Succinoglycan Production Contributes to Acidic pH Tolerance in *Sinorhizobium meliloti* Rm1021

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In this work, the hypothesis that exopolysaccharide plays a role in the survival of *Sinorhizobium meliloti* at low pH levels is addressed. When *S. meliloti* was grown at pH 5.75, synthesis of succinoglycan increased, whereas 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 $\beta(1-2)$ 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 $\beta(1-2)$ glucan, it was found that the inability to produce cyclic $\beta(1-2)$ 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.

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 that could be prohibitive to the establishment of an effective symbiosis (Ferguson et al. 2013; von Uexküll and Mutert 1995). Increased acidity is known to negatively affect the growth of both symbiotic rhizobia and legume plants (Howieson et al. 1988; Lowendorf et al. 1981). Understanding how microbes can 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

(Geddes and Oresnik 2016). 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 that encode the enzymes necessary to synthesize Nod factor (NF). NF is recognized by receptors on plant root hairs, resulting in a complex signal cascade in the plant (Oldroyd 2013). The end result of this cascade is twofold: i) the division of inner-cortical plant cells that form the nodule primordium and ii) the curling 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. meliloti* Rm1021 unable to produce the exopolysaccharide (EPS) succinoglycan do not form effective symbiotic associations with the host plant, and become trapped in the CCRH (Cheng and Walker 1998a; Glazebrook and Walker 1989; Leigh et al. 1985).

S. meliloti is able to produce two main types of EPS, succinoglycan (EPS-I) 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 that 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*. Strains that have an intact *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 (Mendrygal and González 2000; Oresnik et al. 1994).

The gene *exoH*, which encodes the enzyme responsible for succinylation of EPS-I, has been shown to be necessary for symbiosis (Cheng and Walker 1998b; Leigh et al. 1987). 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 result in only a minor decrease in overall plant growth compared with 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.

Many roles for EPS in symbiotic establishment have been suggested, including dampening of the plant immune response and

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protection from reactive oxygen species (Donati et al. 2011; Jones and Walker 2008; 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 (Hawkins and Oresnik 2017; Mendrygal and González 2000; Miller-Williams et al. 2006). It has also hypothesized that EPS plays a role in pH stress tolerance (Cunningham and Munns 1984), though this has never been directly investigated.

Within the rhizosphere, low pH is a function of the plant roots secreting H^+ and organic acids that can decrease the pH by up to 2 pH units (Faget et al. 2013). In addition, it has been shown that the cell wall of elongating roots is acidic and the development of root hairs is preceded by a further localized drop in pH at the site of initiation (Bibikova et al. 1998). It has also been shown that symbiotic compartments such as the bacteroid and the CCRH are acidic (Fedorova et al. 1999; Geddes et al. 2014; Pierre et al. 2013). 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.

RESULTS

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 rhizobium minimal medium (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 Vincent's minimal medium (VMM) 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 (data not shown). 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 (Glazebrook and Walker 1989; Leigh and Lee 1988). 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*).

Fig. 1. Exopolysaccharide (EPS) production under neutral and acidic pH. **A**, Visual mucoidy of strains grown at pH 5.75 or pH 7. **B**, EPS was precipitated from 50 ml of culture supernatant and was dried and weighed after 72 h of growth. The white bar indicates EPS produced at pH 5.75; the 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 that at pH 7. All numbers are an average of three independent biological replicates. EPS weights are normalized to the optical density at 600 nm of the culture.

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 (Fig. 1A). 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 (wild type *expR*⁺) at pH 5.75 compared with that at pH 7 (Fig. 1B and C). When production of EPS was determined for strains capable of only producing either EPS-I or EPS-II (*expA1/expR*⁺ and *exoY55/expR*⁺, respectively), 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 (Fig. 1C). This suggests that production of EPS-I is important under acidic conditions.

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

EPS-I consists of both HMW and LMW fractions. Changes in 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 pH 5.75. 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 1). 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 a ratio, the data show that the EPS-I produced at a low pH is consistent with it being enriched with the LMW fraction (Table 1). We note that, although the estimated chain length for the *exoH* mutant strain is much higher than the wild type, the estimated chain length in this strain does change (Table 1).

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. meliloti* 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 (Fig. 2; Supplementary Fig. S1). Strains that

Table 1. Effect of pH on EPS-I polymerization chain length

Strain (genotype)	pH 7 ^a			pH 5.75 ^a		
	EPS	Reducing ends ^a	Ratio	EPS ^a	Reducing ends	Ratio
Rm1021 (wild type)	286 ± 8	17 ± 1	17	489 ± 20*	46 ± 8	10*
Rm7225 (<i>exoH</i>)	218 ± 13	1 ^b ± 0	300	298 ± 19*	5 ± 1	61*

^a EPS and reducing ends values are presented in milligrams per liter and are an average of three independent biological replicates that 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 to reducing ends; an asterisk (*) means the number is significant compared with pH 7 values based on *P* < 0.05 using Student's *t* test.

Fig. 2. EPS-I mutant sensitivity to acidic pH as calculated in CFU per milliliter from spot dilutions at pH 5.75 (white) and pH 7 (black). Results are an average of three independent biological replicates. One asterisk (*) indicates *P* value < 0.05, compared with wild type, two (**) indicate *P* value < 0.05, compared with *exoY* mutant values.

carried an *exoY* mutation in either *expR* background showed a 1,000-fold decrease in the number of colony forming units (CFU) 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 CFU compared with 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 (Fig. 2). This suggested that the LMW fraction of EPS-I and not the succinylation status of HMW EPS-I confers the ability for bacteria to survive on low pH medium.

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, the transcription of genes involved in the biosynthesis of EPS-I, EPS-II (*expA1*), cyclic β (1-2) glucans (*ndvB*), glycogen (*glgA1*), lipopolysaccharide

(LPS) (*kdsB2*, *lpsL*, and *lpsB*), and capsular polysaccharide (KPS) (*rkpA*, *kdsB2*) was determined in either the wild type or an *exoY* mutant strain (Becker et al. 1997; Dylan et al. 1990; Kereszt et al. 1998; Müller et al. 2009; Wang et al. 2007).

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 (Fig. 3; Table 2). 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 (Fig. 1C), *expA1* was found to be downregulated 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 2).

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 Tn5 insertion in the mutant strain, we still observed transcription of *exoY* in Rm7055 (Fig. 3). The regulators *chvI* and *exoR* were no

Fig. 3. Changes in gene expression due to acidic medium. Data expressed as 2^{ΔΔCt} and represents fold change in 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 three independent biological replicates. Standard deviations for all numbers are within 1 Ct (cycle threshold). NC = no change.

longer observed to have increased transcription at pH 5.75. The transcription of *exoN*, which codes for the protein necessary to catalyze formation of UDP-glucose from glucose-1-phosphate, was found to decrease twofold. 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 with 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.

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

The ability to produce both glycogen and cyclic $\beta(1-2)$ glucans has been linked with changes in EPS-I biosynthesis (Dylan et al. 1990; Wang et al. 2007). To determine if the ability to produce EPS-I affects the intracellular accumulation of glycogen or cyclic $\beta(1-2)$ glucans, both polysaccharides were quantified in strains carrying mutations in either *exoY* or *exoH* and were compared with wild-type production. The data showed that a mutation in *exoY* led to greater amounts of glycogen in both low and neutral pH conditions (Fig. 4A). However, cyclic $\beta(1-2)$ glucans isolated from cells grown at either pH or in either mutant background did not show any significant changes when compared with wild-type concentrations (Fig. 4B).

Cyclic $\beta(1-2)$ glucan production plays an auxiliary 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 the importance of these genes in response to acidic pH. To determine if either glycogen or cyclic $\beta(1-2)$ glucans play a role in surviving low pH, the *exoY*:Tn5 mutation was transduced into strains Rm11482 ($\Delta glgA1$, *glgA2*:: Ω SpSm) and SRmD377 (*ndvB*:Tn5-233) 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 $\Delta glgA1$ Ω SpSm::*glgA2* mutations were not compromised in their ability to survive at pH 5.75 (Fig. 5). 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 $\beta(1-2)$ glucans does not produce a pH sensitive phenotype, when combined with an *exoY* mutant background, the strain becomes impaired in pH tolerance to the same degree as an *exoH* mutant.

A double endoglycanase mutation leads to 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 that 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 (Becker et al. 1993a and b; Leigh et al. 1985), 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

Table 2. Additional quantitative reverse transcription-polymerase chain reaction results

Gene	Function	Fold change ^a	
		Rm1021	Rm7055
<i>exoF</i>	Polysaccharide export	10.85	3.0
<i>exoX</i>	Posttranscriptional regulator	2.0	NC
<i>exoD</i>	Putative transmembrane protein	5.94	NC
<i>rkpA</i>	Putative fatty acid synthase	NC	NC
<i>kdsB2</i>	CMP-Kdo transferase	NC	NC
<i>lpsB</i>	LPS mannosyltransferase	NC	NC
<i>lpsL</i>	UDP-glucuronate 5'-epimerase	NC	NC
<i>expE1</i>	ABC transporter	0.4	NC
<i>expE2</i>	Glycosyltransferase	NC	NC
<i>wggR</i>	Transcriptional activator	NC	NC
<i>flbT</i>	Putative flagellar repressor protein	0.18	0.12
<i>flgB</i>	Flagellar protein	NC	0.14

^a Fold change in gene expression. Data are 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 three independent replicates with standard error within one cycle. NC = no change.

Fig. 4. Production of glycogen and cyclic $\beta(1-2)$ glucans at pH 7 and pH 5.75. Each polysaccharide was extracted after 3 days growth in mannitol/glutamate/salts at the indicated pH. Both **A**, glycogen and **B**, cyclic $\beta(1-2)$ glucans were quantified using the anthrone assay. Results are an average of three independent biological replicates.

was a change in the rate of nodule formation (Fig. 6A). Initial appearance of nodules occurred at about 5 days postinoculation for the wild type under our growth conditions, whereas the *exsH/exoK* mutant strain was consistently delayed by at least 1 day (Fig. 6A). 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 (Fig. 6B).

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 (Fig. 6C). Consistent with previous 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 with wild type-inoculated plants (Fig. 6D). 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.

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 that 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 (Fig. 1A and C). These observations were corroborated through quantitative reverse transcription-polymerase chain reaction (qRT-PCR) expression data of key genes that encode enzymes necessary for biosynthesis of each EPS (Fig. 3; Table 2). 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 h, 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).

The response of *S. meliloti* 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). ExoS acts as a sensor kinase that 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). Homologs 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* and *S. meliloti* has revealed that at acidic pH the repressor ExoR is degraded, resulting in increased EPS-I synthesis (Heckel et al. 2014; Lu et al. 2012). Mutants lacking *chvI* in *S. meliloti* have also been shown to be sensitive to acidic pH (Wang et al. 2010). Combined with our results, it is likely this is due to the inability of the *chvI* mutant to produce EPS-I.

A shift to acidic pH also influences the degree of polymerization of EPS-I to favor lower molecular weight forms (Table 2). Unexpectedly, a decrease in chain length polymerization was also observed in the *exoH* mutant strain that produces nonsuccinylated EPS-I, which cannot be cleaved by the *exsH/exoK* endoglycanases (Table 1). The degree of polymerization of EPS-I has been shown to be under control of 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 value of EPS-I from *S. meliloti* was determined through protonation of its carboxylic groups and was 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,

Fig. 5. Role of cellular polysaccharides in pH tolerance. Calculated in CFU per milliliter from spot dilutions at pH 5.75 (white) and pH 7 (black). Results are an average of three independent biological replicates. One asterisk (*) indicates P value < 0.05, compared with wild type, two (**) indicate P value < 0.05, compared with *exoY* mutant values.

it is unclear if this could be playing a role, since the buffering capacity of EPS-I at pH 5.75 should be negligible. It has also been shown that production of EPS and its localization to the surface of *Rhizobium leguminosarum* bv. *trifolii*, results in a greater electronegative charge, increased hydrophobicity, and changes in the apparent dissociation constant for the cell (Cieřla et al. 2016). It is possible that cell-surface modification from EPS production in *S. meliloti* may have similar effects that play into acidic pH tolerance. While our results showed the inability to produce LMW EPS-I is detrimental to survival at low pH (Fig. 2), 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 $\beta(1-2)$ glucans in pH tolerance when EPS-I is absent was unexpected, since intracellular concentrations were not affected under the conditions that were tested (Fig. 4B). 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 $\beta(1-2)$ glucans may play complementary physiological roles. If this is the case, cyclic $\beta(1-2)$ glucans may also provide a buffering system or a physiological barrier when accumulated in the periplasm. The observation that loss of cyclic $\beta(1-2)$ 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 $\beta(1-2)$ 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 EPSs in an *exoY* mutant can affect the concentration of other macromolecular compounds.

In *S. meliloti*, both glycogen and poly-hydroxybutyrate (PHB) are produced as major carbon storage compounds under growth-limiting conditions (Zevenhuizen 1981). In contrast, EPSs are generally produced after accumulation of these intracellular polysaccharides (Zevenhuizen 1981). The carbon flow between PHB and glycogen is highly interconnected and has been shown to be under similar regulatory control (Dunn et al. 2002; Povolito and Casella 2000). It has been 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 has been inextricably linked with 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

Fig. 6. Symbiotic phenotypes of an *exsH/exoK* mutant strain. **A**, Percentage of plants nodulated over time after inoculation with Rm1021 or Rm8826. Data are the sum of over 50 plants over four biological replicates. The average standard deviation of each point is 8 and 9%, respectively, for Rm1021 and Rm8826. **B**, Average number of days postinoculation before 50% of plants became inoculated. **C**, Rm8826 competed against Rm1021 for nodule occupancy of *Medicago 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 three independent biological replicates. **D**, Average dry weight per *M. sativa* plant inoculated with Rm1021 or Rm8826. Data are an average of three independent biological replicates consisting of 10 plants each. For B, C, and D, significance was determined using Student's *t* test. One asterisk (*) indicates *P* < 0.05.

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 convert HMW EPS-I to the LMW form results in a sensitivity to low pH as well as a delay in nodule development (Figs. 2 and 6A). Importantly, the nodules that were formed by the double endoglycanase mutant appeared to be normal, yet the plants had accumulated less dry matter (Fig. 6D), presumably due to less nitrogen being reduced by the microsymbiont. Although we attempted to repeat these experiments at a low pH, we were unsuccessful due to poor nodulation. We note that it has been previously shown that root-hair development is adversely affected by the pH of the growth medium (Bibikova et al. 1998). The reduction in the amount of dry matter accumulated by the double endoglycanase mutant 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, together with the supporting literature, suggests 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 (Geddes et al. 2014; Jones 2012). The inability to make LMW succinoglycan would decrease the bacterial viability in the CCRH and lead to a delay, but not an abortion, of infection thread formation and visible nodule development (Fig. 6). The visualization of activity from an *exoF::TnphoA* 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 (Fedorova et al. 1999; Reuber et al. 1991). 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 and 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.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

Bacterial strains and plasmids created and used in this work are listed in Table 3. *S. meliloti* was grown at 30°C on either Luria-Bertani or tryptone yeast extract as complex medium (Sambrook et al. 1989). 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 was used at a final

Table 3. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype ^a	Source or reference
<i>Sinorhizobium meliloti</i>		
Rm1021	SU47 <i>str-21</i> , Sm ^r	Meade et al. 1982
SRmA363	Rm1021 <i>expR</i> ⁺ , Sm ^r	Miller-Williams et al. 2006
Rm7055	Rm1021 <i>exoY55::Tn5</i> , Sm ^r , Nm ^r	Leigh et al. 1985
SRmD394	SRmA363 <i>exoY55::Tn5</i> , Sm ^r , Nm ^r	This work
SRmA775	Rm1021 <i>expA1::Tn5</i> , Sm ^r , Nm ^r	This work
SRmA777	SRmA363 <i>expA1::Tn5</i> , Sm ^r , Nm ^r	This work
Rm7225	Rm1021 <i>exoH::Tn5-233</i> , Sm ^r , Gm ^r	Leigh et al. 1987
SRmD445	SRmA363 <i>exoH::Tn5-233</i> , Sm ^r , Gm ^r	This work
Rm8826	Rm1021 <i>exsH::Tn5 exoK::Tn5-233</i> , Sm ^r , Nm ^r , Gm ^r	York and Walker 1997
SRmD377	Rm1021 <i>ndvB::Tn5-233</i> , Sm ^r , Gm ^r	J. Gonzalez
Rm11482	Rm1021 <i>glgA1ΔPstI glgA2::ΩSpSm</i> , Sm ^r , Sp ^r	Wang et al. 2007
SRmD455	Rm7055 <i>glgA1ΔPstI glgA2::ΩSpSm</i> , Sm ^r , Sp ^r , Nm ^r	This work
SRmD456	Rm7055 <i>ndvB::Tn5-233</i> , Sm ^r , Nm ^r , Gm ^r	This work
<i>Escherichia coli</i>		
MM294A	<i>Pro-82 this-1 hsdR17 supE44</i>	Finan et al. 1986
MT607	MM294A; <i>recA56</i>	Finan et al. 1986
MT616	MT607(pRK600)	Finan et al. 1986
DH5α	λ ⁻ φ80d <i>lacZ</i> ⁺ M15 ⁺ (<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> (<i>r_K⁻ m_K⁻</i>) <i>supE44 thi-1 gyrA relA1</i>	Hanahan 1983
Plasmids		
pBluescriptII SK ⁺	Cloning vector ColE1 <i>oriV</i> Amp ^r	Stratagene
pRK600	pRK2013 <i>npt::Tn9</i> Cm ^r	Finan et al. 1986
pMW22	Km ^r fragment cloned as <i>SmaI</i> fragment into pBlueScript	Pickering and Oresnik 2008
pRK415	Tc ^r IncP broad-host-range cloning vector	Keen et al. 1988
pPH1J1	IncP plasmid Gm ^r	Beringer 1974
pMW43	<i>expA1</i> in pBluescriptII SK ⁺	This work
pMW44	Km ^r cassette from pMW22 cloned into <i>expA1</i> in pMW43	This work
pMW45	<i>expA1::Km^r</i> from pMW44 cloned into pRK415	This work

^a Sm^r, Nm^r, Gm^r, Sp^r, Cm^r, Amp^r, and Km^r = resistance to streptomycin, neomycin, gentamicin, spectinomycin, chloramphenicol, ampicillin, and kanamycin, respectively.

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: streptomycin, 200 µg/ml; neomycin, 200 µg/ml; and tetracycline, 5 µg/ml. Gentamicin was used at 20 µg/ml for *E. coli* or 60 µg/ml for *S. meliloti*.

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* (*HindIII* site underlined) (5'-AGTCAAGCTTATGTCTTCTAACGTGAGGCAG-3') and *expA1RV* (*EcoRI* site underlined) (5'-AGTCCGATCCTTATTCCGGACATTGGACGC-3') and was cloned into pBS SK⁺ as a *HindIII/BamHI* fragment creating pMW43. Following this, the kanamycin cassette from pMM22 was isolated as a *SmaI* fragment and blunt end ligated into an *EcoRI* site in *expA1* yielding pMW44. The *expA1* fragment with the kanamycin cassette was then subcloned into pRK415 as a *BamHI/KpnI* fragment to create pMW45. pMW45 was subsequently conjugated into Rm1021, and integration of the mutation was carried out using pPH1J1, 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.

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 three times before being used.

RNA isolation, cDNA synthesis, and qRT-PCR.

For bacterial RNA isolation, cultures were grown as described for polysaccharide isolation with minor modification (discussed below). Cultures were inoculated in 5 ml of RMM with mannitol, and were subcultured into 50 ml of MGS medium buffered at pH 5.75 or pH 7. Cultures were grown overnight to midlog phase. A volume of 10 ml of this culture was removed and was 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 of cDNA was utilized as template for qRT-PCR. Reactions were performed using the SYBR green RT-PCR kit from Invitrogen, as indicated by the kit. RT-PCR reactions were performed as previously described (Geddes et al. 2010). 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 cycle threshold value 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 (Geddes et al. 2014; Glenn et al. 2007; Hoang et al. 2008). Primers used but not previously described are as indicated in Supplementary Table S1.

Isolation and quantification of EPS.

Isolation of EPS, glycogen, and cyclic $\beta(1-2)$ 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 1 day at 30°C in a volume of 50 ml of RMM containing 15 mM mannitol as a carbon source. Cultures were then resuspended in RMM to an optical density

at 600 nm (OD_{600}) of 0.1, then, pelleted and washed twice with double distilled (dd)H₂O. 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 3 days at 30°C. After 3 days, the OD_{600} of the cultures was determined. To facilitate pelleting of the cells, the culture was diluted 1:1 with ddH₂O. The supernatant was removed and used to determine the EPS, whereas the cell pellet was used for quantification of glycogen and cyclic $\beta(1-2)$ 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 was centrifuged at 20,000 $\times g$ for 10 min at 4°C to pellet the EPS. The supernatant was decanted and the resulting pellet was dried at 40°C for 20 min and was then weighed. Weight of EPS was normalized to the OD_{600} of the culture. Student's *t* test was used to determine significance of data.

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 was compared with a glucose standard curve (Lever 1972). A ratio of the concentration of total EPS-I to the 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.

Glycogen and cyclic $\beta(1-2)$ glucan isolation and quantification.

Isolated cell pellets were washed once with 10 ml of ddH₂O and were then resuspended in 1 M KOH and boiled at 100°C in a water bath for 20 min, to allow for cell lysis. Cell debris was removed by centrifugation (13,000 rpm for 10 min) 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 $\beta(1-2)$ glucans (Breedveld et al. 1993). Quantification of glycogen and cyclic $\beta(1-2)$ glucans was carried out using the anthrone-H₂SO₄ 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 used.

Acidic pH tolerance.

Cultures were started in 5 ml of RMM with mannitol and were grown overnight at 30°C. Cultures were then diluted to an OD_{600} of 0.1 in RMM. Then, 1 ml of each culture was removed, was washed with 1 ml of ddH₂O, and was resuspended in 1 ml of saline (0.85% wt/vol). Cultures were then serially diluted from 10⁰ 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 30°C for 4 days and were then quantified by calculating CFU per milliliter. At pH 5.75, single colonies were not visible, so a dissecting microscope was utilized to count microcolonies to calculate CFUs per milliliter.

Plant dry weights and competition for nodule occupancy.

Alfalfa seeds were surface-sterilized with 1% bleach, were washed with at least 10 volumes of sterile ddH₂O, and were subsequently germinated on water agar plates for 48 h 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 10⁷ 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 three independent biological replicates consisting of 10 plants each. Nodule competition was determined, as previously described (Poysti and Oresnik 2007), 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 three independent biological replicates consisting of 50 examined nodules each.

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 μ l of water as described above. The rate of nodule formation was assessed over a period of 21 days. Numbers are an average of five independent biological replicates consisting of 10 plants each.

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