A locus necessary for the transport and catabolism of erythritol in Sinorhizobium meliloti

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In this work we have genetically defined an erythritol utilization locus in Sinorhizobium meliloti. A cosmid containing the locus was isolated by complementation of a transposon mutant and was subsequently mutagenized using Tn5::B20. The locus was found to consist of five transcriptional units, each of which was necessary for the utilization of erythritol. Genetic complementation experiments using genes putatively annotated as erythritol catabolic genes clearly showed that, of the 17 genes at this locus, six genes are not necessary for the utilization of erythritol as a sole carbon source. The remaining genes encode EryA, EryB, EryC and TpiB as well as an uncharacterized ABC-type transporter. Transport experiments using labelled erythritol showed that components of the ABC transporter are necessary for the uptake of erythritol. The locus also contains two regulators: EryD, a SorC class regulator, and SMc01615, a DeoR class regulator. Quantitative RT-PCR experiments showed that each of these regulators negatively regulates its own transcription. In addition, induction of the erythritol locus was dependent upon EryD and a product of erythritol catabolism. Further characterization of polar mutations revealed that in addition to erythritol, the locus contains determinants for adonitol and L-arabitol utilization. The context of the mutations suggests that the locus is important for both the transport and catabolism of adonitol and L-arabitol.

INTRODUCTION

Sinorhizobium meliloti is a Gram-negative soil microbe that can exist either as a free-living organism in the soil or in a symbiotic relationship with legume plants. S. meliloti can interact with the legume alfalfa to form root nodules, and within these nodules it can reduce nitrogen gas and provide this fixed nitrogen to the plant (Spaink, 2000). Prior to the colonization of the root and the initiation of infection threads that lead to the release of the bacteria into the root nodule, the bacteria must be able to survive in the rhizosphere. It has been shown that the ability to use organic compounds can strongly influence competition in the rhizosphere (Triplett & Sadowsky, 1992). In Rhizobium leguminosarum, the inability to utilize erythritol has been shown to affect a strain’s ability to effectively compete for nodule occupancy (Yost et al., 2006).

The ability to utilize erythritol as a sole carbon source is not universal among the Rhizobiaceae (Jordan, 1984; Stowers, 1985). Evidence has been presented that suggests the erythritol catabolic locus may have been horizontally transferred from Brucella to R. leguminosarum (Yost et al., 2006). In addition to the eryABCD locus that had been previously shown to be necessary for erythritol catabolism (Sangari et al., 2000), it was shown that two sets of genes flanking eryABCD of R. leguminosarum were also necessary for erythritol catabolism. These are eryEFG, encoding three ABC transporter genes, and an operon consisting of a DeoR type regulator, a triose phosphate isomerase and a putative ribulose phosphate isomerase (Yost et al., 2006).

The inability to utilize erythritol by Brucella abortus strain S19 has been correlated with attenuated virulence (Meyer, 1966, 1967). This association prompted the elucidation of a biochemical pathway for its catabolism (Sperry & Robertson, 1975a, b). Subsequently, the genes for the first three steps of this biochemical pathway were characterized (eryA, eryB and eryC) and found to appear in an operon with a negative regulator eryD (Sangari et al., 1998, 2000). More recent work, using proteomic as well as comparative genomic approaches, has reinforced the role erythritol transport and catabolism may play in virulence (Burkhardt et al., 2005).

In S. meliloti, screening for mutants unable to use rhamnose as a sole carbon source identified two regions: the genes homologous to the R. leguminosarum genes necessary for rhamnose catabolism, rhaDI and rhaRSTPQMK (Richardson et al., 2004; Richardson & Oresnik, 2007; Richardson et al., 2008), and a gene encoding a triose-phosphate isomerase (tpiA) (Poysti &
The S. meliloti genome contains another gene, tpiB, that also encodes triose-phosphate isomerase activity (Poysti & Oresnik, 2007). Although both genes encode functional triose-phosphate isomerases, they appear to play distinct biochemical roles in the organism. TpiA is necessary for glycerol catabolism and has been hypothesized to play a role in central metabolism, whereas TpiB is necessary for the catabolism of erythritol (Poysti & Oresnik, 2007). It is of note that the overexpression of the wild-type tpiB can complement tpiA-associated mutant phenotypes whereas it has been shown that in S. meliloti a tpiB mutation was unable to be genetically complemented by tpiA (Poysti & Oresnik, 2007). Since only a tpiA/B double mutant, and not a tpiA or tpiB single mutant, is impaired for autotrophic and gluconeogenic growth, tpiB is expressed at low levels in the absence of erythritol (Pickering & Oresnik, 2008; Poysti & Oresnik, 2007).

Our interest in erythritol stems from our initial studies with tpiB (Poysti & Oresnik, 2007). Additionally, as noted by Yost et al. (2006), the genetic content and arrangement of the putative erythritol locus adjacent to tpiB is drastically different from that previously described in R. leguminosarum and B. abortus (Galibert et al., 2001; Halling et al., 2005; Young et al., 2006). Therefore, the goal of this work was to genetically characterize the locus and to determine what components are necessary for the transport and catabolism of erythritol.

**METHODS**

**Bacterial strains, plasmids and media.** Bacterial strains and plasmids used in this work are listed in Table 1. S. meliloti strains were grown routinely at 30 °C on complex Luria-Bertani medium (Sambrook et al., 1989) or on defined Vincent’s minimal medium (VMM) (Vincent, 1970). Carbon sources were filter-sterilized and added to VMM to a final concentration of 15 mM. When required, S. meliloti and Escherichia coli strains were grown with the following antibiotics (concentrations in μg ml⁻¹): chloramphenicol (Cm) 20; gentamicin (Gm) 20 or 60; kanamycin (Km) 20; neomycin (Nm) 200; rifampicin (Rf) 50; streptomycin (Sm) 200; tetracycline (Tc) 5. All antibiotics were filter-sterilized before use.

**DNA manipulations and constructions.** Standard techniques were used for plasmid isolation, restriction enzyme digests, ligations, transformations and agarose gel electrophoresis (Sambrook et al., 1989). Oligonucleotide primers used for PCR amplification are listed in Supplementary Table S1, available with the online version of this paper.

To create an eryD mutation, pBG1 was first constructed by amplification of a 400 bp internal fragment of eryD by PCR using Rm1021 genomic DNA as a template and primers 1 and 2. The amplicon was restricted and ligated into the suicide vector pKNOCK-Gm (Alexeyev, 1999) using BamHI and Clal. pBG1 was transformed into competent E. coli S17-1 and selected for using Gm (20 μg ml⁻¹). Single crossover eryD mutants, SMaRA855 and SMaRA856, were isolated by conjugating pBG1 into Rm1021 and SMaRA449, respectively, and selecting for Sm⁶ and Gm⁶ transconjugants as previously described (Poysti & Oresnik, 2007; Richardson et al., 2004). Colonies were screened for phenotype and mutations were verified by sequencing.

To construct a strain carrying a mutation in SMc01615, the gene upstream of tpiB that putatively encodes a DeoR-type regulator, a 1053 bp product was amplified from Rm1021 genomic DNA by PCR with primers 9 and 10, digested with BamHI and Stul, and cloned into pBluescript II SK to yield pBP149. pBP149 was digested with SmaI into which a SmaI-digested nptII cassette from pMM22 was ligated to generate pBP151. pBP151 was subsequently recloned into pIQ200SK to produce pBP173. pBP173 was mobilized into Rm1021 with MT616 and selected for double recombinants. Colonies were screened onto selective media and sequenced to verify the insertion of nptII in SMc01615. One such mutant was designated SRmA966.

Two different methods were used to construct constitutively expressed erythritol genes. ORFs of eryA, eryB and eryD were amplified by PCR using primers 5 and 6, 7 and 8, and 3 and 4, respectively, and cloned into the broad-host-range vector, pRK7813. The 5’ primers 5, 7 and 3 were all designed to have a ribosome-binding site approximately 8 bp upstream of the annotated ATG to allow constitutive expression. pBG2 was constructed by using PstI and BamHI restriction sites that were added to the primers to digest and ligate the 960 bp eryD ORF into pRK7813 such that it would be expressed from the lacZ promoter. pBG3 and pHY112 were constructed similarly using the eryA ORF with HindIII and EcoRI restriction sites and the eryB ORF with PstI and BamHI restriction sites, respectively. Additionally, the ORFs for eryA, eryC and eryD were recombined into pCO37 from the S. meliloti ORFome using the methods described previously (House et al., 2004; Jacob et al., 2008; Schroeder et al., 2005).

**Genetic manipulations.** Conjugations between E. coli strains and S. meliloti were performed as described previously using the mobilizing strain MT616 (Finan et al., 1985). Transposon mutagenesis of Rm1021 using pRK602 was carried out as described previously (Finan et al., 1985). Mutants were routinely single-colony purified three times and subsequently transduced into Rm1021 with phage ΦM12 to ensure that the mutation was 100% genetically linked to the transposon (Finan et al., 1985). The erythritol-complementing cosmids pEL6 was isolated by conjugating a cosmids bank (Wang et al., 2006) of S. meliloti into SmRmA723 and plating on defined medium using erythritol as a sole carbon source to select for complementation. The cosmids pEL6 was then mutagenized by mating the cosmids into the Tn5::B20 carrying EcaA101 (Clark et al., 2001) and selecting for co-transfer of the cosmids and a Tn5::B20 marker when mated out of EcaA101 and into DH5α yielding pHC1–pHC12 essentially as described previously (Poysti et al., 2007). The position of Tn5::B20 insertions was verified using arbitrary PCR as described previously (Miller-Williams et al., 2006; Poysti et al., 2007). Insertions confirmed to be in the erythritol locus were recombined into Rm1021 as previously described (Glazebrook & Walker, 1991). The inserts were confirmed using arbitrary PCR and were transduced into Rm1021 yielding SmRA78−SmRA787.

**β-Galactosidase assays.** Initial expression studies were carried out using lacZ fusions that were isolated in pEL6 (Table 1). The assays were essentially carried out as described previously (Clark et al., 2001; Oresnik et al., 1998). These data, although not presented, were corroborated by quantitative RT-PCR data (Table 4).

**RNA isolation and cDNA synthesis.** Bacterial cultures were grown to an OD₆₀₀ of approximately 0.8 in VMM containing either 15 mM erythritol and 15 mM glycerol or 15 mM glycerol alone. Cells were harvested by centrifugation and resuspended in TE buffer with lysozyme (0.4 mg ml⁻¹). RNA was then isolated using the Qiagen RNA isolation technique described previously (Barnett et al., 2004). All RNA samples were treated twice with Qiagen on-column RNase-free DNase kit during the DNase step to remove DNA contamination. The RNA was analysed spectrophotometrically and the absorbance ratio of the sample at 260 nm and 280 nm was compared to ensure
### Table 1. Bacterial strains and plasmids

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<th>Strain or plasmid</th>
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the purity of the RNA sample. The concentration of RNA was determined using the absorption coefficient for RNA [0.027 (µg mL\(^{-1}\)) cm\(^{-1}\)]. First strand cDNA synthesis was performed using 1 µg RNA as suggested by the supplier (Invitrogen). The quality of the cDNA synthesis was checked by electrophoresis and quantified spectrophotometrically.

**Junction PCR.** To determine the extent of transcripts, PCRs across putative junctions in the erythritol locus were performed using the cDNA from either induced cells grown on erythritol/glycerol or non-induced cells grown on glycerol. An identical PCR using the RNA samples that were used for the cDNA synthesis as a template was carried out to control for possible genomic DNA contamination. A similar reaction was also performed using genomic template as a positive primer control. PCRs across three gene junctions were performed using primers 21 and 22, 23 and 24, and 25 and 26 (Supplementary Table S1). PCR conditions were as follows: 95 °C for 60 s, 30 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 90 s, and 72 °C for 300 s.

**Quantitative RT-PCR (qRT-PCR).** Approximately 100 ng cDNA sample was used as a template for qRT-PCR. Reactions were performed using the SYBR green RT-PCR kit from Invitrogen as recommended by the manufacturer. Primers 11 and 12 were used to analyse SMc01628 transcription; 13 and 14 were used to analyse eryD transcription; 15 and 16 were used to analyse eryA transcription; 17 and 18 to analyse eryC transcription and finally 19 and 20 to analyse SMc01615 transcription. Primers were designed upstream of all the partial insertion mutations in strains in this work. The qRT-PCR was performed using a Cepheid Smart Cycler with the following program: stage 1, 95 °C for 120 s, once; stage 2, 95 °C for 15 s followed by 60 °C for 30 s, repeated 40 times; stage 3, melting curve analysis of PCR products.

**Transport assay.** Transport assays were carried out essentially as previously described (Poysti et al., 2007). Labelled d-l-[1-14C] erythritol (370 MBq mmol\(^{-1}\)) was purchased from American Radiolabeled Chemicals. Transport assays were initiated by the addition of [14C] erythritol to a final concentration of 2 µM to the samples and aliquots of 0.25 or 0.5 ml were withdrawn at appropriate time points and rapidly filtered through a Millipore 0.45 µm Hv filter on a Millipore Ls6500. The radioactivity retained by the cells was quantified by a liquid scintillation counter (Beckman LS6500).

**Competition assay.** Alfalfa plants were grown as previously described except that Leonard jar assemblies were made from Magenta jars (Sigma) (Oresnik et al., 1994). Competition for nodule occupancy assays were carried out as described previously (Oresnik et al., 1999).

**RESULTS**

**Isolation of erythritol transport and catabolic mutants**

Comparison of the putative erythritol utilization locus of *S. meliloti* with that of *R. leguminosarum* shows that the organization of the catabolic genes is very different (Yost et al., 2006). In addition, the ABC-type transporter genes that are found downstream from eryD in *S. meliloti* (SMc01628–SMc01624) do not appear to be orthologues of *eryEFG* in *R. leguminosarum*, suggesting that components involved in erythritol transport may not always be found in close proximity to the putative erythritol catabolic genes *eryA*, *eryB* and *eryC*. In an attempt to isolate genes necessary for the transport of erythritol, Tn5 mutagenesis of the wild-type was carried out.

Approximately 3000 transposon mutants were initially screened for their inability to grow on erythritol as a sole carbon source. The single mutant that was isolated was purified and designated SRmA428 (Table 1). The transposon was subsequently transduced into Rm1021 and the transductants were retested for phenotype. The transduced strain was designated SRmA465. The transposon was found to be within SMc01627, which is predicted to encode a permease that appears to be part of an ABC-type transporter located between the putative negative regulator *eryD* and the gene encoding the putative erythritol kinase, *eryA*.

Based on the close proximity of the putative start and stop codons found immediately upstream and downstream of each of the genes annotated as being necessary for erythritol catabolism, it was initially assumed that all the genes from *eryD* to *eryC* constituted a single operon (Fig. 1). Combined with the ABC gene transporter gene locus, this suggests close proximity of all the erythritol utilization genes. Attempts to complement SRmA465 with the complementing cosmid for *tpiB*, pPNP163 (Poysti & Oresnik, 2007), were unsuccessful, suggesting that pPNP163 did not contain the entire locus necessary for erythritol catabolism (data not shown). To obtain a complementing cosmid for the erythritol catabolic locus, a cosmid bank was mated en masse into SRmA465 (Wang et al., 2006) and the *S. meliloti* transconjugants that had regained their ability to use erythritol as a sole carbon source were isolated. One such isolate, that had the ability to complement the SMc01627 mutation in SRmA465 as well as the *tpiB2* allele in SRmA584, was retained and designated pEL6 (Table 1).

To generate additional mutations in the erythritol locus, a saturation mutagenesis of pEL6 was performed using Tn5::B20 to generate representative insertions across pEL6. Inserts were identified by generating a PCR fragment from the IS50 element on the Tn5::B20 using an arbitrary PCR protocol, and sequencing the products using a primer designed to match the IS50 element (Poysti et al., 2007). The plasmids carrying insertions within the erythritol locus were designated pHCl12 (Table 1, Fig. 1). Each of these insertions was marker exchanged into the chromosome of Rm1021 to yield the corresponding genomic mutation as described in Methods. These mutations were designated SRm778–787 (Table 1). Each of these strains was unable to use erythritol as a sole carbon source. Also, each strain containing an insertion in a gene downstream of *eryA* was unable to grow when erythritol was present with a secondary carbon source such as glycerol. Similar toxic effects have been previously noted for erythritol as well as other sugars in both *R. leguminosarum* and *E. coli* (Adhya & Shapiro, 1969; Power, 1967; Richardson et al., 2004; Sperry & Robertson, 1975a; Yost et al., 2006).

Although a good distribution of transposon insertions across the transport and catabolic genes of the locus were
isolated, insertions in the two putative regulators were not (Fig. 1). Therefore single crossover mutations in \( \text{eryD} \) and \( \text{SMc01615} \) were constructed giving rise to SRmA885 and SRmA966 respectively. Neither strain was able to use erythritol as a sole carbon source. In addition it was found that SRmA966 was unable to grow on glycerol in the presence of erythritol. Since \( \text{eryD} \) and \( \text{SMc01615} \) are annotated as negative regulators, these phenotypes suggested polarity on downstream genes and were consistent with our hypothesis that the entire locus was transcribed as a single unit.

**Erythritol utilization genes are in different complementation groups**

To address the hypothesis that the erythritol locus in \( S. \text{meliloti} \) was a single operon, plasmids containing inserts within genes that were presumed to be necessary for erythritol catabolism were conjugated into each of SRmA778–SRmA787 to test for complementation. In addition, these plasmids were also conjugated into SRmA885 (\( \text{eryD} \)), SRmA966 (\( \text{SMc01615} \)) and SRmA584 (\( \text{tpiB} \)). The resulting transconjugants were screened for their ability to utilize erythritol as a sole carbon source. The results show that contrary to our initial assumptions, the erythritol locus in \( S. \text{meliloti} \) does not consist of a single transcript from \( \text{eryD} \) to \( \text{eryC} \) (Table 2). The locus appears to consist of at least five transcripts: a transcript containing \( \text{eryD} \), a transcript encoding the components for one or more ABC transporters (\( \text{SMc01628–SMc01624} \)), a transcript that spans \( \text{eryA} \) through \( \text{eryB} \), a transcript containing \( \text{eryC} \) and a transcript containing the genes \( \text{SMc01615, tpiB} \) and \( \text{rpiB} \). Interestingly, since \( \text{eryD} \) appears to be transcribed independently, the inability of SRmA885 to use erythritol as a sole carbon source cannot be explained by polar effects on the ABC transporter. Similarly, SRmA428 (\( \text{SMc01627} \)) has no polar effects on the downstream erythritol catabolic genes.

**\( \text{eryA} \) and \( \text{eryB} \) are contained in a single transcript**

Although the complementation data are consistent with \( \text{eryA} \) and \( \text{eryB} \) being in a single transcript (Table 2), the data could be viewed as ambiguous with respect to \( \text{eryA} \) and \( \text{eryB} \) (Table 2). We note that SRmA782, containing \( \text{eryA} \), as well as SRmA784, containing \( \text{eryB} \), often grew poorly when carrying pHC6 or pHC9. We reasoned that if \( \text{eryA} \), \( \text{SMc01622, fucA1} \) and \( \text{eryB} \) constituted a single transcript, they should all be present on a single mRNA. To test this, total RNA from erythritol-grown Rm1021 was isolated and used to create cDNA. Primers to the junctions of each of these genes were designed. In each case, we were able to generate a PCR product from the cDNA suggesting

**Table 2. Erythritol catabolic genes are in separate complementation groups**

Growth is as follows: +, wild-type; +/-, marginal growth; -, no growth.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>none pHC10 pHC3 (1628) pHC6 (1627) pHC9 (eryA) pHC12 (eryB) pEL6 (eryC) Wild-type</td>
</tr>
<tr>
<td>Rm1021</td>
<td>Wild-type</td>
<td>+                  +                        +                  +                  +                  +                  +                  +</td>
</tr>
<tr>
<td>SRmA855</td>
<td>( \text{eryD} )</td>
<td>–                  +                        +                  +                  +                  +                  +                  +</td>
</tr>
<tr>
<td>SRmA785</td>
<td>( \text{SMc01628} )</td>
<td>–                  –                        +                  +                  +                  +                  +                  +</td>
</tr>
<tr>
<td>SRmA780</td>
<td>( \text{SMc01627} )</td>
<td>–                  –                        –                  +                  +                  +                  +                  +</td>
</tr>
<tr>
<td>SRmA782</td>
<td>( \text{eryA} )</td>
<td>–                  +                        +                  +                  +/-               +                  +                  +</td>
</tr>
<tr>
<td>SRmA784</td>
<td>( \text{eryB} )</td>
<td>–                  +                        +                  +/-               +                  +                  +                  +</td>
</tr>
<tr>
<td>SRmA787</td>
<td>( \text{eryC} )</td>
<td>–                  +                        +                  +                  +                  +                  +                  +</td>
</tr>
<tr>
<td>SRmA966</td>
<td>( \text{SMc01615} )</td>
<td>–                  +                        +                  +                  +                  +                  +                  +</td>
</tr>
<tr>
<td>SRmA584</td>
<td>( \text{tpiB} )</td>
<td>–                  +                        +                  +                  +                  +                  +                  +</td>
</tr>
</tbody>
</table>
that these genes were indeed within a single transcript (Fig. 2). Primers (27 and 28) designed to amplify the junction between eryB and SMc01619 did not generate a PCR product from cDNA suggesting that eryB was the final gene in this transcript (data not shown).

The erythritol locus does not contain uncharacterized genes involved in erythritol catabolism

The metabolic processes necessary for the breakdown of erythritol in B. abortus have been shown to consist of five steps (Sperry & Robertson, 1975b). Of the five reactions, the genes for only three of these, eryA, eryB and eryC have been discovered (Sangari et al., 2000). The two transcripts that contain eryA, eryB and eryC in S. meliloti include five other genes (Fig. 1). These genes encode a putative oxidoreductase (SMc01622), a putative aldolase (FucA1), a putative class II aldolase/adducin protein (SMc01619), a putative sugar kinase (SMc01618) and a putative hydrolase (SMc01617). To determine if any of these genes encoded proteins that play a role in the catabolism of erythritol, eryA, eryB and eryC were introduced and overexpressed in each of the mutants that we had previously isolated (Table 3).

When eryB was overexpressed in SRmA781, SRmA779 and SRmA784, carrying mutations in SMc01622, fucA1 and eryB, respectively, each strain was complemented for the inability to utilize erythritol as a sole carbon source (Table 3). SRmA782, carrying an eryA mutation, was not complemented by the introduction of eryB. Taken together, these data strongly suggest that only eryA and eryB are necessary for erythritol breakdown and that the insertions in SRmA781 and SRmA779 have polar effects on eryB. Similarly, it was found that SRmA783 and SRmA778 were complemented by the introduction of eryC on a plasmid suggesting that proteins encoded by SMc01619, SMc01618 and SMc01617 do not play a role in erythritol catabolism (Table 3). These data suggest that the transcript that contains eryC also contains SMc01617–SMc01619 (Tables 2 and 3).

Introduction of a plasmid containing eryA into SRmA782 did not complement the ability to utilize erythritol (Table 3). Although this is not surprising since all the data presented support the hypothesis that an eryA mutation has a polar affect on eryB (Tables 2 and 3, Fig. 2), it is worth noting that when eryA was expressed in SRmA782, the strain was unable to grow on a medium that contained erythritol with a second carbon source that could support growth (Table 3). This correlates with previous suggestions that toxic effects are the result of the build-up of a phosphorylated intermediate (Adhya & Shapiro, 1969; Power, 1967; Richardson et al., 2004; Sperry & Robertson, 1975a; Yost et al., 2006).

Components of the ABC-type transporter defined by SMc01628–SMc01624 are necessary for transport of erythritol

BLAST analysis using Hyp-EryEFG from R. leguminosarum VF39SM against the S. meliloti Rm1021 database identified SMb20349–SMb20352 as having the highest similarity at the amino acid level (about 67% for EryEFG, about 47% for Hyp). Data from our transposon mutagenesis experiments show that inserts that affect the SMc01628–SMc01624 lead to an inability to use erythritol as a sole carbon source (Table 2). Since duplication of function is not uncommon in Rhizobium (Oresnik et al., 1998; Renalier et al., 1987; Schlüter et al., 1997; Schwedock & Long, 1992), we wanted to determine if erythritol transport was inducible in S. meliloti and if the transporter genes found between eryA and eryD were indeed necessary for erythritol transport.

Rm1021 grown in defined medium containing glucose or glycerol was not capable of taking up labelled erythritol at levels above those observed in dead-cell controls (data not shown). In contrast, Rm1021 grown in medium containing...
either erythritol or erythritol and glycerol displayed robust transport rates (Fig. 3). To determine if the ABC transporter genes between \( \text{eryA} \) and \( \text{eryD} \) were necessary, SRmA465, carrying a Tn5 insertion in SMc01627, was grown in medium containing erythritol and glycerol. The data demonstrate that SRmA465 was unable to transport labelled erythritol into the cell suggesting that this gene(s) encodes a protein(s) used in an ABC transporter involved in the transport of erythritol (Fig. 3).

**EryD and SMc01615 negatively regulate their own transcripts and affect the expression of other ery transcripts**

We had previously shown that \( \text{tpiB} \) could be induced by low concentrations of erythritol, thus allowing it to complement a \( \text{tpiA} \) mutation in Rm1021 (Poysti & Oresnik, 2007). Since \( \text{tpiB} \) is in an operon that includes a DeoR-type negative regulator, encoded by SMc01615, it was postulated that this regulator would regulate \( \text{tpiB} \) expression and that second-site mutations that allowed phenotypic suppression of \( \text{tpiA} \) would be isolated readily (Poysti & Oresnik, 2007). Although more than \( 10^{10} \) cells were plated, this class of second-site mutations was never isolated, which led to the suggestion that \( \text{tpiB} \) may not be solely under the regulation of SMc01615 (Poysti & Oresnik, 2007). To address the regulation of \( \text{tpiB} \) and the other components of the erythritol catabolic genes, RT-PCR experiments were carried out.

Consistent with preliminary \( \beta \)-galactosidase assays using genetic fusions (data not shown), it was found that, in the presence of erythritol, components of the erythritol catabolic pathway were induced 5–10-fold when compared with transcript levels found in glycerol-grown cells (Table 4). Similar analysis with the \( \text{eryD} \) mutant, SRmA885, showed greater than 10-fold increased expression of \( \text{eryD} \) in glycerol conditions compared with wild-type levels demonstrating that, consistent with the annotation, EryD is a negative regulator and that it regulates the expression of its own transcript (Table 4). Intriguingly, every other transcript in the erythritol locus was expressed at, or below, background levels when SRmA885 (\( \text{eryD} \)) was grown under inducing conditions; a phenotype more commonly associated with a positive regulator (Table 4). The inability of SRmA885 to induce erythritol transport and catabolic genes is consistent with its inability to grow using erythritol as a sole carbon source (Table 2).

To address the role that SMc01615 plays in the regulation of erythritol catabolic genes, a strain (SRmA966) containing a mutation in this gene was constructed. Since this strain is unable to grow in the presence of erythritol, only the effect of the mutation under non-inducing conditions could be analysed. The results clearly show that lack of SMc01615 resulted in a greater than 10-fold induction of its own transcript under non-inducing conditions. We note

![Fig. 3. Erythritol transport assays of S. meliloti wild-type (Rm1021, ○) and the erythritol transport mutant SRmA465 (SMc01627, ■) induced with defined medium containing erythritol and glycerol. Where not shown error bars are smaller than the symbol. Error bars represent SD (n=3).](image)

**Table 3. Complementation of mutations in erythritol catabolic genes**

Growth is as follows: +, wild-type; +/-, marginal growth; –, no growth. Ery/glyc, media contained both 15 mM erythritol and 15 mM glycerol.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Plasmid</th>
<th>Ery</th>
<th>Ery/glyc</th>
<th>Glyc</th>
<th>Ery</th>
<th>Ery/glyc</th>
<th>Glyc</th>
<th>Ery</th>
<th>Ery/glyc</th>
<th>Glyc</th>
<th>Ery</th>
<th>Ery/glyc</th>
<th>Glyc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rm1021</td>
<td>Wild-type</td>
<td>None</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SRmA782</td>
<td>( \text{eryA} )</td>
<td>pBG3 (( \text{eryA}^{+} ))</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SRmA781</td>
<td>( \text{Smc01622} )</td>
<td>pBG14 (( \text{eryB}^{+} ))</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SRmA779</td>
<td>( \text{fucA1} )</td>
<td>pHY112 (( \text{eryC}^{+} ))</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>SRmA784</td>
<td>( \text{eryB} )</td>
<td>None</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>( \text{Smc01619} )</td>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>-</td>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
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<td>( \text{eryC} )</td>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 4. Regulation of gene expression by eryD and SMc01615 analysed by qRT-PCR

Data are expressed as $2^{\Delta Ct}$ and represent fold expression over uninduced Rm1021 grown in glycerol. The experiment also included SMc00128 as an internal control (Krol & Becker, 2004). The table shows representative data from a single experiment. The experiment was repeated three times, showing consistent results. Ery/glyc, media contained both 15 mM erythritol and 15 mM glycerol.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Carbon source</th>
<th>$\Delta Ct$</th>
<th>SMc01615</th>
<th>$\Delta Ct$</th>
<th>SMc01615</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rm1021</td>
<td>Wild-type</td>
<td>Glyc</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Rm1021</td>
<td>Wild-type</td>
<td>Ery/glyc</td>
<td>11.0</td>
<td>19.4</td>
<td>16.9</td>
<td>11.9</td>
</tr>
<tr>
<td>SRmA885</td>
<td>eryD</td>
<td>Glyc</td>
<td>14.7</td>
<td>0.8</td>
<td>0.1</td>
<td>0.9</td>
</tr>
<tr>
<td>SRmA885</td>
<td>eryD</td>
<td>Ery/glyc</td>
<td>18.6</td>
<td>0.3</td>
<td>0.1</td>
<td>1.1</td>
</tr>
<tr>
<td>SRmA966</td>
<td>SMc01615</td>
<td>Glyc</td>
<td>5.5</td>
<td>0.7</td>
<td>0.6</td>
<td>1.4</td>
</tr>
<tr>
<td>SRmA782</td>
<td>eryA</td>
<td>Glyc</td>
<td>0.3</td>
<td>0.3</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>SRmA782</td>
<td>eryA</td>
<td>Ery/glyc</td>
<td>1.47</td>
<td>0.8</td>
<td>0.4</td>
<td>0.7</td>
</tr>
</tbody>
</table>

that in SRmA966 we observed an increased level of transcription of eryD suggesting that SMc01615 may also play a role in regulating eryD (Table 4).

The inability to catabolize erythritol affects gene expression

It is unclear if the regulators of ery catabolic genes respond to either erythritol and/or an erythritol metabolite as an effector molecule (Sangari et al., 2000; Yost et al., 2006). Since the organization of the operons containing the ery catabolic and transport genes is so different from that of either B. abortus or R. leguminosarum, it was thought that investigating the effect of erythritol catabolic mutations on the regulation of these genes may provide some insight. Analysis of transcription in SRmA782 (eryA), revealed that only background levels of transcription occurred across the entire erythritol region when it was grown on media containing erythritol (Table 4). This suggests that in addition to its own induction, a functional EryA is essential for the induction of the entire erythritol locus including tpiB and eryD. In addition, it suggests that the regulators of the erythritol locus probably respond to an intermediate of erythritol catabolism.

The erythritol locus contains determinants for adonitol and arabitol utilization

The transcripts that contain the genes necessary for erythritol catabolism (eryA, eryB, eryC and tpiB) also contain six other genes that are annotated as being involved in small-molecule metabolism. Moreover, the genes that encode the components of the ABC transporter contain two ABC proteins and two permease components, formally making it possible that this may represent more than one transport system. To attempt to define what role the other genes in this cluster may play, representative insertion mutants from each of the transcripts (except eryD) were analysed for carbon utilization phenotypes using Biolog PM1 and PM2 plates. The results from the PM1 and PM2 plates that suggested an inability to use a compound as a sole carbon source were verified by streaking the strains on defined media containing these as sole carbon sources. Intriguingly, these results show that mutants with insertions in the ABC transporter transcript (SRmA783), the transcript containing eryA and eryB (SRmA782) as well as the transcript containing eryC (SRmA783) were unable to use adonitol or L-arabitol as a sole carbon source (Table 5). In contrast, the transcript that contains tpiB was only necessary for erythritol catabolism. In an effort to more precisely define which genes were necessary for the utilization of which sugar, other insertions in these transcripts were also tested. The majority of the strains carrying inserts in these transcripts were unable to use adonitol and L-arabitol in addition to erythritol (Table 5). This includes SRmA855 which contains an eryD mutation, suggesting that EryD also regulates the locus during adonitol or L-arabitol catabolism. SRmA787, containing an insertion in eryC, was able to use adonitol and L-arabitol as sole carbon sources (Table 5). It is noteworthy that the analysis also showed that although these strains were unable to grow on L-arabitol, they were still capable of utilizing D-arabitol (Table 5). It is also worth noting that Rm1021 could not grow using the closely related sugar alcohol xylitol as a sole carbon source (data not shown).

Lack of erythritol, adonitol, and arabitol utilization does not affect competition for nodule occupancy in S. meliloti

It has recently been shown that erythritol affects competition for nodule occupancy in R. leguminosarum (Yost et al., 2006). In addition, erythritol utilization has been associated with avirulence in various Brucella strains (Köhler et al., 2002). SRmA465 was used as a representative strain since it lacked the ability to take up erythritol and was unable to grow on adonitol or arabitol (Table 1). Two different inoculation ratios (mutant: wild-type) were tried and in every trial, the ratio of strains isolated from the nodules was identical to the inoculation ratio, suggesting that the ability to utilize erythritol, adonitol or arabitol does not play a role in competition for nodule occupancy (data not shown).
**DISCUSSION**

Our genetic and physical data show that the erythritol locus in *S. meliloti* consists of five transcripts. Of the catabolic genes present, only *eryA*, *eryB*, *eryC* and *tpiB* appear to encode proteins that are necessary for erythritol catabolism (Tables 2 and 3). In addition, genes that encode the determinants for an ABC transporter are essential for the transport of erythritol into the cell (Fig. 3). It is of note that genes encoding Smb20349–51, which were predicted to be necessary for erythritol transport in *S. meliloti* on the basis of identity to the *R. leguminosarum* erythritol transporter (Yost *et al.*, 2006), do not have erythritol transport function based on our ability to show lack of erythritol transport in our mutants.

The original work outlining the catabolic pathway for erythritol catabolism provided evidence for five biochemical steps yielding dihydroxy-acetone phosphate (Sperry & Robertson, 1975b). Analyses done on *B. abortus* and *R. leguminosarum*, as well as the data presented here, only account for three of these genes as well as a dedicated triose-phosphate isomerase (Sangari *et al.*, 2000; Yost *et al.*, 2006). Complementation of each of *eryA*, *eryB*, *eryC* and *tpiB* clearly shows that the other genes at this locus do not encode enzymes that have the missing activities (Table 3). Although our limited mutagenesis did provide evidence for the transporters necessary for erythritol, it did not identify either of these two missing activities. It should also be noted that although a mutation in the gene annotated as *rpiB* (*Smc01613*) did not affect the ability to use erythritol as a sole carbon source, the corresponding mutant in *R. leguminosarum* was unable to use erythritol (Poysti & Oresnik, 2007; Yost *et al.*, 2006). It is not known at this time how *rpiB* does, or does not, contribute to erythritol catabolism in either *R. leguminosarum* or *S. meliloti*. Conjugation of pEL6 (containing the entire *S. meliloti* ery locus) into *R. leguminosarum* strain Rlt100, a strain that does not have the capability of utilizing erythritol as a sole carbon source, allowed it to utilize erythritol (data not shown). We suggest that perhaps the biochemical steps that were originally described in *B. abortus*, that are not accounted for by the genes present in the erythritol locus, may be due to enzymes that have enzymic activity on a broad number of substrates and may not be specific for erythritol. Alternatively, some of the erythritol-associated proteins may have more than one enzymic activity.

The regulation of erythritol catabolic genes in *R. leguminosarum* and *B. abortus* has been shown to be carried out by EryD which has been annotated as a negative regulator that falls into a DeoR category (Sangari *et al.*, 2000; Yost *et al.*, 2006). Consistent with this annotation, an *R. leguminosarum* strain carrying an *eryD* mutation was not diminished in its ability to grow on erythritol (Yost *et al.*, 2006). In contrast, we found that *S. meliloti* carrying an *eryD* mutation was unable to use erythritol as a sole carbon source (Table 2). In addition, if an *eryD* mutation was moved into a strain containing a *tpiA* mutation, it was unable to phenotypically suppress the inability to utilize glycerol associated with a *tpiA* mutation if grown on glycerol with 0.5 mM erythritol (Poysti & Oresnik, 2007).

Taken together, these data prompted a more thorough analysis of regulation that may be occurring at this locus. Our data clearly show that EryD is a negative regulator (Table 4). In contrast, the transcription of the other operons at this locus appears to be at, or below, its basal level when *eryD* is absent (Table 4). These data are inconsistent with the hypothesis that *eryD* negatively regulates the genes encoding the ABC transporter, *eryA*, *eryB*, *eryC* and *tpiB*. The data support the involvement of a positive regulator in erythritol catabolism in *S. meliloti.*

**Table 5.** Polyol utilization phenotypes of mutations across the erythritol locus

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Carbon source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Erythritol</td>
</tr>
<tr>
<td>Rm1021</td>
<td>Wild-type</td>
<td>+</td>
</tr>
<tr>
<td>SRmA885</td>
<td>eryD</td>
<td>–</td>
</tr>
<tr>
<td>SRmA785</td>
<td>Smc01628</td>
<td>–</td>
</tr>
<tr>
<td>SRmA782</td>
<td>eryA</td>
<td>–</td>
</tr>
<tr>
<td>SRmA781</td>
<td>Smc01622</td>
<td>–</td>
</tr>
<tr>
<td>SRmA779</td>
<td>fucA1</td>
<td>–</td>
</tr>
<tr>
<td>SRmA784</td>
<td>eryB</td>
<td>–</td>
</tr>
<tr>
<td>SRmA783</td>
<td>Smc01619</td>
<td>–</td>
</tr>
<tr>
<td>SRmA778</td>
<td>Smc01618</td>
<td>–</td>
</tr>
<tr>
<td>SRmA787</td>
<td>eryC</td>
<td>–</td>
</tr>
<tr>
<td>SRmA966</td>
<td>Smc01615</td>
<td>–</td>
</tr>
<tr>
<td>SRmA584</td>
<td>tpiB</td>
<td>–</td>
</tr>
</tbody>
</table>

Growth is as follows: +, wild-type; +/-, marginal growth; –, no growth.
DalR, the regulator of arabitol utilization in Klebsiella pneumonia is also considered a member of this family (Heuel et al., 1998). SorC has been shown to act as both a negative and a positive regulator of sorbitol utilization in K. pneumonia (Wöhrl et al., 1990). It has been shown that SorC is able to negatively regulate its own transcription. In addition Wöhrl et al. (1990) were also able to demonstrate lack of transcription of the sorbose operon in a non-polar background lacking sorC. Complementation of this mutation with a sorC+ allele restored gene expression of the sorbitol operon. More recently the crystal structure of SorC has been solved, and based on the structural model, a model of the positive and negative transcriptional regulation has been proposed (de Sanctis et al., 2009). The SorC data are analogous to what we observed with eryD (Table 4). Attempts at complementing the eryD mutation with pbG2 and pbG18 (eryD+) gave ambiguous results; in some cases transcription was observed, whereas in other cases it was not (data not shown). Further work is being carried out to try to resolve this issue.

qRT-PCR data also showed that SMc01615 negatively affects the regulation its own transcript, as well as the transcription of eryD (Table 4). We note that SMc01615 contains a sugar-phosphate binding site (cl0039 sugarP), suggesting that it does not respond directly to erythritol, but to a phosphorylated metabolite of the pathway. This is consistent with the data that show only basal levels of transcription of SMc01615 in an eryA background. It is also supported by observation that tpiB is only necessary for erythritol catabolism in S. meliloti and that a strain carrying a tpiA mutation had its inability to grow on glycerol phenotypically suppressed when it was grown on glycerol in the presence of 0.5 mM erythritol, but not 0.5 mM adonitol or L-arabitol (data not shown). The constitutive expression of the SMc01615 transcript in an SMc01615 background suggests that although EryD is required for its induction, the effect is probably indirect and is a consequence of its effect on the catabolic genes (Table 4). The effect of SMc01615 on the transcription of eryD however, is likely to be direct.

In SRm782 (eryA), transcription at the erythritol locus was abolished (Table 4). These data strongly support the hypothesis that transcription is dependent upon a metabolite from the erythritol pathway since even transcription of eryD and SMc01615 were unable to be induced (Table 4). We note that EryD and SMc01615 contain different putative domains. EryD contains a domain that is classified as a part of the sugar-binding super family (cl04446) and SMc01615 contains a domain related to the sugar-phosphate isomerase super family (cl10039). Taken together, this suggests that the regulators may not recognize the same metabolite.

The apparent role of this locus in erythritol catabolism was expected since eryA, eryB and eryC are all homologues of genes previously shown to be necessary for erythritol catabolism in R. leguminosarum and B. abortus (Sangari et al., 2000; Yost et al., 2006). The role of the locus in adonitol and L-arabitol metabolism is surprising, as it was not suggested by the annotation of genes in the locus. The annotation suggests that the genes interspersed among the ery catabolic genes encode an oxidoreductase (SMc01622), an aldolase (fucaI), a putative class II aldolase/adducin protein (SMc01619), a sugar kinase (SMc01618) and a hydrolase (SMc01617). Based on the currently understood pathways of adonitol and arabitol catabolism as they were defined in Aerobacter aerogenes (Wood et al., 1961), as well as what is known about polyol metabolism in R. leguminosarum (Primrose & Ronson, 1980; Ronson & Primrose, 1979; Stowers, 1985; Yost et al., 2006), it is unclear which of these genes are necessary for the catabolism of either arabitol or adonitol. It may be pertinent to point out that orthologues of SMc01617–19 appear to form an independent operon in Mesorhizobium loti (mlr3601, mlr3603 and mlr3604), suggesting that these genes may in fact be a separate catabolic entity (Kaneko et al., 2000).

An inability to catabolize erythritol has been shown to affect competition for nodule occupancy in R. leguminosarum bv. viciae (Fry et al., 2001; Yost et al., 2006), whereas the ability to catabolize adonitol in R. leguminosarum bv. trifolii did not affect competition for nodule occupancy (Oresnik et al., 1998). Competition experiments indicated that a strain unable to transport erythritol, adonitol and L-arabitol was as effective as the wild-type at competing for nodule occupancy on alfalfa. It would be of interest to determine whether the ability to catabolize erythritol by other rhizobia affects competition for nodule occupancy or whether there are host-plant-specific effects of erythritol catabolism on competition.

We are currently investigating the erythritol locus with respect to its make-up, gene arrangement and polyol catabolic ability throughout the rhizobia and other bacterial species. In addition, we are attempting to determine the precise functions encoded by the genes that are necessary for adonitol and arabitol catabolism in S. meliloti. It is hoped that defining the roles of these genes will be of benefit to both the development of S. meliloti as a model organism for the study of plant–microbe interactions as well as to provide insight into basic metabolic pathways.

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Erythritol catabolism in *S. meliloti*


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