An Investigation of Disaccharide-Induced Osmoprotection in Sinorhizobium Meliloti

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AN INVESTIGATION OF DISACCHARIDE-INDUCED OSMOPROTECTION IN *SINORHIZOBIUM MELiloti*

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

*UNIVERSITY OF Redlands*

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Table of Contents

Abstract .............................................................................................................. 3
Introduction ......................................................................................................... 4

Media Experiments ........................................................................................... 8
Mutant Experiments ......................................................................................... 9

General Materials and Methods ..................................................................... 10

E1. Media Selection .......................................................................................... 11
E2. Cellobiose, Glucose, and Sucrose with strain 1021 .................................................... 13
E3. Energy Equivalence .................................................................................... 16
E4. Strain 1021 and mutants that fail to grow on glucose or cellobiose .............. 18
E5. Osmotic Shock by Dry Down and Mannitol ...................................................... 21

Discussion ......................................................................................................... 22

References ......................................................................................................... 26

Appendix: Media and Solution Detail ................................................................. 28

Figures and Tables

Disaccharide Osmoprotectants ..................................................................... 8

Figure 1 .................................................................................................................. 14

Table 1 .................................................................................................................. 14

Figure 2 .................................................................................................................. 15

Table 2 .................................................................................................................. 15

Table 3 .................................................................................................................. 17

Table 4 .................................................................................................................. 18

Table 5 .................................................................................................................. 19

Figure 3 .................................................................................................................. 20

Figure 4 .................................................................................................................. 20

Figure 5 .................................................................................................................. 23
AN INVESTIGATION OF DISACCHARIDE-INDUCED OSMOPROTECTION IN SINORHIZOBIUM MELILOTI

The project sought to investigate the mechanism of disaccharide-induced osmoprotection in S. meliloti. It has been shown that sucrose, trehalose, cellobiose, gentiobiose, palatinose, maltose, turanose and maltotriose can act as non-accumulated osmoprotectants in several strains of S. meliloti (8). Sucrose was shown to provide full recovery of growth in salt shocked cultures of S. meliloti strain 1021, quantified by measuring light scattering at 600 nm. However cellobiose and maltose were found to be ineffective osmoprotectants for strain 1021. Osmoprotection by sucrose may proceed via a separate pathway than that of cellobiose and maltose. A mutant strain, 307620, which can grow on neither cellobiose nor glucose, was also studied. In preliminary experiments the mutant strain displayed osmosensitivity and was not rescued in 0.45M NaCl by the sucrose, cellobiose, or maltose. In the absence of salt, strain 307620 had significantly stunted growth but recovered with supplemental amounts of sucrose, maltose, or glucose. Further experiments are needed for conclusive identification of strain 307620.


Introduction

_Sinorhizionum meliloti_ is one of a type of bacteria known as rhizobia. Rhizobia are classified by their close association with legumes. When infected by rhizobia the roots of legumes produce an outgrowth called a root nodule. Within the root nodule the plant provides the bacteria with a favorable nutrient-rich environment (1). Through this symbiosis the legume can capitalize on the rhizobia’s ability to fix nitrogen. The process of nitrogen fixation converts atmospheric nitrogen (N\textsubscript{2}) to ammonia (NH\textsubscript{3}). Ammonia is a form of nitrogen biologically available to plants for incorporation into proteins. Nitrogen supply is often a limiting factor for plant growth and nitrogen fixation is energetically expensive for the bacteria (1). Thus, the symbiotic relationship results in abundant nitrogen supply for the legume and a ready supply of carbon and energy sources for the rhizobia.

The study of _S. meliloti_ and other rhizobia is of interest because many legumes are important crop species. The most studied host of _S. meliloti_ is _Medicago sativa_, commonly known as alfalfa. More than 7 billion dollars worth of alfalfa is grown annually in the United States, making it the nation’s third largest crop (2). Most alfalfa is destined for animal feed; however, many other legumes are edible and grown for direct human consumption. Alfalfa crops also benefit agricultural production indirectly. Rhizobia when in association with a legume can produce more ammonia than the plant can readily use and as a result ammonia builds up in the soil and is available for subsequent crops. This symbiosis is usually a cheaper source of adequate nitrogen for an alfalfa pasture than is the application of fertilizer (3). In this way crop rotations including alfalfa can increase yield of crops which follow it and reduce fertilizer use. These factors contribute to the alfalfa’s large role as the most produced crop in the western states (2).

The relationship of _S. meliloti_ to _M. sativa_ has been well studied. _M. sativa_ producers frequently utilize dry-down inocula of _S. meliloti_ when planting new crops to gain the benefits of nitrogen fixation
in their fields. Dry down inocula consist of *S. meliloti* which have been grown in liquid culture before being dried down very rapidly to preserve the cells. Often the cells are then mixed with clay and acacia gum and the resulting mixture coated onto the surface of alfalfa seeds prior to planting (13). The goal of this technique is to provide a large enough amount of viable rhizobia to ensure root nodulation. For root nodulation to occur the rhizobia must be capable of surviving and growing in four sets of conditions: the bulk soil, the rhizosphere, the infection thread, and inside the root nodule (1,16). This research primarily focuses on conditions which mimic the conditions experienced in the bulk soil where the rhizobia do not encounter significant amounts of organic molecules secreted by plant roots or from the breakdown of plant material.

Rhizobia living in the bulk soil are exposed to many environmental fluctuations, but this project focuses on osmotic stress. Osmotic stress arises from fluctuating solute concentrations. Osmotic stress is often caused by seasonal weather variations or by gradual salting of soil from irrigation (4). The symbiosis of rhizobia and legumes also requires that the bacteria survive several osmotic shifts. As roots penetrate the soil they have a dramatic effect on the soil conditions within a short distance of the root system, the area defined as the rhizosphere. The presence of cellular material and excreted compounds would constitute a major osmotic shift relative to the environment of free living bacteria in the bulk soil. Thus an ability to adapt to osmotic shifts is essential for the rhizobia to survive free in the bulk soil and for symbiosis with legumes.

*S. meliloti* is of further interest because it adapts naturally to mild osmotic stress (5). The plasma membrane of cells is selectively permeable, as only certain compounds may pass through it. Ions from salts and large organic molecules are unable to pass through; however water diffuses through the membrane very quickly. When the concentration of solutes which cannot cross the plasma membrane is greater on one side than the other, then water will flow through the membrane until the concentrations
reach equilibrium. The primary effect on bacteria is the outflow of water by diffusion to balance the solute concentration on either side of the cellular membrane in hyperosmotic environments. Because the cell wall of bacteria is not an absolutely rigid structure a positive internal osmotic pressure, or turgor pressure, is required to maintain cell structure. Under osmotic stress the loss of cell turgor, which is also the driving force for cell elongation, greatly inhibits growth (6). A large efflux of water causes considerable structural damage and is often fatal. If the solute imbalance is caused by salts, damage by ionic potential can exacerbate the problem by disrupting enzyme activity.

Bacteria cope with osmotic stress by balancing the concentration of solutes in their environment with compatible compounds (6). Compatible compounds are essentially substances that the bacteria can synthesize, with the exception of potassium, and accumulate in the cytosol in high concentrations without disrupting cellular metabolism. A combination of potassium and glutamate is a common method of balancing both solute concentration and ionic potential (5). This normal process of adaptation can be facilitated by the presence of compounds classified by their effect as osmoprotectants. Classical osmoprotectants are conceived as compounds, such as proline, that when present in the environment can be absorbed and accumulated inside the cell in place of compatible compounds the bacteria usually produces. The natural sources of osmoprotectants for rhizobia are other organisms which synthesize the compounds. Such organisms utilize osmoprotectants as internal solutes which are then expelled when the solute concentration in the soil drops. Alternatively such compounds appear in the soil after cell decay.

*S. meliloti* is particularly interesting because it appears somewhat more salt tolerant than other rhizobia. *S. meliloti* also has a divergent response to osmotic stress. Soil osmoticum are balanced using a combination of potassium, glutamate and a dipeptide, N-acetyl glutaminyl glutamine amide or NAGGN, during growth phase and trehalose during the stationary phase (5). The counter ion pair of glutamate
and potassium has been suggested as the primary response (7). Like other bacteria the normal
capabilities of this response can be bolstered by osmoprotectants. However in \textit{S. meliloti} the more
common osmoprotectants are not accumulated within the cell to balance external solutes. In fact only a
single compound, dimethyl sulfoxidepropionate (DMSP), has been shown to be utilized in that fashion
(5). Regardless of the lack of accumulation, most normal osmoprotectants still increase the osmotic
tolerance of \textit{S. meliloti}. Compounds which confer tolerance to osmotic stress but do not appear with
increased concentration within the cytosol are termed non-accumulated osmoprotectants.

\textit{S. meliloti} is also unique in its utilization of some disaccharides as osmoprotectants (8). Disaccharides shown to have this effect are all non-accumulated osmoprotectants and thus increase
tolerance indirectly; the mechanism is unknown. The research group of Gouffi et. al. presented
disaccharides as a new class of osmoprotectants and noted sucrose, trehalose, cellobiose, gentiobiose,
palataneose, maltose, turanose and maltotriose as compounds which conferred significant tolerance (8).
Interestingly all of the disaccharides included in this list contain glucose at the non-reducing end. In
addition the research group of Barra et al. showed that glucose-6-phosphate dehydrogenase (G6PDH)
activity is required for this disaccharide induced osmoprotection to occur (9). G6PDH is one of the first
enzymes in the glucose catabolic pathway in \textit{S. meliloti} (10). If this important enzyme of glucose
metabolism is required for osmoprotection and all the disaccharides which induce osmotic tolerance
contain glucose, it seems possible that glucose may itself increase tolerance to osmotic shock. However
the research group which introduced disaccharides as osmoprotectants found no monosaccharides,
including glucose, that acted as an osmoprotectants. These experiments were conducted in a specific
minimal lactate and aspartate medium (8).
This project sought further information about the mechanism for disaccharide-induced osmoprotection in *S. meliloti*. Experiments with varied media tested the hypothesis that glucose can act as an osmoprotectant in some conditions. Mutant experiments sought mutants with impaired osmoprotection to identify specific genes essential to disaccharide-induced osmoprotection.

**Media Experiments**

This portion of the project looked at the effect of varied media selection in *S. meliloti*. In effect this is a study of the regulation of disaccharide-induced osmoprotection by components of the growth medium. The direction of these experiments arose from the specifics of the minimal lactate and
aspartate medium used by Gouffi et. al. when describing this effect. The medium is very similar to M9: containing phosphate buffer and a supply of minerals needed for healthy growth of the bacteria. In this case the typical nitrogen source of ammonium chloride has been replaced with aspartate and both lactate and aspartate act as carbon sources (8,11). These experiments were conducted using sucrose as a positive control for osmoprotection. Sucrose is a well established non-accumulated disaccharide osmoprotectant which was used in the evaluation of the role of G6PDH in osmoprotection (9). To test for osmoprotection by glucose all glucose experiments were run in tandem with sucrose.

If osmoprotection by disaccharides is linked to the activity of G6PDH a minimal medium, such as lactate and aspartate, may not provide ideal conditions for osmoprotection to occur. We proposed that in conditions under which G6PDH activity is obligatory osmoprotection may be automatically afforded. To test the hypothesis S. meliloti strain 1021 was grown in minimal media with glucose as a sole carbon source.

Similarly the importance of G6PDH, an enzyme central to glucose metabolism, seems to suggest that conditions which inhibit flux through the glucose catabolic pathway may also inhibit osmoprotection by disaccharides. This principle was examined by inclusion of succinate in the growth media. Succinate is known to cause catabolic repression of glucose metabolism (17). If glucose catabolism and G6PDH are repressed the osmoprotection by disaccharides may also be inhibited. Growth media with glucose and sucrose provided as osmoprotectants and supplemental succinate were used to test for catabolite repression of osmoprotection. The hypothesis was that if glucose metabolism enzymes are needed for osmoprotection then osmoprotection will be inhibited by catabolite repressors.

Mutant Experiments

A collection of S. meliloti strains with mutations in sugar metabolism was studied in order to gain more specific information on what enzymes are involved in osmoprotection. A set of random Tn5
mutants were screened for failed growth on either cellobiose or glucose and several of the mutations overlap these two categories and are available for the project (12). Cellobiose was previously mentioned as a disaccharide shown to provide non-accumulated osmoprotection in *S. meliloti* (8). Cellobiose is of particular interest because it has a simple structure of two linked glucose molecules. This structure may be of interest in comparison with glucose data obtained in the media selection experiments. Cellobiose has also received less attention as compared to other disaccharides which confer osmoprotection. Utilizing the available mutants and describing any changes in osmoprotection conferred to the strain by cellobiose or sucrose could help elucidate the mechanism of osmoprotection.

**General Materials and Methods**

The primary media used in these experiments was a minimal lactate and aspartate medium as used by Gouffi et. al. Throughout the paper this medium will be referred to as LA9. As previously mentioned the medium is very similar to M9 as it contains phosphate buffer and a supply of minerals needed for healthy growth of the bacteria. It should be noted that the typical nitrogen source of ammonium chloride used for M9 medium has been replaced with aspartate and that both lactate and aspartate act as carbon sources (8, 11). Luria-Bertani broth with calcium and magnesium (LBCM) was also used as a rich yeast extract media alternative (18). Yeast extract experiments and starter cultures requiring a minimal media were grown in yeast extract mannitol broth or YMB. (11) For this project lactate often replaced mannitol as the carbon source and the resulting media was notated as YML. Unless otherwise noted, carbon sources were provided at a final concentration of 10 mM, and disaccharides provided as osmoprotectants or inhibitors were provided at a final concentration of 1mM. (Detailed preparation methods and contents of media in appendix I)

Media selection experiments and all controls utilized the strain Rm 1021. Rm 1021 was also the parent strain for all mutant strains. Mutant strains were created by random Tn5 mutagenesis and
screened for inability to grow on glucose or cellobiose as lone carbon sources but retained ability to
grow on succinate by Dr. Dan Wacks. (12) Liquid cultures were grown aerobically at 30° C in a rotary
shaker at 200 RPM. Cultures grown on agar plates were incubated at 25 - 30° C as equipment was
available. The growth of liquid cultures was quantified by the absorbance at 600 nm (A600). Starter
cultures were grown in either M9 mannitol or YML media. M9 mannitol starter cultures were utilized
after a 48 hour growth period, 24 hours was used for YML cultures. In both cases starter cultures were
used for inoculation when the A600 of the culture was between 0.8 and 1.1 absorbance units to confirm
that the cultures did not reach maximum optical density and were thus readily dividing. Inoculations
were made by a 1:1000 fold dilution of the inocula into a sterile flask for each medium tested. Following
inoculation each medium was vortexed for 20 seconds and divided into three test tubes to provide
replicates. Growth was evaluated after 48 hours.

E1. Media Selection

Objective

This set of experiments sought an ideal medium for observing osmoprotection by disaccharides
in Sinorhizobium meliloti. A suitable medium needed to provide consistent growth of S. meliloti over a
useful time period and allow osmotic shock to be readily observed. It was also necessary that the carbon
selected did not result in catabolite repression which could have affected past data. Thus it was essential
the osmotic shock could be induced in the chosen media in a similar range to that in lactate and
aspartate medium (LA9). Initially, M9 salts with Mannitol, YMB, and LBCM media were compared to
LA9.
Experimental Method

Starter cultures were grown in LBCM liquid culture in test tubes and were used for the following week. Replicate tubes were prepared independently and inoculated with 15 μl of starter culture. Maximum density of the culture obtained by measuring the $A_{600}$ at 4 to 7 days following inoculation was used to evaluate bacterial growth. Tests of osmotic sensitivity used 0.6 M NaCl. Disaccharides were provided as described in general methods section.

Results

Salt stress as observed by the inhibition of growth by sodium chloride was observed in all media. M9 mannitol media provided the most consistent maximum growth which was reached between 6 and 7 days. Strain 1021 also seems particularly osmosensitive in this media which was further used in the project. Growth in YMB media was less consistent but moderately fast with maximum growth being reached in 3 to 4 days. The growth of 1021 was still drastically reduced in the presence of 0.6 M NaCl in this medium but less so than with M9 mannitol. These characteristics make YMB a good media for starter cultures when experiment time must be shortened. *S. meliloti* grows very well in LBCM. Maximum growth reached a much greater density than the other cultures in just 2 days. However, rapid growth causes small differences in inoculation amounts or time of measurement to result in greater variability. Osmotic shock in LBCM was very slight with the addition of 0.6 M NaCl and remains so until 1.2 M NaCl is added. The hypothesis for this result is the presence of the potent osmoprotectant glycine betaine, a common osmoprotectant, in yeast extract which is provided in large amounts in this rich medium (17).
E2. Cellobiose, Glucose and Sucrose with 1021

Objective and Method

These experiments sought to repeat the observation of cellobiose and sucrose conferred osmoprotection by Gouffi et. al. so that the glucose and cellobiose mutants could be tested for the impairment of this effect. Glucose was also tried as an osmoprotectant. Osmoprotection by sucrose has been further studied and provided a positive control. (9) As previously mentioned the observation that a disaccharide of glucose resulted in osmoprotection but glucose did not was surprising so an additional examination was of interest. During this stage of the project the experimental method was still in development transitioning from the method described for media selection to the general method. Growth of 1021 in LA9 media with glucose, cellobiose, and sucrose was studied. An additional medium M9 glucose forced the uptake and metabolism of glucose by providing 10mM glucose as a lone carbon source. Cultures of Sm 1021 were grown with and without NaCl provided at either 0.6 M or 0.45 M concentration.

Results

While specific experiments were variable, osmoprotection by glucose either by addition to LA9 in trace amounts or as the sole carbon source in M9 Glucose was not observed. A comparison of salt stress by 0.45 M NaCl in M9 mannitol and in M9 glucose in presented in figure 1. This data should be considered qualitative as the experiment was not repeated with the same concentration of salt. Rather, a variety of media conditions were varied in attempt to find conditions in which glucose could act as an osmoprotectant. Results for all of these experiments were also negative; a list of media tested follows in table 2.
Osmoprotection by both cellobiose and sucrose was observed in LA9 with strain 1021. The effect of sucrose was apparent while that of cellobiose was marginal. Though statistically significant based on a two tailed t-test with a p-value of 0.0318 the effect is not robust enough to be useful in the characterization of mutants. These results were repeated with techniques described in the general methods and data from a representative experiment presented in table 1 and figure 2.

| Table 1 |
|------------------|------------------|------------------|------------------|
| Culture          | Average A<sub>600</sub> | Tube 1          | Tube 2          | Tube 3          |
| LA 9 Control     | 0.9765           | 0.9398          | 0.9864          | 1.0033          |
| 0.60M NaCl       | 0.3556           | 0.3417          | 0.3255          | 0.3997          |
| 0.60M NaCl +Cellobiose | 0.4335         | 0.4435          | 0.4451          | 0.4119          |
| 0.60M NaCl +Sucrose | 0.8509          | 0.9128          | 0.8416          | 0.7983          |

Table 1: Growth of Sm 1021 in LA9 medium measured as absorbance at 600nm recorded using JASCO V-530 spectrometer at after 48 hour growth period at 30°C.
Table 2

<table>
<thead>
<tr>
<th>Medium</th>
<th>Concentrations</th>
<th>Additions</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBCM</td>
<td>1 g Bacto© tryptone, 0.5 g Bacto© Yeast Extract, and 1 g NaCl added to 100 ml of deionized water. The solution was autoclaved at 250 °C and 15 psi for thirty minutes for sterilization.</td>
<td>NaCl (0.45-1.5 M), cellobiose, glucose, sucrose, manitol, ethanol</td>
</tr>
<tr>
<td>M9 + Mannitol</td>
<td>10 mM Mannitol, 1 mM MgSO4, 0.25 mM CaCl2, 0.5 μg/mL D-Biotin, and M9 salts.</td>
<td>NaCl(0.15-0.60M), cellobiose, glucose, sucrose, succinate, trehalose</td>
</tr>
<tr>
<td>LA9</td>
<td>10 mM Lactate, 10 mM Aspartate, 1 mM MgSO4, 0.25 mM CaCl2, 0.5 μg/mL D-Biotin, and M9 salts.</td>
<td>NaCl(0.15-0.60M), cellobiose, glucose, sucrose, succinate, trehalose</td>
</tr>
<tr>
<td>M9 + Lactate</td>
<td>10 mM Lactate, 1 mM MgSO4, 0.25 mM CaCl2, 0.5 μg/mL D-Biotin, and M9 salts.</td>
<td>NaCl(0.15-0.60M), cellobiose, glucose, sucrose, succinate, trehalose</td>
</tr>
<tr>
<td>M9 + Succinate</td>
<td>10 mM succinate, 1 mM MgSO4, 0.25 mM CaCl2, 0.5 μg/mL D-Biotin, and M9 salts.</td>
<td>NaCl(0.15-0.60M), cellobiose, glucose, sucrose, trehalose</td>
</tr>
<tr>
<td>M9 + Glucose</td>
<td>10 mM or 20 mM glucose, 1 mM MgSO4, 0.25 mM CaCl2, 0.5 μg/mL D-Biotin, and M9 salts.</td>
<td>NaCl(0.15-0.45M), cellobiose, asparte sucrose, succinate, trehalose</td>
</tr>
</tbody>
</table>

Figure 2: Data in table 1 presented graphically. Growth of Sm 1021 in LA9 medium measured as absorbance at 600nm recorded using JASCO V-530 spectrometer after after 48 hour growth period at 30 °C. Error bars represent standard error.
E3. Energy Equivalence

Objective

Because the inclusion of possible osmoprotectants could be considered as a change in medium it was important to confirm that other medium changes did not have a significant effect. Utilization of a wide variety of media led to the questioning of whether or not the various media were too divergent for valid comparison, a primary concern being carbon supply. This experiment sought to evaluate the growth of *Rm* 1021 on a variety of carbon sources in which the amount of energy afforded by each carbon source had been standardized.

Experimental Method

The number of ATP equivalents produced by each carbon source was estimated based on the known catabolic pathways utilized by *S. meliloti*. The primary factors include how each carbon source enters into the glucose catabolic pathway or is converted into a citric acid cycle intermediate. The path of these intermediates was then traced through the Entner-Doudoroff and citric acid cycle that *S. meliloti* primarily uses for carbon catabolism. (10, 14) Several assumptions were made to obtain these approximations. First all carbon afforded to the rhizobia was assumed to be fully catabolized to form ATP and not used for the synthesis of other molecules. Secondly the first assumption implies that one hundred percent of the carbon source provided in medium was absorbed by the bacteria. Finally the high energy electron carriers FADH$_2$ and NADH$_2$ were assumed to yield 1.5 and 2.5 ATP equivalents per mole, respectively, after donating those electrons to the electron transport chain. These assumptions are very broad and thus the estimates provided are very rough. However we presume that the resulting growth patterns can give some idea of the importance of the relative energy richness of the carbon source to rhizobial growth.
Once the estimates were made Rmi 1021 was grown in liquid cultures adjusted to the standard ATP potential of 6.00x10^{-7} M. M9 mannitol, LA9, and M9 succinate media were tested in this fashion. The liquid culture experiment followed the general methods.

Results

The estimated ATP equivalents produced for each mol of carbon source and the concentration which provided for a standardized ATP potential are listed in table 3. The standardized concentration for lactate and aspartate were calculated to result in a lactate and aspartate media with 6.00x10^{-7} M ATP potential when two carbon sources were provided in equal amounts.

<table>
<thead>
<tr>
<th>Carbon Source</th>
<th>Entry Point</th>
<th>ATP yield/ mol</th>
<th>Standardized Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td>pyruvate</td>
<td>28.5</td>
<td>21 mM</td>
</tr>
<tr>
<td>Succinate</td>
<td>Succinate</td>
<td>16.5</td>
<td>36.3 mM</td>
</tr>
<tr>
<td>Lactate</td>
<td>pyruvate</td>
<td>15</td>
<td>21.8 mM</td>
</tr>
<tr>
<td>Aspartate</td>
<td>oxalacetate</td>
<td>16.5</td>
<td>21.8 mM</td>
</tr>
</tbody>
</table>

Use of the standardized carbon source concentration values did not result in uniform growth of S. melliloti in the four growth media as evaluated by the final optical density reached in each culture. The absorbance at 600 nm from a representative experiment appears in table 4. These results were very similar to final growth values for media without standardized ATP potentials as observed in experiments outlined by table 2. Experiments in table 2 were completed in triplicate, however, only a single stater culture was use din each case. Confirmation of these experiments with a separate starter culture would be preferred so quantitative data is not reported.
### Table 4

<table>
<thead>
<tr>
<th>Culture</th>
<th>Tube 1 $A_{600}$</th>
<th>Tube 2 $A_{600}$</th>
<th>Tube 3 $A_{600}$</th>
<th>Average $A_{600}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>M9 Mannitol</td>
<td>1.7421</td>
<td>1.8597</td>
<td>1.9575</td>
<td>1.8532</td>
</tr>
<tr>
<td>LA9</td>
<td>0.74145</td>
<td>1.0941</td>
<td>0.8994</td>
<td>0.9027</td>
</tr>
<tr>
<td>M9 Succinate</td>
<td>0.9867</td>
<td>0.7536</td>
<td>0.8488</td>
<td>0.8630</td>
</tr>
<tr>
<td>M9 Lactate</td>
<td>1.2707</td>
<td>1.4109</td>
<td>1.1499</td>
<td>1.3663</td>
</tr>
</tbody>
</table>

E4. **Comparison of strain 1021 with mutants that fail to grow on glucose or cellobiose**

**Objectives**

Strains of *S. meliloti* with mutations in sugar metabolism may be very useful in determining importance of specific enzymes in the mechanism of disaccharide-induced osmoprotection. As previously mentioned a collection of mutations screened for impaired growth on cellobiose and/or glucose was available (12). Observation of these mutants in liquid culture with and without osmotic shock induced by NaCl allowed for mutations that impaired the rhizobia’s ability to cope with such stress to be identified. Following identification of mutant strains of interest enzyme assays was planned to determine the affected genes.

**Experimental Method**

The screening of glucose and cellobiose metabolism mutants was confirmed by growth on M9 glucose plates compared with growth on LA9 plates. A qualitative assessment of the mutations effect on osmoprotection was made by comparison of growth on LA9 plates with and without 0.45 M NaCl and
compared to plates with cellobiose and sucrose provided as osmoprotectants. These plates were prepared as liquid cultures in the general method with the addition of 1.5% granulated agar. \emph{S. meliloti} strains were streaked from LBCM plates.

Once confirmed, the growth of mutant strains was compared to the growth of strain \textit{Rm 1021} in liquid culture. LA9, LA9 with sucrose, LA9 with maltose, and LA9 with glucose media were compared. Each of above media was also observed with and without 0.45 M NaCl. Liquid cultures were prepared as outlined in general methods.

Results

The lack of growth of mutant strains on M9 glucose plates confirmed the screening process. The qualitative growth on LA9 plates is summarized in table 5. The growth plates suggest that the glucose minus mutants may have an impaired ability to adapt to conditions of osmotic shock. Strains 307620 and 207119 have very similar behavior and may contain mutations in the same gene. Strains 307620 and 207119 were more salt sensitive than the wild type and were not rescued from salt stress by the addition of 1 mM sucrose. This result suggests that mutations may have impaired a gene required for disaccharide-induced osmoprotection. Strain 20749 recovered from salt stress when provided with 1 mM sucrose and 1 mM cellobiose may have been more effective than in the wildtype strain.

<table>
<thead>
<tr>
<th>Culture</th>
<th>1021</th>
<th>307620</th>
<th>207119</th>
<th>20749</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA9 + 0.45 M NaCl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LA9 cellobiose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LA9 sucrose + 0.45 M NaCl</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 5: Growth of \emph{S. meliloti} evaluated after 4 days. (+) indicates formation of extensive colonies, (+-) indicates colonies formed with significantly smaller or less in number than 1021 control and (-) indicated no growth.
Data using strain 307620 is presented in figure 3. Interestingly strain 307620 seems to be very sensitive to osmotic shock. Disaccharides do not appear to confer osmoprotection even in the case of sucrose which normally provides a very robust effect. Strain 307620 grows poorly in LA9 medium when compared to the wild type (strain 1021) but growth recovers when glucose is provided.

**Figure 3**

Growth of strain 307620 after 72 hours evaluated by absorbance at 600nm recorded using JASCO V-530 spectrometer. Data shown represents the average reading from three cultures.

**Figure 4**

Growth of strain 1021 after 48 hours evaluated by absorbance at 600nm recorded using JASCO V-530 spectrometer. Data shown represents the average reading from three cultures.
E5. Osmotic Shock by Dry Down and Mannitol

Objectives

Interesting results with strain 307620 using treatment with 0.45 M NaCl in liquid culture may indicate that the mutation has disrupted an enzyme essential for the adaptation of the rhizobia to osmotic shock. However to confirm that osmotic shock, not the ionic shock was causing this decrease in growth additional experiments are necessary. Testing the survival of the strain following dry-down and with the non-ionic osmolyte mannitol could confirm previous observation as a result of a general osmotic effect.

Experimental Method

The method for osmotic shock by mannitol experiment closely followed the general method for liquid cultures in LA9. However 0.8 M mannitol was used to induce the shock and the whole culture solution was sterile filtered (0.2μm) after addition of the mannitol. Growth conditions and data acquisition followed the general method.

The dry-down experiment utilized round portions of filter paper with 1 cm diameter that were autoclaved inside a test tube. Strains 1021 and 307620 were grown in LA9 media with and without 1mM sucrose provided as an osmoprotectant. After a 48 hr growth period at 30 °C 10 μl amounts of the cultures were transferred to the sterile filter paper rounds. Control samples were created by the immediate transfer of the rounds to 3ml of LB medium in sterile test tubes. The tubes were resuspended and 100 and 1000 fold dilutions with LB medium were made. 25 μl amounts of each dilution were spread on LB plates. Samples for dry-down were created by allowing the inoculated filter rounds to incubate in sterile Petri dishes for 24 hours at 30 °C. After the dry down period the dilution and
resuspension scheme was repeated as with the controls. After allowing the plates to grow for several days colony counts were used to assess the viability of the rhizobia following dry down.

Results

Due to contamination these experiments were not completed.

Discussion

Media Based Experiments

Disaccharide induced osmoprotection in *S. meliloti* strain 1021 did not follow the expected patterns based on the published observations of Gouffi et. al. This group reported complete recovery of several strains of *S. meliloti* in conditions of osmotic shock when sucrose, trehalose, cellobiose, gentiobiose, palatanose, maltose, turanose or maltotriose were provided. Strain 1021 was not used in that work. The group summarized the structural requirements for the effect in *S. meliloti* as the presence of two glucosyl subunits or a glucosyl subunit and a fructosyl subunit (8). In this study sucrose afforded robust osmoprotection to *Sm* 1021 but cellobiose and maltose afforded very little or no advantage in LA9 medium (Figures 2,3). This suggests the hypothesis that strain 1021 cannot efficiently utilize disaccharides consisting of two glucosyl subunits such as cellobiose and maltose while supplemental disaccharides such as sucrose which consist of a glucosyl and a fructosyl subunit still provide full recovery. In order to assess this hypothesis further experiments with the other disaccharides reported as osmoprotectants in 1021 are needed. If these experiments confirm the hypothesis it would suggest that disaccharide-induced osmoprotection in *S. meliloti* occurs via two different pathways depending on the presence or absence of the fructosyl subunit. The possible subunit dependence of disaccharide osmoprotection is illustrated by figure 5.
All experiments testing for osmoprotection by glucose were negative. These observations are in agreement with published data that monosaccharides do not act as osmoprotectants (8). The functionality of disaccharides consisting of two glucosyl subunits, specifically cellobiose, provided the rationale for reexamining glucose as a possible osmoprotectant. As significant osmoprotection by cellobiose was not observed the lack of osmoprotection by glucose is not surprising.

The inability of cellobiose and maltose to provide osmoprotection for *Rm* 1021 may be attributed to a second difference in their structure as compared with sucrose. Both cellobiose and maltose are consist of two glucosyl subunits connected by a link between carbon one and carbon 4. The bond utilizes the anomeric carbon of only one of the subunits. The availability of the anomeric position make these diasaccharides reducing sugars. Conversely sucrose has no free anomeric carbon and is not a reducing sugar. Sucrose and trehalose are the only non-reducing sugars amongst those diasaccharades known to be osmoprotectants in other stains of *S. meliloti*. It is possible that strain 1021 cannot utilize reducing sugars as osmoprotectants and that the presence of a fructosyl subunit is unimportant. Thus it
is important that both the possible subunit dependence and the possible need for a non-reducing sugar in strain 1021 are examined so that a pattern due to the availability of an anomeric carbon is not mistaken as a subunit dependence. Further experiments with trehalose and palatinose could better define the structural requirements for osmoprotection in Rm 1021. Further confirmation could be obtained using a derivative of cellobiose or maltose with a methylated anomeric position.

**Glucose and cellobiose minus mutants**

Evaluation of osmoprotection with the mutants which fail to grow on glucose and cellobiose as sole carbon sources is still incomplete. However preliminary results with strain 307620 have proven interesting and can give some suggestions of the identity of the mutation. Supplemental amounts of sucrose, maltose (figure 4) and cellobiose (data not shown) do not provide osmoprotection but do increase growth in cultures without NaCl. This clearly indicates that the mutation does not interfere with the uptake of glucose or cellobiose. This observation makes the identification of the affected gene of great interest.

Comparison of strain 307620 with the mutation of glucose-6-phosphate dehydrogenase (*zwf* gene) described by Barra et. al. suggests that 307620 is not the same mutation. Strain 307620 is unable to grow on glucose as a single carbon source as expected of a *zwf* mutant. However inhibition of growth, attributed to a buildup of glucose-6-phosphate, when supplemental amounts of glucose are provided in media which contain an alternative carbon source is characteristic of *zwf* mutants (15). This characteristic was observed by Barra et. al. in their mutant with impaired disaccharide induced osmoprotection (9). Strain 307620 however does not demonstrate this trait as illustrated in figure 4.

The recovery of strain 307620 in comparison to the growth of wild type strain 1021 in the presence of supplemental glucose, sucrose, or maltose is also informative. This provides suggestive but not conclusive evidence that gluconeogenesis is impaired in strain 307620. If the mutation did impair
the synthesis of glucose providing a small amount could increase growth by providing glucose to serve metabolic function such as in nucleotide synthesis. However the preliminary data available thus far does not provide conclusive evidence for identification of strain 307620.
References


Appendix I

Media and Solution Details

Stock Solutions

5x M9 Salts 5.67 g of Na$_2$HPO$_4$ • 7 H$_2$O, 1.5 g of KH$_2$PO$_4$, 0.25 g of NaCl, and 0.5 g NH$_4$Cl salts were dissolved in gently warmed deionized water. The solution was brought to brought to a final volume of 100 mL with deionized water. The solution was autoclaved at 250°C and 15 psi for thirty minutes or passed through a 0.2 micron Nalgene filter for sterilization.

M9 salts solution for use in M9 + LA media was prepared as above with the exception of NH$_4$Cl which was omitted.

100 mM Aspartate 1.72 g of Sodium L-aspartic acid sodium salt monohydrate dissolved in 60 mL of deionized water. Solution brought to final volume of 100 mL with deionized water. The solution was passed through a 0.2 micron Nalgene filter for sterilization.

100 mM Lactate 1.12 g of Sodium L-Lactate dissolved in 60 mL of deionized water. Solution brought to final volume of 100 mL with deionized water. passed through a 0.2 micron Nalgene filter for sterilization.

100 mM Succinate 2.70 g Sodium succinic acid salt hexahydrate (Sigma Ultra) dissolved in 60 mL of heated deionized water. Solution brought to final volume of 100 mL with deionized water. The solution was filtered for sterilization.

10% mannitol 20 grams of mannitol dissolved in 150 mL of deionized water. The solution was stirred and with a stir bar and gently heated to aide in dissolving. Solution brought to final volume of 200 mL with deionized water. A 0.2 micron Nalgene filter was used for sterilization.

D-Biotin 100µg/ mL 20 µg of D-Biotin added to 200 mL of ultrapure. Hot plate and stir bar used for twenty minutes to aide in dissolving the D-Biotin. A 0.2 micron Nalgene filter was used for sterilization. D-Biotin stock was replenished every 6-8 weeks to ensure efficacy.

Media

LBCM 1 g Bacto® tryptone, 0.5 g Bacto® Yeast Extract, and 1 g NaCl added to 100 mL of deionized water. The solution was autoclaved at 250°C and 15 psi for thirty minutes for sterilization. (add calcium and magnesium concentration here)

M9 + Mannitol Using the sterilized standard solutions outlined above M9 + Mannitol cultures were prepared to contain: 10.0 mM Mannitol, 1mM MgSO$_4$, 0.25 mM CaCl$_2$, µg/mL D-Biotin, and M9 salts.

M9 + Lactate + Aspartate Using the sterilized standard solutions outlined above M9 + Mannitol cultures were prepared to contain: 10.0 mM Lactate, 10.0 mM Aspartate, 1mM MgSO$_4$, 0.25 mM CaCl$_2$, 0.5 µg/mL D-Biotin, and M9 salts.

M9 + Lactate Using the sterilized standard solutions outlined above M9 + Mannitol cultures were prepared to contain: 10.0 mM Lactate (6.00x10$^{-7}$ M ATP eq.), 1mM MgSO$_4$, 0.25 mM CaCl$_2$, µg/mL D-Biotin, and M9 salts.
M9 + Succinate  Using the sterilized standard solutions outlined above M9 + Mannitol cultures were prepared to contain:

10.0 mM Succinate (6.00x10^-7 M ATP eq.), 1mM MgSO_{4}, 0.25 mM CaCl_{2}, 1 µg/ml D-Biotin, and M9 salts.