Sinorhizobium meliloti pSymB carries genes necessary for arabinose transport and catabolism

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Arabinose is a known component of plant cell walls and is found in the rhizosphere. In this work, a previously undeleted region of the megaplasmid pSymB was identified as encoding genes necessary for arabinose catabolism, by Tn5-B20 random mutagenesis and subsequent complementation. Transcription of this region was measured by β -galactosidase assays of Tn5-B20 fusions, and shown to be strongly inducible by arabinose, and moderately so by galactose and seed exudate. Accumulation of [3 H]arabinose in mutants and wild-type was measured, and the results suggested that this operon is necessary for arabinose transport. Although catabolite repression of the arabinose genes by succinate or glucose was not detected at the level of transcription, both glucose and galactose were found to inhibit accumulation of arabinose when present in excess. To determine if glucose was also taken up by the arabinose transport proteins, [14 C]glucose uptake rates were measured in wild-type and arabinose mutant strains. No differences in glucose uptake rates were detected between wild-type and arabinose catabolism mutant strains, indicating that excess glucose did not compete with arabinose for transport by the same system. Arabinose mutants were tested for the ability to form nitrogen-fixing nodules on alfalfa, and to compete with the wild-type for nodule occupancy. Strains unable to utilize arabinose did not display any symbiotic defects, and were not found to be less competitive than wild-type for nodule occupancy in co-inoculation experiments. Moreover, the results suggest that other loci are required for arabinose catabolism, including a gene encoding arabinose dehydrogenase.

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INTRODUCTION

Sinorhizobium meliloti is a Gram-negative soil bacterium capable of forming a symbiotic nitrogen-fixing relationship with legumes such as alfalfa. *S. meliloti* infects and forms nodules on plant roots, in a process involving complex signal exchange between the plant and bacteria (Spaink, 2000). Surrounding the roots of plants is a thin zone called the rhizosphere, where plant secretions enrich the soil environment with organic compounds (Bowen & Rovira, 1976). The ability to effectively utilize energy sources found in the rhizosphere may play an important role in the long-term survival of soil microbes such as *S. meliloti*.

Arabinose supports the growth of many rhizobia (Stowers, 1985), but is catabolized by different pathways in slow- and fast-growing species. In both groups, the first two enzymic reactions are conserved. Following the initial reactions, the pathways diverge, producing pyruvate and glycoaldehyde in the slow growers, and 2-oxoglutarate in the fast growers (Dilworth *et al.*, 1986; Duncan & Fraenkel, 1979; Duncan, 1979; Pedrosa & Zancan, 1974; Stowers, 1985). As *S. meliloti* is a member of the fast-growing rhizobia, arabinose is

catabolized to 2-oxoglutarate (Duncan & Fraenkel, 1979). To our knowledge, the only characterized gene involved in this pathway of arabinose catabolism is the arabinose dehydrogenase of *Azospirillum brasiliense* (Watanabe *et al.*, 2006).

Several sugars, including the pentose arabinose, have been detected in the exudate of pea plants (Knee *et al.*, 2001). In fact, arabinose makes up a large proportion of the sugars found in pea plant exudate, and is present at physiologically relevant concentrations, shown to be sufficient to support the growth of *Rhizobium leguminosarum* (Knee *et al.*, 2001). As well as being secreted, arabinose has also been found as a component of plant cell walls (McNeil *et al.*, 1984). Despite the prominence of this sugar around plant roots, genetic characterization of the determinants necessary for *S. meliloti* arabinose catabolism has not been described in the literature.

Loss of the ability to catabolize carbon sources has been correlated with defective symbiosis or decreased ability to compete for nodule occupancy in both *S. meliloti* and *R. leguminosarum*. Although non-competitive strains retain the ability to form a symbiotic relationship with the plant host, wild-type parent strains dominate for nodule occupancy when co-inoculated with less competitive mutant strains. Examples of the latter are the myo-inositol and rhamnose catabolism mutants of *R. leguminosarum* (Fry

Abbreviations: ABC, ATP-binding cassette; Nm, neomycin; Nal, nalidixic acid; Sm, streptomycin; Tc, tetracycline.

et al., 2001; Oresnik *et al.*, 1998) and the proline dehydrogenase mutant of *S. meliloti* (Jiménez-Zurdo *et al.*, 1995, 1997). In addition to arabinose, rhamnose has also been reported to be a constituent of plant secretions and cell walls (Knee *et al.*, 2001; McNeil *et al.*, 1984), suggesting a possible link between the ability to utilize rhizosphere carbon sources and nodulation competency. It has also been reported that an *S. meliloti* Tn5 arabinose catabolism mutant has a delayed and compromised symbiotic phenotype (Duncan, 1981), although the nature of this mutation appears to remain uncharacterized.

In an effort to characterize *S. meliloti* arabinose catabolism genetically, mutants unable to utilize arabinose as a sole carbon source were isolated, and we report the existence of a locus necessary for arabinose transport and catabolism on

the megaplasmid pSymB. In addition to identifying an arabinose catabolism operon, experiments were performed to determine the effects of catabolite repression on arabinose gene expression, the possibility of gene induction by seed exudate, and the symbiotic competence associated with arabinose catabolism mutations.

METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used and generated in this work are listed in Table 1. *S. meliloti* strains were routinely grown at 30 °C on complex Luria–Bertani (LB) medium or defined Vincent’s minimal medium (VMM), as previously described (Sambrook *et al.*, 1989; Vincent, 1970; Richardson *et al.*, 2004). Carbon sources used in defined media were filter-sterilized and used at a final concentration of

Table 1. Bacterial strains and plasmids

For antibiotic abbreviations, see Methods.

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
<i>S. meliloti</i>		
Rm1021	SU47 <i>str-21</i> , Sm ^r	Meade <i>et al.</i> (1982)
RmG212	Rm8501 <i>lac</i> , Sm ^r	Glazebrook & Walker (1991)
SRmA240	RmG212 <i>araF1::</i> Tn5-B20, Nm ^r	This work
SRmA482	RmG212 <i>araD2::</i> Tn5-B20, Nm ^r	This work
SRmA502	Rm1021 <i>araD3::</i> Tn5-B20, Nm ^r	This work
SRmA503	Rm1021 <i>araA4::</i> Tn5-B20, Nm ^r	This work
SRmA505	Rm1021 <i>araF5::</i> Tn5-B20, Nm ^r	This work
SRmA506	Rm1021 <i>araE6::</i> Tn5-B20, Nm ^r	This work
SRmA507	Rm1021 <i>araD7::</i> Tn5-B20, Nm ^r	This work
SRmA508	Rm1021 <i>araB8::</i> Tn5-B20, Nm ^r	This work
<i>E. coli</i>		
MM294A	<i>pro-82 thi-1 hsdR17 supE44</i>	Finan <i>et al.</i> (1986)
MT607	MM294A <i>recA-56</i>	Finan <i>et al.</i> (1986)
MT616	MT607(pRK600)	Finan <i>et al.</i> (1986)
MT620	MT607(pRK600ΩTn5-B20)	T. Finan, McMaster University, via T. Charles
EcA100	MT607 Rif ^r derivative	This work
EcA101	MT607ΩTn5-B20, Kan ^r	Clark <i>et al.</i> (2001)
HB101	<i>hsd20 recA13 rpsL20</i>	Laboratory collection
DH5α	<i>gyrA96</i> , Nal ^r	Invitrogen
Plasmids		
pRK600	pRK2013 <i>npt::</i> Tn9, Cm ^r	Finan <i>et al.</i> (1986)
pRK602	pRK600ΩTn5, Cm ^r , Nm ^r	Finan <i>et al.</i> (1985)
CX1	<i>S. meliloti</i> cosmid bank	T. Charles
pPH1JI	IncP plasmid, Gm ^r	Beringer <i>et al.</i> (1978)
pZW1	CX1-derived arabinose-complementing cosmid, Tc ^r	This work
pZW2	pZW1 <i>araD2::</i> Tn5-B20	This work
pZW3	pZW1 <i>araB8::</i> Tn5-B20	This work
pZW4	pZW1 <i>araD3::</i> Tn5-B20	This work
pZW5	pZW1 <i>araA4::</i> Tn5-B20	This work
pZW7	pZW1 <i>araF5::</i> Tn5-B20	This work
pZW8	pZW1 <i>araE6::</i> Tn5-B20	This work
pZW9	pZW1 <i>araD7::</i> Tn5-B20	This work

15 mM. Seed exudate was prepared as previously described, and filter-sterilized (Mulligan & Long, 1985). Two independent preparations of exudate were used in this work, at 10% (v/v). When required, antibiotics were used at the following concentrations: chloramphenicol (Cm) 20 $\mu\text{g ml}^{-1}$, gentamicin (Gm) 20 or 60 $\mu\text{g ml}^{-1}$, kanamycin (Kan) 50 $\mu\text{g ml}^{-1}$, nalidixic acid (Nal) 15 $\mu\text{g ml}^{-1}$, neomycin (Nm) 200 $\mu\text{g ml}^{-1}$, rifampicin (Rif) 100 $\mu\text{g ml}^{-1}$, streptomycin (Sm) 200 or 600 $\mu\text{g ml}^{-1}$, tetracycline (Tc) 5 or 10 $\mu\text{g ml}^{-1}$. Growth in broth culture was routinely monitored spectrophotometrically at OD₆₀₀.

Genetic techniques. Conjugations of *S. meliloti* and *Escherichia coli* were carried out using the mobilizing strain *E. coli* MT616, as described by Finan *et al.* (1988). Mutagenesis of *S. meliloti* RmG212 was performed by introducing pTH270 by conjugation from *E. coli* strain MT620 into RmG212. Transposon-induced mutants were selected with Sm and Nm, and screened for lack of ability to grow with arabinose as a sole carbon source. Cosmids complementing the resulting arabinose mutations were isolated from the *S. meliloti* cosmid bank CX1 by conjugating the bank en masse into SRmA240, and plating transconjugants on minimal medium containing arabinose and Tc. Cosmids were rescued from complemented arabinose mutant strains by conjugation with *E. coli* DH5 α , and subsequent Nal and Tc selection. Saturation mutagenesis was carried out by introducing complementing cosmid pZW1 into *E. coli* EcA101, subsequently conjugating the cosmid into *E. coli* EcA100, and selecting for co-transfer of the transposon and the cosmid. Individual mutagenized cosmids were screened for loss of their ability to complement arabinose catabolism mutants. Marker exchange of cosmid-borne arabinose Tn5-B20 mutations was performed as described by Glazebrook & Walker (1991). Putative arabinose catabolism mutations were subsequently transduced into *S. meliloti* RmG212 or Rm1021 prior to being used experimentally. Transductions were carried out using phage Φ M12, as described by Finan *et al.* (1984).

Mutant identification. To identify the point of insertion for individual Tn5-B20 mutations, a modification of an arbitrary PCR protocol was utilized (Caetano-Anollés, 1993; Raffa & Raivio, 2002). Strains containing Tn5-B20 insertions were purified, and the genomic DNA was used as a template. A low-stringency PCR amplification (annealing temperature of 45 °C) using the primers IS50(1) (5'-CACGATGAAGAGCAGAAG-3') and DGEN(1) (5'-GGCCACGCGTCGACTAGTCAGNNNNNNNNNACGCC-3') was carried out. The products of this reaction were subsequently amplified with a higher-stringency PCR reaction (annealing temperature of 60 °C) using the primers IS50(2) (5'-TAGGAGGTACATGGAAGTCAGAT-3') and DGEN(2) (5'-GGCCACGCGTCGACTAGTCAG-3'). The primers IS50(1) and IS50(2) are complementary to the end of the IS50 region. The DGEN(2) primer is complementary to the 5' end of DGEN(1), such that the second PCR reaction amplifies weak products generated in the first reaction. The products of the second PCR reaction were gel-isolated and sequenced using the IS50(2) primer. Sequencing reactions were performed with an ABI automated sequencer at the University of Calgary Core DNA Services. Sequence data were trimmed of IS50 sequences and localized within the *S. meliloti* genome using the BLASTN program (Altschul *et al.*, 1997) and the *S. meliloti* genome database (<http://bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime>).

Arabinose dehydrogenase assay. Cultures were grown in flasks containing defined medium with 15 mM glycerol and 15 mM arabinose as carbon sources. Cultures were grown overnight with shaking, pelleted by centrifugation (4000 g, 10 min), and resuspended in 2 ml (per gram pellet) extraction buffer containing 100 mM Tris/HCl (pH 7.6), 5 mM β -mercaptoethanol and 1 mM MgCl₂. Cells were disrupted by two passages through a French pressure cell [16 000 p.s.i. (110 400 Pa)]. The extract was subsequently pelleted (10 000 g, 30 min, 5 °C), retaining the supernatant. Aliquots of

cell-free extracts were frozen at -70 °C. Assays for arabinose dehydrogenase were performed as previously described (Pedrosa & Zancan, 1974). Protein content of extracts was measured using Bradford's protein determination reagent and BSA as a standard.

β -Galactosidase assays. Cells to be assayed were induced by growing cultures overnight in defined media containing the appropriate carbon source(s). Overnight cultures at mid-exponential phase were then subcultured into fresh medium of the same type, and grown to OD₆₀₀ ~0.3, before being assayed as described by Miller (1972).

Arabinose and glucose transport assays. Sugar uptake was measured using [³H]arabinose (370 GBq mmol⁻¹; American Radiolabelled Chemicals), [¹⁴C]arabinose (2.035 GBq mmol⁻¹; American Radiolabelled Chemicals), and [U-¹⁴C]glucose (11.8 GBq mmol⁻¹; New England Nuclear). Assays were performed as described by Richardson *et al.* (2004), with the following modifications. Cells were grown in defined media containing the appropriate carbon source(s), washed twice, and then resuspended in defined media with the original carbon source(s) (omitting arabinose or glucose as necessary) to OD₆₀₀ 0.1–0.3. Uptake of labelled sugars was measured by adding label to bacterial suspensions (final concentrations of 4 nM or 1 μM [³H]arabinose, 1 μM [¹⁴C]arabinose and 120 nM [¹⁴C]glucose), and filtering 0.25 ml samples through a Millipore 0.45 μm HV filter using a Millipore vacuum manifold. Samples were withdrawn at 10–40 s. Radioactivity retained on the filters was counted using a Beckman LS 6500 liquid scintillation counter. Radioactive uptake was normalized to total cell protein, measured by an enhanced Lowry protein assay (Stoscheck, 1990).

Symbiotic proficiency assays. Plant experiments were carried out as previously described (Oresnik *et al.*, 1994). Briefly, alfalfa seeds (*Medicago sativa* cv. Rangelander) were surface-sterilized by a 20 min treatment with 70% ethanol, followed by 30 min in 1% sodium hypochlorite. The seeds were then washed with at least 10 volumes of sterile distilled water and sown onto 1.5% water agar plates. After 2 days, germinating seeds were aseptically planted into a sterile sand/vermiculite mixture containing nitrogen-free Jensen's plant nutrient solution (Glazebrook & Walker, 1991). Plants were inoculated after another 2–3 days with cultures of *S. meliloti* that had been grown overnight in LB broth, and diluted 100-fold with sterile distilled water to approximately 10⁴–10⁵ bacteria per plant. Plants were scored 28–35 days after inoculation. Symbiotic proficiency was determined visually by comparison with wild-type-inoculated and uninoculated control plants. Bacteria were isolated from several randomly selected surface-sterilized nodules (1 min exposure to 1% sodium hypochlorite) to confirm the presence of appropriate genetic markers, ensuring that the inoculated strain was present in the plant nodules.

Symbiotic competition assays. Symbiotic competition was assessed by germinating and planting seeds as described above, and then inoculating with a mixture of two bacterial strains containing a total of 10⁴–10⁵ bacteria per plant. Cultures of wild-type and mutant were grown and diluted to the same OD₆₀₀. A mixed inoculum was made by combining equal volumes of the diluted cultures, then diluting the mixture 100-fold with sterile distilled water. Serial dilutions of the mixed inoculum were spread-plated on LB agar plates. At least 100 colonies were patched from the spread plates onto LB agar plates containing the appropriate antibiotics for strain differentiation. After 28–35 days, plants were harvested, and root nodules were manually removed. At least 50 individual nodules per experiment were sterilized (1 min exposure to 1% sodium hypochlorite), and rinsed in sterile distilled water. Individual nodules were then crushed in 100 μl sterile distilled water. Aliquots (10 μl) of each nodule extract were spotted onto LB agar plates containing the appropriate antibiotics for strain differentiation. Differences in the

percentage of the mutant strain found in the inoculum and nodule extracts were used to assess competitive ability. Data from the competition experiments were evaluated for statistical significance using both binomial probability and Fisher's least significant difference (LSD), assuming that $P < 0.05$ indicated a difference in strain competitiveness.

RESULTS

Isolation of arabinose catabolism mutants

As part of an ongoing interest in *S. meliloti* functional genomics and carbon catabolism pathways that may play a role in rhizosphere survival, strain RmG212, a Lac⁻ derivative of Rm1021 (Glazebrook & Walker, 1991), was subjected to random mutagenesis with the transposon Tn5-B20 that is capable of generating transcriptional *lacZ* fusions (Simon *et al.*, 1989). Mutants containing transposon insertions were screened for a lack of ability to grow on a number of sugars as a sole carbon source, including arabinose. Strain SRmA240 was isolated from this mutagenesis, and the transposon insertion point was identified by sequencing PCR-amplified DNA spanning the 3' end of the Tn5-B20 IS50 element and the flanking genomic DNA. A subsequent BLASTN search of the *S. meliloti* genome database showed that Tn5-B20 was inserted as a transcriptional fusion in *ilvD5* (systematic identifier SMb20890), suggesting that this gene is necessary for the catabolism of arabinose (data not shown).

Subsequent complementation of strain SRmA240 with the CX1 cosmid bank, and saturation mutagenesis of the isolated cosmid pZW1 with Tn5-B20 provided the rest of the arabinose catabolism mutations described in this work (Table 1). Each cosmid mutation that was unable to complement strain SRmA240 for growth on arabinose was integrated into either strain Rm1021 or RmG212, and subsequently retransduced into a wild-type to eliminate the plasmid pPH1JI and the possibility that a secondary mutation arose during marker exchange. Transductants were purified and characterized with respect to carbon utilization phenotype. An arbitrary PCR method was used to generate a fragment which was sequenced to verify the point of insertion. Fig. 1 shows a schematic diagram of the isolated

mutational insertion points and the predicted ORFs from the *S. meliloti* genome sequence database. All mutants were tested for the ability to grow on defined media with the following sugars as sole carbon sources: arabinose, glucose, fucose, lyxose, xylose, ribose, glycerol, galactose, rhamnose, succinate or erythritol. No growth defects other than those for arabinose were detected in the isolated mutants.

Analysis of arabinose transcriptional unit

Each non-complementing mutagenized arabinose cosmid was mated back into every arabinose mutant isolated, and tested for the ability to complement arabinose utilization. As none of the arabinose mutant strains was complemented by any of the mutagenized cosmids, we conclude that the predicted genes from *chvE* to *ilvD5* are part of a single transcriptional unit that is necessary for the catabolism of arabinose in *S. meliloti*. Since this appears to be an operon necessary for arabinose utilization, with no other associated phenotypes, we suggest that the current systematic names derived from genome annotation be changed to conform to standard nomenclature (Demerec *et al.*, 1966). The suggested replacement names are *araABCDEF*, replacing the names *chvE*, *gguA*, *gguB*, *gguC*, *Y20891* and *ilvD5* (systematic identifiers SMb20895, SMb20894, SMb20893, SMb20892, SMb20891 and SMb2089, respectively) that currently exist in the genome database. Throughout this work, these loci will be referred to using our suggested naming scheme. There is another predicted gene less than 200 bp downstream of *araF* (*Y20889*), in which we did not isolate a mutation. Although *Y20889* could be transcribed with the rest of the *ara* operon, it is also possible that it has an independent promoter. Our data could not resolve whether or not this is part of the *ara* operon.

In order to attempt to correlate the components of the *ara* operon with the biochemically elucidated arabinose catabolic pathway, InterProScan (Quevillon *et al.*, 2005) was used to assign functions to each predicted ORF, based on the deduced amino acid sequences. The gene *araA* is a homologue of *chvE* in *Agrobacterium tumefaciens*, which has been shown to couple sugar binding with the induction of plant virulence genes (Huang *et al.*, 1990; Cangelosi *et al.*, 1990), and is thought to be the periplasmic sugar-binding

Fig. 1. Predicted ORFs and isolated mutants in the arabinose catabolism transcriptional unit. Boxes indicate predicted ORFs, with pointed ends indicating the direction of transcription. Triangles indicate positions of Tn5-B20 insertions for a given allele. Filled triangles indicate that the Tn5-B20 insert is a transcriptional fusion. Putative gene functions were assigned by homology; see text for details.

protein of an ATP-binding cassette (ABC) transporter (Kemner *et al.*, 1997). An InterProScan search showed that AraA is a member of the periplasmic binding protein family (InterPro accession number IPR001761).

Both *araB* and *araC* appear to encode parts of an ABC transport system, specifically, the ATP-binding and permease proteins, respectively (IPR003439 and IPR001851). Based on the proximity of these putative transport genes to a locus necessary for arabinose catabolism, we hypothesize that these genes are necessary for arabinose transport. ABC transporters function with two ABC proteins and two permeases that contain transmembrane domains (Locher, 2004). That this operon contains only a single permease gene suggests that the permease functions as a homodimer within the ABC transporter.

AraD is part of a family of proteins of unknown function (IPR009645). Conserved domain analysis using the Conserved Domain Architecture Retrieval Tool (CDART) (Geer *et al.*, 2002) shows that AraD contains domains that are related to COG0179, and members of this family have been shown to have hydratase activity (Arai *et al.*, 1999). AraE is a member of a family of aldehyde dehydrogenases (IPR002086), and AraF is a member of a dehydratase family (IPR000581). The sequence of Y20889 was also queried, since it may be part of the *ara* operon; however, the results of the searches were uninformative.

Based on the biochemically elucidated arabinose catabolism pathway in *S. meliloti* and other fast-growing *Rhizobium* species (Dilworth *et al.*, 1986; Duncan & Fraenkel, 1979; Stowers, 1985), arabinose is likely catabolized to 2-oxoglutarate via five enzymic steps: L-arabinose 1-dehydrogenase, L-arabinonolactonase, L-arabinonate dehydratase, 2-keto-3-deoxy-L-arabinonate dehydratase and 2-oxoglutarate semialdehyde dehydrogenase. Based on the results of database queries, we hypothesize that *araE* encodes 2-oxoglutarate semialdehyde dehydrogenase and *araF* encodes L-arabinonate dehydratase, while *araABC* are involved in arabinose transport. It seems plausible that *araD* also encodes a hydratase. Taken altogether, the *ara* operon does not appear to encode all the enzymes for arabinose catabolism; in particular, the genes encoding L-arabinonolactonase, and L-arabinose dehydrogenase may be present elsewhere in the genome. Searches of the *S. meliloti* database did not yield any unambiguous candidate genes (data not shown). We did not undertake enzyme assays to confirm these putative functions because the required substrates are not commercially available.

Arabinose dehydrogenase activity is present in an *araD* mutant

To test our hypothesis that not all the dehydrogenase enzymes involved in arabinose catabolism are encoded by the *ara* operon, arabinose dehydrogenase activity was measured in cell-free extracts of strains SRmA502 and Rm1021. SRmA502 (*araD2::Tn5-B20*) was chosen because

it has a mutation predicted to have a polar effect on all downstream genes, while still allowing the possibility of arabinose transporter (*araABC*) gene expression. Cultures were grown in defined medium with either arabinose/glycerol or glycerol as carbon sources, lysed, and assayed for arabinose dehydrogenase activity. Arabinose dehydrogenase activities for Rm1021 and SRmA502 from arabinose/glycerol-grown cultures were 8.7 ± 0.4 and 33 ± 3 nmol min⁻¹ mg⁻¹, respectively. Activity for glycerol-grown Rm1021 was 4.8 ± 1.6 nmol min⁻¹ mg⁻¹; measured activity from glycerol-grown SRmA502 was not significantly different. Consistent with our hypothesis, no loss of activity was detected in SRmA502. Taken together, these data provide evidence that arabinose dehydrogenase is encoded by an arabinose-inducible locus elsewhere in the genome. This is similar to the report by Watanabe *et al.* (2006) of an arabinose-inducible arabinose dehydrogenase in *Az. brasilense* which does not appear to be within a locus necessary for arabinose catabolism. We noted that the level of inducibility of *S. meliloti* arabinose dehydrogenase, as measured by enzyme activity, appeared to be greater in an *araD* background.

Induction of the *ara* operon by arabinose, seed exudate and galactose

Arabinose has been shown to be a component of pea plant root secretions (Knee *et al.*, 2001) and plant cell walls (McNeil *et al.*, 1984), leading us to hypothesize that alfalfa seed exudate induces *ara* operon transcription. The *araA* homologue in *A. tumefaciens*, *chvE*, has been shown to be regulated by a negative regulator, *gpbR*, and inducible by arabinose, fucose and galactose (Doty *et al.*, 1993). Moreover, the transcriptional regulator *gpbR* that is associated with the *ara* operon is also a homologue of the *A. tumefaciens* *gpbR* (Fig. 1). This led us to test the ability of fucose, galactose and arabinose to induce *ara* operon expression. To carry out this experiment, the *araF1::Tn5-B20* fusion strain SRmA240 was grown with different carbon sources, and the levels of gene expression were measured via β -galactosidase activity (Table 2).

Similar to *A. tumefaciens* *chvE*, our results showed that the operon containing *araA* appears to be strongly induced by arabinose, and moderately induced by seed exudate and galactose (Table 2). Unlike in *A. tumefaciens*, however, the expression of the operon was unaffected by fucose (Table 2). Although galactose induced the operon, none of the arabinose mutants isolated was compromised in its ability to utilize galactose or any other sugar tested as a sole carbon source. It is interesting to note that strain RmG212, which has been used as a Lac⁻ strain (Glazebrook & Walker, 1991), had a much higher background level of β -galactosidase activity when it was assayed from exudate-grown RmG212. We note that it has previously been shown that native gels of *S. meliloti* induced with lactose and stained for β -galactosidase activity show two bands of activity (Charles *et al.*, 1990), and that galactosides have been shown to be present in the seed exudate (Bringhurst *et al.*, 2001).

Table 2. Induction of the arabinose operon by several carbon sources

Strains were grown overnight in either glycerol or glycerol and the carbon source listed at a concentration of 15 mM, except for exudate, which was used at 10% (v/v). The values shown represent β -galactosidase activity expressed in Miller units and are the means of three independent replicates $\pm$ SD.

Carbon source	Strain	
	SRmA240	RmG212
Arabinose	2049 $\pm$ 181*	6 $\pm$ 2
Exudate	409 $\pm$ 45*	65 $\pm$ 17
Galactose	468 $\pm$ 58*	4 $\pm$ 2
Fucose	86 $\pm$ 10	8 $\pm$ 2
Glycerol	87 $\pm$ 18	9 $\pm$ 1
Glucose	73 $\pm$ 8	8 $\pm$ 2
Succinate	73 $\pm$ 8	10 $\pm$ 4

*These values are significantly different (>95% confidence interval) from the glycerol control value using Student's *t* test.

Together, these results probably account for the reproducibly high background activities that were found with strain RmG212 grown on seed exudate.

Table 3. The arabinose operon is not subject to catabolite repression by succinate or glucose

The values shown represent β -galactosidase activity expressed in Miller units following overnight growth in defined media with the carbon source(s) listed at a concentration of 15 mM. Values are the means of three independent replicates and are presented as mean $\pm$ SD.

Carbon source(s)		Strain	
		SRmA240	RmG212
Galactose	—	593 $\pm$ 188	11 $\pm$ 3
Glucose	—	85 $\pm$ 17	10 $\pm$ 2
Glycerol	—	102 $\pm$ 22	10 $\pm$ 4
Succinate	—	80 $\pm$ 2	10 $\pm$ 1
Arabinose	Glucose	1069 $\pm$ 261*	9 $\pm$ 5
Arabinose	Glycerol	2254 $\pm$ 299	7 $\pm$ 2
Arabinose	Succinate	897 $\pm$ 92*	10 $\pm$ 1
Galactose	Glucose	416 $\pm$ 108	9 $\pm$ 2
Galactose	Glycerol	495 $\pm$ 39	8 $\pm$ 1
Galactose	Succinate	274 $\pm$ 4†	11 $\pm$ 1

*These values are significantly different from those of cells grown with arabinose and glycerol, at the 95% confidence level using Student's *t* test.

†This value is significantly different from that of cells grown with galactose and glycerol at the 95% confidence level using Student's *t* test.

Table 4. Accumulation of [3 H]arabinose by *S. meliloti* Rm1021 and arabinose catabolism mutants

Accumulation is reported as picomoles arabinose accumulated over 10 s per milligram total protein. Formaldehyde-killed controls accumulated 0.6 ± 0.1 pmol mg $^{-1}$. Values are the means of three independent replicates and are presented as mean $\pm$ SD. ND, Not determined.

Growth medium	Label accumulation		
	Rm1021	SRmA503	SRmA240
Glycerol	2.6 $\pm$ 1.3	ND	ND
Arabinose	10.0 $\pm$ 2.4	ND	ND
Glucose	0.6 $\pm$ 0.3	ND	ND
Succinate	3.9 $\pm$ 1.6	ND	ND
Arabinose + glycerol	7.6 $\pm$ 0.5	2.0 $\pm$ 0.3	15.1 $\pm$ 0.7
Arabinose + glucose	0.6 $\pm$ 0.3	1.3 $\pm$ 0.2	0.5 $\pm$ 0.1
Arabinose + succinate	12.1 $\pm$ 2.4	1.4 $\pm$ 0.6	13.0 $\pm$ 5.1

Expression of the *ara* operon is moderately repressed by succinate and glucose

Succinate is a preferred carbon source for *S. meliloti*, and has been shown to repress catabolism and/or transport of other carbon sources such as lactose and raffinose (Ucker & Signer, 1978; Gage & Long, 1998; Bringham & Gage, 2002). However, glucose has also been shown to repress the expression of catabolic genes in *R. leguminosarum* (Oresnik *et al.*, 1998; Richardson *et al.*, 2004). To determine if either succinate or glucose catabolite repression plays a role in arabinose catabolism, strain SRmA240 was grown with arabinose or galactose in combination with succinate, glycerol or glucose, and gene expression was measured by β -galactosidase activity (Table 3).

The results showed that, although transcription appears to be decreased in both succinate- and glucose-grown cultures when combined with the inducing sugars arabinose or galactose, transcriptional activity is not abolished. The observed trends for galactose- or arabinose-induced cells with the addition of glucose or succinate appeared similar. The addition of glycerol, however, did not affect expression, suggesting that the effect of the addition of glucose and succinate was not related to the overall concentration of carbon in the medium (Table 3). Taken together, the data showed that, although glucose and succinate moderate the transcriptional activity of this operon, it is not subject to complete catabolite repression by either succinate or glucose.

The *ara* operon is required for arabinose transport

Three putative genes encoding transport components were identified in the arabinose operon: *araA*, *araB* and *araC*. The requirement of this operon for arabinose catabolism suggests that the products of these genes may be involved in arabinose transport. Although the first step in arabinose

catabolism is thought to lead to the loss of the hydrogen atom from the C-1 carbon, by a dehydrogenase (Duncan & Fraenkel, 1979; Duncan, 1979), we initially attempted to measure arabinose uptake using the only commercially available labelled arabinose, [1-³H]arabinose. Strains Rm1021, SRmA503 and SRmA240 were tested for the ability to accumulate [1-³H]arabinose (Table 4). Three independent trials were performed, and SRmA503 (*araA4::Tn5-B20*), with a disruption at the beginning of the operon, consistently showed lowered levels of label accumulation, while SRmA240 (*araF1::Tn5-B20*), which carries an insert at the end of the operon, was similar to wild-type (Table 4). Although we observed measurable arabinose accumulation, the values did not reflect the rate of arabinose transport. We observed that label accumulation reached a maximal level within 10 s of label addition, regardless of the amount of label added to the cells (data not shown). Therefore, we were unable to find a concentration of substrate that showed a linear increase in label accumulation with time. Following our initial work, [1-¹⁴C]arabinose became available commercially. Using this label, we were able to measure linear rates of uptake for strain Rm1021; however, the rates were very low and were only measurable over tens of minutes (remaining linear for at least 20 min; data not shown). This suggests that using [1-¹⁴C]arabinose, we measured the incorporation of labelled arabinose into cell materials, rather than rates of transport. We hypothesize that the differences observed between accumulation of [1-³H]- and [1-¹⁴C]-labelled arabinose were due to the predicted biochemical modifications of arabinose upon entry into the cell; dehydrogenation of arabinose by arabinose dehydrogenase, and decarboxylation of 2-oxoglutarate by 2-oxoglutarate dehydrogenase. Despite this, our results clearly indicate that the *ara* operon encodes arabinose transport components. Whether or not the very low levels of arabinose uptake measured for strain SRmA503 reflect true transport is not known. It is possible that arabinose is taken up at a low level by some other mechanism, while the *ara* operon is the cell's primary transport mechanism for arabinose.

The fact that glucose-grown cells showed the lowest levels of arabinose accumulation was unexpected, and did not correlate with the observation that glucose did not completely repress transcription of the *ara* operon (Table 3). It is hypothesized that either glucose-grown cells expressed a protein capable of inhibiting arabinose accumulation, or that glucose present in the culture medium directly inhibited uptake of arabinose. The addition of glucose at the time of assay to arabinose-grown cells was found to instantaneously prevent arabinose accumulation, suggesting that it directly interfered with arabinose accumulation (data not shown). In addition to glucose, this effect was found to be caused by galactose. We noted that this effect was only observed at very high excess concentrations of glucose or galactose compared to that of labelled arabinose.

Glucose uptake is not compromised in arabinose catabolism mutants

Following our initial observation that high levels of glucose abolished arabinose transport, it was hypothesized that perhaps glucose could also be transported by the arabinose transporter, thus competing with [³H]arabinose. To test this, rates of [¹⁴C]glucose uptake were measured for strains Rm1021, SRmA240 and SRmA503. It was reasoned that if glucose were to be taken up by AraABC, then there should be a clear difference in the measured rate of glucose uptake in the absence of this ABC transporter. To test this, strains were grown using glucose alone or arabinose plus glycerol as carbon sources. A linear rate of glucose uptake for Rm1021 was measured at $8.3 \pm 0.9 \text{ nmol min}^{-1} (\text{mg total protein})^{-1}$. In all cases, there was no significant difference in the observed rate of growth or the measured glucose uptake rates for Rm1021, SRmA240 and SRmA503 ($P > 0.7$). This suggests that although excess glucose inhibits arabinose transport, it is not through competitive uptake by the *ara* components of the *ara* transport system.

Plant symbiosis and competition for nodule occupancy

Delayed and variable nodulation has been reported in the literature for an *S. meliloti* Tn5 arabinose catabolism mutant (Duncan, 1981). In addition, the *araA* homologue in *A. tumefaciens* (*chvE*) has been shown to be necessary for the induction of plant virulence factors by various sugars (Huang *et al.*, 1990; Cangelosi *et al.*, 1990). These reports led us to ask whether mutations in the *ara* operon would affect the symbiotic ability of *S. meliloti*. To test this, strains SRmA240 (*araF::Tn5-B20*) and SRmA503 (*araA::Tn5-B20*) were assayed for the ability to form nitrogen-fixing nodules, and in addition, SRmA503 was tested for the ability to compete for nodule occupancy with the wild-type.

Plants grown in nitrogen-free media and inoculated with strains SRmA240 or SRmA503 were green and healthy, and there were no significant differences in either the plant dry matter accumulated, or the number or location of the nodules that were formed on the root system of the host plant. Moreover, bacteria isolated from surface-sterilized nodules retained their genetic markers. Examination of the nodule occupancy of plants simultaneously inoculated with both SRmA503 and wild-type Rm1021 showed a decrease in the ratio of SRmA503 isolated relative to the inoculation ratio, when SRmA503 was inoculated at a concentration equal to that of the wild-type. Whereas SRmA503 made up $52 \pm 9\%$ of colonies present in the inoculant for three independent competition experiments, the percentage of SRmA503 recovered from nodule extracts was only $26 \pm 9\%$. Although this decrease in nodule occupancy was statistically significant ($P = 0.0051$), further experiments in which SRmA503 was present at a higher concentration in the inoculum than wild-type did not reveal a significant decrease in the proportion of mutant recovered (Fig. 2).

Fig. 2. Recovery ratios of *chvE* mutant SRmA503 to wild-type Rm1021 from alfalfa nodules were not significantly different from inoculum ratios. Bars indicate the mean amount of SRmA503 present in the inoculum or recovered from nodules over three independent experiments; error bars indicate SD. Light-grey bars, percentage mutant in inoculum; dark-grey bars, percentage mutant recovered.

Taken together, there was no evidence for a competitive difference between SRmA503 and the wild-type Rm1021.

DISCUSSION

In this work, we have suggested a new function-based nomenclature for an operon of genes that are necessary for growth with arabinose as a sole carbon source. The current name for *araA* in the database is *chvE*, named for chromosomal virulence (Huang *et al.*, 1990) in *A. tumefaciens*. We have shown that mutations in *araA* have no apparent defects in symbiotic proficiency or in the ability to compete for nodule occupancy with the wild-type, indicating that there is no reason to keep the name *chvE*. Moreover, the *ggu* mnemonic, glucose and galactose uptake, is misleading and does not reflect the physiological function which we have demonstrated (Table 4). Since the only phenotype for mutations in any of the other *ara* genes is loss of the ability to transport and/or catabolize arabinose, we suggest that these genes should be annotated as *ara* genes in *S. meliloti*. Renaming this region as the *ara* operon is consistent with bacterial nomenclature and reflects the phenotype of mutations in these genes.

Although arabinose catabolism mutations have been described in the past (Duncan & Fraenkel, 1979; Duncan, 1981), to our knowledge no mutation specific for arabinose catabolism has been physically localized in the *S. meliloti* genome. The arabinose utilization operon we have identified is present on the megaplasmid pSymB. Although an extensive genetic map and an elegant *in vivo* deletion strategy have been used to characterize this replicon (Charles & Finan, 1990, 1991), some regions have been

found to be recalcitrant to deletion (Charles & Finan, 1991). Since the utilization of arabinose was screened in those analyses, we conclude that the *araABCDEF* region that we describe is within one of the regions that has not been previously deleted. This is likely since the *ara* operon is located only about 12 kbp away from the sole arginine-specific tRNA-encoding locus of the genome (Finan *et al.*, 2001).

Based on the previous enzymic characterization of arabinose catabolism in *S. meliloti*, there are five hypothesized steps in the conversion of arabinose to 2-oxoglutarate (Duncan & Fraenkel, 1979). The *ara* operon includes genes necessary for uptake of arabinose, leaving only *araDEF* to encode the necessary enzymes for catabolism. Based on our bioinformatic analysis, *araDEF* are likely to encode a semi-aldehyde dehydrogenase and two dehydratases. The observation that arabinose dehydrogenase activity was not affected by mutations in the *ara* operon, and appeared to be inducible in an *ara* insertion mutant likely to have polar effects on downstream genes, provides evidence that the genes necessary for encoding arabinose dehydrogenase activity, and presumably lactonase activity, are not present at this locus and exist elsewhere in the genome. The location of these genes is unknown at this time.

Previous work on arabinose utilization has been carried out in *S. meliloti* strain L5-30 (Duncan, 1981). The results of this analysis suggest that a mutant unable to utilize arabinose has a delayed and variable ability to fix nitrogen symbiotically, and that its lesion is not in a gene that affects either arabinose dehydrogenase or 2-oxoglutarate semialdehyde dehydrogenase activity. It is concluded that the lesion must be in a gene that affects either dehydratase or arabinolactonase activity. Taken together, the genomic sequence of strain Rm1021, the phenotypes of the mutants we generated, and the demonstration that arabinose dehydrogenase activity is not found at the *araABCDEF* locus suggest that the mutation previously characterized may affect arabinolactonase activity. We note that, whereas the phenotypes of the mutants we report here are 100 % linked in transduction with the associated transposon markers, previous researchers did not have access to a generalized transducing phage, and were therefore unable to show that the inability of their mutant to use arabinose was solely due to a transposon insertion (Duncan, 1981). The isolation and identification of the genes necessary for both arabinose dehydrogenase and arabinolactonase activity would be helpful in this regard.

Expression of metabolic genes is affected by the carbon source upon which they are grown. A very clear example of this is *pckA* in *S. meliloti*. The expression of *pckA* is induced when grown using a gluconeogenic carbon source such as succinate or arabinose, whereas no expression is observed if a glycolytic carbon source such as glucose is utilized (Østerås *et al.*, 1995). It has been observed that, although *pckA* expression is reduced by ~50 % if the cells are grown on both glucose and succinate, growth on arabinose and glucose results in a >90 % reduction (Østerås *et al.*, 1995).

Since arabinose is catabolized to a tricarboxylic acid (TCA) intermediary, it was suggested that glucose may either repress arabinose catabolism or uptake. Our findings suggest a combination of both of these effects, as we observed that glucose can inhibit arabinose uptake when present in excess, and glucose moderately represses transcription of the arabinose operon (Tables 3 and 4).

A number of mutations that affect competition for nodule occupancy have been reported in the literature (Aneja *et al.*, 2005; Fry *et al.*, 2001; Jiménez-Zurdo *et al.*, 1995; Oresnik *et al.*, 1998; Phillips *et al.*, 1998; Rosenblueth *et al.*, 1998; Soedarjo & Borthakur, 1998; Streit *et al.*, 1996). The magnitude of these phenotypes appears to vary from severe to statistically significant but subtle. Initially, it appeared that the arabinose catabolism mutant SRmA503 was less competitive than wild-type when co-inoculated in approximately equal amounts, and the results were statistically significant. Further experiments with SRmA503 present at a higher ratio in the inoculum showed no significant decrease in the number of cells recovered. Performing assays of nodule occupancy competition with several ratios of mutant to wild-type is therefore recommended. The ability to thrive and compete in a complex environment such as the rhizosphere, or within the infection thread, is likely affected by many determinants. The challenge ahead is not only to identify which genes affect this complex phenotype but also to ascribe a proper function to them, so that the physiology and regulation of the process will be better understood. Moreover, it is imperative that we begin to understand at what point during the interaction with the host plant the effects of different non-competitive mutants are manifested.

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