The mid genes of Rhizobium sp. strain TAL1145 are required for degradation of mimosine into 3-hydroxy-4-pyridone and are inducible by mimosine

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Mimosine is a toxin present in the tree-legume leucaena (Leucaena leucocephala), including its root nodules and the root exudates. The leucaena-nodulating Rhizobium sp. strain TAL1145 degrades mimosine (Mid+) and utilizes it as a source of carbon and nitrogen. Twelve TAL1145 mutants defective in mimosine degradation (Mid-) were made through Tn3Hogus, TnphoA or kanamycin-resistance-cassette insertions. A 5·0 kb PstI fragment of TAL1145, subcloned from a cosmId clone containing mid genes for mimosine degradation, complemented most of the Mid- mutants. Sequencing this fragment and the adjacent 0·9 kb PstI fragment identified five genes, midA, midB, midC, midD and midR, of which the first three genes encode ABC transporter proteins involved in mimosine uptake, while midD encodes an aminotransferase required for degrading mimosine into 3-hydroxy-4-pyridone, and midR is a regulatory gene encoding a LysR-type transcriptional activator. The location of MidA in the periplasm was shown by making two midA::phoA fusions, which make active alkaline phosphatase in the periplasm. The various mid::gus and mid::phoA fusions were inducible by mimosine, and a mid::gus fusion mutant showed \( \beta \)-glucuronidase activity in the leucaena nodules, indicating that midD is expressed in the nodules. Similarly, a midA::phoA fusion expressed alkaline phosphatase activity in the leucaena nodules, indicating that mimosine induces midA transcription in the bacteroids. mid genes are specific for the Mid+ strains of leucaena Rhizobium and are absent in strains of other Rhizobium, Sinorhizobium and Bradyrhizobium spp.

INTRODUCTION

Mimosine [\( \beta \)-N-(3-hydroxy-4-pyridone)-\( \alpha \)-aminopropionic acid] is a free amino acid present in the tree-legume leucaena (Leucaena leucocephala). Leucaena contains mimosine in all parts of the plants, 4–10% in the shoots (Jones, 1979), 1–1·5% in the roots (Mathews & Rai, 1985) and approximately 0·5% in the nodules (Soedarjo & Borthakur, 1998). Mimosine is toxic to animals, and therefore farm animals grazing on leucaena foliage often suffer from physiological disorders such as thyroid enlargement and loss of hair (Jones, 1979). Some ruminants contain certain bacteria in their rumen that degrade mimosine and utilize it as a source of nutrients (Jones & Megarry, 1986).

Roots and germinating seeds of leucaena secrete mimosine, which may inhibit growth of rhizobia (Soedarjo & Borthakur, 1998). However, some Rhizobium strains isolated from the nodules of Leucaena spp. in different parts of the world degrade mimosine and utilize it as a source of carbon and nitrogen (Soedarjo et al., 1994). Although the ability to catabolize mimosine is not required for nodulation and nitrogen fixation, it provides a competitive nodulation advantage to the mimosine-degrading (Mid+) Rhizobium in the rhizosphere of leucaena by providing a selective source of nutrients and at the same time inhibiting the growth of other micro-organisms and rhizobia (Soedarjo & Borthakur, 1998). Rhizobium sp. strain TAL1145 is one such Mid+ strain, which is known to be competitive for nodule occupancy on leucaena (Moawad & Bohlool, 1984).

Abbreviations: Gus, \( \beta \)-glucuronidase; HP, 3-hydroxy-4-pyridone; MU, 7-hydroxy-4-methylcoumarin; MUG, 4-methylumbelliferyl \( \beta \)-D-glucuronide; RM, Rhizobium mimosine; YEM, yeast extract mannitol.

The GenBank accession number for the nucleotide sequence reported in this paper is AF312768.
Previously, we isolated a cosmid, pUHR181, containing genes for mimosine degradation from a clone library of TAL1145 (Borthakur & Soedarjo, 1999). When this cosmid was transferred to non-mimosine-degrading (Mid\(^{-}\)) strains such as TAL182 and CIAT899, the transconjugants degraded mimosine, and the degradation product, 3-hydroxy-4-pyridone (HP), accumulated in the culture medium. This suggested that pUHR181 contained genes for the degradation of mimosine to HP. Plasmid pUHR191 is a derivative of pUHR181 containing a 12–6 kb insert, which was constructed by deleting approximately a 10 kb fragment from pUHR181. The transconjugants of TAL182 and CIAT899 containing pUHR191 converted mimosine into HP. Therefore, the 12–6 kb cloned DNA in plasmid pUHR191 contains mid genes involved in the first step of mimosine degradation in Rhizobium. Another cosmid, pUHR263, which overlaps with pUHR181 or pUHR191 and contains genes for mimosine degradation, was recently isolated (Fox & Borthakur, 2001). The objectives of the present investigation are to identify and characterize the mid genes present in the 12–6 kb fragment of TAL1145, which are required for the degradation of mimosine to HP.

**METHODS**

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1.

### Table 1. *Rhizobium* strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Characteristics*</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td><strong>Rhizobium</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAL1145</td>
<td>Wild-type <em>Rhizobium</em> sp. strain that nodulates leucaena, Mid(^{+})</td>
<td>George et al. (1994)</td>
</tr>
<tr>
<td>PF6, PF7, PF8,</td>
<td>Tn3Hogus insertion mutants of TAL1145, Mid(^{-}), Kan(^{R})</td>
<td>This study</td>
</tr>
<tr>
<td>PF24, MS1009,</td>
<td></td>
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<td>MS1103, MS1144,</td>
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<td>MS1246, MS1353</td>
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<tr>
<td>RUH128, RUH133</td>
<td>TnphoA insertion mutants of TAL1145, Mid(^{-}), Kan(^{R})</td>
<td>This study</td>
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<tr>
<td>RUH129</td>
<td>TnphoA insertion mutant of TAL1145 that does not show detectable PhoA activity, used as a control for comparison</td>
<td>This study</td>
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<td>RUH121</td>
<td>Kan(^{R}) cassette insertion mid(A) mutant of TAL1145, Mid(^{-}), Kan(^{R})</td>
<td>Borthakur &amp; Soedarjo (1999)</td>
</tr>
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<td>TAL182</td>
<td>Wild-type <em>Rhizobium</em> etli strain, Mid(^{-})</td>
<td>Borthakur &amp; Gao (1996)</td>
</tr>
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<td>CIAT899</td>
<td>Wild-type <em>Rhizobium tropici</em>, Mid(^{-})</td>
<td>Martinez-Romero et al. (1991)</td>
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<tr>
<td>NGR234</td>
<td><em>Rhizobium fredii</em> that nodulates a wide range of host legumes including leucaena</td>
<td>Trinick (1980)</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pLAFR3</td>
<td>Wide-host-range P1-group cosmid vector, Tet(^{R})</td>
<td>Staskawicz et al. (1987)</td>
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<tr>
<td>pRK404</td>
<td>Wide-host-range P1-group cloning vector, Tet(^{R})</td>
<td>Ditta et al. (1985)</td>
</tr>
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<td>pH1JI</td>
<td>P1-group plasmid used to eliminate other P1-group recombinant plasmids from <em>Rhizobium</em> strains, Gen(^{R})</td>
<td>Beringer et al. (1978)</td>
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<td>pUHR181</td>
<td>pLAFR3 cosmid clone isolated from the genomic library of TAL1145; contains mid genes</td>
<td>Borthakur &amp; Soedarjo (1999)</td>
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<td>pUHR263</td>
<td>pLAFR3 cosmid clone isolated from the genomic library of TAL1145; overlaps with pUHR181 and contains mid genes</td>
<td>This study</td>
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<td>pUHR191</td>
<td>A deletion derivative of pUHR181 containing mid genes in a 12-6 kb insert</td>
<td>Borthakur &amp; Soedarjo (1999)</td>
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<td>The 8-4 kb BamHI fragment of pUHR191 containing mid genes was subcloned in pRK404</td>
<td>This study</td>
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<td>pUHR208</td>
<td>5 kb <em>Pst</em>I fragment of pUHR192 subcloned in pRK404</td>
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<tr>
<td>pUHR227</td>
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</tr>
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<td>pUHR208midC::Tn3Hogus-6</td>
<td>This study</td>
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<tr>
<td>pUHR208::gus-7</td>
<td>pUHR208midC::Tn3Hogus-7</td>
<td>This study</td>
</tr>
<tr>
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<td>pUHR208midA::Tn3Hogus-8</td>
<td>This study</td>
</tr>
<tr>
<td>pUHR208::gus-24</td>
<td>pUHR208midD::Tn3Hogus-24</td>
<td>This study</td>
</tr>
<tr>
<td>pUHR208::phoA-128</td>
<td>pUHR208midA::TnphoA-128</td>
<td>This study</td>
</tr>
<tr>
<td>pUHR208::phoA-133</td>
<td>pUHR208midA::TnphoA-133</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Gen, gentamycin; Kan, kanamycin; Tet, tetracycline.*
**Media and growth conditions.** Rhizobium strains were grown in yeast extract mannitol (YEM) (Vincent, 1970) at 28°C. The ability of the Rhizobium strains to degrade mimosine was tested on Rhizobium mimosine (RM) medium (Soedarjo et al., 1994).

**Tn3Hogus mutagenesis.** Plasmids pUHR191 and pUHR263 were mutagenized with random insertion of the transposon Tn3Hogus, which is a derivative of Tn3-HoHo1 (Stachel et al., 1985) constructed in the laboratory of Brian Staskawicz, University of California, Berkeley, USA. This 6-62 kb transposon has a kanamycin resistance marker and a promoterless gus gene near the left inverted repeat (B. Staskawicz, personal communication). The Tn3Hogus insertions in the plasmids were made following the same method as for Tn3-HoHo1 mutagenesis (Stachel et al., 1985), except that kanamycin was used for selection of the transposon. The derivatives of pUHR191 or pUHR263 carrying Tn3Hogus insertions were transferred to the wild-type Rhizobium strain TAL1145. The Tn3Hogus insertions were integrated into the TAL1145 chromosome by marker exchange (Ruvkun & Ausubel, 1981) using a P1-group incompatible plasmid, pPH11. The kanamycin-resistant mutant colonies were screened for the mid phenotype by growing on RM containing 3 mM mimosine and 1 mM FeCl₃.

The mid::Tn3Hogus insertions in mutants PF6, PF7, PF8 and PF24 were transferred from the chromosome to the corresponding homologous position in the recombinant plasmid pUHR208 by double homologous recombination as described previously (Parveen & Borthakur, 1994). pUHR208::gus-6, pUHR208::gus-7, pUHR208::gus-8 and pUHR208::gus-24 were constructed in this way.

**TnphoA mutagenesis.** Escherichia coli strain LE392(pUHR208) was infected with bacteriophage λ::TnphoA (Manoil & Beckwith, 1985) at an approximate m.o.i. of 1 and plated, selecting for tetracycline (10 μg ml⁻¹) and kanamycin (50 μg ml⁻¹) resistance. Kanamycin-resistant colonies were combined and used to isolate plasmid DNA, which was then transformed into the phoA mutant strain CC118 (Manoil & Beckwith, 1985) and plated on LB agar containing tetracycline, kanamycin and 5-bromo-4-chloro-3-indolyl phosphate (40 μg ml⁻¹) to screen for TnphoA insertions showing PhoA activity. The pUHR208mid::phoA derivatives were transferred to TAL1145, and the insertions were integrated into the TAL1145 chromosome by marker exchange as described above.

**Alkaline phosphatase and β-glucuronidase (Gus) activity assay.** The assay for alkaline phosphatase activity in permeabilized cells was done according to Manoil & Beckwith (1985). A fluorometric assay for Gus activity using 4-methylumbelliferonyl β-D-glucuronide (MUG) (Sigma) was done using a protocol based on the method of Jefferson et al. (1987). The fluorescent product 7-hydroxy-4-methylcoumarin (MU) is produced through hydrolysis of MUG by Gus. To measure the Gus activity of the whole nodule, 4-week-old leucaena plants were harvested, and nodules were surface-sterilized by submerging in 70% ethanol for 30 s and 1% sodium hypochlorite for 30 s, and then washing with sterile distilled water several times. Five nodules from each plant were transferred to a 1.5 ml microtube, weighed and crushed in 9 vols Gus extraction buffer containing 50 mM Na₂HPO₄ (pH 7-0), 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% sodium lauryl sarcosine and 0.1% Triton X-100. Lysozyme was added to a concentration of 1 mg ml⁻¹ and incubated for 30 min at 37°C. One-hundred microtitre aliquots were added to a 400 μl volume of MUG assay buffer containing 1 mM MUG in Gus extraction buffer and incubated at 37°C. One-hundred microtitre samples were taken after 1 h, and the reaction was stopped by adding the sample into 900 μl of a buffer containing 0.2 M Na₂CO₃. Fluorometric measurements were made using a Hitachi F2500 fluorescence spectrophotometer, which was precalibrated with 50, 100, 500, 1000 and 3500 nM MU, corresponding to 140, 312, 1590, 3145 and 9400 fluorescence units, respectively. The wavelength of the spectrophotometer was set to 365 nm for excitation and 455 nm for emission. The fluorescence readings of the samples were automatically converted to nM MU by the spectrophotometer. The MU concentrations of a few samples were measured at 30 min intervals for 3 h, and the enzyme activity was found to be linear. The Gus activity was measured as the amount of MU produced per hour by Gus per milligram of nodule fresh weight.

**Plant test.** L. leucocephala variety K8 plants were grown for nodulation and nitrogen fixation assays in modified Leonard jar assemblies containing nitrogen-free nutrient solution as described previously (Borthakur & Gao, 1996). One-week-old seedlings were inoculated with 10⁶ rhizobia diluted with sterile plant nutrient solution from a 2-day-old culture. Nodulation and nitrogen fixation were assayed as described previously (George et al., 1994).

**Histochemical staining of nodules.** Nodules were harvested 4–5 weeks after inoculation of plants and fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), followed by post-fixation in buffered 2% osmium tetraoxide. These were dehydrated in ethanol and embedded in Epon. Sections (20 μm thick) were cut with a Sorval microtome and stained overnight at 37°C in a solution containing 0.1 M Tris (pH 9.0), 1 mM MgCl₂ and 1-6 mg Xl⁻¹ MP ml⁻¹.

**Mimosine and HP determination.** The mimosine and HP concentrations in a sample were determined by HPLC using a C18 column as described previously (Soedarjo et al., 1994). Mimosine was purchased from Sigma. The HP used was a gift from Dr Thomas Hemscheidt, Department of Chemistry, University of Hawaii, Honolulu.

**RESULTS**

**Isolation of Mid− mutants of TAL1145**

Tn3Hogus mutants of TAL1145 were isolated using cloned DNA in pUHR191 or pUHR263. The resulting mutants were screened for the loss of the ability to degrade mimosine (Mid−). In this way, nine Mid− mutants, PF6, PF7, PF8, PF24, MS1009, MS1103, MS1144, MS1353 and MS1246, were selected. These mutants degraded HP, suggesting that only one of the steps in mimosine degradation was blocked in these mutants. The approximate positions and the direction of the Tn3Hogus insertions in these mutants were determined either by Southern analysis of the mutants using pUHR191 or by restriction analysis of pUHR263::Hogus insertions. The position and the direction of the Tn3Hogus insertions were later accurately determined by sequencing the flanking DNA segments after PCR amplification in which one of the two primers used was from the gus gene in the Tn3Hogus transposon. The Tn3Hogus insertions in mutants PF6, PF7, PF8, PF24, MS1009 and MS1246 are located on a 5 kb PstI fragment, while those in MS1103, MS1144 and MS1353 are located on the adjacent 0-9 kb PstI fragment (Fig. 1). The direction of the Tn3Hogus insertion in mutants PF6, PF7, PF8, PF24, MS1009 and MS1103 and MS1353 is from left to right, whereas it is opposite in mutants MS1144 and MS1246. Another Mid− mutant RUH121 was constructed from TAL1145 by site-directed
insertion of a kanamycin resistance cassette into the HindIII site in the mid gene cluster (Fig. 1).

**Growth of the Mid** mutants is inhibited by mimosine in YEM medium

The Mid mutants do not grow on RM medium containing mimosine as the sole source of carbon and nitrogen. These mutants do not have auxotrophic defects since they grow like TAL1145 in minimal medium containing succinate and potassium nitrate as the source of carbon and nitrogen, respectively. Like TAL1145, these mutants utilize amino acids l-glutamine, l-glutamate, l-arginine and l-histidine, but not l-tyrosine and l-phenylalanine. Four mutants containing Tn3Hogus insertions on midA, midC and midD genes (see below) were used for comparing growth in YEM broth containing mimosine (Fig. 2). Their growth in YEM medium containing mimosine was inhibited to different extents. PF24 was the mutant most inhibited by mimosine. This mutant grew to about 30% of the cell density of TAL1145 in the presence of mimosine.

**Complementation of Mid** mutants

The 12-6 kb DNA fragment in pUHR191 contains an 8-4 kb BamHI fragment and other subcloned fragments from the 12-6 kb region fragments in plasmids pUHR192, pUHR208, pUHR227 and pUHR228 are shown. H, HindIII; E, EcoRI; B, BamHI; P, PstI; S, SacI. (B) Dimensions and directions of five mid genes identified within the 5-9 kb fragment shown with open arrows. The short horizontal arrows attached to the vertical lines above the 5-9 kb fragment indicate the positions and directions of Tn3Hogus insertions in nine Mid mutants of TAL1145. Similarly, the position and direction of the TnphoA insertion in mutants RUH128 and RUH133 are indicated with a dotted vertical line with short horizontal arrows on the top. The position of the cassette insertion in the Mid mutant RUH121 is shown with an inverted triangle.
The insertions in the Mid ORFs, four in the left-to-right orientation and one in the opposite direction (Fig. 1). The insertions in the Mid ORFs, four in the left-to-right orientation and one in the opposite direction (Fig. 1). The insertions in the Mid ORFs, four in the left-to-right orientation and one in the opposite direction (Fig. 1). The insertions in the Mid ORFs, four in the left-to-right orientation and one in the opposite direction (Fig. 1). The insertions in the Mid ORFs, four in the left-to-right orientation and one in the opposite direction (Fig. 1).

DNA sequence analysis and identification of five mid genes

Analysis of the nucleotide sequence of the 5.9 kb region comprising the 5.0 and 0.9 kb PstI fragments showed five ORFs, four in the left-to-right orientation and one in the opposite direction (Fig. 1). The insertions in the Mid mutants described above are located within these putative genes, midA, midB, midC, midD and midR (Fig. 1). The possible functions of these genes were assigned based on their homology with other known genes in the database.

The midA ORF is 846 bp long and may encode a polypeptide of 28989 Da. There is a Shine–Dalgarno-like sequence AAAGG 9 bp upstream of the ATG start site. There are three additional ATG sites at 66, 129 and 153 bp upstream of this start site; however, these ATG sites are not preceded by Shine–Dalgarno sequences and therefore are unlikely to be the initiation sites of translation. The deduced amino acid sequence showed 28–29% identity and 45–46% similarity with E. coli and Salmonella typhimurium periplasmic glutamine-binding precursor proteins, indicating that MidA may be a periplasmic mimosine-binding precursor protein. Hydropathy analysis of the deduced amino acid sequence showed that it has a hydrophobic region at the amino terminus, which may be the transit peptide involved in the transport of the protein into the periplasm (data not shown). When the MidA sequence was scanned using the SignalP program (Nielsen et al., 1997), it predicted an N terminus leader peptide with a cleavage site after the alanine residue at position 23. To determine if MidA is a periplasmic protein, midA::phoA fusions were constructed by using TnphoA, which is a transposon probe for periplasmic proteins (Manoil & Beckwith, 1985). The derivatives of TAL1145 containing pUHR208::midA::phoA fusions showed high levels of phosphatase activity, indicating that MidA is a periplasmic protein. Two fusion alleles, midA::phoA-128 and midA::phoA-133, were also integrated into TAL1145 chromosome to obtain mutants RUH128 and RUH133, respectively. The positions of the midA::phoA-128 and midA::phoA-133 fusion alleles in RUH128 and RUH129 were determined by sequencing to be at the 576 and 579 bp positions of the midA ORF (Fig. 1). The Tn3Hogus insertion in mutant PF8 is also located on the midA ORF, 957 bp downstream from the start site of translation (Fig. 1). Similarly, the kanamycin resistance cassette in mutant RUH121 was inserted into the unique HindIII site located 761 bp downstream from the start site of midA.

The midB ORF is 705 bp long, located 345 bp downstream of the TGA stop codon for midA, and may encode a protein with a molecular mass of 25707 Da. To verify that the intergenic gap between midA and midB is real and not due to any sequencing error, this region was sequenced several times in both orientations. There is a putative Shine–Dalgarno sequence GGTCGG 16 nt upstream of the ATG codon. It has up to 47% identity and 65% similarity with various ABC transporter permease proteins, including AtrB of Streptomyces coelicolor. Hydropathy analysis of the deduced amino acid sequence shows that MidB has five hydrophobic membrane-spanning domains, suggesting that it is a membrane-associated permease involved in mimosine transport. The Tn3Hogus insertion in mutant MS1009 is located after nucleotide position 505 of the midB sequence (Fig. 1).

The midC ORF is located at the end of midB, and the ATG start codon for midC overlaps with the TGA stop codon for midB. It is preceded by the putative ribosome-binding site GAGGG, 6 nt upstream of the translation initiation site. midC is 798 bp and encodes a protein of 265 aa with a predicted molecular mass of 29257 Da. It shows up to 63% identity and 78% similarity with ATP-binding proteins of glutamine and many other ABC transporters in different bacteria, including the atrC gene product of S. coelicolor. MidC may be the ATP-binding protein involved in the transport of mimosine into Rhizobium. Hydropathy analysis shows that it has a transmembrane domain between residues 33 and 54, suggesting that an amino-terminal segment of the protein is membrane-associated, and a large segment of the protein from residues 55 to 265 may be located in the cytoplasm. The carboxy-terminal segment of MidC from residues 121 to 265 may be located in the periplasm. It also
has the conserved consensus ‘A’ site (at 49–57), ‘B’ site (at 189–193) and a highly conserved region between these two sites (at 162–178), which are known to be involved in the hydrolysis of ATP (Walker et al., 1982). The Tn3Ho insertions in mutants PF6 and PF7 are located after nucleotide positions 51 and 256, respectively, of the midC sequence (Fig. 1).

The midD ORF is 1125 bp long, located 38 bp downstream of midC, and is preceded by a ribosome-binding site GGAa 5 bp upstream of the ATG start site. Based on the deduced amino acid sequence, it is predicted to encode a protein of 45 901 Da. MidD shows 35 % identity and 54 % similarity with the Bacillus subtilis patB gene product, which is a member of the pyridoxal-phosphate-dependent aminotransferase family. It also has similarity with a 46 kDa haemolysin encoded by the hly gene in Treponema denticola (50 %), the Corynebacterium glutamicum accD gene product C–S lyase with α- and β-elimination activity that degrades aminohydroxycysteine (47 %), the brcQ gene product required for degradation of cysteine in C. glutamicum (47 %) and a β-cystathionase encoded by malY in the marine chitinolytic bacterium Vibrio furnissii (45 %). Similarities of MidD with these proteins from various organisms were spread over almost the entire length of MidD. Based on these homologies, MidD appears to be an aminotransferase that removes the amino group from the alanyl side chain of mimosine. Hydrophobicity analysis shows that MidD is a cytoplasmic protein without any transmembrane domain (data not shown). The Tn3Ho insertions in mutants PF24 and MS1246 are located after nucleotide positions 573 and 1028, respectively, of the midD gene (Fig. 1).

The midR ORF is located 248 bp apart from midA in the opposite direction. It is 963 bp extending from nucleotide position 536 to 1498, and it may encode a protein of 35 683 Da. The ATG start codon is preceded by a Shine–Dalgarno-like sequence, GAGAGG, 8 nt upstream of midR. The deduced amino acid sequence shared homology (up to 34 % identity and 52 % similarity) with several LysR-type transcriptional activator proteins, including NodD1 of Bradyrhizobium sp. NC92, NodD2 of Bradyrhizobium elkanii and NodD3 of Rhizobium leguminosarum bv. phaseoli. A putative helix–turn–helix DNA-binding motif comprising 26 residues was observed in the amino terminus of MidR, from position 27 to 52. The Tn3Ho insertions in the mutants MS1103, MS1144 and MS1353 are located after nucleotide positions 48, 282 and 339, respectively, of the midR sequence (Fig. 1). The Tn3Ho insertion in MS1144 is in the same direction as the midR gene sequence, while those of MS1103 and MS1353 are in the opposite direction.

**mid genes are induced by mimosine**

None of the mutants showed Gus activity in the absence of mimosine. The midD::gus mutant PF24 showed high Gus activity in the presence of mimosine (Table 2). The transconjugants of TAL1145 containing pUHR263midD::gus-24 also showed a very high level of mimosine-inducible Gus activity. In MS1246, the Tn3Ho was inserted in the midD gene in the opposite orientation. The midA::gus mutant PF8 and the midB::gus mutant PF6 showed Gus activity inducible by mimosine. Similarly, the transconjugants of TAL1145 containing pUHR263midA::gus-8 and

### Table 2. Induction of Gus activity by mimosine in Rhizobium carrying mid::gus fusions

<table>
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<tr>
<th>Rhizobium strain</th>
<th>Description</th>
<th>Copy number of mid::gus</th>
<th>Copy number of midR</th>
<th>Gus activity (nM MU ml⁻¹ h⁻¹)</th>
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<td></td>
<td></td>
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</tr>
<tr>
<td>TAL1145</td>
<td>Wild-type</td>
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<td>1</td>
<td>0</td>
</tr>
<tr>
<td>MS1353</td>
<td>midR::gus-1353 mutant</td>
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<td>0</td>
</tr>
<tr>
<td>PF24</td>
<td>midD::gus-24 mutant</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>TAL1145(pUHR208midA::gus-8)</td>
<td>TAL1145 transconjugant</td>
<td>4–7</td>
<td>1</td>
<td>1 230 ± 300</td>
</tr>
<tr>
<td>TAL1145(pUHR208midC::gus-6)</td>
<td>TAL1145 transconjugant</td>
<td>4–7</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>TAL1145(pUHR208midD::gus-7)</td>
<td>TAL1145 transconjugant</td>
<td>4–7</td>
<td>1</td>
<td>100 ± 44</td>
</tr>
<tr>
<td>TAL1145(pUHR208midD::gus-24)</td>
<td>TAL1145 transconjugant</td>
<td>4–7</td>
<td>1</td>
<td>1 530 ± 710</td>
</tr>
<tr>
<td>MS1353(pUHR208midA::gus-8)</td>
<td>MS1353 transconjugant</td>
<td>4–7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MS1353(pUHR208midC::gus-6)</td>
<td>MS1353 transconjugant</td>
<td>4–7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MS1353(pUHR208midC::gus-7)</td>
<td>MS1353 transconjugant</td>
<td>4–7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MS1353(pUHR263midD::gus-24)</td>
<td>MS1353 transconjugant</td>
<td>4–7</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*The copy numbers of pUHR208 and pUHR263 are based on estimates for plasmids with the RK2 replicon (Figurski & Helinski, 1979).
pUHR263midB::gus-6 showed Gus activities in the presence of mimosine, indicating that midA and midC are also induced by mimosine. However, the presence of mimosine induced only a very low level of Gus activity in the midB mutant MS1009 and in the midC mutant PF7. The transconjugant of TAL1145 containing pUHR263midC::gus-7 also showed a low level of mimosine-inducible Gus activity.

The phosphatase activity in the midA::phoA fusion mutants RUH128 and RUH133 and the transconjugants of TAL1145 containing pUHR208midA::phoA-128 and pUHR208midA::phoA-133 was inducible by mimosine (Table 3). Mutant RUH129 containing a phoA insertion in midA in the opposite direction did not show any detectable level of phosphatase activity either in the presence or in the absence of mimosine.

Induction of mid genes by mimosine requires MidR

When the transconjugants of the midR mutant MS1363 containing pUHR263midD::gus-24, pUHR263midA::gus-8 or pUHR263midB::gus-6 were tested for Gus activity after growing them in the presence and absence of mimosine, they did not show induction of Gus activity by mimosine (Table 2). This is in contrast to the mimosine-inducible Gus activity observed in the transconjugants of TAL1145 containing these plasmids. Similarly, the phosphatase activity in the transconjugants of MS1363 containing pUHR208midA::phoA-128 and pUHR208midA::phoA-133 was not inducible by mimosine, whereas the transconjugants of TAL1145 containing any of these plasmids showed high levels of mimosine-inducible alkaline phosphatase activity (Table 3). These results indicate that midR is required for induction of mid genes by mimosine.

Concentrations of mimosine required for mid genes induction

To determine the concentrations of mimosine that are required for induction of mid genes, we induced Gus activity in the midD::gus mutant PF24 and the transconjugant TAL1145pUHR263midD::gus-24 with different concentrations of mimosine in YEM broth. Concentrations between 50 and 100 nM were found to be optimum for induction of these gus fusions, although detectable levels of induction were observed with a concentration of mimosine as low as 10 nM (Fig. 3).

midA and midD are expressed in leucaena nodules

The Mid\textsuperscript{−} mutants of TAL1145 formed normal-looking nitrogen-fixing nodules on leucaena like TAL1145, suggesting that the ability to degrade mimosine is not required for

### Table 3. Induction of alkaline phosphatase activity by mimosine in Rhizobium carrying midA::phoA fusions

<table>
<thead>
<tr>
<th>Rhizobium genotype</th>
<th>Copy number of midA::phoA*</th>
<th>Copy number of midR</th>
<th>Alkaline phosphatase activity (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No mimosine</td>
</tr>
<tr>
<td>TAL1145 (wild-type)</td>
<td>0</td>
<td>1</td>
<td>40 ± 8</td>
</tr>
<tr>
<td>MS1363 (midR mutant of TAL1145)</td>
<td>0</td>
<td>0</td>
<td>36 ± 3</td>
</tr>
<tr>
<td>RUH128 (midA::phoA-128 mutant of TAL1145)</td>
<td>1</td>
<td>1</td>
<td>38 ± 5</td>
</tr>
<tr>
<td>RUH133 (midA::phoA-133 mutant of TAL1145)</td>
<td>1</td>
<td>1</td>
<td>34 ± 4</td>
</tr>
<tr>
<td><strong>Transconjugant strains</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAL1145(pUHR208midA::phoA-128)</td>
<td>4–7</td>
<td>1</td>
<td>198 ± 9</td>
</tr>
<tr>
<td>TAL1145(pUHR208midA::phoA-133)</td>
<td>4–7</td>
<td>1</td>
<td>196 ± 2</td>
</tr>
<tr>
<td>MS1353(pUHR208midA::phoA-128)</td>
<td>4–7</td>
<td>0</td>
<td>197 ± 20</td>
</tr>
<tr>
<td>MS1353(pUHR208midA::phoA-133)</td>
<td>4–7</td>
<td>0</td>
<td>198 ± 8</td>
</tr>
</tbody>
</table>

\*The copy numbers of pUHR208 and pUHR263 are based on estimates for plasmids with the RK2 replicon (Figurski & Helinski, 1979).
nodulation and nitrogen fixation. Since there is mimosine in the leucaena nodule, we expected that the midA gene would be induced in the nodule. As expected, sections of leucaena nodules formed by the midA::phoA-fusion mutant RUH1128 showed phosphatase activity indicating that midA is expressed in the nodules (data not shown). Mutant RUH129, which has a phoA insertion but no mimosine-inducible PhoA activity, did not show any significant level of alkaline phosphatase activity in the nodule. Similarly, nodules formed by the two midD mutants PF24 and MS1246 were also assayed for Gus activity. In the mutant PF24, the promoterless gus gene in the Tn3Hgus transposon made a translational fusion with midD, whereas in the mutant MS1246, the transposon was inserted into the midD gene in the opposite orientation. The Gus activity of the 4-week-old leucaena nodules formed by PF24 was 6657 ± 387 nM MU h⁻¹ (mg nodule fresh wt)⁻¹, while that of nodules formed by MS1246 was 16 ± 14 nM MU h⁻¹ (mg nodule fresh wt)⁻¹, indicating that midD is expressed in the leucaena nodules.

mid genes show no DNA homology with Mid\textsuperscript{−} rhizobia

We used the 5-0 kb PstI fragment of pUHR208 containing mid genes of TAL1145 as a probe against strains of several Rhizobium, Sinorhizobium and Bradyrhizobium spp. and various Mid\textsuperscript{−} and Mid\textsuperscript{−} isolates of leucaena-nodulating rhizobia. The DNA from the Mid\textsuperscript{+} strains hybridized strongly with the probe, suggesting that mid genes are conserved in mimosine-degrading Rhizobium strains (data not shown). The probe did not hybridize with the DNA from any of the Mid\textsuperscript{−} strains tested, including R. tropici strain CIAT899 and Rhizobium strain NGR234 that nodulate leucaena. This suggests that mid genes are specific for only the Mid\textsuperscript{+} strains of leucaena-nodulating Rhizobium.

DISCUSSION

In this study, we have isolated a cluster of five mid genes involved in the first step of mimosine degradation within a 5-9 kb DNA fragment from TAL1145. Twelve Mid\textsuperscript{−} mutants were made using Tn3Hgus, TnphoA and a kanamycin-resistance cassette, which were inserted into these five genes. The inability to catabolize mimosine by these mutants is not associated with any pleotropic amino acid utilization defects. The growth of the midD mutant was very much inhibited by mimosine in YEM medium because this mutant lacked aminotransferase required for mimosine degradation. The three transport mutants, PF6, PF7 and PF8, did not grow as well as TAL1145 because they could not uptake mimosine and consequently failed to utilize mimosine, whereas TAL1145 utilized both YEM and mimosine as nutrients and therefore grew to higher cell densities. These mutants, however, could degrade HP, suggesting that they are blocked in the first step of mimosine degradation. Mimosine degradation by Rhizobium may involve at least two major steps. In the first step, the alanyl side chain is utilized, converting mimosine into HP, which is degraded into pyruvate, formate and ammonia in the subsequent steps. The 12-6 kb cloned DNA in plasmid pUHR191 contains genes for only the first step of mimosine degradation. In addition to the five mid genes in the 5-9 kb PstI fragment, there should be additional genes in the adjacent fragments of pUHR191 that may be also involved in the first step of mimosine degradation. The genes for the second step of mimosine degradation are located in DNA cloned in the cosmid pUH263, which overlaps with pUHR181 (Fox & Borthakur, 2001).

Mimosine induces a mimosine-degrading enzyme activity in the Rhizobium strain TAL1145, and the enzymes for mimosine degradation in this strain are located in the cytoplasm (Soedarjo et al., 1994). We have identified the midD gene encoding mimosine aminotransferase, which is located in the cytoplasm and may be involved in the transfer of the α-amino group of mimosine to an amino-acceptor such as 2-oxoglutarate. The midD::gus fusion in Rhizobium was inducible by mimosine, which supports our previous observation that mimosine induces a mimosine-degrading enzyme activity in Rhizobium TAL1145. The midD::gus fusion mutant PF24 showed Gus activity in the leucaena nodules, indicating that mimosine present in the leucaena nodules induces the expression of midD in the nodules. Similarly, the midA::phoA fusions in mutants RUH128 and RUH133 and the transconjugants of TAL1145 carrying pUHR208midA::phoA showed mimosine-inducible alkaline phosphatase activity. Leucaena nodules formed by the mutants RUH128 and RUH133 showed detectable levels of alkaline phosphatase activity, suggesting that midA is expressed in the nodules. Different gus fusions showed different levels of mimosine-inducible Gus activities, indicating that the four genes may be transcribed from more than one promoter. The two insertions in midC in PF6 and PF7 showed different levels of inducible Gus activities, which may be the result of position effects of gus insertion. The midD::gus fusion in mutant PF24 and the transconjugant TAL1145pUHR263midD::gus-24 expressed at much higher levels than the midB::gus and midC::gus fusions in the presence of mimosine, which may be due to either position effects of the gus fusions or the presence of a secondary promoter upstream of midD.

MidR is required for transcription of midA, midB, midC and midD. Gus activities were observed when transconjugants of TAL1145 containing various pUHR208mid::gus derivatives were grown in the presence of mimosine. When the transconjugants of MS1353 containing these pUHR208mid::gus derivatives were grown in the presence of mimosine, Gus activity was not induced. This shows that expression of the mid genes requires both mimosine and MidR. MidR is a LysR-type transcription activator with conserved DNA-binding motifs, and it has homology with many transcriptional activators including NodD of Rhizobium and Bradyrhizobium. Thus, the transcription of mid genes is
similar to that of nod genes, requiring an inducer molecule and a transcriptional activator protein.

Root exudates of leucaena contain mimosine, which induces mid genes in Rhizobium sp. strain TAL1145. This is somewhat similar to induction of the rha genes for rhamnose catabolism in Rhizobium leguminosarum bv. trifoli for root exudates of clover (Oresnik et al., 1998) and induction of rmmrAB genes for multidrug efflux pump in Rhizobium etli (Gonzalez-Pasayo & Martinez-Romero, 2000). Similarly, the tau genes of Rhizobium tropici are induced by root exudates of bean (Rosenblueth et al., 1998).

In the present study, by using several mid::gus and mid::phoA fusions, we have shown that mid genes are also induced in the leucaena nodules. Recently, Allaway et al. (2001) used plasmid-based promoter probe vectors containing gfp as the reporter gene to identify several rhizosphere-induced genes from environmental bacteria, demonstrating that certain bacterial genes are induced by plant root exudates.

Although the major plant substrates available to the bacteroids are known to be malate, fumarate and succinate (Miller et al., 1988), the carbon supplied to bacteroids may also include amino acids such as asparagine (Huerta-Zepeda et al., 1988) and aspartate (Rastogi & Watson, 1991). High asparaginase and aspartase activities were found in the bacteroids of Rhizobium lupini asparaginase and aspartase activities were found in the bacteroids of Rhizobium lupini (Kretovich et al., 1981) and R. etli (Huerta-Zepeda et al., 1996), indicating that asparagine and aspartate are catabolized by bacteroids. The genes involved in the catabolism of trigonelline, a secondary metabolite present in alfalfa, are expressed in both the free-living rhizobia and the bacteroid of S. meliloti (Boivin et al., 1990). Like trigonelline catabolism genes, mid genes are also expressed in the free-living rhizobia as well as the bacteroids of TAL1145 in the presence of mimosine. Unlike many genes that are expressed in the free-living rhizobia, but are turned off in the bacteroids, induction of mid genes by mimosine is not repressed in the bacteroids. Since mimosine is naturally present in the nodules, Mid + bacteroids gain the benefit of the available nutrient source by not repressing the mimosine-inducible mid genes. The bacteroids use an active transport system encoded by midABC genes to transfer mimosine into the cytoplasm. In the Mid + R. tropici strains, such as CIAT899, in which such an uptake system is absent, only a small amount of mimosine may enter the bacteroid through diffusion. Therefore, in the nodules formed by such strains, there should be a higher gradient of mimosine outside the bacteroid in the host cytoplasm and the symbiosome space. This has probably no adverse effects on the bacteroid and symbiotic nitrogen fixation. However, since mimosine is a strong chelator of iron (Kato et al., 1992), if the leucaena plant is under iron stress, mimosine may inhibit nitrogen fixation by chelating iron and reducing its availability for the nitrogenase enzyme in the bacteroid cytoplasm or for leghaemoglobin biosynthesis in the host cytoplasm. Thus, under iron-limiting conditions, Mid + Rhizobium may be ineffective or partially effective for nitrogen fixation. However, in bacteroids formed by a Mid + strain such as TAL1145, such a mimosine gradient will not form outside the bacteroid membrane since mimosine will be continuously degraded, providing fuel for nitrogen fixation and releasing Fe from any iron–mimosine complex that may also be present in the leucaena root nodule. We believe that the leucaena tree originally evolved in soils that were probably deficient in available iron. Mimosine released in the root exudates of leucaena chelates iron to form Fe–mimosine complexes. These complexes are then taken up by Mid + rhizosphere bacteria, which then provide Fe to the plant in an available form. Rhizobium strains that can degrade mimosine might have co-evolved with leucaena and are the true symbiont for this tree-legume. Other Mid + rhizobia such as R. tropici CIAT899 or R. fredii NGR234 may be members of the opportunistic rhizobia that nodulate leucaena in the absence of the true symbionts and are not equipped with mid genes to utilize mimosine inside the leucaena nodule.

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