

# Investigation of *myo*-Inositol Catabolism in *Rhizobium leguminosarum* bv. *viciae* and Its Effect on Nodulation Competitiveness

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Three discrete loci required for growth on *myo*-inositol in *Rhizobium leguminosarum* bv. *viciae* have been characterized. Two of these are catabolic loci that code for malonate semialdehyde dehydrogenase (*iolA*) and malonate semialdehyde dehydrogenase (*iolD*). *IolD* is part of a possible operon, *iolDEB*, although the functions of *IolE* and *IolB* are unknown. The third locus, *int*, codes for an ABC transport system that is highly specific for *myo*-inositol. LacZ analysis showed that mutation of *iolD*, which codes for one of the last steps in the catabolic pathway, prevents increased transcription of the entire pathway. It is likely that the pathway is induced by an end product of catabolism rather than *myo*-inositol itself. Mutants in any of the loci nodulated peas (*Pisum sativum*) and vetch (*Vicia sativa*) at the same rate as the wild type. Acetylene reduction rates and plant dry weights also were the same in the mutants and wild type, indicating no defects in nitrogen fixation. When wild-type 3841 was coinoculated onto vetch plants with either catabolic mutant *iolD* (RU360) or *iolA* (RU361), however, >95% of the nodules were solely infected with the wild type. The competitive advantage of the wild type was unaffected, even when the mutants were at 100-fold excess. The *myo*-inositol transport mutant (RU1487), which grows slowly on *myo*-inositol, was only slightly less competitive than the wild type. The nodulation advantage of the wild type was not the result of superior growth in the rhizosphere. Instead, it appears that the wild type may displace the mutants early on in the infection and nodulation process, suggesting an important role for *myo*-inositol catabolism.

*Additional keywords:* bacteria, competition.

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*myo*-Inositol is abundant in pea and soybean nodules and is the most common compound inside isolated bacteroids of *Bradyrhizobium japonicum* (Sköt and Egsgaard 1984; Streeter 1987). The recent detection of inositol dehydrogenase activity, an enzyme induced specifically by inositol, in *Rhizobium loti* and *Rhizobium fredii* strains grown in solution extracted from a range of soils provides evidence that inositol also is present in soil and serves as an important substrate (Wood and

Stanway 2001). Given the presence of large amounts of *myo*-inositol in legume nodules and soil, an important question about carbon flux concerns the regulation of *myo*-inositol utilization.

The importance of *myo*-inositol catabolism is illustrated by the synthesis of the *myo*-inositol derivatives known as rhizopines. For example, in alfalfa nodules, *Sinorhizobium meliloti* strains L5-30 and Rm220-3 have been shown to produce rhizopines L-3-*O*-methyl-*scyllo*-inosamine (3-*O*-MSI) and *scyllo*-inosamine, respectively (Murphy et al. 1987; Murphy et al. 1988; Murphy et al. 1993; Saint et al. 1993). In strain L5-30, the genes for synthesis and catabolism of 3-*O*-MSI are located on the *nod-nif sym* plasmid (Murphy et al. 1987; Murphy et al. 1988). Furthermore, the genes for synthesis of 3-*O*-MSI (but not its catabolism) are regulated symbiotically by *nifA*. It has therefore been hypothesized that rhizopines synthesized in the nodule by bacteroids may be catabolized by free-living bacteria in the rhizosphere. In pea nodules, the synthesis of ononitol and *O*-methyl-*scyllo*-inositol, which also belong to the inositol class of compounds, has been shown to depend on the strain of *R. leguminosarum* bv. *viciae* present, and strains of *R. leguminosarum* bv. *viciae* catabolize 3-*O*-MSI (Murphy and Saint 1992; Sköt and Egsgaard 1984; Wexler et al. 1995). The catabolism of rhizopines is dependent upon a functional *myo*-inositol catabolic pathway for *S. meliloti* and *R. leguminosarum* (Bahar et al. 1998; Galbraith et al. 1998).

The rhizopine-producing strain *S. meliloti* L5-30 had a competitive advantage for the nodulation of alfalfa (*Medicago sativa*) in soil when coinoculated with a mutant strain, even though when inoculated alone, the mutant had a similar rate of growth and nodulation to the wild type (Gordon et al. 1996). The mutant occupied less than 30% of nodules. This competitive advantage remained in soil 4 years after inoculation, even though there had been turnover of nodules in that time (Heinrich et al. 1999). Only approximately 10% of *S. meliloti* and *R. leguminosarum* strains, however, produce rhizopines (Wexler et al. 1995). Given that rhizopines must be catabolized via the *myo*-inositol catabolic pathway, we considered that *myo*-inositol also may be important in nodulation competition generally. There also is some evidence that disruption of *myo*-inositol dehydrogenase in *S. meliloti* may alter yields of alfalfa (Bosworth et al. 1994; Scupham et al. 1996). We therefore initiated a study to determine whether the ability to utilize *myo*-inositol is important for

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nodulation competitiveness in a strain of *R. leguminosarum* that does not make rhizopines.

## RESULTS

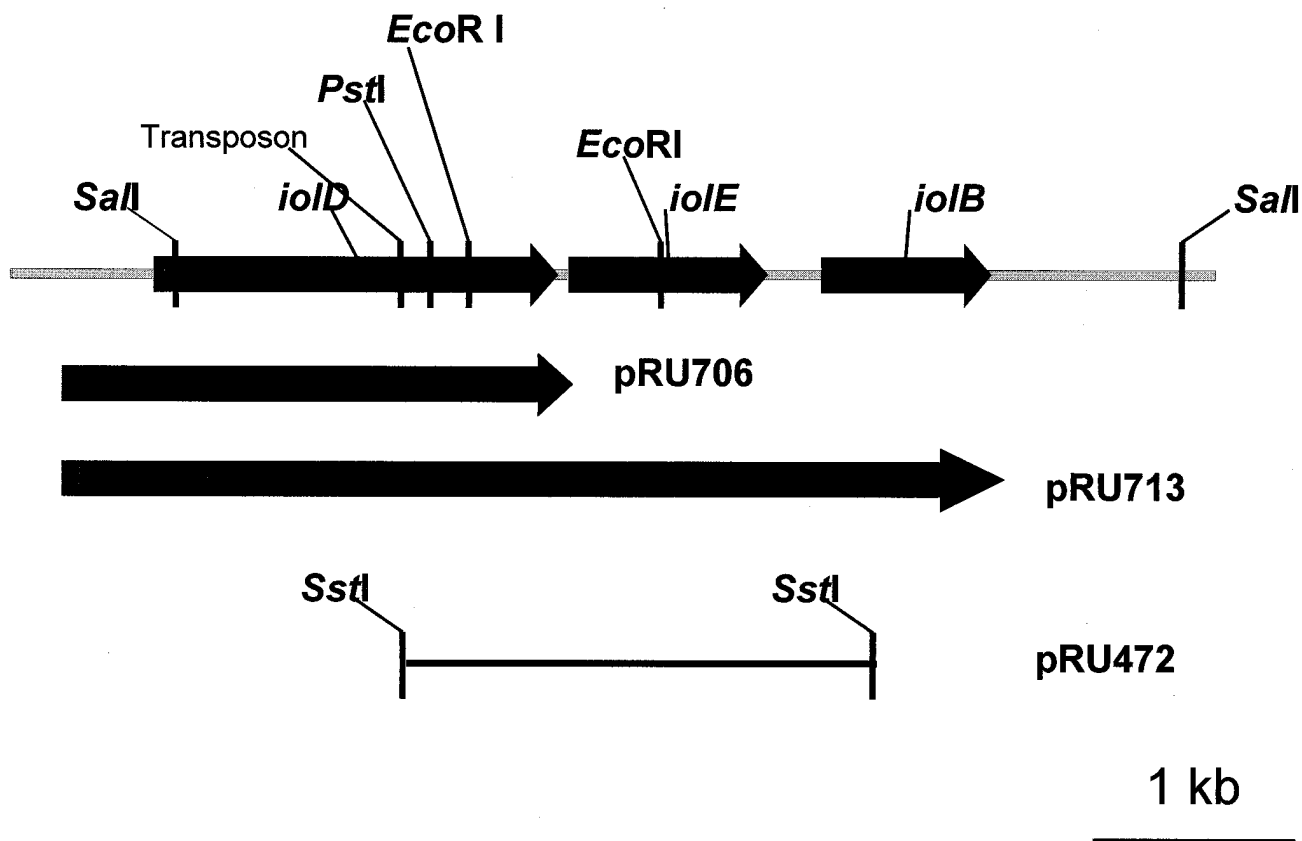
### Characterization of *myo*-inositol catabolic mutants.

The catabolic mutants RU360 and RU361 were identified previously by their inability to grow when *myo*-inositol was the sole carbon source (Poole et al. 1994a). The transposon insertions in these mutants were cloned, and the junction between Tn5 and strain 3841 DNA sequenced (Bahar et al. 1998). The transposons in strains RU360 and RU361 inserted into *iolD* and *iolA*, respectively. *IolD* is a putative acetolactate synthase, whereas *IolA* is a putative malonate semialdehyde dehydrogenase. These inositol mutants were then used to show that the rhizopine 3-*O*-methyl-*scyllo*-inosamine requires the inositol catabolic pathway for breakdown in *R. leguminosarum*.

Two cosmids, pRU3078 and pRU3079, which complement RU360 but not RU361, had been isolated (Poole et al. 1994a). This suggests that these mutations may be widely separated from each other in the genome of strain 3841. As part of our interest in the role of *myo*-inositol in rhizosphere growth and nodulation competition, we considered it essential to better define the nature of the insertions and operon structures. A cosmid library of strain 3841 DNA was introduced into strain RU361, and cosmid pRU3111, which complements growth, was isolated. pRU3111 did not complement strain RU360,

however, which is consistent with the lack of complementation of strain RU361 by cosmids pRU3078 and pRU3079. All three cosmids also were Southern blotted with the Tn5 clones from strains RU360 (pRU472) and RU361 (pRU476). As expected, pRU472 hybridized to pRU3078 and pRU3079 but not to pRU3111, whereas pRU476 hybridized only to pRU3111.

To fully define the mutation in strain RU360, the entire region was sequenced on both strands using plasmid pRU472 (Fig. 1). The region was then extended by direct sequencing of cosmid pRU3078. A total region of 5,606 bp was sequenced on both strands (EMBL accession no. AJ276296). BLASTX analysis of the deduced amino acid sequences revealed homology with protein *IolDEB* encoded by genes in the *myo*-inositol degradation operon of *Bacillus subtilis* (Yoshida et al. 1997). A 9-bp repeat was created in RU360 by the insertion of Tn5-*lacZ* (1,775 to 1,784 bp of EMBL accession no. AJ276296, i.e., GGCAGCACT). The highest identity of the first gene in this putative operon was 41% (*p* value of  $10^{-127}$ ) with *IolD* (acetolactate synthase) in the *myo*-inositol catabolic *iol* operon of *B. subtilis*. Downstream of *iolD* was an open reading frame (ORF) whose deduced amino acid sequence had homology with three proteins, MocC of *R. leguminosarum* *bv. viciae*, MocC of *S. meliloti*, and *IolE* of *B. subtilis*, in the databases. These proteins have no known function. The highest amino acid identity was 42% (*p* value of  $3 \times 10^{-62}$ ) over the entire amino acid sequence of MocC of *R. leguminosarum* *bv. viciae*. The ORF was designated *iolE* and began 46 bp downstream of the end of *iolD*. Downstream (246 bp) of the *iolE*



**Fig. 1.** *iolDEB* genes from *Rhizobium leguminosarum* 3841. Plasmid pRU472 was used for sequencing and is derived from strain RU360. *SstI* site is derived from Tn5-*lacZ* (left) and the chromosome of strain RU360 (right).

gene was an ORF with 62% identity ( $p$  value of  $5 \times 10^{-58}$ ) with *iolB* of *B. subtilis*. This protein has no other homologs in the databases and no known function. All three genes are predicted to be transcribed in the same direction as the  $\beta$ -galactosidase of Tn5-*lacZ* in strain RU360, but they are not organized in the same order as in *B. subtilis*. Putative Shine-Dalgarno sequences were identified for *iolD* (GAGGGG, 3 bp upstream) and *iolB* (AGGAGA, 8 bp upstream), but the only good match for *iolE* (GGAGGA) was too far away (21 bp) from the putative ATG start.

The *iolD* gene is 1,881 bp and encodes a predicted protein of 626 amino acids with a relative molecular mass of 66,730 and an isoelectric point of 6.1. The *iolE* gene is 939 bp and encodes a predicted protein of 312 amino acids with a relative molecular mass of 34,456 and an isoelectric point of 5.6. The *iolB* gene is 795 bp and encodes a predicted protein of 264 amino acids with a relative molecular mass of 29,046 and an isoelectric point of 5.6.

Further sequence was obtained for 1 kb on either side of the *iol* genes. A putative ORF that began 407 bp downstream of *iolB* was identified. Sequencing did not extend far enough to give the end of the putative ORF. BLASTX analysis of the deduced amino acid sequence of the region surrounding the *iol* genes revealed no homology with known sequences. Sequencing of the region upstream of *iolD* did not reveal any homology with sequences in the databases. The sequence data suggest that the putative genes *iolD*, *iolE*, and *iolB* comprise an operon that is distinct from other genes involved in *myo*-inositol utilization.

In order to determine whether all three genes identified in RU360 are necessary for *myo*-inositol catabolism, polymerase chain reaction (PCR) amplification was carried out on the *iol* region with cosmid pRU3078. Plasmids pRU706 (*iolD*) and pRU713 (*iolDEB*) were conjugated into RU360. Six colonies from each conjugation were streaked onto AMA medium containing 10 mM *myo*-inositol as the sole carbon source. None of the colonies of strain RU360/pRU706 were able to grow on *myo*-inositol, indicating that the presence of the entire *iolD* gene was not sufficient to restore growth. All six colonies containing RU360/pRU713 grew on *myo*-inositol as the sole carbon source, indicating that the *iolD*, *iolE*, and *iolB* genes enable complementation. This suggests that the transposon insertion in *iolD* has a polar effect on the downstream genes (*iolEB*) and, therefore, one or both of these genes is essential for *myo*-inositol catabolism. It is consistent that *iolDE* and, perhaps, *iolDEB* are in an operon with a promoter upstream of *iolD*. The large intergenic region between *iolE* and *iolB* (246 bp) suggests that *iolB* may have its own promoter, although this was not investigated.

### Characterization of the *iolA* gene.

An intact Tn5-*lacZ* with flanking chromosomal DNA was cloned previously from RU361 as an approximately 12,400-bp *Sall* fragment into pBluescript SK<sup>-</sup> (pRU438) (Bahar et al. 1998). The clone contained 230 bp of chromosomal DNA upstream of the transposon and approximately 3,800 bp downstream. Whereas the junctions of the transposon had been sequenced (Bahar et al. 1998), suggesting an insert in acetolactate synthase (*iolA*), the entire region needed to be sequenced fully. Therefore, a *NotI* subclone of pRU438 was constructed in pBluescript SK<sup>-</sup> (pRU476). Where appropriate,

the sequence also was extended by direct sequencing from cosmid pRU3111, giving a complete sequence of 3,309 bp (EMBL accession no. AJ276297). A 9-bp repeat was created in RU361 by the insertion of Tn5-*lacZ* (1,525 to 1533 bp in accession no. AJ276297, TAGCCTGCG). BLASTX analysis of the deduced amino acid sequence of the DNA revealed an ORF with homology to several methylmalonate semialdehyde dehydrogenases, including *IolA* of the *B. subtilis* *iol* operon. Highest identity was 68% with MmsA of *Mycobacterium tuberculosis* ( $p$  value of  $1 \times 10^{-145}$ ). The ORF, designated *iolA*, is predicted to be transcribed in the opposite direction to the  $\beta$ -galactosidase in the transposon. The *iolA* gene is 1,497 bp and has a putative Shine-Dalgarno sequence (AGGAGA) 6 bp upstream of the start site. It encodes a predicted protein of 498 amino acids and has a relative molecular mass of 53,639 and an isoelectric point of 5.8.

Downstream of *iolA*, an ORF was identified whose deduced amino acid sequence had identity to Aau3 of *S. meliloti* (EMBL accession no. AF193764). The Aau3 protein is predicted to be required for growth on polyhydroxybutyrate cycle intermediates. There was 74% identity ( $p$  value of  $6 \times 10^{-54}$ ) over the amino acid sequence. Other homologs included Yhde of *B. subtilis* and YjeB of *Escherichia coli*. None of the other homologs have any known function.

Sequencing was continued 1,206 bp upstream of the *iolA* gene with custom primers. An ORF was identified beginning at 405 bp upstream of the start of *iolA*. BLASTX analysis of the deduced amino acid sequence showed that the ORF had 35% identity ( $p$  value of  $9 \times 10^{-12}$ ) over the first 173 amino acids of 282 with a hypothetical gene from *A. tumefaciens*. The gene does not have any known function. The next highest homology was 31% ( $p$  value of  $3 \times 10^{-9}$ ) with a hypothetical transcriptional activator gene, *act*, from *Pseudomonas aeruginosa*. The ORF, designated *orf1*, is predicted to be transcribed in the opposite direction to *iolA*. Sequencing was not extended further because our primary aim was to see whether any other genes involved in *myo*-inositol catabolism were likely to be present. The sequence clearly shows that *iolA* is isolated from other obvious *ino* genes. This is unlike the clustering of the entire *ino* operon in *B. subtilis* (Yoshida et al. 1997) but is consistent with the presence of the *myo*-inositol gene by itself in *S. meliloti* (Galbraith et al. 1998).

### LacZ fusion analysis of *ino* gene expression.

In a previous study, we had shown that strains RU360 and RU361 did not induce the first two enzymes of the *myo*-inositol catabolic pathway, *myo*-inositol dehydrogenase and 2-ketoinositol dehydratase (Poole et al. 1994a). The data in this study show that the failure to induce these first enzymes occurs in strains that are mutated in two of the last steps of the *myo*-inositol catabolic pathway. This suggests that induction of the pathway does not occur as the result of the presence of *myo*-inositol itself but may require elevated levels of later intermediates in the pathway. From the sequencing we conducted in this study, it is apparent that the Tn5-*lacZ* fusion in *iolD* (strain RU360) should be active. When  $\beta$ -galactosidase activity was originally measured in RU360, however, the fusion was inactive (Poole et al. 1994a). It was decided to repeat this assay and to test whether the fusion was active in RU360 when complemented by pRU3078 and pRU3079 (Table 1). Strain RU361

was not tested because Tn5-*lacZ* is transcribed in the opposite direction to the interrupted gene.

The data show that  $\beta$ -galactosidase activity was very low in RU360 grown on 20 mM pyruvate alone. The presence of 10 mM *myo*-inositol, in addition to pyruvate in the growth medium, did not cause the induction of expression of *lacZ*. When complemented with cosmids pRU3078 or pRU3079, however, there were two- and fivefold increases, respectively, in *lacZ* activity when *myo*-inositol was present, in addition to pyruvate. When RU360/pRU3078 and RU360/pRU3079 were grown on *myo*-inositol as the sole carbon source, there was an increase in the expression of *lacZ* of 12- and 20-fold, respectively. These data indicate that the genes interrupted by Tn5-*lacZ* in RU360 are *myo*-inositol inducible but that a complete catabolic pathway is required for induction and that pyruvate partially represses induction.

### Isolation of a new inositol transport mutation.

We show below that *myo*-inositol catabolic mutations (RU360 and RU361) are affected severely in their ability to compete for nodulation of peas and vetch. Because the effects of blocking catabolism can be varied, we conducted a new screen for Tn5 mutants in an attempt to isolate one that is blocked in *myo*-inositol uptake. Approximately 10,000 Tn5 mutants were screened on minimal medium with either glucose or *myo*-inositol as the sole carbon source. Of these, strain RU1487 was identified because it is severely impaired in its ability to grow on *myo*-inositol as the sole carbon source. Its growth rate when *myo*-inositol is the sole carbon source is almost four times slower than that of 3841 (mean generation time of 13.4 h for RU1487 and 3.4 h for 3841). To ascertain whether the reduced growth capability on *myo*-inositol also was the result of the insertion of Tn5, the transposon was transduced with the phage RL38 from RU1487 into 3841 by selecting for kanamycin resistance. Eleven transductants were tested for growth on AMA medium with either 10 mM *myo*-inositol or glucose as the sole carbon source. No growth was visible for any of the transductants on *myo*-inositol at least 8 days after they had been streaked out, whereas they all grew normally on glucose. This confirms the tight linkage of the transposon in strain RU1487 with the very poor ability to grow on *myo*-inositol.

An intact Tn5 was cloned from RU1487 into pBluescript SK<sup>-</sup>, but this proved unstable. Thus, a 7,900-bp *EcoRI* fragment with approximately 2,100 bp of chromosomal DNA flanking both ends of the transposon was cloned into the low-copy-number vector pACYC184 producing pRU426. The two ends of the transposon were PCR amplified with a Tn5 primer (p120) and primers that flank the *EcoRI* site of pACYC184 (p87 and p89). The PCR products were then sequenced with p113 (Tn5 specific primer) and either p114 or p115, which

bind to the PCR products of p120/p87 and p120/p89, respectively. Sequencing was carried out over 2,100 bp, with a gap of approximately 400 bp in the middle. A 9-bp overlap was created in RU1487 by the insertion of Tn5 (AGCTGAACC). BLASTX analysis of the deduced amino acid sequence revealed that the transposon interrupted an ORF with identity to the ATP binding component of ABC transport systems involved in the uptake of D-galactose and methyl-galactoside (Mgl). The identity was 54% over the entire sequence of MglA of *E. coli* (*p* value of  $2 \times 10^{-71}$ ). The gene encoded by the ORF was designated *intA* (*myo*-inositol transport). A putative Shine-Dalgarno sequence (AAGG) was identified 3 bp upstream of the ATG start. Ending 5 bp upstream of the putative start of the *intA* gene was an ORF whose deduced amino acid sequence had 75% identity (*p* value of  $1 \times 10^{-38}$ ) over the final 100 amino acids of 309 of MocB of *S. meliloti* and *R. leguminosarum* bv. *viciae*. This gene encodes a periplasmic binding protein that binds to rhizopines and is thought to be part of a rhizopine transport system. The gene encoded by the ORF was designated *intB* and is predicted to be transcribed in the same direction as *intA*. There also was high homology with D-galactose binding proteins, which also was the case for the *myo*-inositol binding protein of the *Pseudomonas* species (Deshusses and Belet 1984). On the basis of the sequence data, it can be postulated that strain RU1487 is mutated in an operon of genes encoding an ABC transport system that might be responsible for the uptake of *myo*-inositol or a derivative. The high identity of *intB* with MocB (75% identity to the last 100 amino acids of MocB), also suggests the possibility that the *int* ABC uptake system may provide the membrane complex to which MocB binds to promote rhizopine uptake.

### Characterization of *myo*-inositol uptake.

Various strains were grown on 20 mM pyruvate or 20 mM pyruvate plus 10 mM *myo*-inositol as the sole carbon sources, and the uptake of *myo*-inositol was measured (Table 2). Uptake also was measured for 3841, and RU1487 when grown on 10 mM *myo*-inositol alone. All uptake experiments were carried out with <sup>14</sup>C-labeled glucose as a control.

There was a basal rate of *myo*-inositol uptake by each strain grown on 20 mM pyruvate. This did not increase significantly in RU360 and RU1487 grown on pyruvate plus *myo*-inositol or RU1487 grown on *myo*-inositol alone. When strain 3841 was grown on 20 mM pyruvate and 10 mM *myo*-inositol or 10 mM *myo*-inositol alone, however, there were 2.5- and sevenfold increases in the rate of uptake of *myo*-inositol, respectively, compared with the rate of uptake when grown on pyruvate. These data indicate that there is an inducible system for the transport of *myo*-inositol in 3841 but not in the mutants. There was no significant difference in the rate of glucose uptake by the strains on different carbon sources (data not shown).

**Table 1.**  $\beta$ -Galactosidase activity of RU360<sup>a</sup>

Strain	$\beta$ -Galactosidase activity/nmol/min/mg of protein grown on different carbon sources		
	Pyruvate	Pyruvate + <i>myo</i> -inositol	<i>myo</i> -Inositol
RU360	160.3 $\pm$ 15.5	132.2 $\pm$ 4.1	—
RU360/pRU3078	125.7	264.8	1,453.6
RU360/pRU3079	90.0 $\pm$ 4.3	459.4 $\pm$ 35.3	1,871.4 $\pm$ 315.9

<sup>a</sup> Each value represents the mean of three cultures with standard deviation. RU360/pRU3078 is the mean of two cultures. RU360 cannot be grown on *myo*-inositol as the sole carbon source.

The deduced amino acid sequence of the putative *intA* gene of RU1487 has high homology to MglA, a component of the galactose and Mgl uptake system. Therefore, the uptake system might not be specific for *myo*-inositol. To determine the specificity of the transport system, *myo*-inositol uptake by 3841 was measured after the addition of a fivefold excess of different carbon compounds (Table 2). The inducible system is

**Table 2.** Transport of *myo*-inositol<sup>a</sup>

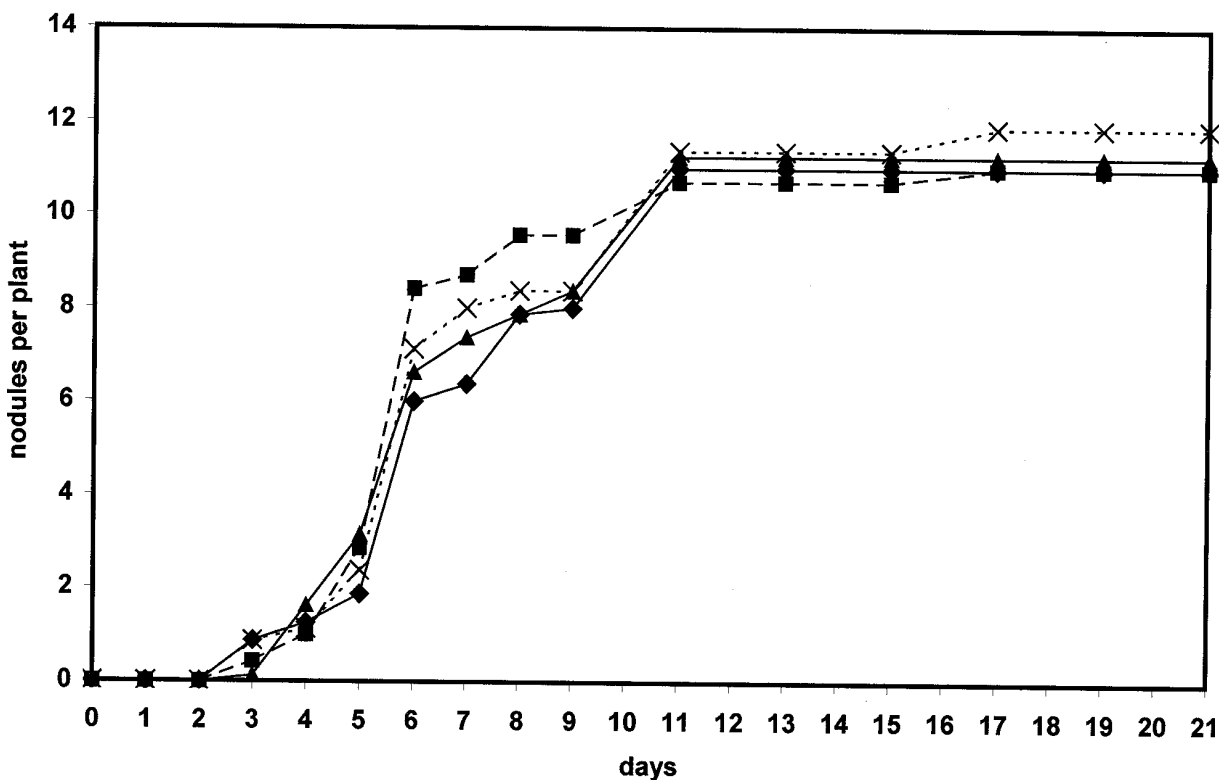
Strain	Transport rate/nmol/min/mg of protein		
	Grown on	Competitor	Transport
3841	Pyruvate	–	6.4 ± 1.4
3841	Pyruvate/inositol	–	15.1 ± 3.5
3841	Inositol	–	47.0 ± 1.7
3841	Inositol	Mannitol	37 ± 5.6
3841	Inositol	Sorbitol	35.2 ± 12.0
3841	Inositol	Glucose	41.6 ± 2.9
3841	Inositol	Fructose	36.0 ± 5.8
3841	Inositol	Pyruvate	40.2 ± 10.7
RU1487	Pyruvate	–	5.9 ± 0.8
RU1487	Pyruvate/inositol	–	4.7 ± 1.3
RU1487	Inositol	–	7.4 ± 1.5
RU360	Pyruvate	–	5.9 ± 0.2
RU360	Pyruvate/inositol	–	5.7 ± 1.5
RU361	Pyruvate	–	5.4 ± 0.2
RU361	Pyruvate/inositol	–	9.2 ± 1.6

<sup>a</sup> Transport rates are shown as the mean of three independent cultures ± standard deviation. *myo*-Inositol was at 25 μM, and competitors were at a fivefold excess. All cultures were grown on acid minimal salts minimal medium, with carbon sources indicated.

clearly specific for *myo*-inositol. The presence of a basal background rate of uptake, presumably the result of a second transport system, explains the poor growth of RU1487 on *myo*-inositol. It also is notable that the absence of *myo*-inositol transport elevation in strains RU360 or RU361 grown on pyruvate/*myo*-inositol indicates an absence of induction, unless there is a complete catabolic pathway. This is the same pattern as observed with LacZ fusion analysis of *iolD* (Table 1).

#### Nodulation ability of the mutants.

Strains RU360, RU361, and RU1487 were able to nodulate vetch at the same rate as 3841, with the same numbers of nodules formed when 10<sup>3</sup> cells were applied to the roots of 7-day-old seedlings (Fig. 2). Low inocula were used because high levels of inocula can bias competition experiments, particularly where there are growth rate differences between strains (Streit et al. 1996). Strain RU360 was shown previously to reduce acetylene at the same rate as 3841 on vetch (Poole et al. 1994a). Strains 3841, RU361, and RU1487 reduced acetylene on pea at 2.4 μmole per h per plant (standard error of the mean [SEM] 0.45), 2.4 μmole per h per plant (SEM 0.1), and 2.9 μmole per h per plant (SEM 0.7), respectively, indicating that these strains are not affected in their ability to fix nitrogen. The strains also were inoculated onto common vetch (*Vicia sativa*), which is a small seeded legume that shows severe nitrogen deficiency if nitrogen fixation is impaired. Average plant dry weights for vetch inoculated with 3841, RU361, and RU1487 were 0.64 (SEM 0.14), 0.65 (SEM 0.19), and 0.78 g (SEM 0.13), respectively, indicating no significant effects.



**Fig. 2.** Nodulation kinetics of vetch by several strains of *Rhizobium leguminosarum*. X, 3841; ▲, RU1487; ■, RU361; and ◆, RU360. Each strain (10<sup>3</sup> CFU) was inoculated onto eight plants. Analysis of variance showed that none of the differences are statistically different.

### Nodulation competitiveness of the mutants.

The above data as well as the previous demonstration that the first two enzymes of *myo*-inositol catabolism are not induced in mature pea bacteroids demonstrates that *myo*-inositol is not important as a carbon source to fuel nitrogen fixation in either pea or vetch. This is hardly surprising given the importance of dicarboxylic acids in this process (Poole and Allaway 2000). The abundance of *myo*-inositol in legume nodules as well as the rhizosphere and the role of the *myo*-inositol catabolic pathway in rhizopine degradation, however, suggests it may be important in earlier steps in growth in the rhizosphere or in nodulation (Bahar et al. 1998; Galbraith et al. 1998; Sköt and Egsgaard, 1984; Streeter 1987; Wood and Stanway 2001). We therefore examined the effect of *myo*-inositol catabolic pathway mutation on growth in the rhizosphere and in competitiveness for nodulation.

When inoculated alone, 3841, RU360, RU361, and RU1487 were the sole occupants of all nodules, as appropriate (Table 3). No nodules were obtained on plants inoculated with sterile acid minimal salts (AMS) alone. When 3841 and RU360 were coinoculated onto vetch in equal numbers ( $10^3$  CFU), 3841 occupied 97.6% of nodules as the sole occupant. Strain 3841 maintained dominance when RU360 was inoculated in tenfold and 100-excess, with 3841 the sole occupant in 99.4 and 95.9% of nodules, respectively (Table 3). When 3841 and RU361 were coinoculated onto vetch in equal numbers ( $10^3$  CFU), 3841 occupied 100% of nodules as sole occupant. Strain 3841 occupied 89.5 and 98% of nodules as sole occupant when RU361 was in ten- and 100-fold excess, respectively (Table 3).

Even though very few nodules containing any kanamycin-resistant bacteria were obtained, the possibility of co-residence in nodules was checked in 12 of these rare nodules from plants, which had been inoculated with equal numbers ( $10^3$ ) of either RU360/3841 or RU361/3841 (72 colonies for each combination). For those plants inoculated with RU360/3841, seven of the 12 nodules had a mixture of kanamycin-resistant (RU360) and -sensitive bacteria (3841). Strain 3841 made up 9% of the colonies isolated from the mixed occupancy nodules. For those plants inoculated with RU361/3841, nine out of 12 nodules had a mixture of kanamycin-resistant (RU361) and -sensitive bacteria (3841). Strain

3841 made up 19% of the colonies isolated from the mixed occupancy nodules. The mutants predominate in these nodules but the wild type is still present in a majority of mixed-occupancy nodules. This contrasts with the vast majority of nodules that were occupied solely by 3841. RU360 and RU361 containing complementing cosmids were competed with the wild type, however, whereas the number of nodules containing a mutant increased dramatically, although the cosmid retention was too low to draw firm conclusions (data not shown). Bacteria isolated from control plants inoculated only with mutants had 100% retention of the kanamycin marker, demonstrating that the presence of kanamycin-sensitive wild-type bacteria in coinoculated plants is not the result of reversion.

The same trends were observed for pea coinoculated with 3841 and RU360 or RU361 (data not shown). These data demonstrate a profound competitive advantage for strains able to catabolize *myo*-inositol. Whereas any single mutation may cause a loss of competition for allele-specific reasons, these mutations are in genes that are not part of the same operon and encode proteins for separate reactions. This makes it highly likely that *myo*-inositol catabolism, per se, is important in nodulation competitiveness. Disrupting a large metabolic pathway such as that for *myo*-inositol, however, may have a range of effects on metabolism, so we considered it important to isolate a transport mutant (see above) and test its effects on nodulation competitiveness.

When vetch plants were coinoculated with RU1487 and 3841 in equal numbers ( $10^3$  CFU), 3841 was the sole occupant in 52.9% of the nodules. When RU1487 was inoculated in ten- and 100-fold excess, 3841 was the sole occupant in 30.7 and 19.1% of the nodules, respectively (Table 3). Co-residence was assessed in 12 nodules that contained kanamycin-resistant bacteria. Nine of the twelve nodules had a mixture of kanamycin-resistant (RU1487) and -sensitive bacteria (3841), whereas three contained RU1487 alone. Strain 3841 made up 19% of the bacteria. Overall, the data indicate that with equal inocula, strain 3841 is present as sole occupant in 53% of nodules, RU1487 is the sole occupant in approximately 12% of nodules, and 35% of nodules have at least some co-residence. This indicates a slight competitive advantage for 3841 over RU1487, a result supported by the fact that a 100-

**Table 3.** Competition for nodule occupancy<sup>a</sup>

Strains	Inoculum Ratio	Number of nodules				
		Total	3841	RU360	RU361	RU1487
None		0				
3841		72	72			
RU360		72		72		
RU361		72			72	
RU1487		72				72
RU360/3841	1:1	84	82	2		
RU360/3841	10:1	176	175	1		
RU360/3841	100:1	194	186	8		
RU361/3841	1:1	116	116		0	
RU361/3841	10:1	191	171		20	
RU361/3841	100:1	159	156		3	
RU1487/3841	1:1	172	91			81
RU1487/3841	10:1	189	58			131
RU1487/3841	100:1	183	35			148

<sup>a</sup> A  $1 \times$  inoculum was  $10^3$  CFU on vetch plants. Nodules scored as containing the wild-type bacteria did not contain any detectable mutants, although nodules containing mutant strains often contained wild type as well (see text).

fold excess of RU1487 reduces sole 3841 occupancy only to 19.1%. This is dramatically different from the *myo*-inositol catabolic mutants RU360 and RU361, which show almost complete dominance by the wild type, even when the mutants are inoculated in 100-fold excess.

The growth of RU360, RU361, RU1487, and 3841 in the rhizosphere of vetch was monitored at 2, 4, 6, and 8 days, immediately following inoculation. Analysis of variance showed that there were no significant differences in the final numbers of any strains ( $P > 0.05$ ). Thus, differences in nodule occupancy are very unlikely to be the result of different growth rates in the rhizosphere.

We also considered the possibility that the presence of *myo*-inositol itself may be toxic to *ino* mutants. Such a possibility might explain the nodulation competition difference between the transport mutant RU1487 and the catabolic mutants RU360 and RU361. Strains 3841, RU1487, RU360, and RU361, however, grew with mean generation times of 4.1, 4.1, 3.9, and 3.9 h, respectively, on *myo*-inositol plus pyruvate minimal medium.

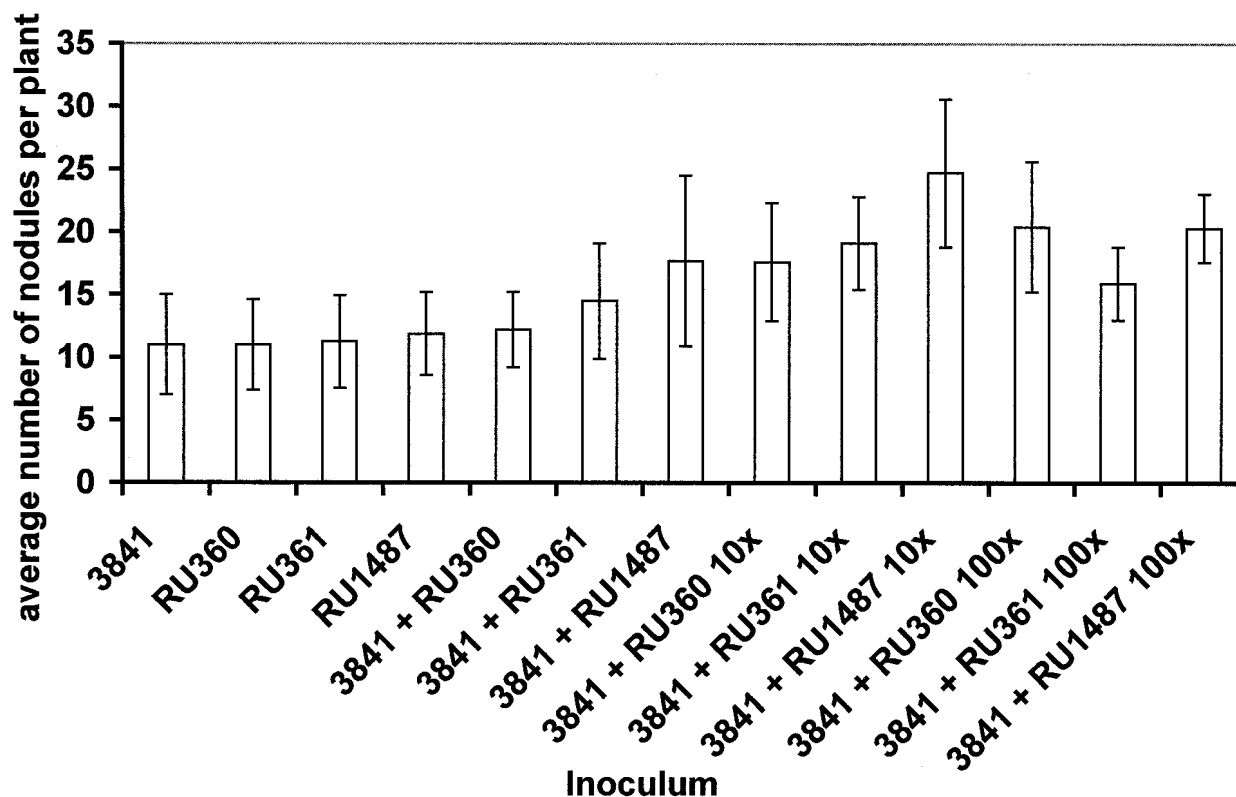
#### Wild type apparently displaces the mutant during development.

The effect of different inoculum levels on the total number of nodules formed on vetch was investigated (Fig 3). There were no significant differences between the number of nodules formed by 3841, RU1487, RU360, or RU361 when inoculated ( $10^3$  CFU) alone. When a 10-fold excess of mutant was added ( $10^4$  CFU) with wild type ( $10^3$  CFU), there was a statistically significant ( $P < 0.1$ ) increase in the total number

of nodules formed. In the case of RU360 and RU361 excess, this result is remarkable because the increased nodulation is in response to the presence of mutant bacteria that occupy few of the mature nodules. These results suggest that the plant is able to detect the presence of extra bacteria in the rhizosphere, perhaps as a result of elevated levels of lipochitooligosaccharides, which then elicit greater numbers of nodules. The absence of the mutants in mature nodules suggests that they have been displaced by the wild type at an early stage of nodule formation or during infection thread formation. It seems relevant that in all situations where mutants of RU360, RU361, or even RU1487 were recovered from nodules, there also was a high incidence of co-resident wild-type bacteria.

#### DISCUSSION

The data presented here indicate that mutants RU360, RU361, and RU1487 are able to nodulate pea and vetch at the same rate as 3841. Bacteroids formed by all the mutants fix nitrogen at the same rate, and the average plant dry weights are the same. The *myo*-inositol catabolic mutants RU360 and RU361, however, suffer a severe disadvantage in competition for nodulation when coinoculated with 3841. Strain RU1487, which is not a catabolic *myo*-inositol mutant, retains the ability to catabolize *myo*-inositol at a low rate and is only at a very slight competitive disadvantage when coinoculated with 3841. This suggests that there is a requirement to utilize *myo*-inositol in order to be competitive for nodulation. A key question is, at what stage does this competitive advantage manifest itself? The *ino* mutants RU360 and RU361 grew as fast as the



**Fig. 3.** Effect of inoculation level on nodulation frequency of vetch. Standard bacterial inoculum was  $10^3$  CFU. Higher inocula are indicated as 10x ( $10^4$  CFU) and 100x ( $10^5$  CFU). A minimum of six plants were harvested for each treatment. Results are shown plus and minus the standard deviation.

wild type in the rhizosphere and elicited nodule formation at a similar rate to that of the wild type (Fig 2). Plant nodulation also responded to the size of the mutant inoculum in the presence of wild type, suggesting that there is no problem with lipochitoooligosaccharide generation. What was notable, however, was that whereas the total number of nodules increased 10:1 ratio of mutant to wild type, the nodules contained wild-type bacteria and not the mutant. This indicates that the *ino* mutants are not competitive with the wild type during the early stages of nodule development and infection thread formation. This possibility also is supported by a high incidence of co-residence of the wild type with the mutant in any nodules that contained mutant bacteria. The apparent ability of the wild type to displace the mutant suggests that the wild type may grow slightly faster at a critical stage such as during infection thread development or, perhaps, at a particular developmental stage. One possibility that seems unlikely is that *myo*-inositol is the sole carbon for growth in the early stages of infection. This is because the transport mutant RU1487 is severely impaired for growth on *myo*-inositol (MGT 13.4 h compared with 3.4 h for the wild type), yet it is still moderately competitive. Overall, the poor growth of RU1487 on *myo*-inositol is compatible with its slight reduction in competitiveness, supporting the idea that *myo*-inositol has a real role in the early stages of nodule development and that the lack of competitiveness of the catabolic mutants is not simply the result of *myo*-inositol toxicity.

Mutants of *R. leguminosarum* bv. *trifolii* that are unable to catabolize rhamnose are at a severe disadvantage for clover nodulation, although this did not occur in sorbitol or adonitol mutants (Oresnik et al. 1998). Whereas the effect of rhamnose and *myo*-inositol may be specific for each biovar of *R. leguminosarum* and plant host, it is possible that multiple carbon sources might affect competition. For example, several different compounds may contribute to the growth rates of strains.

One simple possibility is that infection threads containing *myo*-inositol mutants become aborted, leading to a dominance of the wild type. Certainly, many more infection threads are formed than lead to nodule formation (Bauer 1981). The increased nodulation response of vetch plants when an excess of mutants are added with the wild type, however, indicates that the effects seen here are not so simple. The abortion of infection threads containing the mutant alone should not lead to more nodules; instead, the wild type appears to displace the mutant. In many ways, this study and that of Oresnik et al. (1998) highlights our lack of knowledge concerning early events in nodulation other than those concerned directly with flavonoid detection and lipochitoooligosaccharide synthesis.

## MATERIALS AND METHODS

### Bacterial strains and culture conditions.

Bacterial strains and plasmids used in this study are described in Table 4. *R. leguminosarum* strains were grown at 28°C on either TY (Beringer 1974) or AMS medium (pH 7.2) (Poole et al. 1994b). All carbon and nitrogen sources were at 10 mM concentrations unless otherwise stated. Antibiotics were used at the following concentrations (per ml): 40 µg of kanamycin; 500 µg of streptomycin; 2 µg of tetracycline in AMS medium and 5 µg in TY medium; and 20 µg of gentamicin. *E. coli* strains were grown at 37°C on Luria-Bertani medium, with antibiotic concentrations as follows (per ml): 25 µg of kanamycin; 10 µg of tetracycline; 5 µg of gentamicin; and 50 µg of ampicillin.

### DNA and genetic manipulations.

All routine DNA analysis was performed essentially as described by Sambrook et al. (1989). Transduction was performed with bacteriophage RL38, as described by Buchanan-Wollaston (1979). Tn5 was transduced from RU1487 into

**Table 4.** Bacterial strains and plasmids used

Strain or plasmid	Description	Source or reference
<i>Rhizobium leguminosarum</i> strains		
3841	Str <sup>r</sup> derivative of <i>R. leguminosarum</i> bv. <i>viciae</i> strain 300	Glenn et al. 1980
RU360	Strain 3841 <i>iolD</i> ::Tn5- <i>lacZ</i>	Poole et al. 1994a
RU361	Strain 3841 <i>iolA</i> ::Tn5- <i>lacZ</i>	Poole et al. 1994a
RU1487	Strain 3841 <i>intA</i> ::Tn5	This work
Plasmids		
pACYC184	Low-copy-number cloning vector; chloramphenicol and tetracycline resistant	New England Biolabs Beverly, MA, U.S.A.
pBluescript SK <sup>-</sup>	Phagemid; F <sub>1</sub> <sup>-</sup> origin of replication; <i>ColE1</i> replicon; ampicillin resistant.	Stratagene, La Jolla, CA, U.S.A.
PCR2.1 TOPO	T-overhang cloning vector; ampicillin and kanamycin resistant	Invitrogen, San Diego, CA, U.S.A.
pRU426	12.4-kb <i>EcoRI</i> fragment from RU1427 ( <i>intA</i> ) in pACYC184	This work
pRU438	11.5-kb <i>SalI</i> fragment containing all of Tn5- <i>lacZ</i> and part of <i>iolA</i> from RU361 in pBluescript SK <sup>-</sup>	This work
pRU472	9-kb <i>SstI</i> fragment containing 6 kb of Tn5- <i>lacZ</i> from RU360 in pBluescript SK <sup>-</sup>	This work
pRU476	1.2-kb <i>NotI</i> fragment containing IS50R of Tn5- <i>lacZ</i> and part of <i>iolA</i> from pRU438 in pBluescript SK <sup>-</sup>	This work
pRU706	2.5-kb polymerase chain reaction (PCR) product of <i>iolD</i> (primers p213 and p228) from pRU3078 cloned in pOT1	This work
pRU713	4-kb PCR product of <i>iolDEB</i> (primers p230 and p228) from pRU3078 cloned in pOT1	This work
pRU3078	pLAFR1 cosmid that complements RU360; tetracycline resistant	Poole et al. 1994a
pRU3079	Cosmid that overlaps pRU3078 and complements RU360	Poole et al. 1994a
pRU3111	pLAFR1 cosmid that complements RU361; tetracycline resistant	This work

3841. Eleven kanamycin-resistant transductants were tested for growth on AMA medium with 10 mM *myo*-inositol as the sole carbon source. Chromosomal DNA from strain RU1487 was digested with *EcoRI*, and a 7.9-kb fragment was cloned into Bluescript II SK<sup>-</sup> by selecting for kanamycin resistance. The left and right arms of transposon Tn5 were isolated by *Bam*HI and *Hind*III digestion, followed by self-ligation. Both arms were sequenced with a primer that binds to the ends of IS50 and SK and KS primers in the plasmid.

Primers p213 and p228 were used to amplify a 2,308-bp fragment containing the entire *iolD* gene. Primer p228 contains a *SpeI* site. This fragment was then cloned into plasmid pCR2.1 TOPO and then into pOT1 as an *XbaI*–*SpeI* fragment, generating pRU706. Primers p230 and p216 were used to amplify a 4,343-bp fragment containing the *iolDEB* genes, ending 38 bp beyond the end of *iolB*. This fragment was cloned into pCR2.1 TOPO and then into pOT1 with *PmeI* and *SpeI* restriction enzyme sites that were included in p230 and p216, respectively. The resulting plasmid was named pRU713.

Primers used in this study were p87, TTAAATCAAAA-CTGGTGAAGCTC; p89, GCTAAAATGGAGAAAAAAT-CACTGG; p113, AGGTCACATGGAAGTCAGATC; p114, ACGTTTCAGTTTGTCTCATGG; p115, AAAGACCGTAA-AGAAAAATAAGC; p120, TTGATTTACCAGAATATTTT-GCC; p213, TTTTTTTTCTGCAGCGAGCTGATTTCCCT-GCTTCG; p216, AAAAAAAAAGTACTTTTCGCGCTGTC-AGATTATT; p228, TTTTTTTTACTAGTGCGGAGGA-GCGGTGCCG; p230, TTTTTTTTGTAAACCGAGC-TGATTTCCCTGCTTCG.

All sequencing was performed by the cycle-sequencing method with an ALF automated DNA sequencer (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, U.K.). Genetics Computer Group software (Madison, WI, U.S.A.) and BLASTX analysis were used for computer-assisted sequence analysis. The EMBL accession nos. for *iolA* and *iolDEB* are AJ276297 and AJ276296, respectively.

### Plant assays.

Nodulation and acetylene reduction were determined with common vetch (*V. sativa*) or pea (*Pisum sativum*) plants. Plant growth and acetylene reductions were carried out as described previously (Poole et al. 1994a). For nodulation competition experiments, plants were grown in sterile vermiculite and watered with sterile nitrogen-free rooting solution, as described previously (Poole et al. 1994a). An inoculum of 10<sup>3</sup>, 10<sup>4</sup>, 10<sup>5</sup>, or 10<sup>6</sup> CFU was applied to each plant. Cell numbers were ascertained by a measurement of optical density (OD) at 600 nm and confirmed by plate counts. Plants were harvested 6 weeks postinoculation. To determine nodule occupancy, nodules were surface sterilized, as previously described (Poole et al. 1994a), then crushed and plated on TY medium and then on TY medium containing kanamycin. A minimum of six plants were harvested for each treatment. Samples also were purified from the original TY medium plates to check the resistance marker in individual bacteria and to determine their ability to grow on *myo*-inositol as the sole carbon source. Total bacterial growth in the rhizosphere was determined by harvesting plants at 2, 4, 6, and 8 days. Roots and total vermiculite were ground, and bacteria was serially diluted and plate counted on TY medium, giving the total number of viable rhizosphere and root-associated bacteria.

### Transport assays.

Cells were prepared for transport assays, as previously described (Poole et al. 1985). The total *myo*-inositol concentration was 25 μM, with 0.125 μCi of D[U-<sup>14</sup>C]*myo*-inositol (310 MBq per mmol) added. Samples of 0.1 ml were taken at intervals of 1 min, for up to 5 min. Millipore was filtered and scintillation counted. For competition assays, a fivefold excess (125 μM) of unlabeled solute was added 5 s prior to the addition of the *myo*-inositol.

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