Plasmid-Encoded Catabolic Genes in *Rhizobium leguminosarum* bv. *trifolii*: Evidence for a Plant-Inducible Rhamnose Locus Involved in Competition for Nodulation

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Cosmids carrying genes involved in utilization of rhamnose, sorbitol, and adonitol were isolated from a genomic library of *Rhizobium leguminosarum* by complementation of plasmid-cured derivatives of strain Rlt100 that were unable to grow on these carbon sources. Transposon mutagenesis was used to identify regions of each cosmid necessary for catabolism of the respective carbon source; partial DNA sequencing, as well as analysis of gene fusions created with transposon Tn5-B20, helped to determine the orientation and possible function of genes required for growth on the three substrates. Representative Tn5 insertions in the cosmids were recombined into the wild-type strain Rlt100 by gene replacement to generate isogenic strains unable to use either rhamnose, sorbitol, or adonitol. These strains were tested for their nodulation competitiveness compared with Rlt100 in co-inoculation experiments on clover plants. While sorbitol and adonitol catabolic mutants were unaltered in their competitive behavior, the nodulation competitiveness of three different rhamnose utilization mutants was significantly impaired. This result, coupled with the fact that the rhamnose catabolic genes were inducible by clover root extracts, suggests an important role for rhamnose catabolism in the early stages of the interaction of *R. leguminosarum* with clover plants. Hybridization studies with probes derived from the rhamnose, sorbitol, and adonitol catabolic loci demonstrated that these genes are plasmid encoded in virtually all *R. leguminosarum* strains, including representatives from all three biovars from a variety of different geographic locations.

Additional keyword: rhizosphere.

Rhizobium spp. are gram-negative bacteria that can exist as free-living soil bacteria or as nitrogen-fixing symbionts within

legume root nodules. The interaction of the bacteria and the host plant during nodulation occurs through a well-characterized process involving an exchange of signals (Long 1996; Dénarié et al. 1992). Considerably less is known, especially at the molecular level, about how the bacterium survives in the soil and root environment, and how individual strains achieve a symbiotic association by outcompeting other strains in the rhizosphere (Triplett and Sadowsky 1992; Vlasak and Vanderleyden 1997).

Rhizobium spp. typically contain between two and eight stable plasmids, ranging in size from 100 to 1,700 kb, which can constitute up to 40% of the bacterial genome (Honeycutt et al. 1993). These plasmids have been shown to carry genes necessary for nodulation and nitrogen fixation (Johnston et al. 1978; Hooykaas et al. 1981; Banfalvi et al. 1981; Rosenberg et al. 1981), and a variety of other genes including those involved in exopolysaccharide production (Hynes et al. 1986; Finan et al. 1986), thiamine biosynthesis (Finan et al. 1986), and C₄ dicarboxylate transport (Watson et al. 1988; Finan et al. 1988; Yarosh et al. 1989) in *Sinorhizobium meliloti*. Bacteriocin production and resistance (Hirsch 1979; Hirsch et al. 1980), a restriction and modification system (Rochepeau et al. 1997), and melanin production (Hynes et al. 1988) have all been reported on plasmids in *Rhizobium leguminosarum*. The catabolism of a variety of carbon compounds has been shown to be plasmid encoded for both *R. leguminosarum* and *S. meliloti* (Charles et al. 1990; Charles and Finan 1991; Baldani et al. 1992).

The presence of plasmids has been linked to competitive ability in rhizobia. Introduction of a cryptic plasmid from an *R. tropici* strain into an *R. etli* strain improved nodulation competitiveness of *R. etli* through an unknown mechanism (Martinez-Romero and Rosenblueth 1990). Plasmid-curing experiments in *R. leguminosarum* and *R. etli* resulted in strains that were significantly reduced in their ability to compete with the wild-type organism (Hynes 1990; Brom et al. 1992). When soil was co-inoculated with *R. leguminosarum* bv. *trifolii* W14-2 and derivatives cured of one of the four different plasmids, the wild type formed a population in the clover rhizosphere that was 10 times larger than that of the derivatives (Moënne-Loccoz and Weaver 1995). These data

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suggest that competitiveness may be influenced by the loss or gain of determinant(s) that allow the bacteria to be more vigorous in the rhizosphere. Since the plasmids tend to be large (>200 kb), and most probably code for a large variety of different functions, the challenge now is to determine which specific plasmid-encoded genes play a significant role in the rhizosphere and in competition for nodulation.

One area of interest focuses on genes involved in carbon catabolism. For example, the four plasmids of *R. leguminosarum* bv. *trifolii* W14-2 have a role in growth of the strain on rhamnose, sorbitol, adonitol, lactose, glycerol, and catechol (Baldani et al. 1992). It was hypothesized that some of these plasmid-encoded catabolic genes may play a role in rhizosphere and nodulation competitiveness. To test this hypothesis, the plasmid-encoded determinants for adonitol, sorbitol, and rhamnose were cloned and strains carrying mutations in each of these loci were tested against the isogenic wild-type strain with a nodulation competition assay.

RESULTS

Isolation of complementing cosmids.

Three cosmids, designated pLFWA (adonitol clone), pW3A (rhamnose clone), and pW3C (sorbitol clone), were isolated by complementation of Rlt101 and Rlt102 for growth on the respective carbon sources. Further complementation studies revealed that pW3C complemented for both sorbitol and rhamnose utilization in Rlt101. pW3A, however, did not complement Rlt101 for growth on sorbitol. Southern blot analysis suggested that pW3A and pW3C contain approximately 10 to 15 kb of common DNA (data not shown).

To identify the region(s) necessary for the complementation, Tn5 and Tn5B20 mutageneses were carried out on these cosmids. These mutageneses yielded two independent mutants unable to utilize adonitol (*rbt-1* and *rbt-2*), three independent mutants unable to utilize sorbitol (*srl-1*, *srl-2*, and *srl-3*), and seven independent mutants unable to use rhamnose (*rha-1*, *rha-2*, *rha-5*, *rha-12*, *rha-13*, *rha-14*, and *rha-44*). The insertions affecting rhamnose utilization were isolated in both pW3A and pW3C (Table 1).

Restriction mapping and Southern blot analysis of representative inserts in each of the loci allowed the construction of restriction maps and the determination of the putative direction of transcription of each of the catabolic regions. Inserts that gave an adonitol negative phenotype were found to be 3 kb apart, and on the basis of the transcriptional fusion *rbt-1*, a putative direction of transcription was assigned (Fig. 1A). Since both inserts *rbt-1* and *rbt-2* were unable to com-

plement Rlt102, and they were 3 kb apart, it was of interest to determine if both inserts were in the same transcriptional unit. To address this, complementation analysis was carried out by introducing pLFWA carrying *rbt-1* into Rlt103, which contained the allele *rbt-2*. The resultant construct was not complemented for growth on adonitol, suggesting that the locus defined by *rbt-1* and *rbt-2* consists of a single transcriptional unit.

The sorbitol region was found to span about 1 kb and the direction of transcription was inferred from partial sequence analysis and comparison to sequences obtained from data bases. Complementation experiments with Rlt104 (carrying *srl-3*) and pW3C carrying either *srl-1* or *srl-2* did not result in growth on sorbitol, suggesting that all the inserts are in a single transcriptional unit (Fig. 1B).

Mutations affecting rhamnose catabolism were found in two areas within a 10-kb region of DNA. This is based on the mapping of inserts in pW3A and pW3C that no longer allowed the deletion derivative Rlt101 to use rhamnose as a sole carbon source. Complementation experiments with Rlt105 and Rlt106 (carrying *rha-1* and *rha-2*, respectively) with rhamnose-negative inserts on pW3A and pW3C1 show that this region consists of at least two complementation groups (Table 1). In all cases the mutants were complemented by the cosmid pW3C, which contained the wild-type rhamnose region, showing that these were not dominant negative mutations (Table 1 and data not shown). The direction of transcription of the operons represented by these complementation groups was determined by the orientation of the Tn5B20 inserts that gave rise to *rha-1* and *rha-2* (Fig. 1C).

Putative identification of catabolic loci.

To identify the genes that may be involved in the catabolism of adonitol, rhamnose, and sorbitol, the DNA flanking the Tn5 and Tn5B20 inserts was sequenced with a primer specific for the IS50 elements in Tn5. In most cases, BLASTX analysis of the DNA sequences showed similarity to proteins involved in sugar transport or metabolism (Table 2). The DNA flanking *rbt-1*::Tn5B20 was analyzed and showed very high homology to *rbtD* from *Klebsiella pneumoniae* (Table 2). This gene codes for the enzyme ribitol 2-dehydrogenase, which is necessary for growth of *K. pneumoniae* on adonitol as a sole carbon source and is consistent with the observation that *rbt-1* affects adonitol utilization. BLASTX analysis of the DNA flanking regions to *rbt-2*::Tn5B20 did not give significant homologies to any sequenced region in the data base.

BLASTX analysis of the DNA sequences flanking *srl-1* and *srl-2* in the sorbitol catabolic locus gave high homologies to genes that encode various dehydrogenases as well as xylitol

Table 1. Complementation of rhamnose mutants^a

Strain	Relevant characteristics	Plasmid							
		pW3A <i>rha</i> ⁺	pW3C <i>rha</i> ⁺	pW3A1 <i>rha-1</i>	pW3R44 <i>rha-44</i>	pW3AR2 <i>rha-2</i>	pWCRIB <i>rha-12</i>	pW3CR4 <i>rha-13</i>	pW3CR5 <i>rha-14</i>
Rlt100	Wild type	+	+	+	+	+	+	+	+
Rlt101	<i>rha</i> ⁻	+	+	-	-	-	-	-	-
Rlt105	<i>rha-1</i>	+	+	-	-	+	+	+	+
Rlt107	<i>rha-5</i>	+	+	-	-	+	+	+	+
Rlt106	<i>rha-2</i>	+	+	+	+	-	-	-	-

^a Designations are as follows: +, like wild-type growth; -, no growth.

dehydrogenase and sorbitol dehydrogenases from bacteria and eukaryotes (Table 2 and data not shown). Similarly, sequence analysis of the DNA flanking several inserts in the rhamnose locus showed homology to genes for various ABC type transporters (Table 2). The DNA flanking *rha-1*::Tn5B20 showed very high homology with genes encoding dehydrogenases whereas the DNA flanking *rha-44*::Tn5B20, which was in the same complementation group as *rha-1* (Table 1), did not show significant homology to any sequences in the data base (Table 2).

Wild-type derivatives carrying representative Tn5 or Tn5B20 inserts from each of the catabolic regions were tested for carbon utilization to demonstrate that these mutations did not result in pleiotropic phenotypes affecting the catabolism of several sugars. In all cases, the mutants that were analyzed were shown to be specifically defective for growth on either adonitol, sorbitol, or rhamnose. Although the DNA flanking the inserts in the rhamnose locus showed homology to genes involved in arabinose and sorbitol utilization, mutations at this locus, when recombined into the wild type, were defective only in rhamnose utilization.

Table 2. Detected homologies for sequence flanking IS50_R of representative inserts

Insert ^a	Sequence (bases)	Best homology ^b	Statistical significance ^c
<i>rbt-1</i>	350	<i>Klebsiella pneumoniae</i> ribitol 2-dehydrogenase	2.8×10^{-39}
<i>rbt-2</i>	300	ND ^d	—
<i>srl-1</i>	250	<i>Morganella morganii</i> 2-xylitol dehydrogenase	2.7×10^{-29}
<i>srl-2</i>	250	<i>M. morganii</i> 2-xylitol dehydrogenase	2.7×10^{-29}
<i>srl-5</i>	250	ND	—
<i>rha-1</i>	251	<i>Escherichia coli</i> sorbitol 6 phosphate 2 dehydrogenase	4.4×10^{-9}
<i>rha-44</i>	245	ND	—
<i>rha-12</i>	347	<i>E. coli</i> ribose transport ATP binding protein (RbsA)	3.6×10^{-27}
<i>rha-13</i>	135	<i>E. coli</i> ribose transport ATP binding protein (RbsA)	1×10^{-4}
<i>rha-14</i>	345	<i>E. coli</i> L-arabinose transport ATP binding protein (AraG)	1.3×10^{-20}
<i>rha-2</i>	300	<i>E. coli</i> L-arabinose transport (AraH)	3.8×10^{-17}

^a Inserts are defined in Table 5.

^b From a search of nonredundant protein data base with BLASTX.

^c The possibility of finding the homology by chance is shown.

^d No homology detected.

Fig. 1. Restriction maps of (A) adonitol, (B) sorbitol, and (C) rhamnose regions in *Rhizobium leguminosarum* strain Rlt100. Tn5 insertions are represented by vertical lines; Tn5B20 inserts by vertical lines with arrowheads. Direction of arrowheads indicates orientation of the *lacZ* on Tn5B20. Filled arrowheads indicate transcriptional fusions. Heavy broken horizontal line with arrow below the map indicates putative direction of transcription of genes or operons. Lighter broken line below represents regions used as probes. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; S, *Sal*I.

Adonitol and sorbitol utilization are plasmid-encoded properties in many wild-type *R. leguminosarum* strains.

To ensure that each cosmid clone corresponded to the appropriate plasmid in Rlt100, the DNA flanking some of the Tn5 inserts or a wild-type fragment that contained the flanking regions were cloned and used as probes (Fig. 1). These were used to probe Southern blotted Eckhardt gels containing diverse *R. leguminosarum* wild types.

The sorbitol probe hybridized to the "c" plasmid (pRleW14-2c) of Rlt100, showing that the DNA that complemented the

rhamnose and sorbitol deficiencies in Rlt101 was from the "c" plasmid. Similarly, the adonitol probe hybridized to the "b" plasmid (pRleW14-2b) of Rlt100 (Fig. 2B, lane K). The adonitol probe also hybridized to a single band in Rlt100 and VF39SM genomic DNA.

Southern analysis of Eckhardt gels containing various *R. leguminosarum* wild-type and lab strains demonstrated that in all cases the adonitol probe hybridized to plasmid DNA (Fig. 2). Similarly, the sorbitol and rhamnose probes also hybridized to plasmid DNA (Fig. 2C). Moreover, in all strains tested

Fig. 2. Eckhardt gel, Southern analysis, and schematic representation of sorbitol, adonitol, and rhamnose regions in various wild-type *Rhizobium leguminosarum* strains. **A**, Ethidium bromide-stained Eckhardt gel. **B**, Representative Southern blot of the Eckhardt gel in **A** with a probe from adonitol region. **C**, Schematic representation of an Eckhardt gel showing plasmid bands that hybridized with sorbitol (asterisk) or adonitol (black dot) probe. **A** and **B**: Lane A, T3CF; lane B, T3CB; lane C, 3841; lane D, VF39SM; lane E, GF160; lane F, 336; lane G, 162Y10; lane H, 3855; lane I, B151; lane J, 248; lane K, Rlt100; lane L, P9; lane M, P7; lane N, P4; lane O, P1. **C**: Lane A, T3CF; lane B, T3CB; lane C, 3841; lane D, VF39SM; lane E, GF160; lane F, 336; lane G, 162Y10; lane H, 3855; lane I, B151; lane J, 248; lane K, Rlt100; lane L, 306; lane M, 309; lane N, P9; lane O, P7; lane P, P4; lane Q, P1.

it appeared that loci corresponding to the rhamnose and sorbitol probes were on the same plasmid whereas the probe for the adonitol region hybridized to separate plasmids. A single exception was with strain P4, in which case it was not possible to get strong hybridization, and we were therefore unable to show plasmid localization unambiguously.

Additional adonitol region is present in VF39SM.

An attempt was made to introduce insert *rht-1::Tn5B20* into wild-type strain VF39SM with pJQ200SK. However, no adonitol mutants were isolated by this strategy. Southern analysis showed that many of the Nm^r colonies arose via transposition. Interestingly, however, it was noted that although the adonitol probe only hybridized to pRleVF39d, strains cured of this plasmid could still grow on adonitol. VF39SM derivatives cured of both pRleVF39c and pRleVF39d, however, could not grow on adonitol, suggesting the presence of a second, non-homologous locus on pRleVF39c involved in adonitol catabolism, which is obviously absent in Rlt100.

Adonitol and rhamnose loci are inducible.

To determine if the rhamnose and adonitol loci were inducible, cosmids carrying *lacZ* fusions to either the putative rhamnose or adonitol catabolic genes were introduced into the wild-type Rlt100 and grown in defined media in the presence or absence of rhamnose or adonitol. Fusions to both the adonitol and rhamnose loci were induced by the presence of the carbon source for which they were specific (Table 3). The adonitol fusion *rht-1* showed an 11-fold induction whereas the rhamnose fusion, *rha-5*, was induced threefold. To get a better representation of rhamnose regulation, short-term induction assays were carried out with strain Rlt107, which was derived by introduction into Rlt100 of *rha-5::Tn5B20* by gene replacement. When present in single copy, fusion *rha-5* was induced fourfold by rhamnose (Table 3).

Rhamnose locus is induced by rhamnose and root exudate.

To examine other factors that might potentially regulate the rhamnose locus, more extensive β -galactosidase assays were carried out with the *rha-5* allele insert. Cultures of Rlt107

were induced for 3 h with a variety of carbon sources (Table 4). In these experiments, the *rha-5* fusion was found to be induced approximately sixfold by addition of rhamnose. The addition of equimolar amounts of rhamnose and glucose, or glucose alone, gave values that were similar to the basal activity assayed with glycerol as sole C-source. The addition of equimolar concentrations of succinate and rhamnose, however, did not repress transcriptional activity of the *rha-5* fusion, and, in fact, addition of succinate as a sole carbon source also showed levels of *rha-5* activity greater than in glucose-treated cells (Table 4).

From nodule competition assays it was clear that mutants in rhamnose catabolism were less competitive than the wild type (see below). To test whether this region was induced in the rhizosphere, fusions in the rhamnose region were tested by adding clover root exudate (1/10 final volume of culture) and incubating for 3 h. The *rha-5* fusion was induced approximately threefold over this time period (Table 4). Under similar conditions Rlt100 carrying the *rha-5* allele on a plasmid was also induced by root exudate whereas the Rlt100 carrying the *rht-1* allele did not show any activity over background (data not shown). This demonstrates that some compound(s) that are exuded from the plant affect the expression of the rhamnose locus in Rlt105.

Mutants unable to use rhamnose are noncompetitive with respect to nodulation.

To carry out nodule competition assays, *rht-2*, *srl-1*, and *rha-1* were originally selected to represent the adonitol, sorbitol, and rhamnose regions, respectively. These inserts were recombined by gene replacement into the wild-type strain and the proper location of the insert was verified by catabolic phenotype and by Southern blot analysis (data not shown).

To assess whether the plasmid-encoded adonitol, sorbitol, or rhamnose loci affected nodule occupation, it was first determined that these mutations did not affect the development of nodules. Strains Rlt103, Rlt104, Rlt105, and Rlt106 each formed Fix⁺ nodules on clover that were visually indistinguishable from those inoculated with the wild type. Nodule numbers on plants inoculated with mutants were also not significantly different.

To determine if these mutants affected the ability of the strain to compete against the wild type in forming effective nodules, the mutant strains were co-inoculated with the wild-type strains in approximately equal numbers (Fig. 3). The results clearly showed that mutations in the adonitol locus, rep-

Table 3. Induction of rhamnose and adonitol *lacZ* fusions^a

Strain	Carbon added ^b			Fold induction ^c
	Glycerol	Glycerol + adonitol	Glycerol + rhamnose	
Rlt100 ^d	2 ± 1	2 ± 2	2 ± 1	–
Rlt100/pLFWA5	150 ± 20	1667 ± 350	NT ^e	11
Rlt100/pW3AR5	75 ± 9	NT	243 ± 13	3
Rlt107 ^f	138 ± 20	NT	506 ± 36	4

^a β -Galactosidase activity was assayed as described in Materials and Methods; the data are expressed as Miller units (Miller 1972). Induction time was 16 h except where otherwise noted. In each case, the data represent mean values ± SE from three independent cultures. Each experiment was performed at least two times.

^b Carbon concentrations are as follows: glycerol, 7.5 mM, adonitol, 15 mM; rhamnose, 15mM.

^c Fold induction was calculated by dividing glycerol + adonitol or glycerol + rhamnose activity by glycerol activity alone.

^d Values for Rlt100 with either 16-h or 3-h induction times were identical.

^e Not tested.

^f These values are from a 3-h induction.

Table 4. Expression of *rha-5::Tn5B20* in Rlt107^a

Additions to media	Strains	
	Rlt100	Rlt107
Glycerol	1 ± 1	108 ± 5
Glucose	2 ± 1	165 ± 14
Succinate	3 ± 1	222 ± 27
Rhamnose	2 ± 1	615 ± 36
Rhamnose + glucose	0	112 ± 15
Rhamnose + succinate	0	409 ± 27
Root exudate	2 ± 1	314 ± 14

^a β -Galactosidase activity expressed as described in Materials and Methods; data are expressed as Miller units (Miller 1972). Induction time was 3 h. In each case, data represent mean values ± SE of three independent cultures.

represented by strain Rlt103, and the sorbitol locus, represented by strain Rlt104, did not affect the strain's ability to compete against the wild type for nodule occupancy. This, however, was not the case with the representative rhamnose mutant, Rlt105. In this case, the mutant was significantly outcompeted by the wild type such that nodules containing Rlt105 made up less than 10% of the total nodule population (75 to 100 nodules were isolated per experiment), and in some experiments this value was lower (Fig. 3).

To determine what inoculum ratio of Rlt105/Rlt100 was necessary to achieve a 1:1 nodule occupancy ratio, inocula containing 10-fold and 100-fold excess of Rlt105 were used. It was found that an inoculum containing 10-fold excess of Rlt105 was necessary to achieve a nodule occupancy ratio (wild type/mutant) of 2.1 ± 0.4 ($n = 3$) whereas an inoculum containing 100-fold excess of Rlt105 resulted in a nodule occupancy ratio of 0.6 ± 0.1 ($n = 3$).

Since complementation, mapping, and sequencing data suggested that the rhamnose locus contained at least 2 transcriptional units that are separated by at least 4 kb, the *rha-2* and *rha-5* alleles were also recombined into Rlt100 and tested in a similar manner (Fig. 3). The data were consistent with the results with Rlt105, as both of these mutants were significantly less competitive than the wild-type strain (Fig. 3). This suggests that the entire region may be necessary for competition as assayed by nodule occupancy.

Attempts to complement this nodule competition phenotype on plants by introducing pW3C, which carries the wild-type rhamnose region, into either Rlt105 or Rlt106, and competing the complemented transconjugants against the wild type were unsuccessful. The data from these experiments showed that the transconjugants lost the complementing cosmid so frequently that interpretation of the data was impractical (data not shown).

Fig. 3. Nodule competition assays of catabolic mutants. Wild-type strain Rlt100 was inoculated onto clover plants in an approximately 1:1 ratio with isogenic strains carrying mutations in catabolic genes as indicated in X axis label. Numbers are expressed as number of wild type divided by number of mutant. Open bars = inoculation ratio as determined by plate counts of inoculum; solid bars = ratio of nodules occupied by each strain as determined by bacterial isolation from nodules. Experiments were performed at least three times for each strain; data are presented as the mean $\pm$ SE. When not present, error bars were too small to be represented. Asterisk = significant difference between inoculation ratio and isolation ratio with chi square test at $>95\%$ confidence interval. ($n = 5$ for Rlt105 and Rlt106; $n = 3$ for Rlt107).

Under normal laboratory conditions (on agar plates or in broth cultures) the rhamnose mutants grew at the same rate and to the same culture density as the wild type. To determine if the competition phenotype was simply due to slow growth under the conditions used for the nodulation experiments, Rlt105 was tested for the ability to proliferate under these experimental conditions. Since the nodule occupancy phenotype was also demonstrated on plant nutrient agar (data not shown), and it is easier to maintain homogeneous conditions, it was decided to use plant nutrient agar to carry out colonization experiments. Overnight cultures of Rlt105 or Rlt100 were diluted to approximately 100 CFU and were inoculated onto seedlings grown on plant nutrient agar. After 4 days (92 h) seedlings were removed, placed in microfuge tubes, vortexed (1 min), sonicated to remove tightly bound bacteria (10 min in sonicating water bath), and plated to determine the total population size. Under these conditions Rlt105 attained a population size not significantly different from that of the wild type, Rlt100, both in the media surrounding the root and on the root itself (data not shown). In co-inoculation experiments, no evidence indicated that the proportion of Rlt100/Rlt105 found on the root or in the growing media exceeded the proportions that were in the original inoculum (data not shown). Together these data suggest mutations in the rhamnose region do not result in growth defects under the conditions used for the nodule competition experiments.

DISCUSSION

The presence of plasmid-encoded loci for the catabolism of adonitol, sorbitol, and rhamnose in strain Rlt100 had been indicated by systematic plasmid-curing experiments (Baldani et al. 1992). However, the organization, nature, and biological role of these genes remained unstudied. In order to determine the role of individual catabolic genes in competition, it was necessary to clone these genes, mutate them, and analyze the behavior of isogenic strains carrying mutations at these loci in nodule competition assays.

Based on the mapping of inserts *rht-2* and *rht-1*, the adonitol locus was found to span at least 3 kb. The DNA sequence flanking the IS50 element of insert *rht-1* yielded high homology to the ribitol dehydrogenase gene from *Klebsiella aerogenes* (Charnetzky and Mortlock 1974). Sequence analysis of the DNA flanking the IS50 insert *rht-2*, however, did not yield a significant match to any sequences in the data base. Complementation analysis indicated that both inserts are in a single transcriptional unit. Based on the size of this region, the complementation data, and the differing results from the data base searches, it appears that this locus may contain several genes, although not all are necessarily involved in the catabolism of adonitol. In addition we found that all *R. leguminosarum* strains probed with our probe from the adonitol catabolism locus carried a homologous locus on a plasmid. In strain VF39SM, however, an additional plasmid-encoded locus is also involved in adonitol catabolism.

The sorbitol locus extends approximately 2 kb based on restriction mapping and partial sequencing from the IS50 elements of various inserts (Fig. 1B). Based strictly on sequence analysis of the DNA flanking the inserts that gave sorbitol negative phenotypes we were unable to ascribe potential biochemical function to all the inserts, but all the inserts appear

to be within one complementation group. Inserts *srl-1* and *srl-2* both gave very high homologies to sorbitol dehydrogenases from a variety of sources including gram-negative and gram-positive bacteria as well as eukaryotic organisms. Together with the phenotypic data that show insert *srl-5* to be specific for sorbitol catabolism, this strongly suggests that this locus encodes a sorbitol dehydrogenase as well as other genes that may be involved in the catabolism of sorbitol (Fig. 1B).

The rhamnose locus located on the "c" plasmid of Rlt100 appears to be quite large and to contain multiple genes and transcriptional start sites. By complementation analysis and restriction mapping we have been able to map the rhamnose negative inserts to a 10-kb region, and divide them into at least two putative complementation groups. By sequencing the DNA flanking some of the rhamnose negative inserts, we have been able to deduce that this locus encodes genes for a putative dehydrogenase as well as a putative ABC type transport system. The growth of *R. leguminosarum* Rlt100 on a defined medium containing rhamnose or succinate led to the induction of transcription whereas expression was repressed by the addition of glucose. Moreover, fusions *rha-1* and *rha-5* were induced by root exudate. At present it is not known whether the induction of these fusions by root exudate is due to rhamnose or whether it may be due to some other compound(s) found in the root exudate.

Mutants unable to catabolize adonitol or sorbitol catabolism were not affected in their competitive ability (Fig. 3). In contrast, mutants unable to catabolize rhamnose were compromised in their ability to compete for nodule occupancy (Fig. 3). It is clear, however, that the rhamnose mutants do not appear to be pleiotropic with respect to carbon source utilization, nor do they appear to be impaired with respect to their capacity to nodulate or fix nitrogen. Consistent with the inability of the *rha* mutants to compete for nodule occupancy, *rha* gene fusions were induced by root exudate. Together, these data suggest that the ability to utilize or degrade rhamnose must play a role either in the rhizosphere or perhaps during infection.

Interestingly, however, Rlt100 derivatives carrying deletions in the "c" plasmid (contains the rhamnose catabolic region), or mutations in the rhamnose region present on the "c" plasmid, appear to grow at the same rate and to the same population density as the wild type both in the root environment containing clover seedlings (Moënné-Loccoz and Weaver 1995) and on roots of the seedling itself (data not shown). Moreover, when the "c" plasmid deletion derivative was inoculated with the wild type in a 1:1 ratio onto clover seedlings planted in sand, the final population sizes were very similar (Moënné-Loccoz and Weaver 1995). These data suggest that the utilization of rhamnose does not bring about changes in the total population size in the rhizosphere environment and their uncompetitive nodulation phenotype is not due to a growth phenotype. An alternative or complementary hypothesis that may account for the inability for rhamnose catabolic mutants to compete effectively would be that rhamnose is important during infection thread initiation or development.

Quantitation of infection threads clearly shows that many more infection threads are formed than go on to make nodules (Bauer 1981). How the plant controls which infection threads result in nodules is at present poorly understood. Ultrastruc-

tural studies have shown that the host cell wall is continuous with the infection thread (Newcomb 1981). Rhamnose is commonly found as a component of dicotyledonous plant cell walls in the polysaccharide rhamnogalacturonan (McNeil et al. 1984). If rhamnogalacturonan is a component of the infection thread wall, then it would be reasonable to expect rhamnogalacturonan subunits or degradation products to be present in the infection thread. The inability of a strain to utilize these sources of carbon may result in a strain that grows more slowly during infection thread development. If the mechanism of autoregulation is dependent upon the stage of nodule/infection thread development then it might be expected that a disproportionate number of infection threads formed by rhamnose catabolic mutants might be aborted, thus leading to a situation in which greater than 90% of the nodules would be occupied by the wild type. At present our data do not allow us to distinguish between these various possibilities.

The soil environment is generally nutrient limiting. The present study was undertaken to investigate the hypothesis that plasmid-encoded catabolic genes play a role in determining nodule competition. Our initial goal was not to characterize these loci completely but to identify loci that may be important either in the rhizosphere or during early stages of nodulation. Our data clearly show that catabolic genes can play a role in nodule competition. The work also demonstrates that some catabolic loci do not have a role in nodule competition when assessed with a nodule occupancy assay as a measure of strain competitiveness. We are currently focusing our work on the role of rhamnose catabolism with respect to rhizosphere competition as well as undertaking a detailed molecular and biochemical characterization of this locus to better understand why rhamnose catabolism is important in the rhizosphere.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

Bacterial strains and plasmids used in this work are listed in Table 5. *R. leguminosarum* strains were grown at 28°C on TY as a complex medium (Beringer 1974) and VMM (Vincent 1970) as a defined medium. Carbon sources were added to defined media to a final concentration of 15 mM or 0.4% (wt/vol). For *R. leguminosarum* cultures antibiotics were added at the following concentrations to solid or liquid media: tetracycline (Tc), 5 µg ml⁻¹; neomycin (Nm), 100 µg ml⁻¹; streptomycin (Sm), 600 µg ml⁻¹; gentamicin (Gm), 30 µg ml⁻¹. For *Escherichia coli* cultures antibiotics were added at the following concentrations to solid or liquid media: ampicillin (Ap), 100 µg ml⁻¹; Tc, 10 µg ml⁻¹; kanamycin (Km); 50 µg ml⁻¹; Gm, 15 µg ml⁻¹. For solid media, agar was added to a final concentration of 1.25% (wt/vol).

DNA manipulation, cosmid bank construction, and Southern blot analysis.

Standard techniques were used for plasmid isolation, restriction enzyme digestion, and agarose gel electrophoresis (Sambrook et al. 1989). For Southern analysis and cosmid bank construction, genomic DNA was isolated by a slightly modified version of the protocol outlined by Meade et al. (1982), as has been previously described (Hynes et al. 1986). A cosmid library of strain Rlt100 was constructed with

Table 5. Bacterial strains, plasmids, and bacteriophage^a

Strain, plasmid, or phage	Genotype or phenotype	Reference or source
<i>Rhizobium leguminosarum</i>		
Rlt100	= W14-2, <i>R. leguminosarum</i> bv. <i>trifolii</i> Sm ^r wild type	Baldani et al. 1992
Rlt101	= W14-2 b, cured of plasmid a and d, deletion in plasmid c	Moënne-Loccoz et al. 1995
Rlt102	= W14-2 c, W14-2 derivative cured of plasmids a, b, and d.	Moënne-Loccoz et al. 1995
Rlt103	Rlt100 <i>rbt-2</i> ::Tn5B20, unable to grow on adonitol as sole carbon source	This work
Rlt104	Rlt100 <i>srl-1</i> ::Tn5B20, unable to grow on sorbitol as sole carbon source	This work
Rlt105	Rlt100 <i>rha-1</i> ::Tn5B20, unable to grow on rhamnose as sole carbon source	This work
Rlt106	Rlt100 <i>rha-2</i> ::Tn5B20, unable to grow on rhamnose as sole carbon source	This work
Rlt107	Rlt100 <i>rha-5</i> ::Tn5B20, unable to grow on rhamnose as sole carbon source	This work
248	<i>R. leguminosarum</i> bv. <i>viciae</i>	Hirsch 1979
306	<i>R. leguminosarum</i> bv. <i>viciae</i>	Hirsch 1979
309	<i>R. leguminosarum</i> bv. <i>viciae</i>	Hirsch 1979
336	<i>R. leguminosarum</i> bv. <i>viciae</i>	Hirsch 1979
3841	<i>R. leguminosarum</i> bv. <i>viciae</i> Sm ^r derivative of RI300	Poole et al. 1994
GF160	<i>R. leguminosarum</i> bv. <i>viciae</i> Sm ^r isolate	Finan et al. 1981
VF39SM	<i>R. leguminosarum</i> bv. <i>viciae</i> Sm ^r isolate	Hynes et al. 1988
162Y10	<i>R. leguminosarum</i> bv. <i>trifolii</i> Sm ^r isolate	Moënne-Loccoz et al. 1994
3855	<i>R. leguminosarum</i> bv. <i>viciae</i>	Brewin et al. 1982
T3CF	T3 cured of plasmids f, b, and pJB5JI	Hynes et al. 1989
T3CB	T3 cured of plasmids b and pJB5JI	Hynes et al. 1989
B151	3855 cured of pSym	Brewin et al. 1983
P1	<i>R. leguminosarum</i> bv. <i>viciae</i>	S. Alberta pea nodule isolate
P4	<i>R. leguminosarum</i> bv. <i>viciae</i>	S. Alberta pea nodule isolate
P7	<i>R. leguminosarum</i> bv. <i>viciae</i>	S. Alberta pea nodule isolate
P9	<i>R. leguminosarum</i> bv. <i>viciae</i>	S. Alberta pea nodule isolate
<i>Escherichia coli</i>		
MM294a	<i>pro-82 thi-1 hsdR17 supE44</i>	Finan et al. 1986
S17-1	<i>recA</i> derivative of MM294A with RP4-2 (Tc::Mu-Km::Tn 7) integrated into the chromosome, Sm ^r , Sp ^r	Simon et al. 1983
MT607	MM294A <i>recA56</i>	Finan et al. 1986.
MT614	MT607 with a Tn5 insert in the chromosome	
MT616	MT607(pRK600)	Finan et al. 1986
DH10B	F ⁻ , <i>mcrA</i> Δ(<i>mrr-hsdRMSmcrcBC</i>) φ80dlacZΔM15 Δ <i>lacX74 endA1 recA1 doeRA</i> (<i>ara, leu</i>)7697 <i>araD139 galU galK nupG rpsL</i>	BRL, Gaithersburg, MD
DH5α	<i>endA hsdR17 supE44 thi-1 recA1 gyrA96 relA1</i> Δ(<i>argF-lacZYA</i>)U169 φ80dlacZΔM15	BRL
Plasmids		
pUC19	Cloning vector, ColE1 <i>oriV</i> , Ap ^r	Yanisch-Perron et al. 1985
pBlueScript SK ⁺	Cloning vector, ColE1 <i>oriV</i> , Ap ^r	Stratagene, La Jolla, CA
pJQ200SK	Suicide vector for homologization, P15a <i>ori, mob, sacB</i> , Gm ^r , P15a <i>ori, mob, sacB</i> , Gm ^r	Quandt and Hynes 1993
pRK600	pRK2013 <i>npt</i> ::Tn9 Cm ^r Nm-Km ^r	Finan et al. 1986
pRK7813	RK2 derivative carrying pUC9 polylinker and lambda <i>cos</i> site, Tc ^r	Jones and Guttererson 1987
pPH1JI	IncP plasmid, Gm ^r	Beringer et al. 1978.
pW3C	pRK7813 cosmid from Rlt100 wild-type cosmid bank, complements Rlt101 for rhamnose and sorbitol utilization	This work
pW3A	pRK7813 cosmid from Rlt100 wild-type cosmid bank, complements Rlt101 for rhamnose utilization	This work
pLFWA	pRK7813 cosmid from Rlt100 wild-type cosmid bank, complements Rlt102 for adonitol utilization	This work
pLFWA5	pLFWA <i>rbt-1</i> ::Tn5B20, unable to complement Rlt102 for adonitol utilization	This work
pLFWA66	pLFWA <i>rbt-2</i> ::Tn5B20, unable to complement Rlt102 for adonitol utilization	This work
pW3CS1	pW3C <i>srl-1</i> ::Tn5, unable to complement Rlt101 for sorbitol utilization	This work
pW3CS2	pW3C <i>srl-2</i> ::Tn5, unable to complement Rlt101 for sorbitol utilization	This work
pW3CS5	pW3C <i>srl-4</i> ::Tn5, unable to complement Rlt101 for sorbitol utilization	This work
pW3AR1	pW3A <i>rha-1</i> ::Tn5B20, unable to complement Rlt101 for rhamnose utilization	This work
pW3AR2	pW3A <i>rha-2</i> ::Tn5B20, unable to complement Rlt101 for rhamnose utilization	This work
pW3AR5	pW3A <i>rha-5</i> ::Tn5B20, unable to complement Rlt101 for rhamnose utilization	This work
pW3AR44	pW3A <i>rha-44</i> ::Tn5B20, unable to complement Rlt101 for rhamnose utilization	This work
pW3CR1B	pW3C insert <i>rha-12</i> ::Tn5, unable to complement Rlt101 for rhamnose utilization	This work
pW3CR3A	pW3C insert <i>rha-13</i> ::Tn5, unable to complement Rlt101 for rhamnose utilization	This work
pW3CR5A	pW3C insert <i>rha-14</i> ::Tn5, unable to complement Rlt101 for rhamnose utilization	This work
Transposons		
Tn5	Nm ^r , Sm ^r	Berg and Berg 1986
Tn5B20	Nm-Km ^r , promoterless <i>lacZ</i>	Simon et al. 1989
Bacteriophage		
λ221cI857Pam80	Contains an integrated Tn5B20 used for transposon mutagenesis in S17-1	Simon et al. 1989

^a Abbreviations for antibiotics are as follows: Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Nm, neomycin; Sm, streptomycin; Tc, tetracycline.

genomic DNA that was partially digested with *Sau3AI*, and then was fractionated on a low melting point agarose gel; fractions corresponding to sizes between 30 and 40 kb were subsequently isolated. This DNA was ligated to the cosmid vector pRK7813, which had previously been restricted with *Bam*HI and dephosphorylated. Lambda packaging was accomplished with a commercially prepared packaging mix (Promega, Madison, WI), by the protocol specified by the manufacturer.

Genomic DNA for Southern hybridizations was restricted to completion and electrophoresed through a 0.8% agarose gel with 1× Tris-borate-EDTA (TBE) buffer. DNA was transferred onto nylon membranes (Boehringer Mannheim, Laval, Quebec) overnight by alkaline transfer (Sambrook et al. 1989), neutralized with 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and hybridized overnight with digoxigenin-labeled probes. Unless otherwise stated, hybridizations were done at 67°C overnight and washed 2 × 5 min with 2× SSC, 0.1% sodium dodecyl sulfate (SDS) at room temperature, 2 × 15 min at 67°C with 0.1% SSC, 0.1% SDS. Detection was done with CSPD (Boehringer Mannheim) reagent as specified by the manufacturer.

Probes were constructed by subcloning DNA that was determined to be flanking Tn5 inserts in each of the catabolic regions. The fragments used as probes were gel isolated and labeled with digoxigenin (Boehringer Mannheim) as suggested by the manufacturer. Fragments used are shown in Figure 1.

DNA sequencing and sequence analysis.

DNA flanking Tn5 and Tn5B20 inserts was sequenced by subcloning inserts into pBlueScript with a restriction enzyme that did not cut within the transposon (*Sa*I for Tn5B20) or by recloning the IS50R portion of the transposon and flanking region for Tn5 (with *Bam*HI and *Eco*RI and selecting Km^r and Ap^r). T3 and T7 primers were used to sequence the ends of the subclones and an IS50 primer (5' TAGGAGGTCACATGGA AGTCAGAT 3') was used to sequence the DNA flanking the IS50. Sequencing reactions were done with dye terminators and detected at the University of Calgary Core DNA Services with an ABI automated sequencer.

Sequence data were analyzed with DNASIS (Hitachi Software Engineering, San Bruno, CA) and data base searches were done with the BLASTX program (Altschul et al. 1990).

Genetic manipulations.

To isolate cosmids capable of complementing cured strains for growth on sorbitol, rhamnose and adonitol, the cosmid library was conjugated into either Rlt101 (unable to utilize rhamnose or sorbitol) or Rlt102 (unable to utilize adonitol). Putative complementing cosmids were conjugated back into *E. coli*, retested for complementation, and analyzed by restriction.

Tn5B20 mutagenesis experiments were carried out by infecting *E. coli* S17-1 containing the cosmid (Tc^r) to be mutagenized with lambda mutant λ221cI857Pam80 containing Tn5B20 (Km^r) (Simon et al. 1989). Km^r and Tc^r colonies were subsequently mobilized with pRK600 into either Rlt101 (Sm^r) (for sorbitol and rhamnose) or Rlt102 (for adonitol). Sm^r, Nm^r, and Tc^r colonies were subsequently screened for loss of complementation on the appropriate carbon source.

Cosmids containing inserts of interest were mobilized back into *E. coli* for further analysis. Tn5 mutagenesis was carried out essentially as described by Yarosh et al. (1989).

Gene replacement experiments were accomplished either by the use of pJQ200SK (Quandt and Hynes 1993) or by the use of the incompatible plasmid pPH1JI (Beringer et al. 1978) essentially as described by Ruvkun and Ausubel (1981). Correct replacement of the wild-type gene by the Tn5 mutated one was confirmed by Southern blot analysis.

β-Galactosidase activity.

Rhizobium spp. cultures containing active fusions were assayed by either an overnight or a 3-h induction. Overnight induction consisted of growing cultures overnight in TY and using this culture to inoculate a fresh broth culture of VMM containing the appropriate carbon sources. Cells were grown overnight in VMM broth and were assayed as described by Miller (1972). Three-hour inductions were carried out by growing overnight cultures in TY, washing the cells once with VMM, and resuspending the cells in VMM. Appropriate carbon sources were added and the cultures were incubated for 3 h at 28°C. Cultures were assayed in triplicate as described by Miller (1972) and β-galactosidase activity from fusions was shown to be linear over the duration of the assay (data not shown). In some experiments, lysed cell debris was pelleted, and the cleared supernatants were used to minimize light scattering by the cells. Root exudate was prepared essentially as described by Mulligan and Long (1985) and added to 1/10 final volume.

Modified Eckhardt gel electrophoresis.

To verify plasmid contents of *R. leguminosarum* strains, a modification of the Eckhardt (1978) technique designed for horizontal gels was used as previously described (Hynes and McGregor 1990).

Plant tests.

Plant tests were carried out by the methods of Hynes et al. (1988). Briefly, seeds (*Trifolium repens* cv. Ladino) were surface sterilized by 5 min of 70% ethanol treatment followed by 10 min in a 1/5 dilution of hypochlorite (final concentration 1%, vol/vol). Seeds were then rinsed in at least four changes of sterile, distilled, deionized water. Seeds were germinated on half-strength TY and only those that did not have any bacterial growth were aseptically planted into sterile vermiculite that had been presoaked with plant growth solution (Vincent 1970). Plants were inoculated with strains that were grown overnight in TY, pelleted, and resuspended to a uniform density in either sterile water or plant nutrient solution. The bacteria were then mixed in 1:1 ratio and diluted, and 10 ml of approximately 10⁵ CFU/ml was inoculated onto the seedlings. The inoculation mix was further diluted and used to determine the actual inoculation ratio that was used for each experiment.

Nodules were harvested after about 4 to 5 weeks in a growth chamber. Nodules were surface sterilized, crushed, and streaked out onto TY plates as described by Hynes and O'Connell (1990). Four colonies from each crushed nodule were tested and the identity of strains in nodules was determined by antibiotic resistance and the ability to grow on the appropriate carbon source. Data from nodule competition assays were analyzed by the chi square test.

Root colonization assays.

Overnight cultures of *R. leguminosarum* were grown in TY and diluted in sterile water to approximately 100 CFU/ml. These diluted cultures were used to inoculate five to six sterile clover seedlings grown on plant nutrient agar slants. Inoculated seedlings were then placed into growth chambers for 4 days.

To estimate the relative population present on a given slant, a sample of the fluid at the bottom of the agar slants was plated onto TY agar. To estimate the rhizosphere population, seedlings were aseptically removed from the agar slant and 1 to 2 cm of the root was cut and placed into a microfuge tube containing 1 ml of sterile water. The root was vortexed for 1 min and sonicated for 10 min in a sonicating water bath to remove bacteria adhering to the root surface. Bacteria were quantitated by plating onto TY agar and screening for the appropriate antibiotic resistances.

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