Characterization of Mutations That Affect the Nonoxidative Pentose Phosphate Pathway in *Sinorhizobium meliloti*

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ABSTRACT *Sinorhizobium meliloti* is a Gram-negative alphaproteobacterium that can enter into a symbiotic relationship with *Medicago sativa* and *Medicago truncatula*. Previous work determined that a mutation in the *tkt2* gene, which encodes a putative transketolase, could prevent medium acidification associated with a mutant strain unable to metabolize galactose. Since the pentose phosphate pathway in *S. meliloti* is not well studied, strains carrying mutations in either *tkt2* and *tal*, which encodes a putative transaldolase, were characterized. Carbon metabolism phenotypes revealed that both mutants were impaired in growth on erythritol and ribose. This phenotype was more pronounced for the *tkt2* mutant strain, which also displayed auxotrophy for aromatic amino acids. Changes in pentose phosphate pathway metabolite concentrations were also consistent with a mutation in either *tkt2* or *tal*. The concentrations of metabolites in central carbon metabolism were also found to shift dramatically in strains carrying a *tkt2* mutation. While the concentrations of proteins involved in central carbon metabolism did not change significantly under any conditions, the levels of those associated with iron acquisition increased in the wild-type strain with erythritol induction. These proteins were not detected in either mutant, resulting 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 the carbon flow necessary for carbon metabolism and the establishment of symbiosis.

IMPORTANCE *Sinorhizobium meliloti* is a model organism for the study of plant-microbe interactions and metabolism, especially because it effects nitrogen fixation. The ability to derive the energy necessary for nitrogen fixation is dependent on an organism's ability to metabolize carbon efficiently. The pentose phosphate pathway is central in the interconversion of hexoses and pentoses. This study characterizes the key enzymes of the nonoxidative branch of the pentose phosphate pathway by using defined genetic mutations and shows the effects the mutations have on the metabolite profile and on physiological processes such as the biosynthesis of exopolysaccharide, as well as the ability to regulate iron acquisition.

KEYWORDS *Sinorhizobium meliloti*, carbon metabolism, nitrogen fixation

Nitrogen makes up approximately 1 to 4% of all living cells and is a critical limiting element for the growth of all life (1). In agriculture, the major natural input of nitrogen occurs through symbiotic nitrogen fixation, which is predicted to provide 40 Tg of reduced nitrogen annually (2). 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 (3).

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

Received 7 July 2017 Accepted 21 October 2017

Accepted manuscript posted online 30 October 2017

Citation Hawkins JP, Ordonez PA, Oresnik IJ. 2018. Characterization of mutations that affect the nonoxidative pentose phosphate pathway in *Sinorhizobium meliloti*. J Bacteriol 200:e00436-17. <https://doi.org/10.1128/JB.00436-17>.

Editor Anke Becker, Philipps-Universität Marburg

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S. meliloti and *M. sativa*, which requires constant production of both Nod factor and exopolysaccharides (EPSs) (4, 5). Overall, this results in *S. meliloti* becoming endocytosed into plant cells, followed by terminal differentiation into a bacteroid capable of biological nitrogen fixation (5, 6).

The ability of rhizobia to catabolize various carbon sources has been shown to be integral to the establishment of an effective association. The inability of rhizobia to metabolize specific carbon sources has been shown to result in ineffective competition for nodule occupancy or the inability to fix nitrogen (7–9). This has led 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* contains many genes necessary for the transport and catabolism of carbon sources, enabling it to grow on a diverse array of substrates (10, 11). Central carbon metabolism in *S. meliloti* occurs through the Entner-Doudoroff, Embden-Meyerhof-Parnas, pentose phosphate, and tricarboxylic acid (TCA) pathways (12). *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 (13, 14). 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 enzymatic branches, i.e., the oxidative branch and the nonoxidative branch (15). The initial two enzymatic reactions of the Entner-Doudoroff and oxidative pentose phosphate pathways are shared and convert glucose-6-phosphate 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 (16–18). 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 (18).

The nonoxidative pentose phosphate pathway is ubiquitous among organisms and is primarily used to interconvert hexose and pentose sugars (19, 20). Labeling experiments in *S. meliloti* have indicated the presence of enzymes associated with the nonoxidative pentose phosphate pathway and have suggested the importance of this pathway in cycling carbon to polysaccharides (21, 22). The interconversion of carbon is necessary for the production of ribose-5-phosphate and erythrose-4-phosphate, precursors for provision of nucleic acids and synthesis of aromatic amino acids, respectively (15). Metabolism of a number of carbon sources, including erythritol, ribose, and xylulose, has been shown to feed into central carbon metabolism through the nonoxidative pentose phosphate pathway (23–25). While previous work suggested that erythritol metabolism might feed in through dihydroxyacetone phosphate in *S. meliloti* (26, 27), more recent work has determined that erythrose-4-phosphate is the point of introduction in *Brucella abortus* (25).

The two major enzymes of the nonoxidative 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 glyceraldehyde-3-phosphate (28). Transketolase utilizes a ketose donor and aldose acceptor to carry out the reversible reactions of ribose-5-phosphate and xylulose-5-phosphate to sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate and erythrose-4-phosphate to fructose-6-phosphate and glyceraldehyde-3-phosphate (29). 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 (20). In *Escherichia coli*, both *tktA* and *tktB* are known to encode transketolases (30, 31). Strains lacking *tktA* exhibit auxotrophy for aromatic amino acids, due to disruption of the shikimate pathway, and are unable to grow on pentose sugars (31, 32). Although TktB has been shown to have transketolase activity, strains carrying a mutation in *tktB* do not exhibit any

FIG 1 Loci of putative transketolase and transaldolase genes. A map of the genetic region surrounding *tkt2*, *tkt1*, *cbbT*, and *tal* is shown. Boxes indicate open reading frames. Vertical lines indicate the approximate site of the insertion mutation in *tkt2* or *tal*. Gene names and the colors of boxes are based on curated annotations found at <https://iant.toulouse.inra.fr/bacteria/annotation/cgi/rhime.cgi>. The color denotes the putative class of each ORF. Dark gray, hypothetical; turquoise, central intermediary metabolism; blue, small molecule metabolism; yellow, cell processes; green, macromolecule metabolism; pink, regulator; red, insertion element.

deleterious phenotypes (30, 33). This has led to the suggestion that TktB plays a minor role in the pentose phosphate pathway.

The pentose phosphate pathway in *S. meliloti* remains poorly defined. Previous work described the isolation of a mutation in *tkt2*, encoding a putative transketolase (34). The goals of this work were to determine the genes that encode the primary transketolase and transaldolase and to characterize how these mutations affect symbiosis and the overall physiology of *S. meliloti*.

RESULTS

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 (12). Transketolase and transaldolase represent critical proteins in the nonoxidative pentose phosphate pathway. Since a mutation in a putative transketolase (*tkt2*) was isolated previously (34), it was reasoned that creating a mutation in the transaldolase gene would allow us to characterize the nonoxidative pentose phosphate pathway.

BLASTP analysis showed that Tkt2 from *S. meliloti* has 51% and 49% identity with TktA and TktB, respectively, from *E. coli* K-12, which was shown to be utilized in the pentose phosphate pathway (32). In addition to Tkt2, genes encoding two other putative transketolase proteins were identified in the *S. meliloti* genome; these were Tkt1 and CbbT, which showed 60% and 50% identity with Tkt2, respectively. An examination of the loci encoding these proteins revealed that *tkt2* was 70 bp from an operon containing two genes involved in the Embden-Meyerhof-Parnas pathway (*gap*, encoding glyceraldehyde-3-phosphate dehydrogenase, and *pgk*, encoding phosphoglycerate kinase) and *SMc03980* (encoding a conserved hypothetical protein) (Fig. 1A). The *tkt1* gene appears to be localized within a group of uncharacterized genes, and it has the potential to form an operon and to encode proteins involved in the metabolism of a small compound (Fig. 1B). 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 (35, 36) (Fig. 1C). Due to the genomic context of *cbbT* and *tkt1*, as well as the preliminary characterization of SRmD397, it was decided not to focus directly on these genes.

TABLE 1 Strains and plasmids

Strain or plasmid	Genotype or phenotype ^a	Reference
Strains		
<i>S. meliloti</i>		
Rm1021	SU47 <i>str-21</i> Sm ^r	73
SRmD338	Rm1021 Δ <i>dgok1</i>	This work
SRmD373	SRmD338 <i>tkt2::Tn5</i> Sm ^r Nm ^r	This work
SRmD397	Rm1021 <i>tkt2::Tn5</i> Sm ^r Nm ^r	This work
SRmD480	Rm1021 <i>tal::pKnock-Gm</i> Sm ^r Gm ^r	This work
SRmA723	Rm1021 <i>SMC01627::Tn5-233</i> Sm ^r Gm ^r Sp ^r	27
SRmD479	Φ (SRmD723) $\rightarrow$ SRmD397 Sm ^r Nm ^r Gm ^r	This work
SRmD486	Rm1021 <i>rpiB::pKnock-Gm</i> Sm ^r Gm ^r	This work
SRmD488	Φ (SRmD486) $\rightarrow$ SRmD397 Sm ^r Nm ^r Gm ^r	This work
RmFL2950	RmP110 <i>rhbB::pTH1522</i> Sm ^r	39
SmA818	Rm1021 Δ pSymB Sm ^r	74
<i>E. coli</i>		
MM294A	<i>pro-82 thi-1 hsdR17 supE44</i>	57
MT607	MM294A <i>recA56</i>	57
MT616	MT607(pRK600)	57
DH5 α	λ^- ϕ 80 <i>dlacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> (r _k ⁻ m _k ⁻) <i>supE44 thi-1 gyrA relA1</i>	Lab strain
DH5 α Rif	Rifampin-resistant variant of DH5 α	61
DH5 α λ pir	λ pir lysogen of DH5 α	61
Plasmids		
pJH117	400-bp internal fragment of <i>tal</i> cloned into pKnock-Gm; Gm ^r	This work
pJH120	400-bp internal fragment of <i>rpiB</i> cloned into pKnock-Gm; Gm ^r	This work
pJH109	pCO37/ <i>tkt2</i> Tc ^r	This work
pJH110	pCO37/ <i>tkt1</i> Tc ^r	This work
pJH113	CX1 cosmid complementing <i>tkt2</i>	This work
pJH116	pCO37/ <i>cbtT</i> Tc ^r	This work
pJH118	pCO37/ <i>tal</i> Tc ^r	This work
pRK600	pRK2013 <i>npt::Tn9</i> Cm ^r	57
pRK602	pRK600 Ω Tn5 Cm ^r Nm ^r	57
pKnock-Gm	Suicide vector for insertional mutagenesis; R6K ori RK4 <i>oriT</i> Gm ^r	59
pRK7813	Broad-host-range cloning vector; Tc ^r	75
pCO37	pRK7813 containing <i>attB</i> sites; Gateway-compatible destination vector	62
pMK2014	FRT- <i>ccdB</i> -Cm ^r -FRT cassette Pen ^r	61
pXINT129	<i>λint</i> and <i>xis</i> driven by P _{lacI} ; Km ^r	61

^aSm^r, streptomycin resistant; Nm^r, neomycin resistant; Gm^r, gentamicin resistant; Sp^r, spectinomycin resistant; Cm^r, chloramphenicol resistant; Tc^r, tetracycline resistant; Pen^r, penicillin resistant; Km^r, kanamycin resistant; FRT, flippase recognition target.

Examination of the *S. meliloti* genome revealed one putative transaldolase, which was encoded by *tal* (Fig. 1D). HAMAP-Scan analysis of the amino acid sequence of Tal identified a single trusted match to the MF_00494 family, 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 (Table 1).

Mutations in *tkt2* result in an inability to grow on defined medium. To determine how carbon metabolism was affected by a mutation in either *tkt2* or *tal*, each strain was assessed for its ability 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 (31).

The *tkt2* mutant (SRmD397) was unable to grow in defined medium (Table 2). When the medium was supplemented with tryptophan, tyrosine, and phenylalanine, full growth was restored when glucose, glycerol, or succinate was used as the sole carbon source. However, SRmD397 was unable to utilize either ribose or erythritol, regardless of supplementation with aromatic amino acids (Table 2). A mutation in *tkt1*, encoding the other putative transketolase, did not result in any observable carbon metabolism phenotypes, consistent with phenotypes observed for a *tktB* mutant in *E. coli* (33).

When a plasmid overexpressing *tkt2* (pJH109) was introduced in *trans* into SRmD397, growth on all carbon sources was restored (Table 2). Although the intro-

TABLE 2 Carbon metabolism phenotypes

Strain	Genotype	Growth ^a									
		Glc	Glc FYW	Gly	Gly FYW	Ery	Ery FYW	Suc	Suc FYW	Rib	Rib FYW
Rm1021	Wild type	++	++	++	++	++	++	++	++	++	++
SRmD480	<i>tal::pK-Gm</i>	++	++	++	++	+/-	+/-	++	++	+	+
SRmD480 (pJH120)	<i>tal::pK-Gm (tal⁺)</i>	++	++	++	++	++	++	++	++	++	++
SRmD397	<i>tkt2::Tn5</i>	—	++	—	++	—	—	—	+	—	—
SRmD397 (pJH109)	<i>tkt2::Tn5 (tkt2⁺)</i>	++	++	++	++	+	+	++	++	+	+
SRmD397 (pJH110)	<i>tkt2::Tn5 (tkt1⁺)</i>	++	++	++	++	—	—	+	+	—	—
SRmD397 (pJH116)	<i>tkt2::Tn5 (cbbT⁺)</i>	—	++	—	++	—	—	—	++	—	—
SRmD397 (pJH113)	<i>tkt2::Tn5 (tkt2 cosmid)</i>	++	++	++	++	++	++	++	++	++	++

^aGrowth phenotypes were as follows: —, no growth; +/-, weak growth; +, moderate growth; ++, wild-type growth. Glc, glucose; Gly, glycerol; Ery, erythritol; Suc, succinate; Rib, ribose; FYW, supplemented with phenylalanine, tyrosine, and tryptophan.

duction of pJH109 restored growth, wild-type growth was not observed with medium containing either erythritol or ribose. To determine whether the other putative transketolase genes could complement the *tkt2* mutation, plasmids overexpressing either *tkt1* (pJH110) or *cbbT* (pJH116) were introduced into SRmD397. While pJH116 was unable to complement the growth of SRmD397, pJH110 restored growth using glucose, glycerol, and succinate, which is consistent with *tkt1* encoding a product with minor transketolase activity. The increased expression of *tkt1* might result in higher overall transketolase activity, which could provide enough erythrose-4-phosphate to overcome *tkt2*-mutant-associated auxotrophy. However, the increased activity of *tkt1* alone would not be enough to alleviate increased concentrations of phosphorylated intermediates with carbon sources such as ribose and erythritol, which feed directly into the pentose phosphate pathway. The introduction of pJH114, a cosmid that contained *tkt2*, into SRmD397 restored growth on all carbon sources tested. Comparison of the restriction pattern of the cosmid with the restriction fragments found in the genomic region of *tkt2* showed that *tkt2*, *gap*, *SMc03980*, and *pgk* are all found on a 26-kbp BamHI fragment that is found in pJH114.

A mutation in transaldolase should result in less severe carbon phenotypes than a strain carrying a transketolase mutation. Whereas transketolase is necessary for the formation of erythrose-4-phosphate, which is subsequently needed for the biosynthesis of aromatic amino acids, there are no major metabolites that cannot be produced in a strain carrying a transaldolase mutation. Consistent with this, SRmD480 was unaffected when grown on defined medium with glucose, glycerol, or succinate as the sole carbon source (Table 2). However, SRmD480 grew poorly, relative to the wild-type strain, with ribose or erythritol. This could be due to the fact that both ribose and erythritol feed directly into the pentose phosphate pathway, thus making the activity of transaldolase more important, to metabolize the phosphorylated intermediates. These growth defects were fully complemented with the introduction of pJH120, which constitutively overexpressed the *tal* gene.

Erythritol metabolism is dependent on SMc01613. The ability to metabolize erythritol is a relatively rare phenotype (12). Erythritol metabolism has been best characterized in *B. abortus*, *Rhizobium leguminosarum*, and *S. meliloti* (9, 23, 25). Although it was 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 (25). While previous work in *S. meliloti* showed the involvement of TpiB in erythritol metabolism, the need for SMc01613, which is tentatively annotated as *rpiB*, had not been demonstrated (37). However, the gene *eryl*, which is a *rpiB* homolog and catalyzes the reaction of erythulose-4-phosphate to erythrose-4-phosphate, was shown to be necessary for erythritol metabolism in *B. abortus* (25). To address this, a mutation in *SMc01613* was constructed, yielding SRmD486. When tested, SRmD486 could grow using glucose as the sole carbon source but was unable to utilize erythritol. This strongly suggested conservation of the metabolic pathway between *B. abortus* and

S. meliloti and suggested that erythritol metabolism feeds into central carbon metabolism through erythrose-4-phosphate in *S. meliloti* (see Table S1 in the supplemental material). For consistency, we propose that the genes annotated as *tpiB* and *rpiB* in *S. meliloti* be renamed *eryH* and *eryI*, respectively, to maintain consistency with findings in *B. abortus*.

Supplementation of growth medium with erythritol can 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 for phenylalanine, tryptophan, and tyrosine, SRmD397 was grown on defined medium containing both glucose and erythritol as carbon sources. The results showed that SRmD397 was unable to grow with this combination of carbon sources, even when supplemented with aromatic amino acids (Table S1). However, when the concentration of erythritol was reduced to 200 μ M, the growth of SRmD397 on minimal medium could be partially restored (Table S2). To determine whether the inability to grow was dependent on the presence of erythritol, a mutant allele of *mptB* (SMc01627), which encodes part of the transporter necessary for erythritol uptake, was transduced into SRmD397 (23). The results showed that mutation of the erythritol transporter alleviated the inability of a *tkt2* mutant to grow in the presence of erythritol (Table S1). Taken together, these data suggest that, while low concentrations of erythritol could alleviate the auxotrophy associated with SRmD397, the inability of the strain to grow with 15 mM erythritol could be the result of accumulation of a phosphorylated intermediate (or intermediates) derived from the metabolism of erythritol in SRmD397.

Levels of pentose phosphate pathway-associated metabolites are altered in *tkt2* and *tal* mutants. The ability to measure enzyme activity directly is often used to confirm the functions encoded by genes. Measurement of transketolase and transaldolase activities in 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 would likely affect metabolite pool sizes (Fig. 2). To provide evidence that *tkt2* and *tal* function in the pentose phosphate pathway, the concentrations of pentose phosphate pathway metabolites in each mutant strain were examined and compared to Rm1021 concentrations (Fig. 3).

Consistent with the absence of transketolase activity, a 5-fold increase in the pentose phosphate pool and a 20-fold reduction in the sedoheptulose-7-phosphate concentration were observed when SRmD397 was grown on glucose, compared to the wild-type strain (Fig. 3A). With erythritol supplementation, the concentrations of the pentose phosphate pool and sedoheptulose-7-phosphate increased 50- and 100-fold, respectively, compared to Rm1021 grown under the same conditions (Fig. 3B). These increases are consistent with carbon flowing into the pentose phosphate pathway but having no exit point when transketolase activity is not present, since reactions are not reversible past ribulose-5-phosphate (Fig. 2).

Analysis of metabolites in SRmD480 also showed elevated pentose phosphate pools but, unlike in the *tkt2* mutant strain, the concentration of sedoheptulose-7-phosphate was increased to 100 times greater than the wild-type level when the cells were grown on glucose (Fig. 3A). Supplementation with erythritol resulted in decreases in both of the pools. The observed increases in metabolite levels with growth on glucose are consistent with carbon pooling in the pentose phosphate pathway due to the limited ability of mutant strains to process the carbon. However, it is unclear why growth on erythritol decreased the concentrations of pentose phosphate pathway intermediates. These results support the hypothesis that *tkt2* and *tal* encode the transketolase and transaldolase enzymes, respectively, necessary for the pentose phosphate pathway and erythritol feeds directly into the pentose phosphate pathway. It is noteworthy that,

FIG 2 Simplified map of the central carbon metabolism in *S. meliloti*. Arrowheads indicate the predominate direction of each reaction and are colored to indicate inclusion in a specific metabolic pathway. Dark blue, Embden-Meyerhof-Parnas pathway; gray, Entner-Doudoroff pathway; light blue, pentose phosphate pathway; orange, TCA pathway; red, erythritol metabolism. P, phosphate; G3P, glyceraldehyde-3-phosphate; KDPG, 2-keto-3-deoxy-phosphogluconate; PEP, phosphoenolpyruvate; 3-PG, 3-phosphoglycerate; DHAP, dihydroxyacetone phosphate; α -KG, α -ketoglutarate; CoA, coenzyme A.

although erythrose-4-phosphate was a detectable metabolite, we did not find this metabolite under any of the conditions tested (Table S2).

Mutations in *tkt2* lead to changes in metabolite pools. To assess changes in central carbon metabolism, concentrations of the metabolites involved in the 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 S2). Principal-component analysis (PCA) showed that all strains were distinct from each other in two-dimensional space, with respect to components PC1 and PC2, when induced with either glucose or erythritol (Fig. S1). However, PCA graphing of Rm1021 and SRmD480 findings in response to erythritol suggested similar responses of the strains to growth on erythritol.

FIG 3 Intracellular concentrations of the pentose phosphate pathway intermediates sedoheptulose-7-phosphate (A) and ribose/ribulose-5-phosphate (B) in *S. meliloti*. Bars indicate growth on glucose and erythritol. Concentrations are normalized to the cell pellet weight and are averages of three biological replicates. Metabolite concentrations are compared to the wild-type (WT) values for each condition. *, significant difference ($P < 0.05$) based on Student's *t* test.

Hierarchical clustering grouped metabolites based on concentrations with each condition (Fig. 4). 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 pathway and the upper TCA pathway, were positively correlated in Rm1021 and SRmD480 but were negatively correlated in SRmD397. Cluster II contained the remaining metabolites, and levels were generally increased in SRmD397 while being decreased in the other two strains; the exception to this was cluster IIa metabolites (including the hexose phosphates, malate, and fumarate), which were found to be increased in Rm1021 and decreased in SRmD397 when cells were grown with glucose. The response of SRmD397 to erythritol was separated by metabolites in clusters IIIa and IIIb. Metabolites in cluster IIIb were mostly involved in the pentose phosphate pathway and were increased in SRmD397 in response to erythritol, whereas lactate and α -ketoglutarate in cluster IIIa were increased when cells were grown with glucose. Consistent with the PCA results, no major changes between Rm1021 and SRmD480 were observed when cells were induced with erythritol. Overall, these results showed that a mutation in *tkt2* had greater effects on metabolites associated with central carbon metabolism than did a mutation in *tal*.

Proteome analysis of *tkt* and *tal* mutant strains. Changes in metabolite concentrations can be associated with altered transcription or translation of proteins. To determine whether changes in protein expression were correlated with metabolite concentrations, the proteome of each strain was determined when cells were grown on glucose and when they were induced with erythritol. Overall, 2,469 proteins were detected using one-dimensional (1D) liquid chromatography (LC)-mass spectrometry (MS) analysis. Of the detected proteins, 1,880 were consistent and were considered for quantification.

FIG 4 Clustered heat map of metabolites in central carbon metabolism from strains 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 colored boxes were generated with MetaboAnalyst 3.0. The color scale represents fold changes with respect to the average value of each metabolite. Colors indicate fold increases or decreases in concentrations, as indicated in the key. 2-PG, 2-phosphoglycerate; 3-PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate; G6P, glucose-6-phosphate; G1P, glucose-1-phosphate; F6P, fructose-6-phosphate; α-KG, α-ketoglutarate; G3P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; R5P, ribose-5-phosphate; Ribu5P, ribulose-5-phosphate; S7P, sedoheptulose-7-phosphate.

Other than increases in the levels of proteins involved in erythritol metabolism when cells were grown with erythritol, no major changes in proteins involved in central carbon metabolism were observed (Tables S3 and S4). Peptides from Tkt2 and Tal were detected in decreased concentrations (451- and 67-fold decreases in SRmD397 and SRmD480, respectively), consistent with these strains carrying mutations in the genes encoding these proteins (Table S4). The proteins Gap and Pkg, which are encoded just downstream of *tkt2* (Fig. 1A), were decreased in SRmD397. This finding suggested that the Tn5 mutation has some polar effects on these proteins.

Clusters of orthologous groups (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 3). Furthermore, proteins involved in hemin binding and transport and iron transport were all found to be induced by erythritol in Rm1021. However, proteins associated with rhizobactin were not detected in either mutant strain. The iron-binding and uptake proteins found to be upregulated in Rm1021 were also not detected or were found to be decreased in production in the mutant strains, compared to the wild-type strain.

Erythritol negatively affects rhizobactin production in *tkt2* mutant strains. The metabolism of erythritol was shown previously to influence the production of siderophores in *Brucella* (38). A common way to assess changes in siderophore production is

TABLE 3 Expression of proteins involved in iron acquisition in *tkt2* and *tal* mutants

			Pnet ^a		
Enzyme	Gene	Locus tag	Rm1021	SRmD397	SRmD480
Rhizobactin					
Diaminobutyrate-2-oxoglutarate aminotransferase	<i>rhbA</i>	SMa2400	+	—	—
RhsB, L-2,4-diaminobutyrate decarboxylase	<i>rhbB</i>	SMa2402	2.69 ^b	—	—
RhbC, rhizobactin siderophore biosynthesis	<i>rhbC</i>	SMa2404	5.42	—	—
RhbD, rhizobactin siderophore biosynthesis	<i>rhbD</i>	SMa2406	+	—	—
RhbE, rhizobactin siderophore biosynthesis	<i>rhbE</i>	SMa2408	3.76 ^b	—	—
RhbF, rhizobactin siderophore biosynthesis	<i>rhbF</i>	SMa2410	5.06	—	—
RhrA, transcriptional activator	<i>rhrA</i>	SMa2412	—	—	—
RhtA, rhizobactin receptor precursor	<i>rhtA</i>	SMa2414	2.04 ^b	—	—
Other iron-associated proteins					
Putative hemin-binding periplasmic transmembrane protein	<i>hmuT</i>	SMc01512	4.88	—	+
Putative iron transport protein	<i>shmR</i>	SMc02726	3.22	—	+
Putative hemin transport protein	<i>hmuS</i>	SMc01513	2.94	—	−1.39
Putative ferrichrome iron receptor precursor	<i>foxA</i>	SMc01657	2.61	—	NC
Putative iron uptake periplasmic ABC transporter		SMb21432	2.61	—	+
Putative iron-binding protein	<i>fbpA</i>	SMc00784	1.85	NC	NC
Iron-binding periplasmic protein		SMc04317	1.02	NC	1.58
Probable bacterioferritin (cytochrome c ₁)	<i>bfr</i>	SMc03786	+	−2.87	−3.55
Putative iron transport ATP-binding ABC transporter	<i>sitB</i>	SMc02508	NC	NC	−1.45

^aLog₂ expression (Pnet) of proteins in erythritol-induced strains versus glucose-induced strains. NC, no change (log₂ expression between 1 and −1); —, not detected; +, detected but not quantifiable.

^bChange from not detected to detected, with the exact fold change not being statistically significant.

to determine the ability of strains to compete for a limited source of iron in the medium. Bacteria seeded onto a plate that are unable to produce rhizobactin are unable to grow near rhizobactin-producing strains, which manifests as a zone of clearance. To assess whether the *tkt2* and *tal* mutant strains showed altered rhizobactin production, each strain was used in a rhizobactin competition assay with SmA818, which is unable to produce or to take up rhizobactin due to the loss of pSymA, which carries the operon necessary for rhizobactin synthesis. Strains were impaired in rhizobactin production (39). RmFI2950, which has a mutation in the gene *rhbB* and cannot produce rhizobactin, was used as a negative control. In the presence of glucose, Rm1021 and the *tal* mutant strain had similar zones of clearance in the growth of SmA818 (Fig. 5). The zone of inhibition decreased from 20 mm to 12 mm when the *tkt2* mutant strain was assessed for rhizobactin production. When the medium was supplemented with erythritol, a minor increase in zone clearance size was observed for Rm1021, although no change in clearance was seen for SRmD480. Interestingly, the presence of erythritol abolished the zone of inhibition observed for the *tkt2* mutant. These results

FIG 5 Siderophore production in *tkt2* and *tal* mutant strains. Strains were spotted onto VMM agar seeded with SmA818 and containing either glucose (white bars) or erythritol (black bars). Diameters were compared to the wild-type strain grown under the same conditions. 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.

FIG 6 EPS I production in *tkt2* and *tal* mutants. EPS I was quantified from the culture supernatant or cetrimide-perceptible material from each strain grown in YEM medium. Concentrations were normalized to the OD₆₀₀ of the cell culture and are averages of three independent biological replicates. *, $P < 0.05$, compared to Rm1021, using Student's *t* test. WT, wild type.

suggested that erythritol affects rhizobactin production and only SRmD397 is impaired in the production of this siderophore.

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 (21, 22). Since changes in the hexose phosphate pools were found for each strain grown in glucose (Table S3), the production of succinoglycan was assessed. When the *tkt2* and *tal* mutant strains were streaked onto agar containing calcofluor white, it appeared that both Rm1021 and SRmD480 produced EPS I, while the *tkt2* mutant produced a negligible amount (data not shown). To corroborate this observation, EPS was quantified as either cetrimide-precipitable EPS or the total amount of glucose found in the culture supernatant (Fig. 6). Quantification of the material in the culture supernatant would include any polysaccharide that contains a reducing sugar, whereas cetrimide-precipitable material is assumed to be primarily EPS I. The results show that, in each case, the amount of EPS isolated by cetrimide precipitation is smaller than that determined by measuring anthrone-reactive material found in the supernatant. This is indicative of anthrone-reactive polysaccharides that are not cetrimide precipitable being produced and secreted into the culture supernatant. Overall, it was observed that a mutation in either *tkt2* or *tal* resulted in smaller amounts of synthesized EPS I. While the mutation in *tkt2* severely impaired EPS I production, the *tal* mutant was less affected than the wild-type strain. These results indicate that mutation of the nonoxidative pentose phosphate pathway has a direct effect on overall polysaccharide synthesis, including EPS I production.

Mutations in *tkt2* result in defects in early symbiotic establishment. The ability to acidify the growth medium and produce increased amounts of EPS I is correlated with increased competitiveness for nodule occupancy (40). The *tkt2* mutant was isolated on the basis of being unable to acidify its growth medium (34) and was shown to be impaired in EPS I production (Fig. 6). Since each of these determinants is linked to effective symbiosis, both the *tkt2* mutant and the *tal* mutant were assessed for symbiotic phenotypes. Dry weights of plants inoculated with each strain showed that the *tkt2* mutant was ineffective in fixing nitrogen for alfalfa (Fig. 7A). However, plants inoculated with SRmD480 showed dry matter accumulation that was comparable to that of plants inoculated with Rm1021.

The rate of nodule formation was also determined for each strain when it was inoculated onto alfalfa roots (Fig. 7B and C). Over a period of 14 days, alfalfa inoculated with the *tkt2* mutant was observed to be severely affected with respect to nodule formation, compared to Rm1021, whereas the *tal* mutant showed kinetics similar to those of the wild-type strain. Nodule formation was first visible at 5 days and showed

FIG 7 Nitrogen fixation and nodulation kinetics of *tkt2* and *tal* mutant strains. Strains were inoculated onto alfalfa plants, and the plant dry weight (A), the number of nodules formed per plant (B), and the proportion of nodulated plants (C) were measured. Each dry weight measurement (in panel A) is an average from three biological replicates, each containing 10 plants. Nodulation kinetics experiments (in panels B and C) represent five biological replicates, each containing at least 20 plants. The average standard deviations were 11% for Rm1021 and SRmD480 and 9% for SRmD397. WT, wild type; DPI, days postinoculation.

consistent development over time in the wild-type strain, while strains carrying the *tkt2* mutation did not start nodulating until day 7 and showed markedly slower nodule formation. The total number of nodules was also greatly decreased (0.5 versus 2.5 nodules/plant) (Fig. 7C), and the nodules formed by SRmD397 were small cyst-like formations on the roots, instead of the cylindrical pink nodules that were found on the Rm1021-inoculated plants. Taken together, these results indicated that a mutation in *tkt2* inhibits the establishment of symbiosis at an early stage.

DISCUSSION

In this work, we provide evidence that Tkt2 and Tal act as the primary transketolase and transaldolase, respectively, in the pentose phosphate pathway. This conclusion is based on the similarity of the encoded products to characterized transketolase and transaldolase proteins, carbon metabolism phenotypes (Table 2), the concentrations of pentose phosphate intermediates for each mutant grown on glucose (Fig. 3), and the behavior of the metabolite pools when the bacteria were supplemented with erythritol (Fig. 4; also see Table S2 in the supplemental material). These phenotypes are all consistent with previous findings for other organisms (15, 41, 42).

The genome of *E. coli* encodes two transketolase proteins, TktA and TktB (30, 33). TktA has been shown to provide 70 to 90% of transketolase activity, with the rest being provided by TktB (30). Our data showed that, although a *S. meliloti tkt2* mutant strain had a greatly decreased sedoheptulose-7-phosphate pool, sedoheptulose-7-phosphate

was still measurable (Fig. 3). Also, when *tkt1* is overexpressed in *trans*, it can partially complement a *tkt2* mutation (Table 1); in our hands, a strain with mutations in both *tkt2* and *tkt1* was unable to be rescued through amino acid supplementation on defined medium (data not shown). These data are all consistent with findings for *E. coli* (31, 32). We suggest that *tkt2* and *tkt1* should be renamed *tktA* and *tktB*, respectively, to provide consistency with previously published literature.

The pentose phosphate pathway has been implicated in the cycling of carbon to metabolites necessary for EPS biosynthesis (21, 22). Our results also suggest the importance of the pentose phosphate pathway in EPS production, since a strain carrying a mutation in *tkt2* produced less EPS I than a strain carrying a *tal* mutation, and both produced less EPS I than the wild-type strain (Fig. 6). When the amount of EPS I isolated from each of these strains was compared with the intracellular concentrations of the metabolites we determined, EPS production was positively correlated with both the hexose phosphate ($R^2 = 0.77$) and citrate ($R^2 = 0.99$) pools, whereas it was 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* also showed that there was a correlation between EPS I production and the size of the hexose phosphate pool (43, 44). Enzymatic activities can be affected by allosteric effectors and posttranslational modifications such as phosphorylation events. The latter modifications can occur in response to metabolite pools or environmental stimuli (45–47). In *S. meliloti*, the activity of ExoN, which is required for the synthesis of UDP-glucose, was shown to be modulated by its phosphorylation state (48). Taken together, these findings suggest that the changes in EPS I production in the mutant strains 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 *tal* mutation is consistent with the hypothesis that the genes that encode these activities are continually expressed and alterations in their activities occur at the level of metabolite concentrations (49). The finding that proteins involved in iron acquisition were more abundant in the wild-type strain than in either the *tkt2* or *tal* background was unexpected, and the finding that rhizobactin activity appeared to be affected by erythritol was surprising, since the defined medium that was used is not iron limited (50).

In *B. abortus*, growth using erythritol and iron acquisition have been shown to be interrelated (38, 51). 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 environment, such as that found in pregnant ruminants, may explain why mutants that are unable to utilize erythritol tend to be avirulent (38). In rhizobia, the genes necessary for erythritol metabolism are thought to have been acquired through horizontal gene transfer (9, 27). 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 (9, 23). It is tempting to speculate that vestiges of how erythritol metabolism is regulated in *B. abortus* can still be found in rhizobia. It is also noteworthy that the *tkt2* mutation was originally isolated in a mutant that did not acidify its growth medium (52), 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 (Fig. 7 and Table 2). The inability of a *tkt2* mutant strain to produce erythrose-4-phosphate would affect its ability to generate chorismate, preventing the biosynthesis of tryptophan, phenylalanine, and tyrosine. The role that the biosynthesis of aromatic amino acids plays in the establishment of symbiosis seems to vary by association (53).

Anthranilate synthase is the first dedicated enzyme in the biosynthesis of tryptophan and converts chorismate to anthranilate. This enzyme is encoded by *trpE(G)* in *S. meliloti*, and insertions in this gene were found to result in symbiotically ineffective strains (54). However, mutations in the genes encoding proteins for the latter steps of the pathway (*trpD*, *trpF*, *trpC*, *trpA*, and *trpB*) were symbiotically effective, leading to the

conclusion that only anthranilate synthesis, and not tryptophan synthesis, is necessary for effective establishment of symbiosis (54). The nodules formed by *trpE(G)* mutants have been described as elongated (54). In contrast to this, *tkt2* mutants appear to have a more severe symbiotic phenotype. They form fewer nodules, which are delayed in their appearance, and have an altered morphology (Fig. 7 and data not shown).

Interestingly, like the *trpE(G)* mutant strain, second-site suppressor mutations that partially restore symbiosis have 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 may be similar to those reported previously for the *trpE(G)* mutants and the severe symbiotic phenotype we found may be a result of both direct and indirect effects of strains carrying a *tkt2* mutation. It is hoped that an 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 of symbiosis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids constructed and used in this work are listed in Table 1. *S. meliloti* was grown at 30°C on either Luria-Bertani (LB) medium or yeast extract-mannitol (YEM) medium, as a complex medium (55). Vincent's minimal medium (VMM) was used as a defined medium (50). Carbon sources in defined medium were filter sterilized prior to use and were 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; 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; 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.

Genetic techniques, plasmid construction, and mutant generation. Conjugations and transductions were carried out as described previously (7, 53). Standard techniques for plasmid isolation, ligation, transformation, restriction, and gel electrophoresis were used (55). Tn5 mutagenesis of Rm1021 was carried out using the self-mobilizable plasmid pRK602, as described previously (27, 56, 57). The location of the Tn5 insertion was determined using arbitrary PCR and subsequent sequencing of the isolated PCR products (58).

To construct SRmD480, a single crossover mutation was created utilizing the plasmid pKnock-Gm (59). A 400-bp internal fragment from *tal* was PCR amplified using the primers (restriction sites underlined) pKtal_Fw (5'-AGTCGGATCCGAGTTACCAAGGAAATCTG-3') and pKtal_Rv (5'-AGTCCTCGAGCTTCCTTACGTGGTTGAC-3'). The amplified fragment was gel isolated and 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 resistance on LB agar plates with Gm. Isolated colonies were single colony purified three times on LB agar with Gm before use. Knockout of the *tal* gene was confirmed by PCR amplification and sequencing using the primers ptaConf_Fw (5'-TCGTTGATACCGCGATGTG-3') and ptaConf_Rv (5'-TCAGGCGATCTTCTGGCCG-3'), with SRmD480 genomic DNA as the template.

Construction of SRmD486 was carried out in a similar manner. A 370-bp fragment from *rpiB* was amplified using the primers pKrpIB_FW (5'-AGTCGGATCCCATCTCGCCAAGAGAAGCGA-3') and pKrpIB_RV (5'-AGTCCTCGAGGCTTCGACATTTGCCGCC-3'), followed by cloning of the fragment into pKnock-Gm using the introduced BamHI and XhoI sites to create pJH120. This plasmid was then conjugated into Rm1021 and selected 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'-TTACTTCGCTGATCCAGGC-3') with 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* were constructed by using the *S. meliloti* open reading frames (ORFs), as described previously (60–62). Briefly, each ORF was recombined into the destination vector pCO37 to create pJH109, pJH110, pJH116, and pJH118. A cosmid complementing *tkt2* (pJH113) was also isolated through conjugation of a *S. meliloti* cosmid bank (63) into strain SRmD397, followed by selection for growth on VMM using erythritol as the sole carbon source.

Sample preparation for proteomic and metabolomic analyses. 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 optical density at 600 nm (OD₆₀₀) of 0.4, using fresh LB broth, and 5 ml of the culture was used to start a 500-ml culture in VMM with glucose supplemented with tryptophan, tyrosine, and phenylalanine. The 500-ml cultures were grown to OD₆₀₀ values of 0.1 to 0.2, at which point they were induced with the addition of 15 mM glucose or erythritol. The cultures were grown for 4 h to OD₆₀₀ values of approximately 0.4. Cells were then pelleted by centrifugation at 12,000 × *g* for 1 min, washed with double-

distilled water, and finally pelleted again by centrifugation at $12,000 \times g$ for 1 min. Cultures were decanted, and bacterial cell pellets were weighed and subsequently frozen in liquid nitrogen.

Metabolite extraction. Bacterial cell pellets were washed with cold 150 mM ammonium formate solution (pH 7.4) and then extracted with 600 μ l of 31.6% methanol (MeOH)–36.3% acetonitrile (ACN) in water (vol/vol). Cells were lysed and homogenized by bead beating for 2 min at 30 Hz, 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 the sample temperature maintained at -4°C (Labconco, Kansas City, MO). Pellets were subsequently resuspended in 30 μ l of water as the injection buffer.

LC-MS method. All LC-MS-grade solvents and salts were purchased from Fisher (Ottawa, Canada), including DCM, water, ACN, MeOH, and ammonium acetate. The authentic standards for metabolites of interest were purchased from Sigma-Aldrich Co. (Oakville, Canada). For targeted metabolite analysis and determination of relative concentrations of metabolites, samples were injected into an Agilent 6430 triple-quadrupole (QQQ)-LC-tandem mass spectrometry (MS/MS) system. The mass spectrometer was equipped with an electrospray ionization (ESI) source, and samples were analyzed in positive-ion or negative-ion mode. Multiple reaction monitoring (MRM) transitions were optimized on standards for each metabolite quantitated. The gas temperature and flow rate were set at 350°C and 10 liters/min, respectively, the nebulizer pressure was set at 40 lb/in², and the capillary voltage was set at 3,500 V. Relative concentrations were determined by using external calibration curves. Data were analyzed using MassHunter Quant (Agilent Technologies).

For the measurement of lactate, succinate, fumarate, and malate levels, chromatographic separation was performed on a Scherzo SM-C₁₈ column (3 μ m, 3.0 by 150 mm; 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/glucose-1-phosphate/fructose-6-phosphate, gluconolactone, gluconate, 6-phosphogluconate, glyceraldehyde-3-phosphate/dihydroxyacetone phosphate, 2-phosphoglycerate/3-phosphoglycerate, phosphoenolpyruvate, fructose-1,6-bisphosphate, ribose-5-phosphate/ribose-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 at 100% mobile phase B and a subsequent reequilibration period (6 min) before the next injection. For all LC-MS analyses, 5 μ l of sample was 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) and comparing the result with the external calibration curve.

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 three independent biological replicates, which were measured twice. Principal-component analysis and cluster analysis were performed using MetaboAnalyst 3.0 (<http://www.metaboanalyst.ca>), as described previously (64). 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* values of <0.05 and FDR values of <0.1 were met.

Protein isolation, digestion, and peptide purification. Proteins were extracted from frozen cell pellets as described previously, with some modifications (65). Cell pellets were thawed and resuspended in an extraction buffer containing 100 mM HEPES, 100 mM dithiothreitol (DTT), and 1 mM MgCl₂. Cells were then lysed by two passages through a French pressure cell at 16,000 lb/in². The extract was then pelleted at $10,000 \times g$ for 10 min, and the supernatant was decanted into a fresh tube. The cell debris pellet was resuspended in 1 ml of 4% SDS, 100 mM DTT, and subsequently boiled at 90°C for 20 min to extract any remaining proteins. The extract was then pelleted at $12,000 \times g$ for 10 min to remove cell debris, and the supernatant was pooled with the initial extract to create a pooled protein extract.

One milliliter of protein was transferred to an Amicon Ultra-15 filter device (pore size, 10,000 Da; Millipore, Billerica, MA), following the purification, digestion, and subsequent peptide purification steps described previously (66), with modification of the trypsin/protein ratio to 1:100. The purified peptides were lyophilized and redissolved in 0.1% formic acid in water for subsequent 1D LC-MS analysis.

Protein identification and quantification. One-dimensional LC-MS was used to identify proteins and to quantify extracts as described previously (66). 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. The total ion count (TIC), which is the sum of all collision-induced dissociation (CID) fragment intensities of member peptides, was recorded.

Data analysis and validation using UNITY. Comparative analyses of the gathered expression data were conducted using the in-house system UNITY (66). Data were compared with those for Rm1021 to determine the effect of the mutation on protein expression or were compared with those obtained when cells were grown with glucose to examine the effect of growth with erythritol. Differential expression values were calculated for cross states (Znet) (exponential-phase mutant strain culture versus exponential-phase wild-type culture or exponential-phase erythritol-induced culture versus exponential-phase glucose-induced culture) and among biological replicates (Rnet). A quality control assessing biological variation between any two comparison groups (cross state) versus system noise among

biological replicates (intrareplicative viability) was conducted. Transformation of different populations into final $\log_2$ expression values (Pnet) for differential expression analyses of cross-state samples was conducted as described previously (67). Cutoff scores of ± 1.0 were used to represent proteins observed to be upregulated or downregulated by at least 2-fold versus the comparison condition. Mutant- or condition-specific protein expression was then filtered by searching the Pnet values of ± 1.0 using COG categories for annotation (68).

The signal/noise (S/N) ratio for each quantified protein was determined as described previously, by comparing the means of cross-state and intrareplicate samples (69). On an individual protein level, the S/N ratio is the ratio of the protein expression across the experimental states and intrareplicate normalized values scaled by the overall system S/N ratios. A Monte-Carlo model was used to derive functions relating FDRs to a defined S/N cutoff value. All proteins with S/N ratios of >2.8 were found to have FDR values of $\leq 10\%$. This threshold was used as the cutoff level for reporting significant changes in protein expression. Overall proteomic results are presented in Data Set S1 in the supplemental material.

Rhizobactin competition assay. Rhizobactin assays were performed as described previously, using SmA818 as an indicator strain, with minor modifications (39). 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 of VMM (0.6% [wt/vol] agar) at approximately 50°C and was supplemented with aromatic amino acids. Either glucose or erythritol was added as a carbon source. 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, the supernatant was decanted, and the remaining pellet was resuspended in 50 μ l of 0.85% saline. Ten microliters was subsequently spotted onto the seeded plates and allowed to dry. Plates were then incubated for 4 days at 30°C before clearance zones were recorded. The diameters of the zones of inhibition were recorded, and reported numbers are averages of three independent biological replicates.

EPS quantification. Quantification of EPS was carried out using the anthrone assay described previously (70, 71), with the following modifications. Strains were grown in 5 ml of YEM medium overnight at 30°C. Cultures were then subcultured to an OD_{600} of 0.1 in YEM medium and grown for another 3 days at 30°C. The OD_{600} of the culture was measured and used for normalization of results. Cell cultures were then pelleted at $12,000 \times g$ for 10 min, and the supernatant was decanted and used for quantification of EPS.

To measure total anthrone-reactive material produced, 1 ml of the culture supernatant was assayed directly using the anthrone assay (70). EPS I was also precipitated from the supernatant by the addition of 300 μ l of 1% cetrimide to 1 ml of supernatant. The mixture was centrifuged at $12,000 \times g$ for 10 min to pellet EPS, and the remaining supernatant was removed. Pelleted EPS was resuspended in 10% NaCl. One milliliter of the resuspended EPS was then directly assayed with the anthrone assay. All reported numbers are averages of three independent biological replicates. Significance was determined using Student's *t* test.

Plant dry weights and nodulation kinetics. Plant assays were carried out as described previously (37). Alfalfa seeds were germinated for 2 days on water agar plates. Seedlings were then 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 (72). After 2 days of growth, seedlings were inoculated with approximately 10^7 bacteria resuspended in 10 ml of sterile water. Plants were harvested after 28 days, and the dry weights of the plants were determined. Numbers are average dry weights of 10 plants/pot. All results are averages of three independent biological replicates.

Nodule kinetics was assessed as described previously (40). 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 μ l. The formation of nodules was measured over a period of 14 days. Overall, 100 plants over five biological replicates were used to determine the rate of nodulation for each strain.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00436-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

SUPPLEMENTAL FILE 2, XLSX file, 1.5 MB.

ACKNOWLEDGMENTS

This work was funded by a Natural Sciences and Engineering Research Council of Canada Discovery grant awarded to I.J.O. J.P.H. acknowledges support from a University of Manitoba Faculty of Science Award and the University of Manitoba Faculty of Graduate Studies GETS Program. Proteome analysis was performed at the Manitoba Center for Proteomics and Systems Biology, with the help of Peyman Ezzati. The Manitoba Center for Proteomics and Systems Biology is funded by the Canadian Institutes for Health Research. Metabolite measurements were performed at the Rosalind and Morris Goodman Cancer Research Centre. The Metabolomics Core Facility is supported by the Canada Foundation for Innovation, the Dr. John R. and Clara M. Fraser

Memorial Trust, the Terry Fox Foundation (TFF Oncometabolism Team grant 116128), and McGill University.

We thank George diCenzo and Turlough Finan for bacterial strains.

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