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Detection of Ruminal Bacteria That Degrade Toxic Dihydroxypyridine Compounds Produced from Mimosine

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***Leucaena leucocephala*, a tropical leguminous shrub, contains a toxic amino acid, mimosine. Successful utilization of leucaena as a ruminant forage depends on colonization of the rumen by bacteria that degrade dihydroxypyridines (DHP), which are toxic intermediates in the metabolism of mimosine. Populations in the rumina of animals in some parts of the world, however, do not include bacteria that are able to carry out this degradation. We thus describe tests for the presence of DHP degraders in ruminal populations that are based on degradation (loss) of DHP compounds from culture media. Results obtained with the tests indicate that DHP degraders were not part of microbial populations in the rumina of cattle, sheep, and goats in Iowa, while most rumen samples examined from animals from the Virgin Islands and Haiti contained DHP degraders. These results confirm and extend the findings of others about geographic limits to the distribution of these important ruminal bacteria.**

The tropical shrub leucaena (*Leucaena leucocephala*) is a persistent legume that is native to Central America and is now widely distributed. Leucaena has many uses, but its value in the human diet (18) and as a forage (14) has been limited by the presence of the free amino acid mimosine [β-(3-hydroxy-4-oxopyridyl) α-amino-propionic acid]. The toxicity of mimosine for ruminants is mainly due to 3-hydroxy-4-(1H)-pyridone (3,4-DHP), a goitrogen, that is produced from mimosine in the rumen (5).

After noting differences in tolerance to leucaena by animals in different geographic areas, it was proposed that tolerance was related to the presence or absence of ruminal microbes able to degrade 3,4-DHP (8). Support for this hypothesis was found when DHP-degrading capacity was transferred with the transfer of ruminal fluid from a leucaena-adapted Indonesian goat to Australian goats (11). Subsequently, Jones and co-workers demonstrated the acquisition of tolerance to leucaena diets by ruminants in Australia through ruminal inoculations with DHP-degrading bacteria that were part of a mixed culture that had been obtained from the rumen of a goat in Hawaii (12, 16). In view of this finding, it is clear that knowledge is needed about the presence of DHP-degrading bacteria in microbial populations that colonize ruminants in different geographic locales where leucaena is being fed and in areas where it may be introduced. This paper describes a simple method for making this determination.

MATERIALS AND METHODS

Media used for screening for the presence of DHP-degrading bacteria were modifications of medium 98-5 (2), a medium that contained 30% ruminal fluid and designed to cultivate the predominant bacteria in the rumen. Carbohydrates were deleted from medium 98-5, and either 3,4- or 2,3-DHP was added (Table 1).

The 2,3-DHP used was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis). To prepare 3,4-DHP, mimosine (Sigma Chemical Co., St. Louis, Mo.) was hydrolyzed in 0.2 N HCl in the presence of 2,4-dinitrophenyl-hydrazine, and the 3,4-DHP product of that reaction was subsequently purified by chromatography on Dowex-50 AG50 H^+ as described by Hart et al. (4). The quantity of 3,4-DHP prepared was estimated from measurements of A_{600} of $FeCl_3$ solutions as compared with solutions from recrystallized 3,4-DHP that had a melting point of 241°C.

Ruminal fluid used as an ingredient in culture media was from a bovine fed a hay-grain diet, and it was clarified as described previously (6). The B-vitamin solution was that described by Pittman and Bryant (15), and phytone peptone was obtained from BBL Microbiology Systems, Div. Becton Dickinson and Co., Cockeysville, Md. Before carbonate and reducing agents were added, the pH was adjusted to 6.4 and oxygen was displaced by boiling under a stream of O_2-free CO_2. All subsequent manipulations, including autoclave sterilization in rubber-stoppered tubes or serum bottles, were under an atmosphere of CO_2, using procedures of Hungate (7) and Holdeman and Moore (6). For field studies, syringes were used to inoculate media that had been prepared in serum bottles closed with butyl rubber septum-type stoppers (Bellco Glass, Inc., Vineland, N.J.). Ruminal fluid to be tested for DHP-degrading capacity was obtained from animals prepared with ruminal cannulae, from samples obtained by stomach tube, or from animals at slaughter. Inocula for tests of field samples (0.1 ml/5 ml of medium) were injected through the rubber stoppers, with care being taken to minimize injection of air. All incubations were at 37 to 39°C. In some instances, media were inoculated at a remote site, and there was a delay of 1 to 9 days while samples were shipped before incubation was started.

Degradation of the DHP compounds (or of mimosine) in culture media was routinely monitored through use of a colorimetric test. This test involved mixing 0.25 ml of sample with 5 ml of a ferric chloride reagent (0.6 g of $FeCl_3 \cdot 6H_2O$, 1 ml of concentrated HCl, diluted to 1 liter with H_2O). The presence of 3,4-DHP or mimosine (purple color) or 2,3-DHP (blue color) was readily detected visually, and a semiquan-

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TABLE 1. Culture media used to screen for the presence of DHP-degrading ruminal bacteria

Medium	Component (g or ml/liter)										
	Mineral solution		Resazurin solution[c]	Ruminal fluid	Phytone peptone	2,3-DHP	3,4-DHP	B-vitamin solution	Na_2CO_3	Cysteine hydrochloride · H_2O	$Na_2S \cdot 9H_2O$
	1[a]	2[b]									
2,3A	37.5	37.5	1	300		0.5		20	4	0.5	0.5
3,4A	37.5	37.5	1	300			0.5	20	4	0.5	0.5
3,4B	37.5	37.5	1		30		0.3	20	4	0.5	0.5

[a] Mineral solution 1: K_2HPO_4, 6 g/liter.
[b] Mineral solution 2: KH_2PO_4, 6; $(NH_4)_2SO_4$, 12; NaCl, 12; $MgSO_4 \cdot 7H_2O$, 1.2; $CaCl_2 \cdot 6H_2O$, 1.2 g/liter.
[c] Resazurin solution: 1 g/liter.

titative estimation of these compounds was provided from measurements of A_{600}. For more definitive estimates of degradation and for measurements of 3,4-DHP prepared as described above, samples were analyzed by high-pressure liquid chromatography (17).

The mixed culture used for much of this work was a subculture (in medium 2,3A; Table 1) of a mixed population of ruminal bacteria from a goat in Hawaii. This mixed culture (designated herein as culture A) included organisms that protected cattle and goats in Australia against mimosine-DHP toxicity (13). A bacterial population that was capable of degrading both 2,3- and 3,4-DHP was maintained during serial transfers of culture A (1 or 2% inoculum) in medium 2,3A or 3,4A (Table 1). Transfers were made when the colorimetric test indicated that most or all of the DHP had been degraded. The usual interval between transfers was 3 to 5 days. Strain 78-1, a bacterium isolated from culture A that degrades both 2,3- and 3,4-DHP, also was used for some of the experiments described here. Some of the properties of this isolate have been described before (1).

Ruminal samples (taken by stomach tube from 17 steers) were tested for mimosine-degrading capacity by the method described by Kudo et al. (13). The animals had been fed cracked-corn diets that contained various amounts of corn gluten feed (S. K. Duckett and A. H. Trenkle, J. Anim. Sci. 67[Suppl. 2]:78, 1989). Ruminal samples from these steers were also incubated with 3,4-DHP rather than mimosine (19 ml of ruminal contents plus 1 ml of 44 mM 3,4-DHP). Tests (colorimetric and with the high-pressure liquid chromatography analysis system) for loss of mimosine and DHP were made after 70 h of incubation. These ruminal samples were also tested for DHP-degrading bacteria by inoculating culture medium 3,4A and incubating for 12 weeks.

RESULTS

Modifications of media for detection of DHP degraders. Several modifications of medium 2,3A were made in attempts to enrich DHP-degrading organisms and to test the adequacy of media for detection of DHP degraders. Rates of degradation of 2,3-DHP by bacteria in mixed culture A were more rapid in media that contained 20 or 30% clarified ruminal fluid than with higher or lower levels of ruminal fluid (Fig. 1), and DHP degradation by the mixed population was not detected even after 20 days of incubation in a medium without clarified ruminal fluid. When the initial pH of medium 2,3A was adjusted to 4.5, 5.0, 5.5, or 7.1, the amount of DHP degraded by bacteria in mixed culture A during 21 days of incubation was not enough to be detectable by the colorimetric test. With initial pH of the medium set at 6.0, 6.5, or 6.8, however, all of the DHP had been degraded within 12 days of incubation (data not shown).

Bacteria in mixed culture A were able to degrade 2,3-DHP when ruminal fluid in the culture medium was replaced by yeast extract, Trypticase (BBL), and a mixture of volatile fatty acids as described for medium 10 (3). The rate of 2,3-DHP degradation was increased when the Trypticase concentration in the medium was increased from 2 to 10 g/liter and was decreased when Trypticase was replaced by casein hydrolysate (Fig. 2). These findings agreed with results obtained with the DHP-degrading pure culture, strain 78-1, isolated from mixed culture A (1). Strain 78-1 grew better when Trypticase or phytone peptone was added to culture media, and medium 3,4B, prepared with this property in mind, was used in some experiments for detection of 3,4-DHP-degrading bacteria in ruminal populations. Until more is known about the kinds of bacteria responsible for DHP degradation, however, it may be better to use medium 3,4A as a screening medium as it would probably be less selective than medium 3,4B.

Sensitivity of cultural method for detection of DHP degraders. To test the sensitivity of the cultural method for detection of DHP degraders, test media (2,3A and 3,4B) were inoculated with dilutions of a culture of strain 78-1 plus 0.1 ml of freshly collected ruminal contents from a cow in Iowa. Concentrations of viable colony-forming units of strain 78-1 in cultures added to the mixed ruminal population were determined from colony counts in agar roll tube cultures. When strain 78-1 cells were not added to the ruminal bacteria in the inoculum, DHP degradation was not detected during incubation for as long as 17.5 weeks.

In one experiment when the inoculum was estimated to

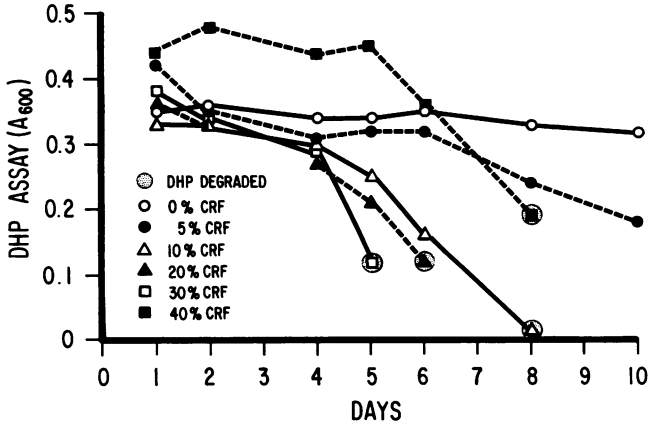

FIG. 1. Degradation of 2,3-DHP as affected by clarified ruminal fluid (CRF) in the culture medium. The encircled symbol designates that DHP had been degraded to the extent that a blue color was not detected in the $FeCl_3$ test.

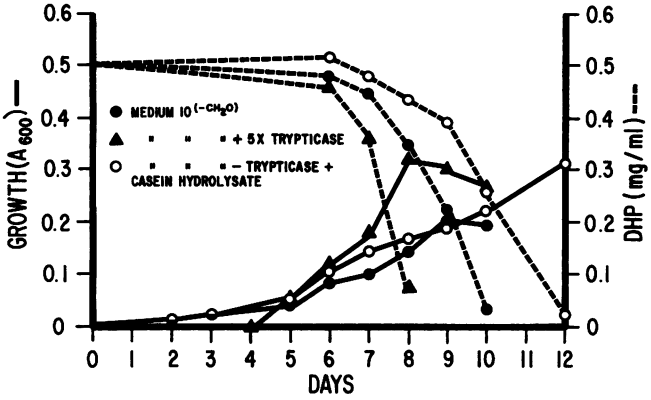

FIG. 2. Degradation of 2,3-DHP as affected by sources of organic nitrogen in the culture medium.

contain 5 to 6 CFU of strain 78-1, the 2,3-DHP (in medium 2,3A) had been degraded to the extent that the $FeCl_3$ test was negative in three of three tubes after 6 weeks (but not after 4 weeks) of incubation. With 10- or 100-fold-greater concentrations of strain 78-1, only 4 weeks of incubation were required for degradation of the DHP. Results of a similar experiment in Table 2 support the hypothesis that this cultural test is able to detect DHP-degrading bacteria even when they are a minor component of the ruminal bacterial population. Incubation periods required for detection of low concentrations of DHP degraders may, however, need to be long when numbers of DHP degraders are low.

Estimates of the concentration of DHP-degrading bacteria in mixed population A were made from measurements of DHP degradation in culture media that had been inoculated with 10-fold dilutions of the mixed population. In one such experiment, based on three-tube most-probable-number estimates (which assume each viable unit is detected in the test system), the concentration of DHP degraders was 1.5×10^7/ml, while the total concentration of viable cells (based on positive growth in peptone-yeast extract-glucose medium) was 21×10^7/ml.

Tests for mimosine and DHP degradation in ruminal contents incubated in vitro. When ruminal fluid samples from feedlot steers (17 animals) were incubated with 3,4-DHP (19 ml of ruminal fluid plus 1 ml of 44 mM 3,4-DHP), no evidence for degradation of DHP during incubation for 70 h was found either by the colorimetric $FeCl_3$ test or by measurements of 3,4-DHP, using high-pressure liquid chromatography. When the incubations were with mimosine, as described by Kudo et al. (13), rather than 3,4-DHP, minor losses of substances that absorbed at 535 nm in the $FeCl_3$ test were detected in 3 of the 17 samples. Analysis of these samples by high-pressure liquid chromatography suggested that changes could have been related to conversion of mimosine to 3,4-DHP, but no evidence for loss of mimosine plus 3,4-DHP in these in vitro incubations was found. Cultural tests for DHP degraders, using screening medium 3,4A, also indicated that DHP degraders were not part of the microbial populations in the rumen of any of these animals.

TABLE 2. Cultural detection of DHP-degrading ruminal bacteria

Inoculum (CFU)[a]	Wk of incubation for positive DHP degradation[b]		
	4 tubes[c]	3 tubes[c]	0[d]
7×10^5	4		
7×10^4	5.5		
7,000	6.5		
700	7.8		
70		8.3	
7		11.5	
None			(>17.5)

[a] Quantity of strain 78-1 added to 0.1 ml of fresh bovine ruminal contents and then used to inoculate each tube of culture medium.
[b] Values are the mean of incubation time required for degradation of DHP in the test media (two tubes of medium 2,3A and two tubes of 3,4B) to the extent that DHP was no longer detected by the $FeCl_3$ test.
[c] Number of tubes with DHP degraded from four tubes that were similarly inoculated.
[d] DHP was not degraded in any of the four tubes.

TABLE 3. DHP-degrading bacteria in ruminal contents from animals at different locations

Species (n)	Location	No. of animals		
		DHP degraded[a]		DHP not degraded (neither 2,3- nor 3,4-DHP)
		2,3- and 3,4-DHP	2,3-DHP only	
Cattle (24)	Iowa	0	0	24[b]
Cattle (6)	Texas	0	0	6
Cattle (20)	Florida-1[c]	0	6	14
Cattle (20)	Florida-2[d]	0	20	0
Cattle (5)	Virgin Islands	4	1	0
Cattle (6)	Haiti	2	1	1
Sheep (4)	Iowa	0	0	4
Sheep (5)	Virgin Islands	5	0	0
Goats (4)	Iowa	0	0	4
Goats (7)	Haiti	4	2	1

[a] Populations that degraded 3,4-DHP, but not 2,3-DHP, were not detected.
[b] Ruminal populations from 17 of these animals failed to degrade 3,4-DHP but were not tested for 2,3-DHP degradation.
[c] Hereford cattle at Sub-Tropical Agricultural Research Station, Brooksville, Fla.
[d] Senepol cattle at Sub-Tropical Agricultural Research Station that had been imported from St. Croix, U.S. Virgin Islands, 3 years prior to these tests.

Cultural tests for the presence of DHP-degrading bacteria in ruminal contents from animals at different geographic locations. The results of tests for the presence of DHP-degrading bacteria in samples of ruminal contents from cattle, sheep, and goats in Iowa, Texas, Florida, the U.S. Virgin Islands, and Haiti are summarized in Table 3. When media 2,3A and 3,4A were inoculated with ruminal contents from goats and cattle from herds in Iowa, there was no evidence for degradation of DHP even after long (16 weeks) incubation. Similar results were obtained with media inoculated with ruminal contents from six head of cattle in south Texas.

When tests were conducted with ruminal contents from cattle at the Sub-Tropical Agricultural Research Station in Florida, 2,3-DHP was degraded in 20 of 20 samples of medium 2,3A that had been inoculated with ruminal contents from a group of Senepol cattle. This group of cattle had been brought to Florida from St. Croix, U.S. Virgin Islands, 3 years prior to our test. Tests for 3,4-DHP degradation were negative with all of these samples. When the inoculum was ruminal contents from Hereford cattle (from the same Florida station, but which had been maintained in an area separate from the Senepol cattle), 2,3-DHP was degraded in 6 of 20 samples. The 3,4 isomer of DHP was not degraded in any of the cultures inoculated with ruminal microbes from animals in Florida (Table 3). These results indicate that the level of separation of the Hereford cattle was not adequate to completely prevent transmission of 2,3-DHP-degrading bac-

teria from the Senepol cattle to some of the Herefords. Additional details concerning tests with cattle in Florida are given by Hammond et al. (3a).

Ruminal samples obtained in the U.S. Virgin Islands were all from animals that had been maintained on pastures where the leucaena presence was characterized as either heavy or moderate. Microbes from ruminal contents from five of five sheep and five of the five cattle tested degraded 2,3-DHP. The 3,4 isomer of DHP was degraded by microbes from all of these sheep and also by microbes from four of the five cattle (Table 3).

Ruminal samples from four of seven goats and from two of four cattle in Haiti degraded both 2,3- and 3,4-DHP, while samples from one of the cattle and two of the goats degraded only 2,3-DHP. Microbes in ruminal contents from one of the Haitian cattle and from one of the goats failed to degrade either of the DHP isomers (Table 3).

Tests for DHP degraders in fecal samples. Samples from cattle at the Sub-Tropical Agricultural Research Station, Brooksville, Fla., were tested to determine whether or not colonization of the intestinal tract by 2,3-DHP-degrading bacteria could be detected with fecal samples as well as with ruminal contents. Three groups (five animals each) were sampled: Senepol cattle and two groups of Hereford cattle. Prior tests of ruminal contents indicated that 2,3-DHP-degrading bacteria were present in ruminal contents of the Senepol cattle, while they were not detected in ruminal contents of the Hereford cattle. One group of Herefords was maintained separately as a control group, while the other group of "contact Herefords" was held in the same pasture with the positive Senepol cattle. Positive tests for degradation of 2,3-DHP from both ruminal and fecal samples were obtained from three Senepols and two Hereford contact animals. Positive tests for 2,3-DHP degraders in ruminal, but not in fecal, samples were obtained with two Senepol cattle, two of the contact cattle, and one of the Herefords that was not known to have been in contact with the Senepols. There were no examples of a positive fecal sample when the corresponding ruminal sample was negative.

DISCUSSION

Aerobic bacteria that degrade DHP compounds have been studied (19), but only recently have anaerobic bacteria that degrade DHP been isolated from ruminal contents, and information about the isolates is still meager (1). Results we obtained support the findings of Jones and co-workers (9, 11, 12) in that DHP-degrading bacteria are a component of microbial populations that colonize the rumina of animals in some, but not all, parts of the world. Details concerning the geographic distribution of DHP degraders and reasons for the limits to their distribution are not yet known. Important questions about factors that affect the transmission and colonization of rumina by DHP-degrading bacteria, possible reservoirs of DHP degraders in gastrointestinal populations of other herbivores, etc., also remain to be answered.

One way to test for the presence of DHP-degrading bacteria in ruminants that are feeding on leucaena is to examine urine for the presence of high levels of DHP, either free or as the glucuronide (5, 12). The cultural test we describe, however, is able to detect DHP degraders that persist in animals long after leucaena has been removed from the diet (3a). Other advantages of this cultural method are that it is relatively simple to perform, the sensitivity of the test is high, and falsely positive tests for the presence of DHP degraders do not seem to be a problem. A disadvantage of this cultural test is that long incubation times are required when concentrations of DHP degraders in mixed populations are low. Reduction of the amount of DHP in the culture media may serve to reduce the incubation time required.

We propose medium 3,4A (Table 1) as the medium of choice for screening microbial populations for the presence of bacteria that degrade 3,4-DHP. In our experience, this medium in butyl rubber-stoppered serum bottles has a shelf life of at least 6 months. If inoculated medium is sent to a laboratory for incubation, the only materials needed in the field would be medium in serum bottles, sterile syringes, and needles. If it is desirable to complete tests at a field station, the only additional material needed for the colorimetric test would be the ferric chloride solution, test tubes, and an incubator.

Preparation of culture medium 3,4A could also be accomplished with little special training or microbiological laboratory facilities. Ruminal fluid and 3,4-DHP are ingredients that are not commercially available. In some of our experiments, ruminal fluid was replaced by Trypticase, yeast extract, a volatile fatty acid mixture, and hemin, as described for medium 10 (3), or by phytone peptone (Table 1). Degradation of DHP by microbes in mixed culture A and by other ruminal populations occurred in such media, but until more is known about nutritional requirements of the functional DHP degraders, we believe that screening results may be more reliable with a medium that contains ruminal fluid than with substitutes for it. The B-vitamin mixture (Table 1) is probably not needed when ruminal fluid is a prominent constituent of the culture medium.

Media used in our first experiments and those of Jones (9) contained 2,3-DHP, which is commercially available, rather than 3,4-DHP. A pure culture isolated from mixed culture A degrades both 2,3- and 3,4-DHP (1), and while 2,3-DHP is an intermediate in the metabolism of 3,4-DHP (10), results reported here and by Hammond et al. (3a) indicate that ruminal bacteria able to degrade 2,3-DHP but not 3,4-DHP also exist. Since the 3,4 isomer is the immediate product of mimosine degradation, it seems unlikely that protection from toxicity could be provided by bacterial populations able to degrade the 2,3 but not the 3,4 isomer of DHP. Thus, a screening medium to detect bacterial populations able to provide ruminants protection from leucaena toxicity should contain mimosine or 3,4-DHP rather than 2,3-DHP.

To simplify screening for DHP degraders, we demonstrated that DHP degraders could also be detected with inocula prepared from bovine feces. Bacteria that degraded 2,3-DHP were detected in fecal samples from 5 of 10 cattle that were positive for the presence of DHP degraders based on tests of ruminal fluid. Thus, the sensitivity of the cultural method for detection of colonization was less with fecal than ruminal samples, and with ruminal samples DHP degradation was detected after shorter incubation time than was required for fecal samples (data not shown). Although it would be more reliable to use ruminal samples, our data indicate that, if samples are taken from a number of animals, it should be possible to use fecal samples as inocula in tests to detect whether or not a group of animals is colonized by DHP degraders. While we do not have enough data to make recommendations on the number of animals that should be sampled from a given population (either by ruminal or fecal samples), experience in Australia (16) and in Florida (3a) indicates that introduced DHP-degrading organisms are readily distributed among animals in a group.

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