pyd Genes of *Rhizobium* sp. Strain TAL1145 Are Required for Degradation of 3-Hydroxy-4-Pyridone, an Aromatic Intermediate in Mimosine Metabolism

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Rhizobium sp. strain TAL1145 degrades the *Leucaena* toxin mimosine and its degradation product 3-hydroxy-4-pyridone (HP). The aim of this investigation is to characterize the *Rhizobium* genes for HP degradation and transport. These genes were localized by subcloning and mutagenesis on a previously isolated cosmid, pUHR263, containing *mid* genes of TAL1145 required for mimosine degradation. Two structural genes, *pydA* and *pydB*, encoding a metacleaveage dioxygenase and a hydrolase, respectively, are required for degradation of HP, and three genes, *pydC*, *pydD*, and *pydE*, encoding proteins of an ABC transporter, are involved in the uptake of HP by TAL1145. These genes are induced by HP, although both *pydA* and *pydB* show low levels of expression without HP. *pydA* and *pydB* are cotranscribed, while *pydC*, *pydD*, and *pydE* are each transcribed from separate promoters. PydA and PydB show no homology with other dioxygenases and hydrolases in *Sinorhizobium meliloti*, *Mesorhizobium loti*, and *Bradyrhizobium japonicum*. Among various root nodule bacteria, the ability to degrade mimosine or HP is unique to some *Leucaena*-nodulating *Rhizobium* strains.

Many soil bacteria can degrade various toxic aromatic compounds, such as benzene, toluene, ethylbenzene, and xylene (10). Mimosine [β -N-(3-hydroxy-4-pyridone)- α -aminopropionic acid] is one such aromatic toxin produced by *Leucaena* and *Mimosa* spp. (6, 16). Mimosine is known to be toxic to both microorganisms and eukaryotic cells (12, 20). Mimosine is a strong chelator of iron, with a binding constant of 10^{36} (11). Its degradation product 3-hydroxy-4-pyridone (HP) also chelates iron (14). Chelating action by mimosine and HP inhibits iron-dependent enzymes and thereby blocks biochemical activities by depriving the cells of available iron (17). Mimosine present in the root exudates of *Leucaena* inhibits the growth of some root nodule bacteria (19). However, some *Leucaena*-nodulating *Rhizobium* strains are able to utilize mimosine as a source of carbon and nitrogen (18, 20).

HP is an intermediate of mimosine degradation in *Rhizobium* (4). The *mid* genes, required for conversion of mimosine into HP, are located within a 12.6-kb fragment of the TAL1145 chromosome, cloned in cosmids pUHR181 and pUHR263 (5). When pUHR181 was transferred to a *Mid*[−] strain, such as TAL182, the transconjugants degraded mimosine into HP, whereas the transconjugants of TAL182 containing pUHR263 degraded mimosine completely (8). Cosmid pUHR263 contains a 12-kb fragment, which is absent in pUHR181. It was hypothesized that genes for HP degradation are located in this 12-kb region of pUHR263. The objective of this study is to characterize the genes for HP degradation in pUHR263.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are described in Table 1. The *Escherichia coli* and *Rhizobium* strains were grown as described previously (20, 21). The growth rates were determined in yeast extract-mannitol (YEM) medium supplemented with 3 mM mimosine or HP and 1 mM FeCl₃ and with the pH adjusted with 1 N HCl to 6.8. Cultures (25 ml) were grown in 250-ml flasks and sampled at 10- to 14-h intervals. Cell density was measured with a spectrophotometer (Spectronic Instruments, Rochester, NY) at 600 nm until cultures reached stationary growth phase. Media samples were analyzed through high-performance liquid chromatography (HPLC) as described below.

Detection of mimosine and HP. Cell cultures were centrifuged (5,000 × g), and the supernatants were filtered through 0.2- μ m nylon filters. Analysis was done on a Dionex HPLC with Chromeleon software and performed with a reverse-phase C₁₈ column (250 by 4.6 mm; Phenomenex, Torrance, CA) at room temperature with a 0.2% orthophosphoric acid mobile phase at a flow rate of 1 ml/min. A diode array detector monitored absorbance at a range of 200 to 300 nm. The wavelength for the detection of mimosine and HP was determined to be 275 nm. Mimosine and HP had a retention time of 2.8 and 4.8 min, respectively.

DNA manipulation, analysis, and sequencing. Plasmid DNA isolation and manipulations were performed by standard procedures (13). Nucleotide sequence was determined by automated sequencing at the Molecular Biology and Biotechnology Facilities, University of Hawaii at Manoa. Both strands of the DNA fragment were sequenced using a primer-walking strategy.

Tn3Hogus mutagenesis. Transposon insertion mutagenesis was done as described previously (5). Locations of the Tn3Hogus transposon insertions were determined by sequencing the flanking region of the transposon. For PCR amplification of the flanking region, a reverse *gus* primer (5'-AATTCCACAGT TTTTCGCGATC3') from the 5' region of the *gus* gene and different forward primers from the sequence of the 5.2-kb fragment were used such that they would produce a PCR product of approximately 500 to 1,000 bp. The PCR products were purified and sequenced using the reverse *gus* primer. From the sequence data, the junctions of the Tn3Hogus insertions were determined.

GUS activity assay. β -Glucuronidase (GUS) activity assay was performed according to a previously described method (8). Methoxyppyridone and pyridone were gifts from Thomas Hemscheidt (Department of Chemistry, University of Hawaii at Manoa), and 1,2 dimethyl-3-hydroxypyrid-4-one was a gift from Hiromu Matsumoto (Department of Molecular Biosciences and Bioengineering, University of Hawaii at Manoa).

Expression and purification of PydA and PydB. pUHR304 and pUHR305 were constructed using primer pairs *pydA*-UP (5'-CGCACATATGAGGAGAA AGTTAATGGCTG 3') and *pydA*-DWN (5'-CGTTAGATCTTATGCTGCTGC

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TABLE 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Characteristics	Reference or source
Bacterial strains		
TAL1145	Wild-type <i>Rhizobium</i> sp. strain that nodulates leucaena, Mid ⁺ HP ⁺	9
PF9	Mutant of TAL1145 containing a Tn3Hogus insertion on <i>pydA</i> , Kan ^r , Mid ⁺ HP ⁻	8
JA139	Mutant of TAL1145 containing a Tn3Hogus insertion on <i>pydB</i> , Kan ^r , Mid ⁺ HP ⁻	This study
JA140	Mutant of TAL1145 containing a Tn3Hogus insertion on <i>pydC</i> , Kan ^r , Mid ⁺ HP ⁻	This study
PF106	Mutant of TAL1145 containing a Tn3Hogus insertion on <i>pydD</i> , Kan ^r , Mid ⁺ HP ⁻	This study
PF101	Mutant of TAL1145 containing a Tn3Hogus insertion on <i>pydE</i> , Kan ^r , Mid ⁺ HP ⁻	This study
TAL182	Wild-type <i>Rhizobium etli</i> strain, Mid ⁻ HP ⁻	3
EHA105	Wild-type <i>Agrobacterium tumefaciens</i> , Mid ⁻ HP ⁻	
NZP4013	Wild-type <i>Sinorhizobium meliloti</i> , Mid ⁻ HP ⁻	9
BURK25	Wild-type <i>Burkholderia</i> sp., Mid ⁻ HP ⁻	Leary and Borthakur, unpublished
Plasmids		
pRK404	Wide-host-range P1-group cloning vector, Tet ^r	7
pPHIJI	P1-group plasmid used to eliminate other P1-group recombinant plasmids from <i>Rhizobium</i> strains, Gen ^r	2
pUHR263	pLAFR3 cosmid clone isolated from the genomic library of TAL1145, contains <i>mid</i> and <i>pyd</i> genes	8
pUHR281	5.2-kb EcoRI fragment from pUHR263 cloned in pRK404	This study
pUHR283	5.2-kb EcoRI fragment from pUHR263 cloned in pRK404	This study
pUHR284	3.1-kb PstI fragment from pUHR263 cloned in pRK404	This study
pUHR322	2.2-kb EcoRI-HindIII fragment from pUHR263 cloned in pRK404	This study
pUHR328	1.1-kb EcoRI-PstI fragment from pUHR263 cloned in pRK404	This study
pUHR329	1.4-kb EcoRI-PstI fragment from pUHR263 cloned in pRK404	This study
pUHR330	1.4-kb fragment cloned in pRK404 containing <i>pydB</i>	This study
pUHR304	<i>pydA</i> cloned in pET14b	This study
pUHR305	<i>pydB</i> cloned in pET14b	This study

GTCGTAG 3') and *pydB*-UP (5'GCACCATATGCCTCATTTTGAAGACCG AG3') and *pydB*-DOWN (5'GTTGAGATCTATATTGTGGTTGCGGGAA GAGC 3'). Underlining indicates restriction sites. Upstream primers for each contained an NdeI site, and downstream primers contained a BglII site. Amplified products were digested with NdeI and BglII and ligated into pET14b (Novagen, Madison, WI), which produced a His₆ tag at the N terminus. Transformed Origami DE3 *E. coli* cells (Novagen) containing pUHR304 and pUHR305 were grown in Luria broth at 37°C up to an optical density of 0.4. Cells were induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and grown for an additional 4 h. Origami cells containing *PydA*-His₆ and *PydB*-His₆ proteins were denatured with 6 M guanidine-HCl and purified according to the manufacturer's protocol using immobilized metal affinity chromatography (Clontech, Palo Alto, CA). Purified *PydA*-His₆ and *PydB*-His₆ were dialyzed against 0.2 M NaCl-50 mM sodium phosphate buffer overnight at 4°C to remove guanidine-HCl.

Protein analysis. *E. coli* and *Rhizobium* proteins were separated with sodium dodecyl sulfate-10% polyacrylamide gels. Protein gels were then transferred to a nitrocellulose membrane (Bio-Rad, Richmond, CA), and Western blotting was performed with an ECL Western blotting analysis system (Amersham Pharmacia

Biotech). Primary polyclonal antibodies were developed from purified *PydA*-His₆ and *PydB*-His₆ and prepared by Alpha Diagnostic International (San Antonio, TX). The polyclonal antibodies were used at a concentration of 1:3,000.

RT-PCR. TAL1145 was cultured in 100 ml of tryptone-yeast extract broth at 28°C for a total of 36 h. After 24 h, 3 mM mimosine or HP was added to the medium, and the culture was grown for an additional 12 h. The culture was centrifuged, and the cells were washed with 100 ml of 0.5 M NaCl. Total RNA was isolated with QIAGEN's (Valencia, CA) RNeasy mini kit according to the manufacturer's protocol. Total RNA was treated with DNase I, incubated at room temperature for 15 min, and heat deactivated for 10 min at 65°C. Dnase I-treated total RNA was used for reverse transcriptase PCR (RT-PCR). RT-PCR was done with 1 µg of total RNA using Promega's (Madison, WI) reverse transcription system according to the manufacturer's recommendations. PCR was performed with 5 µl of the RT-PCR mixture with primers flanking the intergenic regions of *pydAB*, *pydBC*, *pydCD*, and *pydDE*. Expected PCR products were the following: 500 bp for *pydAB* using primers *pydA*-F (5'GGCGGCCTG TCGCATTGGCCT3') and *pydB*-R (5'ACCAGGCCCGGATCCGTGGAT3'), 300 bp for *pydBC* with primers *pydB*-F (5'CGTTGGATCACTTTTCGCGT3')

FIG. 1. Growth of *Rhizobium* strains in YEM containing 3 mM mimosine and 1 mM FeCl₃ (a) and with 3 mM HP and 1 mM FeCl₃ (b). The data points and error bars represent the means and standard deviations of three replicates, respectively.

FIG. 2. Restriction map of cosmid pUHR263 isolated from *Rhizobium* sp. strain TAL1145 library containing genes for mimosine and HP degradation. A 5.2-kb fragment within pUHR263 contained *pydA*, *pydB*, *pydC*, *pydD*, and *pydE* (open arrows). Solid arrows indicate Tn3Hogus insertions and directions of TAL1145 mutants defective in HP degradation. B, BamHI; E, EcoRI; H, HindIII; P, PstI.

and *pydC*-R (5'-GCTTGAGCTGGGATCGTAAC3'), 750 bp for *pydCD* with primers *pydC*-F (5'-CCGTTGATCAAGAACGACCG3') and *pydD*-R (5'-GAC TGGCAGCCTGATATCGATC3'), and 700 bp for *pydDE* with primers *pydD*-F (5'-TTCACAATCTACTGCGCGAATG3') and *pydE*-R (5'-CCAGATCGGTAT ATTCGCCG3'). Additional PCR analyses were performed to determine *pydA*, *pydC*, and *pydD* expression in 3 mM HP-induced and uninduced cultures. The primers RTA-F (5'-CATTCTGTCTATCCGATCTACAC3') and RTA-R (5'-G GCAATTCGCGTTCAACGATA3') yielded an expected 111-bp fragment, primers RTC-F (5'-GTTGGATCACTTTTCGCGTCG3') and RTC-R (5'-CGTT GAGATGGACGACGTTA3') produced a 124-bp product, and primers RTD-F (5'-CGCTCTCTGTTGAGTGCATCA3') and RTD-R (5'-GACCTGGCT CTGTGACCGAT3') amplified a 103-bp fragment. Sigma factor, *sigA*, was used as a reference gene in PCR to control for the quantity of cDNA produced by RT-PCR. *sigA* is a housekeeping gene that encodes a sigma factor of an RNA polymerase required for viability under normal growth. Reference primers *sigA*-F (5'-AGGCGCTGATCATCTGGC3') and *sigA*-R (5'-GATCTCTCAGG GCTCTGGAA3') generated an expected 131-bp PCR product. PCR was also conducted with total RNA not treated with reverse transcriptase as a negative control.

Plant test. *Leucaena leucocephala* K636 seeds were surface sterilized and germinated according to Parveen et al. (15). The plant experiments were conducted in Leonard jar assemblies containing nitrogen-free plant nutrient solution. Each Leonard jar contained four to five *leucaena* seedlings, and three replicates were grown for each treatment. One-week-old seedlings were inoculated with approximately 10^6 rhizobia. Uninoculated control plants were grown

to check for cross-contamination. The *Leucaena* plants were harvested 6 weeks after inoculation to examine nodulation.

Nucleotide sequence accession number. The nucleotide sequence reported was submitted to GenBank and given accession number AY729020.

RESULTS

Isolation of *pyd* mutants of TAL1145. Tn3Hogus insertion mutants of TAL1145 were constructed using pUHR263::Tn3Hogus derivatives. The pUHR263::Tn3Hogus derivatives were transferred to TAL1145, and the disrupted genes were marker exchanged into the chromosome to obtain mutations in the *mid* and *pyd* genes. The mutants were first screened for loss of the ability to grow on mimosine as the sole source of carbon and nitrogen. In this way, 26 mutants that could not utilize mimosine were identified. Restriction mapping of the corresponding pUHR263::Tn3Hogus derivatives for these mutants indicated that the Tn3Hogus insertions in most of the mutants were located on the previously characterized *mid* genes. However, several mutants in which the Tn3Hogus insertions were located outside the *mid* gene cluster were identified. These

TABLE 2. Characteristics of proteins encoded by the *pyd* genes

Protein	Size (amino acids)	Conserved domain	Homology with known proteins
PydA	339	LigB	Up to 45% similarity with extradiol dioxygenases from many bacteria
PydB	275	Alpha/beta hydrolase fold	Up to 52% similarity with hydrolases for degradation of aromatic compounds
PydC	307	RbsB family of periplasmic proteins	Up to 67% similarity with periplasmic substrate-binding proteins of various ABC transport systems
PydD	509	Transmembrane helix at position 281–304	Up to 54% similarity to ABC transport ATP-binding proteins
PydE	304	AraH family of permeases	Up to 67% similarity to ABC transporter permeases

FIG. 3. Analysis of the *pyd* gene cluster by RT-PCR. (a) Gel electrophoresis of the RT-PCR products using primers designed to amplify the intergenic regions of *pydA-pydB* (lane 1), *pydB-pyDC* (lane 2), *pyDC-pyDD* (lane 3), and *pyDD-pyDE* (lane 4). Negative control PCRs containing total RNA without addition of reverse transcriptase were included in this assay (data not shown). RT-PCR amplified a fragment within *pydA*, *pydB*, *pyDC*, *pyDD*, and *pyDE* in lanes 5 to 9, respectively. Positive control PCR products for *pydD-pyDE* (lane 10), *pyDC-pyDD* (lane 11), and *pydB-pyDC* (lane 12) using TAL1145 genomic DNA as the template are also shown. The sizes of the DNA fragments in the marker lanes (M) are indicated. (b) Organization of the *pyd* gene cluster, with arrows showing the approximate positions of the primers designed for RNA amplification in RT-PCR assays. (c) Transcriptional organization of the *pyd* genes. Bold arrows represent the transcriptional units deduced from RT-PCR experiments.

mutants were further screened for the ability to catabolize HP. Five mutants (PF9, JA139, JA140, PF106, and PF101) were selected on the basis of their inability to utilize HP.

Growth of PF9, JA139, JA140, PF106, and PF101 in media containing mimosine and HP. Mutants PF9, JA139, JA140, PF106, and PF101 do not have any auxotrophic defects and can grow as well as TAL1145 in YEM. PF9 and JA140 do not grow in minimal media containing either mimosine or HP as the sole source of carbon and nitrogen. PF106 and PF101 also do not grow on HP but grow slightly with mimosine as the sole source of carbon and nitrogen. When they were grown in YEM supplemented with 3 mM mimosine and 1 mM FeCl₃, they showed growth inhibition to various extents (Fig. 1a). HPLC analysis of the culture supernatants of the mutants at the end of the growth experiment showed that they degraded 90 to 95% of the mimosine in the media and accumulated up to 2 mM HP.

None of the above five mutants could utilize HP in minimal medium, and the presence of HP in a complete medium inhibited the growth of three of these mutants to different extents. In YEM medium supplemented with 3 mM HP, mutants PF101 and PF106 grew as well as TAL1145. On the other

FIG. 4. PydA and PydB in *Rhizobium* detected by Western blot analysis using polyclonal antibodies specific for PydA (a) and PydB (b). Fifty micrograms of total protein was loaded for each sample. Lane 1, TAL1145 induced with mimosine; lane 2, uninduced TAL1145; lane 3, TAL1145 induced with HP.

hand, 3 mM HP caused complete growth inhibition of JA139 and JA140 and partial growth inhibition of PF9 in YEM (Fig. 1b). HPLC analysis showed that over 90 to 98% of HP still remained in the media after the stationary growth phase of these mutants, including PF101 and PF106, which were not inhibited by HP. These results establish that these five mutants are defective in HP degradation or transport.

Complementation of mutants PF9, JA139, JA140, PF106, and PF101. Restriction mapping showed that the Tn3Hogus insertions in mutants PF9, JA139, JA140, PF106, and PF101 were located on a 5.2-kb EcoRI fragment in cosmid pUHR263 but not present in cosmid pUHR181. This 5.2-kb EcoRI fragment of pUHR263 was subcloned in both orientations to obtain plasmids pUHR281 and pUHR283. Both pUHR281 and pUHR283 complement PF9, JA139, JA140, PF106, and PF101 for mimosine and HP degradation. The complemented mutants could grow on minimal medium containing mimosine or HP as the sole source of carbon and nitrogen. In YEM containing 3 mM mimosine or HP, these strains grew as well as TAL1145 and degraded mimosine or HP completely. A 3.1-kb PstI fragment from pUHR263 that overlaps with the 5.2-kb EcoRI fragment can also complement PF9 but not PF101, PF106, JA139, or JA140 (Fig. 2). EcoRI-PstI fragments subcloned from the 5.2-kb EcoRI fragment in plasmids pUHR328 and pUHR329 (1.1 kb and 1.4 kb, respectively) also complemented PF9 but not the other four mutants for mimosine degradation. However, a 2.2-kb EcoRI-HindIII subcloned fragment (pUHR322) complemented PF9, JA139, and JA140.

Nucleotide sequence analysis. Sequencing of the 5.2-kb EcoRI fragment in pUHR281 revealed five open reading frames in the same direction (Fig. 2). Since these genes complemented mutants defective in HP degradation (*pyridone* degradation), these open reading frames were named *pydA*, *pydB*, *pydC*, *pydD*, and *pydE*. The characteristics of the proteins encoded by these genes are shown in Table 2.

RT-PCR analysis of *pyd* genes. To establish the operon structure of the *pyd* genes, RT-PCR was conducted using total RNA extracted from TAL1145 and primers developed for amplification of intergenic regions as well as within *pydA*, *pydB*, *pydC*, *pydD*, and *pydE*. PCR products were amplified within all of the *pyd* genes (Fig. 3). Primers flanking the intergenic region of *pydAB* successfully amplified PCR products. However, no PCR products were observed for *pydBC*, *pydCD*, and *pydDE*. This implicates that *pydAB* is transcribed as a single transcriptional unit, whereas *pydC*, *pydD*, and *pydE* are transcribed as separate transcriptional units. No PCR products were amplified from RNA samples alone.

TABLE 3. The induction of GUS activities in TAL1145 mutants containing *pydA::gus*, *pydB::gus*, *pydC::gus*, and *pydD::gus* fusions by mimosine, HP, or HP analogs

Compound	GUS activity (nM MU/ml/h) ^a				
	TAL1145	PF9	JA140	PF106	PF101
None	0	3,696 ± 2	0	0	0
Mimosine	0	143,068 ± 57	5,534 ± 3	706 ± 6	708 ± 6
HP	0	416,407 ± 141	1,168 ± 7	580 ± 5	641 ± 8
1,2 Dimethyl-3-hydroxypyrid-4-one	0	29,224 ± 132	569 ± 2	695 ± 29	582 ± 38

^a MU, 7-hydroxy-4-methylcoumarin.

Induction of *pydA*, *pydB*, *pydC*, and *pydD* by mimosine, HP, and HP analogs. Western analysis revealed that *pydA* and *pydB* are expressed at low levels and induced to higher levels in the presence of mimosine and HP (Fig. 4). Therefore, GUS activity assays were performed to examine the expression levels of these genes induced by mimosine, HP, and HP analogs. The *pydA::gus* fusion mutant (PF9) showed low levels of GUS activity in the absence mimosine or HP. It showed a 39-fold increase in GUS activity in the presence of mimosine and a 113-fold increase in the presence of HP (Table 3). On the other hand, the *pydB::gus* fusion mutant (JA140) had no GUS activity in the absence of mimosine or HP. It showed induced GUS activity in the presence of mimosine or HP (Table 3). Mimosine and HP also induced GUS activities in the *gus* fusion mutants of *pydC* (PF106) and *pydD* (PF101). Both mutants had an increase in GUS activity, suggesting that HP activates the transcription of these genes (Table 3). To verify the induction of the *pyd* genes in the presence of HP, RT-PCR was performed on total RNA extracted from TAL1145 using primers developed for amplification within *pydA*, *pydC*, and *pydD*. Amplification of *pydB* was not performed because *pydA* and *pydB* are cotranscribed. Increased amplification of PCR products was detected for *pydA*, *pydC*, and *pydD* in the HP-induced cultures (Fig. 5). PCR analysis further supports the induction of the *pyd* genes by HP.

Induced GUS activity was also examined with 1 mM concentrations of the HP analogs shown in Fig. 6. Among these compounds, only 1,2 dimethyl-3-hydroxypyrid-4-one induced *pydA*, *pydB*, *pydC*, and *pydD* (Table 3). The other three compounds did not induce the transcription of these *pyd* genes.

Induction of *pydA*. To determine if mimosine, HP, or both are inducers of *pydA*, the induction of *pydA* was determined by

measuring GUS activity of the *pydA::gus* mutant, PF9, at sequential time intervals: 0, 2, 6, 8, and 12 h after mimosine induction (Fig. 7). PF9 converts mimosine into HP that accumulates in the growth medium. If the GUS activity of PF9 correlates with the presence of mimosine in the medium and decreases with the disappearance of mimosine, this would indicate that mimosine induces *pydA* transcription, whereas if the GUS activity coincides with the accumulation of HP as a result of mimosine degradation, this would indicate that the transcription of *pydA* is induced by HP. As seen in Fig. 7b, the mimosine concentration in the medium decreased with time and completely disappeared after 8 h. The disappearance of mimosine corresponded with the appearance and gradual accumulation of HP in the medium. The GUS activities of the PF9 cells started to increase sometime between 2 and 6 h, with the accumulation of HP in the medium. After 8 h, mimosine completely disappeared and HP accumulated to a concentration of 690 μ M in the medium. The highest GUS activity was observed at 12 h in the presence of 780 μ M HP and no mimosine in the medium (Fig. 7a). Similar induction results were

FIG. 5. RT-PCR analysis of RNA extracted from uninduced (u) and HP-induced (i) cultures of TAL1145 using primers specific for *pydA*, *pydC*, *pydD*, and *sigA*. Sizes of the fragments in the marker lane (M) are shown.

FIG. 6. The structures of mimosine, HP, and HP analogs.

FIG. 7. The change in GUS activity in the *pydA::gus* mutant PF9 at different times following induction with mimosine, HP, or both mimosine and HP. In graphs a, c, and e, the induced GUS activity due to mimosine (open squares), HP (open triangles), both mimosine and HP (crosses), or glucose (open circles) in the PF9 cells at the end of a specific time period was calculated by subtracting the GUS activity in the preceding period. The PF9 cultures were grown in minimal media containing 1 mM concentrations of mimosine, HP, both mimosine and HP, or glucose. Graphs b, d, and f show the concentrations of mimosine (solid squares) and HP (solid triangles) in the culture supernatants of PF9 at different times corresponding to the GUS activities shown in graphs a, c, and e, respectively. Data and error bars represent the means and standard deviations of three replicates.

observed by measuring GUS activity of the *pydA::gus* mutant in the presence of HP (Fig. 7c and d). In another parallel experiment, both mimosine and HP were added initially to the medium, and 200 μM mimosine was added every 2 h to sustain the amount of mimosine in the culture (Fig. 7e and f). The GUS activities of PF9 cells had similar induction results from 0 to 8 h for both the mimosine and HP treatments. However, from 8 to 12 h there was no increase in GUS activity, implying that mimosine may act as a repressor of *pydA*. A GUS activity assay of the *pydA::gus* mutant with 1 mM, 2 mM, and 3 mM HP

resulted in no observed suppression of GUS activity at 8 to 12 h (data not shown), suggesting that mimosine, but not HP, repressed the transcription of *pydA*.

Expression of *pyd* genes in other rhizobia. Cosmid pUHR263 was transferred to other closely related soil bacteria, such as *Agrobacterium tumefaciens* EHA105, *Rhizobium etli* TAL182, *S. meliloti* N2P4013, and *Burkholderia* sp. BURK25, to determine if the *mid* and *pyd* genes are expressed in these bacteria. All cultures were grown at 28°C in YEM supplemented with 1 mM mimosine or HP. The transconjugants of

TABLE 4. The ability of *R. etli* and *A. tumefaciens* transconjugants to degrade mimosine and HP

Plasmid	Gene	Ability to <i>R. etli</i> transconjugants to degrade:		Ability of <i>A. tumefaciens</i> transconjugants to degrade:	
		Mimosine	HP	Mimosine	HP
No plasmid	None	—	—	—	—
pUHR263	<i>mid</i> + <i>pyd</i>	+	+	+	+
pUHR322	<i>pydAB</i>	—	+	—	±
pUHR329	<i>pydA</i>	—	+	—	—
pUHR330	<i>pydB</i>	—	—	—	—

Rhizobium etli TAL182 and *A. tumefaciens* EHA105 completely degraded both mimosine and HP in the media. The transconjugants of *S. meliloti* and *Burkholderia* could not degrade mimosine or HP, although their growth was not inhibited by these compounds. When the transconjugants of *R. etli* and *A. tumefaciens* containing only *pydA* and *pydB* in a plasmid were grown in the presence of HP, the *R. etli* transconjugants could degrade HP completely, whereas the transconjugants of *A. tumefaciens* degraded HP only partially (Table 4). However, among the transconjugants of these bacteria containing either *pydA* or *pydB*, only the *R. etli* transconjugants containing *pydA* in a plasmid could utilize HP. Thus, *pydA* alone could confer the ability to degrade HP in *R. etli* but not in other bacteria.

DISCUSSION

This investigation provides functional analyses of the genes involved in the degradation and transport of HP in *Rhizobium*. HP dioxygenase and HP hydrolase, encoded by *pydA* and *pydB*, are the two enzymes that are required for HP degradation. Dioxygenase and hydrolase are also found in all bacteria that degrade other aromatic compounds. Based on the homologies with other metacleave dioxygenases, the HP dioxygenase appears to be a class III metacleave enzyme involved in an extradiol cleavage reaction. *S. meliloti*, *M. loti*, and *B. japonicum* contain five, four, and nine genes, respectively, encoding dioxygenases in the genome. *PydA* has no similarities with any of these rhizobial dioxygenases. A possible metacleave degradation model for HP is presented in Fig. 8.

Downstream of the *pydA* and *pydB* genes, there are three genes, *pydC*, *pydD*, and *pydE*, encoding proteins of an ABC transporter. Mutations in these genes make the *Rhizobium* strain unable to degrade HP, suggesting that these genes are

required for HP transport. Mimosine is transported into the *Rhizobium* cells through an ABC transporter encoded by the *midABC* genes (5). When mimosine is used as a nutrient in the growth medium, the *pydC* and *pydD* mutants accumulate a small amount of HP in the medium. This indicates that after conversion of mimosine into HP inside the *Rhizobium* cells, a small amount of HP is released into the medium through diffusion. HP seems to be cycled back into the cells through the transporter encoded by *pydC*, *pydD*, and *pydE*. Like mimosine, HP also chelates iron. Therefore, HP cycling may allow *Rhizobium* to take up a second iron molecule during the degradation of a single mimosine molecule. Moreover, some amounts of mimosine may be naturally degraded to HP in the rhizosphere of *Leucaena*. The *pyd* transporter in *Rhizobium* may have evolved for transporting the available HP from the environment.

All five *pyd* genes identified in this study are required for HP degradation by TAL1145. However, the transconjugants of *A. tumefaciens* strain EHA105 containing only *pydA* and *pydB* can degrade HP, suggesting that this strain can transport HP through other transporters. Surprisingly, the transconjugant of *R. etli* TAL182 containing *pydA* alone can utilize HP, suggesting that this strain has other genes that substitute the functions of both the transporter and hydrolase required for HP utilization. On the other hand, the transconjugants of *S. meliloti* containing all five *pyd* genes or even the entire cluster of *mid* and *pyd* genes could not degrade HP or mimosine. It is possible that besides *mid* and *pyd* genes, other genes encoding a transcription factor or a cofactor may be involved in mimosine degradation, and these genes may be missing in *S. meliloti*. The cleavage product of HP by dioxygenase may be toxic to *Rhizobium* in the absence of a hydrolase required for complete breakdown of HP into pyruvate and formate. It is likely that the *pydB* mutant accumulated this toxic intermediate and therefore did not survive in the presence of HP.

Based on RT-PCR results, *pydA* and *pydB* appear to be transcribed as a single transcriptional unit, whereas *pydC*, *pydD*, and *pydE* are each transcribed as a separate transcriptional unit. Western analysis shows that both *pydA* and *pydB* are expressed at low levels. In the presence of HP, the expression of *pydA* is increased 113-fold, suggesting that *pydA* is induced by HP. Similarly, the expression of *pydB* is also induced by HP. Although mimosine seems to enhance the expression of *pydA*, our time course analysis shows that HP, but not mimosine, induces *pydA*. Degradation of mimosine produced HP, which induced *pydAB*. However, if mimosine per-

FIG. 8. The proposed pathway of HP degradation determined by the *pyd* genes in *Rhizobium* sp. strain TAL1145.

sists in the media even in the presence of HP, mimosine appears to repress the transcription of *pydA*.

There are other mimosine-degrading soil bacteria in the rhizosphere of *L. leucocephala*. We have isolated several strains of a *Klebsiella* sp. that degrade mimosine. *Synergistes jonesii* is a gram-negative rumen bacterium from cattle that also degrades HP (1). However, genes for mimosine or HP degradation have not been isolated from these bacteria. Characterization of the *pydAB* genes from these bacteria will establish whether they contain similar HP dioxygenase and hydrolase enzymes. The root exudates of different plants may have many aromatic compounds that are utilized by rhizosphere bacteria. Mimosine is a good example of a specific aromatic compound secreted by *Leucaena* roots. The elucidation of mimosine or HP degradation pathways by *Rhizobium* will enhance our understanding of the role of a specific root-exuded compound in rhizosphere colonization and ecology.

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