Characterization and identification of environmental stress tolerance mechanisms in *Sinorhizobium meliloti*

By

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Abstract

The process of biological nitrogen fixation by rhizobial inoculum provides an environmental beneficial alternative to nitrogen acquisition compared to artificial fertilizers. *Sinorhizobium meliloti* is a gram-negative α-proteobacteria which can fix nitrogen when in a symbiotic relationship with its legume hosts *Medicago sativa* and *Medicago truncatula*. The ability of rhizobium to carry out this process however is dependent on rhizobium being able to thrive in the diverse conditions present in both soil and during symbiosis. This thesis describes the use of bacterial genetics to characterize how metabolism and the production of polysaccharides affect the survival of strains under salt and acidic pH stress. The gene *SMc00722* was characterized and shown to be integral for tolerance of high magnesium concentrations, though was not necessary for symbiotic establishment. The production of low molecular weight succinoglycan was shown be directly involved tolerating acidic pH, and directly influences the ability of the strain to compete for nodule occupancy. The gene *tkt2* was characterized as being the primary transketolase involved in the non-oxidative pentose phosphate pathway. The activity of this protein was shown to directly influence the production of succinoglycan, and tolerate acidic pH. Mutants in this gene were also shown to be impaired in biological nitrogen fixation, and the rate of nodule formation. Overall, these results are consistent with they hypothesis that mechanisms used by rhizobia to tolerate diverse environmental conditions directly interact with the symbiotic process. These data may lead to further understanding of the complex signaling process between rhizobia and legumes, and an understand of how rhizobial inoculums could be applied in more diverse soil conditions.

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Dedication

This thesis is dedicated my family and friends. To my parents, Andrew and Raquel, who have always encouraged me to go as far as I could go. From day one, they have always taught me to strive for my best and pursue my dreams as far as I can. It has been a long time since I first started university, and it has not always been easy, but they have always been there for me to help me when I needed it and I can never be thankful enough for it. To my Grandparents, who gave me the support and help I needed when times were tough, without you I never could have continued into grad studies so thank you so much. To my sister and brother in-law, Taylor and Bobby, and to my niece Jane, thank you for always being there for me, and always reminding me about things I'm prone to forgetting. And to all my family, I know I don't call or visit as much as I should, but you have all shown your love and support for me always, and I will never be able to show enough appreciation for it.

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List of Abbreviations

µg: Microgram
µL: Microliter
µM: Micromolar
6PG: 6-Phosphogluconate
Amp: Ampicillin
ATP: Adenosine triphosphate
BNF: Biological nitrogen fixation
bp: Base pair
CBB: Calvin-Benson-Bassham
CID: Collision induced dissociation
CCRH: Curled colonized root hair
cDNA: Complementary DNA
Cm: Chloramphenicol
Ct: Cycle threshold
DHAP: Dihydroxyacetone-3 phosphate
DNA: Deoxyribonucleic acid
DOC: Deoxycholate
E4P: Erythrose-4-phosphate
ED: Entner-Doudoroff
EMP: Embden–Meyerhof–Parnas
EPS: Exopolysaccharide
EPS-I: Succinoglycan
EPS-II: Galactoglucan
ESI: Electrospray ionization
F: Phenylalanine
F6P: Fructose-6-phosphate
FDR: False discovery rate
g: Gram
G1P: Glucose-1-phosphate
G3P: Glyceraldhyde-3-phosphate
G6P: Glucose-6-phosphate
Gm: Gentamycin
H_2O: Water
H_2SO_4: Sulfuric acid
HMW: High molecular weight
HPLC: High-pressure liquid chromatography
IT: Infection thread

K^+: Potassium cation
Km: Kanamycin
KPS: Capsular polysaccharide
L: Liter
LB: Luria-Bertani
LC: Liquid chromatography
LMW: Low molecular weight
LPS: Lipopolysaccharide
M: Molar
mg: Milligram
Mg^{2+}: Magnesium cation
MGS: Mannitol-Glutamate-Salts
$MgSO_4$: Magnesium sulfate
min: Minutes
mL: Milliliter
mM: Millimolar
MRM: Multiple reaction monitoring
MS: Mass spectrophotometry
N_2: Dinitrogen
Na: Sodium
Na^+: Sodium cation
NaOH: Sodium hydroxide
NC: No change
NF: Nod factor
NH_4^+: Ammonia
Nm: Neomycin
OD: Optical density
P: Phosphate
PC: Principle component
PCA: Principle component analysis
PEP: Phosphoenolpyruvate
PHB: Polyhydroxy-butyrate
PP: Pentose phosphate
QQQ: Triple quadrupole
qRT-PCR: Quantitative real-time PCR
R5P: Ribose-5-phosphate
Ribu5P: Ribulose-5-phosphate
RMM: Rhizobium minimal medium
RNA: Ribonucleic acid
S/N: Signal to noise

S7P: Sedoheptulose-7-phosphate
Sm: Streptomycin
Sp: Spectinomycin
TCA: Tricarboxylic acid cycle
Tg: Teragram
TIC: Total ion count
Tn: Transposon
UV: Ultraviolet
V: Volts
VMM: Vincent's minimal medium
W: Tryptophan
X5P: Xyulose-5-phosphate
Y: Tyrosine

Chapter 1:

Literature Review

1.1 Biological nitrogen fixation

Nitrogen is a critical limiting element for growth of all life (Woodmansee et al. 1978). While nitrogen accounts for approximately 78% of our atmosphere, it is present as inert N_2 and can not be utilized by plants and animals. Overall, N_2 represents a pool of $4x10^9$ Tg of nitrogen which can be potentially utilized for growth of living organisms (Hirsch and Mauchline 2015). N_2 must be converted to a useable form through either reduction or oxidation to be utilizable by plants and animals. It is estimated that 413 Tg of N_2 is fixed into useable forms annually (Fowler et al. 2013). This can occur from atmospheric fixation, biological fixation, or through industrial processes.

Artificial human input of reduced nitrogen into soils is estimated to be 121 Tg annually, accounting for approximately 30% of nitrogen input into soil (Hirsch and Mauchline 2015). This is carried out through the Haber-Bosch process, developed in the 1900s. The Haber-Bosch process is an energy intensive two-step process. First, methane and gaseous water are converted to H_2 and CO_2 in the presence of a nickel catalyst. Then N_2 and $3H_2$ are converted to $2NH_3$ using heat, pressure, and an iron catalyst (Ertl 1991). NH_3 produced in the process is then applied in various chemical compounds in fertilizer. However, it is predicted that only 10-17% of artificially introduced nitrogen is incorporated into plants, with the rest being lost to the environment through leaching (Leach et al. 2012). Loss of nitrogen to the surrounding environment has negative consequences, ranging from soil acidification to eutrophication, and has lead to a focus on utilizing biological means to fix and apply nitrogen to plants.

Atmospheric fixation primarily occurs through lightning fixation. Energy from lightning drives the production of NO_x from N_2, with NO_2 being primarily formed (Tie et al. 2002). NO_2 is

subsequently deposited into the soil through rainfall. This process is estimated to input 5 Tg of nitrogen annually into the nitrogen cycle (Tie et al. 2002).

Biological nitrogen fixation (BNF) occurs through the reduction of molecular nitrogen to NH_3 through diazotrophic microorganisms. Diazotrophs are microorganisms which can fix atmospheric N_2 to a useable form such as NH_3 and are capable of growth without external forms of reduced nitrogen. This reaction is carried out by the enzyme nitrogenase, converting N_2 and $8H^+$ to $2NH_3$ and H_2, using 16 ATP molecules in the process. Overall, BNF is predicted to contribute a total of 118 Tg of reduced nitrogen annually to soil (Fowler et al. 2013). 60 Tg of this amount is predicted to occur from the incorporation of nitrogen into legume plants through the process of symbiotic nitrogen fixation (SNF) (Herridge et al. 2008). Since nitrogen fixed in this manner is incorporated directly into the legume and not added to the soil, emphasis has been placed on studying SNF.

1.1.1 Rhizobia

Rhizobia are characterized as α- and β-proteobacteria which are capable of reducing atmospheric nitrogen to ammonia while in a symbiotic relationship with legume plants. Nod factor (NF) secreted by rhizobia in response to flavonoids from legumes can induce the formation of a micro-aerobic structure called the nodule on the roots of legume plants. Formed nodules can be either spherical (determinant nodules) or cylindrical (indeterminant nodules), and this is dependent upon each specific legume (Oldroyd et al. 2011). Rhizobia that inhabit nodules carry out BNF here using the enzyme nitrogenase. The genes necessary for symbiotic establishment and BNF are primarily encoded for by the *nod, nif,* and *fix* genes, and are found in most rhizobia. However, some rhizobia have been shown to be able to establish symbiosis in the

absence of *nod* genes, which encode for proteins that synthesize NF, through the use of type-III secretion systems (Giraud et al. 2007, Okazaki et al. 2013).

1.1.2 Sinorhizobium meliloti Rm1021

The symbiotic relationship between *Sinorhizobium meliloti* Rm1021 and *Medicago sativa* (Alfalfa) is one of the most studied examples of rhizobia-legume symbiosis, and is used as a model system for studying the interaction. *S. meliloti* is a gram-negative α-proteobacterium belonging to the *Rhizobiacea* family. In addition, *S. meliloti* is closely related to the animal and plant pathogens, *Brucella* and *Agrobacterium*, which has allowed for easy comparison between the two species. In addition to alfalfa, *S. meliloti* is known to be able to establish a symbiotic relationship with other legumes such as *M. truncatula*.

S. meliloti Rm1021 has a multipartite genome which was sequenced in 2001. The genome is split into the chromosome (3.6 Mbp) and two megaplasmids, pSymA (1.35 Mbp) and pSymB (1.68 Mbp) (Capela et al. 2001, Finan et al. 2001, Galibert et al. 2001). Each part of the *S. meliloti* genome is suggested to be maintained for their involvement in cell growth, metabolism, and symbiosis (diCenzo et al. 2014). The majority of genes essential for the growth of *S. meliloti* are located on the chromosome, which includes those necessary for transcription, translation, and DNA repair (Capela et al. 2001). pSymB has been suggested to be important for its growth in the diverse environment of the soil, since genes for exopolysaccharide synthesis, and transport and catabolism of small molecules are located on this plasmid (Finan et al. 2001). The function of pSymA has generally been associated with symbiosis, since genes necessary for nodule formation and nitrogen fixation are located on this plasmid. pSymA is not necessary for growth of *S. meliloti* since strains lacking this plasmid are still able to grow (Oresnik et al. 2000).

1.1.3 Symbiotic establishment between rhizobia and legumes

Symbiotic establishment occurs through a complex signal exchange. This begins from the secretion of plant derived flavonoids or iso-flavonoids, which are recognized by compatible rhizobia species (Oldroyd and Downie 2008, Oldroyd et al. 2011). Flavonoids are perceived by rhizobia by binding to the NodD transcriptional regulator (Fisher and Long 1993). In the case of the *S. meliloti – M. sativa* relationship, the recognized flavonoid is luteolin. Recognition of luteolin by NodD1 results in the induction of the *nod* genes, which encode the proteins necessary to synthesize NF (Fisher and Long 1993). The structure of NF is similar to chitin, and is comprised of 3 to 5 β(1-4) linked N-acetylglucosamine residues with a fatty acid tail on the first residue. Nod factor can have various modifications to the n-acetylglucosamine residues which are specific between each symbiotic interaction (Mylona et al. 1995). Purified Nod factor from *S. meliloti* has been shown to have both acetyl and sulfate modifications on its n-acetylglucosamine residues. Enzymes responsible for these modifications have been shown to be encoded for by *nodL* and *nodHPQ*, respectively (Spaink 2000). The initial recognition of NF is essential for symbiotic establishment, as strains unable to produce NF are unable to enter symbiosis (Spaink 2000).

After NF is synthesized, it is secreted into the environment where it can be recognized by the lysine motif (LysM) domain of receptor-like kinases on the host plant (Gust et al. 2012). Perception of NF by is usually by a pair of these proteins, and in *M. sativa* these proteins are MtNFP and MtLYK3 (Limpens et al. 2015). Recognition of NF by these proteins, in association with the protein DMI2, triggers nuclear calcium oscillations through a still yet undiscovered secondary messenger. Calcium spiking in the nucleus has been shown to be dependent upon the nuclear membrane localized proteins DMI1, CNG15C, and MCA8, which are responsible for

regulating calcium and potassium transport (Charpentier et al. 2013, 2016, Oldroyd 2013). These calcium oscillations are decoded by DMI3 (CCamK). Overall, this results in the production of transcription factors necessary for regulating root hair curling, and division of inner cortical cells, which form the nodule primordia (Mitra et al. 2004, Capoen et al. 2011).

1.1.4 Root hair colonization

Root hairs can curl around rhizobia already attached to the plant membrane forming an apoplastic space in which rhizobia can continue to proliferate. This structure is known as the curled colonized root hair. Colonization is initiated by the degradation of the plant cell wall, and a localized calcium influx at the root hair tip (Muñoz et al. 1998, Morieri et al. 2013). This results in the formation of an invaginated plasma membrane in the root hair called the infection thread (IT). The IT progresses through the root hair cell, and will continue to penetrate cell layers until it reaches the dividing inner cortical cells which form the nodule primordia (Oldroyd et al. 2011). Only rhizobia at the tip of the IT continue to replicate down the thread, resulting in only one clonal population reaching the forming nodule (Gage 2002).

IT formation and penetration requires constant production of both NF and exopolysaccharides (EPS) by *S. meliloti.* Mutants of *S. meliloti* Rm1021 unable to produce EPS-I, or add proper decoration to the forming polysaccharide result in the abortion of the symbiotic process (Long et al. 1988, Cheng and Walker 1998a, Limpens et al. 2003, Mendis et al. 2016). While the role of NF is clear, the exact role of exopolysaccharide production, specifically succinoglycan, is still unknown. It has been suggested that succinoglycan either plays a role in stress tolerance, or in immune system evasion (Miller-Williams et al. 2006, Jones and Walker 2008, Lehman and Long 2013). Recently, it has also been shown that EPS produced by *Mesorhizobium loti* are recognized by a receptor on *Lotus japonicus* called EPR3, and has been

shown to be directly involved in symbiotic signaling (Kawaharada et al. 2015). However, an EPS receptor in *M. sativa* has not yet been isolated. In addition to EPS, it has also been shown that another polysaccharide, cyclic β(1-2) glucan, is necessary for symbiotic establishment, but the role they play during symbiosis remains unclear (Dylan et al. 1990b).

1.1.5 Bacteroid formation

Rhizobia at the tip of the infection thread eventually become endocytosed into inner cortical cells which make up the nodule primordium. When endocytosed, bacteria become surrounded by a plant derived membrane, forming a structure termed the symbiosome. The space between the plant derived membrane and the bacteria is termed the peribacteroid space. Estimates using FACS have shown that there are thousands of symbiosomes present in a single plant cell (Udvardi and Poole 2013). In the *S. meliloti – M. sativa* symbiotic relationship, rhizobia undergo a process of terminal differentiation into bacteroids. This involves the replication of the *S. meliloti* genome up to 24 times without the division of the cell (Oldroyd et al. 2011). Recent work has shown that terminal differentiation is due to activity of plant derived nodule cysteine rich peptides (NCR). *M. truncatula* unable to produce NCRs have been shown to be defective in bacteroid formation (Van de Velde et al. 2010, Horváth et al. 2015). However, terminal differentiation only occurs in a subset of legume species which has been termed the inverted repeat-lacking clade (IRLC) legumes. Formed bacteroids are then capable of performing biological nitrogen fixation, providing nitrogen to the plant in the form of either amides or purines (Geddes and Oresnik 2016).

1.2 Central carbon metabolism

Rhizobia will encounter numerous carbon sources during saprophytic growth. Therefore, the ability to metabolize numerous carbon sources could provide a competitive advantage to

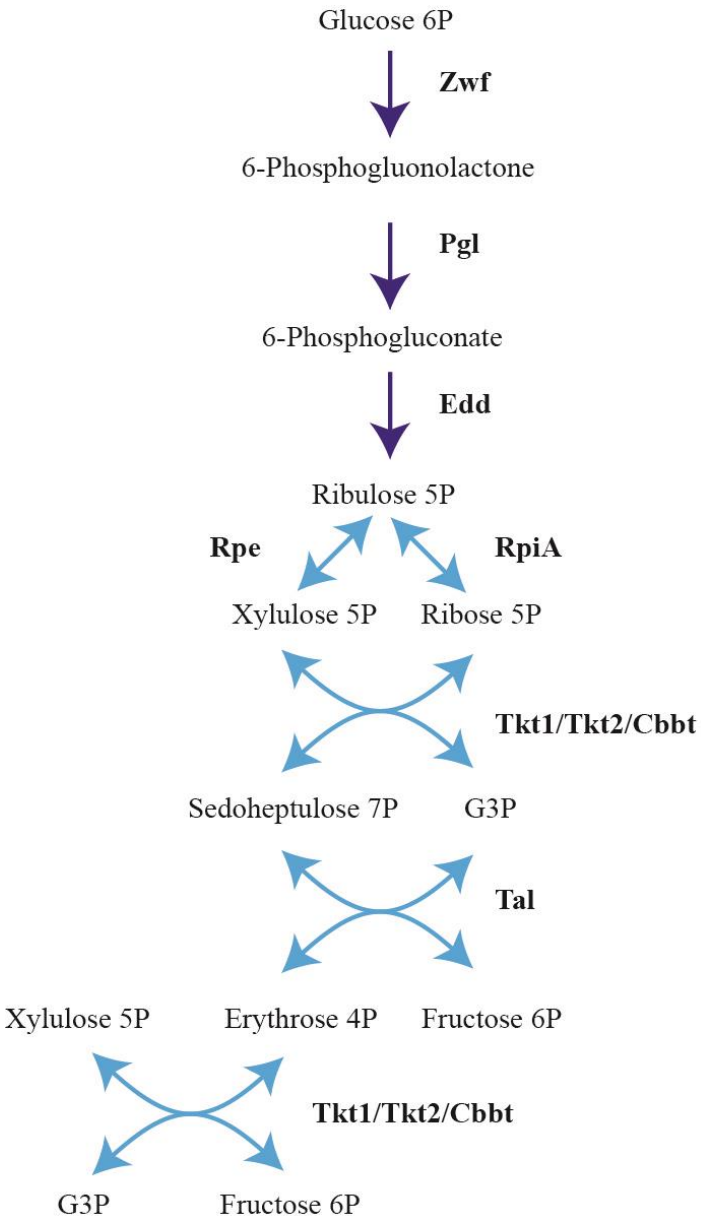
rhizobia during symbiotic establishment. The genome of *S. meliloti* encodes for a wide array of genes for transport and catabolism of carbon sources and small molecules, and as such has the ability to grow on a diverse number of substrates (Stowers 1985, Galibert et al. 2001). The inability of rhizobia to metabolize specific carbon sources has been shown to affect competition for nodule occupancy, or result in the inability to symbiotically fix nitrogen for their respective hosts (Finan et al. 1988, Oresnik et al. 1998, Yost et al. 2006, Geddes and Oresnik 2012a). This has led to the hypothesis that carbon metabolism is a key determinant in symbiotic efficiency.

Central carbon metabolism in *S. meliloti* occurs through the Entner-Doudoroff (ED), Embden-Meyerhof-Parnas (EMP), pentose phosphate (PP), and Tricarboxylic Acid (TCA) pathways (Geddes and Oresnik 2014). *S. meliloti* primarily utilizes the ED, and the lower half of the EMP pathway for catabolism of hexoses. Due to the absence of phosphofructokinase, which catalyzes the formation of fructose-1,6-bP from fructose-6P (F6P), the EMP pathway is primarily utilized for gluconeogenesis (Irigoyen et al. 1990, Capela et al. 2001). *S. meliloti* also encodes for enzymes involved in the Calvin-Benson-Bassham (CBB) pathway in the *cbb* operon, allowing for autotrophic growth (Pickering and Oresnik 2008).

1.2.1 Pentose phosphate pathway

The pentose phosphate pathway is critical for generating metabolites necessary for the nucleotide synthesis (Ribose-5P), and aromatic compounds (Erythrose-4P). Activity associated with carbon cycling through the pentose pathway has previously been shown in *S. meliloti*, and has been suggested to be important in producing EPS (Portais et al. 1999, Gosselin et al. 2001). The PP pathway is divided into the oxidative and non-oxidative branches which act in subsequent order (Figure 1).

Figure 1 Pentose phosphate pathway



Enzymatic reactions of the pentose phosphate pathway. Arrows in purple are part of the oxidative pentose phosphate pathway, and arrows in blue are occur in the non-oxidative portion of pathway. Enzymes carrying out each reaction are indicated in bold. Zwf: Glucose-6P dehydrogenase, Pgl: 6PG lactonase, Edd: 6PG dehydrogenase, Rpe: Ribulose-5P epimerase, RpiA: Ribose-5P isomerase, Tkt1/Tkt2/Cbbt: Transketolase, Tal: Transaldolase

The oxidative pentose phosphate pathway is largely shared with the ED pathway, and overall converts glucose-6P (G6P) to ribulose-5P. In yeast and mammalian cell lines, the oxidative PP pathway also has suggested importance in producing the cellular reductant NADPH (Gorsich et al. 2006, Krüger et al. 2011). The first step is considered the rate limiting step in many organisms, and involves converting glucose-6P to 6P-gluconolactone using G6P dehydrogenase, generating one NADPH from $NADP^+$ in the process (Stincone et al. 2015). This substrate is then converted to 6P-gluconate (6PG) by 6P-gluconolactonase. The first dedicated enzyme of the pentose phosphate pathway is 6PG dehydrogenase, which converts 6PG to ribulose-5P, producing 1 NADPH in the process. This reaction is not reversible and is also considered the committal step of the PP pathway (Geddes and Oresnik 2014). The non-oxidative part of the pathway is largely involved in interconverting pentoses and hexoses through the reactions of transketolase and transaldolase. Enzyme activities associated with the activity of these proteins have been detected in *S. meliloti*, but these proteins have not yet been identified (Cervenansky and Arias 1984).

1.2.2 Transketolase and transaldolase

The two major enzymes of the non-oxidative pentose phosphate pathway are transketolase and transaldolase. Transketolase is found ubiquitously among organisms, and is the rate limiting enzyme of the non-oxidative pentose phosphate pathway (Kochetov and Solovjeva 2014). The transketolase protein is generally found as a homodimer with two catalytic sites. Each site also incorporates a binding site for its two co-factors, thiamine diphosphate and calcium, which help coordinate the reaction of transketolase, (Kochetov and Philippov 1970). While other divalent cations may also be utilized in different organisms, this only affects the rate of the reaction and not the overall reaction (Heinrich et al. 1972). Transketolase utilizes a ketose donor

and aldose acceptor to carry out the reversible reactions: ribose-5-phosphate and xylulose-5-phosphate to sedoheptulose-7-phosphate and glyceraldhyde-3-phosphate, and xylulose-5-phosphate and erythrose-4-phosphate to fructose-6-phosphate and glyceraldhyde-3-phosphate (Schenk et al. 1998). While in *Escherichia coli*, both *tktA* and *tktB* are known to encode for transketolases (Josephson and Fraenkel 1974, Iida et al. 1993), *S. meliloti* is predicted to encode 3 transketolases: Tkt1, Tkt2, and CbbT.

Like transketolase, transaldolases are also found among all organisms (Samland and Sprenger 2009). Transaldolases do not use any cofactors for their activity, and their mechanism of action involves the transfer of dihydroxyacetone between sugar phosphates. In the pentose phosphate pathway, transaldolase catalyzes the reversible reaction of fructose-6-phosphate and erythrose-4-phosphate to sedoheptulose-7-phosphate and glyceraldhyde-3-phosphate (Samland and Sprenger 2009).

1.3 Exopolysaccharides

1.3.1 Succinoglycan

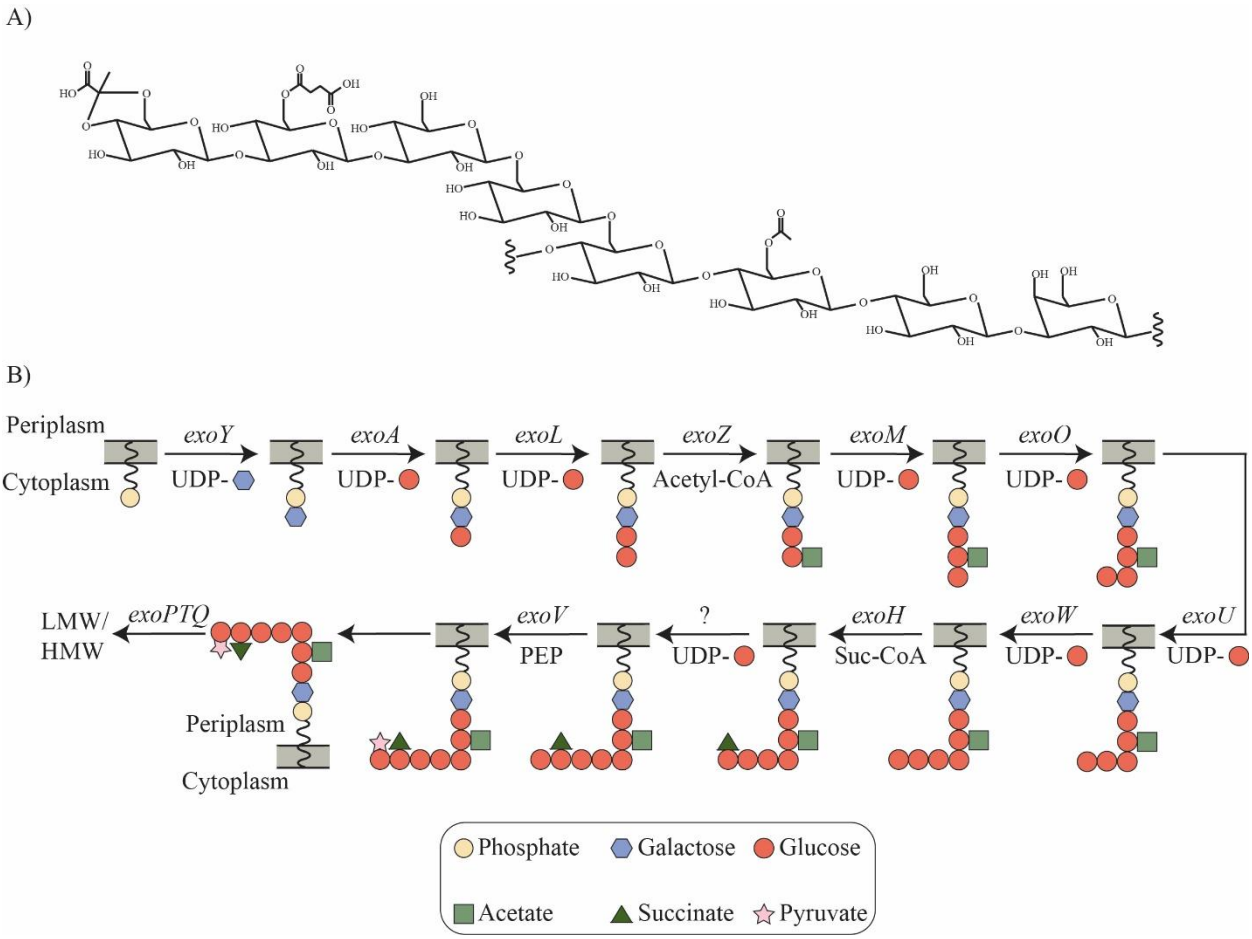
S. meliloti is able to produce two main types of EPS; succinoglycan (EPS-I) and galactoglucan (EPS-II) (Skorupska et al. 2006). Succinoglycan is comprised of a repeating polymer that is made up of an eight sugar core consisting of one galactose and seven glucose units that can be modified with succinyl, pyruvyl, and acetyl groups (Reinhold et al. 1994). The overall process involved in EPS-I biosynthesis has been extensively characterized (Reuber and Walker 1993), and is outlined in Figure 2. Genes necessary for the overall synthesis of EPS-I are found in the *exo* gene cluster localized on pSymB. A mutation in the gene *exoY*, which encodes a galactosyltransferase necessary for the initial transfer of galactose to the phosphoprenol carrier, is unable to produce any EPS-I (Cheng and Walker 1998b).

Succinoglycan is produced in both high molecular weight (HMW) and low molecular weight (LMW) forms. The overall chain length of succinoglycan can be modified in two primary ways; cleavage of the polysaccharide by endoglycanases, or regulation by the *exoPTQ* system. While some work has been done on characterizing *exoPTQ*, little is yet known about how it influences EPS-I polymer chain length (Becker et al. 1995, González et al. 1998). The endoglycanases ExsH and ExoK are involved in directly cleaving HMW EPS-I into LMW forms. Strains lacking the endoglycanases ExsH and ExoK have been shown to not produce any LMW EPS-I (Mendis et al. 2016). In addition, *exoH* mutant strains, which lack the ability to add the succinyl group to the EPS-I polymer, have been shown to produce only HMW EPS-I which cannot be cleaved to LMW EPS-I by the endoglycanases (York and Walker 1997). Interestingly, while an *exsH/exoK* double mutant strain is symbiotically active, a strain mutated in *exoH* is unable to establish functional symbiosis (Leigh et al. 1987, Cheng and Walker 1998b, Mendis et al. 2016). This has led to the hypothesis that suggests that succinylation of EPS-I may be critical for symbiosis.

1.3.2 Galactoglucan

Galactoglucan is comprised of a repeating polymer of a single glucose linked to a galactose (Skorupska et al. 2006). EPS-II can be modified with acetyl and pyruvyl groups (Her et al. 1990). The genes necessary for EPS-II biosynthesis are found in the *exp* (*wga)* loci localized on pSymB (Finan et al. 2001). Mutation of *expA* has been shown to prevent production

Figure 2. EPS-I biosynthesis



Overview of succinoglycan biosynthesis. A) The repeating monomer of succinoglycan used to make both high and low molecular weight forms of the polysaccharide. B) Representation of the succinoglycan synthesis pathway in *S. meliloti*. Each indicated gene encodes the protein necessary to catalyze the addition of each indicated substrate. Succinoglycan is synthesized in the cytoplasm before being exported into the periplasm.

of EPS-II (Glazebrook and Walker 1989). However, the exact biosynthetic pathway of EPS-II remains unclear. *S. meliloti* strain Rm1021 is unable to produce EPS-II under normal conditions due to an insertion element in the regulator *expR*. However, production of EPS-II can be restored in Rm1021 under specific medium conditions (Oresnik et al. 1994, Mendrygal and González 2000). Stains which have an intact *expR* gene are able to produce EPS-II under normal laboratory conditions and are termed *expR*$^+$ (Glazebrook and Walker 1989). The production of EPS-II in the *expR*$^+$ background of *S. meliloti* has been shown to rescue the symbiotic defect associated with the inability to produce EPS-I production (Glazebrook and Walker 1989).

1.3.3 Cyclic β(1-2) glucans

In addition to the two primary exopolysaccharides, the production of cyclic β(1-2) glucans has been shown to be necessary for symbiosis. This polysaccharide is a cyclic chain of 17-25 β(1-2) linked glucose units (Spaink 2000). Similarly to succinoglycan, cyclic β(1-2) glucans can also be decorated with succinyl groups (Miller et al. 1988), and they can also have phosphoglycerol additions. The production of cyclic β(1-2) glucans is encoded by 3 genes: *ndvA*, *ndvB*, and *opgC*. NdvA and NdvB are involved in the export and biosynthesis of the polysaccharide respectively (Galibert et al. 2001). While not yet characterized, *opgC* is putatively annotated as a glucan succinyl transferase involved in adding the succinyl group to the forming polysaccharide. These polysaccharides primarily accumulate in the periplasm, but can also be excreted (Abe et al. 1982). Mutant strains unable to produce this polysaccharide are unable to colonize alfalfa, being found to be deficient in attachment to plant root hairs or end up forming abortive infection threads (Dylan et al. 1990b).

1.4 Stress tolerance mechanisms and symbiotic signaling

Rhizobia encounter different stresses during its growth in the soil, and during symbiotic establishment. These stresses need to be successfully managed to establish a symbiotic relationship with legumes. These conditions include: changes in osmolarity, decreasing pH, lowered oxygen concentration, and reactive oxygen species. In addition, symbiotic bacteria must be able to manage the plant immune response in order to successfully colonize legumes. The ability to tolerate these conditions has shown to be closely related to the ability of rhizobia to establish symbiosis, or successfully compete for nodule occupancy. The tolerance to these stresses can be observed to promote the production of determinants necessary for symbiotic establishment and nitrogen fixation. This has led to the hypothesis that conditions present during symbiosis may act as a signal for symbiotic establishment, rather than obstacles that rhizobia simply must tolerate to successfully colonize the plant. The following lists examples of how the stress response of both legumes and bacteria have been co-opted to be involved in symbiotic signaling.

1.4.1 Plant immune response

Plants are subject to numerous pathogens throughout their life cycle, and have developed a signaling pathway to respond to invading pathogens. Activation of plant immunity results in the production of phytoalexins (Ren et al. 2008), reactive oxygen species (ROS) formation (Ren et al. 2002), changes in gene expression (Denoux et al. 2008), and sialic acid accumulation (Martinez-Abarca et al. 1998). The plant immune response is activated when microbe-associated molecular patterns (MAMPs) are recognized by plant receptors (Dangl and Jones 2001), resulting in MAMP triggered immunity (MTI). Two MAMPs largely studied for triggering MTI are bacterial flagellae and cell wall chitin. Bacterial flagellae are detected by a conserved amino

acid motif on the flagella known on the as Flg22. In *Arabidopsis thaliana,* this motif has been shown to be recognized by a leucine rich receptor-like kinase on the plasma membrane called FLS2 (Gómez-Gómez and Boller 2000). Mutation of the gene encoding for this receptor was shown to abolish the oxidative burst associated with immune system activation (Gomez-Gomez, 2000).

Chitin is a MAMP located primarily on fungal cell walls, and is made up of a polymer of repeating β(1-4) linked n-acetyl glucosamine residues which can be variably modified. Application of chitin to *Medicago truncatula* root hairs has been shown to elicit an immune response through an increased production of ROS (Kim et al. 2006). It has been shown that application of purified chitotetraose, which is comprised of 4 β(1-4) linked n-acetylglucosamine residues, induces production of sialic acid and jasmonic acid in *M. truncatula* (Leitner et al. 2008). Though a receptor for chitin has not been found in *M. truncatula,* a transmembrane LysM receptor kinase called CERK1 in *A. thaliana* has been identified to be involved in recognition of chitin (Miya et al. 2007). CERK1 was determined to be essential for activating the mitogen activated protein kinase response, and thus the immune response, in *A. thaliana* to chitin recognition (Miya et al. 2007).

It is theorized that rhizobium-legume symbiosis evolved from a pathogenic relationship with a bacteria (Nakagawa et al. 2011). There are many similarities in how plants respond to both symbiotic bacteria and pathogens (Berrabah et al. 2015). In accordance with the theory that symbiosis evolved from pathogenicity, inoculation of *Lotus japonicus* with *Mesorhizobium loti* still results in an induction of plant defence responses (Kouchi et al. 2004). Parts of the immune response, such as the creation of oxidative stress, are known to be necessary for symbiotic establishment (Puppo et al. 2013). Mutants over-expressing catalase resulted in slower

nodulation and malformed infection threads (Jamet et al. 2007). This shows that a fine tuning of the immune response is necessary to create proper conditions for symbiotic establishment. In addition, molecules such as flavonoids have both anti-microbial activity and are necessary for symbiotic establishment.

1.4.2 Flavonoids

The biosynthesis of flavonoids in plants is generally well understood (Ferrer et al. 2008). They are produced as secondary metabolites, and to date thousands of different flavonoids have been isolated (Ferrer et al. 2008). The biochemical diversity of flavonoids is achieved through biochemical modification of a limited number of base structures. Flavonoids play diverse roles in plant biology ranging from affecting flower colour, auxin transport, and anti-microbial defences (Winkel-Shirley 2001). Flavonoids are localized to a number of different locations within the plant including the flower, seeds, and roots, and can be secreted from the root into the rhizosphere (Peer 2001, Morris and Djordjevic 2006, Cesco et al. 2010).

Flavonoids are known to inhibit the growth of a number of soil pathogens including both bacteria and fungi (Hassan and Mathesius 2012). The production of flavonoids has long been known to increase in response to pathogen invasion, and have been shown to be directly involved in plant defence response (Cramer et al. 1985). One group of flavonoids, the iso-flavonoids, are found exclusively in legumes (Hirsch et al. 2001). Iso-flavonoids were originally thought to be involved in defense against fungi, and were later shown to also have toxic effects on some isolated bacteria. Following the isolation of iso-flavonoids from field grown legumes, work to formally assess the effect of a number of iso-flavonoids on various rhizobia species was carried out (Pankhurst and Bigs 1980). It was found that numerous iso-flavonoids harboured anti-

microbial activity against rhizobia species, and it was concluded that these compounds deserved more attention with respect to their effect on nodule development.

The role of flavonoids in symbiosis was determined by purifying compounds from plant root exudates which could induce the transcription of *nodABC,* which encode proteins necessary for NF synthesis for symbiosis (Peters et al. 1986, Hartwig et al. 1990). Characterization of the induction showed that luteolin induced *nodABC* when applied to bacteria in nM concentrations. Secretion of luteolin was also shown to occur in distinct areas of the developing root (Peters and Long 1988). Recognition of specific flavonoids was also shown to play a role in plant-host specificity during symbiosis (Hartwig et al. 1990). Taken together, it seems that flavonoids secreted from plants were primarily used as anti-microbial agents (Cowan 1999). However, rhizobia have been able to utilize very specific portions of this defence response as a signal indicating the presence of a compatible host, and respond through the production of Nod factor to initiate symbiotic signaling.

1.4.3 Nod factor

The initial bacterial signal that occurs in the rhizobia-legume symbiosis is the production of a lipochito-oligosaccharide termed Nod factor (Lerouge et al. 1990). The structure of NF is comprised of 3 to 5 β(1-4) linked n-acetylglucosamine residues, with a fatty acid tail on the first residue, and can have various modifications to the n-acetylglucosamine residues (Mylona et al. 1995). While structurally similar to chitin, Nod factor contains shorter n-acetylglucosamine chain lengths.

In the *S. meliloti- M. truncatula* model, NF is recognized by the LysM receptors MtNFP and MtLYK3 (Oldroyd 2013). This recognition induces a number of responses from *M. truncatula* which are necessary for successful symbiotic establishment. Transcriptomic studies

have revealed that Nod factor recognition can regulate genes involved in the plant immune response (El-Yahyaoui et al. 2004). Interestingly, recent studies have also shown that isolated Nod factor from rhizobia can affect the immune response of both legumes and non-legumes. The innate immune response due to recognition of Flg22 in *Arabidopsis thaliana* was observed to be decreased in response when NF isolated from *B. japonicum,* or chitotetraose was also applied (Liang et al. 2013). NF also restored bacterial growth on *A. thaliana* leaves during application of Flg22, which is known to stimulate the innate immune system. This suggested that Nod factor mediated suppression of the plant's innate immune system is necessary for successful colonization of plants.

Nod factor is known to activate some innate defence responses from the plant. Transcriptomic studies indicate that the plant immune response is initially activated due to *S. meliloti* inoculum (Lohar et al. 2006). Application of purified Nod factor results in production of H_2O_2, which in turn induces the expression of the *rip1* peroxidase in *M. truncatula,* (Ramu et al. 2002). Increased production of H_2O_2 has also been observed to occur around root hair tips during Nod factor exposure (Cardenas et al. 2008). So, while the suppression of the immune system is necessary for growth, the initial immune response may provide a signal for rhizobia to undergo physiological changes necessary for a symbiotic lifestyle through oxidative stress and plant immune peptides. Observations from early signaling involving the interplay of flavonoids and Nod factor production is a clear example of how a potential stress, flavonoids, induce a bacterial signal, Nod factor, that have become completely necessary for symbiotic interaction. However, outside of the plant defence response, various symbiotic stresses are predicted to occur during symbiotic establishment, which are also observed to play a role in potential symbiotic signaling.

1.4.4 Osmotic stress

Genes involved in adaptation to varying osmotic conditions have been shown to be critical for the establishment of a functional symbiotic relationship. Osmotic conditions before symbiosis are fully dependent on salts and exudates present in the soil. Bulk soil generally has a low osmolarity with some exceptions, due to the ability of soil to bind solutes (Miller and Wood 1996). However, the area of the rhizosphere is predicted to have a higher osmolarity due to plant exudate. Plant roots take up water, essential nutrients, and minerals rom the rhizosphere which are necessary for their growth. However, non-essential ions such as sodium and aluminum are either left in the rhizosphere, or excreted, which leads to localized areas of high osmolarity (Jungk 2002). While the osmotic conditions throughout symbiosis in the rhizobia-legume interaction are unknown, current research is consistent with the hypothesis that both high and low osmolarity conditions exist throughout symbiosis (Botsford and Lewis 1990, Dylan et al. 1990a). Since tolerance mechanisms for high and low osmolarity have been shown to be necessary for symbiosis, it is necessary to understand the roles osmoprotectants may play in symbiosis (Dylan et al. 1990b, Robledo et al. 2012).

The ability to tolerate high osmolarity conditions in rhizobia has been studied extensively. Bacteria can increase cytoplasmic osmotic strength through the import or synthesis of cytoplasmic carbohydrates (Yancey et al. 1982). This prevents the loss of cell volume to the external environment. Under high osmolarity conditions, rhizobia have also been shown to accumulate n-acetylglutaminylglutamine (NAGGN), proline-betaine, and glycine-betaine as osmoprotectants. However, *S. meliloti* is the only rhizobium species known to accumulate NAGGN (Smith et al. 1994, Miller and Wood 1996). Rhizobia are also known to increase

glutamate production in response to osmotic stress, which is predicted to act as a counter-ion for potassium accumulation (Botsford et al. 1994).

Bacteria also exhibit the ability to modify intracellular osmotic conditions through ion flux. In both *Escherichia coli* and *S. meliloti* it has been shown that intracellular levels of potassium increase due to osmotic stress (Csonka 1989, Botsford and Lewis 1990). Interestingly, increased potassium levels lead to an increase in nitrogenase activity in *Bradyrhizobium* sp 32H1 when grown under low oxygen conditions (Gober and Kashket 1987). As the bacteroid is predicted to be an area of elevated osmotic stress (Miller and Wood 1996), this provides a link showing that osmotic stress tolerance may be a signal for the regulation of nitrogenase in the bacteroid through the regulation of potassium concentration.

Adaptation to hypoosmotic conditions has also been shown to important in the plant-microbe interaction. To adapt to hypotonic conditions, many bacteria have been shown to accumulate periplasmic glucans. For instance, *E. coli* accumulates membrane derived oligosaccharides in response to either high salt concentrations or sucrose (Rumley et al. 1982). Interestingly, mutations preventing the accumulation of membrane derived oligosaccharide (MDO) in the periplasm did not affect the ability of *E. coli* to grow in hypotonic media (Bohin and Kennedy 1984). However, evidence suggests that the accumulation of periplasmic oligosaccharides is an adaptive response that modulates the osmolarity of the periplasm in both *E. coli* and *S. meliloti*.

The majority of the organisms in the family *Rhizobiaceae* produce a cyclic β(1-2) linked glucan (York et al. 1980, Breedveld and Miller 1994). While primarily located in the periplasmic space, cyclic β(1-2) glucans can also be secreted into surrounding media (Abe et al. 1982). As with MDO accumulation in *E. coli*, cyclic β(1-2) glucans are observed to accumulate in both

rhizobia and *Agrobacterium* (Dylan et al. 1986). Accumulation of these periplasmic glucans can be observed when grown under hypotonic conditions, and suppressed in hypertonic media (Miller et al. 1986, Dylan et al. 1990a, Breedveld and Miller 1995). The apparent osmoregulation of these glucans led to the hypothesis that cyclic β(1-2) glucans may be involved in hypotonic stress adaptation in rhizobia. Using a mutant which was impaired for the ability to produce cyclic β(1-2) glucans, it was observed that the inability to produce these cyclic glucans impaired the ability to grow on hypotonic medium, unlike in *E. coli* (Dylan et al. 1990a).

The important link between hypotonic stress tolerance and symbiotic signaling became apparent when strains unable to produce cyclic β(1-2) glucans were examined for the ability to enter into an effective symbiotic relationship with their host plant. A mutation in *S. meliloti ndvB*, which is essential for production of cyclic β(1-2) glucans, prevented *S. meliloti* from establishing a symbiotic relationship with *M. truncatula* (Dylan et al. 1990a). Similarly, *A. tumefaciens* strains unable to produce these cyclic glucans are also impaired in virulence, and are observed to be unable to attach to plants to initiate virulence (Douglas et al. 1985, Swart et al. 1994). While increasing media osmolarity restored virulence of *A. tumefaciens*, this has not been observed in rhizobial symbiotic relationship with legumes (Swart et al. 1994). In an attempt to restore symbiosis in a *S. meliloti ndvB* mutant, extracellular cyclic β(1-2) glucan was added to media, in lines with the hypothesis that cyclic β(1-2) glucans are involved in attachment to the plant. However, addition of external cyclic β(1-2) glucan did not restore symbiosis between *S. meliloti* and *M. sativa* (Dylan, 1990b).

Taken together, this suggests that the role of cyclic β(1-2) glucan production in rhizobia is involved in multiple steps in the symbiotic process. In support of this, increased amounts of cyclic β(1-2) glucan can be isolated from *S. meliloti* bacteroids (Abe et al. 1982). This suggests a

role for cyclic glucans outside of hypoosmotic stress adaptation since the bacteroid is predicted not to be an area of hypoosmotic stress. One suggested role could be that cyclic glucans play a role in symbiotic signaling. This may indicate that areas of hypotonic stress throughout symbiosis increase the production of cyclic β(1-2) glucans to be in high enough concentration to act as a signal for establishing symbiosis.

1.4.5 Low oxygen content

During symbiotic establishment, rhizobia encounter areas of low oxygen concentration in the nodule. Control of oxygen concentration is important for symbiosis since oxygen inhibits the activity of nitrogenase (Hunt and Layzell 1993). However, oxygen is also needed for bacterial cells to produce optimal levels of ATP for cellular function (Hunt and Layzell 1993). Oxygen levels are controlled in the nodule through a diffusion barrier, formed by the plant below the nodule cortex, to create optimal oxygen concentrations for nitrogen fixation (Hunt et al. 1987). Tight regulation of oxygen concentration in bacteroids also leads to a number of signaling and physiological changes in bacteria, which promote symbiosis and nitrogen fixation. It has been well documented that low oxygen concentrations activates the two component system FixJL, which in turn increases the transcription of the majority of the genes involved in nitrogen fixation (David et al. 1988, Virts et al. 1988).

Oxygen concentration has also been shown to regulate LPS synthesis and decoration (Kannenberg and Brewin 1989, Tang and Hollingsworth 1998). This is thought to have a role in adaptation to the low oxygen environment. Production and modification of LPS is strain specific and is thought to be involved in determining host range for symbiosis in some rhizobia (Via et al. 2016). The ability to produce or properly modify LPS has been linked to defects in symbiotic establishment (Keating et al. 2002). As LPS content and decoration is dynamic based upon its

environment, it is expected that LPS modification would change during symbiosis. Recent work has also shown that flavonoids can induce changes in decoration of LPS, and that these changes are necessary for symbiosis (Broughton et al. 2006). Overall, it seems that low oxygen concentration can bring about a change in LPS production which is necessary for both symbiosis and survival in these conditions, in addition to regulating transcription of genes necessary for nitrogen fixation.

1.4.6 Reactive oxygen species

In addition to low oxygen concentration, rhizobia encounter ROS during symbiotic establishment. This includes the generation of both H_2O_2 and peroxide radicals through the innate immune response of the plant. The production of ROS can be found throughout symbiotic compartments ranging from the IT to mature nodules (Santos et al. 2001). ROS are generated upon Nod factor recognition, and is thought to predominantly occur from the activity of NADPH oxidase (Lohar et al. 2007). Rhizobia utilize a number of mechanisms to deal with potential damage from ROS (Boscari et al. 2013). The importance of ROS scavenging during symbiosis is highlighted by the finding that strains which carry mutations in the genes *katB/C,* which encode for catalases, are impaired in infection thread formation (Jamet et al. 2003). However, a positive role for ROS in symbiosis has also been observed. When catalase is over-expressed in *S. meliloti*, aberrant IT formation and delayed nodule development are observed (Jamet et al. 2007). While it is unknown exactly how ROS may contribute to symbiosis, two main suggestions have been made; either ROS plays a role in IT development, or ROS induces physiological changes in *Rhizobium* that are necessary for symbiosis (Pauly et al. 2006).

In line with the thinking that ROS may act as a signal to bacteria for symbiotic establishment, it has been shown *B. japoncium* exposed to oxidative stress produces an increased

amount of EPS (Donati et al. 2011). The production of EPS has long been suggested to be involved in the tolerance of various stresses encountered by bacteria. In *Pseudomonas syringae*, mutants unable to produce EPS have been observed to be sensitive to ROS (Király et al. 1997). Studies have also shown that mutants unable to produce either EPS-I or EPS-II in *S. meliloti* are sensitive to oxidative stress as well (Lehman and Long 2013). Both EPS-I and EPS-II were shown to decrease the amount of H_2O_2 detectable in culture. Furthermore, it was shown that LMW EPS-I is the responsible fraction which scavenges H_2O_2 from media. Taken together, oxidative stress is seen to promote the production of exopolysaccharides which are necessary for the tolerance of ROS. Since production of EPS is necessary for symbiosis in *S. meliloti*, this provides another link in how tolerance of stress promotes symbiotic establishment.

1.4.7 pH stress

The ability to tolerate acidic pH conditions has largely been studied from the perspective of tolerating acidic soils in the environment. The area of the rhizosphere is predicted to be an area of increased acidic stress, as throughout their life cycle, plants can excrete acidic compounds into the surrounding soil, decreasing the pH of the soil by as much as 2 pH units (Faget et al. 2013). This occurs from the secretion of protons to maintain the net charge across the root membrane, and from the secretion of organic compounds (Marschner 1991, Jones et al. 2003). During the symbiotic interaction between rhizobia and legumes, it has been hypothesized that many plant derived compartments have an acidic pH. The bacteroid has been predicted to be an acidic compartment due to the activity of ATPases in the peribacteroid membrane which create a pH gradient (Fedorova et al. 1999). This gradient can create a pH as low as 4.5 in the peribacteroid space, and has been suggested that the acidification is a mark of functional symbiosis (Pierre et al. 2013). Recent studies in our lab have also determined that the CCRH is

also an area of localized acidic pH stress (Geddes et al. 2014). These findings are particularly important as *S. meliloti* is known to have poor survival when medium pH decreases below 6 (Hellweg et al. 2009). To establish successful symbiosis, rhizobia need to be able to tolerate acidic pH during symbiotic establishment, and many of these responses are directly involved in symbiosis.

Two regulatory systems have been shown to be involved in the tolerance of pH stress, *actRS* and *exoS/exoR/chvI* (Dilworth et al. 2000, Fenner et al. 2004). However, the mechanism of pH tolerance in rhizobia varies between species, and it tends to involve one of two main mechanisms; the maintenance of cytoplasmic pH, or the modification and production of extracellular and membrane components (Cunningham and Munns 1984a, Chen et al. 1993). Early studies on acidic pH tolerance screened mutagenized strains of *S. meliloti* for sensitivity to acidic pH. Results showed that strains unable to maintain a cytoplasmic intracellular pH became sensitive to acidic medium conditions (O'Hara et al. 1989). This suggested that the ability to maintain a large pH gradient between the cytoplasm and extracellular medium is critical for pH tolerance. One mechanism used to create an intracellular pH gradient was the utilization of ion transporters. In *E. coli* it has been shown that the use of a K+/H+ antiporter is critical for maintaining intracellular pH (Kroll and Booth 1981). When investigating pH tolerant and sensitive strains of *R. leguminosarum,* it was observed that pH tolerant strains accumulated high cytoplasmic concentrations of potassium at low pH, whereas pH sensitive strains did not (Aarons and Graham 1991).

In addition to antiporter activity, regulation of potassium efflux proteins has been shown to be important for pH tolerance as well. The potassium efflux system in *S. meliloti,* has been shown to be regulated by glutathione, since mutants in glutathione synthesis were unable to

accumulate intracellular potassium (Riccillo et al. 2000). Glutathione has been shown to be involved in tolerating many environmental stressors, including pH and ROS stress, and has been shown to be increased in production under acidic conditions (Riccillo et al. 2000, Muglia et al. 2007). Glutathione is assumed to regulate cytoplasmic pH by controlling the cytoplasmic concentrations of potassium in the cell. Interestingly, mutations in the synthesis pathway for glutathione result in either a fix^- or delayed nodulation phenotype (Harrison et al. 2005). Additionally, K^+ concentrations have been shown to regulate nitrogenase activity so this accumulation of K^+ in acidic conditions may act as a symbiotic signal (Gober and Kashket 1987). These are two prime examples of how stress management act to alter the physiological state of rhizobia to permit symbiosis.

Recently, our lab has also been able to correlate the increased production of exopolysaccharides with increasing medium acidity (Geddes et al. 2014). This is consistent with studies showing that the expression of the *exo* gene cluster is up-regulated due to acidic pH (Hellweg et al. 2009). In addition, it has also been observed that acid tolerant strains of rhizobia produce more exopolysaccharides than acid sensitive strains under non-stress conditions (Cunningham and Munns 1984a). These observations have led to the suggestion that the production of polysaccharides may play a role in tolerating acidic pH, though this has never been directly investigated. Interestingly, mutations that resulted in an increased production of exopolysaccharide in *R. leguminosarum* and *S. meliloti* did not result in an increased tolerance to acidic media (Howieson et al. 1988, Reeve et al. 1997). These observations combined suggest that in terms of stress tolerance, the production of exopolysaccharides may serve an on/off function rather than a gradient of tolerance, and that the increased production of exopolysaccharides due to pH stress may have another role.

The response to low pH is largely mediated through the ExoR/ExoS/ChvI (RSI) system, which has been shown to be upregulated due to acidic pH in *S. meliloti* (Hellweg et al. 2009). The RSI system is well studied for its ability to regulate the production of EPS-I and flagella (Cheng and Walker 1998b, Heavner et al. 2015). It is long known that the production of EPS-I is important for symbiotic interaction (González *et al.*, 1996; York and Walker, 1997; Cheng and Walker, 1998). The protein ExoS acts as a sensor kinases which directly phosphorylates the response regulator ChvI in response to a yet unknown signal (Cheng and Walker 1998b, Yao et al. 2004). This system is regulated through direct binding of the repressor ExoR to ExoS in the periplasm (Chen et al. 2008). Homologues of this system in *Agrobacterium tumefaciens* have been shown to be involved in gene regulation due to acidic pH, and it has been suggested that acidity is a key signal in establishing virulence with plants (Li et al. 2002). Further study of the RSI regulon in *A. tumefaciens* has revealed that at acidic pH the repressor ExoR is degraded, resulting in increased EPS-I synthesis (Heckel et al. 2014). A mechanism for degradation of ExoR in *S. meliloti* has also been shown (Lu et al. 2012). Degradation of ExoR could account for the increase in transcription of *exoR* at lower pH. Taken together, this suggests that the acidic conditions found in the CCRH leads to the production of both HMW and LMW EPS-I which are necessary for symbiotic signaling and stress tolerance. This also suggests that pH may be used as a signal to initiate EPS-I production for symbiotic establishment.

1.4.8 NCRs

Recent research has also revealed that the role of the plant innate immune system plays a critical role in symbiotic establishment in legumes which form indeterminant nodules, serving as another example as how a stress response from plants has been co-opted into a symbiotic signal. Plants produce antimicrobial peptides (AMPs) as part of the innate immune response. AMPs are

prevalent among eukaryotic organisms, and are well studied for their anti-microbial activity (Maroti et al. 2011). The mechanism of action of AMPs involves the disruption of bacterial membranes through interaction with the cell surface, and ribosome inactivation (Endo and Tsurugi 1986, Shai 2002, Brogden 2005). One of the most well studied families of AMPs are the cysteine rich peptides (CRP). These are characterized as having conserved cysteine residues which are involved in disulfide bridge formation, which promotes folding typical of the CRP class AMPs (Tailor et al. 1997). In addition to their anti-microbial activity, it has been suggested that these CRPs play a role in signaling as well (Schopfer 1999).

A subset of CRPs has been found in the nodules of select legume plants and are referred to as nodule specific cysteine rich peptides (NCRs). NCRs are structurally and functionally similar to AMPs; they are predicted to be around 100 amino acids long, contain the conserved cysteine residues for disulfide bridge formation, and are predicted to be largely cationic (Mergaert et al. 2003). These peptides have also been show to have anti-microbial activity against a number of organisms, including rhizobia (Haag et al. 2011). However, the presence of the protein BacA in *S. meliloti* is observed to be involved in tolerating the challenge with NCRs, as mutants in *bacA* are observed to be rapidly killed off *in planta* (Haag et al. 2011). In *M. truncatula*, there are predicted to be upwards of 300 different NCRs produced by around 600 different genes (Mergaert et al. 2003, Zhou et al. 2013). Interestingly, only legumes of the IRLC are observed to produce NCRs (Mergaert et al. 2006). In these legumes, symbiotic bacteria become terminally differentiated into bacteroids while in plant nodules, and cannot revert to normal functioning bacteria. Non-IRLC legumes such as *L. japonicus* do not produce NCRs, and symbiotic bacteria do not become terminally differentiated (Mergaert et al. 2003). It was this observation that first led to the hypothesis that the NCRs of IRLC legumes are involved in both

the defence response of legumes, and involved in the terminal differentiation of symbiotic bacteria necessary for nitrogen fixation (Mergaert et al. 2003).

The localization of NCRs also suggests much about their role in symbiosis. When NCRs are expressed in the nodule, they are targeted to the symbiotic membrane and can also be found within the cytoplasm of bacteroids (Van de Velde et al. 2010). In the same study, it was also shown that a mutation in *M. truncatula dnf-1* prevents targeting of NCRs to the bacteroid, and prevented bacteroids from terminally differentiating. Also, when NCR035 from *M. truncatula* was expressed in *L. japonicum*, which is deficient in NCR production, it localized to the symbiosome of bacteroids, resulting in production of a single elongated bacteroid indicative of terminal differentiation (Alunni et al. 2007, Van de Velde et al. 2010). This highlighted the importance of NCRs for symbiotic establishment in the IRLC legumes. Mutation in the gene *dnf7*, encoding for proteins involved in the production of NCR169, is unable to perform BNF in *M. truncatula* (Horváth et al. 2015). Nodules in this mutant were impaired in elongation, and triggered early senescence. Production of NCR211 has also been shown to be necessary for bacteroid persistence (Kim et al. 2015). These combined studies show the necessity of NCRs in regulating bacteroid differentiation and symbiotic nitrogen fixation.

Microarray analysis implies that NCR recognition may play a role in the bacterial stress response, as well as preventing cell division during symbiosis (Penterman et al. 2014). After exposure to NCRs, the expression of genes involved in bacterial stress response were found to be increased in transcription. This includes *rpoH1;* which is involved in regulating genes for acid and heat tolerance, and the two component systems *exoS-chvI* and *feuP-feuQ;* which are responsible for regulating EPS and cyclic β(1-2) glucan production, respectively (Reuber et al. 1990, Griffitts et al. 2008). This observation led to the conclusion that NCR recognition may be a

bacterial signal that allows for adaptation to *in planta* conditions, and also increase the production of polysaccharides necessary for symbiosis, in addition to its role in bacteroid differentiation (Penterman et al. 2014). This shows that NCRs may have evolved in plants from simply being an AMP, produced as a response to bacterial invasion, to being also involved in symbiotic establishment as a signal which induces physiological and morphological changes in the bacteria necessary for nitrogen fixation.

1.5 Thesis goals and hypothesis

The overall goal of this thesis was to use bacterial genetics to investigate how environmental conditions regulate the production of polysaccharides, and the role this plays in symbiosis and stress tolerance. Understanding how rhizobia tolerate adverse conditions found in the soil and during symbiotic establishment could lead to the development of inoculum which is more competitive for nodule occupancy compared to strains native to the soil. In addition, a large amount of the world's soil exhibits conditions which are prohibitive to the growth of rhizobia, preventing its use as an inoculum in these soils (Ferguson et al. 2013). Understanding how *S. meliloti* is able to tolerate these conditions could lead to development of a strain able to survive in these adverse soil conditions, allowing the use of symbiotic nitrogen fixation as a form of nitrogen in these areas.

The first goal of the thesis was the characterization of the gene *SMc00722*. Previous work had determined that increasing concentrations of potassium and magnesium prohibited production of EPS-II in *S. meliloti* (Miller-Williams et al. 2006). However, little is known about how magnesium, sodium, and phosphate, regulate the production of exopolysaccharides. In an attempt to determine how magnesium regulated EPS-II production, a random mutagenesis was

carried out to screen for mutants able to produce EPS-II. This led to the isolation of a mutation in *SMc00722*, which is characterized in chapter 2.

The second goal of the thesis was to investigate determinants which contributed to pH tolerance in *S. meliloti*. This specifically focused on the role that production of polysaccharides and medium acidification played in the tolerance of acidic pH stress. Previous work had determined that a mutant unable to catabolize galactose would acidify medium, and produce more EPS-I when grown using galactose along with another carbon source for growth (Geddes et al. 2014). These observations led to two primary questions; what role does the production of exopolysaccharides play in pH stress tolerance, and what was the cause of medium acidification in the mutant strain. The role polysaccharides play in pH tolerance was investigated using well defined mutations that prevent production of either EPS-I, EPS-II, cyclic β(1-2) glucans, or glycogen (Chapter 3). Furthermore, using a screen defined in Chapter 4, we isolated a mutation in a putative transketolase which was observed to prevent medium acidification in the galactose mutant strain. Since little is known about the pentose phosphate pathway in *S. meliloti*, mutations in this pathway were characterized (Chapter 5). In understanding how both polysaccharides and metabolism contribute to pH tolerance, we hoped to emphasize the importance of acidic pH stress tolerance during symbiotic establishment.

Chapter 2:

Characterization of a gene encoding a membrane protein that affects exopolysaccharide production and intracellular Mg2+ concentrations in *Sinorhizobium meliloti*

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2.1 Abstract

Exopolysaccharides play an important role in the physiology of a bacterial cell. *Ensifer meliloti* is capable of producing at least two types of exopolysaccharides (EPS); succinoglycan, and galactoglucan. In *E. meliloti* exopolysaccharides are best known for their role in mediating interaction with its symbiotic hosts. It was previously shown that high concentrations of Mg^{2+} or K^{+} were capable of suppressing the mucoid phenotype associated with galactoglucan production in an *expR*$^{+}$ derivative of Rm1021. In an attempt to determine how Mg^{2+} regulates galactoglucan production, SRmD363 was mutagenized and screened for mutants which were visibly mucoid at high concentrations of magnesium. Tn*5* mutations in genes *exoX*, *emmB*, *phoC*, and *SMc00722* were isolated. SMc00722 is annotated as a hypothetical transmembrane protein that is conserved in the α-proteobacteria. Characterization of *SMc00722* in Rm1021 showed that the increased mucoidy was due to succinoglycan. Strains carrying mutations in SMc00722 showed increased biofilm production, and were more sensitive to high Mg^{2+} concentrations and deoxycholate. In addition, we show that strains carrying a mutation in *SMc00722* have elevated intracellular Mg^{2+} concentrations. Taken together the data is consistent with the hypothesis that SMc0722 may play a role in maintaining intracellular magnesium concentration and we suggest that this gene be tentatively annotated as *mhrA* (**m**agnesium **h**omeostasis **r**elated).

2.2 Introduction

S. meliloti is a Gram-negative α-proteobacteria which can exist either as a free living organism in the soil, or in a symbiotic relationship with legumes. When existing in a symbiotic relationship with *Medicago sativa*, *S. meliloti* reduces molecular nitrogen to ammonia, which is provided to the plant, and in exchange receives nutrients from the legume. To establish an

effective symbiosis, a complex signal exchange must take place (Oldroyd 2013). In the initial steps of symbiotic establishment, rhizobia recognize plant-produced organic molecules called flavonoids. Flavonoid recognition results in the activation of the *nod* genes, and subsequently the production of Nod factors by rhizobia (Jones et al. 2007). Nod factor recognition by the plant results in root hair curling, the formation of curled colonized root hairs (CCRH), and replication of plant cells which will form the nodule (Oldroyd et al. 2011). From the CCRH, infection proceeds through a plant derived structure called the infection thread (IT) to the plant cells forming the nodule (Oldroyd, 2011). Here rhizobia are endocytosed and terminally differentiated into bacteroids which fix nitrogen for the legume (Geddes and Oresnik 2016).

In order for symbiosis to progress through the IT, constant production of Nod factor and exopolysaccharides (EPS) is required (Jones et al. 2007). *S. meliloti* is capable of producing two symbiotically active types of EPS; succinoglycan (EPS-I) and galactoglucan (EPS-II) (Skorupska et al. 2006). EPS-I consists of a repeating polymer of a single galactose linked to seven glucose units which can be modified with acetyl, pyruvyl, and succinyl groups (Reinhold et al. 1994). The production of EPS-I is typically visualized by a calcofluor bright phenotype when observed under UV light (Finan et al. 1985, Leigh et al. 1985). EPS-II is a polymer of repeating glucose and galactose units which can be decorated by acetyl and pyruvyl groups (Her et al. 1990). This polysaccharide is not typically produced in Rm1021, the lab wild-type strain, due to an insertion element in the regulator *expR*, though production can be restored through a mutation in the *mucR* gene or in phosphate limited conditions (Oresnik et al. 1994, Keller et al. 1995, Mendrygal and González 2000, Pellock et al. 2000). EPS-II production is generally visualized as a mucoid phenotype (Glazebrook and Walker 1989).

It has been recently shown that succinylated EPS-I is primarily involved in symbiotic

establishment (Mendis et al. 2016). However in strains carrying an intact *expR* gene, it has been shown that LMW EPS-II can also be used in the absence of EPS-I to establish an effective symbiosis (González et al. 1996). Current suggested roles for exopolysaccharides include immune system dampening, stress tolerance, and symbiotic signaling (Jones and Walker 2008, Lehman and Long 2013, Kawaharada et al. 2015).

Salt concentrations have been shown to influence the production of both EPS-I and EPS-II (Lloret et al. 1998, Mendrygal and González 2000, Miller-Williams et al. 2006). Previous work has shown magnesium can suppress EPS-II production in an *expR*$^+$ background (Miller-Williams et al. 2006). This reduction was not observed when either Li^+ or Ca^{2+} was added, suggesting that the non-mucoid phenotype observed was due to magnesium and not the osmolarity of the medium. To this end we mutagenized an *expR*$^+$ strain of *S. meliloti*, screening for mutants which would not be suppressed in EPS-II production under high magnesium concentrations. The majority of the mutants isolated were previously characterized, and shown to be responsible for increased EPS-I production. However, a mutation in the previously uncharacterized gene *SMc00722* was isolated. This mutation was characterized in an attempt to define the role that this gene plays in *S. meliloti*.

2.3 Materials and Methods

2.3.1 ***Bacterial strains and media***

Strains and plasmids used are indicated in Table 1. *S. meliloti* strains were regularly grown at 30ºC in TY (Beringer 1974), GYM (Dylan et al. 1990a) , or LB (Sambrook and Russell 2001). When necessary mannitol was filter sterilized and added to the medium to a final concentration of 15 mM. Antibiotics were added to media when necessary at the following concentrations: streptomycin, 200 µg ml^{-1}; neomycin, 200 µg ml^{-1}; chloramphenicol, 20 µg ml^{-1};

and tetracycline, 5 µg ml^{-1}. When required, 10 mM deoxycholate (DOC) or 0.02% w/v calcofluor were added to growth media as required.

2.3.2 Genetic techniques, plasmids, DNA manipulations and sequencing

Tri-parental matings, Tn*5* transposon mutagenesis, and transductions were carried out essentially as previously described (Finan et al. 1984, 1988). Standard procedures were used for DNA manipulations (Sambrook and Russell 2001). A plasmid (pJH100), constitutively expressing the gene *SMc00722* was constructed by recombining *SMc00722* into pCO37 using the *S. meliloti* ORFeome as previously described (Jacob et al. 2008, Geddes and Oresnik 2012b). The point of insertion in mutants isolated from Tn*5* mutagenesis experiments was determined by the use of an arbitrary PCR protocol as previously described (Miller-Williams et al. 2006).

2.3.3 Biofilm assays

Biofilm assays were adapted from a previously described method (O'Toole and Kolter 1998). Essentially 5 mL cultures were grown in glass test-tubes containing GYM medium at 30ºC for 7 days. Bacterial growth was measured by optical density and the cultures were removed. The test tubes were air dried for 20 minutes then fixed by heating at 60ºC for 30 minutes. The biofilms were then stained for 1 minute with 2 mL of crystal violet solution. Tubes

Table 1 Strains and Plasmids

Strain	Genotype or phenotype[a]	Source or reference
Strains		
S. meliloti		
Rm1021	SU47 *str-21*, Sm^r	(Meade et al. 1982)
SRmA363	Rm1021 *expR*$^+$, Sm^r	(Miller-Williams et al. 2006)
SRmA687	Rm1021 *mhrA*::Tn*5*, Sm^r, Nm^r	This work
SRmA686	SRmA363 *mhrA*::Tn*5*, Sm^r, Nm^r	This work
E. coli		
MM294A	*Pro-82 this-1 hsdR17 supE44*	(Finan et al. 1986)
MT607	MM294A; *recA56*	(Finan et al. 1986)
MT616	MT607(pRK600)	(Finan et al. 1986)
DH5α	λ^- φ80d*lacZ*°M15 °(*lacZYA-argF)U169 recA1 endA1 hsdR17*(r_k^- m_k^-) *supE44 thi-1 gyrA relA1*	(Hanahan 1983)
Plasmids		
pRK600	pRK2013 *npt*::TN*9* Cm^r	(Finan et al. 1986)
pMW22	Km^r fragment cloned as SmaI fragment into pBlueScript	(Pickering and Oresnik 2008)
pRK415	Tc^r IncP broad-host-range cloning vector	(Keen et al. 1988)
pPH1JI	IncP plasmid Gm^r	(Beringer 1974)

[a] Sm^R, Nm^R, Gm^R, Sp^R, Cm^R, Amp^R, Km^R; Resistance to streptomycin, neomycin, gentamicin, spectinomycin, chloramphenicol, ampicillin, and kanamycin

containing the fixed, stained biofilms were washed three times with 5 mL of ddH_2O to remove excess crystal violet and subsequently decolourized using 5 mL 95% ethanol. The crystal violet was quantitated using a spectrophotometer (OD_{570}) and the value was normalized to the optical density of the culture after growth.

2.3.4 EPS determination

Cultures were grown in GYM media containing different concentrations of magnesium at 30ºC for 5 days. Cultures were harvested by centrifugation (4000 x g, 20 minutes), the EPS from the resultant supernatants was precipitated using cetrimide, and was then re-suspended in 10% NaCl. EPS was then quantified using the anthrone-H_2SO_4 method (Morris and Gonzalez 2009). The amount of EPS present was normalized to the OD_{600} of the culture.

2.3.5 Plant assays

Plant experiments were carried out as previously described (Poysti et al. 2007). Briefly, surface sterilized alfalfa seeds were germinated on water agar plates then transplanted into Lenoard jars containing a 1:1 mixture of sand and vermiculite that had been soaked with nitrogen free Jensen's medium. After 3 days, plants were inoculated with 10^5 bacteria per plant. Plants were harvested 28 days after inoculation. Symbiotic proficiency was quantitated by determining plant dry weights as an integrative measure of overall nitrogen fixation when grown in nitrogen deficient conditions (Hunt and Layzell 1993). To determine the effect of salt stress, the concentration of NaCl in Jensen's medium was adjusted to 0, 25, 50, 100, and 150mM.

2.3.6 Measurement of intracellular magnesium accumulation

Intracellular magnesium concentrations were determined using a method previously described for measuring intracellular K^+, with minor modifications

(McLaggan et al. 2002, Lee et al. 2007). Cultures were grown in GYM containing 20 mM Mg^{2+} to an OD_{600} ~ 0.4 and then harvested on 0.4 µm nitrocellulose filters. Cells were then washed dropwise with 5 mL Mg^{2+} free GYM containing 50 mM NaCl to remove externally bound exopolysaccharides, and then re-suspended into 10 mL of Milli-Q water. The OD_{600} of each of the cell suspension was measured, and then cells were boiled at 90ºC for 10 minutes. Cell debris was removed by centrifugation at 13,000 rpm for 20 minutes, and supernatant was removed to a new tube. Released Mg^{2+} was quantified using a High-Resolution Inductively Coupled Plasma Mass Spectrophotometer. Measurements were carried out by Panseok Yang in the Department of Geological Sciences, University of Manitoba. Samples from independent biological samples were measured in triplicate.

2.4 Results

2.4.1 Tn5 mutagenesis and isolation of SMc00722 mutant

Previous work has shown that the mucoid phenotype associated with EPS-II production in an *expR*$^+$ background could be reversed by the addition of $MgSO_4$ (Miller-Williams et al. 2006). In an effort to identify how magnesium regulates EPS-II production, a Tn*5* mutagenesis was carried out on and *expR*$^+$ derivative of Rm1021 and the resulting colonies were screened for mutants which were still mucoid under increased magnesium concentrations. Utilizing this screen, Tn*5* insertions were isolated in genes *exoX*, *emmB*, *phoCDET*, and *SMc00722*, a conserved hypothetical transmembrane protein. The *SMc00722* mutation was further investigated

for its role in responding to magnesium concentrations as mutations in the other genes have been previously characterized (Zhan and Leigh 1990, Voegele et al. 1997, Morris and Gonzalez 2009).

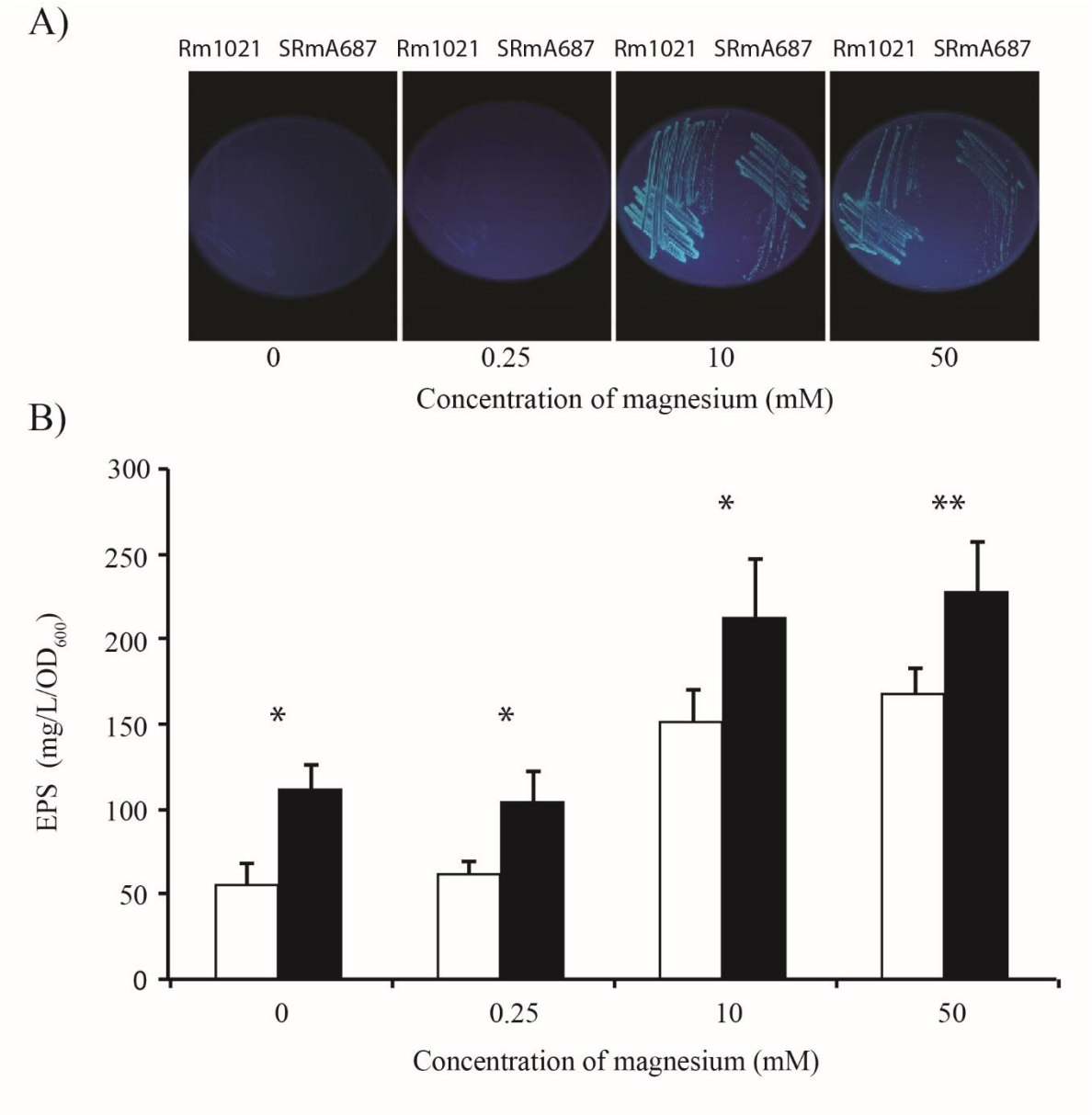
2.4.2 A mutation in SMc00722 causes an increase in EPS-I production

Our goal had been to isolate mutants that affected EPS-II production due to increased magnesium concentrations; however mutations in *exoX* and *emmB* have been shown to increase EPS-I production regardless of magnesium concentration (Zhan and Leigh 1990, Morris and Gonzalez 2009). While mutations in *phoCDET* have been shown to affect EPS-II production (Oresnik et al. 1994), it has been suggested that *phoCDET* transcription could play a role in regulating EPS-I through *phoB* (Mendrygal and González 2000).

The mucoidy associated with a mutation in *SMc00722* could be due to an increase of either EPS-I or EPS-II production. To determine this, mutations preventing production of either EPS-I (*exoA*) or EPS-II (*expA1*) were transduced into the *SMc00722* mutant strain in the *expR*$^+$ background. In addition, the Tn*5* insertion in *SMc00722* was transduced into Rm1021 making strain SRmA687. It was found that mucoidy associated with the *SMc00722* mutation in the *expR*$^+$ background was dependent upon *exoA* and not *expA1*. When Rm1021 and SRmA687 were streaked onto LB containing 50 mM magnesium, Rm1021 was still visibly dry, whereas SRmA687 was slightly mucoid. Taken together, these results indicate that the observed mucoidy was due to increased EPS-I production.

To determine if EPS-I production was altered by increased Mg^{2+} concentrations, strains were streaked on GYM plates supplemented with Calcofluor with increasing magnesium concentrations (Figure 3a). Increasing fluorescence was observed for both strains as Mg^{2+} concentrations increased. No visible difference in fluorescence between the strains was observed.

Figure 3. Mutation in *mhrA* increases EPS-I production



A) Calcofluor fluorescence of Rm1021 and SRmD687 at increasing magnesium concentrations. Images are taken under UV to visualize fluorescence. B) EPS-I production of Rm1021 (White) and SRmA687 (Black) when grown with different concentrations of magnesium. An asterisk denotes a significant difference using Student's T test ($P < 0.05$); A double asterisk denotes a significant difference using Student's T test ($P < 0.1$). Error bars represent standard deviation.

Direct quantitation of produced EPS-I under these conditions showed increased EPS biosynthesis when the concentration of magnesium in medium is increased above 10 mM (Figure 3b). However, SRmA687 was found to produce more EPS-I at all concentrations of magnesium that were tested.

2.4.3 SMc00722 mutants increase biofilm formation

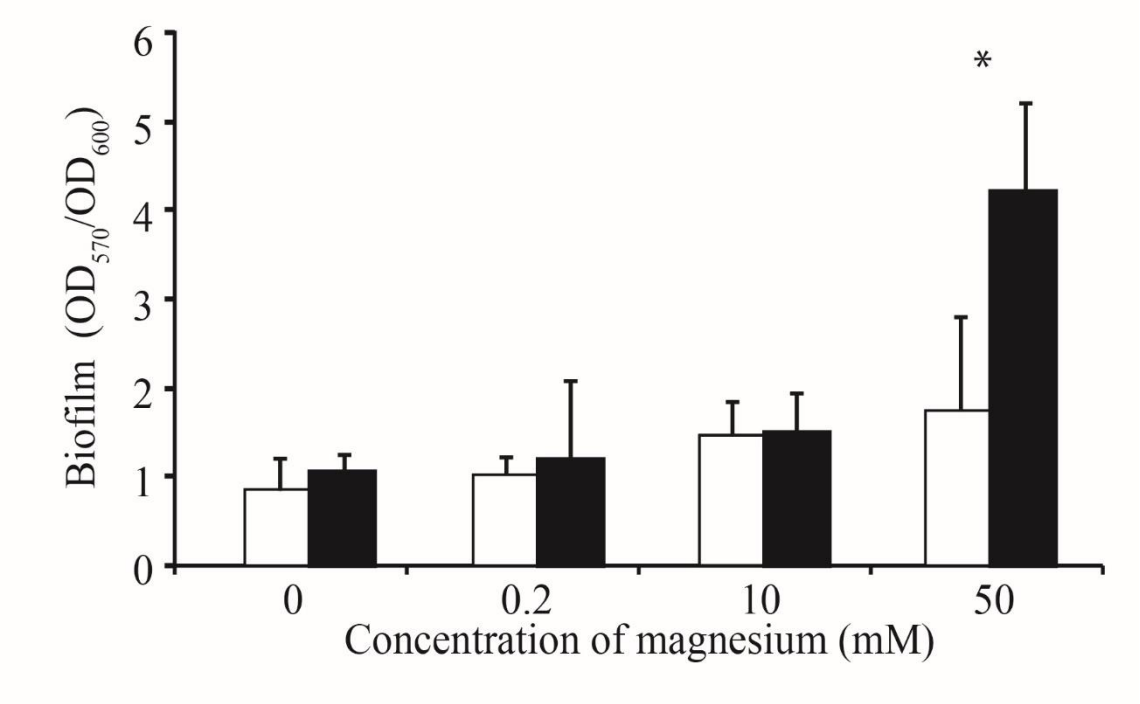
EPS is one of the major components involved in the formation of biofilm. It has been shown that the production of EPS-I and biofilms are both regulated through ExoS/ChvI/ExoR, and increased production of EPS-I has been correlated with increased biofilm formation (Chen et al. 2009, Rinaudi and Gonzalez 2009, Bélanger and Charles 2013). As such, the ability to produce biofilms was also investigated (Figure 4). The amount of biofilm produced by Rm1021 was not significantly different at any of the tested magnesium concentrations. The production of biofilm by SRmA687 was also not found not to be different except at a concentration of 50 mM magnesium, where a significant increase of biofilm formation was observed.

2.4.4 A mutation in SMc00722 decreases stress tolerance

Increased production of EPS and biofilms has been suggested to play a role in the tolerance of many stresses such as detergents, desiccation, salt stress, oxidative stress, and pH stress (Cunningham and Munns 1984a, Miller-Williams et al. 2006, Rinaudi and Giordano 2010, Lehman and Long 2013). To determine if SRmA687 had any sensitivities to applied stress it was tested for tolerance to deoxycholate (DOC), a detergent often used to assay membrane defects, as well as increased concentrations to magnesium.

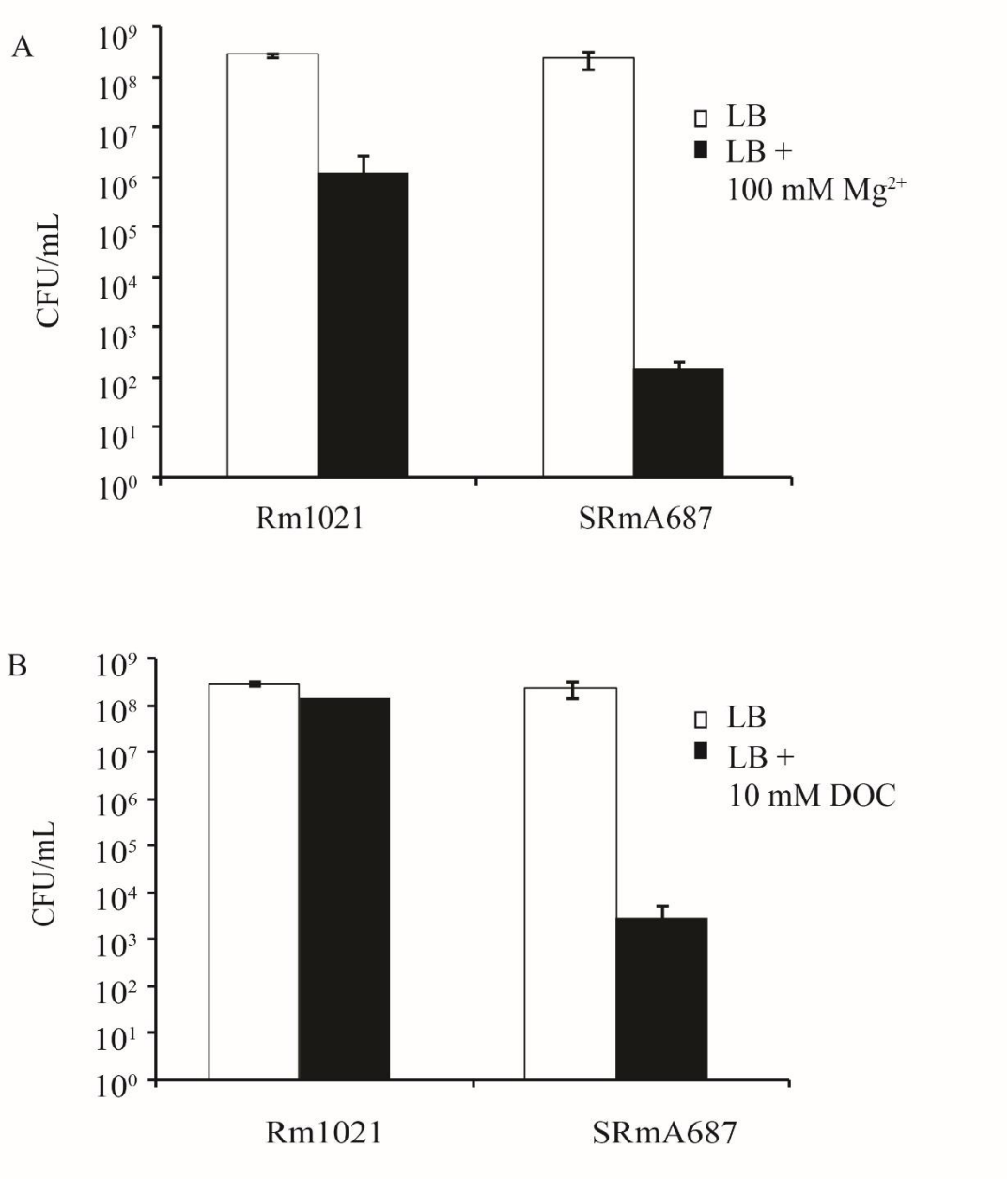
It was found that, whereas Rm1021 showed a 2 log decrease in growth when grown in the presence of DOC, SRmA687 exhibited a 5 log difference when DOC is present in media (Figure 5A). When tested for growth using elevated concentrations of magnesium, Rm1021 was

Figure 4. Biofilm quantification



Quantification of biofilms formed by Rm1021(white) and SRmA687 (black) at different concentrations of magnesium. An asterisk denotes a significant difference using Student's T test ($P < 0.05$).

Figure 5. Sensitivity to potential stressors



Sensitivity of Rm1021 and SRmA687 to A) 10 mM DOC and B) 100 mM magnesium. White bars indicate growth on LB, Black bars indicate LB supplemented with either 100 mM Mg^{2+} (panel A) or 10 mM Deoxycholate (panel B). Data are presented as CFU/mL growing on each plate after 3 days. Changes between LB and LB with DOC are significant using Student's T test ($P < 0.05$).

unaffected by the addition of 100 mM of Mg^{2+}. However, SRmA687 exhibited a 5 log decrease in observable CFU/mL at 100 mM of magnesium (Figure 5b). To test if these phenotypes were directly attributable to the loss of *SMc00722*, a plasmid, pJH100, containing a wild-type copy of *SMc00722* was introduced into SRmA687. The introduction of this plasmid was able to restore growth of SRmA687 on both DOC and 100 mM magnesium.

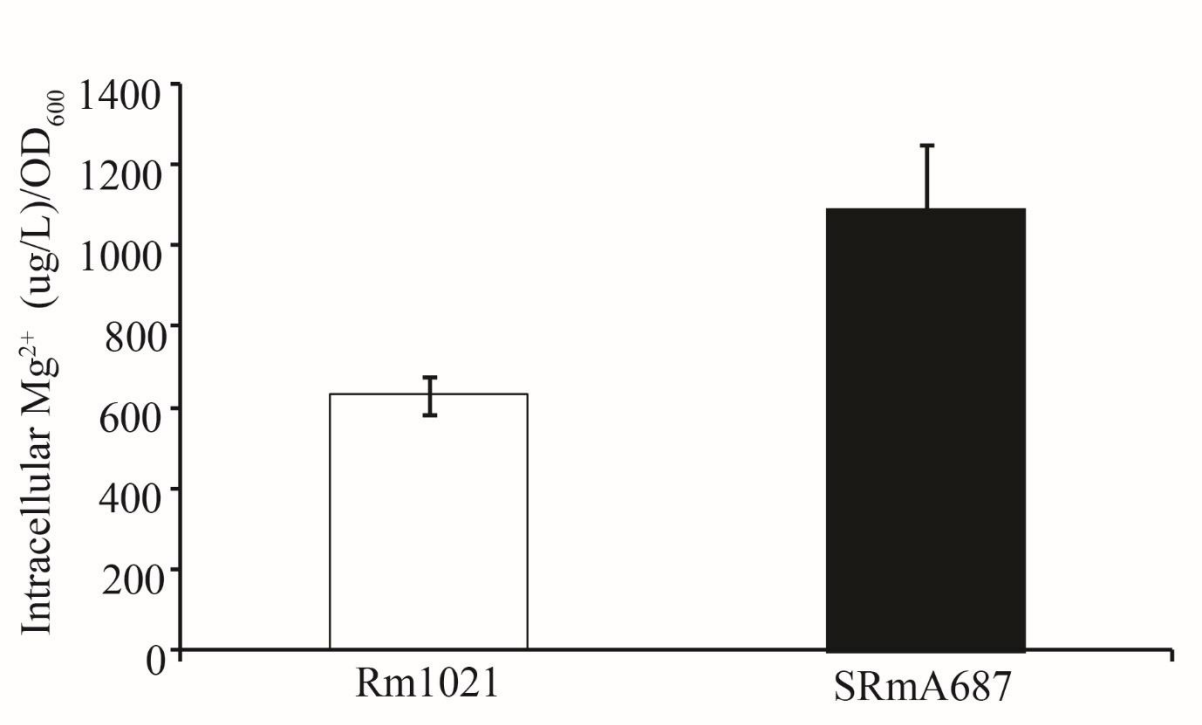
2.4.5 Intracellular magnesium concentrations are increased in SMc00722 mutants

A BLASTP search of *SMc00722* shows homology to a hypothetical transmembrane protein predicted to have 2-3 transmembrane domains. This protein also exhibits weak homology to a magnesium binding domain from CorA, a protein involved in magnesium efflux (Knoop et al. 2005). Increased biofilm formation under magnesium stress, and increased sensitivity to magnesium led to the hypothesis that a mutation in *SMc00722* may indirectly impact intracellular magnesium levels. Intracellular magnesium concentrations were analyzed to determine if SMc00722 plays a role in regulating internal magnesium homeostasis. Cells were grown at 20 mM of magnesium as this concentration was determined to show no adverse effects on SRmA687. It was found that the intracellular concentration of magnesium in SRmA687 was significantly higher than Rm1021 (Figure 6).

2.4.6 A mutation in SMc00722 does not affect symbiosis

Mutations that affect membrane stability, or affect EPS-I production, often affect symbiotic development (Morris and Gonzalez 2009, Ardissone et al. 2011, Jones 2012, Geddes et al. 2014). When SRmA687 was inoculated onto alfalfa it was found that it was as effective as the wild-type based on accumulated plant dry weights under standard conditions. Increasing $MgCl_2$ and NaCl concentrations have been shown to affect EPS-II production in a similar manner (Miller-Williams et al. 2006). Since SRmA687 was found to be sensitive to a number of

Figure 6. Intracellular magnesium concentrations



Intracellular magnesium concentrations of Rm1021 (white) and SRmA687 (black). Magnesium content was determined as describe in Materials and Methods. The P value was determined to be 0.0014 using Student's T-test.

stresses, we wished to determine if adverse soil conditions such as increased salinity could affect symbiosis. Since NaCl did not affect the growth of SRmA687, plants inoculated with Rm1021 and SRmA687 were grown with increasing concentrations of NaCl. While alfalfa dry weights were found to decrease 40% at 150 mM NaCl compared to standard conditions, no statistical difference in dry weight was found in plants inoculated with Rm1021 or SRmA687 at any tested concentration (Figure 7).

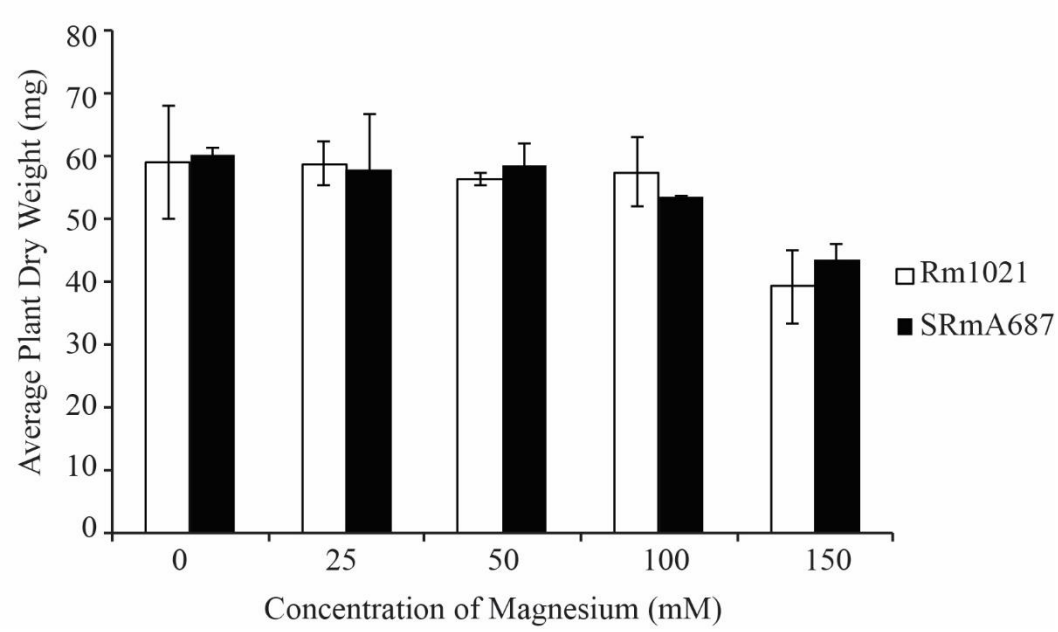
2.5 Discussion

In this work we set out to characterize mutations that would result in the over-production of EPS-II under increased magnesium concentrations. While mutations that had increased EPS-II production were not isolated, a number of mutants that resulted in increased EPS-I production were isolated. Of interest was a mutation in *SMc00722*, a gene that is annotated as encoding a hypothetical transmembrane protein.

Through our characterization it was determined that a mutation in *SMc00722* resulted in a mucoid phenotype that was independent of *expR*$^{+}$ and was due to greater production of EPS-I (Figure 3). In addition, it was found that although a strain carrying this mutation had enhanced biofilms at elevated magnesium concentrations (Figure 4), it was also more sensitive to magnesium at these concentrations, and was generally sensitive to DOC (Figure 5). Of note, a BLASTP search of SMc00722 revealed a weak homology (E-value 1.75e-04) from amino acids 494-650 to the magnesium binding domain of CorA. CorA has been shown to be involved in magnesium efflux (Knoop et al. 2005), and it is noteworthy that when SRmA687 was assayed it was found to have higher intracellular concentrations of magnesium (Figure 6).

The phenotypes associated with a strain carrying a mutation in *SMc00722* suggest general

Figure 7. SRmA687 is unaffected in symbiotic nitrogen fixation



Strains were inoculated onto alfalfa plants and assayed for plant dry weight. Each dry weight measurement is an average of 3 biological replicates, each containing 10 plants. Error bars represent standard deviation of the biological replicates.

changes that are similar to those seen when an organism is coping with stress or has membrane alterations. Increased production of both EPS-I and biofilm have been associated with increased stress tolerance (Cunningham and Munns 1984a, Costerton et al. 1995). The ability of EPS-I to chelate divalent cations such as magnesium has been previously demonstrated (Sutherland 1997), so the isolation mutants with increased EPS-I production due to magnesium or salt can be explained as a direct response to a physiological stress. Previous studies on EPS produced by *Rhizobium etli* and *Alteromonas macleodii* sub *fijensis* show each polysaccharide can bind approximately 80 µg of magnesium or zinc respectively per mg of EPS (Loaëc et al. 1998, Belcarz et al. 2013). Based upon our EPS quantification data (Figure 3), the amount of EPS-I being produced at higher magnesium concentrations would be ineffective at adsorbing enough magnesium to prevent buildup of intracellular magnesium. Thus while increased production of EPS-I is observed in SRmA687, the strain is still sensitive to increased magnesium concentrations.

We note that within the literature a number of reports have shown that EPS and cation concentrations are correlated (Appanna 1989; Mendrygal and González 2000; Miller-Williams, Loewen and Oresnik 2006). Examples of magnesium affecting biofilm formation have also been previously reported for other bacteria (Costerton et al. 1995, Rinaudi et al. 2006, Mulcahy and Lewenza 2011).

Our data show that the intracellular concentration of magnesium is significantly higher in SRmA687. Magnesium is essential to normal cell function and the concentration of this ion is regulated (Romani 2011). Magnesium also appears to play a role in phosphate regulation (Moncrief and Maguire 1999). Our working hypothesis is that SMc00722 plays a role in

maintaining intracellular magnesium concentrations. The aberrant intracellular magnesium levels might be signaling a general stress response that is manifested as changes that we have detected. We suggest that SMc00722 should be annotated as *mhrA* (**m**agnesium **h**omeostasis **r**elated). The paucity of data on any orthologues of SMc00722 makes it difficult to assess whether the elevated intracellular magnesium concentration is a direct effect of *mhrA*. While the precise role MhrA still unknown, this work provides new information that may prove invaluable in ascribing function on this previously uncharacterized protein that is conserved throughout the α-proteobacteria.

Chapter 3:

Succinoglycan directly contributes to pH tolerance in *Sinorhizobium meliloti* Rm1021

Reproduced from Mol. Plant. Microb. Interact., Hawkins, J. P., Geddes, B. A., and Oresnik, I. J. Succinoglycan production contributes to acidic pH tolerance in *Sinorhizobium meliloti* Rm1021, https://doi.org/10.1094/MPMI-07-17-0176-R, Copyright 2017, with permission from Mol. Plant. Microb. Interact. This work was carried out and written by Justin Hawkins. Barney Geddes was involved in initial experimental design.

3.1 Abstract

In this work, the hypothesis that exopolysaccharide plays a role in the survival of *S. meliloti* at low pH is addressed. When *S. meliloti* was grown at pH 5.75 the synthesis of succinoglycan increased, whereas the synthesis of galactoglucan decreased. Succinoglycan that was isolated from cultures grown at low pH had a lower degree of polymerization relative to that which was isolated from cultures grown at neutral pH, suggesting that low molecular weight (LMW) succinoglycan might play a role in adaptation to low pH. Mutants unable to produce succinoglycan, or only able to produce high molecular weight polysaccharide were found to be sensitive to low pH. However, strains unable to produce LMW polysaccharide were 10-fold more sensitive. In response to low pH, transcription of genes encoding proteins for succinoglycan, glycogen, and cyclic β(1-2) glucans biosynthesis increased, while those encoding proteins necessary for the biosynthesis of galactoglucan decreased. While changes in pH did not affect the production of glycogen or cyclic β(1-2) glucan, it was found that the inability to produce cyclic β(1-2) glucan did contribute to pH tolerance in the absence of succinoglycan. Finally, in addition to being sensitive to low pH, a strain carrying mutations in *exoK* and *exsH*, which encode the glycanases responsible for the cleavage of succinoglycan to LMW succinoglycan, exhibited a delay in nodulation and was uncompetitive for nodule occupancy. Taken together the data suggest that the role for LMW succinoglycan in nodule development may be to enhance survival in the colonized curled root hair.

3.2 Introduction

The availability of reduced nitrogen affects plant growth and productivity. In agriculture, symbiotic nitrogen fixation provides over 40 million tons of nitrogen to legume plants annually

(Herridge et al. 2008). The utilization of biological nitrogen fixation is dependent on the ability of both the host plant, and symbiotic rhizobia, to be able to manage environmental stresses such as soil acidity. Approximately 40% of the world's soil is affected by acidic conditions which could be prohibitive to the establishment of an effective symbiosis (von Uexküll and Mutert 1995, Ferguson et al. 2013). Increased acidity is known to negatively affect the growth of both symbiotic rhizobia and legume plants (Lowendorf et al. 1981, Howieson et al. 1988). Understanding how microbes are able to tolerate and survive in acidic conditions could contribute to improving plant yields in these environments.

The interaction of *Sinorhizobium meliloti* with alfalfa is used as a model system for studying symbiosis and nitrogen fixation. The developmental process is initiated upon recognition of a plant-secreted flavonoid by rhizobia in the rhizosphere. Recognition of flavonoids by *S. meliloti* leads to the induction of *nod* genes which encode the enzymes necessary to synthesize Nod factor (NF). NF is recognized by receptors on plant root hairs, this results in a complex signal cascade in the plant (Oldroyd 2013). The end result of this cascade is two-fold: the division of inner-cortical plant cells which form the nodule primordium, and the deformation of plant root hairs (Jones et al. 2007). Root hair curling is one of the earliest observable events associated with an effective symbiotic association. Detailed characterization of root hair curling has shown that the curled root hair gives rise to an apoplastic space in which the bacteria form a microcolony. This compartment has been termed the curled colonized root hair (CCRH). Mutants of *S. melilot* Rm1021 unable to produce the exopolysaccharide succinoglycan (EPS-I) do not form effective symbiotic associations with the host plant, and become trapped in the CCRH (Leigh et al. 1985, Glazebrook and Walker 1989, Cheng and Walker 1998a).

S. meliloti is able to produce two main types of exopolysaccharides (EPS); succinoglycan

and galactoglucan (EPS-II) (Skorupska et al. 2006). Either one of these polysaccharides must be produced for symbiotic establishment to be successful (Glazebrook and Walker 1989). Succinoglycan is comprised of a repeating polymer that is made up of an eight sugar core consisting of one galactose and seven glucose units that can be decorated with succinyl, pyruvyl, and acetyl groups (Reinhold et al. 1994). Galactoglucan is a polymer that is made up of a repeating subunit which contains a single glucose linked to a galactose (Skorupska et al. 2006). EPS-II can be modified with acetyl and pyruvyl groups (Her et al. 1990). *S. meliloti* strain Rm1021 is unable to produce EPS-II under normal conditions due to an insertion element in the regulator *expR*. Stains which have an wild-type *expR* gene are able to produce EPS-II, and are termed *expR*$^{+}$ (Pellock et al. 2000). Production of EPS-II can also be restored in Rm1021 under specific medium conditions (Oresnik et al. 1994, Mendrygal and González 2000).

The gene *exoH*, which encodes the enzyme responsible for succinylation of EPS-I, has been shown to be necessary for symbiosis (Leigh et al. 1987, Cheng and Walker 1998b). In addition, *exoH* mutants have been shown to produce only high molecular weight (HMW) EPS-I that cannot be cleaved into the low molecular weight (LMW) form by the endoglycanases encoded by the genes *exsH* and *exoK* (York and Walker 1997). Strains lacking these endoglycanases have also been shown to not produce any LMW EPS-I (Mendis et al. 2016). Mutant strains carrying both endoglycanase mutations have been shown to be symbiotically active, and only result in a minor decrease in overall plant growth compared to plants inoculated with the wild-type (Mendis et al. 2016). This suggested that succinylation of EPS-I, and not the LMW fraction is essential for symbiosis.

A number of roles for EPS in symbiotic establishment have been suggested, which includes dampening of the plant immune response, and protection from reactive oxygen species

(Jones and Walker 2008, Donati et al. 2011, Lehman and Long 2013). More recently, EPS has also been shown to be directly involved in the symbiotic signaling process between *Mesorhizobium loti* and *Lotus japonicus* (Kawaharada et al. 2015). EPS has also been shown to be involved in tolerating physiological stress such as high salt, phosphate, and magnesium concentrations (Mendrygal and González 2000, Miller-Williams et al. 2006, Hawkins and Oresnik 2017). It has also hypothesized that EPS plays a role in pH stress tolerance (Cunningham and Munns 1984a), though this has never been directly investigated.

Within the rhizosphere, low pH is a function of the plant roots secreting H^+ and organic acids which can decrease the pH by up to 2 pH units (Faget et al. 2013). It has also been shown that symbiotic compartments such as the bacteroid and the CCRH are acidic (Fedorova et al. 1999, Pierre et al. 2013, Geddes et al. 2014). This is prohibitive to the growth of *S. meliloti* Rm1021 since the strain cannot grow below a pH of 5.5 (Hellweg et al. 2009). In testing the response of *S. meliloti* to low pH, a sudden pH shift from pH 7 to pH 5.75 has been shown to give rise to a complex transcriptional response that includes an increased transcription of genes encoding proteins necessary for the biosynthesis of EPS-I (Hellweg et al. 2009). However, the direct effect pH has on EPS production has not been determined, and the role EPS production may play in these environments remains unclear.

In this work, we address the hypothesis that EPS-I plays a direct role in pH tolerance, and that this is an important determinant in establishing symbiosis with legume plants. We show that LMW EPS-I facilitates survival at low pH, and that strains that cannot produce LMW EPS-I show a delay in nodule establishment, and are less competitive for nodule occupancy.

3.3 Materials and Methods

3.3.1 Bacterial strains, plasmids, and media

Bacterial strains and plasmids created and used in this work are listed in Table 2. *S. meliloti* was grown at 30ºC on either Luria-Bertani (LB) or tryptone yeast extract (TY) as complex medium (Sambrook et al. 1989). Rhizobium minimal medium (RMM) (Broughton et al. 1986) or Mannitol/Glutamate/Salts (MGS) (Mendrygal and González 2000) was used as a defined medium. Mannitol was filter sterilized and used at a final concentration of 15 mM. When required, medium was buffered using either 50 mM MOPS or 50 mM MES. Antibiotics were used as indicated for *S. meliloti* and *Escherichia coli* at the following concentrations (µg/mL): streptomycin (Sm) 200 µg/mL; neomycin (Nm) 200 µg/mL; and tetracycline (Tc), 5 µg/mL. Gentamicin (Gm) was used at 20 µg/mL for *E. coli* or 60 µg/mL for *S. meliloti*.

3.3.2 DNA and genetic manipulations

Conjugations and transductions were carried out as previously described (Finan et al. 1984, 1988). Standard techniques for plasmid isolation, ligations, transformation, restrictions, and gel electrophoresis were used (Sambrook et al. 1989).

To construct a knockout of the gene *expA1* (*wgaA*) the gene was PCR amplified from Rm1021 genomic DNA using primers expA1FW (*Hind*III site underlined) (5'-AGTCAAGCTTATGTCTTCTAACGTGAGGCAG-3') and expA1RV (*Eco*RI site underlined) (5'-AGTCGGATCCTTATTTCCGGACATTGGACGC-3') and cloned into pBluescript SK^+ as a *Hin*dIII/*Bam*HI fragment creating pMW43. Following this, the kanamycin cassette from pMM22 was isolated as a *Sma*I fragment and blunt end ligated into an *Eco*RI site in *expA1* yielding

Table 2. Strains and plasmids

Strain	Genotype or phenotype[a]	Source or reference
Strains		
S. meliloti		
Rm1021	SU47 *str-21*, Sm^r	(Meade et al. 1982)
SRmA363	Rm1021 *expR*$^+$, Sm^r	(Miller-Williams et al. 2006)
Rm7055	Rm1021 *exoY55*::Tn*5*, Sm^r, Nm^r	(8)
SRmD394	SRmA363 *exoY55*::Tn*5*, Sm^r, Nm^r	This work
SRmA775	Rm1021 *expA1*::Tn5, Sm^r, Nm^r	This work
SRmA777	SRmA363 *expA1*::Tn*5*, Sm^r, Nm^r	This work
Rm7225	Rm1021 *exoH*::Tn*5-233*, Sm^r, Gm^r	(Leigh et al. 1987)
SRmD445	SRmA363 *exoH*::Tn*5-23,3* Sm^r, Gm^r	This work
Rm8826	*exsH*::Tn*5 exoK*::Tn*5-233*, Sm^r, Nm^r, Gm^r	(York and Walker 1997)
SRmD377	Rm1021 *ndvB*::Tn*5-233*, Sm^r, Gm^r	J. Gonzalez, U. of Texas
Rm11482	Rm1021 *glgA1*Δ*Pst*I *glgA2*::ΩSpSm, Sm^r, Sp^r	(Wang et al. 2007)
SRmD455	Rm7055 *glgA1*Δ*Pst*I *glgA2*::ΩSpSm, Sm^r, Sp^r, Nm^r	This work
SRmD456	Rm7055 *ndvB*::Tn*5-233*, Sm^r, Nm^r, Gm^r	This work
E. coli		
MM294A	*Pro-82 this-1 hsdR17 supE44*	(Finan et al. 1986)
MT607	MM294A; *recA56*	(Finan et al. 1986)
MT616	MT607(pRK600)	(Finan et al. 1986)
DH5α	λ$^-$ φ80d*lacZ*°M15 °(*lacZYA-argF)U169 recA1 endA1 hsdR17*(r_k^- m_k^-) *supE44 thi-1 gyrA relA1*	(Hanahan 1983)
Plasmids		
pBluesciptII SK$^+$	Cloning vector ColE1 *oriV* Amp^r	Stratagene
pRK600	pRK2013 *npt*::TN*9* Cm^r	(Finan et al. 1986)
pMW22	Km^r fragment cloned as SmaI fragment into pBlueScript	(Pickering and Oresnik 2008)
pRK415	Tc^r IncP broad-host-range cloning vector	(Keen et al. 1988)
pPH1JI	IncP plasmid Gm^r	(Beringer 1974)
pMW43	*expA1* in pBluesciptII SK$^+$	This work
pMW44	Km^r cassette from pMW22 cloned into *expA1* in pMW43	This work
pMW45	*expA1*::Km^r from pMW44 cloned into pRK415	This work

[a] Sm^R, Nm^R, Gm^R, Sp^R, Cm^R, Amp^R, Km^R; Resistance to streptomycin, neomycin, gentamicin, spectinomycin, chloramphenicol, ampicillin, and kanamycin

pMW44. The *expA1* fragment with the Km cassette was then sub-cloned into pRK415 as a *Bam*HI/*Kpn*I fragment to create pMW45. pMW45 was subsequently conjugated into Rm1021, and integration of the mutation was carried out using pPH1JI as previously described (Oresnik et al. 1998, Pickering and Oresnik 2008). The resulting mutation was verified by amplifying the insertion junctions by PCR and sequencing the resulting product. The *expA1* mutation was subsequently transduced into Rm1021 or SRmA363, creating SRmA775 and SRmA777 respectively. Transduction of the *expA1* mutation into each strain was confirmed by sequencing.

SRmD445 was created by transducing the *exoH* mutation from Rm7225 into SRmA363. Strains SRmD455 and SRmD456 were constructed by transducing the *exoY* mutation from Rm7055 into Rm11482 and SRmD377 respectively. Transductants were routinely single colony purified 3 times before being used.

3.3.3 RNA isolation, cDNA synthesis, and quantitative RT-PCR

For bacterial RNA isolation, cultures were grown as described for polysaccharide isolation with minor modification (see below). Cultures were inoculated in 5 mL RMM with mannitol, and sub-cultured into 50 mL MGS medium buffered at pH 5.75 or pH 7. Cultures were grown overnight to mid-log phase. A volume of 10 mL of this culture was removed and quenched using a 2:1 volume of Qiagen RNA protect. RNA was then extracted from the quenched cells using the Qiagen RNeasy kit as previously described (Geddes et al. 2010). Approximately 200 ng cDNA was utilized as template for qRT-PCR. Reactions were performed using the SYBR green RT-PCR kit from Invitrogen as indicated by the manufacturer. RT-PCR reactions were preformed as previously described (Geddes et al. 2010). Results are an average of 3 independent biological replicates/ The gene *SMc00128*, encoding a hypothetical protein, was utilized as an internal

control for comparison of data sets (Krol and Becker 2004). Transcription of this gene was observed to not be influenced by pH in Rm1021, showing a mean CT of 23.79 and 23.41 at pH 5.75 and pH 7 respectively. The RT-PCR primers used in this work are previously described (Glenn et al. 2007, Hoang et al. 2008, Geddes et al. 2014). Primers used, but not previously described, are as indicated in Table 3.

3.3.4 Isolation and quantification of EPS

Isolation of EPS, glycogen, and cyclic β(1-2) glucans was carried out essentially as previously described with modifications (Breedveld et al. 1993, Marroquí et al. 2001, Wang et al. 2007). Strains were grown for one day at 30ºC in a volume of 50 mL RMM containing 15 mM mannitol as a carbon source. Cultures were then resuspended in RMM to an OD_{600} of 0.1, then pelleted and washed twice with ddH_2O. The final pellet was then resuspended in 500 mL of MGS buffered at either pH 5.75 using 50 mM MES, or pH 7 using 50 mM MOPS. Cells were then grown for three days at 30ºC. After three days, the OD_{600} of the cultures was determined. To facilitate pelleting of the cells, the culture was diluted 1:1 with ddH_2O. The supernatant was removed and used to determine the EPS, whereas the cell pellet was used for quantification of glycogen and cyclic β(1-2) glucans.

To quantify EPS production, EPS was precipitated from the supernatant using ethanol precipitation as previously described (Marroquí et al. 2001). Essentially the EPS was precipitated using 100% ethanol in a ratio of 2.5:1 with culture supernatant and centrifuged at 13000 RPM for 10 min at 4ºC to pellet the EPS. The supernatant was decanted and the resulting pellet was dried at 40ºC for 20 minutes and was then weighed. Weight of EPS was normalized to the OD_{600} of the culture. Results are an average of 3 independent biological replicates. Student's T-test was used to determine significance of data.

Table 3 Additional primers used in qRT-PCR

Gene	Sense Primer	Antisense Primer
chvI	5’ –AGGGCTACAAGGTCGAAACC-3’	5’ –TGGACGTGAGAAAGATGACG-3’
mucR	5’ –ATGACAGAGACTTCGCTCGG-3’	5’ –TCACTTGCCGCGACGCTTCC-3’
sinI	5’ –CCGGAAATCCGTAGTGCGTC-3’	5’ –ATGCGCGATCCTGGGAGATT-3’
wggR	5’ –GAGAGGGATGAACCACAGGA-3’	5’ –ATATCTTCGACCCCGAGCTT-3’
expA1	5’ –GCAGCTTCTTCCGGTCTCTA-3’	5’ –GATCGGATAGCCGAGTGAAA-3’
glgA1	5’ –GGTCATGACGATCCACAACA-3’	5’ –GGAAGCCGACATCTCCATAA-3’

Concentration of EPS-I in culture supernatant was also determined using the Anthrone assay as previously described (Morris 1948). The concentration of total reducing ends of EPS-I in culture supernatant was determined through the Lever assay, and compared to a glucose standard curve (Lever 1972). A ratio of the concentration of total EPS-I : concentration of reducing ends was used to estimate the change in total chain length of EPS-I. The concentration of EPS was normalized to cell pellet weight.

3.3.5 Glycogen and cyclic β(1-2) glucan isolation and quantification

Isolated cell pellets were washed once with 10 mL ddH_2O and then re-suspended in 1 M KOH and boiled at 100ºC in a water bath for 20 minutes to allow for cell lysis. Cell debris was removed by centrifugation (13000 RPM for 10 minutes), and supernatant was removed to a new tube. Glycogen was precipitated by the addition of 100% ethanol 1:1 (vol/vol) and collected (Wang et al. 2007). The remaining supernatant was quantified for the presence of periplasmic cyclic β(1-2) glucans (Breedveld et al. 1993). Quantification of glycogen and cyclic β(1-2) glucans was carried out using the anthrone-H_2SO_4 method (Breedveld et al. 1990). Results were compared against a glucose standard curve. Concentrations are an average of three independent biological replicates. Concentrations were normalized to the weight of cell pellet.

3.3.6 Acidic pH tolerance

Cultures were started in 5 mL RMM with mannitol and grown overnight at 30ºC. Cultures were then diluted to an OD_{600} of 0.1 in RMM. Then 1 mL of each culture was removed, washed with 1 mL ddH_2O, and resuspended in 1 mL saline (0.85% w/v). Cultures were then serial diluted from 10^0 to 10^{-5} and 10 µL of each dilution was spotted onto MGS agar plates buffered at either pH 5.75 or pH 7. Plates were then incubated at 30ºC for 4 days, and then growth was scored by

calculating CFU/mL. At pH 5.75 single colonies were not visible, so a dissecting microscope was utilized to count colonies to obtain a number to calculate CFU /mL. All results are an average of 3 independent biological replicates.

3.3.7 Plant dry weights and competition for nodule occupancy

Alfalfa seeds were surface sterilized with 1% bleach, washed with at least 10 volumes of sterile ddH_2O, and were subsequently germinated on water agar plates for 48 hours in the dark. Ten seedlings were then planted in sterile Leonard jars containing a 1:1 ratio of sand to vermiculite that had been soaked in nitrogen free Jensen's medium. After 2 days, seedlings were inoculated with approximately 10^7 CFU of bacteria in 10 mL of water. Nodule occupancy and plant dry weights were determined after 30 days. Plant dry weights are an average of 3 independent biological replicates consisting of 10 plants each. Nodule competition was determined by comparing the ratio of the mutant strain to wild-type found in the inoculum, to the same ratio found in isolated nodules. Numbers are an average of 3 independent biological replicates consisting of 50 examined nodules each. Error bars indicate standard deviation.

3.3.8 Nodule kinetics

Sterile alfalfa seedlings were planted on slants containing 10 mL of Jensen's agar. After 2 days, slants were inoculated with 10^6 bacteria in 100 µL of water as described above. The rate of nodule formation was assessed over a period of 21 days. Numbers are an average of 5 independent biological replicates consisting of 10 plants each.

3.4 Results

3.4.1 EPS-I production increases and EPS-II decreases when S. meliloti is grown at low pH.

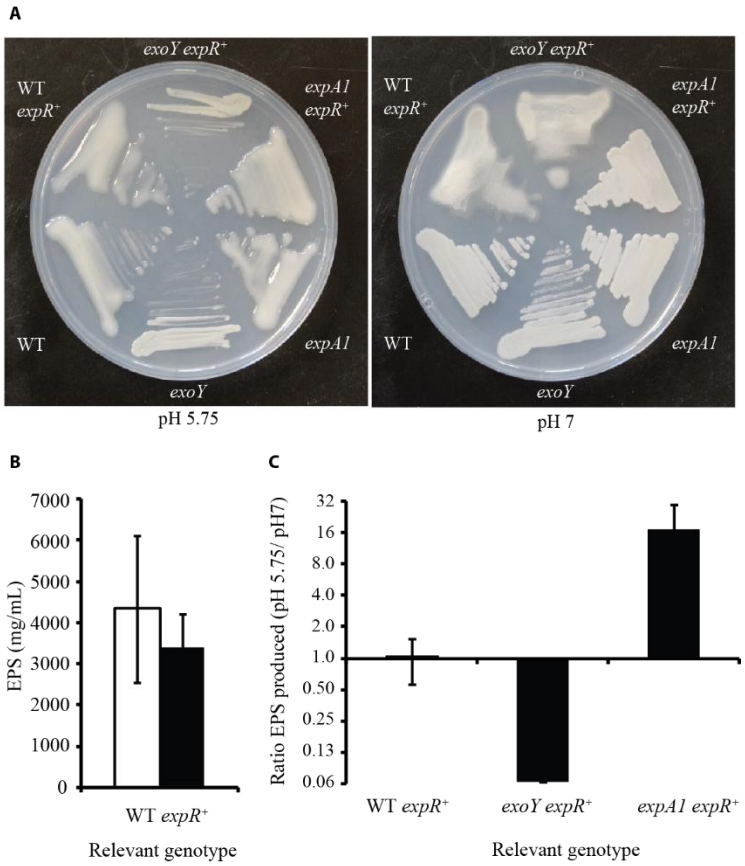
We had previously shown that when a mutant unable to utilize galactose was grown in RMM, containing both galactose and glycerol, it acidified its growth medium and produced EPS-

I (Geddes et al. 2014). The growth of *S. meliloti* has been previously shown to be impaired at pH 5.5, and only slightly affected at pH 5.75 when grown on VMM medium utilizing glucose as a carbon source (Hellweg et al. 2009). We wished to further characterize the role pH had on EPS production using RMM as a defined medium. When characterizing the growth of *S. meliloti* at different pH values using RMM, we found that growth was the same as what had been previously published using VMM. To be consistent with what had been previously determined, we chose to use a pH value of 5.75 to test the effect of medium acidification on EPS production.

To determine the effect of acidic pH on production of both EPS-I and EPS-II, mutants that are unable to produce either EPS-I (*exoY55*), or EPS-II (*expA1*) were utilized (Leigh and Lee 1988, Glazebrook and Walker 1989). Since an insertion element in the regulator *expR* prevents the production of EPS-II in Rm1021, these alleles were transduced into a background that carried an intact *expR* gene to restore production of galactoglucan (SRmD363). This yielded strains SRmD394 (*expR*$^+$, *exoY55*) and SRmA777(*expR*$^+$, *expA1*).

At pH 7, Rm1021 strains carrying either *exoY* or *expA1* mutations were indistinguishable on agar plates with respect to mucoidy. In an *expR*$^+$ background the strains were visually mucoid, except for the strain carrying the *expA1* mutation (Figure 8A). At pH 5.75, Rm1021 and SRmD363 were visibly mucoid and this was dependent on having an intact *exoY* gene. The strain capable of producing only EPS-II (*exoY, expR*$^+$) was found to be dry at pH 5.75. These results suggested low pH repressed the production of EPS-II while increasing production of EPS-I. To confirm these results the total amount of EPS produced by each strain was quantified. The amount of perceptible EPS did not change significantly in SRmD363 (WT *expR*$^+$) at pH 5.75 compared to pH 7 (Figure 8B and 1C). When production of EPS was determined for strains capable of only producing either EPS-I or EPS-II (*expA1/expR*$^+$ and *exoY55/expR*$^+$ respectively)

Figure 8 EPS production under neutral and acidic conditions



EPS production under neutral and acidic conditions. **A)** Visual mucoidy of strains grown at pH 5.75 or pH 7 **B)** EPS was precipitated from 50 mL of culture supernatant, dried, and weighed after 72 H of growth. White bar indicates EPS produced at pH 5.75, black bar indicates EPS produced at pH 7. **C)** Precipitated EPS from strains grown at pH 7 and pH 5.75 expressed as a ratio of EPS produced at pH 5.75 over pH 7. All numbers are an an average of 3 independent biological replicates. EPS weights are normalized to the OD_{600} of the culture.

we observed increased EPS-I, and decreased EPS-II production in response to low pH. At neutral pH EPS-II biosynthesis was increased while EPS-I was decreased (Figure 8C). This suggests that production of EPS-I is important under acidic conditions.

3.4.2 A shift to acidic pH decreases the average chain length of EPS-I

EPS-I consists of both HMW and LMW fractions. Changes in the distribution between HMW and LMW succinoglycan can be detected by using the Lever assay to determine the concentration of reducing ends as a proportion of the total amount of EPS that has been synthesized (York and Walker 1998). To determine if the proportion of HMW and LMW fractions change, the concentration of reducing ends from EPS isolated from Rm1021 was determined at pH 7 and at pH5.75. As a control, we utilized Rm7225 which carries an *exoH* mutation and has been shown to produce only HMW EPS-I (Mendis et al. 2016).

The results show that both Rm1021 and the *exoH* mutant were found to produce approximately equivalent amounts of EPS-I at neutral pH (Table 4) At pH 5.75, the total amount of EPS-I produced by Rm1021 was found to increase by 70%, while the amount produced by Rm7225 only increased by 36%. The concentration of reducing ends found in EPS-I from the wild-type also increased when medium pH decreased. When expressed as ratio, the data show that the EPS-I produced at a low pH is consistent with it being enriched with the LMW fraction (Table 4) We note that although the estimated chain length for the *exoH* mutant strain is much higher than the wild-type, the estimated chain length of the *exoH* mutant strain does change (Table 4)

Table 4. Effect of pH on EPS-I polymerization chain length.

	pH 7			pH 5.75		
Strain (genotype)	EPS[a] (mg/L)	Reducing [a] Ends (mg/L)	Ratio	EPS[a] (mg/L)	Reducing [a] Ends (mg/L)	Ratio
Rm1021 (WT)	286 ± 8	17 ± 1	17	489 ± 20[*]	46 ± 8	10[*]
Rm7225 (*exoH*)	218 ± 13	1[b] ± 0	300	298 ± 19[*]	5 ± 1	61[*]

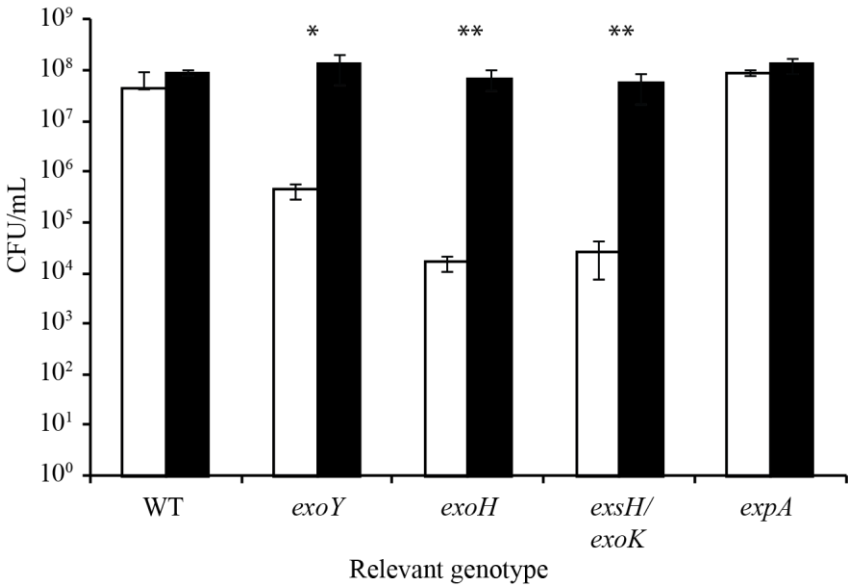
[a] Concentrations are an average of 3 independent biological replicates which have been rounded to the nearest whole number. The ratio between total EPS-I and reducing ends is used as an indicator of the overall change in chain length of EPS-I. [b]Actual value prior to value was 0.7. This value was used to determine the ratio of EPS : reducing ends *; Significant compared to pH 7 values based on $P < 0.05$ using Student's T-test.

3.4.3 LMW EPS-I is involved in acidic pH stress tolerance.

To determine if EPS-I or EPS-II play a direct role in allowing a cell to survive low pH conditions, mutants unable to produce either EPS-I or EPS-II were spotted onto medium buffered at pH 7 or pH 5.75. Since the proportion of LMW EPS-I increases when *S. melilolti* was grown at a low pH, we also tested if mutants unable to produce LMW EPS-I were impaired in pH stress tolerance. To accomplish this, both *exoH* (Rm7225) and *exsH/exoK* (Rm8826) mutant strains, which are incapable of producing LMW EPS-I, were tested for pH sensitivity. Inclusion of the double endoglycanase mutant strain let us examine if either the succinylation of EPS-I, or the LMW fraction was necessary for pH tolerance.

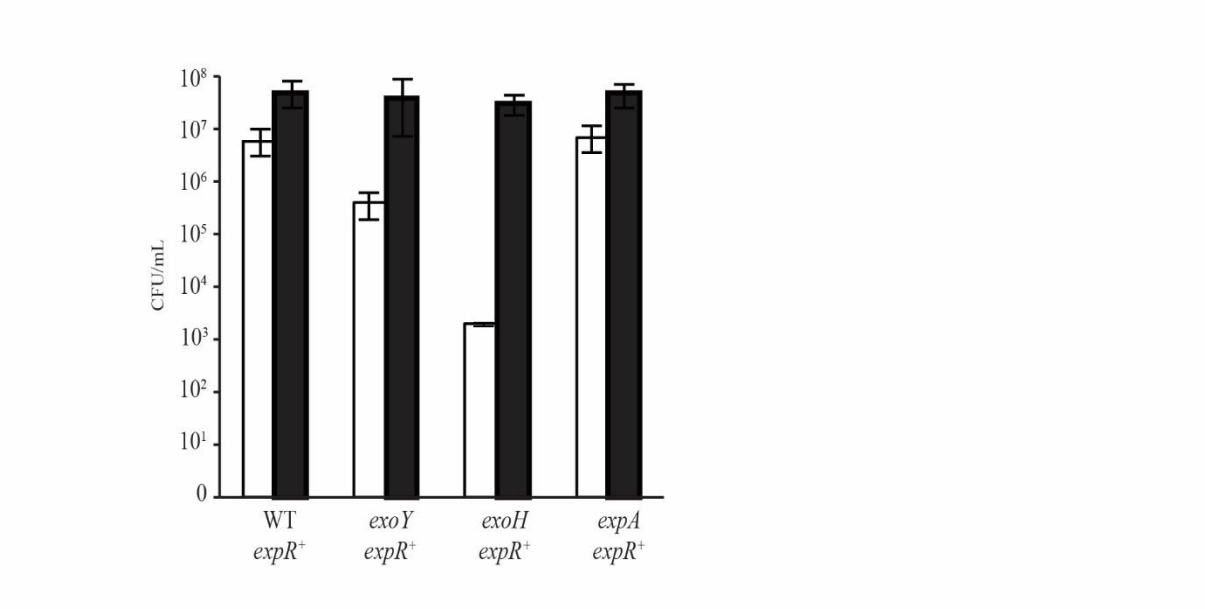
The data from both the *expR*$^{-}$ and *expR*$^{+}$ backgrounds showed the same trends (Figure 9 Figure 10). Strains that carried an *exoY* mutation in either *expR* background showed a 1,000 fold decrease in the number of colony forming units when spotted onto medium at low pH. The presence or absence of EPS-II did not affect overall survivability at low pH. A strain carrying a mutation in *exoH* was found to be even more sensitive to low pH conditions than *S. meliloti* strains unable to produce any EPS-I, resulting in a 10,000 fold decrease in colony forming units compared to wild-type. The pH sensitivity of a strain carrying both *exsH* and *exoK* mutations was found to be the same as the *exoH* mutant strain at pH 5.75 (Figure 9). This suggested that LMW fraction of EPS-I, and not the succinylation status of HMW EPS-I confers the ability for the bacteria to survive on low pH medium.

Figure 9 EPS-I mutant sensitivity to acidic pH.



EPS-I mutant sensitivity to acidic pH. Calculated CFU/ mL from spot dilutions at pH 5.75 (white) and pH 7 (black). Results are an average of 3 independent biological replicates. *; P value < 0.05 compared to WT. **; P value <0.05 compared to *exoY* mutant values.

Figure 10 EPS-I mutant sensitivity to acidic pH in an *expR*$^+$ background



Calculated CFU/ mL from spot dilutions at pH 5.75 (white) or pH 7 (black). Results are an average of 3 independent biological replicates.

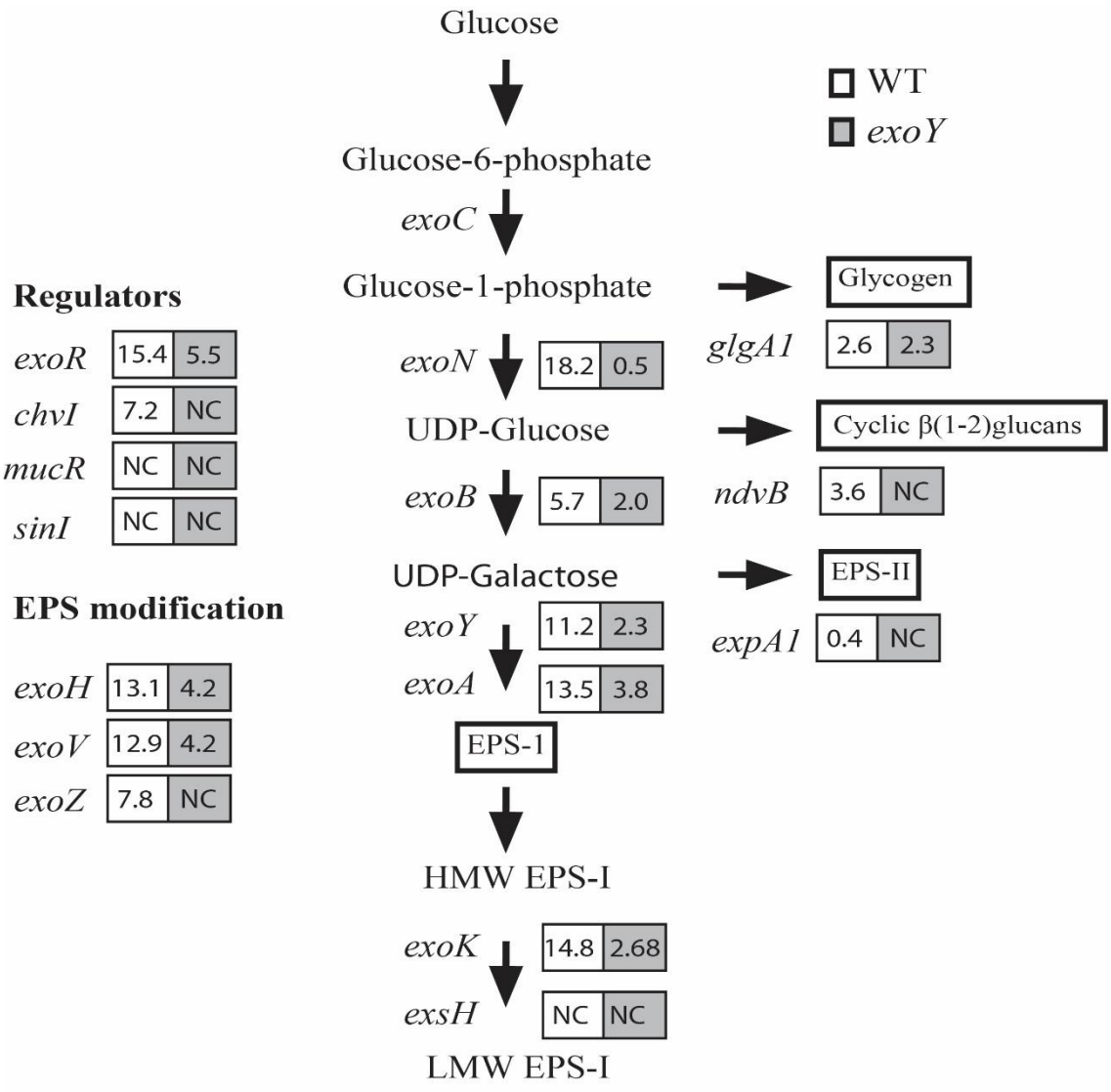
3.4.4 Induction of genes encoding polysaccharide biosynthesis components are altered in an exoY mutant.

The result that a strain carrying an *exoH* mutation was more sensitive to acidic pH than a mutation in *exoY* was unexpected. It was hypothesized that this difference might be due to a redirection of carbon into other polysaccharides, which might also be involved in pH tolerance. To investigate this hypothesis, the effect pH has on the transcription of genes involved in the biosynthesis of EPS-I, EPS-II (*expA1*), cyclic β(1-2) glucans (*ndvB*), glycogen (*glgA1*), LPS (*kdsB2, lpsL* and *lpsB*), and KPS (*rkpA, kdsB2*) was investigated in either the wild-type or an *exoY* mutant strain (Dylan et al. 1990a, Becker et al. 1997, Kereszt et al. 1998, Wang et al. 2007, Müller et al. 2009).

The results showed that *S. meliloti* responds to growth in acidic conditions through increased transcription of genes that encode determinants for both HMW and LMW EPS-I production (Figure 11 Table 5). In addition, both *exoR* and *chvI*, encoding proteins that have been shown to regulate EPS-I biosynthesis, were found to be upregulated. Consistent with our EPS quantification (Figure 8C), *expA1* was found to be down regulated under acidic conditions. In addition, transcription of *glgA1* and *ndvB* were also increased. Transcription of representative genes encoding determinants for LPS and KPS biosynthesis were found to be unaffected by acidic conditions (Table 5).

When the same analysis was carried out in an *exoY* mutant background, it was found that the transcription of most genes involved in EPS-I biosynthesis were greater at pH 5.75 than when grown at pH 7. Since primers used for detecting the transcription of *exoY* were positioned upstream of the Tn*5* insertion in the mutant strain, we still observed transcription of *exoY* in Rm7055 (Figure 11). The regulators *chvI* and *exoR* were no longer observed to have increased

Figure 11. Change in gene expression due to acidic medium



Data expressed as $2^{\Delta\Delta Ct}$ and represents fold change gene expression at pH 5.75 over pH 7. The gene *SMc00128* was included in the experiment as an internal control. All numbers represent an average of 3 independent biological replicates. Standard deviations for all numbers are within 1 cycle threshold.

*NC; No change

Table 5 Additional qRT-PCR results

Gene	Function	Fold Change Rm1021[a]	Fold Change Rm7055[a]
exoF	Polysaccharide export	10.8	3.0
exoX	Post-transcriptional regulator	2.0	NC[b]
exoD	Putative transmembrane protein	5.9	NC
rkpA	Putative fatty acid synthase	NC	NC
kdsB2	CMP-Kdo transferase	NC	NC
lpsB	LPS mannosyltransferase	NC	NC
lpsL	UDP-Glucuronate 5'-epimerase	NC	NC
expE1	ABC transporter	0.4	NC
expE2	Glycosyltransferase	NC	NC
wggR	Transcriptional activator	NC	NC
flbT	Putative flagellar repressor protein	0.2	0.1
flgB	Flagellar protein	NC	0.1

[a] Fold change in gene expression. Data is expressed as $2^{\Delta\Delta Ct}$ representing fold expression of strains grown in medium buffered at pH 5.75 over pH 7. *SMc00128* is used as an internal control. The table represents an average of 3 independent replicates with standard error within 1 cycle.

Kdo: 3-Deoxy-D-manno-oct-2-ulosonic acid

[b]NC; No Change

transcription at pH 5.75. The transcription of *exoN*, which encodes for the protein necessary to catalyze formation of UDP-glucose from glucose-1-phosphate, was found to decrease two-fold. This enzyme is of importance because it serves as a branching point between glycogen synthesis and other polysaccharides. Additionally, of the genes analyzed for synthesis of other polysaccharides, only *glgA1* was observed to be increased in transcription when grown at acidic pH compared to neutral pH in the *exoY* background. Taken together this suggested that the carbon that would normally be utilized to produce EPS-I may be directed toward the synthesis of glycogen in the absence of EPS-I production.

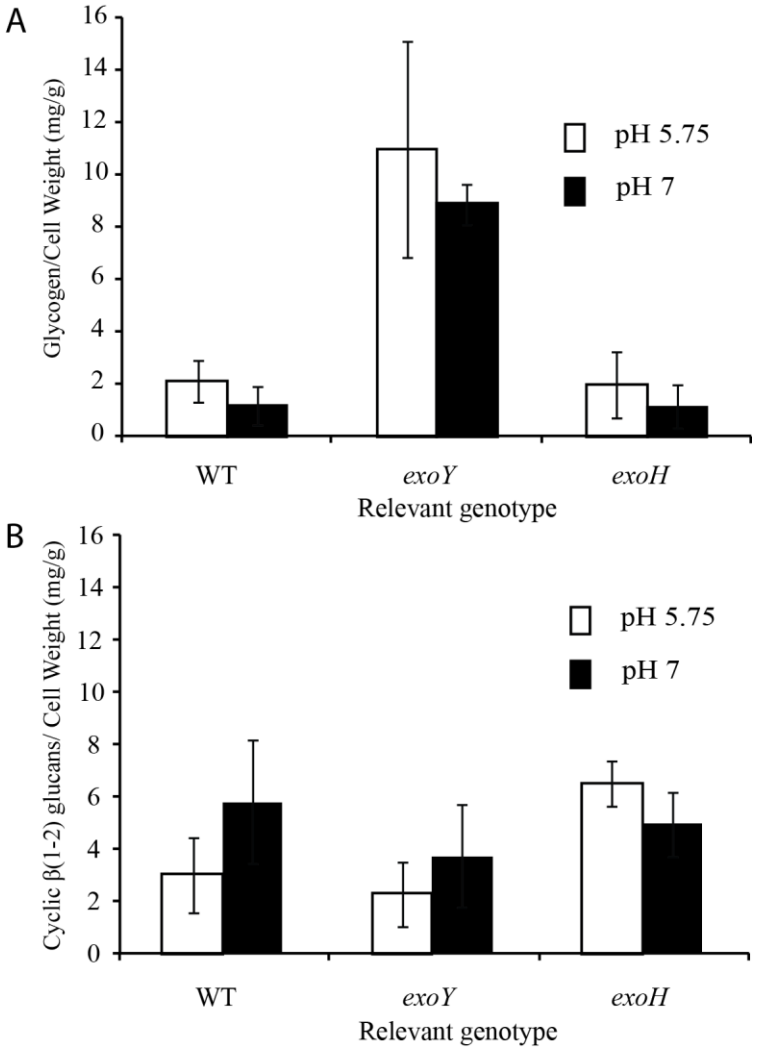
3.4.5 Production of glycogen is affected by the ability to produce EPS-I.

The ability to produce both glycogen and cyclic β(1-2) glucans has been linked with changes in EPS-I biosynthesis (Dylan et al. 1990a, Wang et al. 2007). To determine if the ability to produce EPS-I affects the intracellular accumulation of glycogen or cyclic β(1-2) glucans, both polysaccharides were quantified in strains carrying mutations in either *exoY* or *exoH* and compared to the wild-type production. The data showed that a mutation in *exoY* led to greater amounts of glycogen in both low and neutral pH conditions (Figure 12). However, cyclic β(1-2) glucans isolated from cells grown at either pH, or in either mutant background, did not show any significant changes when compared to WT concentrations (Figure 12).

3.4.6 Cyclic β(1-2) glucan production plays an auxillary role in pH tolerance.

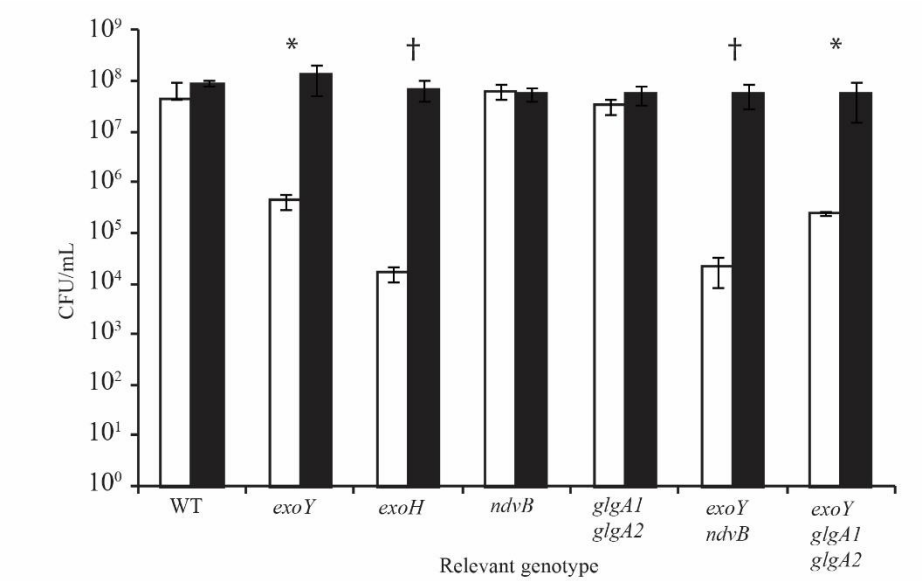
While neither intracellular polysaccharide was shown to be influenced by pH at the time point measured, pH was still shown to influence the transcription of both *ndvB and glgA1*. This may indicate importance of these genes in response to acidic pH. To determine if either glycogen or cyclic β(1-2) glucans play a role in surviving low pH, the *exoY*::Tn*5* mutation was transduced

Figure 12 Production of glycogen and cyclic β(1-2) glucans at pH 7.0 and pH 5.7.



Each polysaccharide was extracted after 3 days growth in MGS at the indicated pH. Both **A)** Glycogen and **B)** Cyclic β(1-2)glucans were quantified using the anthrone assay. Results are an average of 3 independent biological replicates.

Figure 13 Role of cellular polysaccharides in pH tolerance



Calculated CFU/ mL from spot dilutions at pH 5.75 (white) and pH 7 (black). Results are an average of 3 independent biological replicates. *; P value < 0.05 compared to WT. †; P value <0.05 compared to *exoY* mutant values.

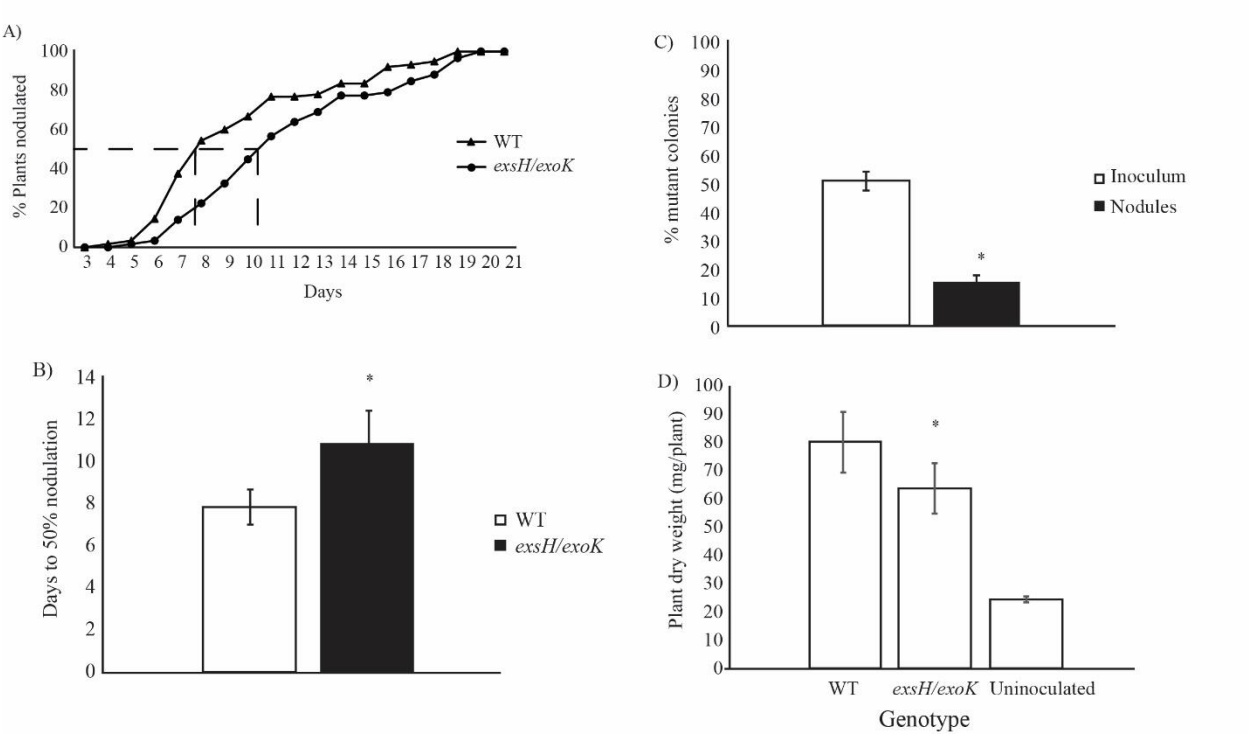
into the strains Rm11482 (Δ*glgA1*, *glgA2*:: ΩSpSm) and SRmD377 (*ndvB*::Tn*5*-233) to create strains SRmD455 and SRmD456 respectively. Each strain was then tested for sensitivity to acidic pH. Strains containing either the *ndvB* mutation, or the Δ*glgA1* ΩSpSm::*glgA2* mutations were not compromised in their ability to survive at pH 5.75 (Figure 13). The *ndvB/exoY* double mutant was found to have an increased sensitivity to acidic pH, while SRmD455 still showed the same pH sensitivity as an *exoY* mutant strain. Overall, the data showed that while the inability to produce cyclic β(1-2) glucans does not produce a pH sensitive phenotype, when combined withan *exoY* mutant background, the strain becomes impaired in pH tolerance to the same degree as an *exoH* mutant.

3.4.7 A double endoglycanase mutation leads a delay in nodule formation and is ineffective in competition for nodule occupancy.

Acidic pH has been shown to be present in a number of plant derived compartments during the nodulation process (Fedorova et al. 1999, Pierre et al. 2013). The finding that strains which acidified their growth medium were more competitive for nodule occupancy in an EPS-I dependent manner suggested that the ability to adapt to a low pH environment is necessary for efficient nodulation (Geddes et al. 2014). While most strains that carry a mutation in genes necessary for EPS-I biosynthesis in *S. meliloti* have severe symbiotic phenotypes (Leigh et al. 1985, Becker et al. 1993a, 1993b), plants inoculated with the *exsH*/*exoK* mutant strain only exhibit a slight decrease in overall plant growth (Mendis et al. 2016). If adaptation to acidic pH is a determinant in symbiotic establishment, it was reasoned that pH sensitive strains should be delayed in nodule formation, and possibly be also uncompetitive for nodule occupancy.

The results show that although plants inoculated with the double endoglycanase mutant achieved 100% nodulation, there was a change in the rate of nodule formation Figure 14A).

Figure 14 Symbiotic phenotypes of an *exsH*/*exoK* mutant strain.



A) Percent of plants nodulated over time after inoculation with Rm1021 or Rm8826. Data are the sum of over 50 plants over 4 biological replicates. The average standard deviation of each point is 8% and 9% for Rm1021 and Rm8826 respectively. **B)** Average number of days post inoculation before 50% of plants became inoculated. **C)** Rm8826 competed against Rm1021 for nodule occupancy of *M. sativa*. Data are presented as the average proportion of Rm8826 found in the inoculum (white), and isolated from nodules (black). Data are the average of 3 independent biological replicates. **D)** Average dry weight per plant of *M. sativa* inoculated with Rm1021 or Rm8826. Data is an average of 3 independent biological replicates consisting of 10 plants each. For panels B, C, and D, significance was determined using Student's T-test. *; $P < 0.05$ compared to WT.

Initial appearance of nodules occurred at about 5 days post inoculation for the wild-type under our growth conditions, whereas the *exsH*/*exoK* mutant strain was consistently delayed by at least one day (Figure 14A). This delay became more prominent when plants reached 50% nodulation; while plants inoculated with Rm1021 were 50% nodulated after 8 days, plants inoculated with the mutant achieved this proportion at 11 days (Figure 14B).

When this strain was tested for its ability to compete for nodule occupancy, it was found that the *exsH*/*exoK* mutant was severely impaired in its ability to compete with the wild-type for nodule occupancy (Figure 14C). Consistent with previously results (Mendis et al. 2016), the ability of plants inoculated with the *exsH*/*exoK* mutant to accumulate dry matter under nitrogen limiting conditions was found to be slightly impaired compared to wild-type inoculated plants (Figure 14D). These data are consistent with the hypothesis that the inability of the double endoglycanase mutant to tolerate acidic pH plays a role in early nodule formation and its ability to compete for nodule occupancy.

3.5 Discussion

In this work we addressed the hypothesis that EPS-I plays a role in allowing *S. meliloti* to survive a low pH environment. By using well characterized *S. meliloti* mutant strains which produce either EPS-I, EPS-II, or both polysaccharides, we provide direct evidence that EPS-I and EPS-II production are regulated by the pH of the medium (Figure 8A, C). These observations were corroborated through qRT-PCR expression data of key genes that encode enzymes necessary for biosynthesis of each EPS (Figure 11, Table 5). Although these results are consistent with previous work, we note that changes in gene expression of *expA1*, *glgA1*, and *ndvB* have not been previously reported. While our results are from cells exposed to each pH for 24 hours, previous work focused on transcriptional changes that occurred after an hour following a sudden

pH change. We note that although this work found a change in expression of *glgA2* (Hellweg et al. 2009), this gene has been shown to not be involved in glycogen synthesis (Wang et al. 2007).

A shift to acidic pH also influences the degree of polymerization of EPS-I to favour lower molecular weight forms (Table 4).A decrease in chain length polymerization was also observed in the *exoH* mutant strain that produces non-succinylated EPS-I, which cannot be cleaved by the *exsH/exoK* endoglycanases (Table 4)

The degree of polymerization of EPS-I has been shown to be under control by ExoP/ExoT/ExoQ, in addition to post processing cleavage by ExsH and ExoK (Becker et al. 1995, González et al. 1998). The transcription of *exoP, exoT,* and *exoQ* have also been shown to increase under acidic conditions (Hellweg et al. 2009). This may explain why we observed a significant difference of polymerization in *exoH* mutant strain. Although the degree of polymerization changed, it did not appear to be great enough to have a physiological effect.

The exact mechanism that LMW EPS-I production plays in pH tolerance is still unknown. The production of this form of succinoglycan could simply provide a physiological barrier, or the increased amount of reducing ends may create a natural buffering system. However, the pK_a of EPS-I from *S. meliloti* was determined through protonation of its carboxylic groups, and shown to be pH 3.8 (Szewczuk-Karpisz et al. 2014). Although the pK_a gives some insight into the biophysical properties of EPS-I, it is unclear if this could be playing a role since the buffering capacity of EPS-I at pH 5.75 should be negligible. While our results showed the inability to produce LMW EPS-I is detrimental to survival at low pH (Figure 9), further studies are necessary to determine if increased production of the LMW fraction of EPS-I could offer increased acidic pH tolerance. Future investigation is also still needed to determine why a mutant

unable to produce LMW EPS-I is more sensitive to acidic pH than a mutant not producing any EPS-I at all.

The involvement of cyclic β(1-2) glucans in pH tolerance when EPS-I is absent was unexpected since intracellular concentrations were not affected under the conditions that were tested (Figure 12B). Previous work has shown that suppression of the symbiotic phenotype associated with an *ndvB* mutant implicates EPS-I associated genes (Nagpal et al. 1992), suggesting that EPS-I and cyclic β(1-2) glucans may play complementary physiological roles. If this is the case, cyclic β(1-2) glucans may also provide a buffering system, or a physiological barrier when accumulated in the periplasm. The observation that loss of cyclic β(1-2) glucan production on its own does not result in a change in pH sensitivity also further suggests that these two polysaccharides play a similar physiological role. The possibility does exist that an increased amount of cyclic β(1-2) glucans could be excreted into medium to provide increased pH tolerance, though this has yet to be investigated.

The production of EPS represents a large carbon sink for *S. meliloti*. Our results are consistent with the hypothesis that carbon can be redirected to another polysaccharide, such as glycogen, in the absence of EPS-I production. While the accumulation of glycogen did not affect pH tolerance between the *exoY* and *exoH* mutant strains, the data clearly demonstrate that carbon that is not used for synthesis of exopolysaccharides in an *exoY* mutant can affect the concentration of other macromolecular compounds.

In *S. meliloti* both glycogen and poly-hydroxybutryarte (PHB) are produced as major carbon storage compounds under growth limiting conditions (Zevenhuizen 1981). In contrast, exopolysaccharides are generally produced after accumulation of these intracellular

polysaccharides (Zevenhuizen 1981). The carbon flow between PHB and glycogen is highly inter-connected, and has been shown to be under similar regulatory control (Povolo and Casella 2000, Dunn et al. 2002). It has determined that the loss of PHB synthesis results in a decrease of EPS-I synthesis, leading to the hypothesis that carbon stored in PHB is used for the production of other polysaccharides during symbiosis (Aneja et al. 2004, Wang et al. 2007). To our knowledge, this may be the first report to demonstrate that the loss of EPS-I production influences either one of these major carbon stores. It is of note that it has been recently shown that continual growth at low pH results in a decreased accumulation of PHB, which may be correlated with the increase in EPS-I production (Draghi et al. 2017).

Succinoglycan is required for nodule development in *S. meliloti* (Leigh et al. 1985). Although it has been shown to be important, its exact role has never been elucidated. More recently, it has been shown in *Lotus japonicus* that defects in EPS could be suppressed by mutations in a LysM-type receptor kinase that can directly bind EPS (Kawaharada et al. 2015). In *S. meliloti*, it has been shown that the succinylation of EPS-I, and not the LMW form of the EPS-I was an important determinant for allowing the establishment of an effective symbiosis. Taken together, these strongly suggest that EPS-I can be a signal that is used to initiate the formation of an infection thread. While this may represent a strong "checkpoint" in the process, we propose that LMW EPS-I plays a role that governs the efficiency of the overall nodulation process.

Fluorescent microscopy utilizing acidotropic dyes has clearly shown that the bacteria encounter low pH environments during the initial colonization of the root hair (Geddes et al. 2014), as well as when the bacteria are actively fixing nitrogen (Fedorova et al. 1999). Our data suggest that the inability to covert HMW EPS-I to the LMW form results in a sensitivity to low

pH as well as a delay in nodule development (Figure 9, Figure 14A). Importantly, the nodules that were formed by the double endoglycanase mutant appeared to be normal, yet the plants had accumulated less dry matter (Figure 14D); presumably due to less nitrogen being reduced by the microsymbiont. The reduction in the amount of dry matter accumulated can either be due to the delay in nodule formation, which also leads to a delay in the maturation of nitrogen fixing bacteroids, or that the nitrogen fixing bacteroids are less efficient due to low pH found in the symbiosome. These two suggestions may not be mutually exclusive.

The results of this study add to the literature and imply that pH plays a strong role in shaping the efficiency of early host-symbiont interaction. Once the colonization of the curled root hair occurs, the compartment becomes acidified (Geddes et al. 2014). Acidification leads to an increase of succinoglycan production via *exoR/exoS/chvI* signaling (Chen et al. 2008, Hellweg et al. 2009). The synthesized EPS plays two roles; the succinylated EPS-I works as a signal, which is critical for infection thread development (Jones et al. 2007, Kawaharada et al. 2015), whereas the LMW fraction enhances the ability of the bacteria to survive in the CCRH. If acidification, and thus EPS synthesis, occurs more rapidly, this can lead to a more rapid progression of the bacteria from the CCRH to the formation of visible nodules (Jones 2012, Geddes et al. 2014). The inability to make LMW succinoglycan would decrease the bacterial viability in the CCRH leading to a reduced amount of bacterial capable of continuing the symbiotic interaction. This would lead to a delay, but not an abortion, of infection thread formation and visible nodule development (Figure 14). The visualization of activity from an *exoF*::Tn*phoA* fusion in the invasion zone of nodules also suggests that succinoglycan may play a role later in nodule development (Reuber et al. 1991). It is pertinent to note that there is ample evidence suggesting that actively fixing symbiosomes are acidic compartments, and that *exo*

genes are not highly expressed bacteroids (Reuber et al. 1991, Fedorova et al. 1999). It is also noteworthy that other mechanisms of tolerating low pH have been described for *S. meliloti* (Chen et al. 1993, Tiwari et al. 1996), suggesting that LMW succinoglycan may not play a major role at later stages of symbiosis.

Overall, our observations support the hypothesis that production of LMW EPS-I is involved in tolerating acidic pH stress, and that this affects symbiosis. The precise mechanism of how EPS-I fulfils this role is currently unclear. Further work to define how EPS-I biosynthesis responds to environmental shifts by measuring transcription, translation, as well as metabolic pools may provide useful data to formulate hypotheses that can be used to address this question and are the focus of future studies.

Chapter 4:

Common Dyes Used to Determine Bacterial Polysaccharides on Agar Are Affected by Medium Acidification

Reproduced from Canadian Journal of Microbiology, Volume 63, Hawkins, J. P., Geddes, B. A., and Oresnik, I. J. Common dyes used to determine bacterial polysaccharides on agar are affected by medium acidification, doi: 10.1139/cjm-2016-0743, Copyright 2017, with permission from the Canadian Journal of Microbiology. Justin Hawkins measured medium acidification and wrote the manuscript. Barney Geddes created the mutant strains and preformed dye phenotype observations.

4.1 Abstract

In this work we highlight effects of pH on bacterial phenotypes when using the bacteriological dyes Aniline blue, Congo red, and Calcofluor white to analyze polysaccharide production. Study of galactose catabolism in *Sinorhizobium meliloti* led to the isolation of a mutation in *dgok1*, which was observed to overproduce exopolysaccharides when grown in the presence of galactose. When this mutant strain was spotted onto plates containing Aniline blue, Congo red, or Calcofluor white, the intensity of the associated staining was strikingly different when compared to the Wild-type. Additionally, a Calcofluor dull phenotype was observed, suggesting production of a polysaccharide other than succinoglycan. Further investigation of this phenotype revealed that these results were dependent on medium acidification, as buffering at pH 6 had no effect on these phenotypes, while medium buffered at pH 6.5 resulted in a reversal of the phenotypes. Screening for mutants of the *dgok1* strain that were negative for the aniline blue phenotype yielded a strain carrying a mutation in *tkt2* which is annotated as a putative transketolase. Consistent with the plate phenotypes, when this mutant was grown in broth cultures it did not acidify its growth medium. Overall this work shows that caution should be exercised in evaluating polysaccharide phenotypes based strictly on the use of dyes.

4.2 Introduction

Bacteriological dyes have been used as an important component in medium to determine production of various polysaccharides and extracellular elements. In particular, Congo red, Calcofluor white, and Aniline blue have seen prominent use in bacteria, plants, and fungi. Congo red and Calcofluor white are typically used as dyes to stain polysaccharides and cellular elements

containing β(1-3) or β(1-4) linkages, and have particularly strong interactions with cellulose and chitin (Hughes and McCully 1975, Kneen and Larue 1983, Wood and Fulcher 1983, Mori and Bellani 1996, Robledo et al. 2012). However, the use of these dyes is not limited to polysaccharides since Congo red can also bind to extracellular fibers called Curli produced by *Escherchia coli* (Reichhardt et al. 2016). The use of these dyes has also had an impact on the ability to isolate symbiotically effective rhizobia. Most *Rhizobium* strains lack the ability to bind Congo red when grown on YEM (Zevenhuizen et al. 1986). However, non-nodulating strains of *Rhizobium leguminosarum bv. trifollii* have been isolated on the basis of binding to Congo red. This has resulted in the use of Congo red as a potential indicator of nodulating strains of *R.leguminosarum bv. trifollii* in mixed cultures (Bromfield and Jones 1980). Calcofluor white is used as an indicator for production of the polysaccharide succinoglycan (EPS-I) in *S. meliloti* Rm1021, and it has long been used as a screening tool to isolate mutants which affect production of this symbiotically important polysaccharide (Leigh et al. 1985, 1987, Long et al. 1988).

The dye Aniline blue is largely utilized to detect the production of callose, a linear β(1-3) glucan found in cell walls in both plants and fungi (Stone et al. 1984). This is visualized through a bright yellow fluorescent phenotype when observed under UV light. Fluorescence is due to the interaction of a fluorochrome from Aniline blue (Sirofluor) interacting with β(1-3) linkages (Evans et al. 1984). While bacteria do not produce callose, production of similar polysaccharides consisting of β(1-3) linked glucans has been observed, including the polysaccharide curdlan (Kenyon and Buller 2002, Stone 2005). Production of curdlan is known to occur in various bacteria including some *Agrobacterium* species (Stasinopoulos et al. 1999). The production of curdlan is visualized in media supplemented with Aniline blue by cell biomass turning a blue colour (Stasinopoulos et al. 1999).

4.3 Methods and Results

The production of polysaccharides can be heavily influenced by many factors such as pH, salt concentration, osmolarity, and the ability to metabolize carbon sources (Dylan et al. 1990b, Miller-Williams et al. 2006, Hellweg et al. 2009, Geddes et al. 2014). Previous work to identify *S. meliloti* mutants unable to catabolize galactose led to the isolation of a Tn*5* insertion mutant in *dgoK1* (SRmD304), which was observed to acidify medium when grown in the presence of galactose (Geddes and Oresnik 2012a, 2012b, Geddes et al. 2014). When SRmD304 was initially streaked on Rhizobium Minimal Medium (RMM) (Broughton et al. 1986) using galactose and glycerol as carbon sources, a mucoid phenotype was observed suggesting an increased production of EPS. However, when the strain was streaked onto RMM containing Calcofluor it exhibited a dull fluorescent phenotype that seemed to be atypical for EPS-I (Figure 1c, top panel).

To further investigate the produced polysaccharide, SRmD304 was screened on medium supplemented with either Aniline blue, Congo red, or Calcofluor white. Rm1021 (Wild-type) and SRmD304 were grown at 30°C overnight in 5 mL cultures of LB broth (Sambrook et al. 1989) to an OD_{600} of 1.0. A 1 mL aliquot of each culture was pelleted and re-suspended in 100 µL of 0.85% w/v saline. Finally, 20 µL of this cell suspension was spotted onto YMA (Yeast Mannitol Agar) agar (Kneen and Larue 1983) supplemented with 15 mM galactose and Congo red (25 µg/ mL), and RMM supplemented with 15 mM galactose and either Aniline blue (50 µg/ mL) or Calcofluor white (0.02%).

The results showed that after 5 days at 30°C Rm1021 had a negative reaction on plates supplemented with either Congo red or Aniline blue (light orange and white respectively) (Figure 15, top panels). However, a Calcofluor bright phenotype was observed suggesting only

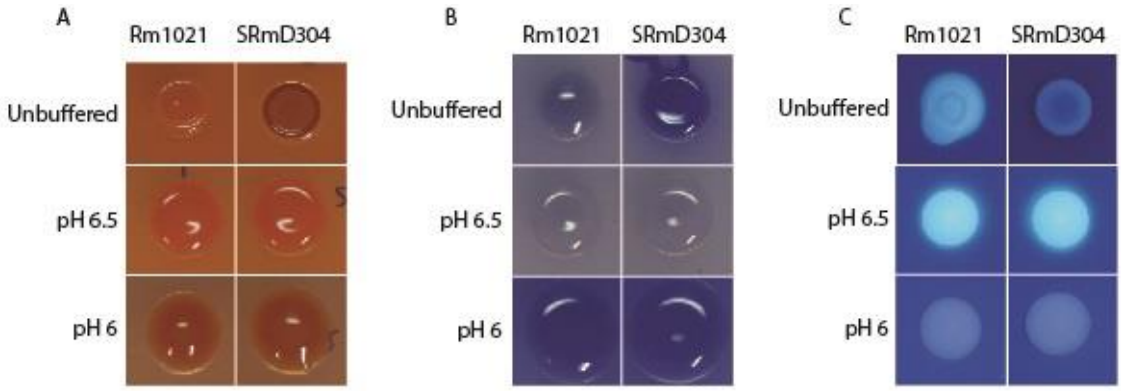
succinoglycan was being produced, which is consistent with previous results (Zevenhuizen et al. 1986, Leigh and Lee 1988).

SRmD304 gave a positive result with both Aniline blue and Congo red, turning blue and red respectively, while displaying a dull fluorescence under UV on plates containing Calcofluor white. A possible interpretation of this result was that in the presence of galactose SRmD304 may be producing a novel EPS that contained β(1-3) linkages; possibly curdlan. The operon *crdASC* encodes enzymes involved in the biosynthesis of curdlan in *Agrobacterium* (Stasinopoulos et al. 1999); however orthologues to these genes are not found in *S. meliloti* Rm1021 genome.

Further characterization of SRmD304 showed that, when the *dgoK1* strain was grown in weakly buffered conditions in the presence of galactose and a secondary carbon source that it could metabolize it would acidify its growth medium to pH 4.5, and that the EPS that was produced had a 7:1 glucose:galactose ratio that was indicative of succinoglycan (Geddes et al. 2014).

Many dyes are known to be affected by media pH, including Congo red, Aniline blue, and Calcofluor (Darken, 1962; Evans *et al.*, 1984; Mera and Davies, 1984). To determine if the observed dye phenotypes associated with *dgoK1* resulted from medium acidification, the mutant

Figure 15. Dye phenotypes in buffered media



Observed dye phenotypes of strains on plates supplemented with **A)** Congo red **B)** Aniline blue, and **C)** Calcofluor white. **A+B)** Positive staining was observed at pH 6, and in SRmD304 in unbuffered medium. Differential colours of medium are due to differences in dye staining the medium in each condition. Bacterial spots were scored after 5 days on media that was buffered as labeled. Calcofluor white images were observed during exposure to UV light.

and the wild-type strains were spotted onto plates that were modified by adding 50 mM MOPS buffered at either pH 6 or pH 6.5. The results show that when the wild-type (Rm1021) and the strain carrying the *dgoK1* mutation (SRmD304) were spotted onto plates buffered at pH 6.5 the phenotypes that were noted for SRmD304 on the weakly buffered plates differed. The intensity of the staining with Congo red and Aniline blue decreased, whereas the fluorescence associated with Calcofluor white increased (Figure 15, middle panels). In contrast, when the strains were spotted onto plates buffered at pH 6 the phenotypes of both the wild-type and the strain carrying the *dgoK1* mutation appeared similar to that seen when SRmD304 was spotted onto the weakly buffered media, resulting in a positive reaction to Aniline blue and Congo red while being Calcofluor dull (Figure 15, bottom panels). Taken together these observations support the hypothesis that the dye phenotypes associated with the *dgoK1* mutation were due to medium acidification.

The influence of pH on Aniline blue phenotypes has not to our knowledge been previously shown at this pH range. It was reasoned that if the positive reaction of Aniline blue is correlated with medium acidification in *S. meliloti*, this property could be exploited to screen for mutant strains unable to acidify their growth medium. To test this hypothesis, SRmD338, which carries an unmarked deletion of *dgok1* (Geddes 2014), was mutagenized with Tn*5* as previously described (Finan et al. 1988). The resulting Tn*5* mutants were screened on RMM supplemented with 15 mM galactose and Aniline blue. Mutants that were phenotypically white were purified, and the site of insertion in the *S. meliloti* genome was determined using arbitrary PCR (Miller-Williams et al. 2006). Two such mutants were isolated in this manner; one in the gene *SMc00588,* which has been previously shown to be necessary for medium acidification in a *dgok1* mutant background (Geddes et al. 2014), and one in the gene *tkt2,* encoding for a putative

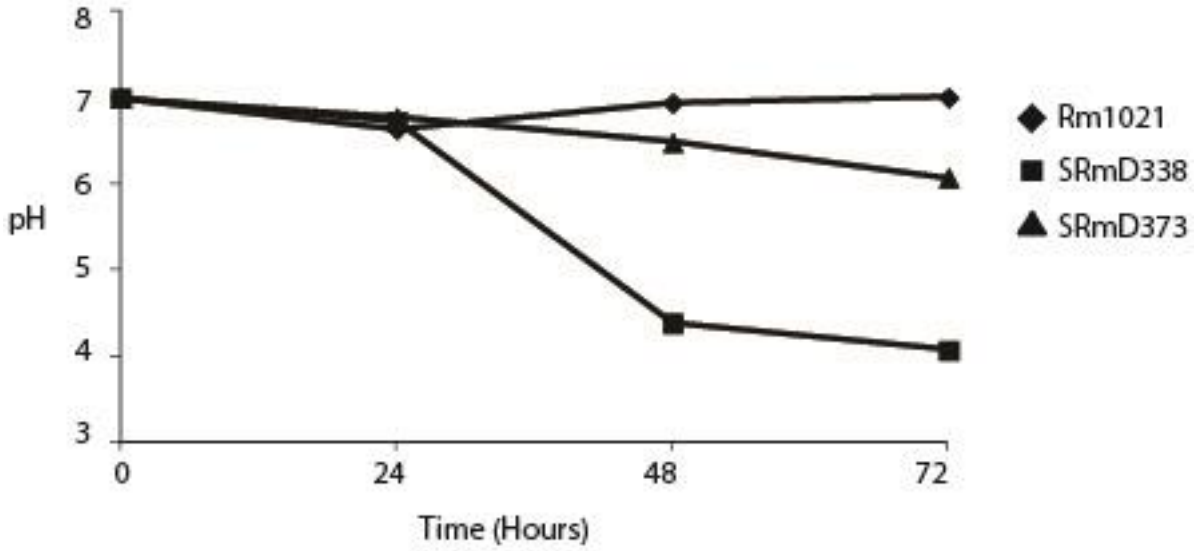
transketolase. The *tkt2*::Tn*5* mutation was transduced into Rm1021 to further investigate phenotypes associated with this mutation. It was determined that a strain carrying this mutation had a carbon utilization phenotype similar to those reported for transketolase mutants in *E. coli* (Zhao and Winkler 1994). The growth phenotypes were fully complemented by the introduction of a plasmid over-expressing *tkt2* (data to be presented elsewhere).

To determine if the Aniline blue phenotype associated with SRmD373 (Δ*dgoK1*, *tkt2*::Tn*5*) was due to an inability to acidify its growth medium, broth cultures of SRmD373 were grown in RMM medium containing both glycerol and galactose as previously described (Geddes 2014). Consistent with what was previously observed, the wild-type (Rm1021) did not acidify its growth medium whereas supernatant of the SRmD338 (Δ*dgoK1*) growth medium dropped to a pH around 4.5 (Figure 16). In contrast, SRmD373 (Δ*dgoK1*, *tkt2*::Tn*5*) was markedly impaired in its ability to acidify its growth medium (Figure 16).

4.4 Discussion

The isolation of a mutation in *tkt2* suggests alteration of central carbon metabolism plays a role in the observed galactose dependent medium acidification observed in SRmD338. This could be due to altered carbon flow to compounds which are secreted and subsequently drop the pH of the medium. Medium acidification in the *dgok1* background has been shown to be dependent on galactose dehydrogenase activity (SMc00588), suggesting that galactonate accumulation plays a role in altering the medium (Geddes and Oresnik 2012a). The observation that a mutation in *tkt2* results in a similar phenotype suggests that medium acidification may not be solely dependent upon SMc00588. We have also observed that a strain carrying only a *tkt2* mutation has an Aniline blue negative phenotype (white) under conditions which Rm1021 stains blue (Data to be presented elsewhere). This suggests a mutation in *tkt2* may affect medium

Figure 16. Measurement of culture pH



Measurement of culture pH for each strain grown in RMM medium with glycerol and galactose over 72 hours. The data presented are an average of 3 independent biological replicates. Where not visible error bars are smaller than the size of data points.

acidification independent of the *dgok1* mutation and galactose. Since the loss of transketolase represents a major block in central metabolism, it seems plausible that altered carbon flow to other metabolites may also affect medium acidification. Of note, it has been recently shown that proteins associated with the pentose phosphate pathway are increased when *S. meliloti* is grown in acidic conditions (Draghi et al. 2016).

The results observed here show previously un-reported dye phenotypes of Aniline blue, Calcofluor white and Congo red. While it is well known that these dyes have pH components, it is usually at extreme pH ranges, and have not been reported around pH 6 – pH 6.5. Although our results suggest that the attributes associated with Aniline blue can be exploited to screen for phenotypes, it is important to emphasize that these results suggest caution should be used when using these dyes to assign polysaccharide phenotypes if they are being used in weakly buffered media.

Chapter 5:

Characterization of the non-oxidative pentose phosphate pathway and its contribution to medium acidification and symbiosis in *Sinorhizobium meliloti* Rm1021

Reproduced from Journal of Bacteriology, (2018), Hawkins, J. P., Onodorez, P.A., and Oresnik, I. J. Characterization of Mutations That Affect the Nonoxidative Pentose Phosphate Pathway in *Sinorhizobium meliloti*. 200(2): e00436-17

Copyright 2018, with permission from the Journal of Bacteriology. Justin Hawkins carried out experiments and wrote the manuscript. Patricia Onodorez performed rhizobactin competition assays.

5.1 Abstract

Sinorhizobium meliloti is a Gram-negative α-proteobacteria that can enter a symbiotic relationship with *Medicago sativa* and *M. truncatula*. Previous work had determined that a mutation in the gene *tkt2*, a putative transketolase, could prevent medium acidification associated with *dgok1*. Since the pentose phosphate pathway in *S. meliloti* is not well studied, strains carrying mutations in either *tkt2* and *tal*, a putative transaldolase, were characterized. Carbon metabolism phenotypes revealed that both mutants are impaired in growth on erythritol and ribose. This phenotype was more pronounced in the *tkt2* mutant strain, which also displayed an auxotrophy for aromatic amino acids. Changes in pentose phosphate pathway metabolite concentrations was also consistent with having a mutation in either *tkt2* or *tal*. Metabolite concentrations in central carbon metabolism were also found to shift dramatically strains carrying a *tkt2* mutation. While proteins involved in central carbon metabolism did not significantly change in concentration under any condition, those associated with iron acquisition increased in the wild-type strain when induced with erythritol. These proteins were not detected in either mutant, and resulted in less observable rhizobactin production in the *tkt2* mutant. While both mutants were impaired in succinoglycan synthesis, only the *tkt2* mutant strain was unable to establish symbiosis with alfalfa. These results suggest that *tkt2* and *tal* play central roles in regulating carbon flow necessary for carbon metabolism and symbiotic establishment.

5.2 Introduction

Nitrogen makes up approximately 1-4% of all living cells, and is a critical limiting element for growth of all life (Woodmansee et al. 1978). In agriculture, the major natural input of nitrogen occurs through symbiotic nitrogen fixation, which is predicted to provide 40 Tg of

reduced nitrogen annually (Herridge et al. 2008). Biological nitrogen fixation occurs when a microsymbiont establishes a symbiotic relationship with a host plant and reduces atmospheric nitrogen to ammonia, provided to the plant in the form of amides or purines (Geddes and Oresnik 2016).

The relationship between *Sinorhizobium meliloti* and *Medicago sativa* has undergone extensive study and is used as a model system for studying plant-microbe interactions. During symbiotic establishment, a complex signal exchange takes place between *S. meliloti* and *M. sativa*, and requires constant production of both Nod factor and exopolysaccharides (Jones et al. 2007, Oldroyd 2013). Overall, this results in *S. meliloti* becoming endocytosed into plant cells, followed by terminal differentiation into a bacteroid capable of biological nitrogen fixation (Oldroyd 2013, Farkas et al. 2014).

The ability of rhizobium to catabolize various carbon sources has been shown to be integral for the establishment of an effective association. The inability of rhizobium to metabolize specific carbon sources has been shown to result in ineffective competition for nodule occupancy, or the inability to fix nitrogen (Finan et al. 1988, Oresnik et al. 1998, Yost et al. 2006). This has lead to the hypothesis that efficient carbon metabolism is critical to the symbiotic process. Rhizobia can catabolize a wide array of carbon sources to proliferate in the numerous environments these bacteria encounter. The genome of *S. meliloti* encodes for many genes necessary for the transport and catabolism of carbon sources, enabling it to grow on a diverse array of substrates (Stowers 1985, Galibert et al. 2001). Central carbon metabolism in *S. meliloti* occurs through the Entner-Doudoroff, Embden-Meyerhof-Parnas, pentose phosphate, and Tricarboxylic Acid (TCA) pathways (Geddes and Oresnik 2014). *S. meliloti* primarily utilizes the Entner-Doudoroff pathway for the catabolism of hexoses due to the absence of

phosphofructokinase, which catalyzes the formation of fructose-1,6-bisphosphate from fructose-6-phosphate (Irigoyen et al. 1990, Capela et al. 2001). This has also led to the suggestion that the upper Embden-Meyerhof-Parnas pathway primarily plays a gluconeogenic role in *S. meliloti*.

The pentose phosphate pathway is classically divided into two different enzymatic branches, the oxidative branch and the non-oxidative branch (Stincone et al. 2015). The initial two enzymatic reactions of the Entner-Doudoroff and oxidative pentose phosphate pathways are shared and convert glucose-6-phosophate to 6-phosphogluconate (6PG). The only dedicated enzyme in this part of the pathway is 6PG dehydrogenase which converts 6PG to ribulose-5-phosphate. The oxidative half of the pentose phosphate pathway is not ubiquitous among organisms, and has been shown to be absent in various organisms including some rhizobia (Martínez-De Drets and Arias 1972, Grochowski et al. 2005, Bräsen et al. 2014). In addition, the presence of enzymes in the oxidative pentose phosphate pathway, such as 6PG dehydrogenase, has been used to separate fast and slow growing rhizobial species (Martínez-De Drets and Arias 1972).

The non-oxidative pentose phosphate pathway is ubiquitous among organisms and is primarily used to interconvert hexose and pentose sugars. (Cervenansky and Arias 1984, Djordjevic 2004). Labeling experiments in *S. meliloti* have indicated the presence of enzymes associated with the non-oxidative pentose phosphate pathway, and suggested the importance of this pathway in cycling carbon to polysaccharides (Portais et al. 1999, Gosselin et al. 2001).The interconversion of carbon is necessary for the production of ribose-5-phosphate and erythrose-4-phosphate, precursors for providing the nucleic acids, and the synthesis of aromatic amino acids respectively (Stincone et al. 2015). Metabolism of a number of carbon sources have been shown to feed into central carbon metabolism through the non-oxidative pentose phosphate pathway,

including erythritol, ribose, and xylulose (Duncan 1981, Geddes and Oresnik 2012b, Barbier et al. 2014). While previous work suggested that erythritol metabolism might feed in through dihydroxyacetone-phosphate in *S. meliloti* (Sperry and Robertson 1975, Geddes et al. 2010), more recent work has determined that erythrose-4-phosphate is the point of introduction in *Brucella abortus* (Barbier et al. 2014).

The two major enzymes of the non-oxidative pentose phosphate pathway are transketolase and transaldolase. Transaldolase, catalyzes the reversible reaction of fructose-6-phosphate and erythrose-4-phosphate to sedoheptulose-7-phosphate and glyceraldhyde-3-phosphate (Samland and Sprenger 2009). Transketolase utilizes a ketose donor and aldose acceptor to carry out the reversible reactions; ribose-5-phosphate and xylulose-5-phosphate to sedoheptulose-7-phosphate and glyceraldhyde-3-phosphate, and xylulose-5-phosphate and erythrose-4-phosphate to fructose-6-phosphate and glyceraldhyde-3-phosphate (Schenk et al. 1998). Proteomic studies have provided evidence that is consistent with the operation of the pentose phosphate pathway when *S. meliloti* is either free living or in the bacteroid (Djordjevic 2004). In *Escherchia coli*, both *tktA* and *tktB* are known to encode for transketolases (Josephson and Fraenkel 1974, Iida et al. 1993). Strains lacking *tktA* exhibit auxotrophy for aromatic amino acids due to the disruption of the shikimate pathway, and are unable to grow on pentose sugars (Josephson and Fraenkel 1969, 1974). Although TktB has been shown to have transketolase activity, strains carrying a mutation in *tktB* do not exhibit any deleterious phenotypes (Iida et al. 1993, Zhao and Winkler 1994). This has led to the suggestion that TktB plays a minor role in the pentose phosphate pathway.

The pentose phosphate pathway remains poorly defined in *S. meliloti*. Previous work has described the isolation of a mutation in *tkt2*, encoding for a putative transketolase (Hawkins et al.

2017). The goal of this work is to determine the genes that encode the primary transketolase and transaldolase, and to characterize how mutations in these genes affect symbiosis and the overall physiology of *S. meliloti*.

5.3 Materials and Methods

5.3.1 Bacterial strains, plasmids, and media.

Bacterial strains and plasmids constructed and used in this work are listed in Table 6. *S. meliloti* was grown at 30ºC on either Luria-Bertani (LB), or yeast extract mannitol (YEM) as a complex medium (Sambrook et al. 1989). Vincent's minimal medium (VMM) was used as a defined medium (Vincent 1970). Carbon sources in defined medium were filter sterilized prior to use and added to and used at a final concentration. Amino acids were supplemented at the following concentrations as necessary: tryptophan, 25 µg/ mL; tyrosine, 20 µg/mL; and phenylalanine, 60 µg/mL. The following antibiotics were used as indicated for *S. meliloti*: streptomycin (Sm), 200 µg/mL; neomycin (Nm), 200 µg/mL; gentamicin (Gm), 20 µg/mL; and tetracycline (Tc), 5 µg/mL. The same concentrations of antibiotics were used for *E. coli*, except for Gm which was used at 60 µg/mL.

5.3.2 Genetic techniques, plasmid construction, and mutant generation.

Conjugations and transductions were carried out as previously described (7, 53). Standard techniques for plasmid isolation, ligation, transformation, restriction, and gel electrophoresis

Table 6. Strains and plasmids

Strain	Genotype or phenotype[a]	Reference
Strains		
S. meliloti		
Rm1021	SU47 *str-21*, Sm^r	(Meade et al. 1982)
SRmD338	Rm1021 Δ*dgok1*	This work
SRmD373	SRmD338 *tkt2*::Tn*5*, Sm^r, Nm^r	This work
SRmD397	Rm1021 *tkt2*::Tn*5*, Sm^r, Nm^r	This work
SRmD480	Rm1021 *tal*::pKnock-Gm, Sm^r, Gm^r	This work
SRmA723	Rm1021 *SMc01627*::Tn*5-233* Sm^r, Gm^r, Sp^r	(Geddes et al. 2010)
SRmD479	Φ (SRmD723) → SRmD397 Sm^r, Nm^r, Gm^r	This work
SRmD486	Rm1021 *rpiB*::pKnock-Gm, Sm^r, Gm^r	This work
SRmD488	Φ (SRmD486) → SRmD397 Sm^r, Nm^r, Gm^r	This work
RmFL2950	RmP110, *rhbB*::pTH1522 Sm^r	(diCenzo et al. 2014)
SmA818	Rm1021 ΔpSymB Sm^r	(Oresnik et al. 2000)
E. coli		
MM294A	*Pro-82 this-1 hsdR17 supE44*	(Finan et al. 1986)
MT607	MM294A; *recA56*	(Finan et al. 1986)
MT616	MT607(pRK600)	(Finan et al. 1986)
DH5α	λ^- φ80d*lacZ*°M15 °(*lacZYA-argF)U169 recA1 endA1 hsdR17*(r_k^- m_k^-) *supE44 thi-1 gyrA relA1*	(Hanahan 1983)
DH5αRif	Rifampicin resistant variant of DH5α	(House et al. 2004)
DH5αλpir	λpir lysogen of DH5α	(House et al. 2004)
Plasmids		
pJH117	400 bp internal fragment of *tal* cloned into pKnock-Gm, Gm^r	This work
pJH120	400 bp internal fragment of *rpiB* cloned into pKnock-Gm, Gm^r	This work
pJH109	pCO37/*tkt2* Tc^R	This work
pJH110	pCO37/*tkt1* Tc^R	This work
pJH113	CX1 cosmid complementing *tkt2*	This work
pJH116	pCO37/*cbbT* Tc^R	This work
pJH118	pCO37/*tal* Tc^R	This work
pRK600	pRK2013 *npt*::TN*9* Cm^r	(Finan et al. 1986)
pRK602	pRK600ΩTn*5*, Cm^r, Nm^r	(Finan et al. 1985)
pKnock-Gm	Suicide vector for insertional mutagenesis; R6K Ori, RK4 *oriT* Gm^r	(Alexeyev 1999)
pRK7813	Broad host range cloning vector, Tc^R	(Jones and Gutterson 1987)
pCO37	pRK7813 containing *attB* sites, Gateway-compatible destination vector	(Jacob et al. 2008)
pMK2014	FRT-*ccdB*-Cam^r-FRT cassette, Pen^R	(House et al. 2004)
pXINT129	λ*int* and *xis* driven by P_{lac}, Km^R	(Platt et al. 2000)

[a] Sm^R, Nm^R, Gm^R, Sp^R, Cm^R, Amp^R, Km^R= Resistance to streptomycin, neomycin, gentamicin, spectinomycin, chloramphenicol, ampicillin, and kanamycin respectively

were used (Sambrook et al. 1989). Tn*5* mutagenesis of Rm1021 was carried out using the self mobilizable plasmid pRK602 as previously described (Finan et al. 1985, Geddes et al. 2010). The location of the Tn*5* insertion was determined using arbitrary PCR and subsequent sequencing of the isolated PCR products (Miller-Williams et al. 2006).

To construct SRmD480, a single crossover mutation was created utilizing the plasmid pKnock-Gm (Alexeyev 1999). A 400 bp internal fragment from *tal* was PCR amplified using the primers pKtal_Fw ($^{5'}$ AGTCGGATCCGAGGTTACCAAGGAAATCTG $^{3'}$) and pKtal_Rv ($^{5'}$ AGTCCTCGAGCTTCCTTCACGTGGTTGAC $^{3'}$). The amplified fragment was gel isolated, then cloned into pKnock-Gm using BamHI and XhoI sites which were incorporated into the primers to create pJH117. The construct was subsequently transformed into *E. coli* strain DH5αλpir. Plasmid pJH117 was then conjugated into Rm1021 using the mobilizing strain MT616, and was selected for Gm^{r} on LB Gm plates. Isolated colonies were single colony purified three times on LB Gm before use. Knockout of the *tal* gene was confirmed by PCR amplification and sequencing using the primers ptalConf_Fw ($^{5'}$TCGTTGATACCGCCGATGTG $^{3'}$) and ptalConf_Rv ($^{5'}$TCAGGCGATCTTCTGGCCG$^{3'}$) and using SRmD480 genomic DNA as template.

Construction of SRmD486 was carried out in a similar manner. A 370 bp fragment from *rpiB* was amplified using primers pKrpiB_FW ($^{5'}$AGTCGGATCCCATCTCGCCAAGAGAAGCGA$^{3'}$) and pKrpiB_RV ($^{5'}$AGTCCTCGAGGCTTCGACATTTGCCGCC$^{3'}$), followed by cloning the fragment into pKnock-Gm using introduced BamHI and XhoI sites to create pJH120. This plasmid was then conjugated into Rm1021 and selected for using Gm. The single crossover mutation was

confirmed by PCR amplification and sequencing, using the primers prpiBC_FW ($^{5'}$AAAATCGCGATTGGAGC$^{3'}$) and prpiBC_RV ($^{5'}$TTACTTCGCCTGATCCAGGC$^{3'}$) and SRmD486 genomic DNA. SRmD488 was constructed by transduction of the *rpiB* mutant allele from SRmD486 into SRmD397 using phage ΦM12

Plasmids overexpressing *tkt2*, *tkt1*, *cbbT*, and *tal* by using the *S. meliloti* ORFeome as previously described (House et al. 2004, Schroeder et al. 2005, Jacob et al. 2008). Briefly, each of the ORFs were recombined into the destination vector pCO37 to create to create pJH109, pJH110, pJH116, and pJH118 respectively. A cosmid complementing *tkt2* (pJH113) was also isolated through conjugation of a *S. meliloti* cosmid bank (Wang et al. 2006) into strain SRmD397, followed by selection for growth on VMM using erythritol as a sole carbon source.

5.3.3 Sample preparation for proteomic and metabolomic analysis.

Strains used for both proteomic and metabolic analysis were grown overnight at 30˚C in 5 mL of LB broth. Each culture was then diluted to an OD_{600} of 0.4 using fresh LB. 5 mL of the culture was used to start 500 mL cultures of VMM glucose supplemented with tryptophan, tyrosine, phenylalanine. This 500 mL culture was then to an OD_{600} between 0.1 – 0.2, at which point they were induced with either the addition of 15 mM glucose or erythritol. The cultures were grown for 4 hours to an OD_{600} of approximately 0.4. 50 mL of culture was then pelleted by centrifugation at 12000g for 1 minute, washed with ddH_2O, and finally pelleted again by centrifugation at 12000g for 1 min for metabolite analysis. The same process was carried out with 500 mL of culture for proteome analysis. Cultures were decanted and bacterial cell pellets were weighed, and subsequently frozen in liquid nitrogen.

5.3.4 Metabolite extraction.

Bacterial cell pellets were washed with cold 150 mM ammonium formate solution at a pH of 7.4 and then extracted with 600 µL of 31.6% MeOH/36.3% acetonitrile in H_2O (v/v). Cells were lysed and homogenized by bead-beating for 2 minutes at 30Hz using ceramic beads (TissueLyser II – Qiagen). Cellular extracts were partitioned into aqueous and organic layers following dichloromethane (DCM) treatment and centrifugation. Aqueous supernatants were dried by vacuum centrifugation with sample temperature maintained at -4^{o}C (Labconco, Kansas City MO, USA). Pellets were subsequently resuspended in 30 µl of H_2O as the injection buffer.

5.3.5 LC/MS Method.

All LC/MS grade solvents and salts were purchased from Fisher (Ottawa, Ontario Canada): DCM, water (H_2O), acetonitrile (ACN), methanol (MeOH) and ammonium acetate. The authentic standards for metabolites of interest were purchased from Sigma-Aldrich Co. (Oakville, Ontario, Canada). For targeted metabolite analysis and relative concentration determination of metabolites, samples were injected onto an Agilent 6430 Triple Quadrupole (QQQ)-LC-MS/MS. The mass spectrometer was equipped with an electrospray ionization (ESI) source and samples were analyzed in positive or negative mode. Multiple reaction monitoring (MRM) transitions were optimized on standards for each metabolite quantitated. Gas temperature and flow were set at 350°C and 10 L/min respectively, nebulizer pressure was set at 40 psi and capillary voltage was set at 3500V. Relative concentrations were determined from external calibration curves. Data was analyzed using MassHunter Quant (Agilent Technologies).

For the measurement of lactate, succinate, fumarate and malate, chromatographic separation was performed on a Scherzo SM-C18 column 3 µm, 3.0×150mm (Imtakt Corp, Japan). The chromatographic gradient started after a 2 min hold at 100% mobile phase A (0.2%

formic acid in water) with a 6 min gradient to 80% phase B (0.2% formic acid in MeOH) at a flow rate of 0.4 mL/min. For the measurement of glucose-6-phosphate/-1-phosphate/fructose-6-phosphate, gluconolactone, gluconate, 6-phosphogluconate, glyceraldhyde-3-phosphate/DHAP, 2-phosphoglycerate/3-phosphoglycerate, phosphoenolpyruvate, fructose-1,6-bisphosphate, ribose-5-phosphate/ribulose-5-phosphate, erythrose-4-phosphate, sedoheptulose-7-phosphate, citrate, cis-Aconitate, isocitrate and α-ketoglutarate, the chromatographic gradient started after a 2 min hold at 100% mobile phase A (100 mM formic acid in water) with a 6 min gradient to 80% phase B (200 mM ammonium formate in 30% ACN, pH 8) at a flow rate of 0.4 mL/min.

All gradients were followed by a 5 min hold time at 100% mobile phase B and a subsequent re-equilibration time (6 min) before the next injection. For all LC/MS analyses, 5 μL of sample were injected. The column temperature was maintained at 10°C. Data were quantified by integrating the area under the curve of each compound using MassHunter Quant (Agilent Technologies, Santa Clara, CA, USA) and comparing to the external calibration curve.

5.3.6 Data handling and statistical analysis.

Metabolite concentrations were normalized to the weight of each cell pellet provided for metabolite extraction. Concentrations are presented as an average of 3 independent biological replicates, which were measured twice. Principle component analysis and cluster analysis were performed using MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/) as previously described (Xia and Wishart 2016). When a metabolite was not detected, a minimal value was assigned based on the minimal detection range for that metabolite. Statistical significance was determined using MetaboAnalyst 3.0 by utilizing Welch's two-sample *t* test, and by determining the false-discovery rate (FDR) between samples. Metabolites were considered to be significantly different if the criteria of $P < 0.05$ and a $FDR < 0.1$ were met.

5.3.7 Protein isolation, digestion, and peptide purification.

Protein was extracted from frozen cell pellets as previously described with some modifications (Poysti et al. 2007). Cell pellets were thawed and resuspended in an extraction buffer containing 100 mM HEPES, 100 mM DTT, and 1 mM $MgCl_2$. Cells were then lysed by two passages through a French pressure cell at 16,000 psi. The extract was then pelleted at 10,000 g for 10 minutes, and the supernatant was decanted into a fresh tube. The cell debris pellet was resuspended in 1 mL of 4% SDS and 100 mM DTT and subsequently boiled at 90°C for 20 minutes to extract any remaining proteins. The extract was then pelleted at 12,000g for 10 minutes to remove cell debris, and the supernatant was then pooled with the initial extract to create a pooled protein extract.

One mL of protein was transferred to an Amicon Ultra-15 10K filter device (Millipore, Billerica, MA), which followed the purification, digestion, and subsequent peptide purification steps as previously described with modification of the trypsin to protein ratio to 1:100 (Gungormusler-Yilmaz et al. 2014). The purified peptides were lyophilized and re-dissolved in 0.1% formic acid in water for subsequent one dimensional liquid chromatography followed by mass spectrometry (1D-LC-MS) analysis.

5.3.8 Protein identification and quantification.

1D-LC-MS was used to identify proteins and quantify extracts by the Manitoba Centre for Proteomics and Systems Biology as previously described (Gungormusler-Yilmaz et al. 2014). A database derived from the *S. meliloti* Rm1021 genome (https://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi) was used for the alignment of detected peptides. Total ion count (TIC), which is the sum of all collision induced dissociation (CID) fragment intensities of member peptides, was recorded.

5.3.9 Data analysis and validation using Unity.

Comparative analyses of the gathered expression data was conducted using the in-house system "UNITY" (Gungormusler-Yilmaz et al. 2014). Data was compared to Rm1021 to determine the effect of the mutation on protein expression, or compared to glucose grown conditions to examine the effect of erythritol induction. Differential expression values were calculated for: cross-state: (Znet) (Exponential phase mutant strain – Exponential phase wildtype culture, or Exponential phase erythritol induced culture – Exponential phase glucose induced culture); and among biological replicates (Rnet) for intra biological replicate differences. A quality control of biological variation between any two comparison groups (cross-state) versus system noise among biological replications (intra-replicative viability) was conducted. Transformation of different populations into final log(2) expression values (Pnet) for differential expression analysis in cross-state samples was conducted as previously described (Verbeke et al. 2014). Cut-off scores of ± 1.0 were used to represent proteins observed to be up or downregulated by at least 2 fold to the comparison condition. Mutant or condition specific protein expression was then filtered by searching the Pnet values ± 1.0 using COG categories for annotation (Tatusov et al. 2000).

The signal to noise (S/N) ratio for each quantified protein was determined as previously described by comparing the means of cross state and intra-replicate samples. (Sharma et al. 2016). On an individual protein level, the S/N is the ratio of the protein expression across the experimental states and intra-replicate normalized values, scaled by the overall system S/N values. A Monte-Carlo Model was used to derive functions relating 'false discovery rates' (FDR) to a defined S/N cut-off. All proteins with a S/N > 2.8 were found to have a FDR of 10% or less.

This threshold was used as the cut-off level for reporting significant changes in protein expression. Overall proteomic results are presented in Datasets S1 and S2.

5.3.10 Rhizobactin competition assay.

Rhizobactin assays were performed as previously described using SmA818 as an indicator strain with minor modification (diCenzo et al. 2014). SmA818 was grown in LB broth overnight at 30°C and diluted to an OD_{600} of 0.1. 3 mL was added to 300 mL VMM (0.6% w/v agar) at approximately 50°C, and was supplemented with aromatic amino acids. Either glucose or erythritol were added as carbon sources. Plates were then poured and allowed to set. Strains being tested for rhizobactin production were grown to an OD_{600} of 0.9. 1 mL of this culture was pelleted, supernatant was decanted, and the remaining pellet was resuspended in 50 µL 0.85% saline. 10 µL was subsequently spotted onto the seeded plates and allowed to dry. Plates were then incubated for 4 days at 30˚C before recording zones of clearance. Diameters of zone of inhibition were recorded and reported numbers are an average of three independent biological replicates.

5.3.11 EPS quantification.

Quantification of EPS was carried out using the anthrone assay as previously described with the following modifications (Morris 1948, Mendrygal and González 2000). Strains were grown in 5 mL of YEM overnight at 30ºC. Cultures were then sub-cultured to an OD_{600} of 0.1 in YEM and then grown for another 3 days at 30ºC. The OD_{600} of the culture was measured and used for normalization of results. Cell cultures were then pelleted at 12,000 g for 10 minutes, and the supernatant was decanted and used for quantification of EPS.

To measure total anthrone reactive material produce, 1 mL of the culture supernatant was assayed directly using the anthrone assay (Morris 1948). EPS-I was also precipitated from the supernatant by the addition of 300 µL of 1% cetrimide to 1 mL of supernatant. The mixture was centrifuged at 12,000 g for 10 minutes to pellet EPS, and remaining supernatant was removed. Pelleted EPS was resuspended in 10% NaCl. One mL of the resuspended EPS was then directly assayed by the anthrone assay. All reported numbers are an average of three independent biological replicates. Significance was determined using Student's T-test.

5.3.12 Plant dry weights and nodule kinetics.

Plant assays were carried out as previously described (Poysti and Oresnik 2007). Alfalfa seeds were germinated for 2 days on water agar plates. Then, seedlings were transferred to Leonard jars containing a 1:1 mixture of sand and vermiculite which had been soaked with nitrogen free Jensen's medium and autoclaved (Glazebrook and Walker 1991). After 2 days of growth, seedlings were inoculated with approximately 10^7 bacteria that were resuspended in 10 mL of sterile water. Plants were harvested after 28 days, and dry weights of the plants were determined. Numbers are an average dry weight of 10 plants per pot. All results are an average of 3 independent biological replicates.

Nodule formation was observed as previously described (Geddes et al. 2014). Seedlings were germinated as described above. After 2 days, seedlings were transplanted to 10 mL Jensen's agar slants and inoculated with approximately 10^5 bacteria in a volume of 100 µL. Formation of nodules was counted over a period of 14 days. Overall, 100 plants over 5 biological replicates were used for determining rate of nodulation for each strain.

5.4 Results

5.4.1 Identification of putative transketolase and transaldolase genes.

While metabolism in *S. meliloti* has been the focus of numerous biochemical and genetic studies, the pentose phosphate pathway remains poorly characterized (Geddes and Oresnik 2014). Transketolase and transaldolase represent critical proteins in the non-oxidative pentose phosphate pathway. Since a mutation in a putative transketolase (*tkt2*) was previously isolated (Hawkins et al. 2017), it was reasoned that creating a mutation in the transaldolase gene would allow us to characterize the non-oxidative pentose phosphate pathway.

BLASTP analysis showed Tkt2 from *S. meliloti* has 51% identity to TktA from *E. coli* K-12, which was shown to be utilized in the pentose phosphate pathway (Josephson and Fraenkel 1969). In addition to Tkt2, two other putative transketolase proteins were also identified in the *S. meliloti* genome. These were Tkt1 and CbbT, which showed 60% and 50% identity to Tkt2 respectively. An examination of the loci encoding these proteins revealed that *tkt2* was 70 bp from an operon encoding two genes involved in the Embden-Meyerhof-Parnas pathway (*gap* and *pgk*), and *SMc03980,* a conserved hypothetical protein (Figure 17a). The gene *tkt1* appears to be localized within a group of uncharacterized genes, and has the potential to form an operon and encode proteins involved in the metabolism of a small compound (Figure 17b). The third putative transketolase in the genome is encoded by *cbbT,* which is found in the *cbb* operon localized on pSymB, and has been shown to be involved in the Calvin-Benson-Bassham (CBB) cycle (Finan et al. 2001, Pickering and Oresnik 2008) (Figure 17c). Due to the genomic context of *cbbT* and *tkt1*, as well as the preliminary characterization of SRmD397, mutations in *cbbT* and *tkt1* were not constructed.

Examination of the *S. meliloti* genome revealed one putative transaldolase which was encoded for by *tal* (Figure 17d). A HAMAP-Scan of the amino acid sequence of Tal identified a single trusted match to family MF_00494, suggesting that it was the transaldolase involved in the pentose phosphate pathway. To carry out our characterization of the pentose phosphate pathway, a strain carrying a mutation in this gene was constructed, yielding SRmD480.

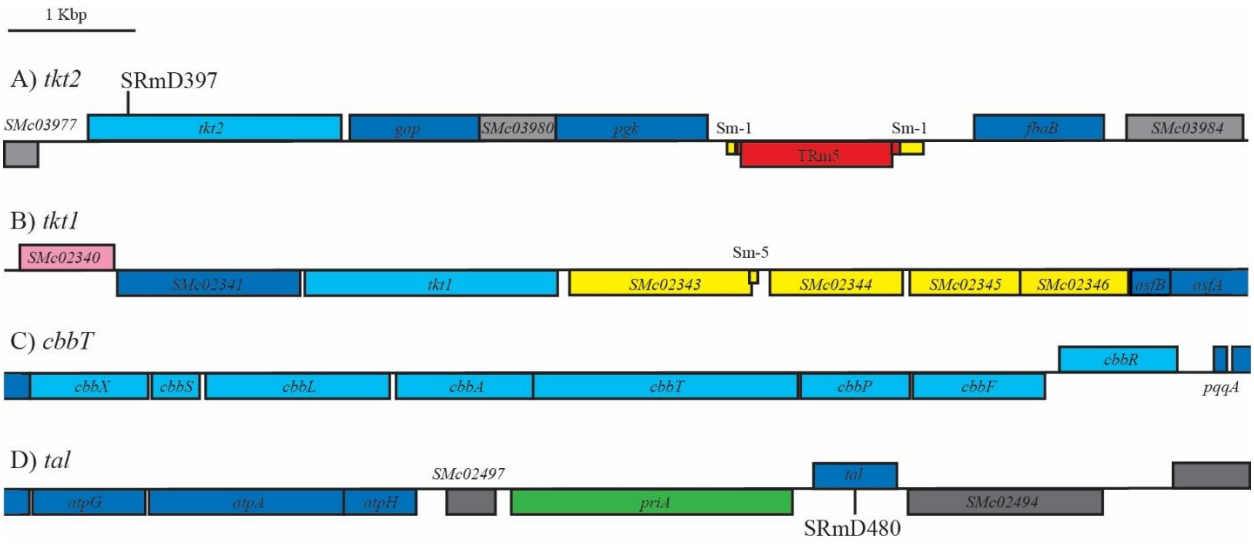
5.4.2 Mutations in tkt2 result in an inability to grown on defined medium.

To determine how carbon metabolism was affected by a mutation in either *tkt2* or *tal*, each strain was assessed for its capability to metabolize some key carbon sources. In *E. coli*, strains carrying a mutation in *tktA* have been shown to be auxotrophic due to an inability to synthesize erythrose-4-phosphate, which is necessary for the biosynthesis of aromatic amino acids, and are unable to grow on pentose sugars (Josephson and Fraenkel 1974).

The *tkt2* mutant (SRmD397) was unable to grown in defined medium (Table 7)When the medium was supplemented with tryptophan, tyrosine, and phenylalanine, full growth was restored when either glucose, glycerol, or succinate were used as sole carbon sources. However, SRmD397 was unable to utilize either ribose or erythritol regardless of supplementation with aromatic amino acids (Table 7).

When a plasmid overexpressing *tkt2* (pJH109) was introduced *in trans* into SRmD397, growth on all carbon sources was restored (Table 7). Although the introduction of pJH109 restored growth, wild-type growth was not observed on medium containing either erythritol or ribose. To determine if the other putative transketolase genes could complement the *tkt2*

Figure 17. Loci of putative transketolase and transaldolase genes



Map of genetic region surrounding *tkt2*, *tkt1*, *cbbT*, and *tal*. Boxes indicate open reading frames. Vertical lines indicate the approximate site of each insertion mutation in *tkt2* or *tal*. Gene names and colour of boxes are based on curated annotation found at https://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi. Colour denotes the putative class of each encoded ORF. Dark grey; hypothetical, Turquoise; central intermediary metabolism, Blue; small molecule metabolism, Yellow; cell processes, Green; macromolecule metabolism.

Table 7. Carbon metabolism phenotypes

Strain	Genotype	Glc	Glc^{FYW}	Gly	Gly^{FYW}	Ery	Ery^{FYW}	Suc	Suc^{FYW}	Rib	Rib^{FYW}
Rm1021	WT	++	++	++	++	++	++	++	++	++	++
SRmD480	*tal*::pK-Gm	++	++	++	++	+/-	+/-	++	++	+	+
SRmD480 (pJH120)	*tal*::pK-Gm (*tal*$^{+}$)	++	++	++	++	++	++	++	++	++	++
SRmD397	*tkt2*::Tn*5*	-	++	-	++	-	-	-	+	-	-
SRmD397 (pJH109)	*tkt2*::Tn*5* (*tkt2*$^{+}$)	++	++	++	++	+	+	++	++	+	+
SRmD397 (pJH110)	*tkt2*::Tn*5* (*tkt1*$^{+}$)	++	++	++	++	-	-	+	+	-	-
SRmD397 (pJH116)	*tkt2*::Tn*5* (*cbbT*$^{+}$)	-	++	-	++	-	-	-	++	-	-
SRmD397 (pJH113)	*tkt2*::Tn*5* (*tkt2* cosmid)	++	++	++	++	++	++	++	++	++	++

Glc; Glucose, Gly; Glycerol, Ery; Erythritol, Suc; Succinate, Rib; Ribose, ^{FYW}; Supplemented with phenylalanine, tyrosine, and tryptophan. Growth phenotypes: - , No growth; +/-, Weak growth (Head-streak only); +, Moderate growth (Some growth after Head-streak) ; ++, Wild-type growth.

mutation, plasmids over-expressing either *tkt1*(pJH110) or *cbbT* (pJH116) were introduced into SRmD397. While pJH116 was unable to complement growth of SRmD397, pJH110 restored growth using glucose, glycerol, and succinate. The introduction of pJH113, a cosmid that contained *tkt2*, into SRmD397 restored growth on all carbon sources tested.

SRmD480 was unaffected when grown on defined medium with glucose, glycerol, or succinate as sole carbon sources (Table 7). However, SRmD480 grew poorly relative to the wild-type when using ribose or erythritol. These growth defects were fully complemented with the introduction of pJH120 which constitutively over-expressed the *tal* gene.

5.4.3 Erythritol metabolism is dependent on Smc01613.

The ability to metabolize erythritol is a relatively rare phenotype (Geddes and Oresnik 2014). Erythritol metabolism has been best characterized in *B. abortus*, *R. leguminosarum*, as well as *S. meliloti* (Yost et al. 2006, Geddes and Oresnik 2012b, Barbier et al. 2014). Although long thought to be converted to dihydroxyacetone-phosphate, a recent reassessment of the erythritol pathway in *Brucella* elegantly demonstrated that erythritol is metabolized to erythrose-4-phosphate, and feeds directly into the pentose phosphate pathway (Barbier et al. 2014). While previous work in *S. meliloti* showed the involvement of TpiB in erythritol metabolism (Poysti and Oresnik 2007), the need for Smc01613, which is tentatively annotated as *rpiB*, had not been demonstrated. To address this, a mutation in *SMc01613* was constructed yielding SRmD486. When tested, SRmD486 was able to grow using glucose as a sole carbon source, but was unable to utilize erythritol. This strongly suggested conservation of the metabolic pathway between *B. abortus* and *S. meliloti* (Table 8).

For consistency, we propose that the genes annotated as *tpiB* and *rpiB* in *S. meliloti* be renamed as *eryH* and *eryI* respectively to maintain consistency with what has been found in *B. abortus*.

Table 8. Growth using glucose and erythritol as dual carbon sources

Strain	Genotype	Glc	Glc^{FYW}	Ery	Ery^{FYW}	Glc/Ery	Glc/Ery^{FYW}
Rm1021	WT	++	++	++	++	++	++
SRmD480	*tal*	++	++	+	+	++	++
SRmD397	*tkt2*	-	++	-	-	-	-
SRmA723	*SMc01627*	++	++	-	-	++	++
SRmD479	*SMc01627/tkt2*	++	++	-	-	-	++
SRmD486	*rpiB*	++	++	-	-	-	-
SRmD488	*rpiB/tkt2*	-	++	-	-	-	-

Glc; Glucose, Gly; Glycerol, Ery; Erythritol, Suc; Succinate, Rib; Ribose, ^{FYW}; Supplemented with phenylalanine, tyrosine, and tryptophan. Growth phenotypes: - , No growth; +/-, Weak growth; +, Moderate growth; ++, Wild-type growth

5.4.4 Supplementation of growth medium with erythritol does not alleviate auxotrophy.

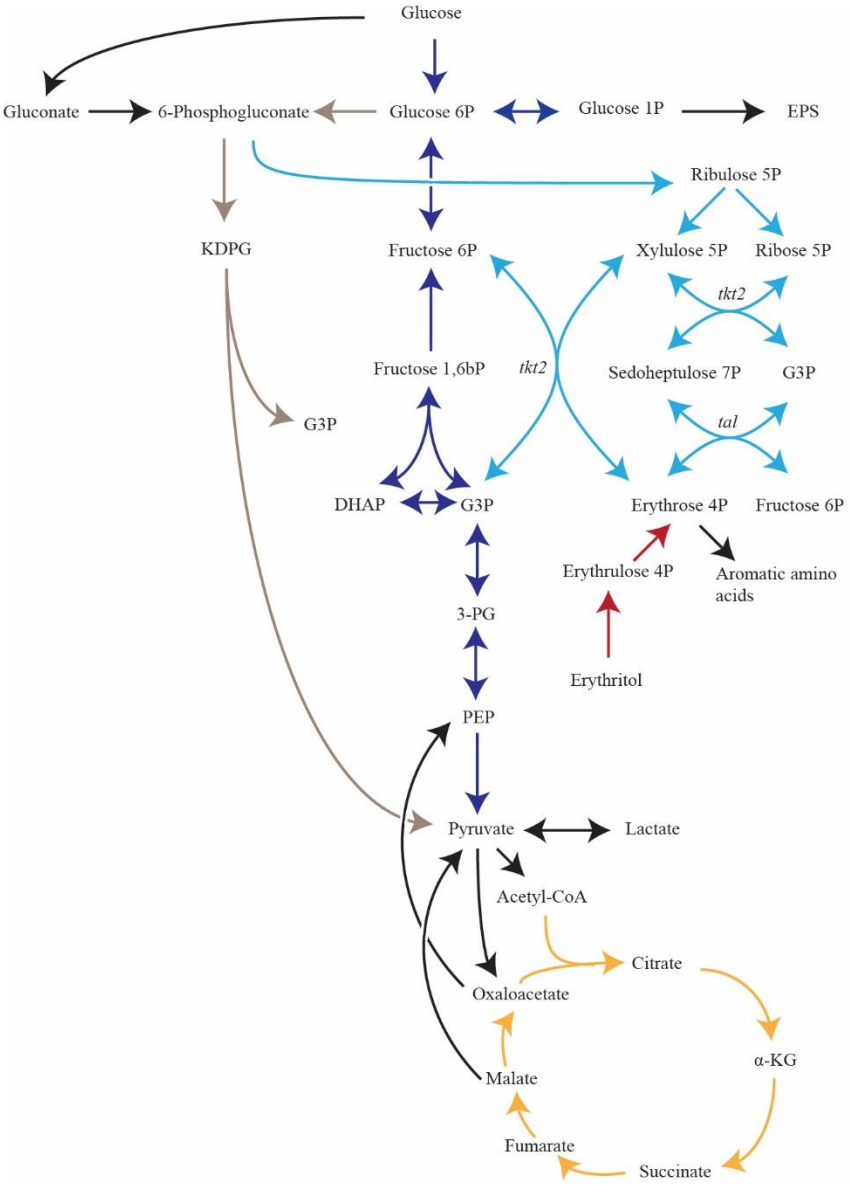
S. meliloti can metabolize erythritol, and thus should be able to generate erythrose-4-phosphate without using the pentose phosphate pathway. In an attempt to bypass the observed auxotrophy, SRmD397 was grown on defined medium containing both glucose and erythritol as carbon sources. The results showed that SRmD397 was unable to grow on this combination of carbon sources, even when supplemented with aromatic amino acids (Table 8).

To determine if the inability to grow was dependent on the presence of erythritol, a mutant allele of *mptB* (encoding part of the transporter necessary for erythritol uptake) was transduced into SRmD397. The results showed that mutation of the erythritol transporter alleviated the inability of a *tkt2* mutant to grow in the presence of erythritol (Table 8). Taken together these data suggest that the inability to grow could be the result of an accumulation of a phosphorylated intermediate(s) derived from the metabolism of erythritol in SRmD397.

5.4.5 Pentose phosphate pathway associated metabolites are altered in tkt2 and tal mutants.

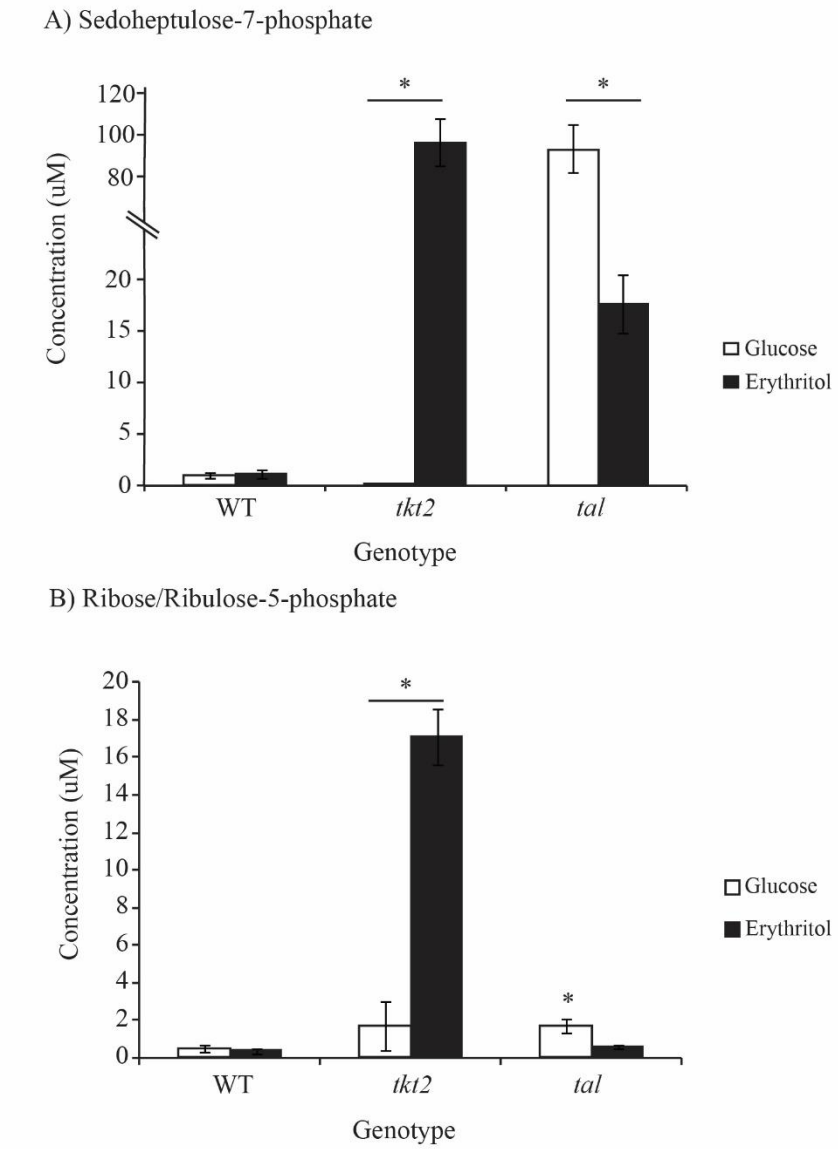
The ability to directly measure enzyme activity is often used to confirm the function that is encoded by genes. Measurement of transketolase and transaldolase activities from crude extracts is dependent on coupled enzyme reactions that rely on substrates whose supply is often inconsistent. Pentose phosphates, erythrose-4-phosphate, and sedoheptulose-7-phosphate, are metabolites that are chiefly found as part of the pentose phosphate pathway. An examination of central carbon metabolism in *S. meliloti* suggests that mutation of either the transketolase or transaldolase should have an impact on the ability of carbon to flow through the pentose phosphate pathway, and thus likely to affect metabolite pool sizes (Figure 18). To provide evidence that *tkt2* and *tal* function in the pentose phosphate pathway, the concentration of

Figure 18 Central carbon metabolism in S. meliloti



Simplified map of central carbon metabolism in *S. meliloti*. Arrow heads indicate predominate direction of each reaction, and are coloured to indicate inclusion in a specific metabolic pathway. Dark blue, EMP; Grey, ED; light blue, PP; yellow, TCA; red, erythritol metabolism. Abbreviations: P, phosphate; G3P, glyceraldehyde-3-phosphate; KDPG, 2-keto-3-deoxy-phosphogluconate; PEP, phosphoenolpyruvate; α-KG; α-ketoglutarate.

Figure 19 Intracellular concentrations of pentose phosphate pathway intermediates



Concentration of sedoheptulose-7-phosphate and ribose/ribulose-5-phosphate in *S. meliloti*. White and black bars indicate induction with glucose and erythritol respectively. Concentrations are normalized to the cell pellet weight, and are an average of 3 biological replicates. Metabolite concentrations are compared to the wild-type for each condition. *; significant difference ($P < 0.05$) based on Student's T-test.

pentose phosphate pathway metabolites in each mutant strain was examined and compared to Rm1021 concentrations (Figure 19).

Consistent with the absence of transketolase activity, when SRmD397 was grown on glucose a 5 fold increase in the pentose phosphate pool, and a 20 fold reduction in the sedoheptulose-7-phosphate concentration were observed when compared to the wild-type (Figure 19a). When supplemented with erythritol, the concentration of the pentose phosphate pool and sedoheptulose-7-phosphate increased 50 and 100 fold respectively when compared to Rm1021 grown using the same conditions (Figure 19b). Analysis of metabolites in SRmD480 also showed elevated pentose phosphate pools, but unlike the *tkt2* mutant strain the concentration of sedoheptulose-7-phosphate was increased 100 times greater than the wild-type when grown it was grown on glucose (Figure 19a). Supplementation with erythritol resulted in a decrease in both these pools. These results support the hypothesis that *tkt2* and *tal* encode the transketolase and transaldolase enzymes necessary for the pentose phosphate pathway, and that erythritol feeds directly into the pentose phosphate pathway. It is noteworthy that although erythrose-4-phosphate was a detectable metabolite, we did not find this metabolite in any of the conditions that were tested (Table 9).

5.4.6 Mutations in tkt2 lead to changes in metabolite pools.

To assess changes in central carbon metabolism, concentrations of the metabolites involved in Entner-Doudoroff, Embden-Meyerhof-Parnas, pentose phosphate, and TCA pathways were examined. Overall, 17 different metabolites were quantified to determine how each mutation and condition affected intracellular metabolite concentrations (Table 9).Principle component analysis (PCA) showed that each strain is distinctly clustered from each other in two-dimensional space with respect to components PC1 and PC2 when induced with either glucose or

Table 9 Metabolite analysis of *tkt2* and *tal* mutant strains

Metabolites (nM/mg)	Rm1021	Rm1021 Ery	SRmD397	SRmD397 Ery	SRmD480	SRmD480 Ery
ED/EMP						
G6P/G1P/F6P	4142	3212	994*	3146	1627*	2694
Gluconate	660	548	1384*	2497	454*	405
6PG	ND	ND	566**	501	199*	ND
G3P/DHAP	199	373	491*	745	161	331
2PG/3PG	259	734	89	151	937*	603
PEP	797	4349	348	889	1962*	3902
Lactate	2495	4216	8029*	4693	2043	4068
F1,6BP	ND	ND	ND	ND	ND	ND
PPP						
R5P/Ribu5P	474	368	2273*	17071	1659*	606
E4P	ND	ND	ND	ND	ND	ND
S7P	1013	1173	50*	112443	108632*	17757
TCA						
Citrate	2293	2365	1223*	1005	1876	2352
Cis-Aconitic acid	213	251	72*	38	166	245
αKG	171	366	1132*	710	278	506
Succinate	1038	783	1065	816	1163	552
Fumarate	1149	747	1029	1128	895	627
Malate	876	618	678	896	563*	549

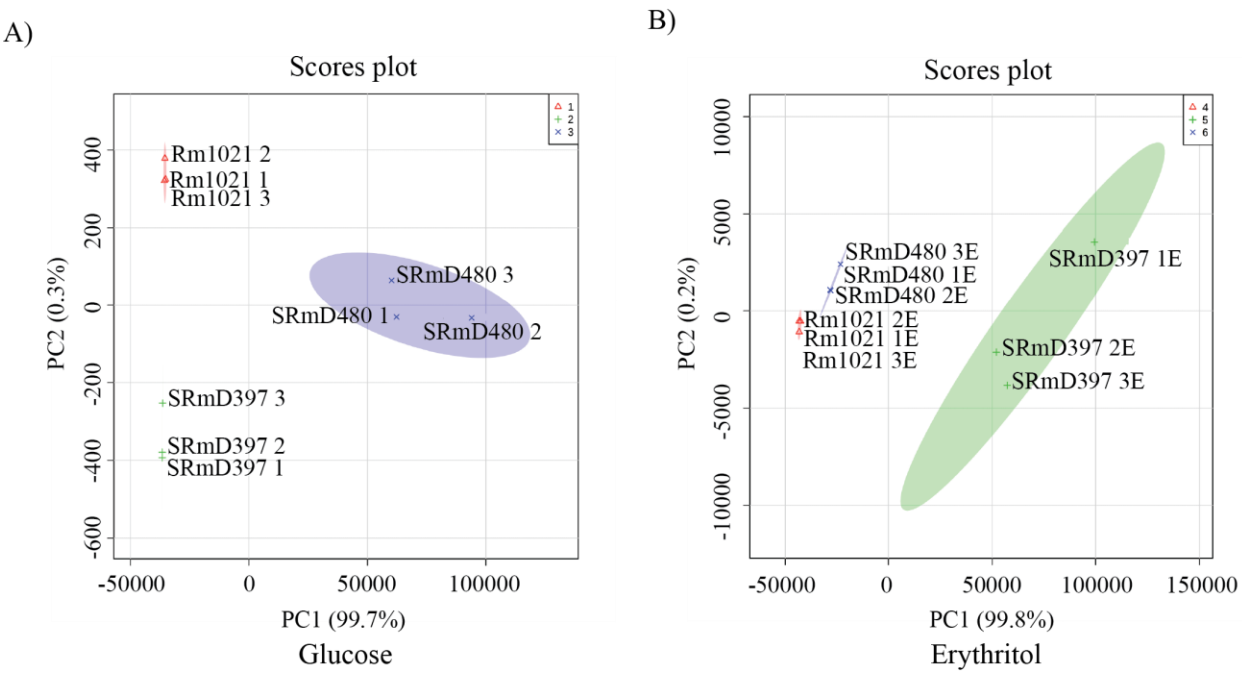
Concentration of metabolites involved in Entner-Doudoroff, Embden–Meyerhof–Parnas, pentose phosphate pathway and the tricarboxylic acid cycle for each strain when grown on glucose or induced with erythritol. Coloured backgrounds are used to highlight either increases (green) or decreases (red) in metabolites when each erythritol induced strain is compared to its glucose grown value. Metabolite abbreviations are as defined in text.

*, denotes significant differences in metabolite concentrations of glucose grown cells when compared to the wild-type. Significance ($P < 0.05$) was determined by Student's T-test. In all cases n = 3.

[a] Metabolite detected, but values between biological replicates variable.

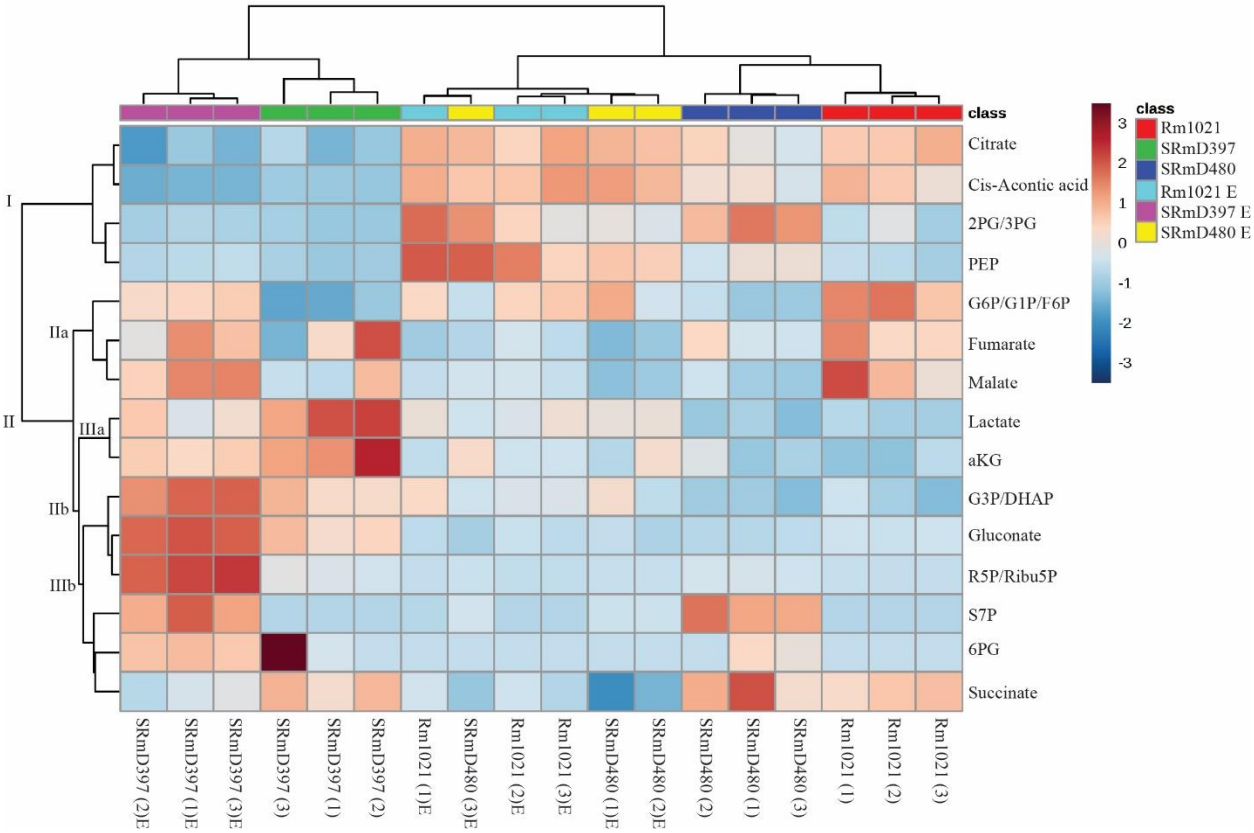
[b] ND; Not detected.

Figure 20 PCA analysis



PCA showing metabolite distribution within PC1 and PC2 for cultures induced with **A)** glucose and **B)** erythritol. The coloured areas surrounding the data points represent 95% confidence limits.

Figure 21. Clustered heat map of metabolites in central carbon metabolism



Cluster display of metabolite data from each strain grown with glucose or induced with erythritol (E). Metabolites are represented by each row, while each replicate is represented by a column. The dendrogram and coloured boxes were generated by the MetaboAnalyst 3.0. The colour scale represents fold change with respect to the average value of each metabolite. Colours indicate increased and decreased fold concentrations as indicated on the legend.

erythritol (Figure 20). However, PCA graphing of Rm1021 and SRmD480 in response to erythritol suggested a similar response of each strain to erythritol induction.

Hierarchical clustering grouped metabolites based on concentrations in each condition (Figure 21). Analysis showed separation of SRmD397 from the other two strains under each condition based on cluster I and II metabolites. Cluster I metabolites, consisting of those in the lower Entner-Doudoroff and upper TCA pathways, were positively correlated in Rm1021 and SRmD480, but were negatively correlated with SRmD397. Cluster II contained the remaining metabolites, and were generally increased in SRmD397 while being decreased in the other two strains. The exception to this was cluster IIa metabolites (containing the hexose phosphates, malate, and fumarate) which were found to be increased in Rm1021 and decreased in SRmD397 when grown with glucose. The response of SRmD397 to erythritol was separated by metabolites in clusters IIIa and IIIb. Metabolites in IIIb were mostly involved in the pentose phosphate pathway and were increased in SRmD397 in response to erythritol, whereas lactate and α-ketoglutarate in IIIa were increased when grown with glucose. Consistent with PCA, no major changes were observed between Rm1021 and SRmD480 when induced with erythritol. Overall, these results showed that a mutation in *tkt2* had a greater affect on metabolites associated with central carbon metabolism than a mutation in *tal*.

5.4.7 Proteome analysis of tkt and tal mutant strains.

Changes in metabolite concentrations can be associated with altered transcription or translation of proteins. To determine if changes in protein expression correlated with metabolite concentrations, the proteome of each strain was determined when they were grown on glucose, and when they were induced with erythritol. Overall, 2469 proteins were detected using 1D-LC-

Table 10 Change in protein concentration due to erythritol induction

Enzyme	Gene	Locus tag	Rm1021 P_{net}	SRmD397 P_{net}	SRmD480 P_{net}
ED					
Glucose 6-phosphate dehydrogenase	*zwf*	SMc03070	NC	NC	NC
6-Phosphogluconolactonase	*pgl*	SMc03069	1.19	NC	1.59
6-Phosphogluconate dehydratase	*edd*	SMc03068	+	+	+
2-Keto-3-Deoxy-6-phosphogluconate aldolase	*eda2*	SMc03153	NC	1.08	NC
EMP					
Glucose 6-phosphate isomerase	*pgi*	SMc02163	NC	NC	NC
Fructose bisphosphatase	*cbbI*	SMc20202	+	+	+
Fructose 6-bisphosphate aldolase	*cbbA*	SMc20199	+	+	+
	cbbA2	SMb21192	NC	NC	NC
	fbaB	SMc03983	NC	NC	NC
Glyceraldehyde 3-phosphate dehydrogenase	*gap*	SMc03979	NC	NC	NC
Phosphoglycerate kinase	*pgk*	SMc03981	NC	-	NC
Phosphoglycerate mutase	*gpmA*	SMc02838	NC	NC	NC
	gpmB	SMc00006	1.91	NC	NC
Enolase	*eno*	SMc01028	NC	NC	NC
Pyruvate kinase	*pykA*	SMc04005	NC	NC	NC
PP					
6-Phosphogluconate dehydrogenase	*edd*	SMc03068	+	+	+
Ribose 5-phosphate isomerase	*rpiA*	SMc00152	-	-	+
Ribulose 5-phosphate epimerase	*rpe*	SMc00511	-	-	-
	ppe	SMb20195	-	-	-
Transketolase	*tkt2*	SMc03978	NC	+	NC
	tkt1	SMc02342	-	-	-
	cbbT	SMb20200	-	-	-
Transaldolase	*tal*	SMc02495	NC	NC	+
Ery					
Erythritol kinase	*eryA*	SMc01623	3.12	1.68	+
Erythritol phosphate dehydrogenase	*eryB*	SMc01620	6.86	6.02	7.74
D-Erythrulose 1-phosphate dehydrogenase	*eryC*	SMc01616	+	+	+
Triose phosphate isomerase	*tpiB*	SMc01614	+	6.62	2.85
Ribose 5-phosphate isomerise B	*rpiB*	SMc01613	5.33	5.95	5.38
TCA					
Pyruvate dehydrogenase	*pdhA*	SMc01030	NC	NC	NC
	pdhB	SMc01031	NC	NC	NC
	pdhC	SMc01032	NC	NC	NC
	lpdA1	SMc01035	NC	NC	NC
Citrate synthase	*gltA*	SMc02087	NC	NC	NC
Aconitase	*acnA*	SMc03896	-	-	-
Isocitrate dehydrogenase	*icd*	SMc00480	NC	NC	NC
a-ketoglutarate dehydrogenase	*sucA*	SMc02482	NC	NC	NC
	sucB	SMc02483	NC	NC	NC
	lpdA2	SMc02487	NC	NC	NC
Succinyl-CoA synthetase	*sucC*	SMc02480	NC	NC	NC
	sucD	SMc02481	NC	NC	NC
Succinate dehydrogenase	*sdhA*	SMc02466	-1.4	NC	NC
	sdhB	SMc02465	NC	NC	NC
	sdhC	SMc02464	-	-	-
	sdhD	SMc02463	-	-	-
Fumerase	*fum*	SMc00149	NC	NC	NC
Malate dehydrogenase	*mdh*	SMc02479	NC	NC	NC
Malic enzyme	*dme*	SMc00169	NC	NC	NC
	tme	SMc01126	NC	NC	NC
Phosphoenolpyruvate carboxykinase	*pckA*	SMc02562	NC	+	+
Pyruvate orthophosphate dikinase	*ppdK*	SMc00025	NC	NC	NC

Pyruvate carboxylase	*pyc*	SMc03895	NC	NC	NC
Isocitrate lyase	*aceA*	SMc00768	-	-	-
Malate synthase	*glcB*	SMc02581	NC	NC	NC

Log(2) change in expression (Pnet) of each indicated protein when induced with erythritol, compared to samples grown in glucose. NC, No change (Pnet between -1 and 1); - , Not detected; +, Detected not quantifiable because of inconsistency in biological replicates.

Table 11. Change in protein expression due to mutation in tkt2 or tal

Enzyme	Gene	Locus tag	397 Glc P_{net}	397 Ery P_{net}	480 Glc P_{net}	480 Ery P_{net}
ED						
Glucose 6-phosphate dehydrogenase	*zwf*	SMc03070	NC	NC	NC	NC
6-Phosphogluconolactonase	*pgl*	SMc03069	NC	NC	NC	NC
6-Phosphogluconate dehydratase	*edd*	SMc03068	+	+	+	+
2-Keto-3-Deoxy-6-phosphogluconate aldolase	*eda2*	SMc03153	NC	NC	NC	NC
EMP						
Glucose 6-phosphate isomerase	*pgi*	SMc02163	NC	NC	NC	NC
Fructose bisphosphatase	*cbbI*	SMc20202	+	+	+	+
Fructose 6-bisphosphate aldolase	*cbbA*	SMc20199	+	+	+	+
	cbbA2	SMb21192	NC	NC	NC	NC
	fbaB	SMc03983	NC	0.98	1.21	-1.73
Glyceraldehyde 3-phosphate dehydrogenase	*gap*	SMc03979	-6.03	-4.85	NC	NC
Phosphoglycerate kinase	*pgk*	SMc03981	-4.44	-2.56	NC	1.27
Phosphoglycerate mutase	*gpmA*	SMc02838	NC	NC	NC	NC
	gpmB	SMc00006	2.1	NC	1.72	NC
Enolase	*eno*	SMc01028	NC	NC	NC	NC
Pyruvate kinase	*pykA*	SMc04005	NC	NC	NC	NC
PP						
6-Phosphogluconate dehydrogenase	*edd*	SMc03068	NC	NC	NC	NC
Ribose 5-phosphate isomerase	*rpiA*	SMc00152	-	-	+	-
Ribulose 5-phosphate epimerase	*rpe*	SMc00511	-	-	-	-
	ppe	SMb20195	-	-	-	-
Transketolase	*tkt2*	SMc03978	-8.82	+	NC	NC
	tkt1	SMc02342	-	-	-	-
	cbbT	SMb20200	-	-	-	-
Transaldolase	*tal*	SMc02495	NC	NC	+	-6.07
Ery						
Erythritol kinase	*eryA*	SMc01623	-	-1.66	-	-1.84
Erythritol phosphate dehydrogenase	*eryB*	SMc01620	NC	NC	NC	NC
D-Erythrulose 1-phosphate dehydrogenase	*eryC*	SMc01616	+	+	+	+
Triose phosphate isomerase	*tpiB*	SMc01614	+	NC	+	-2.28
Ribose 5-phosphate isomerise B	*rpiB*	SMc01613	2.74	NC	-1.47	-1.1
TCA						
Pyruvate dehydrogenase	*pdhA*	SMc01030	NC	NC	NC	NC
	pdhB	SMc01031	NC	NC	NC	NC
	pdhC	SMc01032	NC	NC	NC	NC
	lpdA1	SMc01035	NC	NC	NC	NC
Citrate synthase	*gltA*	SMc02087	NC	NC	NC	NC
Aconitase	*acnA*	SMc03896	-	-	-	-
Isocitrate dehydrogenase	*icd*	SMc00480	NC	NC	NC	NC
a-ketoglutarate dehydrogenase	*sucA*	SMc02482	NC	NC	NC	NC
	sucB	SMc02483	NC	NC	NC	NC
	lpdA2	SMc02487	NC	NC	NC	NC
Succinyl-CoA synthetase	*sucC*	SMc02480	NC	NC	NC	NC
	sucD	SMc02481	NC	NC	NC	NC
Succinate dehydrogenase	*sdhA*	SMc02466	NC	1.32	NC	NC
	sdhB	SMc02465	NC	NC	NC	NC
	sdhC	SMc02464	-	-	-	-
	sdhD	SMc02463	-	-	-	-
Fumerase	*fum*	SMc00149	NC	NC	NC	NC
Malate dehydrogenase	*mdh*	SMc02479	NC	NC	NC	NC
Malic enzyme	*dme*	SMc00169	NC	NC	NC	NC
	tme	SMc01126	NC	NC	NC	NC
Phosphoenolpyruvate carboxykinase	*pckA*	SMc02562	NC	+	+	+
Pyruvate orthophosphate dikinase	*ppdK*	SMc00025	NC	NC	NC	+

Pyruvate carboxylase	*pyc*	SMc03895	NC	NC	NC	NC
Isocitrate lyase	*aceA*	SMc00768	-	-	-	-
Malate synthase	*glcB*	SMc02581	NC	NC	NC	NC

Log(2) change in expression (Pnet) of each indicated protein due to mutation of either *tkt2* or *tal*, compared to Rm1021. NC, No change (Pnet between -1 and 1); - , Not detected; +, Detected not quantifiable because of inconsistency in biological replicates.

MS analysis. Of the detected proteins, 1880 were consistent in biological duplicate and considered for quantification.

Other than an increase of proteins involved in erythritol metabolism when grown with erythritol, no major changes were observed in proteins involved in central carbon metabolism (Table 10, Table 11). Peptides from Tkt2 and Tal were detected in decreased concentrations (451 and 67 fold respectively in SRmD397 or SRmD480); consistent with these strains carrying mutations in these proteins (Table 11). The proteins Gap and Pgk, which are encoded just downstream of *tkt2* (Figure 17), were decreased in SRmD397. This suggested that the Tn*5* mutation has some polar effect on these proteins.

COG analysis of the proteome data showed that proteins involved in inorganic ion transport and metabolism were among the most upregulated groups when cultures were induced with erythritol. Examination of proteins in this group showed that in Rm1021, proteins involved in rhizobactin synthesis increased in concentration (Table 12). Furthermore, proteins involved in hemin binding and transport, and iron transport were all found to be induced by erythritol in Rm1021. However, proteins were not induced by erythritol in either mutant strain. The iron binding and uptake proteins found to be upregulated in Rm1021 were also not detected, or found to be decreased in production in the mutant strains when compared to the wild-type.

5.4.8 Erythritol negatively affects rhizobactin production in tkt2 mutant strains.

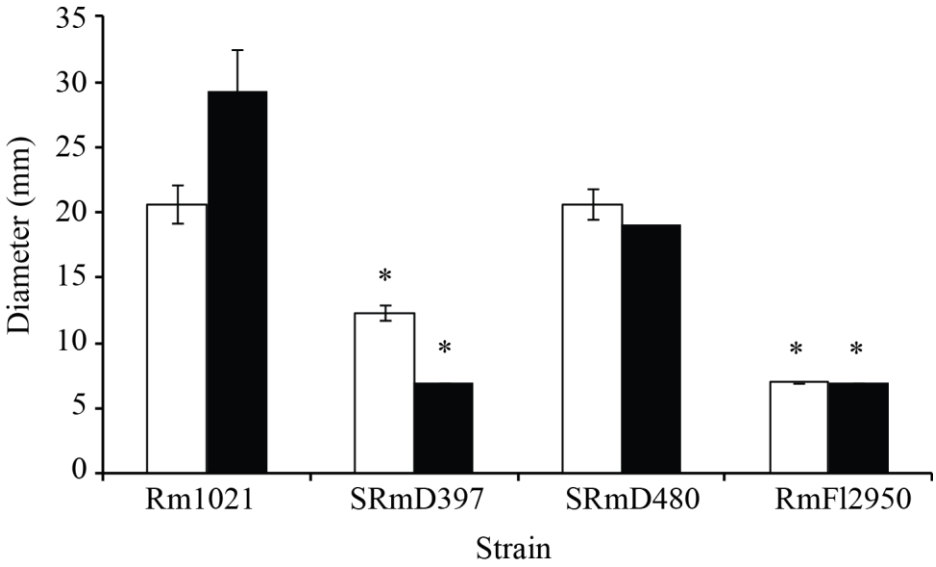
The metabolism of erythritol has been previously shown to influence the production of siderophores in *Brucella* (Bellaire et al. 1999). To assess if these strains were altered in

Table 12. Expression of proteins involved in iron sequestration

Enzyme	Gene	Locus tag	Rm1021 P_{net}	SRmD397 P_{net}	SRmD480 P_{net}
Rhizobactin					
diaminobutyrate-2-oxoglutarate aminotransferase	*rhbA*	SMa2400	+	-	-
RhsB L-2,4-diaminobutyrate decarboxylase	*rhbB*	SMa2402	2.69*	-	-
RhbC rhizobactin siderophore biosynthesis	*rhbC*	SMa2404	5.42	-	-
RhbD Rhizobactin siderophore biosynthesis	*rhbD*	SMa2406	+	-	-
RhbE Rhizobactin siderophore biosynthesis	*rhbE*	SMa2408	3.76*	-	-
RhbF Rhizobactin siderophore biosynthesis	*rhbF*	SMa2410	5.06	-	-
RhrA transcriptional activator	*rhrA*	SMa2412	-	-	-
RhtA Rhizobactin receptor precursor	*rhtA*	SMa2414	2.04*	-	-
Other iron associated proteins					
Putative hemin binding periplasmic transmembrane	*hmuT*	SMc01512	4.88	-	+
Putative iron transport protein	*shmR*	SMc02726	3.22	-	+
Putative hemin transport protein	*hmuS*	SMc01513	2.94	-	-1.39
Putative ferrichrome iron receptor precursor	*foxA*	SMc01657	2.61	-	NC
Putative iron uptake ABC transporter periplasmic		SMb21432	2.61	-	+
Putative iron binding protein	*fbpA*	SMc00784	1.85	NC	NC
Iron binding periplasmic protein		SMc04317	1.02	NC	1.58
Probable bacterioferritin (Cytochrome C1)	*bfr*	SMc03786	+	-2.87	-3.55
Putative iron transport ATP-binding ABC	*sitB*	SMc02508	NC	NC	-1.45

Log(2) expression (Pnet) of proteins for strains induced with erythritol compared to glucose induced strains. NC: No change (Log(2) expression between 1 and -1), -: Not detected, +: Detected but not quantifiable, *: Changes from not detected to detected, exact fold change not statistically significant

Figure 22. Siderophore production in *tkt2* and *tal* mutant strains.



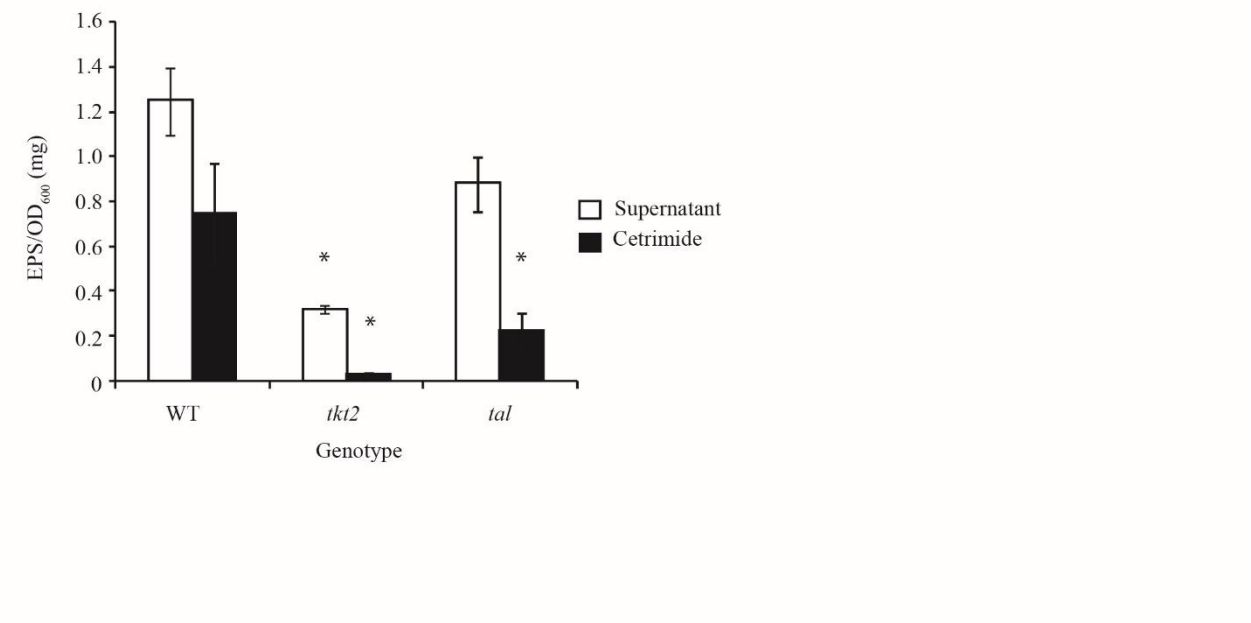
Strains were spotted onto VMM agar seeded with SmA818 and either containing glucose (white) or erythritol. Diameters were compared to the wild-type strain grown under the same condition. A minimal diameter of 6 mm represents the size of the tested culture spot. Significance was determined using Student's T-test. *; $P < 0.05$ compared to Rm1021 at each condition.

rhizobactin production, each strain was used in a rhizobactin competition assay with SmA818, *tkt2* mutant strain was assessed for rhizobactin production. When the medium was supplemented which is unable to produce or uptake rhizobactin (diCenzo et al. 2014). RmFl2950, which has a mutation in the gene *rhbB* and can not produce rhizobactin, was used as a negative control. In the presence of glucose, both Rm1021 and the *tal* mutant strain had similar zones of clearance to the growth of SmA818 (Figure 22). The zone of inhibition decreased from 20mm to 12mm when the with erythritol, a minor increase in zone clearance size was observed in Rm1021, though no change in clearance was seen in SRmD480. Interestingly, the presence of erythritol abolished the zone of inhibition observed in a *tkt2* mutant. These results suggested that erythritol affects rhizobactin production, and only SRmD397 is impaired in production of this siderophore.

5.4.9 Mutation of tkt2 or tal leads to aberrant EPS-I production.

The pentose phosphate pathway is involved in cycling carbon to glucose-6-phosphate, which can be converted to glucose-1-phosphate to be used for EPS-I biosynthesis (Portais et al. 1999, Gosselin et al. 2001). Since there were changes in the hexose phosphate pools found between each strain grown in glucose (Table 9) the production of succinoglycan was assessed. EPS was quantified as either cetrimide precipitable EPS, or as the total amount of glucose found in the culture supernatant. (Figure 23). The results show that in each case, the amount of EPS isolated by cetrimide precipitation is lower than that determined by measuring anthrone reactive material found in the supernatant. Overall, it was observed that a mutation in either *tkt2* or *tal* resulted in lower amounts of synthesized EPS-I. While a mutation in *tkt2* was severely impaired in EPS-I production, the *tal* mutant was less impacted compared to the wild-type. These results indicate that mutation of the non-oxidative pentose phosphate pathway has a direct effect on EPS-I production.

Figure 23. EPS-I production in tkt2 and tal mutants.



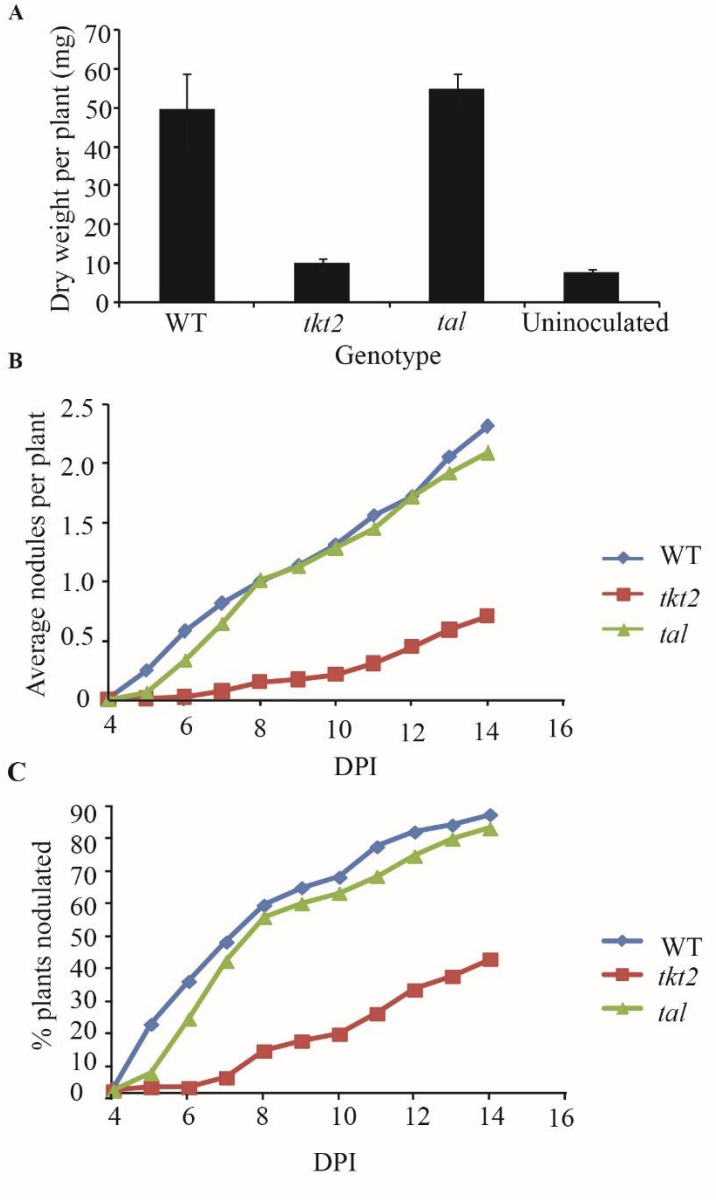
Quantification of EPS-I from culture supernatant (white), or cetrimide perceptible material (black) from each strain grown in YEM medium. Concentrations are normalized to the OD_{600} of the cell culture and are an average of 3 independent biological replicates. *; $P < 0.05$ compared to Rm1021 using Student's T-test.

5.4.10 Mutations in tkt2 result in defects in early symbiotic establishment.

The ability to acidify growth medium and produce increased amounts of EPS-I is correlated with increased competitiveness for nodule occupancy (Geddes et al. 2014). The mutation in *tkt2* was isolated on the basis of being unable to acidify its growth medium (Hawkins et al. 2017), and was shown to be impaired in EPS-I production (Figure 23). Since each of these determinants are linked to effective symbiosis, the ability of both the *tkt2* and *tal* mutant strains were assessed for symbiotic phenotypes. Dry weights of plants inoculated with each strain showed that the *tkt2* mutant was ineffective at fixing nitrogen for alfalfa (Figure 24a). However, plants inoculated with SRmD480 showed dry matter accumulation that was comparable to plants inoculated with Rm1021.

The rate of nodule formation was also determined for each strain when inoculated onto alfalfa roots (Figure 24b,c). Over a period of 14 days, alfalfa inoculated with the *tkt2* mutant was observed to be severely impacted with respect to nodule formation when compared to Rm1021, whereas the *tal* mutant showed similar kinetics as the wild-type strain. In the wild-type, nodule formation was first visible at 5 days and showed consistent development over time, while strains carrying the *tkt2* mutation did not start nodulating until day 7; and the rate of nodule formation was markedly slower. The total number of nodules was also greatly decreased (0.5 vs 2.5 nodules/plant) (Figure 24c). In addition to the speed of nodule formation, the nodules formed by SRmD397 were small cyst-like formations on the roots instead of cylindrical pink nodules that were found on the Rm1021 inoculated plants. Combined, these results indicated that a mutation in *tkt2* inhibits symbiotic establishment at an early stage.

Figure 24. Nitrogen fixation and nodule kinetics of *tkt2* and *tal* mutant strains.



Strains were inoculated onto alfalfa plants and assayed for either plant dry weight (A), the number of nodules formed per plant (B), and the proportion of nodulated plants (C). Each dry weight measurement is an average of 3 biological replicates, each containing 10 plants. Nodulation kinetics experiments (B + C) represent 5 biological replicates, each replicate containing at least 20 plants. The average standard deviation for each point was 11% for Rm1021 and SRmD480, and 9% for SRmD397.

5.5 Discussion

In this work, we provide evidence that Tkt2 and Tal act as the primary transketolase and transaldolase in the pentose phosphate pathway. This is based on the similarity of the encoded products to characterized transketolase and transaldolase proteins, and carbon metabolism (Table 7), the concentration of pentose phosphate intermediates when grown on glucose of each mutant (Figure 19), and the behaviour of the metabolite pools when the bacteria were supplemented with erythritol (Figure 21, Table 9)These phenotypes are all consistent with what has been previously found in other organisms (De Wulf and Vandamme 1997, Ishii et al. 2007, Stincone et al. 2015).

The genome of *E. coli* encodes two transketolase proteins, TktA and TktB (Iida et al. 1993, Zhao and Winkler 1994). TktA has been shown to provide 70-90% of transketolase activity, with the rest being provided by TktB (Iida et al. 1993). Our data shows that although a *S. meliloti tkt2* mutant strain has a greatly decreased sedoheptulose-7P pool, it is still measurable (Figure 19). Also, when *tkt1* is overexpressed *in trans* it can partially complement a *tkt2* mutation (Table 7), and in our hands, a *tkt2*/*tkt1* double mutant strain was unable to be rescued through amino acid supplementation on defined medium. These data are all consistent with what has been seen in *E. coli* (Josephson and Fraenkel 1969, 1974). We suggest that *tkt2 and tkt1* should be renamed to *tktA* and *tktB* respectively, to provide consistency with previously published literature.

A strain carrying a mutation in *tkt2* produces less EPS-I than a strain carrying a *tal* mutation, and both produce less EPS-I than the wild-type (Figure 23). If the amount of EPS-I isolated from each of these is compared to the intracellular concentrations of the metabolites we determined, EPS production is positively correlated with both the hexose phosphate (R^2=0.77)

and citrate (R^2=0.99) pools, whereas it is negatively correlated with the pentose phosphate (R^2=0.88), lactate (R^2=0.83), and gluconate (R^2=0.58) pools. We note that recent metabolomic characterization of acid-tolerant growth of *S. meliloti* has also shown that there is a correlation between EPS-I production, and the size of the hexose phosphate pool (Draghi et al. 2016, 2017). Enzymatic activities can be affected by allosteric effectors and post-translational modifications such as phosphorylation events. These latter modifications can occur in response to metabolite pools of environmental stimuli (Stock et al. 1989, Feria et al. 2008, Shimizu 2013). In *S. meliloti*, the activity of ExoN, which is required for the synthesis of UDP-glucose, has been shown to be modulated by its phosphorylation state (Medeot et al. 2016). Taken together this suggests that the changes seen in EPS-I production in each mutant strain are likely due to changes in the hexose phosphate pools resulting from a mutation in either transketolase or transaldolase.

The lack of changes in the proteins involved in central carbon metabolism in strains carrying either the *tkt2* or the *tal* mutation is consistent with the hypothesis that the genes that encode these activities are continually expressed, and that alteration of their activity occurs at the level of metabolite concentration through allosteric regulation (Hagberg et al. 2016). The finding that proteins involved in iron acquisition were more abundant in the wild-type than in either of the *tkt2* or *tal* background was unexpected, and that rhizobactin activity appeared to be affected by erythritol was surprising since the defined medium that was used is not iron limited (Vincent 1970).

In *B. abortus*, growth using erythritol and iron acquisition have been shown to be interrelated (Bellaire et al. 1999, Jain et al. 2011). Although the reason for this is unknown, it has been speculated that the inability of *B. abortus* to grow in the presence of erythritol in a low iron medium, such as found in pregnant ruminants, may explain why mutants unable to utilize

erythritol tend to be avirulent (Bellaire et al. 1999). In rhizobium, the genes necessary for metabolism of erythritol are believed to have been acquired by horizontal gene transfer (Yost et al. 2006, Geddes et al. 2010). Although erythritol catabolism does not affect the ability of *S. meliloti* to interact with its host plant, *R. leguminosarum* erythritol mutants are compromised in their ability to compete for nodule occupancy (Yost et al. 2006, Geddes and Oresnik 2012b). It is tempting to envision that mechanisms of how erythritol metabolism is regulated in *B. abortus* can be found in Rhizobia. It is also noteworthy that the *tkt2* mutation was originally isolated as a mutant that did not acidify its growth medium (Hawkins and Oresnik 2017), thus making iron less bioavailable.

The inability of a strain carrying a transketolase mutation to enter into an effective symbiotic relationship is most strongly correlated with its amino acid auxotrophy (Figure 24, Table 7). The inability of a *tkt2* mutant strain to produce erythrose-4-phosphate will affect the ability to generate chorismate, preventing biosynthesis of tryptophan, phenylalanine, and tyrosine. The role that the biosynthesis of aromatic amino acids play in the establishment of symbiosis seems to vary by each association (Dunn 2014).

Anthranilate synthase is the first dedicated enzyme in the biosynthesis of tryptophan and converts chorismate to anthranilate. In *S. meliloti*, this enzyme is encoded by *trpE(G)*, and insertions in this gene were found to result in symbiotically ineffective strains (Barsomian et al. 1992). However, mutations in the genes encoding the latter steps of the pathway (*trpD*, *trpF*, *trpC*, *trpA* and *trpB*) were symbiotically effective, leading to the conclusion that only anthranilate, and not tryptophan synthesis, is necessary for an effective symbiotic establishment (Barsomian et al. 1992). The nodules formed by *trpE(G)* mutants have been described as elongated (Barsomian et al. 1992). In contrast to this, *tkt2* mutants appear to have a more severe

symbiotic phenotype. They form fewer nodules that are delayed in their appearance, and have an altered morphology (Figure 24).

Interestingly, like the *trpE(G)* mutant strain, second site suppressor mutations which partially restore symbiosis have also been isolated from the *tkt2* mutant strain. While the suppressor mutations isolated from the *trpE(G)* mutants were not further characterized, we are currently pursuing the nature of second site mutations that occurred in the *tkt2* mutant strain. Our current working hypothesis is that it is likely that some of the suppressors that have been isolated to be similar to those previously reported for the *trpE(G)* mutants, and that the severe symbiotic phenotype we have found may be a result of both direct, as well as indirect effects of strains carrying a *tkt2* mutation. It is hoped that by understanding of how these determinants influence phenotypes associated with a mutation in *tkt2* may lead to insights into how the reactions of the pentose phosphate pathway affect the overall physiology of the organism, and how they affect the early stages in symbiosis.

Chapter 6:

Conclusions

6.1 Thesis Conclusions and Observations

The goal of this thesis was to determine how stress tolerance influences the production of determinants that are necessary for the symbiotic interaction between *S. meliloti* and its legume hosts. The research described in the previous sections on both magnesium and pH stress tolerance led to a number of conclusions that indicated the importance of polysaccharides. These conclusions, in combination with ongoing research in the field, have led us to the hypothesis that conditions found during symbiosis, which are classically defined as stress, have been co-opted to be involved in symbiotic signaling. The overall conclusions and outstanding questions regarding stress tolerance in *S. meliloti* are discussed in this section.

We have determined that the production of succinoglycan is intrinsically linked to the ability of *S. meliloti* to tolerate stress, both in the environment and during symbiosis. The work in chapter 2 on the characterization of *SMc00722* was an extension of previous work, which identified that the production of polysaccharides was necessary for salt stress tolerance (Miller-Williams et al. 2006). The mutation in *SMc00722* was originally isolated by screening for a strain which produced more EPS at increased magnesium concentrations. However, it was determined that a mutation in *SMc00722* resulted in the overproduction of EPS-I under standard conditions as well (Hawkins and Oresnik 2017). The simple argument that could be made is that *SMc00722* directly regulates the production of EPS-I, as a mutation in these gene results in many similar phenotypes to that of a mutation in *emmABC* (Morris and Gonzalez 2009). However, further characterization led to the conclusion that increased EPS production was likely due to a general stress response of the mutant, since the strain's membrane stability is compromised, and is impaired in maintaining intracellular magnesium homeostasis. This could indicate that the production of succinoglycan may also contribute to membrane stability.

Interestingly, despite the many phenotypes associated with a *SMc00722* mutant, a mutation in this gene did not impact its ability to enter an effective symbiotic relationship with alfalfa. This could indicate that membrane stability and magnesium concentrations are not important to the symbiotic relationship.

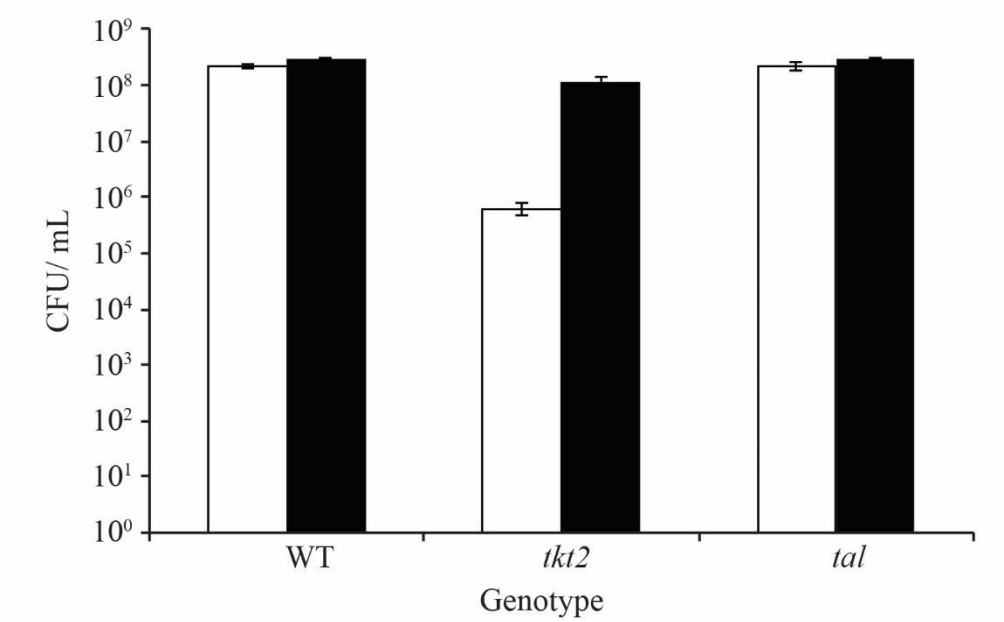
Increasing the concentration of magnesium, or decreasing medium pH was observed to increase production of succinoglycan. With our understanding of how EPS production is involved in stress tolerance (Miller-Williams et al. 2006, Lehman and Long 2013), it seemed likely that the role of increased EPS production was to tolerate these stresses. Our work in chapter 3 has directly provided evidence for LMW EPS-I having a role in pH stress tolerance. In addition, it seems that production of cyclic β(1-2) glucans may also play a role in pH stress tolerance in an overlapping role with EPS-I. Interestingly, the production of both of these polysaccharides are known to be necessary for symbiotic establishment.

Furthermore, these polysaccharides may play similar roles in symbiotic establishment, since suppression of the fix $^{-}$ phenotype associated with the inability to produce cyclic β(1-2) glucans is linked to EPS-I production, (Nagpal et al. 1992). The one striking similarity between these two polysaccharides is that they both become decorated with succinyl modifications (Breedveld and Miller 1994, Jones et al. 2007). Since recent work has indicated the importance of succinylation in symbiotic signaling, the overlap between cyclic β(1-2) glucans and succinoglycan may be related to recognition of succinyl groups on each polysaccharide. This can be further investigated when receptors for EPS production are identified in *M. truncatula* or *M. sativa*. While a receptor for EPS production has been identified in *Lotus japonicous, M. loti* does not produce succinylated EPS (Kawaharada et al. 2015). This would indicate that decoration of polysaccharides for symbiosis would be specific for each host-symbiont interaction.

It has been debated if EPS-I production played a direct role in symbiotic signaling between *S. meliloti* and its hosts, or if EPS-I is involved in stress tolerance and immune system avoidance. Recent work has determined that succinylation of EPS-I is necessary for early symbiotic signaling using *S. meliloti* (Mendis et al. 2016). Whereas our work has indicated a role for LMW EPS-I in pH stress tolerance, and was observed to play a role in competition for nodule occupancy. This is consistent with previous published work which had identified that LMW EPS-I is involved in the tolerance of oxidative stress (Lehman and Long 2013). Taken together, it seems EPS-I plays both a role in symbiotic signaling and in stress tolerance. Signaling from succinylated EPS-I is important in the early parts of the symbiotic interaction, including infection thread development. LMW EPS-I plays a role in tolerating stress found during the symbiotic interaction, and is important in competition for nodule occupancy.

Work done in chapter 5 indicates the importance of metabolism in regulating polysaccharide production and in stress tolerance. It had been determined that the activity of transketolase was necessary for medium acidification in a mutant strain that could not metabolize galactose (Hawkins et al. 2017). Further work determined that a mutation in *tkt2* resulted in sensitivity to acidic pH (Figure 25). Interestingly, the pH sensitivity of the *tkt2* mutant strain was found to be the same as an *exoY* mutant (100 fold decrease in growth at pH 5.75 vs pH 7). Furthermore, a *tkt2* mutant is impaired in EPS-I biosynthesis, which we now know is involved in pH tolerance. This can draw to potential roles for transketolase in pH tolerance, either through medium acidification or EPS production.

Figure 25. pH sensitivity of *tkt2* and *tal* mutants



Growth phenotype of *tkt2* and *tal* mutant strains grown on plates buffered at either pH 7 (black) or pH 5.75 (White). Results are an average of 3 independent biological replicates.

Overall it can be seen that tolerance of acidic pH stress has a major effect on protein expression and metabolism (Draghi et al. 2016, 2017). Combined with our observed data, many of these changes in response to acidic pH result in physiological changes that promote symbiosis. Since acidic pH is observed throughout symbiosis, it is possible that pH acts as a signal to change bacterial physiology for symbiotic establishment. Throughout the literature similar findings can be observed where stress tolerance mechanisms are directly involved in the symbiotic process (

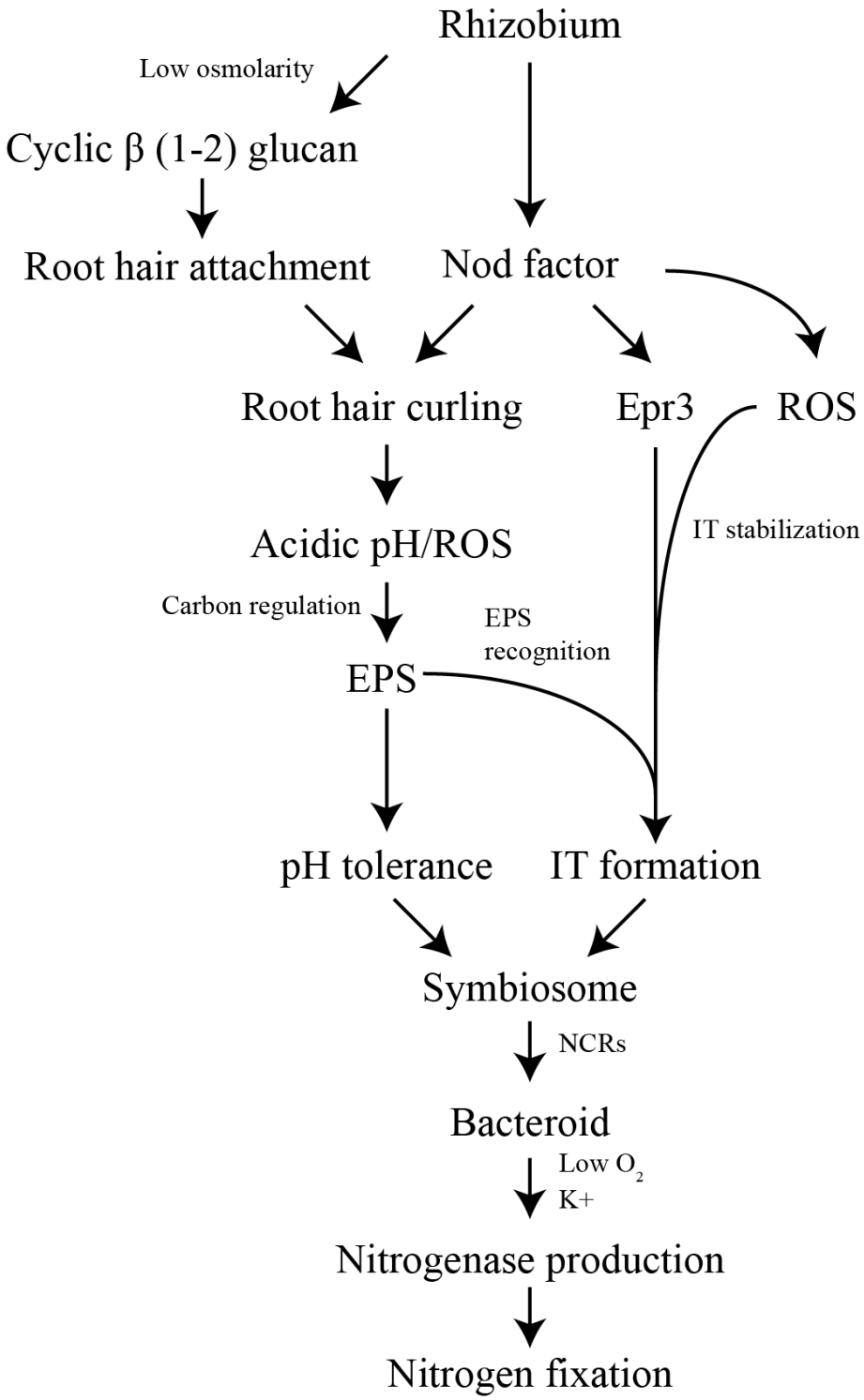
Figure 26). These findings together suggest that environmental conditions are not something that simply must be tolerated, but may be integrally connected to symbiotic establishment.

6.2 Outstanding questions and future work

6.2.1 The role of EPS-II and its regulation

The original goal of the project was to identify genes and determinants which are involved in the production of EPS-II in response to increasing concentrations of magnesium. However, after an extensive screening process, we were unable to identify mutations which could prevent the suppression of EPS-II production in response to increased magnesium concentrations. The production of EPS-II is dependent on the presence of an intact *expR* in *S. meliloti* under standard conditions, or can be induced by phosphate depleted medium (Mendrygal and González 2000, Pellock et al. 2000). EPS-II has been shown to be regulated by acetyl-homoserine lactones (AHLs) produced by the *sinI/R* quorum sensing system, and through the regulator *mucR* (Marketon et al. 2003, Mueller and Gonzalez 2011). The production of EPS-I is positively regulated by *mucR*, whereas production of EPS-II is suppressed. In addition, work done in *S. fredii* has also implicated *mucR* as a regulator of genes involved in ion transport (Jiao et al. 2016). Together this suggests that the EPS response of *S. meliloti* to ion concentration

Figure 26. Role of stress tolerance mechanisms in symbiosis



may be directly or indirectly tied to regulation through *mucR*. It is important to note that while we never isolated a mutation in *mucR* as having increased EPS-II biosynthesis in increased magnesium concentrations, a *mucR* mutant strain has never been directly tested for EPS-II production under these conditions.

The role EPS-II production plays in *S. meliloti* also remains unclear. Its production has been shown to be repressed under conditions which may be found during saprophytic growth or during symbiotic establishment, such as increased salt, phosphate, magnesium concentrations, and acidic pH (Mendrygal and González 2000, Miller-Williams et al. 2006). While production of EPS-II has been shown to somewhat rescue the symbiotic defect of an EPS-I deficient strain, the significant investment of *expR*$^+$ strains in EPS-II production indicates it must serve an important function. One of the clearest indications of EPS-II function may be attributed to the overproduction of this polysaccharide in phosphate deplete medium, and its role in agglutination (Mendrygal and González 2000, Sorroche et al. 2010). One suggestion may be that the production of EPS-II is a form of hypotonic stress tolerance, and EPS-II may provide a physical barrier for *S. meliloti* from the solution, which results in agglutination of cells. Furthermore, EPS-II has also been suggested to have a role in survival protection from predator strains (Pérez et al. 2014). This may also be simply due to its physical presence which can mask the presence of the rhizobium from predators. Overall, it seems that the production of EPS-II probably has significantly more importance in environmental conditions than in laboratory conditions.

6.2.2 Stress tolerance mechanism of EPS-I

The work in chapter 3 indicates the importance of LMW EPS-I in pH stress tolerance. The production of the low molecular weight fraction has also been shown to be involved in the

tolerance of oxidative stress (Lehman and Long 2013). However, it remains unclear how the production of the LMW fraction provides *S. meliloti* with tolerance to oxidative and pH stress. The finding that the *exsH/exoK* double endoglycanase mutation is sensitive to acidic pH also indicates that succinylation of the polysaccharide is not important in survival under these conditions. This may indicate that the increased amount of reducing ends present due to lower chain length is important in stress tolerance, or that acetyl and pyruvyl modifications may be important.

Previous work has shown that the production of polysaccharides does not provide buffering in acidic conditions, which is further corroborated by the pK_a of succinoglycan being 3.8 (Cunningham and Munns 1984b, Szewczuk-Karpisz et al. 2014). Medium acidification has been shown to increase bioavailability of many nutrients and ions which are correlated with either oxidative stress or pH stress damage, such as iron and aluminum. While EPS-I has been suggested to not bind either of these ions (Cunningham and Munns 1984b, Lehman and Long 2013), it does not rule out that EPS-I may bind another molecule that is necessary for acidic pH stress. It is also possible that association of LMW EPS-I with bacterial membranes may provide more structural stability to the cell than HMW EPS-I would. This could explain the incorporation of EPS production as a general stress response, and why strains compromised in membrane integrity produce more EPS-I (Morris and Gonzalez 2009, Hawkins and Oresnik 2017).

6.2.3 The role of carbon flow in pH stress tolerance

One of the more intriguing questions raised from chapter 3 is why a mutant that cannot produce LMW EPS-I more sensitive to acidic pH than a mutant strain unable to produce any EPS-I at all. While our research showed an increased production of glycogen in an EPS-I deficient strain, glycogen was not observed to be involved in pH stress tolerance. While cyclic

β(1-2) glucans did contribute to tolerance; strains deficient in EPS-I production produce the same amount of cyclic β (1-2) glucans as strains only able to produce HMW EPS-I. This indicates that another mechanism is likely the cause of increased pH tolerance in the *exoY* mutant strain.

In our study, we focused our examination on 4 major polysaccharides. Two of the other major polysaccharides, LPS and KPS, were not investigated since pH did not regulate genes associated with their production. It is possible that carbon flow to these polysaccharides may happen independent of gene regulation. Acidic pH has been shown to influence the decoration of rhizobial LPS, which could be important to stress tolerance (Tao et al. 1992). Apart from this, pSymB also encodes many putative operons hypothesized to be involved in polysaccharide synthesis that have not been investigated. It is possible that carbon flow may be directed to a yet uncharacterized polysaccharide which has importance in pH tolerance. One potential candidate yet to be investigated is polyhydroxy-butyrate. Large scale investigation of polysaccharide biosynthesis in an EPS-I deficient strain would be necessary to further investigate this hypothesis.

6.2.4 Involvement of transketolase in symbiosis and medium acidification

Results in chapter 5 indicate that a mutation in the primary transketolase of *S. meliloti* has a number of effects on metabolism and symbiosis. The mutation results in a block of metabolic pathways necessary for aromatic compound synthesis, and affects EPS-I biosynthesis; both of which have been shown to affect symbiotic establishment (Long et al. 1988, Dunn 2014). Due to this, it is hard to pinpoint the exact reason why a mutation in transketolase is impaired in symbiosis. However, a number of suppressor mutations have been isolated which are observed to partially suppress the symbiotic phenotypes associated with the *tkt2* mutation. By investigating

how suppression of this phenotype is accomplished, it is hoped that the exact mechanism *tkt2* and central carbon metabolism overall, plays in symbiotic establishment.

The mutation in *tkt2* was originally isolated by screening for mutants which could prevent galactose dependent medium acidification in a strain unable to catabolize galactose (Hawkins et al. 2017). Now that the function of *tkt2* is understood, the role it plays in medium acidification can now be investigated. Since our observations indicate a dramatic shift in metabolite pools compared to the wild-type strain when a mutation in *tkt2* is present, it seems likely that the decreased production of a metabolite which acidifies medium is the cause of loss of acidification. Previous work had implicated secretion of gluconate as the cause of medium acidification in *S. meliloti* grown in RC medium (Gosselin et al. 2001). Galactose is metabolized through the De Ley – Doudoroff pathway in *S. meliloti*, and a mutation in the gene *dgok1* would result in a build-up of intermediates in this pathway (Geddes and Oresnik 2012a). It is possible that the build-up of galactonate in this pathway is responsible for medium acidification when the *dgoK1* mutation is present. If this is true, altered metabolism in a *tkt2* mutant strain may simply result in altered carbon flow through galactose metabolism, resulting in the observed loss of medium acidification. It is also possible that a mutation in *tkt2* may result in a change in gluconate concentrations which would alter medium acidification. Investigation of extracellular metabolites should be able to elucidate the cause of impaired medium acidification associated with the *tkt2* mutation.

Overall, the work presented in this thesis, in combination with previous work, is consistent with the hypothesis that mechanisms used by bacteria to tolerate adverse environmental conditions become co-opted into the symbiotic process. Further work will

continue to identify the yet unknown mechanisms of how metabolic regulation by the pentose phosphate pathway plays a role in stress tolerance and symbiosis.

6.3 Literature Cited

Aarons, S.R., and Graham, P.H. 1991. Response of *Rhizobium leguminosarum Bv Phaseoli* to acidity. Plant Soil **134**(1): 145–151.

Abe, M., Amemura, A., and Higashi, S. 1982. Studies on cyclic β-1,2-glucan obtained from periplasmic space of *Rhizobium trifolii* cells. Plant Soil **64**(3): 315–324. doi:10.1007/BF02372514.

Alexeyev, M.F. 1999. The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of gram-negative bacteria. Biotechniques **26**(5): 5–7.

Alunni, B., Kevei, Z., Redondo-Nieto, M., Kondorosi, A., Mergaert, P., and Kondorosi, E. 2007. Genomic organization and evolutionary insights on GRP and NCR genes, two large nodule-specific gene families in *Medicago truncatula*. Mol. Plant. Microbe. Interact. **20**(9): 1138–1148. doi:10.1094/MPMI-20-9-1138.

Aneja, P., Dai, M., Lacorre, D.A., Pillon, B., and Charles, T.C. 2004. Heterologous complementation of the exopolysaccharide synthesis and carbon utilization phenotypes of *Sinorhizobium meliloti* Rm1021 polyhydroxyalkanoate synthesis mutants. FEMS Microbiol. Lett. **239**(2): 277–283. doi:10.1016/j.femsle.2004.08.045.

Appanna, V.D. 1989. Exopolysaccharide synthesis in *Rhizobium trifolii* in the presence of manganese and aluminium. Microbios Lett. **40**(157): 31–36.

Ardissone, S., Kobayashi, H., Kambara, K., Rummel, C., Noel, K.D., Walker, G.C., Broughton, W.J., and Deakin, W.J. 2011. Role of BacA in lipopolysaccharide synthesis, peptide transport, and nodulation by *Rhizobium sp*. strain NGR234. J. Bacteriol. **193**(9): 2218–2228. doi:10.1128/JB.01260-10.

Barbier, T., Collard, F., Zúñiga-Ripa, A., Moriyón, I., Godard, T., Becker, J., Wittmann, C., Van Schaftingen, E., and Letesson, J.-J. 2014. Erythritol feeds the pentose phosphate pathway via three new isomerases leading to D-erythrose-4-phosphate in *Brucella*. Proc. Natl. Acad. Sci. U. S. A. **111**(50): 17815–17820. doi:10.1073/pnas.1414622111.

Barsomian, G.D., Urzainqui, A., Lohman, K., and Walker, G.C. 1992. *Rhizobium meliloti* mutants unable to synthesize anthranilate display a novel symbiotic phenotype. J. Bacteriol. **174**(13): 4416–4426.

Becker, A., Kleickmann, A., Keller, M., Arnold, W., and Pühler, A. 1993a. Identification and analysis of the *Rhizobium meliloti exoAMONP* genes involved in exopolysaccharide biosynthesis and mapping of promoters located on the *exoHKLAMONP* fragment. Mol. Gen. Genet. **241**(3–4): 367–379. doi:10.1007/BF00284690.

Becker, A., Kleickmann, A., Küster, H., Keller, M., Arnold, W., and Pühler, A. 1993b. Analysis of the Rhizobium meliloti genes *exoU, exoV, exoW, exoT*, and *exoI* involved in exopolysaccharide biosynthesis and nodule invasion: *exoU* and *exoW* probably encode glucosyltransferases.

Becker, A., Niehaus, K., and Pühler, A. 1995. Low-molecular-weight succinoglycan is predominantly produced by *Rhizobium meliloti* strains carrying a mutated ExoP protein characterized by a periplasmic N-terminal domain and a missing C-terminal domain. Mol. Microbiol. **16**(2): 191–204. doi:10.1111/j.1365-2958.1995.tb02292.x.

Becker, A., Rüberg, S., Küster, H., Roxlau, A.A., Keller, M., Ivashina, T., Cheng, H.-P., Walker, G.C., and Pühler, A. 1997. The 32-kilobase exp gene cluster of *Rhizobium meliloti* directing the biosynthesis of galactoglucan: genetic organization and properties of the encoded gene products. J. Bacteriol. **179**(4): 1375–1384.

Bélanger, L., and Charles, T.C. 2013. Members of the *Sinorhizobium meliloti* ChvI regulon identified by a DNA binding screen. BMC Microbiol. **13**(132): 1–13. doi:10.1186/1471-2180-13-132.

Belcarz, A., Janczarek, M., Kolacz, K., Urbanik-Sypniewska, T., and Ginalska, G. 2013. Do Ca^{2+}chelating polysaccharides reduce calcium ion release from gypsum-based biomaterials. Cent. Eur. J. Biol. **8**(8): 735–746. doi:10.2478/s11535-013-0191-4.

Bellaire, B.H., Elzer, P.H., Baldwin, C.L., and Ii, R.M.R. 1999. Production of the siderophore 2,3-dihydroxybenzoic acid is required for wild-type growth of *Brucella abortus* in the presence of erythritol under low-iron conditions *in vitro*. Infect. Immun. **67**(5): 2615–2618. doi:10.1128/IAI.71.5.2927.

Beringer, J.E. 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. **84**(1): 188–198. doi:10.1099/00221287-84-1-188.

Berrabah, F., Ratet, P., and Gourion, B. 2015. Multiple steps control immunity during the intracellular accommodation of rhizobia. J. Exp. Bot. **66**(7): 1977–1985. doi:10.1093/jxb/eru545.

Bohin, J.P., and Kennedy, E.P. 1984. Regulation of the synthesis of membrane-derived oligosaccharides in *Escherichia coli*. Assay of phosphoglycerol transferase I in vivo. J. Biol. Chem. **259**(13): 8388–8393.

Boscari, A., Meilhoc, E., Castella, C., Bruand, C., Puppo, A., and Brouquisse, R. 2013. Which role for nitric oxide in symbiotic N2-fixing nodules: toxic by-product or useful signaling/metabolic intermediate? Front. Plant Sci. **4**: 1–6. doi:10.3389/fpls.2013.00384.

Botsford, J.L., Alvarez, M., Hernandez, R., and Nichols, R. 1994. Accumulation of Glutamate by *Salmonella typhimurium* in Response to Osmotic-Stress. Appl. Environ. Microbiol. **60**(7): 2568–2574.

Botsford, J.L., and Lewis, T.A. 1990. Osmoregulation in *Rhizobium meliloti*: Production of glutamic acid in response to osmotic stress. Appl. Environ. Microbiol. **56**(2): 488–494.

Bräsen, C., Esser, D., Rauch, B., and Siebers, B. 2014. Carbohydrate metabolism in Archaea: current insights into unusual enzymes and pathways and their regulation. Microbiol. Mol. Biol. Rev. **78**(1): 89–175. doi:10.1128/MMBR.00041-13.

Breedveld, M.W., Canter Cremers, H.C.J., Batley, M., Posthumus, M.A., Zevenhuizen,

L.P.T.M., Wijffelman, C.A., and Zehnder, A.J.B. 1993. Polysaccharide synthesis in relation to nodulation behavior of *Rhizobium leguminosarum*. J. Bacteriol. **175**(3): 750–757.

Breedveld, M.W., and Miller, K.J. 1994. Cyclic β-glucans of members of the family Rhizobiaceae. Microbiol. Rev. **58**(2): 145–61.

Breedveld, M.W., and Miller, K.J. 1995. Synthesis of glycerophosphorylated cyclic (1,2)-b-glucans in *Rhizobium meliloti* strain 1021 after osmotic shock. Microbiology **141**(1): 583–588.

Breedveld, M.W., Zevenhuizen, L.P.T.M., and Zehnder, A.J.B. 1990. Osmotically induced oligo- and polysaccaride synthesis by *Rhizobium meliloti* SU-47. J.Gen.Microbiol. **136**(12): 2511–2547.

Brogden, K.A. 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. **3**(3): 238–250. doi:10.1038/nrmicro1098.

Bromfield, E.S.P., and Jones, D.G. 1980. A strain marker in *Rhizobium trifolii* based on the absorption of congo-red. Zentrabil Bakteriol Naturwiss **135**(4): 290–295.

Broughton, W.J., Hanin, M., Relic, B., Kopcinska, J., Golinowski, W., Simsek, S., Ojanen-Reuhs, T., Reuhs, B., Marie, C., Kobayashi, H., Bordogna, B., Le Quere, A., Jabbouri, S., Fellay, R., Perret, X., and Deakin, W.J. 2006. Flavonoid-inducible modifications to rhamnan O antigens are necessary for *Rhizobium sp*. strain NGR234-legume symbioses. J. Bacteriol. **188**(10): 3654–3663. doi:10.1128/JB.188.10.3654-3663.2006.

Broughton, W.J., Wong, C.H., Lewin, A., Samrey, U., Myint, H., Meyerz, H., Dowling, D.N., and Simon, R. 1986. Identification of *Rhizobium* plasmid sequences involved in recognition of *Psophocarpus,Vigna*, and other legumes. J Cell Biol **102**(4): 1173–1182.

Capela, D., Barloy-Hubler, F., Gouzy, J., Bothe, G., Ampe, F., Batut, J., Boistard, P., Becker, A., Boutry, M., Cadieu, E., Dréano, S., Gloux, S., Godrie, T., Goffeau, A., Kahn, D., Kiss, E., Lelaure, V., Masuy, D., Pohl, T., Portetelle, D., Pühler, A., Purnelle, B., Ramsperger, U., Renard, C., Thébault, P., Vandenbol, M., Weidner, S., and Galibert, F. 2001. Analysis of the chromosome sequence of the legume symbiont *Sinorhizobium meliloti* strain 1021. Proc. Natl. Acad. Sci. U S A **98**(17): 9877–9882. doi:10.1073/pnas.161294398.

Capoen, W., Sun, J., Wysham, D., Otegui, M.S., Venkateshwaran, M., Hirsch, S., Miwa, H., Downie, J.A., Morris, R.J., Ane, J.-M., and Oldroyd, G.E.D. 2011. Nuclear membranes control symbiotic calcium signaling of legumes. Proc. Natl. Acad. Sci. **108**(34): 14348–14353. doi:10.1073/pnas.1107912108.

Cardenas, L., Martinez, A., Sanchez, F., and Quinto, C. 2008. Fast, transient and specific intracellular ROS changes in living root hair cells responding to Nod factors (NFs). Plant J. **56**(5): 802–813. doi:10.1111/j.1365-313X.2008.03644.x.

Cervenansky, C., and Arias, A. 1984. Glucose-6-phosphate dehydrogenase deficiency in pleiotropic carbohydrate-negative mutant strains of *Rhizobium meliloti*. J. Bacteriol. **160**(3): 1027–1030.

Cesco, S., Neumann, G., Tomasi, N., Pinton, R., and Weisskopf, L. 2010. Release of plant-borne flavonoids into the rhizosphere and their role in plant nutrition. Plant Soil **329**(1): 1–25. doi:10.1007/s11104-009-0266-9.

Charpentier, M., Sun, J., Martins, T.V., Radhakrishnan, G. V, Findlay, K., Soumpourou, E., Thouin, J., Véry, A., Sanders, D., Morris, R.J., and Oldroyd, G.E.D. 2016. Nuclear-localized cyclic nucleotide–gated channels mediate symbiotic calcium oscillations. Science . **352**(6289): 1102–1105. doi:10.1126/science.aae0109.

Charpentier, M., Vaz Martins, T., Granqvist, E., Oldroyd, G.E.D., and Morris, R.J. 2013. The role of DMI1 in establishing Ca^{2+} oscillations in legume symbioses. Plant Signal. Behav. **8**(2): e22894. doi:10.4161/psb.22894.

Chen, E.J., Fisher, R.F., Perovich, V.M., Sabio, E.A., and Long, S.R. 2009. Identification of direct transcriptional target genes of *ExoS/ChvI* two-component signaling in *Sinorhizobium meliloti*. J. Bacteriol. **191**(22): 6833–6842. doi:10.1128/JB.00734-09.

Chen, E.J., Sabio, E.A., and Long, S.R. 2008. The periplasmic regulator ExoR inhibits ExoS/ChvI two- component signaling in *Sinorhizobium meliloti*. Mol Microbiol **69**(5): 1290–1303. doi:10.1038/jid.2014.371.

Chen, H., Richardson, A.E., and Rolfe, B.G. 1993. Studies of the physiological and genetic basis of acid tolerance in *Rhizobium leguminosarum biovar trifolii*. Appl. Environ. Microbiol. **59**(6): 1798–1804.

Cheng, H.-P., and Walker, G.C. 1998a. Succinoglycan is required for initiation and elongation of infection threads during nodulation of alfalfa by *Rhizobium meliloti*. J. Bacteriol. **180**(19): 5183–5191.

Cheng, H.-P., and Walker, G.C. 1998b. Succinoglycan production by *Rhizobium meliloti* is regulated through the ExoS-ChvI two-component regulatory system. J. Bacteriol. **180**(1): 20–26.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., and Lappin-Scott, H.M. 1995. Microbial biofilms. Annu. Rev. Microbiol. **49**: 711–745. doi:10.1146/annurev.mi.49.100195.003431.

Cowan, M.M. 1999. Plant products as antimicrobial agents. Clin. Microbiol. Rev. **12**(4): 564–82.

Cramer, C.L., Ryder, T.B., Bell, J.N., and Lamb, C.J. 1985. Rapid Switching of Plant Gene Expression Induced by Fungal Elicitor. Science . **227**(4691): 1240–1243.

Csonka, L.N. 1989. Physiological and genetic responses of bacteria to osmotic stress. Microbiol. Rev. **53**(1): 121–147.

Cunningham, S.D., and Munns, D.N. 1984a. The correlation between extracellular polysaccharide production and acid tolerance in *Rhizobium*. Soil Sci. Soc. Am. J. **48**: 1273–1277. doi:10.2136/sssaj1984.03615995004800060014x.

Cunningham, S.D., and Munns, D.N. 1984b. Effects of rhizobial extracellular polysaccharide on

pH and aluminum activity. Soil Sci. Soc. Am. J. **48**(6): 1276–1279. doi:10.2136/sssaj1984.03615995004800060015x.

Dangl, J.L., and Jones, J.D.G. 2001. Plant pathogens and integrated defence responses to infection. Nature **411**: 826–833.

Darken, M.A. 1962. Absorption and transport of fluorescent brighteners by microorganisms. Appl. Microbiol. **10**: 387–393.

David, M., Daveran, M.L., Batut, J., Dedieu, A., Domergue, O., Ghai, J., Hertig, C., Boistard, P., and Kahn, D. 1988. Cascade regulation of Nif gene-expression in *Rhizobium meliloti*. Cell **54**(5): 671–683.

Denoux, C., Galletti, R., Mammarella, N., Gopalan, S., Werck, D., De Lorenzo, G., Ferrari, S., Ausubel, F.M., and Dewdney, J. 2008. Activation of defense response pathways by OGs and Flg22 elicitors in *Arabidopsis* seedlings. Mol. Plant **1**(3): 423–445. doi:10.1093/mp/ssn019.

diCenzo, G.C., MacLean, A.M., Milunovic, B., Golding, G.B., and Finan, T.M. 2014. Examination of prokaryotic multipartite genome evolution through experimental genome reduction. PLoS Genet. **10**(10). doi:10.1371/journal.pgen.1004742.

Dilworth, M.J., Tiwari, R.P., Reeve, W.G., and Glenn, A.R. 2000. Legume root nodule bacteria and acid pH. Sci. Prog. **83**(4): 357–389.

Djordjevic, M.A. 2004. *Sinorhizobium meliloti* metabolism in the root nodule: A proteomic perspective. Proteomics **4**(7): 1859–1872. doi:10.1002/pmic.200300802.

Donati, A.J., Jeon, J.M., Sangurdekar, D., So, J.S., and Chang, W.S. 2011. Genome-wide transcriptional and physiological responses of *Bradyrhizobium japonicum* to paraquat-mediated oxidative stress. Appl. Environ. Microbiol. **77**(11): 3633–3643. doi:10.1128/AEM.00047-11.

Douglas, C., Halperin, W., Gordon, M., and Nester, E. 1985. Specific attachment of *Agrobacterium tumefaciens* to bamboo cells in suspension cultures. J. Bacteriol. **161**(2): 764–766.

Draghi, W.O., Del Papa, M.F., Barsch, A., Albicoro, F.J., Lozano, M.J., Pühler, A., Niehaus, K., and Lagares, A. 2017. A metabolomic approach to characterize the acid-tolerance response in *Sinorhizobium meliloti*. Metabolomics **13**(6): 71. Springer US. doi:10.1007/s11306-017-1210-2.

Draghi, W.O., Del Papa, M.F., Hellweg, C., Watt, S.A., Watt, T.F., Barsch, A., Lozano, M.J., Lagares, A., Salas, M.E., López, J.L., Albicoro, F.J., Nilsson, J.F., Torres Tejerizo, G.A., Luna, M.F., Pistorio, M., Boiardi, J.L., Pühler, A., Weidner, S., Niehaus, K., and Lagares, A. 2016. A consolidated analysis of the physiologic and molecular responses induced under acid stress in the legume-symbiont model-soil bacterium *Sinorhizobium meliloti*. Sci. Rep. **6**: e29278. doi:10.1038/srep29278.

Duncan, M.J. 1981. Properties of Tn*5*-induced Carbohydrate Mutants in *Rhizobium meliloti*. J.

Gen. Microbiol.: 61–67.

Dunn, M.F. 2014. Key roles of microsymbiont amino acid metabolism in rhizobia-legume interactions. Crit. Rev. Microbiol. **7828**(4): 1–41. doi:10.3109/1040841X.2013.856854.

Dunn, M.F., Araíza, G., Encarnación, S., Vargas, C., Mora, J., and Arai, G. 2002. Effect of *aniA* (carbon flux regulator) and mutations on pyruvate metabolism in *Rhizobium etli*. J. Bacteriol. **184**(8): 2296–2299. doi:10.1128/JB.184.8.2296-2299.2002.Updated.

Dylan, T., Helinski, D.R., and Ditta, G.S. 1990a. Hypoosmotic adaptation in *Rhizobium meliloti* requires beta-(1-2)-glucan. J. Bacteriol. **172**(3): 1400–1408.

Dylan, T., Ielpi, L., Stanfield, S., Kashyap, L., Douglas, C., Yanofsky, M., Nester, E., Helinski, D.R., and Ditta, G.S. 1986. *Rhizobium meliloti* genes required for nodule development are related to chromosomal virulence genes in *Agrobacterium tumefaciens*. Proc. Natl. Acad. Sci. U. S. A. **83**(12): 4403–7. doi:10.1073/pnas.83.12.4403.

Dylan, T., Nagpal, P., Helinski, D.R., and Ditta, G.S. 1990b. Symbiotic pseudorevertants of *Rhizobium meliloti ndv* mutants. J. Bacteriol. **172**(3): 1409–1417.

El-Yahyaoui, F., Kuster, H., Amor, B. Ben, Hohnjec, N., Pu, A., Becker, A., Vernie, T., Gough, C., Niebel, A., Godiard, L., and Gamas, P. 2004. Expression profiling in *Medicago truncatula* pdentifies more than 750 genes differentially expressed during nodulation , including many potential regulators of the symbiotic program. Plant Physiol. **136**: 3159–3176. doi:10.1104/pp.104.043612.the.

Endo, Y., and Tsurugi, K. 1986. Mechanism of action of ricin and related toxic lectins on eukaryotic ribosomes. Nucleic Acids Symp. Ser. **262**(17): 187–190.

Ertl, G. 1991. Elementary Steps in Ammonia Synthesis. *In* Catalytic Ammonia Synthesis: Fundamentals and Practice. *Edited by* J.R. Jennings. Springer US, Boston, MA. pp. 109–132. doi:10.1007/978-1-4757-9592-9_3.

Evans, N.A., Hoyne, P.A., and Stone, B.A. 1984. Characteristics and specificity of the interaction of a fluorochrome from aniline blue (Sirofluor) with polysaccharides. Carbohydr. Polym. **4**(3): 215–230. doi:10.1016/0144-8617(84)90012-2.

Faget, M., Blossfeld, S., von Gillhaussen, P., Schurr, U., and Temperton, V.M. 2013. Disentangling who is who during rhizosphere acidification in root interactions: combining fluorescence with optode techniques. Front. Plant Sci. **4**(392): 1–8. doi:10.3389/fpls.2013.00392.

Farkas, A., Maróti, G., Durgő, H., Györgypál, Z., Lima, R.M., Medzihradszky, K.F., Kereszt, A., Mergaert, P., and Kondorosi, É. 2014. *Medicago truncatula* symbiotic peptide NCR247 contributes to bacteroid differentiation through multiple mechanisms. Proc. Natl. Acad. Sci. U. S. A. **111**(14): 5183–8. doi:10.1073/pnas.1404169111.

Fedorova, E., Thomson, R., Whitehead, L.F., Maudoux, O., Udvardi, M.K., and Day, D.A. 1999. Localization of H+-ATPase in soybean root nodules. Planta **209**(1): 25–32. doi:10.1007/s004250050603.

Fenner, B.J., Tiwari, R.P., Reeve, W.G., Dilworth, M.J., and Glenn, A.R. 2004. *Sinorhizobium medicae* genes whose regulation involves the ActS and/or ActR signal transduction proteins. FEMS Microbiol. Lett. **236**(1): 21–31. doi:10.1016/j.femsle.2004.05.016.

Ferguson, B.J., Lin, M.-H., and Gresshoff, P.M. 2013. Regulation of legume nodulation by acidic growth conditions. Plant Signal. Behav. **8**(3): e23426. doi:10.4161/psb.23426.

Feria, A.-B., Alvarez, R., Cochereau, L., Vidal, J., Garcia-Maurino, S., and Echevarria, C. 2008. Regulation of phosphoenolpyruvate carboxylase phosphorylation by metabolites and abscisic acid during the development and germination of barley seeds. Plant Physiol. **148**(2): 761–774. doi:10.1104/pp.108.124982.

Ferrer, J.L., Austin, M.B., Stewart, C., and Noel, J.P. 2008. Structure and function of enzymes involved in the biosynthesis of phenylpropanoids. Plant Physiol. Biochem. **46**(3): 356–70. doi:10.1016/j.plaphy.2007.12.009.

Finan, T.M., Hartweig, E., LeMieux, K., Bergman, K., Walker, G.C., and Signer, E.R. 1984. General transduction in *Rhizobium meliloti*. J. Bacteriol. **159**(1): 120–124.

Finan, T.M., Hirsch, A.M., Leigh, J.A., Johansen, E., Kuldau, G.A., Deegan, S., Walker, G.C., and Signer, E.R. 1985. Symbiotic mutants of *Rhizobium meliloti* that uncouple plant from bacterial differentiation. Cell **40**(4): 869–877. doi:10.1016/0092-8674(85)90346-0.

Finan, T.M., Kunkel, B., Vos, G.F.D.E., and Signer, E.R. 1986. Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. J. Bacteriol. **167**(1): 66–72.

Finan, T.M., Oresnik, I.J., and Bottacin, A. 1988. Mutants of *Rhizobium meliloti* defective in succinate metabolism. J. Bacteriol. **170**(8): 3396–3403.

Finan, T.M., Weidner, S., Wong, K., Buhrmester, J., Chain, P., Vorhölter, F.J., Hernandez-Lucas, I., Becker, A., Cowie, A., Gouzy, J., Golding, B., and Pühler, A. 2001. The complete sequence of the 1,683-kb pSymB megaplasmid from the N2-fixing endosymbiont *Sinorhizobium meliloti*. Proc. Natl. Acad. Sci. U. S. A. **98**(17): 9889–94. doi:10.1073/pnas.161294698.

Fisher, R.F., and Long, S.R. 1993. Interactions of NodD at the *nod* Box: NodD binds to two distinct sites on the same face of the helix and induces a bend in the DNA. doi:10.1006/jmbi.1993.1515.

Fowler, D., Coyle, M., Skiba, U., Sutton, M.A., Cape, J.N., Reis, S., Sheppard, L.J., Jenkins, A., Grizzetti, B., Galloway, N., Vitousek, P., Leach, A., Bouwman, A.F., Butterbach-bahl, K., Dentener, F., Stevenson, D., Amann, M., Voss, M., and Fowler, D. 2013. The global nitrogen cycle in the twenty- first century. Philisophical Trans. R. Soc. B. doi:http://dx.doi.org/10.1098/rstb.2013.0164.

Gage, D.J. 2002. Analysis of infection thread development using Gfp- and DsRed-expressing *Sinorhizobium meliloti*. J. Bacteriol. **184**(24): 7042–7046. doi:10.1128/JB.184.24.7042-7046.2002.

Galibert, F., Finan, T.M., Long, S.R., Puhler, A., Abola, P., Ampe, F., Barloy-Hubler, F., Barnett, M.J., Becker, A., Boistard, P., Bothe, G., Boutry, M., Bowser, L., Buhrmester, J., Cadieu, E., Capela, D., Chain, P., Cowie, A., Davis, R.W., Dreano, S., Federspiel, N.A., Fisher, R.F., Gloux, S., Godrie, T., Goffeau, A., Golding, B., Gouzy, J., Gurjal, M., Hernandez-Lucas, I., Hong, A., Huizar, L., Hyman, R.W., Jones, T., Kahn, D., Kahn, M.L., Kalman, S., Keating, D.H., Kiss, E., Komp, C., Lelaure, V., Masuy, D., Palm, C., Peck, M.C., Pohl, T.M., Portetelle, D., Purnelle, B., Ramsperger, U., Surzycki, R., Thebault, P., Vandenbol, M., Vorholter, F.-J., Weidner, S., Wells, D.H., Wong, K., Yeh, K.-C., and Batut, J. 2001. The composite genome of the legume symbiont *Sinorhizobium meliloti*. Science . **293**(5530): 668–672. doi:10.1126/science.1060966.

Geddes, B.A. 2014. Characterization of genetic loci for carbon metabolism and competition for nodule occupancy in *Sinorhizobium meliloti*. University of Manitoba.

Geddes, B.A., González, J.E., and Oresnik, I.J. 2014. Exopolysaccharide production in response to medium acidification is correlated with an increase in competition for nodule occupancy. Mol. Plant. Microbe. Interact. **27**(12): 1307–1317. doi:10.1094/MPMI-06-14-0168-R.

Geddes, B.A., and Oresnik, I.J. 2012a. Inability to catabolize galactose leads to increased ability to compete for nodule occupancy in *Sinorhizobium meliloti*. J. Bacteriol. **194**(18): 5044–5053. doi:10.1128/JB.00982-12.

Geddes, B.A., and Oresnik, I.J. 2012b. Genetic characterization of a complex locus necessary for the transport and catabolism of erythritol, adonitol and L-arabitol in *Sinorhizobium meliloti*. Microbiology **158**(8): 2180–2191. doi:10.1099/mic.0.057877-0.

Geddes, B.A., and Oresnik, I.J. 2014. Physiology, genetics, and biochemistry of carbon metabolism in the alphaproteobacterium *Sinorhizobium meliloti*. Can. J. Microbiol. **60**(1): 491–507.

Geddes, B.A., and Oresnik, I.J. 2016. The mechanism of symbiotic nitrogen fixation. *In* The Mechanistic Benefits of Microbial Symbionts, Advances in Environmental Microbiology 2. *Edited by* C.J. Hurst. Springer International Publishing Switzerland. pp. 69–97. doi:10.1007/978-3-319-28068-4.

Geddes, B.A., Pickering, B.S., Poysti, N.J., Collins, H., Yudistira, H., and Oresnik, I.J. 2010. A locus necessary for the transport and catabolism of erythritol in *Sinorhizobium meliloti*. Microbiology **156**(10): 2970–2981. doi:10.1099/mic.0.041905-0.

Giraud, E., Moulin, L., Vallenet, D., Barbe, V., Cytryn, E., Avarre, J.-C., Jaubert, M., Simon, D., Cartieaux, F., Prin, Y., Bena, G., Hannibal, L., Fardoux, J., Kojadinovic, M., Vuillet, L., Lajus, A., Cruveiller, S., Rouy, Z., Mangenot, S., Segurens, B., Dossat, C., Franck, W.L., Chang, W.-S., Saunders, E., Bruce, D., Richardson, P., Normand, P., Dreyfus, B., Pignol, D., Stacey, G., Emerich, D., Vermeglio, A., Medigue, C., and Sadowsky, M. 2007. Legumes symbioses: absence of Nod genes in photosynthetic *Bradyrhizobia*. Science . **316**(5829): 1307–1312. doi:10.1126/science.1139548.

Glazebrook, B.J., and Walker, G.C. 1991. Genetic Techniques in *Rhizobium meliloti*. Methods Enzymol. **204**(1989): 398–418.

Glazebrook, J., and Walker, G.C. 1989. A novel exopolysaccharide can function in place of the calcofluor-binding exopolysaccharide in nodulation of alfalfa by *Rhizobium meliloti*. Cell **56**(4): 661–672. doi:10.1016/0092-8674(89)90588-6.

Glenn, S.A., Gurich, N., Feeney, M.A., and González, J.E. 2007. The ExpR/Sin quorum-sensing system controls succinoglycan production in *Sinorhizobium meliloti*. J. Bacteriol. **189**(19): 7077–7088. doi:10.1128/JB.00906-07.

Gober, J.W., and Kashket, E.V.A.R. 1987. K + regulates bacteroid-associated functions of *Bradyrhizobium*. Proc. Natl. Acad. Sci. U. S. A. **84**(1): 4650–4654.

Gómez-Gómez, L., and Boller, T. 2000. FLS2: An LRR receptor–like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. Mol. Cell **5**(6): 1003–1011. doi:10.1016/S1097-2765(00)80265-8.

González, J.E., Reuhs, B.L., and Walker, G.C. 1996. Low molecular weight EPS II of *Rhizobium meliloti* allows nodule invasion in *Medicago sativa*. Proc. Natl. Acad. Sci. U. S. A. **93**(16): 8636–8641. doi:10.1073/pnas.93.16.8636.

González, J.E., Semino, C.E., Wang, L.X., Castellano-Torres, L.E., and Walker, G.C. 1998. Biosynthetic control of molecular weight in the polymerization of the octasaccharide subunits of succinoglycan, a symbiotically important exopolysaccharide of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. U. S. A. **95**(23): 13477–82. doi:10.1073/pnas.95.23.13477.

Gorsich, S.W., Dien, B.S., Nichols, N.N., Slininger, P.J., Liu, Z.L., and Skory, C.D. 2006. Tolerance to furfural-induced stress is associated with pentose phosphate pathway genes *ZWF1, GND1, RPE1*, and *TKL1* in *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. **71**(3): 339–349. doi:10.1007/s00253-005-0142-3.

Gosselin, I., Wattraint, O., Riboul, D., Barbotin, J.N., and Portais, J.C. 2001. A deeper investigation on carbohydrate cycling in *Sinorhizobium meliloti*. FEBS Lett. **499**(1–2): 45–49. doi:10.1016/S0014-5793(01)02518-2.

Griffitts, J.S., Carlyon, R.E., Erickson, J.H., Moulton, J.L., Barnett, M.J., Toman, C.J., and Long, S.R. 2008. A *Sinorhizobium meliloti* osmosensory two-component system required for cyclic glucan export and symbiosis. Mol. Microbiol. **69**(2): 479–490. doi:10.1111/j.1365-2958.2008.06304.x.

Grochowski, L.L., Xu, H., and White, R.H. 2005. Ribose-5-phosphate biosynthesis in *Methanocaldococcus jannaschii* occurs in the absence of a pentose-phosphate pathway. J. Bacteriol. **187**(21): 7382–7389. doi:10.1128/JB.187.21.7382-7389.2005.

Gungormusler-Yilmaz, M., Shamshurin, D., Grigoryan, M., Taillefer, M., Spicer, V., Krokhin, O.V., Sparling, R., and Levin, D.B. 2014. Reduced catabolic protein expression in *Clostridium butyricum* DSM 10702 correlate with reduced 1,3-propanediol synthesis at high glycerol loading. AMB Express **4**(1): 1–14. doi:10.1186/s13568-014-0063-6.

Gust, A.A., Willmann, R., Desaki, Y., Grabherr, H.M., and Nürnberger, T. 2012. Plant LysM proteins: Modules mediating symbiosis and immunity. Trends Plant Sci. **17**(8): 495–502.

doi:10.1016/j.tplants.2012.04.003.

Haag, A.F., Baloban, M., Sani, M., Kerscher, B., Pierre, O., Farkas, A., Longhi, R., Boncompagni, E., Herouart, D., Dall'Angelo, S., Kondorosi, E., Zanda, M., Mergaert, P., and Ferguson, G.P. 2011. Protection of *Sinorhizobium* against host cysteine-rich antimicrobial peptides is critical for symbiosis. PLoS Biol. **9**(10). doi:10.1371/journal.pbio.1001169.

Hagberg, K.L., Yurgel, S.N., Mulder, M., and Kahn, M.L. 2016. Interaction between nitrogen and phosphate stress responses in *Sinorhizobium meliloti*. Front. Microbiol. **7**: 1–12. doi:10.3389/fmicb.2016.01928.

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**(4): 557–580. doi:10.1016/S0022-2836(83)80284-8.

Harrison, J., Jamet, A., Muglia, C.I., Sype, G. Van De, Aguilar, O.M., Puppo, A., and Frendo, P. 2005. Glutathione plays a fundamental role in growth and symbiotic capacity of *Sinorhizobium meliloti*. J. Bacteriol. **187**(1): 168–174. doi:10.1128/JB.187.1.168.

Hartwig, U.A., Maxwell, C.A., Joseph, C.M., and Phillips, D.A. 1990. Effects of alfalfa nod gene-inducing flavonoids on *nodABC* transcription in *Rhizobium meliloti* strains containing different *nodD* genes. J. Bacteriol. **172**(5): 2769–2773.

Hassan, S., and Mathesius, U. 2012. The role of flavonoids in root-rhizosphere signalling: Opportunities and challenges for improving plant-microbe interactions. J. Exp. Bot. **63**(9): 3429–3444. doi:10.1093/jxb/err430.

Hawkins, J.P., Geddes, B.A., and Oresnik, I.J. 2017. Common dyes used to determine bacterial polysaccharides on agar are affected by medium acidification. Can. J. Microbiol. **63**: 559–562. doi:10.1139/cjm-2016-0743.

Hawkins, J.P., and Oresnik, I.J. 2017. Characterization of a gene encoding a membrane protein that affects exopolysaccharide production and intracellular Mg^{2+} concentrations in *Ensifer meliloti*. FEMS Microbiol. Lett. **364**: 1–7. doi:10.1093/femsle/fnx061.

Heavner, M.E., Qiu, W.G., and Cheng, H. 2015. Phylogenetic co-occurrence of ExoR, ExoS, and ChvI, components of the RSI bacterial invasion switch, suggests a key adaptive mechanism regulating the transition between free-living and host-invading phases in Rhizobiales. PLoS One **10**(8): 1–22. doi:10.1371/journal.pone.0135655.

Heckel, B.C., Tomlinson, A.D., Morton, E.R., Choi, J.H., and Fuqua, C. 2014. *Agrobacterium tumefaciens* ExoR controls acid response genes and impacts exopolysaccharide synthesis, horizontal gene transfer, and virulence gene expression. J. Bacteriol. **196**(18): 3221–3233. doi:10.1128/JB.01751-14.

Heinrich, P.C., Steffen, H., Janser, P., and Wiss, O. 1972. Studies on the reconstitution of apotransketolase with thiamine pyrophosphate and analogs of the coenzyme. Eur. J. Biochem. **30**(3): 533–541. doi:10.1111/j.1432-1033.1972.tb02124.x.

Hellweg, C., Pühler, A., and Weidner, S. 2009. The time course of the transcriptomic response of

Sinorhizobium meliloti 1021 following a shift to acidic pH. BMC Microbiol. **9**(37): 37. doi:10.1186/1471-2180-9-37.

Her, G.R., Glazebrook, J., Walker, G.C., and Reinhold, V.N. 1990. Structural studies of a novel exopolysaccharide produced by a mutant of *Rhizobium meliloti* strain Rm1021. Carbohydr. Res. **198**(2): 305–312. doi:10.1016/0008-6215(90)84300-J.

Herridge, D.F., Peoples, M.B., and Boddey, R.M. 2008. Global inputs of biological nitrogen fixation in agricultural systems. Plant Soil **311**(1–2): 1–18. doi:10.1007/s11104-008-9668-3.

Hirsch, A.M., Lum, M.R., and Downie, J.A. 2001. What makes the rhizobia-legume symbiosis so special? Plant Physiol. **127**(4): 1484–1492. doi:10.1104/pp.010866.

Hirsch, P.R., and Mauchline, T.H. 2015. The Importance of the Microbial N Cycle in Soil for Crop Plant Nutrition. *In* Advances in applied microbiology. Elsevier Ltd. doi:10.1016/bs.aambs.2015.09.001.

Hoang, H.H., Gurich, N., and González, J.E. 2008. Regulation of motility by the ExpR/Sin quorum-sensing system in *Sinorhizobium meliloti*. J. Bacteriol. **190**(3): 861–871. doi:10.1128/JB.01310-07.

Horváth, B., Domonkos, Á., Kereszt, A., Szűcs, A., Ábrahám, E., Ayaydin, F., Bóka, K., Chen, Y., Chen, R., Murray, J.D., Udvardi, M.K., Kondorosi, É., and Kaló, P. 2015. Loss of the nodule-specific cysteine rich peptide, NCR169, abolishes symbiotic nitrogen fixation in the *Medicago truncatula dnf7* mutant. Proc. Natl. Acad. Sci. U. S. A.: 3–8. doi:10.1073/pnas.1500777112.

House, B.L., Mortimer, M.W., and Kahn, M.L. 2004. New recombination methods for *Sinorhizobium meliloti* genetics. Appl. Environ. Microbiol. **70**(5): 2806–2815. doi:10.1128/AEM.70.5.2806-2815.2004.

Howieson, J.G., Ewing, M.A., and D'Antuono, M.F. 1988. Selection for acid tolerance in *Rhizobium meliloti*. Plant Soil **105**(2): 179–188. doi:10.1007/BF02376781.

Hughes, J., and McCully, M.E. 1975. The use of an optical brightener in the study of plant structure. Stain Technol. **50**(5): 319–29. doi:10.3109/10520297509117082.

Hunt, S., King, B.J., Canvin, D.T., and Layzell, D.B. 1987. Steady and nonsteady state gas exchange characteristics of soybean nodules in relation to the oxygen diffusion barrier. Plant Physiol. **84**: 164–172. doi:10.1104/pp.84.1.164.

Hunt, S., and Layzell, D.B. 1993. Gas exchange of legume nodules and the regulation of nitrogenase activity. Annu. Rev. Plant Physiol. Plant Mol. Biol. **44**(1): 483–511. doi:10.1146/annurev.pp.44.060193.002411.

Iida, A., Teshiba, S., and Mizobuchi, K. 1993. Identification and characterization of the *tktB* gene encoding a second transketolase in *Escherichia coli* K-12. J. Bacteriol. **175**(17): 5375–5383.

Irigoyen, J.J., Sanchez-Diaz, M., and Emerich, D.W. 1990. Carbon metabolism enzymes of

Rhizobium meliloti cultures and bacteroids and their distribution within alfalfa nodules. Appl. Environ. Microbiol. **56**(8): 2587–2589.

Ishii, N., Nakahigashi, K., Baba, T., Robert, M., Soga, T., Kanai, A., Hirasawa, T., Naba, M., Hirai, K., Hoque, A., Ho, P.Y., Kakazu, Y., Sugawara, K., Igarashi, S., Harada, S., Masuda, T., Sugiyama, N., Togashi, T., Hasegawa, M., Takai, Y., Yugi, K., Arakawa, K., Iwata, N., Toya, Y., Nakayama, Y., Nishioka, T., Shimizu, K., Mori, H., and Tomita, M. 2007. Multiple high-throughput analyses monitor the response of *E. coli* to perturbations. Science . **316**(April): 593–598. doi:10.1126/science.1132067.

Jacob, A.I., Adham, S.A.I., Capstick, D.S., Clark, S.R.D., Spence, T., and Charles, T.C. 2008. Mutational analysis of the *Sinorhizobium meliloti* short-chain dehydrogenase/reductase family reveals substantial contribution to symbiosis and catabolic diversity. Mol. Plant-Microbe Interact. **21**(7): 979–87. doi:10.1094/MPMI-21-7-0979.

Jain, N., Rodriguez, A.C., Kimsawatde, G., Seleem, M.N., Boyle, S.M., and Sriranganathan, N. 2011. Effect of *entF* deletion on iron acquisition and erythritol metabolism by *Brucella abortus* 2308. FEMS Microbiol. Lett. **316**(1): 1–6. doi:10.1111/j.1574-6968.2010.02186.x.

Jamet, A., Mandon, K., Puppo, A., and Hérouart, D. 2007. H_2O_2 is required for optimal establishment of the *Medicago sativa/Sinorhizobium meliloti* symbiosis. J. Bacteriol. **189**(23): 8741–8745. doi:10.1128/JB.01130-07.

Jamet, A., Sigaud, S., Van de Sype, G., Puppo, A., and Herouart, D. 2003. Expression of the bacterial catalase genes during *Sinorhizobium meliloti-Medicago sativa* symbiosis and their crucial role during the infection process. Mol Plant Microbe Interact **16**(3): 217–225. doi:10.1094/MPMI.2003.16.3.217.

Jiao, J., Wu, L.J., Zhang, B., Hu, Y., Li, Y., Zhang, X.X., Guo, H.J., Liu, L.X., Chen, W.X., Zhang, Z., and Tian, C.F. 2016. MucR is required for transcriptional activation of conserved ion transporters to support nitrogen fixation of *Sinorhizobium fredii* in soybean nodules. Mol. Plant-Microbe Interact. **29**(5): 352–361. doi:10.1094/MPMI-01-16-0019-R.

Jones, D.L., Dennis, P.G., Owen, A.G., and Van Hees, P.A.W. 2003. Organic acid behavior in soils - Misconceptions and knowledge gaps. Plant Soil **248**(1–2): 31–41. doi:10.1023/A:1022304332313.

Jones, J.D.G., and Gutterson, N. 1987. An efficient mobilizable cosmid vector, pRK7813, and its use in a rapid method for marker exchange in *Pseudomonas fluorescens* strain HV37a. Gene **61**(3): 299–306. doi:10.1016/0378-1119(87)90193-4.

Jones, K.M. 2012. Increased production of the exopolysaccharide succinoglycan enhances *Sinorhizobium meliloti* 1021 symbiosis with the host plant *Medicago truncatula*. J. Bacteriol. **194**(16): 4322–4331. doi:10.1128/JB.00751-12.

Jones, K.M., Kobayashi, H., Davies, B.W., Taga, M.E., and Walker, G.C. 2007. How rhizobial symbionts invade plants: the *Sinorhizobium-Medicago* model. Nat. Rev. Microbiol. **5**(8): 619–633. doi:10.1038/nrmicro1705.

Jones, K.M., and Walker, G.C. 2008. Responses of the model legume *Medicago truncatula* to the rhizobial exopolysaccharide succinoglycan. Plant Signal. Behav. **3**(10): 888–890. doi:10.1073/pnas.0709338105.888.

Josephson, B.L., and Fraenkel, D.G. 1969. Transketolase mutants of *Escherichia coli*. J Bacteriol **100**(3): 1289–1295.

Josephson, B.L., and Fraenkel, D.G. 1974. Sugar metabolism in transketolase mutants of *Escherichia coli*. J. Bacteriol. **118**(3): 1082–1089.

Jungk, A.O. 2002. Dynamics of nutrient movement at the soil root interface. *In* Plant Roots. The hidden half, 3rd edition. *Edited by* U. Kafkafi, Y. Waisel, and A. Eshel. CRC Press. pp. 587–616. doi:doi:10.1201/9780203909423.ch35.

Kannenberg, E.L., and Brewin, N.J. 1989. Expression of a cell surface antigen from *Rhizobium leguminosarum* 3841 is regulated by oxygen and pH. J. Bacteriol. **171**(9): 4543–4548.

Kawaharada, Y., Kelly, S., Nielsen, M.W., Hjuler, C.T., Gysel, K., Muszyński, A., Carlson, R.W., Thygesen, M.B., Sandal, N., Asmussen, M.H., Vinther, M., Andersen, S.U., Krusell, L., Thirup, S., Jensen, K.J., Ronson, C.W., Blaise, M., Radutoiu, S., and Stougaard, J. 2015. Receptor-mediated exopolysaccharide perception controls bacterial infection. Nature **523**(7560): 308–312. doi:10.1038/nature14611.

Keating, D.H., Willits, M.G., and Long, S.R. 2002. A *Sinorhizobium meliloti* lipopolysaccharide mutant altered in cell surface sulfation. J. Bacteriol. **184**(23): 6681–6689. doi:10.1128/JB.184.23.6681.

Keen, N.T., Tamaki, S., Kobayashi, D., and Trollinger, D. 1988. Improved broad host range plasmids for DNA cloning in Gram-negative bacteria. Gene **70**: 191–197.

Keller, M., Roxlau, A., Weng, W.M., Schmidt, M., Quandt, J., Niehaus, K., Jording, D., Arnold, W., and Puhler, A. 1995. Molecular analysis of the *Rhizobium meliloti mucR* gene regulating the biosynthesis of the exopolysaccharides succinoglycan and galactoglucan. Mol Plant Microbe Interact **8**(2): 267–277.

Kenyon, W.J., and Buller, C.S. 2002. Structural analysis of the curdlan-like exopolysaccharide produced by *Cellulomonas flavigena KU*. J Ind Microbiol Biotechnol **29**(4): 200–203. doi:10.1038/sj.jim.7000277\r7000277 [pii].

Kereszt, A., Kiss, E., Reuhs, B.L., Carlson, R.W., Kondorosi, A., and Putnoky, P. 1998. Novel *rkp* gene clusters of *Sinorhizobium meliloti* involved in capsular polysaccharide production and invasion of the symbiotic nodule: The *rkpK* gene encodes a UDP-glucose dehydrogenase. J. Bacteriol. **180**(20): 5426–5431.

Kim, M., Chen, Y., Xi, J., Waters, C., Chen, R., and Wang, D. 2015. An antimicrobial peptide essential for bacterial survival in the nitrogen-fixing symbiosis. Proc. Natl. Acad. Sci. **112**(49): 15238–15243. doi:10.1073/pnas.1500123112.

Kim, S.-Y., Sivaguru, M., and Stacey, G. 2006. Extracellular ATP in plants. Visualization, localization, and analysis of physiological significance in growth and signaling. Plant

Physiol. **142**(3): 984–992. doi:10.1104/pp.106.085670.

Király, Z., El-Zahaby, H.M., and Klement, Z. 1997. Role of extracellular polysaccharide (EPS) slime of plant pathogenic bacteria in protecting cells to reactive oxygen species. J. Phytopathol. **145**(2–3): 59–68. doi:10.1111/j.1439-0434.1997.tb00365.x.

Kneen, B.E., and Larue, T.A. 1983. Congo red absorption by *Rhizobium leguminosarum*. Appl. Environ. Microbiol. **45**(1): 1–4.

Knoop, V., Groth-Malonek, M., Gebert, M., Eifler, K., and Weyand, K. 2005. Transport of magnesium and other divalent cations: Evolution of the 2-TM-GxN proteins in the MIT superfamily. Mol. Genet. Genomics **274**(3): 205–216. doi:10.1007/s00438-005-0011-x.

Kochetov, G.A., and Philippov, P.P. 1970. Calcium: Cofactor of transketolase from baker's yeast. Biochem. Biophys. Res. Commun. **38**(5): 930–933. doi:10.1016/0006-291X(70)90810-7.

Kochetov, G.A., and Solovjeva, O.N. 2014. Structure and functioning mechanism of transketolase. Biochim. Biophys. Acta - Proteins Proteomics **1844**(9): 1608–1618. doi:10.1016/j.bbapap.2014.06.003.

Kouchi, H., Shimomura, K., Hata, S., Hirota, A., Wu, G., Kumagai, H., Tajima, S., Suganuma, N., Suzuki, A., Aoki, T., Hayashi, M., Yokoyama, T., Ohyama, T., Asamizu, E., Kuwata, C., Shibata, D., and Tabata, S. 2004. Large-scale analysis of gene expression profiles during early stages of root nodule formation in a model legume, *Lotus japonicus*. DNA Res. **11**(4): 263–274. doi:10.1093/dnares/11.4.263.

Krol, E., and Becker, A. 2004. Global transcriptional analysis of the phosphate starvation response in *Sinorhizobium meliloti* strains 1021 and 2011. Mol. Genet. Genomics **272**(1): 1–17. doi:10.1007/s00438-004-1030-8.

Kroll, R.G., and Booth, I.R. 1981. The role of potassium transport in the generation of a pH gradient in *Escherichia coli*. Biochem. J. **198**(3): 691–8. doi:10.1111/j.1574-6968.1983.tb00172.x.

Krüger, A., Grüning, N.-M., Wamelink, M.M.C., Kerick, M., Kirpy, A., Parkhomchuk, D., Bluemlein, K., Schweiger, M.-R., Soldatov, A., Lehrach, H., Jakobs, C., and Ralser, M. 2011. The pentose phosphate pathway is a metabolic redox sensor and regulates transcription during the antioxidant response. Antioxid. Redox Signal. **15**(2): 311–324. doi:10.1089/ars.2010.3797.

Leach, A.M., Galloway, J.N., Bleeker, A., Erisman, J.W., Kohn, R., and Kitzes, J. 2012. A nitrogen footprint model to help consumers understand their role in nitrogen losses to the environment. Environ. Dev. **1**(1): 40–66. doi:10.1016/j.envdev.2011.12.005.

Lee, C.R., Cho, S.H., Yoon, M.J., Peterkofsky, A., and Seok, Y.J. 2007. *Escherichia coli* enzyme IIANtr regulates the K+ transporter TrkA. Proc Natl Acad Sci U S A **104**(10): 4124–4129. doi:10.1073/pnas.0609897104.

Lehman, A.P., and Long, S.R. 2013. Exopolysaccharides from *Sinorhizobium meliloti* can

protect against H_2O_2-dependent damage. J. Bacteriol. **195**(23): 5362–5369. doi:10.1128/JB.00681-13.

Leigh, J.A., and Lee, C.C. 1988. Characterization of polysaccharides of *Rhizobium meliloti exo* mutants that form ineffective nodules. J. Bacteriol. **170**(8): 3327–3332.

Leigh, J.A., Reed, J.W., Hanks, J.F., Hirsch, A.M., and Walker, G.C. 1987. *Rhizobium meliloti* mutants that fail to succinylate their calcofluor-binding exopolysaccharide are defective in nodule invasion. Cell **51**(4): 579–587. doi:10.1016/0092-8674(87)90127-9.

Leigh, J.A., Signer, E.R., and Walker, G.C. 1985. Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. Proc. Natl. Acad. Sci. U. S. A. **82**(18): 6231–6235. doi:10.1073/pnas.82.18.6231.

Leitner, M., Kaiser, R., Rasmussen, M.O., Driguez, H., Boland, W., and Mithöfer, A. 2008. Microbial oligosaccharides differentially induce volatiles and signalling components in *Medicago truncatula*. Phytochemistry **69**(10): 2029–2040. doi:10.1016/j.phytochem.2008.04.019.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Prome, J.C., and Denarie, J. 1990. Symbiotic host-specificity of Rhizobium meliloti is determined by a sulfated and acylated glucosamine oligosaccharides signal. Nature **344**: 781–784.

Lever, M. 1972. A new reaction for colorimetric determination of carbohydrates. Anal. Biochem. **47**(1): 273–279. doi:10.1016/0003-2697(72)90301-6.

Li, L., Jia, Y., Hou, Q., Charles, T.C., Nester, E.W., and Pan, S.Q. 2002. A global pH sensor: *Agrobacterium* sensor protein ChvG regulates acid-inducible genes on its two chromosomes and Ti plasmid. Proc. Natl. Acad. Sci. U. S. A. **99**(19): 12369–74. doi:10.1073/pnas.192439499.

Liang, Y., Cao, Y., Tanaka, K., Thibivilliers, S., Wan, J., Choi, J., Qiu, J., and Stacey, G. 2013. Nonlegumes respond to Rhizobial nod factors by suppressing the innate immune response. Science . (September): 1384–1387. doi:10.1126/science.1242736.

Limpens, E., Franken, C., Smit, P., Willemse, J., Bisseling, T., and Geurts, R. 2003. LysM Domain Receptor Kinases Regulating Rhizobial Nod Factor-Induced Infection. Science . **302**(5645): 630–633. doi:10.1126/science.1090074.

Limpens, E., van Zeijl, A., and Geurts, R. 2015. Lipochitooligosaccharides Modulate Plant Host Immunity to Enable Endosymbioses. Annu. Rev. Phytopathol. **53**(1): 311–334. doi:10.1146/annurev-phyto-080614-120149.

Lloret, J., Wulff, B.B.H., Rubio, J.M., Downie, J.A., Bonilla, I., and Rivilla, R. 1998. Exopolysaccharide II production is regulated by salt in the halotolerant strain *Rhizobium meliloti* EFB1. Appl. Environ. Microbiol. **64**(3): 1024–1028.

Loaëc, M., Olier, R., and Guezennec, J. 1998. Chelating properties of bacterial exopolysaccharides from deep-sea hydrothermal vents. Carbohydr. Polym. **35**(5): 1171–1179. doi:10.1016/S0144-8617(97)00109-4.

Lohar, D.P., Haridas, S., Gantt, J.S., and VandenBosch, K.A. 2007. A transient decrease in reactive oxygen species in roots leads to root hair deformation in the legume-rhizobia symbiosis. New Phytol. **173**(1): 39–49. doi:10.1111/j.1469-8137.2006.01901.x.

Lohar, D.P., Sharopova, N., Endre, G., Peñuela, S., Samac, D., Town, C., Silverstein, K. a T., and VandenBosch, K. a. 2006. Transcript analysis of early nodulation events in *Medicago truncatula*. Plant Physiol. **140**(1): 221–234. doi:10.1104/pp.105.070326.1.

Long, S.R., Reed, J.W., Himawan, J., and Walker, G.C. 1988. Genetic analysis of a cluster of genes required for synthesis of the calcofluor-binding exopolysaccharide of *Rhizobium meliloti*. J. Bacteriol. **170**(9): 4239–4248.

Lowendorf, H.S., Baya, A.M., and Alexander, M. 1981. Survival of *Rhizobium* in acid soils. Appl. Environ. Microbiol. **42**(6): 951–957.

Lu, H.Y., Luo, L., Yang, M.H., and Cheng, H.-P. 2012. *Sinorhizobium meliloti* ExoR is the target of periplasmic proteolysis. J. Bacteriol. **194**(15): 4029–4040. doi:10.1128/JB.00313-12.

Marketon, M.M., Glenn, S. a, Eberhard, A., and González, J.E. 2003. Quorum Sensing Controls Exopolysaccharide Production in *Sinorhizobium meliloti*. J. Bacteriol. **185**(1): 325–331. doi:10.1128/JB.185.1.325.

Maroti, G.G., Kereszt, A., Kondorosi, E., and Mergaert, P. 2011. Natural roles of antimicrobial peptides in microbes, plants and animals. Res. Microbiol. **162**(4): 363–374. doi:10.1016/j.resmic.2011.02.005.

Marroquí, S., Zorreguieta, A., Santamaría, C., Temprano, F., Soberón, M., Megías, M., and Downie, J.A. 2001. Enhanced symbiotic performance by *Rhizobium tropici* glycogen synthase mutants. J. Bacteriol. **183**(3): 854–864. doi:10.1128/JB.183.3.854-864.2001.

Marschner, H. 1991. Mechanisms of adaptation of plants to acid soils. Plant Soil **134**(1): 1–20. doi:10.1007/BF00010712.

Martinez-Abarca, F., Herrera-Cervera, J. a, Bueno, P., Sanjuan, J., Bisseling, T., and Olivares, J. 1998. Involvement of salicylic acid in the establishment of the *Rhizobium meliloti* - Alfalfa symbiosis. Mol. Plant-Microbe Interact. **11**(2): 153–155. doi:10.1094/MPMI.1998.11.2.153.

Martínez-De Drets, G., and Arias, A. 1972. Enzymatic basis for differentiation of *Rhizobium* into fast and slow-growing groups. J. Bacteriol. **109**(1): 467–470.

McLaggan, D., Jones, M.A., Gouesbet, G., Levina, N., Lindey, S., Epstein, W., and Booth, I.R. 2002. Analysis of the *kefA2* mutation suggests that KefA is a cation-specific channel involved in osmotic adaptation in *Escherichia coli*. Mol. Microbiol. **43**(2): 521–536. doi:10.1046/j.1365-2958.2002.02764.x.

Meade, H.M., Long, S.R., Ruvkun, G.B., Brown, S.E., and Ausubel, F.M. 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. **149**(1): 114–122.

Medeot, D.B., Rivero, M.R., Cendoya, E., Contreras-Moreira, B., Rossi, F.A., Fischer, S.E., Becker, A., and Jofré, E. 2016. *Sinorhizobium meliloti* low molecular mass phosphotyrosine phosphatase SMc02309 modifies activity of the UDP-glucose pyrophosphorylase ExoN involved in succinoglycan biosynthesis. Microbiology **162**(3): 552–563. doi:10.1099/mic.0.000239.

Mendis, H.C., Madzima, T.F., Queiroux, C., and Jones, K.M. 2016. Function of succinoglycan polysaccharide in *Sinorhizobium meliloti* host plant invasion depends on succinylation, not molecular weight. MBio **7**(3): e00606–e00616. doi:10.1128/mBio.00606-16.

Mendrygal, K.E., and González, J.E. 2000. Environmental regulation of exopolysaccharide production in *Sinorhizobium meliloti*. J. Bacteriol. **182**(3): 599–606. doi:10.1128/JB.182.3.599-606.2000.

Mera, S.L., and Davies, J.D. 1984. Differential Congo red staining: the effects of pH, non-aqueous solvents and the substrate. Histochem. J. **16**(2): 195–210. doi:10.1007/BF01003549.

Mergaert, P., Nikovics, K., Kelemen, Z., Maunoury, N., Vaubert, D., Kondorosi, A., and Kondorosi, E. 2003. A novel family in *Medicago truncatula* consisting of more than 300 nodule-specific genes coding for small, secreted polypeptides with conserved cysteine motifs. Plant Physiol. **132**(1): 161–173. doi:10.1104/pp.102.018192.

Mergaert, P., Uchiumi, T., Alunni, B., Evanno, G., Cheron, A., Catrice, O., Mausset, A.-E., Barloy-Hubler, F., Galibert, F., Kondorosi, A., and Kondorosi, E. 2006. Eukaryotic control on bacterial cell cycle and differentiation in the Rhizobium-legume symbiosis. Proc. Natl. Acad. Sci. U. S. A. **103**(13): 5230–5235. doi:10.1073/pnas.0600912103.

Miller-Williams, M., Loewen, P.C., and Oresnik, I.J. 2006. Isolation of salt-sensitive mutants of *Sinorhizobium meliloti* strain Rm1021. Microbiology **152**(7): 2049–2059. doi:10.1099/mic.0.28937-0.

Miller, K.J., Gore, R.S., and Benesi, A.J. 1988. Phosphoglycerol substituents present on the cyclic beta-1,2-glucans of *Rhizobium meliloti* 1021 are derived from phosphatidylglycerol. J. Bacteriol. **170**(10): 4569–4575.

Miller, K.J., Kennedy, E.P., and Reinhold, V.N. 1986. Osmotic adaptation by gram-negative bacteria: possible role for periplasmic oligosaccharides. Science . **231**(4733): 48–51. doi:10.1126/science.3941890.

Miller, K.J., and Wood, J.M. 1996. Osmoadaptation by rhizosphere bacteria. Annu. Rev. Microbiol. **50**: 101–136. doi:10.1146/annurev.micro.50.1.101.

Mitra, R.M., Gleason, C. a, Edwards, A., Hadfield, J., Downie, J.A., Oldroyd, G.E.D., and Long, S.R. 2004. A Ca2+/calmodulin-dependent protein kinase required for symbiotic nodule development: Gene identification by transcript-based cloning. Proc. Natl. Acad. Sci. U. S. A. **101**(13): 4701–4705. doi:10.1073/pnas.0400595101.

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami,

N., Kaku, H., and Shibuya, N. 2007. CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. Proc. Natl. Acad. Sci. U. S. A. **104**(49): 19613–8. doi:10.1073/pnas.0705147104.

Moncrief, M.B.C., and Maguire, M.E. 1999. Magnesium transport in prokaryotes. J. Biol. Inorg. Chem. **4**(5): 523–527. doi:10.1007/s007750050374.

Mori, B., and Bellani, L.M. 1996. Differential staining for cellulosic and modified plant cell walls. Biotech. Histochem. **71**(2): 71–72. doi:10.3109/10520299609117136.

Morieri, G., Martinez, E.A., Jarynowski, A., Driguez, H., Morris, R., Oldroyd, G.E.D., and Downie, J.A. 2013. Host-specific Nod-factors associated with *Medicago truncatula* nodule infection differentially induce calcium influx and calcium spiking in root hairs. New Phytol. **200**(3): 656–662. doi:10.1111/nph.12475.

Morris, A.C., and Djordjevic, M.A. 2006. The *Rhizobium leguminosarum biovar trifolii* ANU794 induces novel developmental responses on the subterranean clover cultivar Woogenellup. Mol. plant-microbe Interact. **19**(5): 471–479. doi:10.1094/MPMI-19-0471.

Morris, D.L. 1948. Quantitative determination of carbohydrates with Dreywood's anthrone reagent. Science . **107**(2775): 254–255.

Morris, J., and Gonzalez, J.E. 2009. The novel genes *emmABC* are associated with exopolysaccharide production, motility, stress adaptation, and symbiosis in *Sinorhizobium meliloti*. J. Bacteriol. **191**(19): 5890–5900. doi:10.1128/JB.00760-09.

Mueller, K., and Gonzalez, J.E. 2011. Complex regulation of symbiotic functions is coordinated by *mucR* and quorum sensing in *Sinorhizobium meliloti*. J. Bacteriol. **193**(2): 485–496. doi:10.1128/JB.01129-10.

Muglia, C.I., Grasso, D.H., and Aguilar, O.M. 2007. *Rhizobium tropici* response to acidity involves activation of glutathione synthesis. Microbiology **153**(4): 1286–1296. doi:10.1099/mic.0.2006/003483-0.

Mulcahy, H., and Lewenza, S. 2011. Magnesium limitation is an environmental trigger of the *Pseudomonas aeruginosa* biofilm lifestyle. PLoS One **6**(8): e23307. doi:10.1371/journal.pone.0023307.

Müller, M.G., Forsberg, L.S., and Keating, D.H. 2009. The *rkp*-1 cluster is required for secretion of Kdo homopolymeric capsular polysaccharide in *Sinorhizobium meliloti* strain Rm1021. J. Bacteriol. **191**(22): 6988–7000. doi:10.1128/JB.00466-09.

Muñoz, J.A., Coronado, C., Pérez-Hormaeche, J., Kondorosi, A., Ratet, P., and Palomares, a J. 1998. MsPG3, a *Medicago sativa* polygalacturonase gene expressed during the alfalfa-*Rhizobium meliloti* interaction. Proc. Natl. Acad. Sci. **95**: 9687–9692. doi:10.1073/pnas.95.16.9687.

Mylona, P., Pawlowski, K., and Bisseling, T. 1995. Symbiotic nitrogen fixation. Plant Cell **7**: 869–885. doi:10.1097/00010694-199511000-00009.

Nagpal, P., Khanuja, S.P., and Stanfield, S.W. 1992. Suppression of the ndv mutant phenotype of *Rhizobium meliloti* by cloned *exo* genes. Mol Microbiol **6**(4): 479–488.

Nakagawa, T., Kaku, H., Shimoda, Y., Sugiyama, A., Shimamura, M., Takanashi, K., Yazaki, K., Aoki, T., Shibuya, N., and Kouchi, H. 2011. From defense to symbiosis: Limited alterations in the kinase domain of LysM receptor-like kinases are crucial for evolution of legume-Rhizobium symbiosis. Plant J. **65**(2): 169–180. doi:10.1111/j.1365-313X.2010.04411.x.

O'Hara, G.W., Goss, T.J., Dilworth, M.J., and Glenn, A.R. 1989. Maintenance of Intracellular pH and Acid Tolerance in *Rhizobium meliloti*. Appl. Environ. Microbiol. **55**(8): 1870–1876.

O'Toole, G.A., and Kolter, R. 1998. Initation of biofilm formation in *Pseudomans fluorescens* WC365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Mol. Microbiol. **28**(3): 449–461.

Okazaki, S., Kaneko, T., Sato, S., and Saeki, K. 2013. Hijacking of leguminous nodulation signaling by the rhizobial type III secretion system. Proc. Natl. Acad. Sci. U. S. A. **110**(42): 17131–6. doi:10.1073/pnas.1302360110.

Oldroyd, G.E.D. 2013. Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. Nat. Rev. Microbiol. **11**(4): 252–63. doi:10.1038/nrmicro2990.

Oldroyd, G.E.D., and Downie, J.A. 2008. Coordinating nodule morphogenesis with rhizobial infection in legumes. Annu. Rev. Plant Biol. **59**: 519–46. doi:10.1146/annurev.arplant.59.032607.092839.

Oldroyd, G.E.D., Murray, J.D., Poole, P.S., and Downie, J.A. 2011. The rules of engagement in the legume-rhizobial symbiosis. Annu. Rev. Genet. **45**: 119–44. doi:10.1146/annurev-genet-110410-132549.

Oresnik, I.J., Charles, T.C., and Finan, T.M. 1994. Second site mutations specifically suppress the Fix- phenotype of *Rhizobium meliloti ndvF* mutations on alfalfa: Identification of a conditional *ndvF*-dependent mucoid colony phenotype. Genetics **136**(4): 1233–1243.

Oresnik, I.J., Liu, S.L., Yost, C.K., and Hynes, M.F. 2000. Megaplasmid pRme2011a of *Sinorhizobium meliloti* is not required for viability. J. Bacteriol. **182**(12): 3582–3586. doi:10.1128/JB.182.12.3582-3586.2000.

Oresnik, I.J., Pacarynuk, L.A., O'Brien, S.A.P., Yost, C.K., and Hynes, M.F. 1998. Plasmid-encoded catabolic genes in *Rhizobium leguminosarum bv. trifolii*: evidence for a plant-inducible rhamnose locus involved in competition for nodulation. Mol. Plant. Microbe. Interact. **11**(12): 1175–1185. doi:10.1094/MPMI.1998.11.12.1175.

Pankhurst, C.E., and Bigs, D.R. 1980. Sensitivity of *Rhizobium* to selected isoflavonoids. Can. J. Microbiol. **26**: 542–545. doi:10.5194/acpd-13-6851-2013.

Pauly, N., Pucciariello, C., Mandon, K., Innocenti, G., Jamet, A., Baudouin, E., Hérouart, D., Frendo, P., and Puppo, A. 2006. Reactive oxygen and nitrogen species and glutathione: Key

players in the legume-Rhizobium symbiosis. J. Exp. Bot. **57**(8): 1769–1776. doi:10.1093/jxb/erj184.

Peer, W.A. 2001. Flavonoid accumulation patterns of transparent testa mutants of *Arabidopsis*. Plant Physiol. **126**(2): 536–548. doi:10.1104/pp.126.2.536.

Pellock, B.J., Cheng, H., and Walker, G.C. 2000. Alfalfa root nodule invasion efficiency is dependent on *Sinorhizobium meliloti* polysaccharides. J. Bacteriol. **182**(15): 4310–4318. doi:10.1128/JB.182.15.4310-4318.2000.Updated.

Penterman, J., Abo, R.P., De Nisco, N.J., Arnold, M.F.F., Longhi, R., Zanda, M., and Walker, G.C. 2014. Host plant peptides elicit a transcriptional response to control the *Sinorhizobium meliloti* cell cycle during symbiosis. Proc. Natl. Acad. Sci. U. S. A. **111**(9): 3561–6. doi:10.1073/pnas.1400450111.

Pérez, J., Jiménez-Zurdo, J.I., Martínez-Abarca, F., Millán, V., Shimkets, L.J., and Muñoz-Dorado, J. 2014. Rhizobial galactoglucan determines the predatory pattern of *Myxococcus xanthus* and protects *Sinorhizobium meliloti* from predation. Environ. Microbiol. **16**(7): 2341–2350. doi:10.1111/1462-2920.12477.

Peters, N.K., Frost, J.W., Long, S.R., Peters, N.K., Frost, J.W., and Long, S.R. 1986. A Plant Flavone , Luteolin , Induces Expression of *Rhizobium meliloti* Nodulation Genes. Science . **233**(4767): 977–980.

Peters, N.K., and Long, S.R. 1988. Alfalfa Root Exudates and Compounds which Promote or Inhibit Induction of *Rhizobium meliloti* Nodulation Genes. Plant Physiol. **88**(2): 396–400. doi:10.1104/pp.88.2.396.

Pickering, B.S., and Oresnik, I.J. 2008. Formate-dependent autotrophic growth in *Sinorhizobium meliloti*. J. Bacteriol. **190**(19): 6409–6418. doi:10.1128/JB.00757-08.

Pierre, O., Engler, G., Hopkins, J., Brau, F., Boncompagni, E., and Hérouart, D. 2013. Peribacteroid space acidification: A marker of mature bacteroid functioning in *Medicago truncatula* nodules. Plant, Cell Environ. **36**(11): 2059–2070. doi:10.1111/pce.12116.

Platt, R., Drescher, C., Park, S., and Phillips, G.J. 2000. Genetic system for reversible integration of DNA constructs and *lacZ* gene fusions into the *Escherichia coli* chromosome. Plasmid **23**: 12–23. doi:10.1006/plas.1999.1433.

Portais, J.C., Tavernier, P., Gosselin, I., and Barbotin, J.N. 1999. Cyclic organization of the carbohydrate metabolism in *Sinorhizobium meliloti*. Eur. J. Biochem. **265**(1): 473–480. doi:10.1046/j.1432-1327.1999.00778.x.

Povolo, S., and Casella, S. 2000. A critical role for *aniA* in energy-carbon flux and symbiotic nitrogen fixation in *Sinorhizobium meliloti*. Arch. Microbiol. **174**(1–2): 42–49. doi:10.1007/s002030000171.

Poysti, N.J., Loewen, E.D.M., Wang, Z., and Oresnik, I.J. 2007. *Sinorhizobium meliloti* pSymB carries genes necessary for arabinose transport and catabolism. Microbiology **153**(3): 727–736. doi:10.1099/mic.0.29148-0.

Poysti, N.J., and Oresnik, I.J. 2007. Characterization of *Sinorhizobium meliloti* triose phosphate isomerase genes. J. Bacteriol. **189**(9): 3445–3451. doi:10.1128/JB.01707-06.

Puppo, A., Pauly, N., Boscari, A., Mandon, K., and Brouquisse, R. 2013. Hydrogen peroxide and nitric oxide: key regulators of the Legume-Rhizobium and mycorrhizal symbioses. Antioxid. Redox Signal. **18**(16): 2202–19. doi:10.1089/ars.2012.5136.

Ramu, S.K., Peng, H.M., and Cook, D.R. 2002. Nod factor induction of reactive oxygen species production is correlated with expression of the early nodulin gene *rip1* in *Medicago truncatula*. Mol. Plant-Microbe Interact. **15**(6): 522–528. doi:10.1094/MPMI.2002.15.6.522.

Reeve, W.G., Dilworth, M.J., Tiwari, R.P., and Glenn, A.R. 1997. Regulation of exopolysaccharide production in *Rhizobium leguminosarum biovar viciae* WSM710 involves *exoR*. Microbiology **143**(6): 1951–1958.

Reichhardt, C., McCrate, O.A., Zhou, X., Lee, J., Thongsomboon, W., and Cegelski, L. 2016. Influence of the amyloid dye Congo red on curli, cellulose, and the extracellular matrix in *E. coli* during growth and matrix purification. Anal. Bioanal. Chem.: 1–9. Analytical and Bioanalytical Chemistry. doi:10.1007/s00216-016-9868-2.

Reinhold, B.B., Chan, S.Y., Reuber, T.L., Marra, A., Walker, G.C., and Reinhold, V.N. 1994. Detailed structural characterization of succinoglycan, the major exopolysaccharide of *Rhizobium meliloti* Rm1021. J. Bacteriol. **176**(7): 1997–2002.

Ren, D., Liu, Y., Yang, K.-Y., Han, L., Mao, G., Glazebrook, J., and Zhang, S. 2008. A fungal-responsive MAPK cascade regulates phytoalexin biosynthesis in *Arabidopsis*. Proc. Natl. Acad. Sci. U. S. A. **105**(14): 5638–5643. doi:10.1073/pnas.0711301105.

Ren, D., Yang, H., and Zhang, S. 2002. Cell death mediated by MAPK is associated with hydrogen peroxide production in *Arabidopsis*. J. Biol. Chem. **277**(1): 559–565. doi:10.1074/jbc.M109495200.

Reuber, T.L., Long, S., and Walker, G.C. 1991. Regulation of *Rhizobium meliloti exo* genes in free-living cells and in planta examined by using Tn*phoA* fusions. J. Bacteriol. **173**(2): 426–434.

Reuber, T.L., Urzainqui, A., Glazebrook, J., Reed, J.W., and Walker, G.C. 1990. Genetic analyses and manipulations of *Rhizobium meliloti* exopolysaccharides. Nov. Biodegrad. Microb. Polym. **12**(4): 285–294.

Reuber, T.L., and Walker, G.C. 1993. Biosynthesis of succinoglycan, a symbiotically important exopolysaccharide of *Rhizobium meliloti*. Cell **74**(2): 269–280. doi:10.1016/0092-8674(93)90418-P.

Riccillo, P.M., Muglia, C.I., De Bruijn, F.J., Roe, A.J., Booth, I.R., and Aguilar, O.M. 2000. Glutathione is involved in environmental stress responses in *Rhizobium tropici*, including acid tolerance. J. Bacteriol. **182**(6): 1748–1753. doi:10.1128/JB.182.6.1748-1753.2000.

Rinaudi, L., Fujishige, N.A., Hirsch, A.M., Banchio, E., Zorreguieta, A., and Giordano, W. 2006.

Effects of nutritional and environmental conditions on *Sinorhizobium meliloti* biofilm formation. Res. Microbiol. **157**(9): 867–875. doi:10.1016/j.resmic.2006.06.002.

Rinaudi, L. V., and Giordano, W. 2010. An integrated view of biofilm formation in rhizobia. FEMS Microbiol. Lett. **304**(1): 1–11. doi:10.1111/j.1574-6968.2009.01840.x.

Rinaudi, L. V., and Gonzalez, J.E. 2009. The low-molecular-weight fraction of exopolysaccharide II from *Sinorhizobium meliloti* is a crucial determinant of biofilm formation. J. Bacteriol. **191**(23): 7216–7224. doi:10.1128/JB.01063-09.

Robledo, M., Rivera, L., Jiménez-Zurdo, J.I., Rivas, R., Dazzo, F., Velázquez, E., Martínez-Molina, E., Hirsch, A.M., and Mateos, P.F. 2012. Role of Rhizobium endoglucanase CelC2 in cellulose biosynthesis and biofilm formation on plant roots and abiotic surfaces. Microb. Cell Fact. **11**: 1-12-2859-11–125. doi:10.1186/1475-2859-11-125.

Romani, A. 2011. Cellular magnesium homeostasis. Arch Biochem biophys **141**(4): 520–529. doi:10.1016/j.surg.2006.10.010.Use.

Rumley, M.K., Therisod, H., Weissborn, A.C., and Kennedy, E.P. 1982. Mechanisms of regulation of the biosynthesis of membrane-derived oligosaccharides in *Escherichia coli*. J. Biol. Chem. **267**(17): 11806–11810. doi:10.1073/pnas.79.4.1092.

Sambrook, J., Fritsch, E.F., and Mantiatis, T.L. 1989. Molecular cloning: a laboratory manual. *In* 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Sambrook, J., and Russell, D.W. 2001. Molecular cloning: a laboratory manual. *In* 3rd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Samland, A.K., and Sprenger, G.A. 2009. Transaldolase: From biochemistry to human disease. Int. J. Biochem. Cell Biol. **41**(7): 1482–1494. doi:10.1016/j.biocel.2009.02.001.

Santos, R., Hérouart, D., Sigaud, S., Touati, D., and Puppo, A. 2001. Oxidative Burst in Alfalfa-*Sinorhizobium meliloti* Symbiotic Interaction. Mol. Plant-Microbe Interact. **14**(1): 86–89. doi:10.1094/MPMI.2001.14.1.86.

Schenk, G., Duggleby, R.G., and Nixon, P.F. 1998. Properties and functions of the thiamin diphosphate dependent enzyme transketolase. Int. J. Biochem. Cell Biol. **30**(12): 1297–1318. doi:10.1016/S1357-2725(98)00095-8.

Schopfer, C.R. 1999. The Male Determinant of Self-Incompatibility in *Brassica*. Science . **286**(5445): 1697–1700. doi:10.1126/science.286.5445.1697.

Schroeder, B.K., House, B.L., Mortimer, M.W., Yurgel, S.N., Maloney, S.C., Ward, K.L., and Kahn, M.L. 2005. Development of a functional genomics platform for *Sinorhizobium meliloti*: construction of an ORFeome. Appl. Environ. Microbiol. **71**(10): 5858–5864. doi:10.1128/AEM.71.10.5858.

Shai, Y. 2002. Mode of action of membrane active antimicrobial peptides. Biopolym. - Pept. Sci. Sect. **66**(4): 236–248. doi:10.1002/bip.10260.

Sharma, P.K., Fu, J., Spicer, V., Krokhin, O. V., Cicek, N., Sparling, R., and Levin, D.B. 2016. Global changes in the proteome of *Cupriavidus necator* H16 during poly-(3-hydroxybutyrate) synthesis from various biodiesel by-product substrates. AMB Express **6**(36): DOI 10.1186/s13568-016-0206-z. doi:10.1186/s13568-016-0206-z.

Shimizu, K. 2013. Regulation systems of bacteria such as Escherichia coli in response to nutrient limitation and environmental stresses. Metabolites **4**(1): 1–35. doi:10.3390/metabo4010001.

Skorupska, A., Janczarek, M., Marczak, M., Mazur, A., and Król, J. 2006. Rhizobial exopolysaccharides: genetic control and symbiotic functions. Microb. Cell Fact. **5**(7): 1–19. doi:10.1186/1475-2859-5-7.

Smith, L.T., Allaith, A.A., and Smith, G.M. 1994. Mechanism of osmotically regulated N-acetylglutaminylglutamine amide production in *Rhizobium meliloti*. Plant Soil **161**(1): 103–108. doi:10.1007/BF02183090.

Sorroche, F.G., Rinaudi, L. V., Zorreguieta, Á˜., and Giordano, W. 2010. EPS II-dependent autoaggregation of *Sinorhizobium meliloti* planktonic cells. Curr. Microbiol. **61**(5): 465–470. doi:10.1007/s00284-010-9639-9.

Spaink, H.P. 2000. Root nodulation and infection factors produced by rhizobial bacteria. Annu. Rev. Microbiol. **54**(1): 257–288.

Sperry, J.F., and Robertson, D.C. 1975. Inhibition of growth by erythritol catabolism in *Brucella abortus*. J. Bacteriol. **124**(1): 391–397.

Stasinopoulos, S.J., Fisher, P.R., Stone, B.A., and Stanisich, V.A. 1999. Detection of two loci involved in (1→3)-β-glucan (curdlan) biosynthesis by *Agrobacterium sp.* ATCC31749, and comparative sequence analysis of the putative curdlan synthase gene. Glycobiology **9**(1): 31–41. doi:10.1093/glycob/9.1.31.

Stincone, A., Prigione, A., Cramer, T., Wamelink, M.M.C., Campbell, K., Cheung, E., Olin-Sandoval, V., Gruning, N., Kruger, A., Alam, M.T., Keller, M.A., Breitenbach, M., Brindle, K.M., Rabinowitz, J.D., and Ralser, M. 2015. The return of metabolism: biochemistry and physiology of the pentose phosphate pathway. Biol Rev Camb Philos Soc **90**(3): 927–963. doi:10.1002/aur.1474.

Stock, J.B., Ninfa, A.J., and Stock, A.M. 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. **53**(4): 450–490.

Stone, B. 2005. Callose and related glucans. Encycl. Life Sci. (Jan): 1–15. doi:10.1038/npg.els.0004111.

Stone, B.A., Evans, N.A., Bonig, I., and Clarke, A.E. 1984. The application of Sirofluor, a chemically defined fluorochrome from aniline blue for the histochemical detection of callose. Protoplasma **122**(3): 191–195. doi:10.1007/BF01281696.

Stowers, M.D. 1985. Carbon metabolism in *Rhizobium* species. Ann. Rev. Microbiol **39**: 89–108.

Sutherland, I. 1997. Microbial exopolysaccharides-structural subtleties and their consequences.

Pure Appl. Chem. **69**(9): 1911–1917.

Swart, S., Lugtenberg, B.J.J., Smit, G., and Kijne, J.W. 1994. Rhicadhesin-mediated attachment and virulence of an *Agrobacterium tumefaciens chvB* mutant can be restored by growth in a highly osmotic medium. J. Bacteriol. **176**(12): 3816–3819.

Szewczuk-Karpisz, K., Wisniewska, M., Pac, M., Choma, A., and Komaniecka, I. 2014. *Sinorhizobium meliloti* 1021 exopolysaccharide as a flocculant improving chromium(III) oxide removal from aqueous solutions. Water Air Soil Pollut **225**(8): 2052–2067. doi:10.1007/s11270-014-2052-4.

Tailor, R., Acland, D.P., Attenborough, S., Cammue, B.P.A., Evans, I.J., Osborn, R.W., Ray, J.A., Rees, S.B., and Broekaert, W.F. 1997. A novel family of small cysteine-rich antimicrobial peptides from seed of *Impatiens balsamina* is derived from a single precursor protein. J. Biol. Chem. **272**(39): 24480–24487.

Tang, Y., and Hollingsworth, R.I. 1998. Regulation of lipid synthesis in *Bradyrhizobium japonicum* : Low oxygen concentrations trigger phosphatidylinositol biosynthesis. Appl. Environ. Microbiol. **64**(5): 1963–1966.

Tao, H., Brewin, N.J., and Noel, K.D. 1992. *Rhizobium leguminosarum* CFN42 lipopolysaccharide antigenic changes induced by environmental conditions. J. Bacteriol. **174**(7): 2222–2229. doi:10.1128/jb.174.7.2222-2229.1992.

Tatusov, R.L., Galperin, M.Y., Natale, D.A., and Koonin, E. V. 2000. The COG database: a tool for genome-scale analysis of protein functions and evolution. Nucleic Acids Res. **28**(1): 33–36. doi:10.1093/nar/28.1.33.

Tie, X., Zhang, R., Brasseur, G., and Lei, W. 2002. Global NOx production by lightning. J. Atmos. Chem. **43**(1): 61–74. doi:10.1023/A:1016145719608.

Tiwari, R.P., Reeve, W.G., Dilworth, M.J., and Glenn, A.R. 1996. Acid tolerance in *Rhizobium meliloti* strain WSM419 involves a two-component sensor-regulator system. Microbiology **142**(7): 1693–1704. doi:10.1099/13500872-142-7-1693.

Udvardi, M., and Poole, P.S. 2013. Transport and metabolism in legume-rhizobia symbioses. Annu. Rev. Plant Biol. **64**(February): 781–805. doi:10.1146/annurev-arplant-050312-120235.

von Uexküll, H.R., and Mutert, E. 1995. Global extent, development and economic-impact of acid soils. Plant Soil **171**: 1–15. doi:10.1007/BF00009558.

Van de Velde, W., Zehirov, G., Szatmari, A., Debreczeny, M., Ishihara, H., Kevei, Z., Farkas, A., Mikulass, K., Nagy, A., Tiricz, H., Satiat-Jeunemaitre, B., Alunni, B., Bourge, M., Kucho, K., Abe, M., Kereszt, A., Maroti, G., Uchiumi, T., Kondorosi, E., and Mergaert, P. 2010. Plant peptides govern terminal differentiation of bacteria in symbiosis. Science . **327**: 1122–1126.

Verbeke, T.J., Spicer, V., Krokhin, O. V., Zhang, X., Schellenberg, J.J., Fristensky, B., Wilkins, J.A., Levin, D.B., and Sparling, R. 2014. *Thermoanaerobacter thermohydrosulfuricus* WC1

shows protein complement stability during fermentation of key lignocellulose-derived substrates. Appl. Environ. Microbiol. **80**(5): 1602–1615. doi:10.1128/AEM.03555-13.

Via, V.D., Zanetti, M.E., and Blanco, F. 2016. How legumes recognize rhizobia. Plant Signal. Behav. **11**(2): e1120396. doi:10.1080/15592324.2015.1120396.

Vincent, J.M. 1970. A Manual for the Practical Study of Root-nodule Bacteria. International Biological Programme by Blackwell Scientific, Oxford, England.

Virts, E.L., Stanfield, S.W., Helinski, D.R., and Ditta, G.S. 1988. Common regulatory elements control symbiotic and microaerobic induction of *nifA* in *Rhizobium meliloti*. Proc Natl Acad Sci U S A **85**(9): 3062–3065.

Voegele, R.T., Bardin, S., and Finan, T.M. 1997. Characterization of the *Rhizobium* (*Sinorhizobium*) *meliloti* high- and low-affinity phosphate uptake systems. J. Bacteriol. **179**(23): 7226–7232.

Wang, C., Meek, D.J., Panchal, P., Boruvka, N., Archibald, F.S., Driscoll, B.T., and Charles, T.C. 2006. Isolation of poly-3-hydroxybutyrate metabolism genes from complex microbial communities by phenotypic complementation of bacterial mutants. Appl. Environ. Microbiol. **72**(1): 384–391. doi:10.1128/AEM.72.1.384-391.2006.

Wang, C., Saldanha, M., Sheng, X., Shelswell, K.J., Walsh, K.T., Sobral, B.W.S., and Charles, T.C. 2007. Roles of poly-3-hydroxybutyrate (PHB) and glycogen in symbiosis of *Sinorhizobium meliloti* with *Medicago* sp. Microbiology **153**(2): 388–398. doi:10.1099/mic.0.29214-0.

Winkel-Shirley, B. 2001. Flavonoid Biosynthesis . A Colorful Model for Genetics , Biochemistry , Cell Biology , and Biotechnology. Plant Physiol. **126**(6): 485–493. doi:10.1104/pp.126.2.485.

Wood, J., and Fulcher, R.G. 1983. A basis for specific detection and histochemistry of polysaccharides. J. Histochem. Cytochem. **31**(6): 823–826.

Woodmansee, R.G., Dodd, J.L., Bowman, R.A., Clark, F.E., and Dickinson, C.E. 1978. Nitrogen budget of a shortgrass prairie ecosystem. Oecologia **34**: 363–376.

De Wulf, P., and Vandamme, E.J. 1997. Production of D-ribose by fermentation. Appl. Microbiol. **48**: 141–148.

Xia, J., and Wishart, D.S. 2016. Using MetaboAnalyst 3.0 for comprehensive metabolomics data analysis. Curr. Protoc. Bioinforma. **55**(14): 1–91. doi:10.1002/cpbi.11.

Yancey, P.H.H., Clark, M.E., Hand, S.C., Bowlus, R.D., and Somero, G.N. 1982. Living with water stress: evolution of osmolyte systems. Science **217**(4566): 1214–1222. doi:10.1126/science.7112124.

Yao, S.Y., Luo, L., Har, K.J., Becker, A., Rüberg, S., Yu, G.Q., Zhu, J.B., and Chenga, H.-P. 2004. *Sinorhizobium meliloti* ExoR and ExoS proteins regulate both succinoglycan and flagellum production. J. Bacteriol. **186**(18): 6042–6049. doi:10.1128/JB.186.18.6042-

6049.2004.

York, G.M., and Walker, G.C. 1997. The *Rhizobium meliloti exoK* gene and *prsD*/*prsE*/*exsH* genes are components of independent degradative pathways which contribute to production of low-molecular-weight succinoglycan. Mol Microbiol **25**(1): 117–134. doi:10.1046/j.1365-2958.1997.4481804.x.

York, G.M., and Walker, G.C. 1998. The *Rhizobium meliloti* ExoK and ExsH glycanases specifically depolymerize nascent succinoglycan chains. Proc. Natl. Acad. Sci. U. S. A. **95**(9): 4912–4917. doi:10.1073/pnas.95.9.4912.

York, W.S., McNeil, M., Darvill, A.G., and Albersheim, P. 1980. Beta-2-linked glucans secreted by fast-growing species of *Rhizobium*. J. Bacteriol. **142**(1): 243–248.

Yost, C.K., Rath, A.M., Noel, T.C., and Hynes, M.F. 2006. Characterization of genes involved in erythritol catabolism in *Rhizobium leguminosarum bv. viciae*. Microbiology **152**(7): 2061–2074. doi:10.1099/mic.0.28938-0.

Zevenhuizen, L.P.T.M. 1981. Cellular glycogen, B-1,2-glucan, poly-B-hydroxybutyric acid and extracellular polysaccharides in fast-growing species of *Rhizobium*. Antonie Van Leeuwenhoek **47**(6): 481–497. doi:10.1007/BF00443236.

Zevenhuizen, L.P.T.M., Bertocchi, C., and Van Neerven, A.R.W. 1986. Congo red absorption and cellulose synthesis by Rhizobiaceae. Antonie Van Leeuwenhoek **52**: 381–386.

Zhan, H.J., and Leigh, J.A. 1990. Two genes that regulate exopolysaccharide production in *Rhizobium meliloti*. J. Bacteriol. **172**(9): 5254–5259.

Zhao, G., and Winkler, M.E. 1994. An *Escherichia coli K-12 tktA tktB* mutant deficient in transketolase activity requires pyridoxine (vitamin B6) as well as the aromatic amino acids and vitamins for growth. J. Bacteriol. **176**(19): 6134–6138.

Zhou, P., Silverstein, K.A., Gao, L., Walton, J.D., Nallu, S., Guhlin, J., and Young, N.D. 2013. Detecting small plant peptides using SPADA (Small Peptide Alignment Discovery Application). BMC Bioinformatics **14**(1): 335. doi:10.1186/1471-2105-14-335.