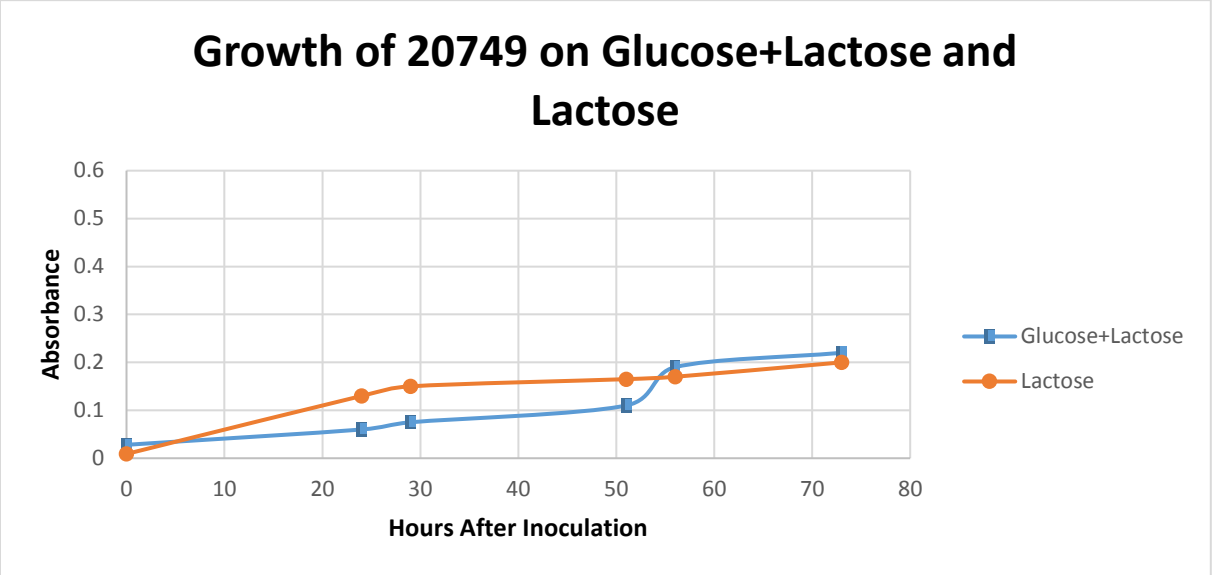

Figure 23: Growth of 20749 strain in glucose and glucose + lactose backgrounds

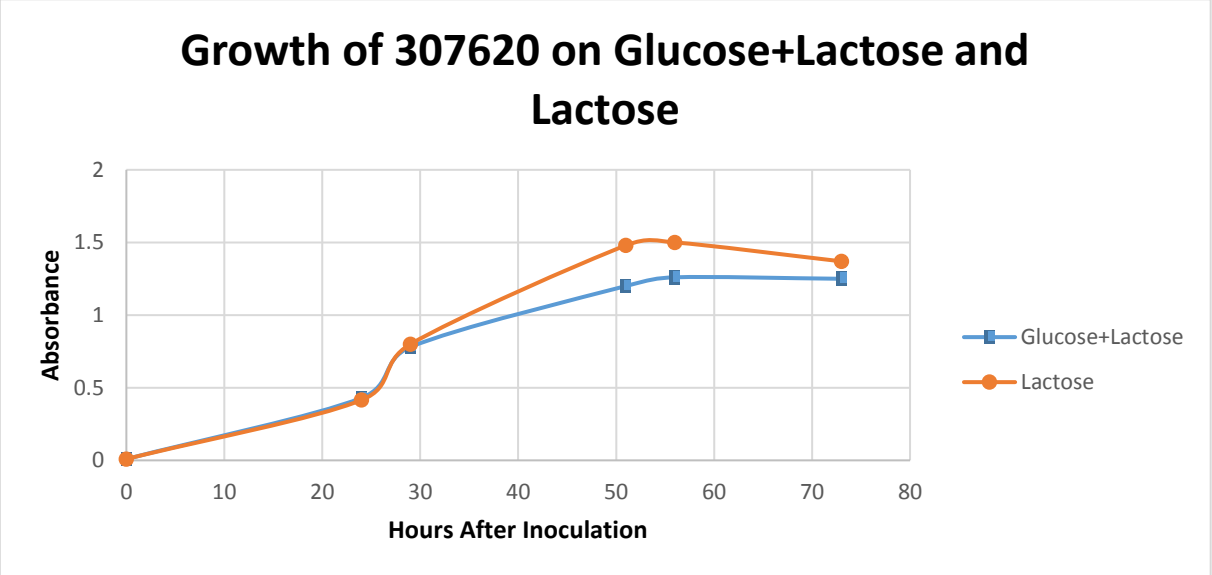

Figure 24: Growth of 307620 strain in glucose and glucose + lactose backgrounds

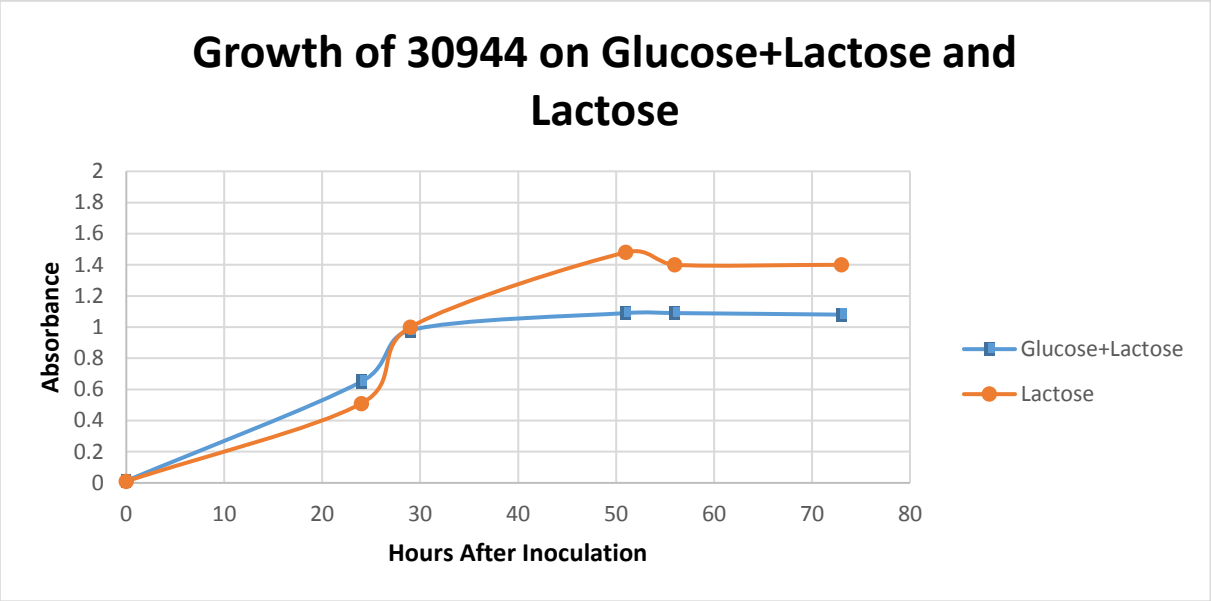

Figure 25: Growth of 30944 strain in glucose and glucose + lactose backgrounds

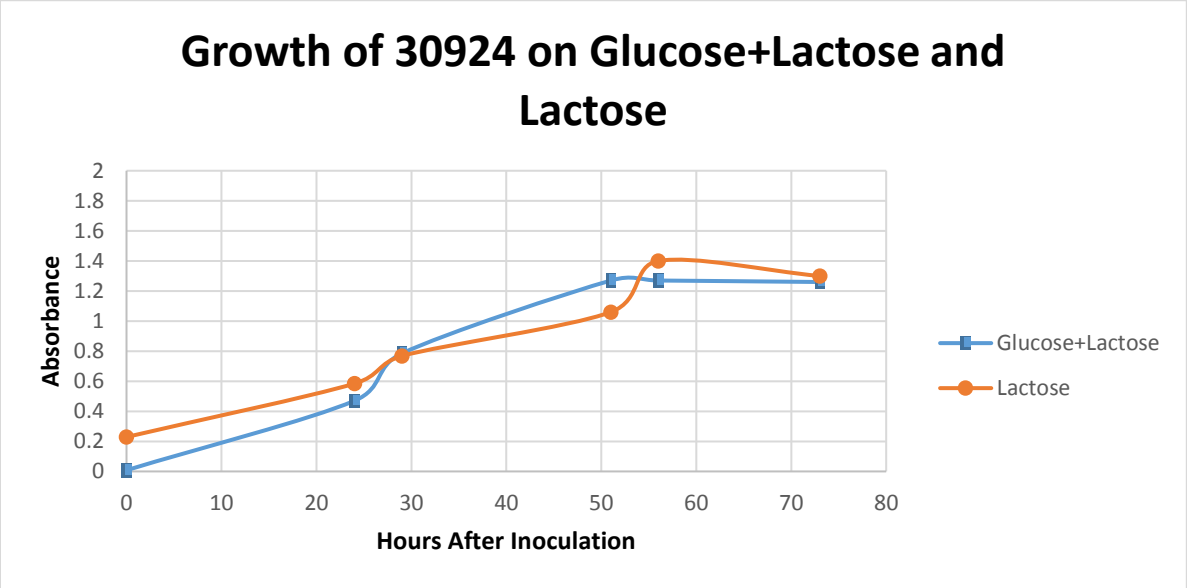

Figure 26: Growth of 30924 strain in glucose and glucose + lactose backgrounds

Growth of SB112 Mutants on Gluconate + Lactose and Lactose:

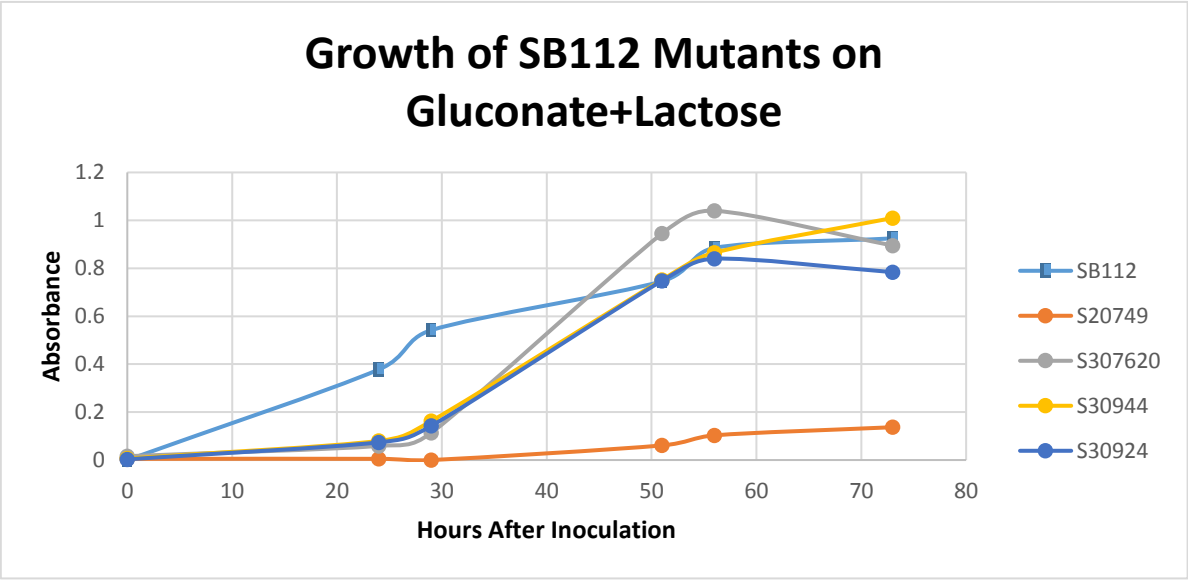

Figure 27: Growth of all five strains in the SB112 background on gluconate and lactose; each color represents a different strain.

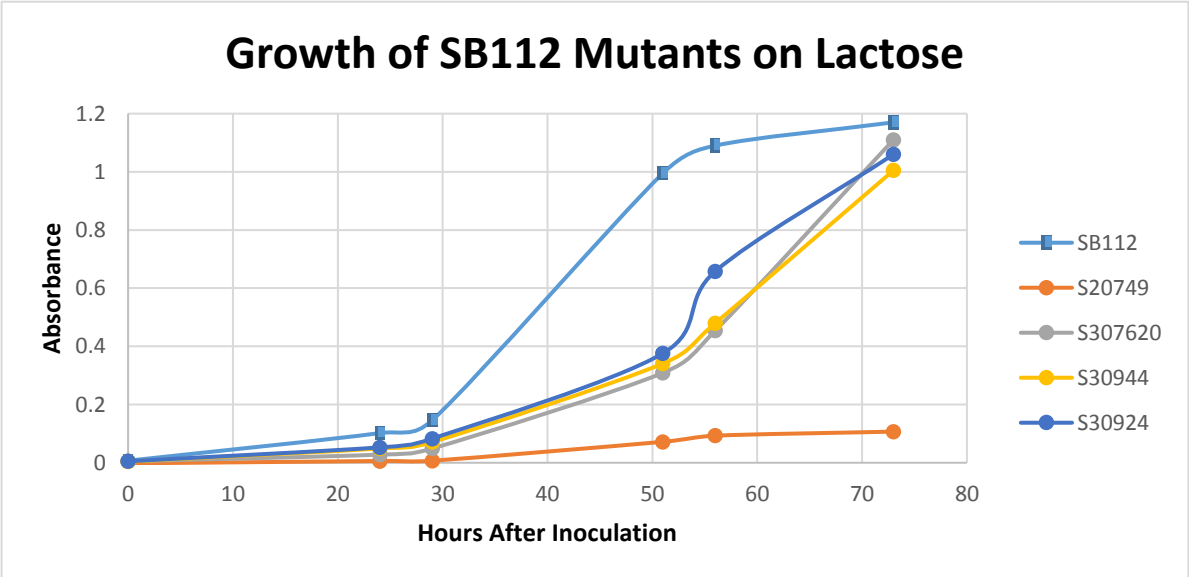

Figure 28: Growth of all five strains in the SB112 background on lactose; each color represents a different strain.

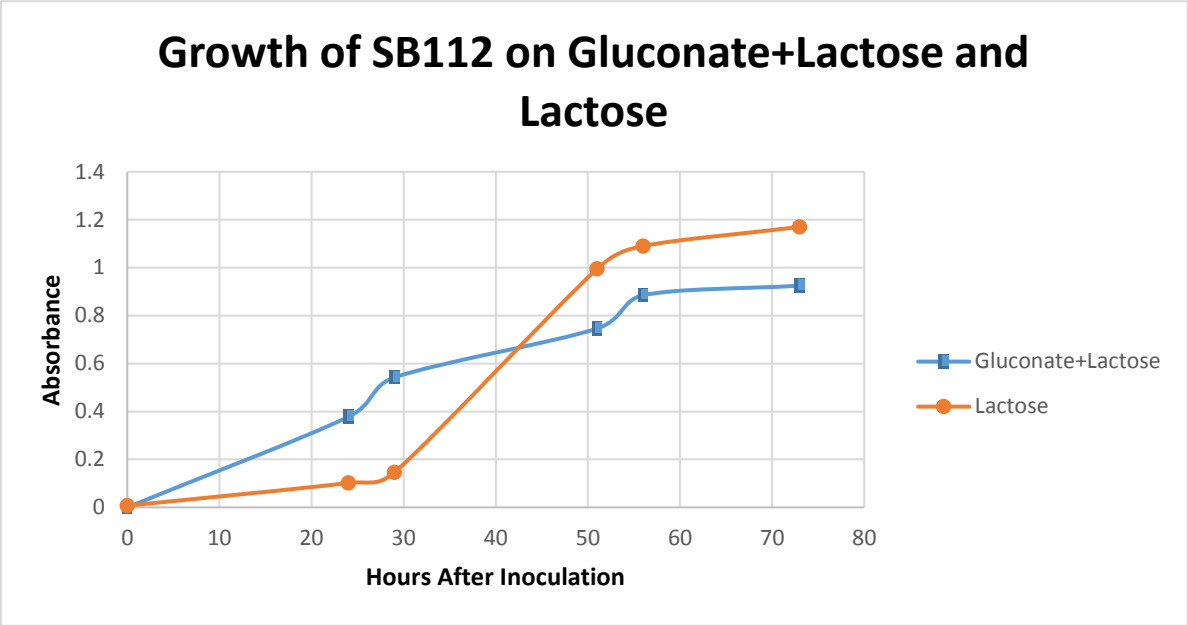

Figure 29: Growth of SB112 strain on gluconate + lactose and lactose

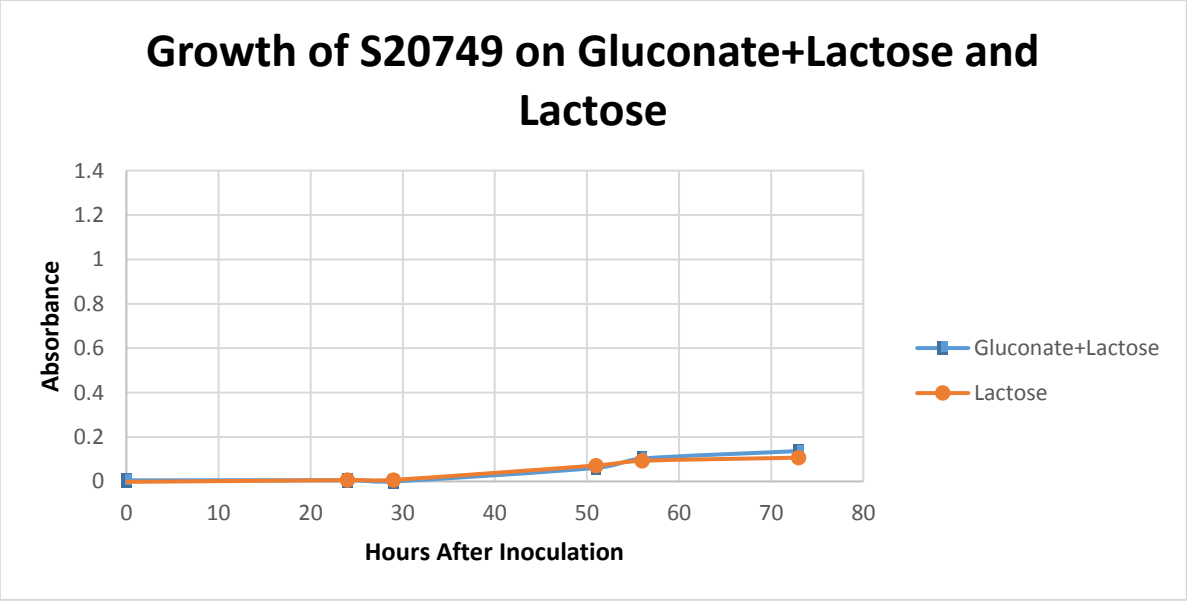

Figure 30: Growth of S20749 strain on gluconate + lactose and lactose

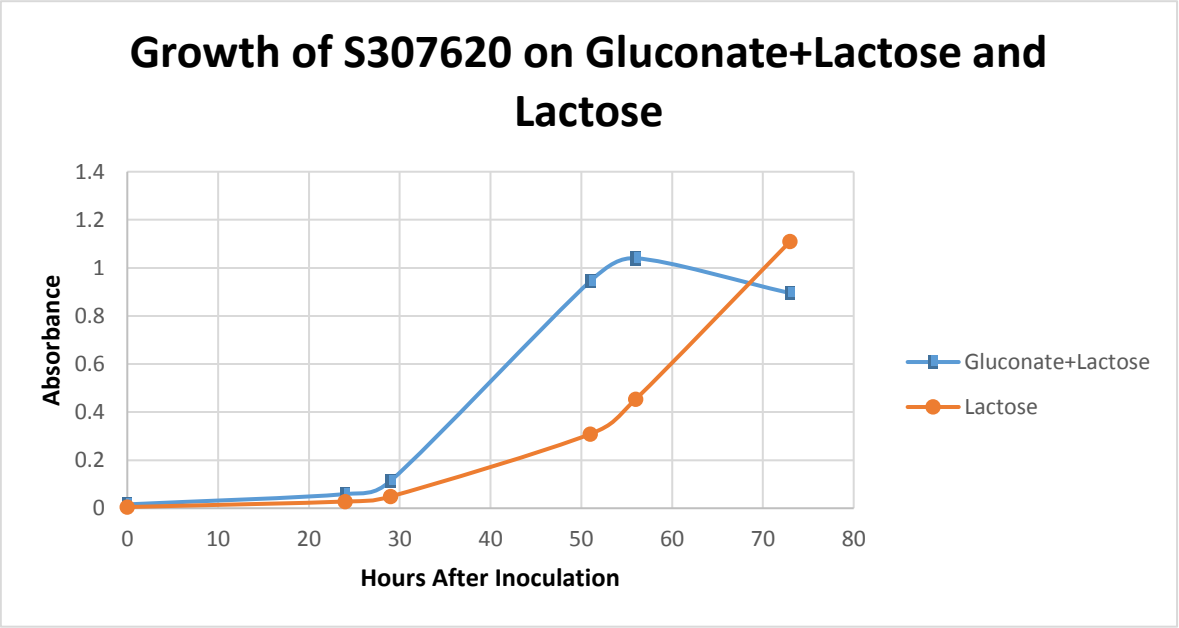

Figure 31: Growth of S307620 strain on gluconate + lactose and lactose

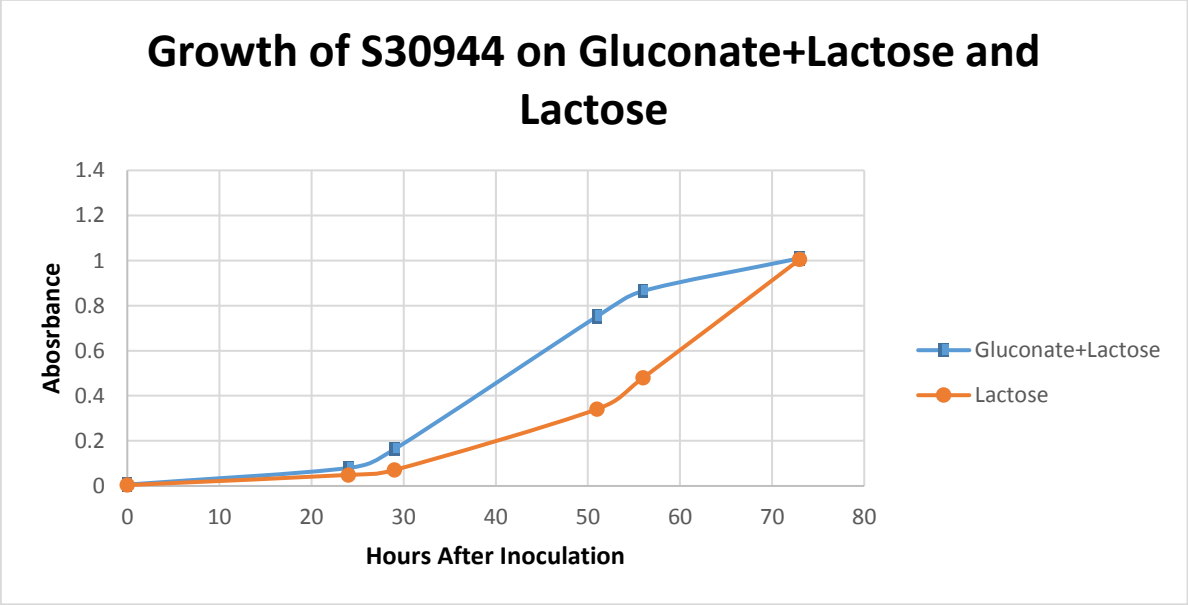

Figure 32: Growth of S30944 strain on gluconate + lactose and lactose

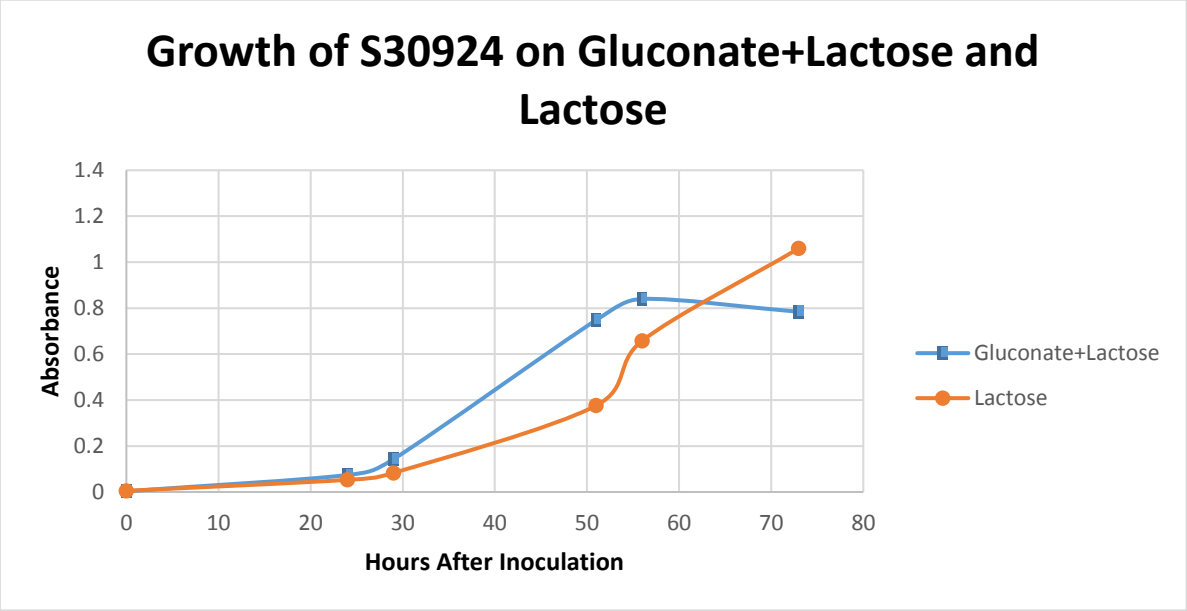

Figure 33: Growth of S30924 strain on gluconate + lactose and lactose

Discussion:

These growth experiments succeeded in giving some insight into what glucose sources each of the mutants can grow on. Each of the growth experiments was repeated a second time to obtain reproducible results and the results were in fact close for each time. Looking at the results of the growth experiments involving the M9t gluconate, M9t glucose, and M9t solutions and the wild type strains, there was not very much cell growth for any of the strains in the M9t media. This was to be expected, because the M9t media does not have a carbon/energy source in it and so it makes sense that the strains did not grow well in this media. The growth of the mutants in the M9t glucose solution showed that only the wild type strain 1021 grew. All the other four mutants in this background had very little growth in this media. This confirmed that these mutants are in fact glucose-minus mutants. None of the

mutants, including the normal wild-type strain 1021, grew very well in the M9t gluconate media. This confirmed that these mutants in the wild-type background were in fact gluconate-minus mutants and are not capable of growing on gluconate.

In the growth experiments involving the M9t gluconate, M9t glucose, and M9t solutions and the strains in the SB112 background, the strains did once again not grow on the M9t liquid media. Once again, this is to be expected because the M9t did not contain a carbon source, and was being used as more of a control. The strains did also not really grow on the M9t glucose, which was also expected, because they were all expected to be glucose-minus mutants. However, the strains did in fact grow in the M9t gluconate, with all of them reaching high absorbance between 1 and 1.5. This was somewhat unexpected, because it showed that these mutants in the SB112 background were not gluconate-minus, and can grow on gluconate. Work by previous research students had shown otherwise, so these results complicate the process of trying to determine where in the Entner-Doudoroff pathway these mutations lie. Furthermore, in the M9t gluconate background, the strain 20749 also grew. This was very unusual and unexpected as well because up until this point, 20749 was a strain that had not been growing on anything in any of the experiments, both from this project as well as previous research students. So, this strain growing on the M9t gluconate solution was very unusual, and these results served as warning that perhaps the experimental design had a flaw.

The next set of growth experiments involved growing the mutants in the wild-type background in M9 media containing glucose and lactose, and just lactose. This experiment was done to obtain cells that could then be used later for the assay experiments, to obtain results about whether catabolite repression was occurring. The strain 20749 did not really grow

in either of the media, which was expected. In the M9t containing only the lactose, the mutants all grew rather rapidly, until they leveled off, but this was due to simply running out of a carbon source. Comparing this growth to the growth of the strains on glucose and lactose, the growth in the media with the glucose was more gradual and not as quick and steep as the growth on just lactose. Even without doing the assay experiments, these results show an indicator that catabolite repression is in fact occurring due to the glucose, which can be seen by looking at the growth on the lactose versus the glucose and lactose. The glucose is most likely repressing the genes for using the lactose, making the mutants only use the glucose, which is why the growth doesn't look the same as on lactose, and is more gradual and slow.

Finally, we can look at the results of the growth of the SB112 mutants on lactose and lactose and gluconate. Despite what had happened previously with the 20749 strain, it was still included in this set of growth experiments. The results showed that the 20749 strain behaved exactly as it had been doing, and not as it did the one previous time where it grew. On one hand, this was a positive result because the 20749 behaved like "normal" again, however, it is still unknown why the 20749 grew previously in the M9t gluconate. Looking at the results of the SB112 mutants in the gluconate and lactose, the SB112 strain started to grow sooner than the 307620, 30944, and 30924 strains, but once these 3 strains started to grow, they grew more than the SB112 strain. First, this suggests that these mutants are in fact gluconate-minus, and therefore they take longer to start growing than the SB112 strain. It also suggests that gluconate-mediated catabolite repression may in fact be occurring. At the beginning of growth, gluconate may be repressing the mutants' ability to grow on lactose and only allowing them to grow on gluconate. However, because they are gluconate-minus mutants, they don't grow very

well at first. However, once the gluconate is used up, they can now use lactose, and this is where their growth greatly and rapidly increases. These results were interesting, but simply made figuring out where the mutations lie in the pathway even more confusing. The cells from both this experiment, and the previous one with the wild-type mutants in glucose and lactose, were harvested by centrifuging and are stored in the freezer. They will be used in beta-galactosidase assays that will be performed.

References:

1. Atlas, Ronald M.; Bartha, Richard. 1987. Microbial Ecology: Fundamentals and applications. The Benjamin/Cummings Publishing Co, Inc. 133-139, 290-298.
2. Bringhurst, Ryan M,; Gage, Daniel J. 2002. Control of Inducer Accumulation Plays a Key Role in Succinate-Mediated Catabolite Repression in Sinorhizobium meliloti, American Society for Microbiology. Vol. 184, No. 19. 5385-5392.
3. Dalton, David. 2007. Nitrogen Fixation. Biology Department at Reed College. http://academic.reed.edu/biology/Nitrogen/.
4. Donegan, Katherine K,; Seidler, Ramon J.; Doyle, Jack D.; Porteous, L. Arlene; Digiovanni, George; Widmer, Franco; Watrud, Lidia S. 1999. A field study with genetically engineered alfalfa inoculated with recombinant Sinorhizobium meliloti: effects on the soil ecosystem. Journal of Applied Ecology. Vol 36, Iss. 6. 920-936.
5. Jelesko, John G,; Leigh, John A. 1994. Genetic characterization of a Rhizobium meliloti lactose utilization locus. Molecular Microbiology. Vol. 11, No. 1. 165-173.
6. Miller, Jeffery H. 1972. Experiments in Molecular Genetics. Cold Springs Harbor Laboratory. Cold Springs Harbor, New York.
7. O' Gara, F.; Birkenhead, K,; Boesten, B.; Fitzmaurice, A.M. 1989. Carbon metabolism and catabolite repression in Rhizobium spp. Federation of European Microbiological Societies: FEMS Microbiology Reviews. Vol. 63. 93-102.
8. Pierce, K. M., J. J. Callan, P. McCarthy, and J. V. O'Doherty. "The Interaction between Lactose Level and Crude Protein Concentration on Piglet Post-weaning Performance, Nitrogen Metabolism, Selected Faecal Microbial Populations and Faecal Volatile Fatty Acid Concentrations." *Animal Feed Science and Technology* 132.3 (2007): 267-82. Web. 27 Mar. 2017.
9. Richardson, A. E., M. A. Djordjevic, R. J. Simpson, and B. G. Rolfe. 1988. The effects of pH, Ca and Al on the exudate from clover seedlings of compounds that induce the expression of nodulation genes in *Rhizobium trifolii*. Plant Soil 109:37-47.
10. Todar, Kenneth. 2004. Regulation and Control of Metabolic Activity. University of Wisconsin-Madison Department of Bacteriology. 09/25/07. http://textbookofbacteriology.net/regulation.html.
11. Ucker, David S.; Signer, Ethan R. 1978. Catabolite-Repression-Like Phenomenon in Rhizobium meliloti. American Society for Microbiology: Journal of Bacteriology. Vol. 136, No. 3. 1197-1200.
12. Van, P., and J. Vanderleyden. "The Rhizobium-plant Symbiosis." *Microbiological Reviews.* U.S. National Library of Medicine, Mar. 1995. Web. 27 Mar. 2017.

Appendix:

Table 1: Growth of 1021, 20749 and 307620 on M9t glucose

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t glucose-1021	0.002	0.095	0.450	0.452	0.452	0.453
M9t glucose-20749	-0.003	0.015	0.086	0.112	0.134	0.082
M9t glucose-307620	0.001	0.033	0.036	0.036	0.037	0.018

Table 2: Growth of 30944, and 30924 on M9t glucose

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t glucose-30944	-0.002	0.006	0.048	0.064	0.064	0.043
M9t glucose-30924	-0.002	0.005	0.064	0.065	0.065	0.038

Table 3: Growth of 1021, 20749, and 307620 on M9t gluconate

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t gluconate-1021	0.026	0.093	0.138	0.161	0.180	0.214
M9t gluconate-20749	-0.008	0.021	0.096	0.106	0.113	0.120
M9t gluconate-307620	-0.002	0.034	0.103	0.121	0.121	0.063

Table 4: Growth of 30944, and 30924 on M9t gluconate

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t gluconate-30944	-0.007	0.024	0.111	0.117	0.134	0.149
M9t gluconate-30924	-0.009	0.016	0.064	0.064	0.065	0.083

Table 5: Growth of 1021, 20749, and 307620 on M9t

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t-1021	0.003	0.057	0.057	0.057	0.058	0.049
M9t-20749	0.000	0.013	0.060	0.060	0.061	0.049
M9t-307620	0.016	0.055	0.059	0.059	0.060	0.043

Table 6: Growth of 30944, and 30924 on M9t

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t-30944	0.007	0.031	0.052	0.053	0.054	0.047
M9t-30924	0.000	0.033	0.064	0.064	0.064	0.051

Table 7: Growth of SB112, S20749, and S307620 on M9t glucose

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t glucose-SB112	-0.001	0.045	0.310	0.340	0.257	0.323
M9t glucose-S20749	-0.003	-0.001	0.152	0.268	0.340	0.284
M9t glucose-S307620	0.003	0.011	0.050	0.032	0.026	0.039

Table 8: Growth of S30944, and S30924 on M9t glucose

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t glucose-S30944	0.003	0.020	0.049	0.113	0.119	0.097
M9t glucose-S30924	0.009	0.067	0.502	0.454	0.412	0.388

Table 9: Growth of SB112, S20749, and S307620 on M9t gluconate

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t gluconate-SB112	0.004	0.073	1.000	0.840	0.650	0.542
M9t gluconate-S20749	-0.004	0.004	0.510	0.985	0.700	0.782
M9t gluconate-S307620	0.001	0.032	0.970	1.400	1.230	0.955

Table 10: Growth of S30944, and S30924 on M9t gluconate

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t gluconate-S30944	0.000	0.031	0.990	1.440	1.050	1.070
M9t gluconate-S30924	0.005	0.064	0.975	1.020	0.880	0.812

Table 11: Growth of SB112, S20749, and S307620 on M9t

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t-SB112	0.011	0.036	0.049	0.030	0.036	0.030
M9t-S20749	0.003	0.001	0.051	0.046	0.044	0.042
M9t-S307620	0.015	0.020	0.058	0.047	0.047	0.047

Table 12: Growth of S30944, and S30924 on M9t

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t-S30944	0.002	0.024	0.050	0.041	0.034	0.039
M9t-S30924	0.008	0.041	0.060	0.048	0.043	0.050

Table 13: Growth of 1021, 20749, and 307620 on glucose and lactose

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 29 Hours	Absorbance at 51 Hours	Absorbance at 56 Hours	Absorbance at 73Hours
1021 G+L	0.392	1.060	1.440	1.700	1.700	1.700
20749 G+L	0.248	0.336	0.346	0.376	0.466	0.550
307620 G+L	0.311	0.965	1.100	1.340	1.400	1.380

Table 14: Growth of 30944, and 30924 on glucose and lactose

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 29 Hours	Absorbance at 51 Hours	Absorbance at 56 Hours	Absorbance at 73Hours
30944 G+L	0.270	0.920	1.055	1.240	1.240	1.170
30924 G+L	0.354	0.995	1.190	1.520	1.480	1.460

Table 15: Growth of 1021, 20749, and 307620 on lactose

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 29 Hours	Absorbance at 51 Hours	Absorbance at 56 Hours	Absorbance at 73Hours
1021 L	0.382	0.634	0.730	1.640	1.620	1.560
20749 L	0.210	0.390	0.400	0.428	0.472	0.528
307620 L	0.226	0.580	0.734	1.720	1.740	1.700

Table 16: Growth of 30944, and 30924 on lactose

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 29 Hours	Absorbance at 51 Hours	Absorbance at 56 Hours	Absorbance at 73Hours
30944 L	0.200	0.622	0.758	1.760	1.740	1.740
30924 L	0.406	0.738	0.905	1.760	1.700	1.600

Repeat of Growth Experiments:

Table 17: Growth of SB112, S20749, and S307620 on M9t glucose

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t glucose-SB112	0.001	0.239	0.660	0.792	0.860	0.885
M9t glucose-S20749	0.001	0.044	0.080	0.096	0.084	0.096
M9t glucose-S307620	0.007	0.080	0.106	0.065	0.112	0.434

Table 18: Growth of S30944 and S30924 on M9t glucose

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t glucose-S30944	0.005	0.054	0.077	0.077	0.079	0.142
M9t glucose-S30924	0.004	0.069	0.069	0.080	0.064	0.068

Table 19: Growth of SB112, S20749 and S307620 on M9t gluconate

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t gluconate-SB112	0.000	0.263	0.965	0.778	0.634	0.552
M9t gluconate-S20749	0.006	0.003	0.000	0.022	0.106	0.126
M9t gluconate-S307620	0.004	0.322	1.440	1.250	1.120	1.050

Table 20: Growth of S30944 and S30924 on M9t gluconate

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t gluconate-S30944	0.003	0.192	1.370	1.190	1.080	0.870
M9t gluconate-S30924	0.010	0.143	1.280	1.330	1.060	0.800

Table 21: Growth of SB112, S20749 and S307620 on M9t

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t-SB112	0.007	0.041	0.030	0.039	0.035	0.034
M9t-S20749	0.000	0.033	0.044	0.047	0.044	0.039
M9t-S307620	0.006	0.041	0.043	0.047	0.042	0.042

Table 22: Growth of S30944 and S30924 on M9t

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t-S30944	0.006	0.044	0.031	0.039	0.030	0.037
M9t-S30924	0.015	0.047	0.047	0.054	0.060	0.068

Table 23: Growth of 1021, 20749 and 307620 on M9t glucose

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t glucose-1021	0.014	0.266	0.400	0.895	0.975	0.965
M9t glucose-20749	0.007	0.042	0.065	0.087	0.100	0.165
M9t glucose-307620	0.000	0.030	0.045	0.053	0.038	0.031

Table 24: Growth of 30944 and 30924 on M9t glucose

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t glucose-30944	0.004	0.050	0.056	0.066	0.057	0.047
M9t glucose-30924	0.006	0.046	0.050	0.020	0.049	0.028

Table 25: Growth of 1021, 20749 and 307620 on M9t gluconate

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t gluconate-1021	0.010	0.004	0.000	-0.003	0.000	-0.002
M9t gluconate-20749	0.001	0.001	0.000	-0.005	0.000	-0.003
M9t gluconate-307620	0.006	-0.002	0.000	-0.005	-0.001	-0.007

Table 26: Growth of 30944 and 30924 on M9t gluconate

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t gluconate-30944	0.015	0.028	0.032	0.037	0.052	0.063
M9t gluconate-30924	0.003	0.005	0.010	0.015	0.025	0.021

Table 27: Growth of 1021, 20749 and 307620 on M9t

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t-1021	-0.001	0.054	0.057	0.045	0.100	0.104
M9t-20749	0.006	0.018	0.038	0.046	0.044	0.035
M9t-307620	0.011	0.062	0.080	0.092	0.103	0.110

Table 28: Growth of 30944 and 30924 on M9t

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 48 Hours	Absorbance at 72 Hours	Absorbance at 96 Hours	Absorbance at 120 Hours
M9t-30944	0.003	0.052	0.052	0.026	0.033	0.027
M9t-30924	0.020	0.081	0.088	0.095	0.104	0.113

Table 29: Growth of 1021, 20749, and 307620 on glucose and lactose

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 29 Hours	Absorbance at 51 Hours	Absorbance at 56 Hours	Absorbance at 73 Hours
1021 G+L	0.006	0.500	0.880	1.300	1.380	1.400
20749 G+L	0.028	0.060	0.075	0.110	0.190	0.220
307620 G+L	0.009	0.430	0.780	1.200	1.260	1.250

Table 30: Growth of 30944, and 30924 on glucose and lactose

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 29 Hours	Absorbance at 51 Hours	Absorbance at 56 Hours	Absorbance at 73 Hours
30944 G+L	0.013	0.650	0.980	1.090	1.090	1.080
30924 G+L	0.008	0.470	0.785	1.270	1.270	1.260

Table 31: Growth of 1021, 20749, and 307620 on lactose

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 29 Hours	Absorbance at 51 Hours	Absorbance at 56 Hours	Absorbance at 73 Hours
1021 L	0.014	0.385	0.702	1.600	1.300	1.270
20749 L	0.009	0.130	0.150	0.165	0.170	0.200
307620 L	0.009	0.415	0.800	1.480	1.500	1.370

Table 32: Growth of 30944, and 30924 on lactose

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 29 Hours	Absorbance at 51 Hours	Absorbance at 56 Hours	Absorbance at 73 Hours
30944 L	0.010	0.510	1.000	1.480	1.400	1.400
30924 L	0.229	0.584	0.768	1.060	1.400	1.300

Table 33: Growth of 1021, 20749, and 307620 on glucose and lactose

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 29 Hours	Absorbance at 51 Hours	Absorbance at 56 Hours	Absorbance at 73 Hours
SB112 G+L	0.000	0.378	0.542	0.746	0.885	0.925
S-20749 G+L	0.005	0.005	0.000	0.061	0.103	0.137
S-307620 G+L	0.016	0.059	0.114	0.945	1.040	0.895

Table 34: Growth of 30944, and 30924 on glucose and lactose

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 29 Hours	Absorbance at 51 Hours	Absorbance at 56 Hours	Absorbance at 73 Hours
S-30944 G+L	0.006	0.080	0.163	0.752	0.865	1.010
S-30924 G+L	0.003	0.074	0.143	0.748	0.840	0.784

Table 35: Growth of 1021, 20749, and 307620 on lactose

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 29 Hours	Absorbance at 51 Hours	Absorbance at 56 Hours	Absorbance at 73 Hours
SB112 L	0.007	0.102	0.146	0.995	1.090	1.170
S-20749 L	-0.002	0.006	0.007	0.072	0.093	0.107
S-307620 L	0.005	0.028	0.049	0.309	0.454	1.110

Table 36: Growth of 30944, and 30924 on lactose

	Absorbance at 0 Hours	Absorbance at 24 Hours	Absorbance at 29 Hours	Absorbance at 51 Hours	Absorbance at 56 Hours	Absorbance at 73 Hours
S-30944 L	0.004	0.049	0.071	0.341	0.480	1.005
S-30924 L	0.005	0.053	0.083	0.376	0.658	1.060