

Mimosine, a Toxin Present in Leguminous Trees (*Leucaena* spp.), Induces a Mimosine-Degrading Enzyme Activity in Some *Rhizobium* Strains

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Thirty-seven *Rhizobium* isolates obtained from the nodules of leguminous trees (*Leucaena* spp.) were selected on the basis of their ability to catabolize mimosine, a toxin found in large quantities in the seeds, foliage, and roots of plants of the genera *Leucaena* and *Mimosa*. A new medium containing mimosine as the sole source of carbon and nitrogen was used for selection. The enzymes of the mimosine catabolic pathway were inducible and were present in the soluble fraction of the cell extract of induced cells. On the basis of a comparison of the growth rates of *Rhizobium* strains on general carbon and nitrogen sources versus mimosine, the toxin appears to be converted mostly to biomass and carbon dioxide. Most isolates able to grow on mimosine as a source of carbon and nitrogen are also able to utilize 3-hydroxy-4-pyridone, a toxic intermediate of mimosine degradation in other organisms.

Mimosine [β -N-(3-hydroxy-4-pyridone)- α -aminopropionic acid] is a toxin found in large quantities in the seeds and foliage of leguminous trees and shrubs of the genera *Leucaena* and *Mimosa*. Structurally, it is an analog of dihydroxyphenylalanine with a 3-hydroxy-4-pyridone ring instead of a 3,4-dihydroxyphenyl ring. The seeds, stems, pods, and leaf tissues of different *Leucaena* species have been shown to contain mimosine in various amounts (4, 5, 10, 13, 19). Seeds contain 4 to 5% mimosine on a dry-weight basis (14). The mimosine contents of different parts of the shoot vary from 1 to 12%; old stems contain the smallest and growing tips contain the largest amounts (13). *Leucaena* root also contains 1 to 1.5% mimosine (19).

Mimosine is a pyridoxal antagonist that inhibits growth and protein synthesis in microorganisms (7, 24). It has general antimitotic activity and is toxic for animals, causing reduction in growth, loss of hair, and general ill-health when *Leucaena* leaves are ingested (6,12,13). One of the degradation products of mimosine, 3-hydroxy-4-pyridone (HP), causes goiters, loss of hair, and reduced productivity when fed to animals (6, 11).

Previous studies of the metabolism of mimosine had established that enzymes present in the green tissues of *Leucaena* spp. can rapidly degrade mimosine to HP in macerated leaves (16, 25, 28). Mimosine is also converted to HP in the rumen of leucaena-fed ruminants (17). The rumens of these animals contain certain bacteria that degrade HP, thus protecting them from the harmful effects of mimosine and HP (14, 15). Allison et al. (1) recently characterized four strains of such a bacterium, *Synergistes jonesii*, originally isolated from the rumen of a goat in Hawaii.

Leucaena spp. are nodulated by root nodule bacteria belonging to the genus *Rhizobium*. In the present work, it is shown that some *Rhizobium* isolates from the nodules of *Leucaena* trees are able to utilize mimosine as their sole source of carbon

and nitrogen. We have shown that at least one of the enzymes involved in mimosine catabolism is inducible by the toxin and is located in the cytoplasm.

MATERIALS AND METHODS

Bacterial strains. Some *Rhizobium* isolates were obtained from the NIFTAL Project, Maui, Hawaii. A further 32 isolates were obtained from *Leucaena* nodules collected from different parts of the island of Oahu, Hawaii.

Media and growth conditions. *Rhizobium* strains were grown in TY (2), yeast extract-mannitol (YEM) (31), and *Rhizobium* mimosine (RM) media. The RM medium contained the following components per liter of deionized water: 250 mg of K_2HPO_4 , 100 mg of KCl, 10 mg of $Na_2EDTA \cdot 2H_2O$, 8 mg of $FeCl_2$, 1 ml of micronutrient solution, 0.5 ml of 1 M $CaCl_2$, 1 ml of 1 M $MgSO_4$, 5 ml of vitamin solution, and 10 ml of mimosine (Sigma Chemical Co, St. Louis, Mo.) stock solution (40 mg/ml). The micronutrient solution contained the following salts per liter of deionized water: 1.5 mg of $MnSO_4$, 1.1 g of $ZnSO_4 \cdot 7H_2O$, 170 mg of $CuCl_2 \cdot 2H_2O$, 50 mg of $Na_2MoO_4 \cdot 2H_2O$, and 10 mg of $CoCl_2 \cdot 2H_2O$. The vitamin solution contained (per liter) 100 mg of biotin, 100 mg of thiamine, and 100 mg of DL-pantothenate. Sterile stock solutions of $CaCl_2$, $MgSO_4$, vitamins, and mimosine were added to the medium after autoclaving. The pH of the medium was adjusted to 6.8 before autoclaving. To prepare a mimosine stock solution (40 mg/ml), 460 mg of mimosine was dissolved in 10 ml of 0.4 M NaOH, and 1.5 ml of 1.0 N HCl was added to adjust the pH to 8.4 and the final concentration of mimosine to 40 mg/ml. At this concentration, mimosine precipitates if the pH of the solution is brought below 8.2. In order to adjust for the high pH of the mimosine stock solution, 150 μ l of 1.0 N HCl was added to the RM medium for each milliliter of mimosine stock solution used. The final pH of the RM medium was approximately 7.0. RM medium appeared light yellow. To make RM agar medium, 15 g of purified agar (Fisher Scientific) per liter of RM broth was added before autoclaving. In some cases, 25 mg of bromothymol blue solution dissolved in 5 ml of ethanol or 25 mg of Congo red per liter of RM agar was added before

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autoclaving. RP medium contained HP (200 wg/ml) instead of mimosine.

Rhizobium isolates were screened for their ability to utilize mimosine as the sole source of carbon and nitrogen (Mid⁺) by streaking them on RM agar plates and incubating the plates at 28°C for 4 days. The growth rates of strains TAL1145 and TAL1566 in liquid RM medium with different mimosine concentrations were determined by inoculating 50 ml of RM medium into 250-ml screw-cap bottles with 0.5 ml of *Rhizobium* culture. Cultures were grown at 28°C with shaking and growth was determined every 6 h by measuring the cell density as optical density at 600 nm (OD₆₀₀) in a Spectronic 20 colorimeter (Bausch and Lomb, Rochester, N.Y.). Aliquots from each culture were also plated to check for possible contamination. Each treatment was replicated three times.

Growth of *Rhizobium* strains on mimosine versus general carbon and nitrogen sources. TAL1145 was grown in minimal medium supplemented with one of three different carbon sources or with mimosine as a standard. Ammonium sulfate served as the source of nitrogen when glucose or galactose was used as the source of carbon. For the treatment in which sodium glutamate was the source of carbon and nitrogen, ammonium sulfate was added to make the medium equal in nitrogen content to RM medium. The concentrations were chosen such that the same amount of carbon and nitrogen was present at the beginning of the incubation in each flask. The growth in each medium was monitored over time by measuring OD₆₀₀. Each treatment was replicated three times.

Mimosine determination. The mimosine or HP concentration in a sample was determined by means of high-performance liquid chromatography (HPLC) by using a C₁₈ column (4.6 by 250 mm; Rainin Instrument Co., Woburn, Mass.) with UV detection at 280 nm (29). Mimosine or HP was eluted by using a solvent system of 0.2% orthophosphoric acid (vol/vol) in filtered, deionized water at a flow rate of 1 ml/min. In this system, mimosine and HP had retention times of 2.7 and 4.8 min, respectively.

The mimosine used in the growth experiments was >95% pure as determined by ¹H nuclear magnetic resonance. It had a melting point of 226°C (decomposition) and a specific optical rotation of [α_D] = -21.4 (H₂O, C = 0.4), in agreement with values given in the literature.

The disappearance of mimosine and HP from the growth media. Media containing 400 μg of mimosine per ml (RM) or 200 μg of HP per ml (RP) were inoculated with TAL1145 (Mid⁺ Pid⁺), MS22 (Mid⁺ Pid⁻), or MS13 (Mid⁻ Pid⁻). The cultures were incubated at 28°C with shaking. Growth rate and the disappearance of mimosine or HP from the growth medium were monitored over time. Each treatment was repeated three times.

Lysis buffer. Lysis buffer was used during cell washing and cell disruption, as well as during incubation of the cell extract with mimosine. The buffer contained 50 mM Tris (pH 7.2), 50 mM NaCl, 10 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol, and 0.5 mM phenylmethanesulfonyl fluoride. Phenylmethanesulfonyl fluoride is added before cell disruption in order to protect the enzymes from endogenous proteases.

Cell disruption and enzymatic assay. The cells were harvested, washed once with lysis buffer, pelleted, and resuspended in 10 ml of lysis buffer before being broken with a French press at a pressure of 1,260 lb/in². The cells were put through the French press twice to ensure that most cells were lysed. The cell debris was removed by centrifugation (3,000 x g for 10 min at 4°C). The membrane fraction was separated by ultracentrifugation at 300,000 x g for 1 h at 4°C. The supernatant containing the soluble proteins was transferred to a test

TABLE 1. Number of Mid⁺ *Rhizobium* isolates originally isolated from nodules of different *Leucaena* plant species from different geographical locations

Location	No. of isolates from:				
	<i>Leucaena leucocephala</i>	<i>Leucaena diversifolia</i>	<i>Leucaena retusa</i>	<i>Leucaena shannoni</i>	<i>Leucaena macrophylla</i>
Maui, Hawaii	6 (5) ^a	2 (1)		1 (0)	
Oahu, Hawaii	16 (12)				
Other ^b	5 (4)	1 (1)	4 (4)		2 (0)

^a The numbers in parentheses indicate the number of isolates that were Pid⁺.

^b Other locations include New Guinea, Australia, the Philippines, Puerto Rico, Colombia, Honduras, and the United States (Tex.).

tube, and the pellet containing the membrane fraction was resuspended in 1 ml of lysis buffer. The protein concentrations in the soluble and membrane fractions were determined according to the method of Bradford (3). For the enzyme assay, the protein concentration in both the soluble and membrane fractions was adjusted to 2 mg/ml. Mimosine was added to the membrane and soluble fractions to a final concentration of 100 μg/ml. Control samples were run with protein boiled for 10 min before incubation with mimosine. Samples were incubated at 28°C with shaking. The reaction was stopped by incubating the samples at 75°C for 10 min and then subjecting them to centrifugation (3,500 x g) for 10 min. The supernatant was filtered through a 0.45-μm-pore-size filter (MSI Inc.) and subjected to HPLC analysis.

Induction of enzyme activity. In order to determine if the mimosine-degrading enzyme activity is inducible, TAL1145 was grown in TY at 28°C for 24 h with shaking, after which the cells were harvested and recultured in RM medium for 12 h. The cells grown in TY or RM medium were disrupted by the procedure described above.

Synthesis of HP. 3-Benzoyloxy-4-pyrone (27) was converted to 3-hydroxy-4-pyridone by the method of Harris (9). Analytical data obtained for the intermediates were in accord with published data.

RESULTS

Identification of Mid⁺ isolates. RM medium contains mimosine as the sole source of carbon and nitrogen. *Rhizobium* isolates that metabolize mimosine (Mid⁺) grew on the RM agar plates in 3 to 4 days. Growth was confirmed in RM broth for those that tested positive on solid medium. A total of 92 isolates were obtained from the nodules of various *Leucaena* species. Only 37 of these were able to use mimosine as a source of carbon and nitrogen at a substrate concentration of 400 μg/ml (Table 1). None of the *Rhizobium* strains isolated from the nodules of *Mimosa invisa*, *Mimosa pigra*, and leguminous trees such as *Acacia spp.*, *Prosopis glandulosa*, *Sesbania spp.*, *Robinia pseudoacacia*, and *Glyricidia sepium* grew on RM medium. *Rhizobium* strain NGR234 (30) and *Rhizobium tropicii* CIAT899 (18), which nodulate *Leucaena spp.*, and *Rhizobium* strains (*Aeschynomene*), including BTail (32), also did not utilize mimosine. None of the strains of *Rhizobium leguminosarum* bv. *viciae*, *R. leguminosarum* bv. *phaseoli*, *R. leguminosarum* bv. *trifolii*, *Rhizobium edi*, *Rhizobium meliloti*, *Rhizobium fredii*, and *Bradyrhizobium* that were tested utilized mimosine.

The final cell density of Mid⁺ *Rhizobium* strains in RM medium depends on concentration of mimosine. When *Rhizobium* strain TAL1145 was grown in RM medium containing 1,000 μg of mimosine per ml (Fig. 1), the cultures turned from

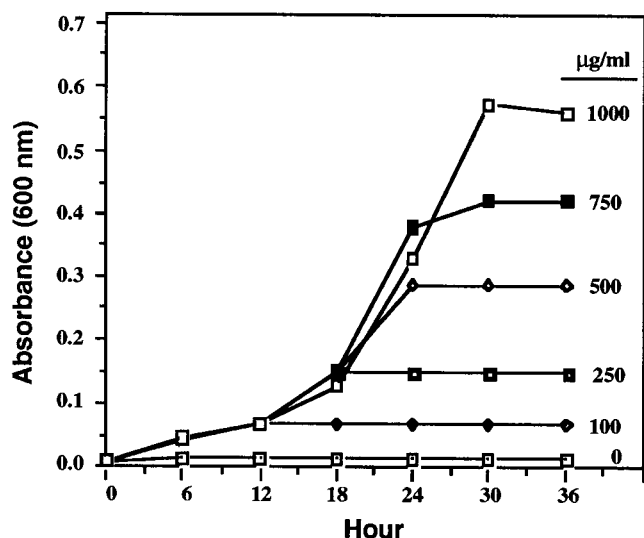


FIG. 1. Growth of *Rhizobium* strain TAL1145 in RM medium containing 0 to 1,000 μg of mimosine per ml. The numbers on the right indicate mimosine concentrations in the RM medium.

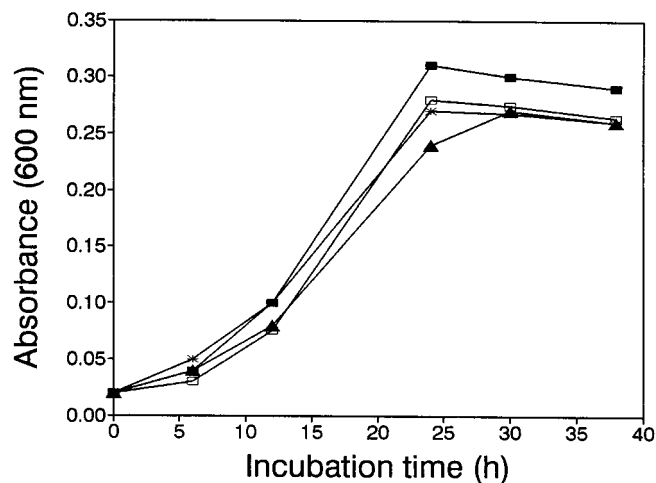


FIG. 2. Growth of *Rhizobium* strain TAL1145 on minimal medium containing 2.0 mM mimosine (solid triangle) or equivalent amounts of the carbon and nitrogen sources: glutamic acid plus ammonium sulfate (asterisk), glucose plus ammonium sulfate (solid square), and galactose plus ammonium sulfate (open square).

light yellow to colorless as they reached the stationary phase, at which point the mimosine in the medium was exhausted as determined by HPLC. At a substrate concentration of 1,000 $\mu\text{g}/\text{ml}$, a final OD_{600} of 0.62 ± 0.03 was reached after 30 h of incubation. At mimosine concentrations below this value, the stationary phase was reached after 12 to 24 h, whereas at higher concentrations mimosine appeared to be bacteriostatic but not bacteriocidal. The final cell density, determined as the OD_{600} , was directly proportional to the mimosine concentration in the RM medium. Qualitatively similar results were obtained with the Mid^+ isolate TAL1566.

Some Mid^+ isolates do not degrade HP. All Mid^+ isolates were screened for their ability to catabolize HP, which is known to be a chemical as well as an enzymatic degradation product of mimosine. Twenty-seven of the 37 Mid^+ isolates tested were able to use HP ($\text{Mid}^+ \text{Pid}^+$). Of the 10 isolates that do not use HP as the source of carbon and nitrogen ($\text{Mid}^+ \text{Pid}^-$), isolate MS22 was studied in more detail. Another isolate, MS13, which does not degrade either mimosine or HP ($\text{Mid}^- \text{Pid}^-$), was used as a negative control. In RM medium, MS22 grew to the same cell density as did TAL1145 ($\text{Mid}^+ \text{Pid}^+$). Under these conditions, HP was not excreted into the medium by MS22 and could not be detected in the cell lysate of cells actively catabolizing mimosine. In RP medium, on the other hand, no cell growth was observed with either MS22 or the $\text{Mid}^- \text{Pid}^-$ isolate MS13, whereas TAL1145 grew until HP was no longer present in the medium as determined by HPLC.

Growth of *Rhizobium* strains on mimosine versus general carbon and nitrogen sources. On the basis of a report in the literature (33), several carbon sources, e.g., citrate, proline, phenylalanine, tyrosine, glucose, galactose, and sodium glutamate, supplemented with ammonium sulfate as the nitrogen source, were screened for their ability to support growth of strain TAL1145 on minimal medium. Growth was observed in medium containing either glucose, galactose, or sodium glutamate. Thus, these carbon sources were chosen to evaluate the efficiency with which TAL1145 catabolizes mimosine. The concentrations of the carbon and nitrogen sources in each treatment were chosen such that each incubation mixture contained the same amount of "carbon" and "nitrogen" as RM

medium with 400 μg of mimosine per ml. The result of this time course experiment is shown in Fig. 2. It appears that mimosine is about as good a source of carbon and nitrogen as are sodium glutamate and the sugars combined with ammonium sulfate. Qualitatively similar patterns were observed in several repetitions of this experiment. In each case, cultures catabolizing sugars showed slightly higher final cell densities than did the one growing on mimosine. With sodium glutamate-ammonium sulfate as the carbon and nitrogen source, the final cell density was virtually the same as it was with mimosine.

The mimosine-degrading enzyme activity is located in the soluble fraction of the cell lysate and is inducible by mimosine. To establish whether the mimosine-degrading enzyme activity is located in the membrane or the soluble fraction and whether the activity is inducible by mimosine, extracts of cells of TAL1145 ($\text{Mid}^+ \text{Pid}^+$) and MS13 ($\text{Mid}^- \text{Pid}^-$) grown on RM as well as TY medium were prepared. Soluble and particulate fractions were separated by high-speed centrifugation, and the protein fractions so obtained were incubated with mimosine (0.5 mM). The consumption of substrate in each reaction mixture was monitored over time. The reaction rates were calculated from the linear portions of these time curves and are as follows. For induced cultures, mimosine degradation occurred at a rate of 5.0 nmol/mg of protein per min in the soluble fraction but was not detectable in the membrane fraction (the limit of detection was 0.2 nmol/mg of protein per min). For uninduced cultures, mimosine degradation was detectable in neither the soluble nor the membrane fraction. It appears that an enzyme system for mimosine catabolism is located in the soluble fraction and is inducible by the toxin, since the substrate is not affected by protein fractions of cells grown on complete medium or the protein in the particulate fraction of RM-grown cells.

DISCUSSION

Unlike opine synthesized by plants upon infection by *Agrobacterium* spp. (22) or rhizopines synthesized by alfalfa nodules infected by *R. meliloti* L5-30 (21), mimosine is naturally present in the shoots and roots of *Leucaena* spp. How-

ever, as with rhizopine utilization, only a limited number of *Rhizobium* isolates can utilize mimosine as a selective growth substance. The ability to catabolize mimosine is not required for nodulation or nitrogen fixation, since many strains that cannot catabolize mimosine, e.g., MS13 (Mid⁻ Pid⁻), can form nitrogen-fixing nodules on *Leucaena* spp. Mimosine utilization may be a specialized mechanism that some rhizobia and other microorganisms living in the rhizosphere of *Leucaena* spp. have developed to survive. It is conceivable that the ability to catabolize mimosine provides a competitive advantage to Mid⁺ strains of *Rhizobium* in the rhizosphere.

It appears, however, that there is no direct relationship between the ability to catabolize mimosine and nitrogen-fixing ability. For instance, TAL1566 degrades mimosine as well as TAL1145 does, but it is not a good nitrogen fixer, since *Leucaena leucocephala* plants inoculated with TAL1566 formed enough nodules but appeared chlorotic after 4 weeks whereas plants nodulated with TAL1145 were dark green (26). TAL 1145, which was reported to be a competitive strain for nodulation of *Leucaena* spp. (20), was identified in this study as a Mid⁺ strain. This strain was chosen for a detailed study of mimosine utilization among 37 Mid⁺ isolates because it is used as an inoculant for *Leucaena* spp. and is genetically characterized (8).

This is the first report to demonstrate that some rhizobia that nodulate *Leucaena* spp. catabolize mimosine and are able to use the toxin as their sole source of carbon and nitrogen. We found that more than one-third of the leucaena-nodulating isolates we studied grow on minimal medium containing mimosine. The final cell densities of cultures of strain TAL1145 grown on mimosine and on other general carbon and nitrogen sources (Fig. 2) matched closely. This suggests that *Rhizobium* strains convert the toxin mostly to cell mass and carbon dioxide and do not transform it to a defined metabolite which accumulates but cannot be detected by our analytical method.

Most Mid⁺ isolates were able to grow on HP (Pid⁺), while some isolates, such as MS22, could grow on mimosine but not on HP (Pid⁻). The latter were expected to accumulate or excrete HP when grown in RM medium, since we hypothesized at the onset of this study that catabolism of mimosine in *Rhizobium* spp. would follow the mechanism that was established for degradation of mimosine in *Leucaena glauca* (25), *L. leucocephala* (16), and animals (11, 12, 23). However, unexpectedly, MS22 growing on mimosine did not accumulate HP in the cells or excrete it into the medium. Furthermore, MS22 grew to the same cell density as did the Mid⁺ Pid⁺ strain TAL1145 in RM medium. This suggests that both strains can derive the same amount of nitrogen and carbon from a given amount of the toxin and that HP is not the end product of mimosine degradation in MS22. If HP were the product of mimosine degradation by Pid⁻ cells, MS22 should be able to extract only half as much nitrogen and carbon from mimosine as TAL1145. This should result in a lower final cell density than that of the Mid⁺ Pid⁺ strain at a given toxin concentration in the medium. This interpretation was supported by results of experiments with cell extracts. When mimosine was added to the cell extracts of MS22 grown in RM medium, mimosine disappeared within 3 h but HP was again not detected. Furthermore, when HP was incubated with a cell extract of MS22 grown in RM medium, no change in HP concentration was observed over 3 h. Even the cell extracts of the Mid⁺ Pid⁺ strain TAL1145, grown in RM medium, did not contain enzyme activity for degradation of HP. We postulate that the enzymes for HP catabolism or transport either are inducible by HP but not by mimosine or are unstable in the extract. In either case, this would suggest that two different

enzyme systems are responsible for mimosine and HP catabolism in *Rhizobium* spp. Whether an enzyme system for HP degradation can be induced in TAL1145 is currently under investigation. In summary, we have identified an inducible enzyme system in *Rhizobium* spp. capable of degrading the toxin mimosine produced in its symbiotic hosts *Leucaena* spp.

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