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PCR Detection of the Pyridinediol-Degrading Ruminal Bacterium, *Synergistes jonesii*, in the Rumen Fluid of Cattle

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Abstract

Synergistes jonesii, a Gram-negative rumen bacterium, can catabolize a toxic pyridinediol, 3-hydroxy-4-pyridone, which is produced from mimosine present in *Leucaena* fodder in the rumen. Thus, it protects ruminants from the harmful effects of this toxin. A PCR-based method was developed to detect the presence of this pyridinediol-degrading bacterium in the rumen fluid of cattle. A unique 0.9-kb DNA sequence was identified by sequencing several fragments from a genomic library of *S. jonesii* strain 78-1 and four pairs of primers were designed and used for PCR amplification of specific fragments. The PCR conditions were optimized with one pair of primers that amplified a 349-bp fragment from *S. jonesii* DNA but not from any other bacterial DNA tested. This method could detect the presence of 1 to 10 bacteria in a sample. The method was tried on rumen fluid samples added with a known number of the bacterium. With a simple pretreatment of the mixed sample, the PCR method reached a sensitivity only 100 fold lower than with pure-culture samples. Application of this method detected the presence of *S. jonesii* in some of the rumen fluid samples from cattle in Hawaii. This method will be a valuable tool to determine the effects of inoculation of cattle with *S. jonesii* and to monitor the population dynamics of this pyridinediol-degrading bacterium in cattle fed with different amounts of *Leucaena*.

Keywords: Mimosine, *Leucaena*, rumen bacteria, symbiosis

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1. Introduction

Leguminous trees and shrubs of the genus *Leucaena* are common in many tropical and subtropical countries. Their high tolerance to drought, resistance to pests and diseases and ability to grow in a wide variety of soil types make them important trees in agroforestry (Brewbaker and Sorensson, 1990). The leaves, young stems, flowers, and pods are all good sources of protein and minerals for livestock. Leaves of *Leucaena* are comparable with alfalfa or lucerne leaf material in terms of protein and minerals (Jones, 1979). *Leucaena* is very easy to grow and its yield potential is comparable to that of alfalfa. The new improved varieties of *Leucaena* spp. developed at the University of Hawaii give some of the highest yields reported for tropical fodder and wood (Brewbaker et al., 1990). However, as a fodder, *Leucaena* may have harmful effects because of the presence of a free amino acid, mimosine, which is known to be toxic to animals (Hegarty et al., 1964). Structurally, mimosine [ß-N(3-hydroxy-4-pyridone)-α-aminopropanoic acid] is an analog of dihydroxyphenylalanine with a 3-hydroxy-4-pyridone ring instead of a 3,4-dihydroxyphenyl ring. The seed, stem, pod and leaf tissues of different *Leucaena* species contain 1 to 12% mimosine (Jones, 1979).

Mimosine has general antimitotic activity that blocks the cell cycle at late G1 (Boehme and Lenardo, 1993; Khanna and Lavin, 1993). It was found to arrest cell division in cultured human cells (Hoffman et al., 1991; Watters et al., 1994; Telfer and Green, 1993) and in Chinese hamster cells (Mosca et al., 1992). It also inhibited replication in yeast (Levenson and Hamlin, 1993) and cultured petunia leaf cells (Perennes et al., 1993). Experiments with tissue and cell cultures suggest that it is particularly the mitotic stages that are most susceptible to mimosine. In hair follicle bulbs, for instance, mimosine induced degeneration of the mitotic region resulting finally in hair loss (Montagna and Yun, 1963; Reis et al., 1975). The underlying molecular mechanisms of blocking the cell cycle by mimosine are not as yet understood. Feldman and Schonthal (1994) have shown that mimosine prevents the serum-stimulated synthesis and activation of histone H1 kinase, a crucial regulator of cell cycle progression. Recently, Gilbert et al. (1995) have shown that mimosine inhibits DNA synthesis preventing the formation of the replication fork by altering deoxyribonucleotide metabolism. It is also known to chelate metals, bind pyridoxal phosphate and inhibit the enzymes tyrosine decarboxylase, tyrosinase (Thomson et al., 1969) and ribonucleotide reductase (Dai et al., 1994).

The toxicity of mimosine has been shown particularly in connection with animal husbandry. Mimosine is hydrolyzed to a toxic pyridinediol, 3-hydroxy-4-pyridone (DHP) by leaf enzymes during ingestion in the rumen

(Hegarty et al., 1979; Lowry et al., 1983). Because of the toxic effect of mimosine, sheep unaccustomed to *Leucaena*, shed their wool approximately 7 to 14 days after feeding on *Leucaena* (Hegarty et al., 1964; Reis et al., 1975). Similarly, mice having 10% *Leucaena* seed or 1% mimosine in the diet showed loss of hair (Crounse et al., 1962). Gross pathological signs related directly to mimosine consumption included ulceration of the lingual and esophageal epithelia, atrophy of the gums, necrotic papillae in the esophagus and stomach, follicular cell hyperplasia and colloid accumulation in the thyroid gland, depressed serum thyroxine level, focal replacement of eye-lens fibers by globular bodies (Reis et al., 1975; Holmes et al., 1981; Megarrity and Jones, 1983).

In spite of the high toxic effects of mimosine on animals, *Leucaena* leaves are used as fodder for sheep and cattle in some countries where the ruminants contain a bacterium that degrades mimosine and DHP to nontoxic products, thus protecting the animals from the harmful effects of these compounds (Jones and Megarrity, 1983). These anaerobic bacteria establish a symbiotic association with ruminants by colonizing the rumen of these animals and help the animals to feed on *Leucaena* without any harmful effect of DHP. Jones and Megarrity (1986) showed that the DHP-degrading rumen bacteria isolated from a goat in Hawaii could be cultured in the laboratory. When these bacteria were used to inoculate the rumen of cattle in Australia and Florida, these animals became colonized with the bacteria and were protected against the harmful effects of mimosine and DHP (Jones and Megarrity, 1986; Hammond et al., 1989). Allison et al. (1992) characterized these bacteria and designated them as *Synergistes jonesii*.

To plan an effective animal feeding program with *Leucaena* as the major fodder, it is important to know if all or certain groups of cattle contain these bacteria and therefore possess the ability to detoxify DHP. The major drawback in identifying *S. jonesii* is that it is an obligate anaerobe and therefore is not easy to grow in the laboratory. Therefore, it was important to develop an easy detection method using small samples of rumen fluid. The purpose of this study was to develop a PCR method for detection *S. jonesii* in the samples of rumen fluids of cattle.

2. Materials and Methods

Bacterial strain

Synergestis jonesii strain 78-1 (ATCC 49833) was grown under anaerobic conditions as described by Allison et al. (1992).

DNA preparation and Southern hybridization

Genomic DNA was prepared using standard procedures (Sambrook et al., 1989). For Southern hybridizations, genomic DNA (~3 μg) was digested with restriction enzymes, electrophoresed on a 1.0% agarose gel and transferred to a nylon membrane. DNA probes were labeled by random priming using the dioxigenin labeling and detection kit from Boehringer Mannheim, Indianapolis, USA. Digoxigenin-labeled probes were used to hybridize at 42°C in a buffer containing 5× SSC, 0.02% SDS, 2% blocking reagent and 50% formamide (1× SSC is 0.15 M NaCl, 15 mM sodium citrate) for 12 h and washed at 60°C with 2× SSC, 0.1% SDS for 30 min. Chemiluminescent alkaline phosphatase substrate Lumi-Phos 530 (Boehringer Mannheim) was used to detect signal by exposure to the X-ray films.

DNA sequence analysis

The 0.9-kb *Pst*I fragment was sequenced in both orientations with an automated DNA sequencer (Model 373A, Applied Biosystems Co., CA) at the Biotechnology and Molecular Biology Instrumentation Facilities, University of Hawaii. The pUC18 forward and reverse primers were used to sequence from the both ends and additional sequence in each direction was obtained by using synthesized primers. The sequencing data were analyzed using the Experimental BLAST Network Service (Altschul et al., 1990).

PCR methods

The PCR reactions were done using the GeneAmp PCR system 2400 (Perkin Elmer). Standard PCR reactions were done in a final volume of 50 μl which included 100 ng of sample DNA, 200 μM each of dATP, dCTP, dGTP and dTTP, 0.2 μM each of specific primers, 2.5 mM $MgCl_2$ and 1.5 U of *Taq* polymerase (Promega, Madison, WI). 'Hot start' at 94°C for 5 min was used to avoid non-specific DNA amplification. Thermal cycling was done as follows for 25–45 cycles: denaturation for 30 sec at 94°C, primer annealing at 60–67°C for 30 sec and extension at 72°C for 30 sec. The following primers were used: 1F : CTT TGGCGTCGCCTACTTCGA; 2F : GCC CCT GAA TCC GGA TAT GAGG; 3R : GCC ATC GCC TAC GCC AAA CAG; 4R : CCA ATT TGA GAA CGA CAA AGC GA.

Preparation of rumen fluid samples

1 ml of rumen fluid was centrifuged in a microfuge tube at 14,000 rpm for 2 min and the supernatant was discarded. Usually the solid materials in the rumen

fluid accumulate in the lower part of the pellet and bacteria precipitate as a loose upper layer. This upper layer of the pellet is resuspended in 1 ml of 1M NaCl and transferred to a new tube and spun for 2 min. The supernatant was discarded and the pellet was washed once with 1 ml NaCl and once with 1 ml dH_2O. Finally the pellet was resuspended in 10 µl of water and 1 µl of this sample was used for each PCR reaction.

Construction of a genomic library of S. jonesii

A genomic library of strain 78-1 was constructed in pLAFR3, a cosmid vector with low copy number and broad host range (Staskawicz et al., 1987). Total genomic DNA from strain 78-1 was partially digested with Sau3A and size-fractionated in 0.7% agarose gel. The 20–30-kb DNA fragments were electroeluted, cloned into the *Bam*HI site of pLAFR3, and packaged *in vitro*. The packaged DNA was transduced into *Escherichia coli* LE392, and the cosmids were selected on LB medium containing tetracycline (5 µg/ml).

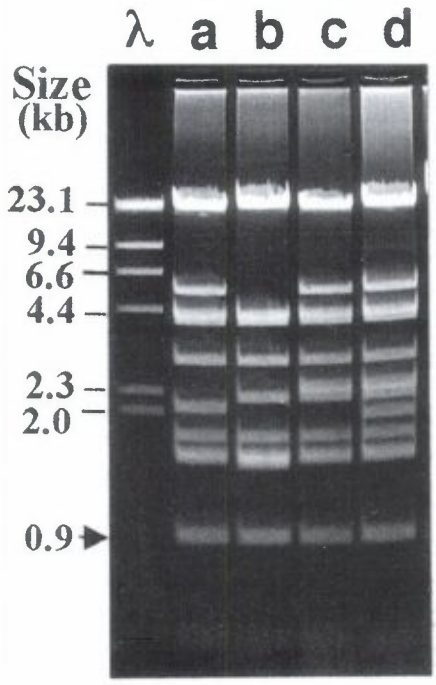

Figure 1. Common DNA fragments in four cosmid clones containing possible DHP-degradation genes from a *S. jonesii* genomic library. The cosmids were digested with *Pst*I and the DNA fragments were separated on a 1% agarose gel. The arrow shows the 0.9-kb fragment.

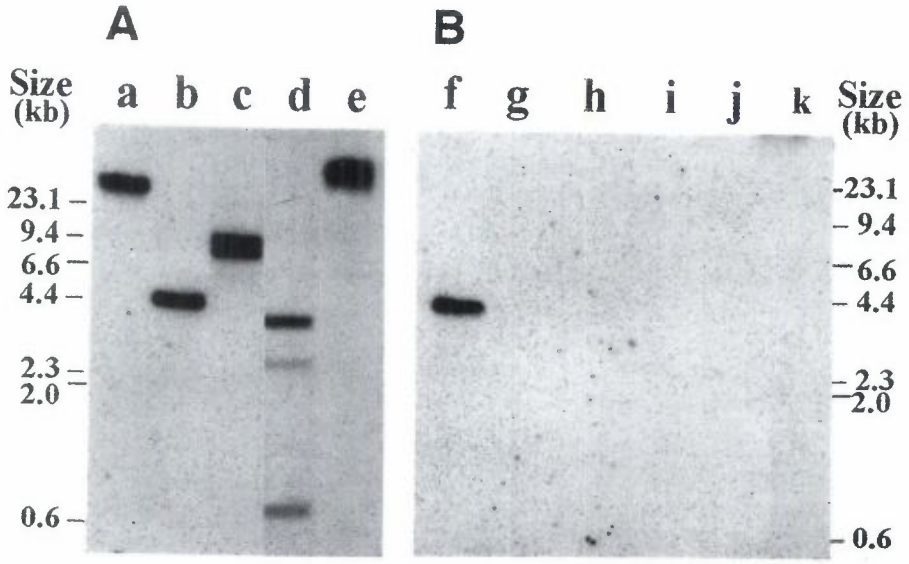

Figure 2. Southern hybridization of genomic DNA of *S. jonesii* (A) and other bacteria (B) with 0.9-kb *Pst*I fragment. The probe hybridized with *S. jonesii* DNA digested with *Bam*HI (lane a), *Eco*RI (lanes b and f), *Hind*III (lane c), *Sal*I (lane d) and *Xba*I (lane e). It did not hybridize with *Eco*RI digested DNA of *Agrobacterium tumefaciens* (lane g), *E. coli* (lane h), *Listeria* monocytogenes (lane i), *Pseudomonas fluorescens* (lane j), and *Rhizobium* sp. strain TAL1145 (lane k).

3. Results

Isolation of overlapping clones

The genomic library in *E. coli* was plated on minimal agar medium containing DHP (400 μg/ml) as the sole source of nitrogen. After 3 days some small colonies grew, a few of which were isolated, restreaked on the same medium and purified. Plasmid DNA isolated from eight such colonies was digested separately with *Eco*RI, *Hind*III and *Pst*I showed overlapping DNA fragments and was classified into four different plasmids (Fig. 1).

Subcloning and partial sequencing of fragments

The four overlapping plasmids contained at least six common *Pst*I fragments. To isolate DNA sequence unique for *S. jonesii*, three common *Pst*I fragments, 0.9 to 3.0 kb in size, were subcloned in pUC18 and partially sequenced from the two ends. When the sequenced data were compared with the GenBank database

```
   1  CTGCAGGTCG ACGGATCTGC GTGGCGACGC GCCGTATTGG TCGGCGCGGG
       PstI
  51  CCTGCGCCTC TTCGGTTATA GCGGCTTTGG CGTCGCCTAC TTCGACGAAA
                                  Primer 1F ──────────►
 101  GCGTTGTCTA TGCGCAGGGC GTAGCGCTTC GTTTTCCGCC GCGCGGACGA

 151  TTTGTTCGGT TTCTATCAGC GCTTTGTTTT CGTTCATCTG GGCGACGAGG

 201  GCTTCGCGTT TCGTCGCTTC GGCGATTTCG TTTTCTTTTA CGCGCGTATC

 251  AAGGTCGCTG TAAAATCCGG CCATCTGCCG GCGTATGGAC TGCTCGCCCC
                                                      Primer
 301  TGAATCCGGA TATGAGG GCG GCTAAATCCG CGTCGCCGGC CGAGTCGTCT
      2F ──────────►
 351  ACGCTCGCTA TCATGGCGTT GTGCATTTTT CCATAGGCTT CGTCTCTGTC

 401  GGCCCGCAGC TCGGCTTCTT CGATTATCGC CGCGCGGTTT GAATCGACGT

 451  CCACTATCAC GCGGTCTACC GAGCGTGGCG ATGGCTTCGT CCTCCCGCGC

 501  GCGCAGTTCT TTTTCGGAGA TGAGCGAGGC GGCGGCGTTT TTTATCTGCG

 551  CTTCGAGTAT GAGCCTCGAC GCCGCTTCGG CTTCGAGTCG CCCGTCGACT

 601  ATGTTGAGCC GCCCGATGAG TTCCTGTTTG GCGTAGGCGA TGGCTTGTCT
                                  ◄────────── Primer 3R
 651  GGTCGCTTTG TCGTTCTCAA ATTGGTTGGA GGCGTTCTGT ATGACGCCTT
       ◄────────── Primer 4R
 701  CCGCGAGCGC GTCGAAGTCG TCCGCGTACT GCCCGACGAA GCCGCTTATG

 751  GTGCCGTTCT GCGCGTCTAT TTTCACTTCG GCCGCCTCTA TGCGCGCCAC

 801  GGCCAGGTCG AGTTCGACGA GGGAGGCTTT GAGCGCTATG GCCGCGTCGA

 851  GCCCCTGCAG
       PstI
```

Figure 3. The nucleotide sequence of the 0.9-kb *Pst*I fragment. The regions of the sequence from where the primer sequences were selected are shown in bold and the directions of the primers are indicated with arrows.

two fragments showed some sequence similarities with other known sequences in the database. However, the complete sequence of the 0.9-kb *Pst*I fragment or its deduced amino acid sequence did not show any similarities with any known sequences. When the 0.9-kb *Pst*I fragment was used as a probe in Southern

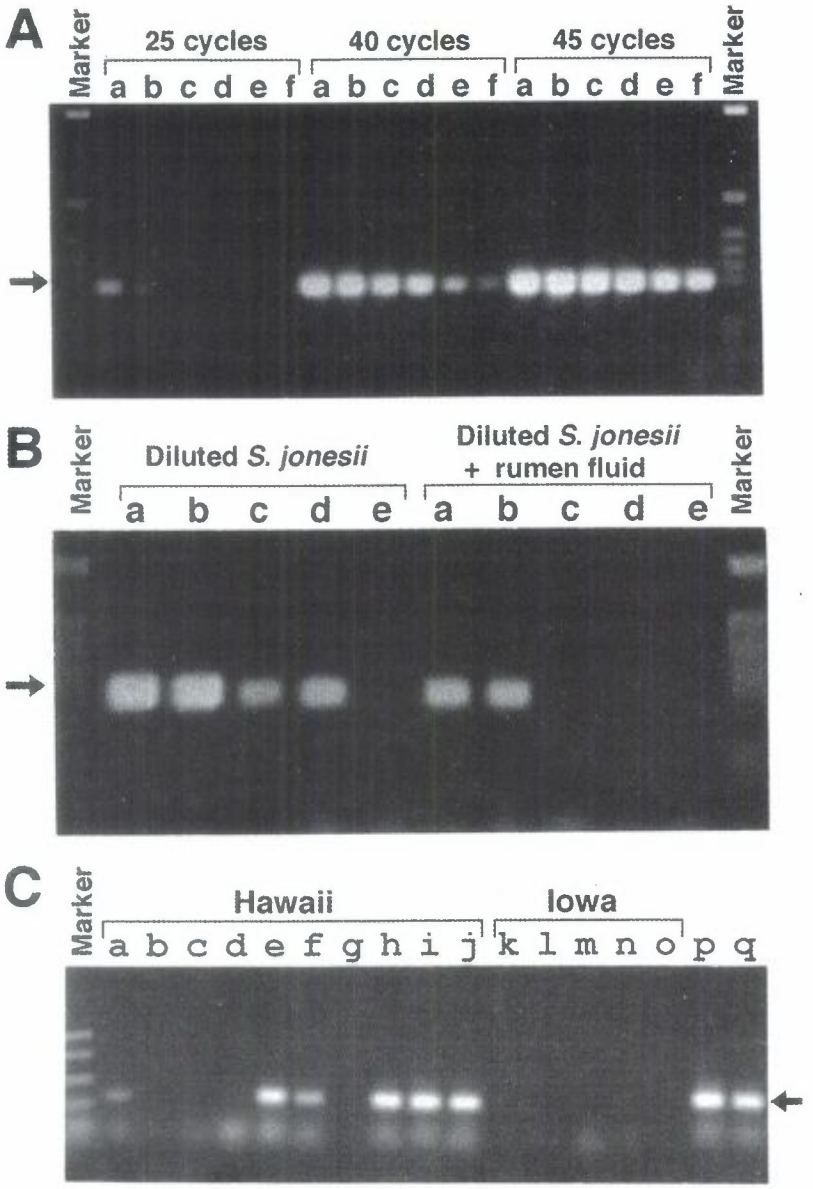

Figure 4. A: PCR detection of *S. jonesii*, using: 25, 40 and 45 PCR cycles different numbers of *S. jonesii* cells. The approximate number of *S. jonesii* cells in lanes a, b, c, d, e and f are 10^6, 10^5, 10^4, 10^2, and 10, respectively. B: Diluted *S. jonesii* cells were mixed with cattle rumen fluid and reisolated before doing PCR. The approximate number of *S. jonesii* cells in lanes a, b, c, d, and e are 1000, 100, 10, 1 and 1, respectively. C: Detection of *S. jonesii* in Hawaiian cattle using PCR method with bacteria isolated from 100 µl of rumen fluid samples.

hybridization, it hybridized strongly with the genomic DNA from *S. jonesii* but not with those from *Agrobacterium tumefaciens*, *E. coli*, *Listeria* monocytogenes, *Pseudomonas fluorescens* and *Rhizobium* sp. strain TAL1145 (Fig. 2). This suggested that the sequence of the 0.9-kb fragment might be unique for *S. jonesii* and might be used for developing a PCR detection method for this organism. The 0.9-kb *Pst*I fragment was completely sequenced in both directions and the nucleotide sequence is shown in Fig. 3.

Optimization of PCR condition for detection of S. jonesii bacteria

Two forward and two reverse primers were designed based on the 0.9-kb sequence of *S. jonesii* (see Materials and Methods). Four different paired combinations of these primers were used to amplify *S. jonesii* DNA through PCR. One pair of these primers (2F : GCC CCT GAA TCC GGA TAT GAGG, and 3R : GCC ATC GCC TAC GCC AAA CAG) which showed the best results in generating the PCR fragments in the preliminary experiments was used for subsequent PCR analysis. These primers generated a fragment of 349 bp from *S. jonesii* DNA but not from the DNA of *E. coli*, *Rhizobium*, *Bradyrhizobium*, *Pseudomonas*, *Agrobacterium* or *Listeria* monocytogenes. A concentrated frozen stock of *S. jonesii* cells with an optical density (at 600 nm) of 1 with approximately 10^9 cells was serially diluted in distilled water and dilutions of 10^{-3} to 10^{-9} were used for PCR analyses. When dilutions between 10^{-3} and 10^{-8}, containing ~10^6 to 10 bacteria, respectively were used in PCR with 25, 40 and 45 cycles, a 349 bp fragment was synthesized in all dilutions at 40 and 45 PCR cycles (Fig. 4A). At 25 PCR cycles bands were not detectable when the number of bacteria was less than 10^5 and at 45 cycles the bands were very well synthesized even with 10 bacteria. To determine the sensitivity of detection when the bacteria are isolated from rumen fluid, the bacterial dilutions between 10^{-6} to 10^{-9}, containing ~1000 to 1 bacterial cell, respectively were spun down, resuspended in 1 ml of rumen fluid of cattle, and repurified before using in PCR analysis with 45 cycles. The reisolation procedure reduced the sensitivity of detection and at least 100 bacteria were necessary to obtain the 349-bp PCR fragment whereas when the sample was not mixed with rumen fluid, 1 to 10 bacteria were sufficient to generate a detectable PCR fragment (Fig. 4B).

Detection of S. jonesii in rumen fluid of cattle

Rumen fluids samples, collected from several cattle in the Waialee Livestock Research Farm, University of Hawaii, were tested for the presence of *S. jonesii* using PCR. Rumen fluid obtained from a cow in Iowa which was never fed with *Leucaena* nor inoculated with *S. jonesii* and which was shown to be

negative for the presence of *S. jonesii* by biochemical test, was used as a negative control. A few of the samples tested were found to be positive for the presence of *S. jonesii* (Fig. 4C). Cows sampled in this study were of different ages and some had never been exposed to *Leucaena*. Samples prepared from the rumen fluid of the cow from Iowa were found to be negative, as expected.

4. Discussion

S. jonesii is an anaerobic Gram-negative bacterium that colonizes the rumens of cattle and goat where it plays an important detoxification role by degrading DHP. The rumen provides the anaerobic environment necessary for the microorganism while the bacterium helps the animal by degrading DHP into nontoxic products. Thus, *S. jonesii* establishes a symbiotic relationship with cattle feeding on *Leucaena*. Mimosine is degraded to a pyridinediol DHP by an enzyme present in the *Leucaena* leaves when the leaves are macerated (Smith and Fowden, 1966). In the *Leucaena* leaves, mimosine and the mimosine-degrading enzyme may be physically separated by membranes which are disturbed by maceration of the leaves in the cattle's rumen. Therefore, mimosine is naturally degraded to DHP in the rumen. Mimosine can also be degraded chemically to DHP by acid hydrolysis (Hegarty et al., 1964).

Mimosine or DHP-degrading ability is not confined to rumen bacteria alone. There are other aerobic soil bacteria in the *Leucaena* rhizosphere that can degrade mimosine. Soedarjo et al. (1994) showed that mimosine induces mimosine-degrading enzyme activity in some strains of *Rhizobium* that nodulate *Leucaena*. These strains can utilize mimosine as a source of carbon and nitrogen. Five genes involved in mimosine transport and degradation in *Rhizobium* have been cloned and sequenced (Soedarjo and Borthakur, unpublished results). It is not known yet whether the genes for mimosine degradation in these *Rhizobium* strains have homology with *S. jonesii* genes.

The four overlapping cosmid clones identified in this study may contain genes involved in DHP catabolism, since the *E. coli* containing these clones could grow on DHP as the sole source of nitrogen. For developing the PCR detection method for *S. jonesii*, we first identified from these overlapping clones a 860-bp region of *S. jonesii* DNA which has no homology with any known sequence. Analysis of the 860-bp sequence in this study did not reveal any open reading frame, suggesting that this sequence may not be a part of a mimosine-degrading operon in the *S. jonesii*. Based on this unique sequence, we developed PCR primers for amplification of the *S. jonesii*-specific DNA fragment. These PCR primers did not amplify or hybridize with other bacterial DNA tested indicating that these primers are specific for this fragment. We have also tried

two pairs of primers based on the 16S rDNA sequence of *S. jonesii*. Although these primers could synthesize specific fragments from *S. jonesii* genomic DNA in PCR reactions, they were not very specific and generated similar fragments from some other bacteria (data not shown).

Although the DHP-degrading bacterium *S. jonesii* was originally isolated from the rumen of goat in Hawaii, it is not known if this ability is ubiquitously distributed throughout the cattle population in Hawaii. This distribution is of particular interest considering that *Leucaena* is not regularly fed to all cattle. In the absence of *Leucaena* in the diet, a *S. jonesii* population may not be maintained in the rumen of cattle. Moreover, the occurrence of the DHP-degrading *S. jonesii* may fluctuate with elevations. Therefore, it is important to examine the occurrence of *S. jonesii* in the cattle populations in order to develop an effective feeding program with *Leucaena*. We have developed an easy detection method for these DHP-degrading bacteria using PCR. Our method could detect the presence of 1 to 10 bacteria in a rumen fluid sample. When rumen fluid from dairy cattle in Hawaii was tested by this method, some of them showed the presence of *S. jonesii*. The PCR negative samples came from young cows that were never been exposed to *Leucaena*. This suggests that *S. jonesii* is not present in all cattle in Hawaii.

Previously, McSweeney et al. (1993) described a detection method for *S. jonesii* using an oligonucleotide probe targeting 16S rRNA which requires RNA isolation and Northern hybridization. The method described in the present study is much simpler and does not require laborious experiments. This method can also estimate the number of bacteria in the rumen fluid sample. This can be done by two or three serial dilutions of the rumen fluid sample for PCR analysis and the PCR positive at the highest dilution can be estimated to contain 1–10 bacteria.

Cattle inoculated with pure cultures of *S. jonesii* strain 78-1 may require 2–3 weeks to colonize the rumen and degrade DHP (Hammond et al., 1989). It is likely, that the presence of *Leucaena* in the diet may also provide a positive selection pressure for this organism to multiply and establish itself in the rumen of ruminant animals. On the other hand, it is also possible that in the absence of *Leucaena* in the diet for several months, a gradual decline may result in the number of *S. jonesii* cells in the rumen leading to an undetectable level. This may be the reason why some of the Hawaiian cattle we have tested did not contain detectable levels of *S. jonesii* in the rumen fluid. The PCR method developed in this study will be a valuable tool to determine effects of inoculation of cattle with *S. jonesii* and to monitor the population dynamics of this pyridinediol-degrading bacterium in cattle fed with different amounts of *Leucaena*.

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