A functional *myo*-inositol catabolism pathway is essential for rhizopine utilization by *Sinorhizobium meliloti*

Mark P. Galbraith,^{1†} Szi Fei Feng,¹ James Borneman,^{2‡} Eric W. Triplett,² Frans J. de Bruijn³ and Silvia Rossbach¹

Author for correspondence: Silvia Rossbach. Tel: +1 616 387 5868. Fax: +1 616 387 2849.
e-mail: Silvia.Rossbach@wmich.edu

¹ Department of Biological Sciences, Western Michigan University, Kalamazoo, MI 49008, USA

² Department of Agronomy and Center for the Study of Nitrogen Fixation, University of Wisconsin-Madison, Madison, WI 53706, USA

³ MSU-DOE Plant Research Laboratory, Department of Microbiology, NSF Center for Microbial Ecology, Michigan State University, East Lansing, MI 48824, USA

Rhizopine (L-3-O-methyl-scylo-inosamine) is a symbiosis-specific compound found in alfalfa nodules induced by specific *Sinorhizobium meliloti* strains. It has been postulated that rhizobial strains able to synthesize and catabolize rhizopine gain a competitive advantage in the rhizosphere. The pathway of rhizopine degradation is analysed here. Since rhizopine is an inositol derivative, it was tested whether inositol catabolism is involved in rhizopine utilization. A genetic locus required for the catabolism of inositol as sole carbon source was cloned from *S. meliloti*. This locus was delimited by transposon Tn5 mutagenesis and its DNA sequence was determined. Based on DNA similarity studies and enzyme assays, this genetic region was shown to encode an *S. meliloti* *myo*-inositol dehydrogenase. Strains that harboured a mutation in the *myo*-inositol dehydrogenase gene (*idhA*) did not display *myo*-inositol dehydrogenase activity, were unable to utilize *myo*-inositol as sole carbon/energy source, and were unable to catabolize rhizopine. Thus, *myo*-inositol dehydrogenase activity is essential for rhizopine utilization in *S. meliloti*.

Keywords: *Sinorhizobium meliloti*, rhizopine catabolism, inositol catabolism, *idhA*, *myo*-inositol dehydrogenase

INTRODUCTION

Rhizopines are known to be nutritional mediators, and have been shown to play a role in nitrogen-fixing symbiotic associations between members of the *Rhizobiaceae* and legume plants (Murphy & Saint, 1992). One specific rhizopine, the inositol compound L-3-O-methyl-scylo-inosamine (3-O-MSI), is synthesized by *Sinorhizobium meliloti* strain L5-30 in nodules of its host plant, *Medicago sativa* (alfalfa). This strain, L5-30, is able not only to synthesize the rhizopine in nodules, but also to degrade and utilize rhizopine under free-living conditions (Murphy *et al.*, 1987; Rossbach *et al.*, 1994a). It has been postulated that the rhizopine represents a nutrient source for bacterial strains that possess the

ability to degrade it. In fact, it has been reported that bacterial strains which are able to synthesize and utilize 3-O-MSI have a fitness advantage and are better able to nodulate their host plant, alfalfa (Gordon *et al.*, 1996).

Interestingly, the occurrence of rhizopine synthesis and catabolism genes is rare among different strains of *Rhizobiaceae*. Only three *S. meliloti* strains of 50 worldwide isolates tested were found to be able to synthesize and catabolize rhizopine and to possess the appropriate rhizopine synthesis (*mos*) and catabolism (*moc*) genes (Rossbach *et al.*, 1995b). In another study, out of 300 strains tested, only 12 *S. meliloti* and nine *Rhizobium leguminosarum* bv. *viciae* strains were shown to be able to catabolize and synthesize rhizopine (Wexler *et al.*, 1995). Based on the availability of cloned *moc* and *mos* genes, we are pursuing two goals. First, we are constructing a selectable marker cassette based on the *moc* genes, since the presence of the rhizopine catabolism genes in an organism endows it with the ability to utilize 3-O-MSI as sole nitrogen and carbon source. Second, by constructing transgenic plants expressing the *mos* genes and secreting rhizopine we will

[†] Present address: Merck & Co., WP 77T, Sumneytown Pike, West Point, PA 19486, USA.

[‡] Present address: Department of Plant Pathology, University of California, Riverside, CA 92521, USA.

Abbreviation: 3-O-MSI, L-3-O-methyl-scylo-inosamine.

The GenBank accession number for the sequence reported in this paper is AF059313.

attempt to create so-called 'biased rhizospheres', in which beneficial soil bacteria, harbouring the *moc* genes, may gain a competitive advantage and thus be selectively enriched in the rhizosphere (Rossbach *et al.*, 1995b).

A detailed knowledge of the structure and expression of the rhizopine synthesis and catabolism genes is required for both these projects. *mos* genes involved in the synthesis of 3-O-MSI and *moc* genes involved in catabolism and utilization of rhizopine have been cloned from *S. meliloti* strain L5-30 and characterized in detail (Murphy *et al.*, 1987, 1988, 1993; Rossbach *et al.*, 1994a). Determination of the DNA sequence of the *moc* locus, coupled to Tn5 mutagenesis, revealed that out of the six ORFs present at this locus four are essential for rhizopine catabolism, *mocABR* and *mocC* (Rossbach *et al.*, 1994a). Comparison of the deduced protein sequences with sequences in the databases allowed a prediction of their putative roles in rhizopine catabolism. MocB appears to be a periplasmic binding protein, probably facilitating the transport of rhizopine into the cell, whereas MocR is a regulatory protein, containing a DNA-binding motif. It is suspected that MocA and MocC are enzymes involved in the degradation of rhizopine (Rossbach *et al.*, 1994a). MocA appears to be a dehydrogenase and both MocA and MocC proteins display similarities to proteins encoded by genes located in the *myo*-inositol catabolism operon of *Bacillus subtilis* (Yoshida *et al.*, 1997).

The rhizopine 3-O-MSI is an inositol derivative. *myo*-Inositol is a common and essential compound present in plants (Loewus, 1990). A variety of soil- and plant-associated bacteria have been described that possess the ability to degrade *myo*-inositol, including *Aerobacter aerogenes* (now *Klebsiella pneumoniae*), *R. leguminosarum* bv. *viciae*, *B. subtilis* and *S. meliloti* (Anderson & Magasanik, 1971a, b; Berman & Magasanik, 1966a, b; Bosworth *et al.*, 1994; Fujita *et al.*, 1991; Loewus, 1990; Poole *et al.*, 1994). In *K. pneumoniae* and *R. leguminosarum* bv. *viciae*, the pathway of inositol degradation has been shown to involve the enzymes *myo*-inositol dehydrogenase and 2-keto-*myo*-inositol dehydratase (Berman & Magasanik, 1966a; Poole *et al.*, 1994). The genes involved in *myo*-inositol degradation have been cloned and sequenced from the Gram-positive bacterium *B. subtilis* (Fujita *et al.*, 1991; Yoshida *et al.*, 1994, 1997).

Mutant strains of *S. meliloti* that are not able to use *myo*-inositol as sole carbon source have been described (Bosworth *et al.*, 1994). These *S. meliloti* mutant strains carry the interposon Ω in a genetic locus responsible for inositol catabolism. These strains were among the first genetically modified micro-organisms released in field experiments, since the inositol locus was used as the integration site for genes that increase the nitrogen-fixation capacity (*nifA*, *dctABD*; Bosworth *et al.*, 1994; Scupham *et al.*, 1996).

We hypothesized that inositol catabolism is involved in rhizopine degradation, and we tested this hypothesis by analysing the *S. meliloti* mutant strains that are unable

to grow on *myo*-inositol as sole carbon source. These *S. meliloti* mutant strains were shown to carry an insertion in a *myo*-inositol dehydrogenase gene and to lack *myo*-inositol dehydrogenase activity. The locus encoding *myo*-inositol dehydrogenase was cloned and delimited via Tn5 mutagenesis. The *myo*-inositol dehydrogenase gene was the only gene in this locus essential for the utilization of *myo*-inositol as sole carbon source. Moreover, *S. meliloti* mutant strains with an insertion in the *myo*-inositol dehydrogenase gene were found to be unable to utilize rhizopine. Thus, we conclude that a functional *myo*-inositol catabolism pathway is essential for the ability to utilize rhizopine.

METHODS

Bacteria, plasmids and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* strains were grown in LB with appropriate antibiotics (Miller, 1992). Minimal medium for *E. coli* was M9 (Miller, 1992). Rich medium for *S. meliloti* was TY (Berliner, 1974), and as minimal medium either GTS (Kiss *et al.*, 1979) or the minimal M medium described by Rossbach *et al.* (1994a) was used. In minimal M medium the carbon source was glucose (final concentration 0.25%) and the nitrogen source was potassium nitrate (final concentration 0.1%). *S. meliloti* strains were grown at 28 °C.

Molecular techniques. Chromosomal and plasmid DNA isolation procedures were carried out essentially as described by Ausubel *et al.* (1992) and Sambrook *et al.* (1989). DNA probes were labelled with digoxigenin-11-dUTP of the DIG DNA Labelling kit and detected with the DIG Luminescent Detection kit, both from Boehringer Mannheim. The construction of the cosmid clone bank of *S. meliloti* strain 1021 used in this work was described previously (de Bruijn *et al.*, 1989). Triparental conjugations, transposon Tn5 mutagenesis of plasmid pMG1000, and gene replacement techniques were carried out as described by de Bruijn & Rossbach (1994). Gene replacements were verified by correct marker exchange and for mutant strains L56, #56, #105 and #113 by Southern blotting and hybridization with the wild-type region. Manual DNA sequencing was carried out with the T7 Sequenase 7-Deaza-dGTP sequencing kit and radioactive [³²S]dATP α S (Amersham). Oligonucleotides were purchased from Only DNA. For DNA sequence analysis the following computer programs were used: IntelliGenetics Molecular Biology Software System 5.4, CodonUse 3.1 (Conrad Halling, University of Chicago, USA), SeqVu 1.0.1 (The Garvan Institute of Medical Research), BLAST programs (Altschul *et al.*, 1990) and the programs BESTFIT, gap, PILEUP, peptidesort from the Wisconsin Package 9.1, Genetics Computer Group (GCG), Madison, WI, USA.

Enzyme assays. Precultures were inoculated 1:100 in 2.8 l Fernbach flasks containing 250 ml minimal medium with 0.2% succinate as carbon source and 0.02% *myo*-inositol as inducer. Mid-exponential-phase cultures were pelleted, washed with 40 mM HEPES buffer (pH 7.0) and resuspended in 5 ml buffer. Cells were disrupted with a Branson W185 sonicator at 50 W with three 10 s sonication periods. Cell debris was pelleted and the supernatant was used directly for enzyme assays. *myo*-Inositol dehydrogenase activity was determined as previously described by Poole *et al.* (1994). Each reaction mixture (pH 10.0) contained, in 1 ml, 50 μ mol sodium carbonate, 50 μ mol ammonium chloride, 0.4 μ mol NAD, 100 μ mol *myo*-inositol and 100 μ l cell-free extract. Increase in

Table 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Reference
Strains		
<i>Escherichia coli</i>		
HB101	<i>supE44 hsdS20 recA13 ara-14 proA2 lacY1 galk2 rpsL20 xyl-5 mtl-1</i>	Sambrook <i>et al.</i> (1989)
<i>Sinorhizobium meliloti</i>		
L5-30	Wild-type, Sm ^R Ino ⁺ Mos ⁺ Moc ⁺	Kowalski (1970)
1021	Wild-type derivative of SU47, Sm ^R Ino ⁺ Mos ⁻ Moc ⁻	Meade <i>et al.</i> (1982)
PC	Wild-type, Ino ⁺ Mos ⁻ Moc ⁻	Bosworth <i>et al.</i> (1994)
RMB7101	Mutant derived from SU47, Sm ^R Sp ^R Ino ⁻ Mos ⁻ Moc ⁻	Bosworth <i>et al.</i> (1994)
RMB7201	Mutant derived from PC, Sm ^R Sp ^R Ino ⁻ Mos ⁻ Moc ⁻	Bosworth <i>et al.</i> (1994)
#56	Mutant derived from 1021, <i>idhA::Tn5</i> , Km ^R Sm ^R Ino ⁻ Mos ⁻ Moc ⁻	This work
L56	Mutant derived from L5-30, <i>idhA::Tn5</i> , Km ^R Sm ^R Ino ⁻ Mos ⁺ Moc ⁻	This work
Plasmids		
pBluescript KS	Ap ^R , cloning vector	Stratagene
pLAFR1	Tc ^R Mob ⁺ Tra ⁻ , IncP, <i>cos</i>	Friedman <i>et al.</i> (1982)
pRK290	Tc ^R Mob ⁺ Tra ⁻ , IncP	Ditta <i>et al.</i> (1980)
pTE3	Tc ^R , broad-host-range expression vector, <i>trp</i> promoter	Egelhoff & Long (1985)
pPM1031	Tc ^R , <i>moc</i> locus of <i>S. meliloti</i> L5-30 in pLAFR1	Murphy <i>et al.</i> (1987)
pSR8612	Tc ^R , <i>mocABC</i> of <i>S. meliloti</i> L5-30 cloned downstream of the <i>trp</i> promoter in pTE3	Rosbach <i>et al.</i> (1994b)
pMG1000	Tc ^R , inositol and arabinose catabolism regions of <i>S. meliloti</i> 1021 cloned into pLAFR1	This work
pMG1002	Tc ^R , 2.0 kb <i>Bam</i> HI fragment of pMG1000 cloned into pLAFR1, contains the <i>idhA</i> gene	This work
pMG1003	Tc ^R , 2.0 kb <i>Bam</i> HI fragment of pMG1000 cloned into pLAFR1, opposite orientation as compared to pMG1002	This work
pMW184	Tc ^R , <i>Eco</i> RI fragment containing <i>S. meliloti</i> 1021 <i>ino::Tn5</i> region, <i>Hpa</i> I and <i>Bal</i> 31 digested, re-ligated with <i>Spe</i> I linker, cloned into pRK290	Williams <i>et al.</i> (1988)
pTB6	Ap ^R , 590 bp <i>Eco</i> RI- <i>Spe</i> I fragment of pMW184 cloned into pBluescript KS	This work

A₃₄₀ was monitored with a Beckman Recording Quartz spectrophotometer. The protein content of cell-free extracts was determined by the method of Lowry. The specific *myo*-inositol dehydrogenase activity was expressed as nmol NADH reduced min⁻¹ (mg protein)⁻¹ ± SEM. The values are the mean of three determinations.

Nodulation assays. Inoculation of alfalfa plants (*M. sativa* cv. Cardinal) with *S. meliloti* strains was carried out as described previously (Rosbach *et al.*, 1995b).

Rhizopine catabolism assays. Preparation of extracts containing the rhizopine and rhizopine catabolism assays were carried out as described by Rosbach *et al.* (1994a). Half-strength minimal M medium without carbon and nitrogen source but mixed 1:1 with nodule extract containing rhizopine was used for the catabolism assays. Rhizopine was detected by high-voltage paper electrophoresis (buffer: 1.1 M acetic acid/0.7 M formic acid, pH 1.9) and subsequent alkaline silver staining (Rosbach *et al.*, 1994a).

RESULTS

S. meliloti mutants unable to catabolize inositol cannot degrade rhizopine

The ability to utilize the rhizopine 3-O-MSI was originally discovered in *S. meliloti* strain L5-30 (Moc⁺). Many other *S. meliloti* wild-type strains tested were not able to degrade rhizopine (Moc⁻; Tempé *et al.*, 1982).

Nevertheless, when the rhizopine catabolism (*moc*) genes were cloned on plasmids and conjugated into Moc⁻ *S. meliloti* wild-type strains, then these strains were able to catabolize rhizopine (Murphy *et al.*, 1987; Rosbach *et al.*, 1994a). However, when plasmids carrying the *moc* genes were introduced into a variety of other soil bacteria, these bacteria were found to be unable to degrade rhizopine, suggesting other crucial genes existed in *S. meliloti* contributing to rhizopine degradation. Since rhizopine is an inositol derivative (3-O-MSI), we reasoned that inositol catabolism may be involved in rhizopine utilization. Therefore, we studied two *S. meliloti* mutant strains (RMB7101 and RMB7201) that are unable to use *myo*-inositol as sole carbon source (Bosworth *et al.*, 1994). Although *S. meliloti* strains RMB7101 and RMB7201 have different genetic backgrounds, they both carry an Ω insertion in the inositol catabolism locus (Table 1).

To test the possibility that inositol catabolism plays a crucial role in rhizopine utilization, we introduced plasmids carrying the *moc* genes (pPM1031 and pSR8612; Table 1), and the vector pLAFR1 into mutant strains RMB7101 and RMB7201, as well as into the corresponding wild-type strains 1021 and PC. The resulting exconjugants were examined for their ability to utilize rhizopine. The *moc* genes carried by plasmids

Fig. 1. High-voltage paper electropherogram of a rhizopine catabolism assay after alkaline silver staining. Half-strength minimal medium was inoculated with different *S. meliloti* strains and incubated for 3 d. Fifteen microlitres of the supernatant of each culture was loaded on the origin of the paper. The cathode is positioned at the bottom of the figure. The reference substances (S) that are loaded in the left and right lanes are mannopine (MOP), mannopinic acid (MOA) and agropine (AGR). Lanes: 1, *S. meliloti* strain PC(pPM1031); 2, PC(pSR8612); 3, PC(pLAFR1); 4, RMB7201(pPM1031); 5, RMB7201(pSR8612); 6, RMB7201(pLAFR1); 7, RMB7101(pPM1031); 8, RMB7101(pSR8612); 9, RMB7101(pLAFR1); 10, L5-30; 11, uninoculated control (medium with rhizopine).

pPM1031 and pSR8612 were found to be sufficient to confer the ability to utilize rhizopine to the *S. meliloti* wild-type strains PC (Fig. 1, lanes 1 and 2) and 1021 (data not shown). However, mutant strains RMB7101 and RMB7201 harbouring these plasmids were found to be unable to utilize rhizopine (Fig. 1, lanes 4, 5, 7 and 8).

As expected, the control plasmid pLAFR1 could not confer the ability to degrade rhizopine to these strains (Fig. 1, lanes 3, 6 and 9). The catabolism assay with wild-type strain PC harbouring plasmid pLAFR1 shows an apparent shift in mobility of the rhizopine spot on the electropherogram (Fig. 1, lane 3). This minor mobility shift is usually observed in catabolism assays with rhizobial Moc⁻ strains (Rossbach *et al.*, 1994a, 1995b), but it is not displayed by other Moc⁻ soil bacteria (S. Rossbach, unpublished observations) or by the mutant strains that are unable to catabolize *myo*-inositol (Fig. 1, lanes 4–9). The reasons for the observed mobility shift are not entirely clear; it could be that the Moc⁻ rhizobial strains are able to partially degrade the rhizopine, but at this point the chemical structure of the intermediates in the rhizopine catabolic pathway is not known. Nevertheless, the catabolism assays carried out with the mutant strains unable to catabolize *myo*-inositol show that these strains are also unable to degrade the rhizopine. Thus, we conclude from these experiments that a functional inositol catabolism pathway is necessary for the ability to utilize rhizopine in *S. meliloti*.

Cloning of the inositol locus from *S. meliloti* 1021

For the construction of mutant strains RMB7101 and RMB7201, the genomic region surrounding a transposon insertion in an inositol catabolism region of *S. meliloti* 1021 had been cloned on plasmid pMW184 (Bosworth *et al.*, 1994; Williams *et al.*, 1988). This mutated DNA region had been further manipulated with exonuclease *Bal31* (Williams *et al.*, 1988). Thus, the mutated region could not serve as source of the wild-type genomic region; however, it could serve as a hybridization probe. A subclone of pMW184, pTB6 (Table 1; Fig. 2), carries genomic DNA adjacent to the

Fig. 2. Correlated physical and genetic map of the inositol locus of *S. meliloti* 1021. The DNA fragments cloned in pMG1000, pMG1002 and pTB6 are shown. B, *Bam*HI; G, *Bgl*II; E, *Eco*RI; H, *Hind*III; X, *Xba*I. (S), *Spe*I linker present in pTB6. The boxed arrow denotes the location and direction of the *idhA* gene. The numbered arrows denote the different locations of the transposon Tn5 insertions in pMG1000. The phenotypes of the *S. meliloti* mutant strains that resulted from the gene-replacement experiment are indicated on the bottom: N, no successful marker exchange; +, able to catabolize carbon source; -, unable to catabolize carbon source. Before determining the rhizopine catabolism phenotype, plasmid pPM1031 carrying the *moc* genes was conjugated into the different mutant strains.

original transposon insertion of the inositol locus on a 490 bp *EcoRI*–*SpeI* fragment. We used this fragment as a probe to clone the corresponding wild-type genomic inositol catabolism locus. A colony hybridization experiment was performed using the cosmid library of *S. meliloti* 1021 constructed in the vector pLAFR1 (de Bruijn *et al.*, 1989). Twelve hundred colonies of the clone bank were transferred onto nylon filters and hybridized with the above-mentioned probe. Seven colonies were identified that yielded positive hybridization signals. Plasmid DNA of these seven colonies was isolated, digested with the restriction endonuclease *EcoRI*, separated by agarose gel electrophoresis and blotted onto nylon filters. As a control, *S. meliloti* total chromosomal DNA restricted with *EcoRI* was included. The blot was hybridized with the 490 bp *EcoRI*–*SpeI* fragment of pTB6, and one of the cosmid clones (pMG1000) was found to contain a hybridizing fragment of 7 kb. A fragment of the same size in the lane loaded with *S. meliloti* 1021 wild-type DNA also hybridized with this probe (data not shown).

Complementation of mutant strains unable to catabolize *myo*-inositol

To verify that pMG1000 indeed carries the inositol catabolism locus, we conjugated pMG1000 into *S. meliloti* strains RMB7101 and RMB7201. The resulting strains were examined for growth on minimal M medium with 0.2% *myo*-inositol as sole carbon source. Strains RMB7101 and RMB7201 containing plasmid pMG1000 were able to grow on minimal medium with *myo*-inositol as sole carbon source, whereas RMB7101 and RMB7201 containing the vector pLAFR1 were not.

Strains of *E. coli* are unable to degrade *myo*-inositol (Holt, 1993). To examine whether pMG1000 could also confer the ability to use inositol as sole carbon source to *E. coli*, strain HB101 was transformed with pMG1000 and its growth on minimal M9 medium with *myo*-inositol as sole carbon source was tested. Strain HB101 harbouring pMG1000 was found to be able to grow on minimal M9 medium with glucose as sole carbon source, but unable to grow on M9 medium with 0.2% *myo*-inositol medium as sole carbon source. Thus, the *S. meliloti* genetic region carried by pMG1000 appears to be insufficient to confer the ability to use *myo*-inositol as sole carbon or energy source upon *E. coli*.

Construction of a correlated physical and genetic map of the *S. meliloti* DNA carried by pMG1000 via transposon Tn5 mutagenesis

In addition to the 7.0 kb *EcoRI* fragment that hybridized with the 490 bp *EcoRI*–*SpeI* fragment of pTB6, plasmid pMG1000 carries four additional *EcoRI* fragments of ~8.1, 4.3, 2.05 and 1.95 kb. To delimit the genetic locus responsible for inositol catabolism, we subjected pMG1000 to Tn5 mutagenesis (de Bruijn & Rossbach, 1994). Fifty out of 120 independent Tn5 insertions were mapped to the DNA segment cloned in pMG1000 (see Fig. 2). Using restriction digests with the endonucleases

HindIII, *BglII* and *BamHI*, these 50 insertions were more precisely mapped to 35 different sites (Fig. 2). To determine the phenotype of these Tn5 insertion mutants, we replaced the wild-type region of *S. meliloti* strain 1021 with the different pMG1000::Tn5-containing fragments. All resulting mutant strains were tested for their growth on minimal medium with *myo*-inositol as sole carbon and energy source. Only mutant strains #56, #105 and #113 were found to be unable to utilize *myo*-inositol as sole carbon and energy source. The corresponding Tn5 insertions were located in a 1 kb region of the 7 kb *EcoRI* fragment previously found to hybridize with the 490 bp *EcoRI*–*SpeI* fragment of pTB6. The ability of the mutant strains to grow on a variety of different carbon sources, including *scyllo*-inositol, sorbitol, mannitol, adonitol, D-glucose, fructose, L-arabinose, D-mannose and succinate, was also examined. All mutant strains (and the wild-type) were found to be capable of utilizing these compounds as sole carbon and energy source, with only one exception. Mutant strains #1, 3, 7, 13, 37, 44, 59 and 78 were found to grow very poorly on L-arabinose as sole carbon source. These mutants map to a 9 kb region, 3 kb upstream from the inositol locus (Fig. 2). This 9 kb region may, therefore, carry a genetic locus directly or indirectly involved in L-arabinose utilization.

Rhizopine utilization phenotype of the *S. meliloti* mutant strains

To examine which areas of the *S. meliloti* region carried by pMG1000 were involved in rhizopine catabolism, we introduced plasmid pPM1031 into mutant strains #3, 55, 56, 61, 64, 65, 68, 96, 103 and 124. The resulting transconjugants were examined for rhizopine utilization. Strain #56(pPM1031) was found to be the only one unable to utilize rhizopine.

Construction of an *S. meliloti* L5-30 strain unable to degrade *myo*-inositol

S. meliloti mutant strains unable to degrade *myo*-inositol were found to be unable to utilize rhizopine, when provided with the *moc* genes in *trans* (see above). To show that the same applied to the *S. meliloti* strain from which the *moc* genes were isolated (strain L5-30), we performed a gene-replacement experiment with strain L5-30 and the transposon-mutated inositol region of *S. meliloti* 1021. Plasmid pMG1000::56 was used for this 'heterologous' gene replacement experiment and gene exchange in strain L5-30 was verified by Southern blotting. The resulting strain, L56, was found to be unable to use *myo*-inositol as sole carbon source or to utilize rhizopine (data not shown).

Subcloning of the region carrying the inositol catabolism region

To further delimit the region conferring the ability to degrade inositol, we constructed a pLAFR1 plasmid derivative carrying a 2.0 kb *BamHI* fragment from

pMG1000 in both orientations, resulting in plasmids pMG1002 and pMG1003 (Table 1). Both plasmids were introduced into *S. meliloti* strain #56 and their ability to complement this mutant strain was examined. Both plasmids pMG1002 and pMG1003 could confer the ability to use *myo*-inositol as sole carbon and energy source upon mutant strain #56, suggesting that an entire inositol degradation gene was present on this 2 kb *Bam*HI fragment.

To further characterize the *myo*-inositol degradation gene, the DNA sequence of a 1200 bp region starting 96 bp upstream of the *Eco*RI site of plasmid pMG1002 was determined. The analysis of the resulting DNA sequence using the CodonUse program by C. Halling

2920

and *yucG* (accession no. Z93940) genes represent ORFs with unknown functions originating from the *B. subtilis* complete genome sequencing project (Kunst *et al.*, 1997), whereas the *B. subtilis* *iolG* gene encodes a *myo*-inositol dehydrogenase (Fujita *et al.*, 1991; Yoshida *et al.*, 1997). The *Str. griseus* *strI* gene is a gene of unknown function located in the operon for the biosynthesis of streptomycin, an inosamine derivative (Mansouri & Piepersberg, 1991). The *mocA* gene product of *S. meliloti* L5-30 is essential for rhizopine utilization (Rossbach *et al.*, 1994a). Common to all these sequences is an N-terminal NAD(H)-binding motif, consisting of the amino acids GXGXXG, which has been proposed to form a $\beta\alpha\beta$ -fold involved in binding the ADP moiety of NAD(H) in dehydrogenases (Thompson & Donkersloot, 1992). Thus, we conclude that these genes potentially all encode NAD(H)-dependent dehydrogenases, similar to the *B. subtilis* *iolG* gene that encodes a functional *myo*-inositol dehydrogenase (Fujita *et al.*, 1991; Yoshida *et al.*, 1997).

Determination of the *myo*-inositol dehydrogenase activity

To examine whether the ORF found on plasmid pMG1000 and its derivatives indeed encodes an enzyme with *myo*-inositol dehydrogenase activity, an enzyme assay was carried out. *S. meliloti* wild-type and mutant strains were grown in minimal medium with 0.2% succinate as carbon source and 0.02% *myo*-inositol. *S. meliloti* strain 1021 harbouring the pLAFR1 vector was found to display a specific *myo*-inositol dehydrogenase activity of 62 ± 3 nmol NADH reduced min^{-1} (mg protein) $^{-1}$. Strain 1021 harbouring pMG1000, however, displayed a four times higher specific *myo*-inositol dehydrogenase activity [255 ± 19 nmol NADH reduced min^{-1} (mg protein) $^{-1}$]. Mutant strain #56 did not display any detectable *myo*-inositol dehydrogenase activity, but when it harboured plasmid pMG1000 it displayed enzyme activity comparable to the wild-type strain containing pMG1000 [241 ± 5 nmol NADH reduced min^{-1} (mg protein) $^{-1}$]. Moreover, mutant strain L56 displayed no detectable *myo*-inositol dehydrogenase activity, whereas its corresponding wild-type strain L5-30 displayed a *myo*-inositol dehydrogenase activity of 25 ± 2 nmol NADH reduced min^{-1} (mg protein) $^{-1}$. No *myo*-inositol dehydrogenase activity could be observed when the wild-type strains were grown in medium without 0.02% *myo*-inositol, suggesting that the *myo*-inositol dehydrogenase activity is inducible in *S. meliloti*.

Nodulation and nitrogen-fixation phenotype of *myo*-inositol degradation mutants

Mutant *S. meliloti* strains #56 and L56 could nodulate *M. sativa* plants and induce pink, nitrogen-fixing nodules on alfalfa indistinguishable from those induced by wild-type strains. These observations confirm that the *myo*-inositol dehydrogenase gene of *S. meliloti* is not essential for the establishment of an effective nitrogen-fixing symbiosis.

DISCUSSION

Inositol derivatives are commonly found in legume nodules. For example, in soybean nodules, *myo*-inositol, D-*chiro*-inositol, 3-O-methyl-D-*chiro*-inositol (D-pinitol) and 4-O-methyl-*myo*-inositol (ononitol) have been found to be the most abundant water-soluble forms of carbon in bacteroids (Streeter, 1987). Ononitol has also been found to be the major soluble carbohydrate in pea nodules induced by *R. leguminosarum* (Scot & Egsgaard, 1984). However, the role of different cyclitol compounds in nitrogen-fixing symbioses is not fully understood. Some of the methyl ethers of inositol increase in plant tissues during salt or water stress (Loewus *et al.*, 1990), leading to the hypothesis that perhaps bacteroids in the nodule are subjected to high osmotic pressure and thus synthesize cyclitol compounds as osmotic protectants.

However, a different role has been assigned to unusual compounds such as the rhizopine 3-O-MSI. It has been postulated that rhizopine plays a role in enhancing the competitiveness of *S. meliloti* strains that are able to synthesize 3-O-MSI during infection of their host, alfalfa, by being able to catabolize it in the nodule or under free-living conditions (Murphy & Saint, 1992; Rossbach *et al.*, 1995a). The hypothesis underlying this concept is that the 3-O-MSI synthesized in nodules is secreted into the rhizosphere and serves as a nutrient source for the strains that are able to catabolize it. For example, strains with the ability to utilize rhizopine are able to nodulate plants at a higher rate (Gordon *et al.*, 1996), and mutant strains unable to catabolize rhizopine are less competitive with respect to nodule occupancy (Rossbach *et al.*, 1995a).

Here, we report that a functional *myo*-inositol catabolism pathway is essential for the ability of *S. meliloti* to utilize rhizopine. To prove the connection between *myo*-inositol catabolism and rhizopine utilization, we made use of *S. meliloti* mutants unable to use *myo*-inositol as sole carbon source, and demonstrated that these mutant strains were unable to catabolize rhizopine. We further delimited the region responsible for this phenotype to a DNA fragment of 2 kb, harbouring an ORF, the predicted product of which shares significant amino acid similarity with the *myo*-inositol dehydrogenase encoded by the *iolG* (*idh*) gene of *B. subtilis*. Moreover, enzyme assays of strains carrying a mutation in this gene revealed that these strains were deficient in *myo*-inositol dehydrogenase activity. Thus, we conclude that the *idhA* gene of *S. meliloti* encodes a functional *myo*-inositol dehydrogenase.

Based on the analysis of the phenotype of the *idh* mutant strains #56, #105 and #113, which were able to utilize *scyllo*-inositol as sole carbon source, we conclude that the *S. meliloti* *myo*-inositol dehydrogenase does not play an essential role in the degradation of *scyllo*-inositol. Therefore, it seems that the activity of the *myo*-inositol dehydrogenase is specific for the *myo*-stereoisomer of inositol. This conclusion is supported by the observation that the purified *myo*-inositol dehydrogenase enzyme of

B. subtilis reacts only with *myo*-inositol as substrate, and not with *scyllo*-inositol (Ramaley *et al.*, 1979).

The results described in this paper reveal that *myo*-inositol dehydrogenase is essential for rhizopine utilization, suggesting that *myo*-inositol is an intermediate in rhizopine catabolism. It has also been speculated that *scyllo*-inosamine might be an intermediate during catabolism of rhizopine (Murphy *et al.*, 1987). It is possible that both compounds, *scyllo*-inosamine and *myo*-inositol, are intermediates in the rhizopine catabolic pathway; however, the exact structure of the degradation products of rhizopine is not known yet, mainly because of the unavailability of chemically synthesized 3-O-MSI.

Whereas the exact pathway of rhizopine degradation remains to be elucidated, the pathway of *myo*-inositol degradation, however, has been studied in detail in *A. aerogenes* (now *K. pneumoniae*; Anderson & Magasanik, 1971a, b; Berman & Magasanik, 1966a, b). In these publications, Magasanik and others have shown that this organism catabolizes *myo*-inositol via the intermediates 2-keto-*myo*-inositol, D-2,3-diketo-4-deoxy-epi-inositol, 2-deoxy-5-keto-D-gluconic acid and 2-deoxy-5-keto-D-gluconic acid 6-phosphate, resulting in dihydroxyacetone phosphate and malonic semialdehyde. Malonic semialdehyde is further degraded by malonic semialdehyde carboxylase to acetyl-CoA, CO₂ and NADH + H⁺. Poole *et al.* (1994) have described two enzymes that are involved in the degradation of *myo*-inositol in *R. leguminosarum* bv. *viciae*, *myo*-inositol dehydrogenase and 2-keto-*myo*-inositol dehydratase. Based on enzyme assay data, Poole *et al.* (1994) have postulated that the degradation of *myo*-inositol in *R. leguminosarum* bv. *viciae* follows a pathway similar to the one described for *K. pneumoniae*.

In fact, *B. subtilis* is the only micro-organism from which the inositol dehydrogenase enzyme has been purified and the corresponding gene has been cloned (Fujita *et al.*, 1991; Ramaley *et al.*, 1979). It is interesting to note that the deduced gene product of the *idhA* gene of *S. meliloti* not only shows similarities to the deduced protein product of the *B. subtilis* *iolG* (*idh*) gene, but also to the deduced protein products of *yrbE* and *yucG* in *B. subtilis* (Kunst *et al.*, 1997). However, it is not known whether the *yucG* or *yrbE* gene products play a role in inositol catabolism.

Another interesting observation is the similarity of the *S. meliloti* *idhA* gene product with the product of the *S. meliloti* *mocA* gene. *MocA* is essential for rhizopine degradation as well (Rossbach *et al.*, 1994a). *MocA* and *IdhA* display 33% identity in their amino acid sequence and both show signatures of dehydrogenases, suggesting that they could carry out related functions. However, despite their similarities at the amino acid level, these two gene products cannot replace each other's function. On the one hand, the *mocA* gene could not complement the *idhA* mutant #56 (S. Rossbach & M. Galbraith, unpublished observation), and on the other hand, the *mocA* mutant unable to utilize rhizopine was generated

in a strain carrying the *idh* wild-type gene (Rossbach *et al.*, 1994a).

It is also interesting to note that the *idhA* gene in *S. meliloti* does not seem to be part of a larger operon involved in *myo*-inositol degradation. All *S. meliloti* mutant strains carrying insertions in the vicinity of the *idhA* gene were found to be able to utilize *myo*-inositol as sole carbon source. This is in contrast to observations made in *B. subtilis*, in which the *iolG* gene is part of an 11-kb-long operon with 12 different genes (Yoshida *et al.*, 1994, 1997). Ten of these genes are essential for inositol catabolism, since *B. subtilis* strains with mutations in these genes have been said to be unable to degrade *myo*-inositol as sole carbon source (Yoshida *et al.*, 1997). The results from our experiments do not exclude the presence of other genes essential for *myo*-inositol degradation elsewhere in the genome of *S. meliloti*.

Clearly, both degradative pathways for *myo*-inositol and rhizopine in *S. meliloti* need further study. Interestingly, Scupham *et al.* (1996) have reported that *S. meliloti* strains with Ω insertions in the inositol locus increase alfalfa yield in field tests. Moreover, since we have shown here that *myo*-inositol catabolism is essential for rhizopine catabolism, further studies on the *S. meliloti* inositol catabolism pathway are clearly warranted and are likely to provide major insights into the molecular microbial ecology of symbiotic plant-microbe interactions.

ACKNOWLEDGEMENTS

We would like to thank Dr Rob Eversole and Uwe Rossbach for help with preparing the figures, and Janeen Holmes for help with the manuscript. This work was funded by the College of Arts and Sciences and the Center for Research into Environmental Signal Transduction (CREST) at Western Michigan University, and at Michigan State University by the US Department of Energy DE-FG02-91ER20021 and the US Department of Agriculture (92-39210-8224).

REFERENCES

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Anderson, W. A. & Magasanik, B. (1971a). The pathway of *myo*-inositol degradation in *Aerobacter aerogenes* – identification of the intermediate 2-deoxy-5-keto-D-gluconic acid. *J Biol Chem* **246**, 5653–5661.
- Anderson, W. A. & Magasanik, B. (1971b). The pathway of *myo*-inositol degradation in *Aerobacter aerogenes* – conversion of 2-deoxy-5-keto-D-gluconic acid to glycolytic intermediates. *J Biol Chem* **246**, 5662–5675.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1992). *Short Protocols in Molecular Biology*. New York: Wiley.
- Beringer, J. E. (1974). R factor transfer in *Rhizobium leguminosarum*. *J Gen Microbiol* **84**, 188–198.
- Berman, T. & Magasanik, B. (1966a). The pathway of *myo*-inositol degradation in *Aerobacter aerogenes* – dehydrogenation and dehydration. *J Biol Chem* **241**, 800–806.
- Berman, T. & Magasanik, B. (1966b). The pathway of *myo*-

- inositol degradation in *Aerobacter aerogenes* – ring scission. *J Biol Chem* **241**, 807–812.
- Bosworth, A. H., Williams, M. K., Albrecht, K. A. & 7 other authors (1994).** Alfalfa yield response to inoculation with recombinant strains of *Rhizobium meliloti* with an extra copy of *dctABC* and/or modified *nifA* expression. *Appl Environ Microbiol* **60**, 3815–3832.
- de Bruijn, F. J. & Rossbach, S. (1994).** Transposon mutagenesis. In *Methods for General and Molecular Bacteriology*, pp. 387–405. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- de Bruijn, F. J., Rossbach, S., Schneider, M., Ratet, P., Messmer, S., Szeto, W., Ausubel, F. M. & Schell, J. (1989).** *Rhizobium meliloti* 1021 has three differentially regulated loci involved in glutamine biosynthesis, none of which is essential for symbiotic nitrogen fixation. *J Bacteriol* **171**, 1673–1682.
- Ditta, G., Stanfield, S., Corbin, D. & Helinski, D. R. (1980).** Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proc Natl Acad Sci USA* **77**, 7347–7351.
- Egelhoff, T. T. & Long, S. R. (1985).** *Rhizobium meliloti* nodulation genes: identification of *nodDABC* gene products, purification of *nodA* protein and expression of *nodA* in *Rhizobium meliloti*. *J Bacteriol* **164**, 591–599.
- Friedman, A. M., Long, S. R., Brown, S. E., Buikema, W. J. & Ausubel, F. M. (1982).** Construction of a broad host range cloning vector and its use in the genetic analysis of *Rhizobium* mutants. *Gene* **18**, 289–296.
- Fujita, Y., Shindo, K., Miwa, Y. & Yoshida, K. (1991).** *Bacillus subtilis* inositol dehydrogenase-encoding gene (*idh*): sequence and expression in *Escherichia coli*. *Gene* **108**, 121–125.
- Gordon, D. M., Ryder, M. H., Heinrich, K. H. & Murphy, P. J. (1996).** An experimental test of the rhizopine concept in *Rhizobium meliloti*. *Appl Environ Microbiol* **62**, 3991–3996.
- Holt, J. G. (1993).** *Bergey's Manual of Determinative Bacteriology*. Baltimore, MD: Williams & Wilkins.
- Kiss, G. B., Vincze, E., Kalman, Z., Forrai, T. & Kondorosi, A. (1979).** Genetic and biochemical analysis of mutants affected in nitrate reduction in *Rhizobium meliloti*. *J Gen Microbiol* **113**, 105–118.
- Kowalski, M. (1970).** Transducing phages of *Rhizobium meliloti*. *Acta Microbiol Pol Ser A* **19**, 109–114.
- Kunst, F., Ogasawara, N., Mozer, I. & 148 others (1997).** The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*. *Nature* **390**, 249–256.
- Loewus, F. A. (1990).** Structure and occurrence of inositols in plants. In *Inositol Metabolism in Plants*, pp. 1–11. Edited by D. J. Morre, W. F. Boss & F. A. Loewus. New York: Wiley-Liss.
- Loewus, F. A., Everard, J. D. & Young, K. A. (1990).** Inositol metabolism: precursor role and breakdown. In *Inositol Metabolism in Plants*, pp. 21–45. Edited by D. J. Morre, W. F. Boss & F. A. Loewus. New York: Wiley-Liss.
- Mansouri, K. & Piepersberg, W. (1991).** Genetics of streptomycin production in *Streptomyces griseus*: nucleotide sequence of five genes, *strFGHIK*, including a phosphatase gene. *Mol Gen Genet* **228**, 459–469.
- Meade, H. M., Long, S. R., Ruvkun, G. B., Brown, S. E. & Ausubel, F. M. (1982).** Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn5 mutagenesis. *J Bacteriol* **149**, 114–122.
- Miller, J. H. (1992).** *A Short Course in Bacterial Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Murphy, P. J. & Saint, C. P. (1992).** Rhizopines in the legume–*Rhizobium* symbiosis. In *Molecular Signals in Plant–Microbe Communication*, pp. 377–390. Edited by D. P. S. Verma. Boca Raton, FL: CRC Press.
- Murphy, P. J., Heycke, N., Banfalvi, Z., Tate, M. E., de Bruijn, F. J., Kondorosi, A., Tempé, J. & Schell, J. (1987).** Genes for the catabolism and synthesis of an opine-like compound in *Rhizobium meliloti* are closely linked and on the Sym plasmid. *Proc Natl Acad Sci USA* **84**, 493–497.
- Murphy, P. J., Heycke, N., Trenz, S. P., Ratet, P., de Bruijn, F. J. & Schell, J. (1988).** Synthesis of an opine-like compound, a rhizopine, in alfalfa nodules is symbiotically regulated. *Proc Natl Acad Sci USA* **85**, 9133–9137.
- Murphy, P. J., Trenz, S. P., Grzemska, W., de Bruijn, F. J. & Schell, J. (1993).** The *Rhizobium meliloti* rhizopine *mos* locus is a mosaic structure facilitating its symbiotic regulation. *J Bacteriol* **175**, 5193–5204.
- Poole, P. S., Blyth, A., Reid, C. J. & Walters, K. (1994).** *myo*-Inositol catabolism and catabolite regulation in *Rhizobium leguminosarum* bv. *viciae*. *Microbiology* **140**, 2787–2795.
- Ramaley, R., Fujita, Y. & Freese, E. (1979).** Purification and properties of *Bacillus subtilis* inositol dehydrogenase. *J Biol Chem* **254**, 7684–7690.
- Rossbach, S., Kulpa, D. A., Rossbach, U. & de Bruijn, F. J. (1994a).** Molecular and genetic characterization of the rhizopine catabolism (*mocABCR*) genes of *Rhizobium meliloti* L5-30. *Mol Gen Genet* **245**, 11–24.
- Rossbach, S., McSpadden, B., Kulpa, D. & de Bruijn, F. J. (1994b).** Rhizopine synthesis and catabolism genes for the creation of ‘biased rhizospheres’ and a marker system to detect (genetically modified) microorganisms in the soil. In *Biotechnology Risk Assessment*, pp. 223–244. Edited by M. Levin, C. Grim & J. S. Angle. College Park, MD: University of Maryland Biotechnology Institute.
- Rossbach, S., McSpadden, B., Ganoff, M. & de Bruijn, F. J. (1995a).** *Rhizobium meliloti* rhizopine catabolism genes: distribution, role in competition and potential as marker gene to track microbes. In *Biotechnology Risk Assessment*, pp. 180–188. Edited by M. Levin, C. Grim & J. S. Angle. College Park, MD: University of Maryland Biotechnology Institute.
- Rossbach, S., Rasul, G., Schneider, M., Eardly, B. & de Bruijn, F. J. (1995b).** Structural and functional conservation of the rhizopine catabolism (*moc*) locus is limited to selected *Rhizobium meliloti* strains and unrelated to their geographical origin. *Mol Plant–Microbe Interact* **8**, 549–559.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Scot, L. & Egsgaard, H. (1984).** Identification of ononitol and O-methyl-scylo-inositol in pea nodules. *Planta* **161**, 32–36.
- Scupham, A. J., Bosworth, A. H., Ellis, W. R., Wacek, T. J., Albrecht, K. A. & Triplett, E. W. (1996).** Inoculation with *Sinorhizobium meliloti* RMBPC-2 increases alfalfa yield compared with inoculation with a nonengineered wild-type strain. *Appl Environ Microbiol* **62**, 4260–4262.
- Streeter, J. G. (1987).** Carbohydrate, organic acid, and amino acid composition of bacteroids and cytosol from soybean nodules. *Plant Physiol* **85**, 768–773.
- Tempé, J., Petit, A. & Bannerot, H. (1982).** Presence de substances semblables a des opines dans des nodosités de luzerne. *C R Acad Sci Paris Ser III* **295**, 413–426.
- Thompson, J. & Donkersloot, J. A. (1992).** N-(carboxyalkyl)

amino acids: occurrence, synthesis, and functions. *Annu Rev Biochem* **61**, 517–557.

Wexler, M., Gordon, D. M. & Murphy, P. J. (1995). The distribution of inositol rhizopine genes in *Rhizobium* populations. *Soil Biol Biochem* **27**, 531–537.

Williams, M. K., Cannon, F., McLean, P. & Beynon, J. (1988). Vector for the integration of genes into a defined site in the *Rhizobium meliloti* genome. In *Molecular Genetics of Plant–Microbe Interactions*, pp. 198–199. Edited by R. Palacios & D. P. S. Verma. St Paul, MN: American Phytopathological Society.

Yoshida, K., Sano, H., Miwa, Y., Ogasawara, N. & Fujita, Y. (1994). Cloning and nucleotide sequencing of a 15 kb region of the *Bacillus subtilis* genome containing the *iol* operon. *Microbiology* **140**, 2289–2298.

Yoshida, K. I., Aoyama, D., Ishio, I., Shibayama, T. & Fujita, Y. (1997). Organization and transcription of the *myo*-inositol operon, *iol*, of *Bacillus subtilis*. *J Bacteriol* **179**, 4591–4598.

Received 14 April 1998; revised 22 June 1998; accepted 26 June 1998.