AN ABSTRACT OF THE THESIS OF

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Bacterial cultures enriched from sheep rumen fluid have demonstrated the ability to detoxify pyrrolizidine alkaloids (seneciphylline and jacobine) in tansy ragwort (*Senecio jacobaea*). The microbes are difficult to isolate using classical anaerobic techniques, therefore, microbes from two different enrichment cultures demonstrating similar degradation activity were identified using their 16S ribosomal RNA genes. Gene sequences from a rich medium enrichment were matched to *Clostridium bifermentans, Prevotella ruminicola, Escherichia coli,* and from a minimal medium enrichment to, *C. clostridiiforme, C. aminophilum, Streptococcus bovis*, and *Butyrivibrio fibrosolvens.* There were no identical organisms between the two libraries, but the common genus was *Clostridium.*

BACTERIAL 16S RIBOSOMAL DNA ANALYSIS OF PYRROLIZIDINE ALKALOID DETOXIFYING ENRICHMENTS FROM THE OVINE RUMEN

by

Diane R. Gray

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CONTRIBUTION OF AUTHORS

Dr. A. Morrie Craig was involved in the development of the project and writing of the manuscript. Dr. Stephen J. Giovannoni and his laboratory workers assisted in the study design and provided technical assistance. Dr. Wade Johnston was involved in providing enrichment cultures.

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DEDICATION

I dedicate this to my husband, Randy, who provides me love, support, and encouragement, and to my Heavenly Father, Creator of all things, who has revealed Himself to all people through His Creation and through His Word. *"Trust in the Lord with all your heart and lean not on your own understanding. In all you ways acknowledge Him and He will make your path straight."* Proverbs 3:5-6 (New American Standard Bible)

BACTERIAL 16S RIBOSOMAL DNA ANALYSIS OF PYRROLIZIDINE ALKALOID DETOXIFYING ENRICHMENTS FROM THE OVINE RUMEN

Chapter 1

A LITERATURE REVIEW

Introduction

Statement of Problem

Microbes from ovine (sheep) rumen fluid have demonstrated the ability to break down pyrrolizidine alkaloids (PAs) and their toxic metabolites. Fastidious growth conditions and requirements have made microbes in PA detoxifying enrichment cultures difficult to isolate and identify using classical anaerobic culturing techniques.

Objective

The purpose of this study was to identify the members of PA detoxifying enrichments using their 16S ribosomal RNA genes, and to compare the compositions of two different enrichments to find microbes they have in common, which may identify the organisms with the detoxifying capabilities.

Background

Pyrrolizidine Alkaloids

Pyrrolizidine alkaloids are chemicals originating naturally from plant sources. PAs are found in over eight thousand plant species, or three percent of the world's flowering plants (Culvenor, 1980). Plants producing toxic and nontoxic PAs grow throughout the world, and main sources include families *Boraginaceae* (all genera), *Compositae* (genera *Senecio, Eupatoriae*), *Leguminosea* (genus *Crotalaria*), and *Scrophulariaceae* (genus *Castillerja*) (WHO, 1988) (figure 1.1).

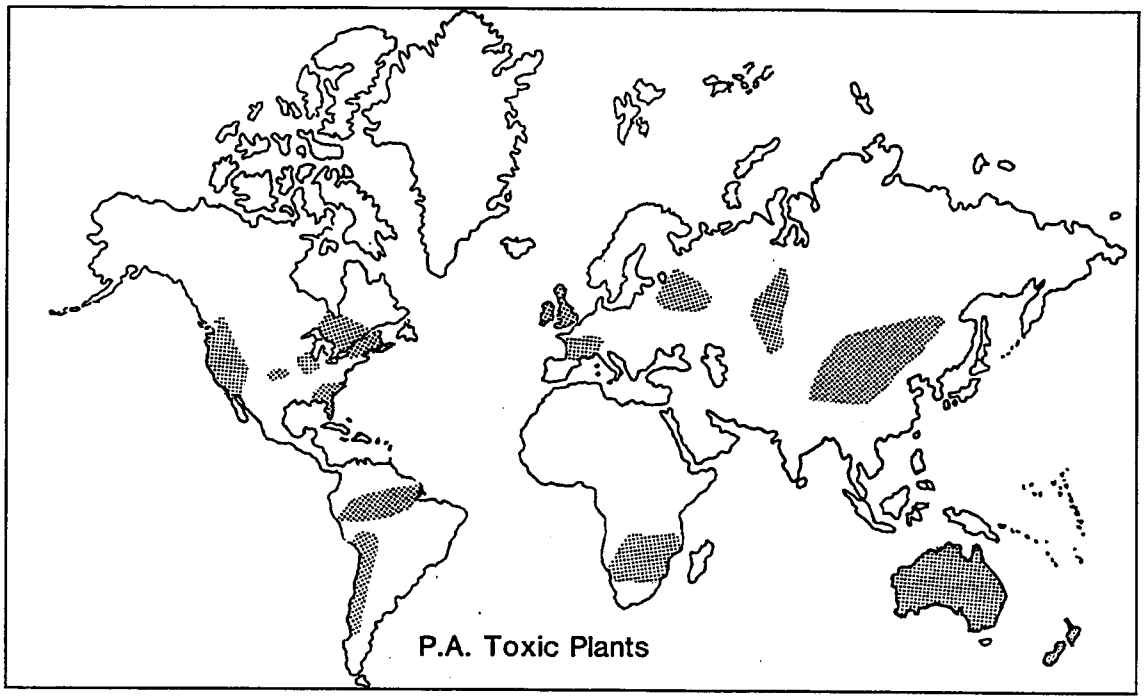

Figure 1.1. Regions of the world occupied by PA toxic plants. PAs are found in over eight thousand species of plants worldwide. The common plant containing PAs in the Northwestern United States is tansy ragwort *(Senecio jacobaea).*

The pyrrolizidine molecule is made up of two 5-membered rings sharing a common nitrogen, and the esters are amino-alcohols derived from the heterocyclic nucleus. The esters may be open branches (e.g., heliotrine, lasocarpine) or macrocyclic diesters (e.g., monocrotaline, retrorsine) (figure 1.2).

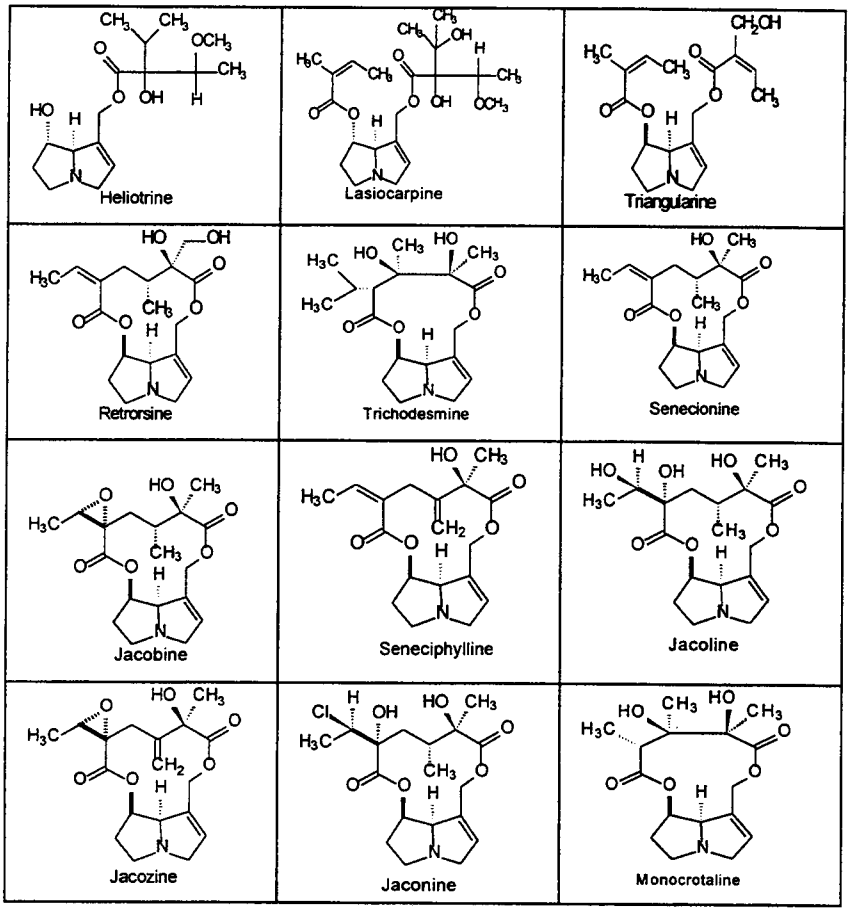

Figure 1.2. Structures of open and closed ringed pyrrolizidine alkaloids. PAs chemical structures include open branches and macrocyclic rings. Heliotrine is an open monoester PA, lasiocarpine and triangularine are open diesters. Macrocyclic PAs are diesters with varying acidic ring groups. One reactive component of the molecules is the double bond in the necine base. Seneciphylline and jacobine are two predominant PAs in tansy ragwort *(Senecio jacobaea)*.

PAs can undergo hydrolysis, N-oxidation, or pyrrole formation in the liver. The products of hydrolysis are the necine base and necic acid that are water soluble and are excreted in the urine. N-oxidation occurs with the nitrogen in the necine base and this product is also water soluble. Pyrrole derivatives (e.g. dehydropyrrolizidine) are formed from mixed function oxidases in the liver and interact with tissues resulting in tissue damage and eventually death. These pyrrole derivatives cross link double-stranded DNA affecting cellular mitosis (Pearson, 1990). Hepatocytes that cannot divide become megalocytes as the cytoplasm expands without nuclear division. The cells die and are replaced by connective tissue instead of hepatocytes, leading to liver cirrhosis. The metabolic derivatives are not only antimitotic, but they also bind to protein and nucleic acids, blocking replication, protein synthesis, and enzymatic pathways. Hepatotoxicity is most often observed with PA toxicosis, but effects in pulmonary and renal tissues have also been observed. PA toxicity is characterized by chronic, progressive, often delayed intoxication after plant consumption. Horses demonstrating PA toxicosis exhibit weight loss, slight-to-moderate icterus, and behavioral abnormalities. Cattle exhibit diarrhea, weight loss, tenesmus, prolapsed rectum, and ascites (Pearson, 1990). PAs have been long considered a health hazard to livestock.

Tansy Ragwort *(Senecio jacobaea)*

In the Pacific Northwest, the plant tansy ragwort is a predominant weed containing six PAs: seneciphylline, jacobine, senecionine, jacozine, jacoline, and jaconine (figure 1.2) (Roitman et al., 1979). This non-native species arrived in grain shipments

from England around the 1880's and spread invasively throughout the Northwest creating problems for livestock and the agricultural industry. Feeding studies indicate cattle and horses are susceptible to hepatic disease after consuming 5-10% of their body weight in tansy plant material (Craig et al., 1991). However, sheep demonstrate resistance to the PAs in tansy, consuming quantities up to 300% of their body weight with no measurable abnormalities (Culvenor, 1978).

Detoxification Studies

Three hypotheses were suggested to explain the metabolic differences observed between cattle and sheep: 1) differences in liver metabolism, 2) differences in ruminal absorption activities, 3) differences in ruminal flora composition and/or activity.

Liver metabolism. One proponent of the liver metabolism hypothesis suggested an increased level of microsomal epoxide hydrolase in sheep accounts for the differences observed between cattle and sheep (Swick et al., 1983; Cheeke, 1977). Cheeke and coworkers cite an experiment in which ground tansy incubated with sheep rumen fluid (from uninduced sheep) for 48 hours, the mixture was freeze-dried, and then fed to rats. Because the mixture was still toxic to rats, Cheeke concluded PA was not broken down by rumen fluid contents. These experiments failed to use the ruminants of interest, but instead used small laboratory animals as models to demonstrate indirectly that sheep did not receive their protection from ruminal microbes (Cheeke, 1983, 1984, 1994; Shull et al., 1976a, 1976b; Buckmaster et al., 1976).

Ruminal absorption. Experiments performed by Craig and coworkers, however, demonstrated ovine ruminal factors affect the degradation of PA (Craig et al., 1985, 1986, 1992; Wachenheim et al., 1991, 1992a, 1992b). The group accomplished this task by completely bypassing the ovine rumen and inserting an indwelling catheter into the right ruminal vein to infuse quantities a sheep would eat daily (Blythe and Craig, 1986). Control sheep consumed tansy orally. The infused sheep developed signs of PA toxicosis and the control sheep did not, indicating an ovine ruminal factor affects PA degradation.

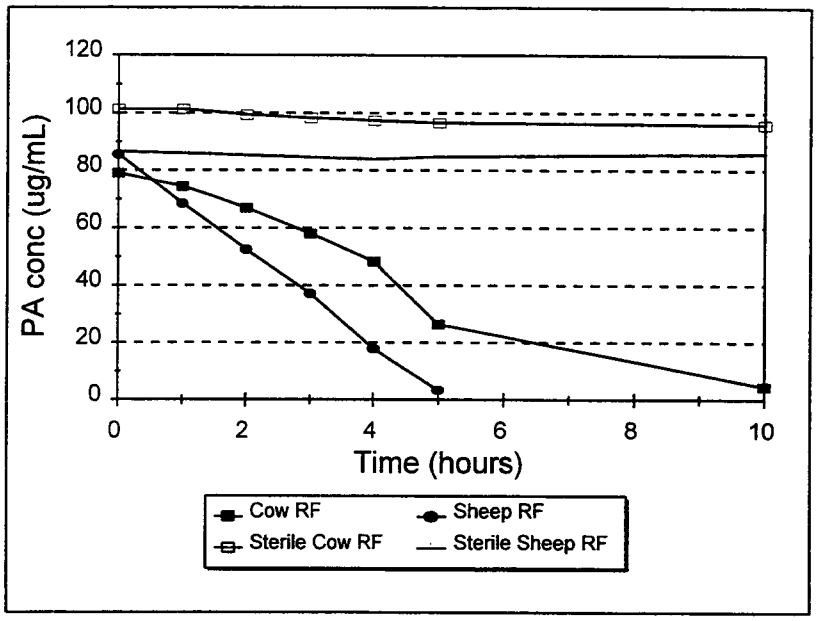

Figure 1.3. ***In vitro*** **PA detoxification over time: comparing steer and sheep rumen fluid activities to sterile controls by HPLC.** (Wachenheim et al., 1992a) PA was not detected after five hour incubation with sheep rumen fluid (closed circles). PA was still detected after ten hour incubation with steer rumen fluid (closed squares). PA was present in sterile controls at initial concentration throughout time course, demonstrating an ovine ruminal factor is responsible for PA detoxification.

Ruminal flora. Furthermore, Craig and coworkers tested the activity of ovine ruminal flora. To determine if ruminal microorganisms could metabolize PA *in vitro,* Hungate artificial rumen fluid was incubated with strained rumen fluid from either sheep or cow, for 48 hours. At time zero, 0.1 mg/ml of PA was added and the concentration quantitated at 12, 24, and 48 hours by HPLC (Wachenheim et al., 1992a). Pasteurized rumen fluid was incubated as a control. The ovine rumen fluid degraded PA within 12 hours while bovine rumen fluid and the pasteurized sample did not (figure 1.3). Ovine rumen fluid flora demonstrated different activity than bovine, therefore, indicating the factor of degradation lies in the rumen and that the flora can degrade PA *in vitro.*

In additional experiments, groups of microorganisms from ovine rumen fluid were collected to determine which type of organism performed the degradation processes. Rumen fluid was centrifuged at different speeds to separate rumen fluid into four fractions containing the following combinations of microorganisms: 1) protozoa, large bacteria, small bacteria; 2) large bacteria, small bacteria; 3) small bacteria; 4) bacteria free. Fractions 1, 2, and 3 had similar degradation rates and fraction 4 showed no degradation, indicating small bacteria, being the common factor among 1, 2, and 3, are responsible for PA degradation (figure 1.4). Therefore, Craig and coworkers demonstrated the difference observed between cattle and sheep is due to microbial detoxification in the sheep rumen (Craig et al., 1992).

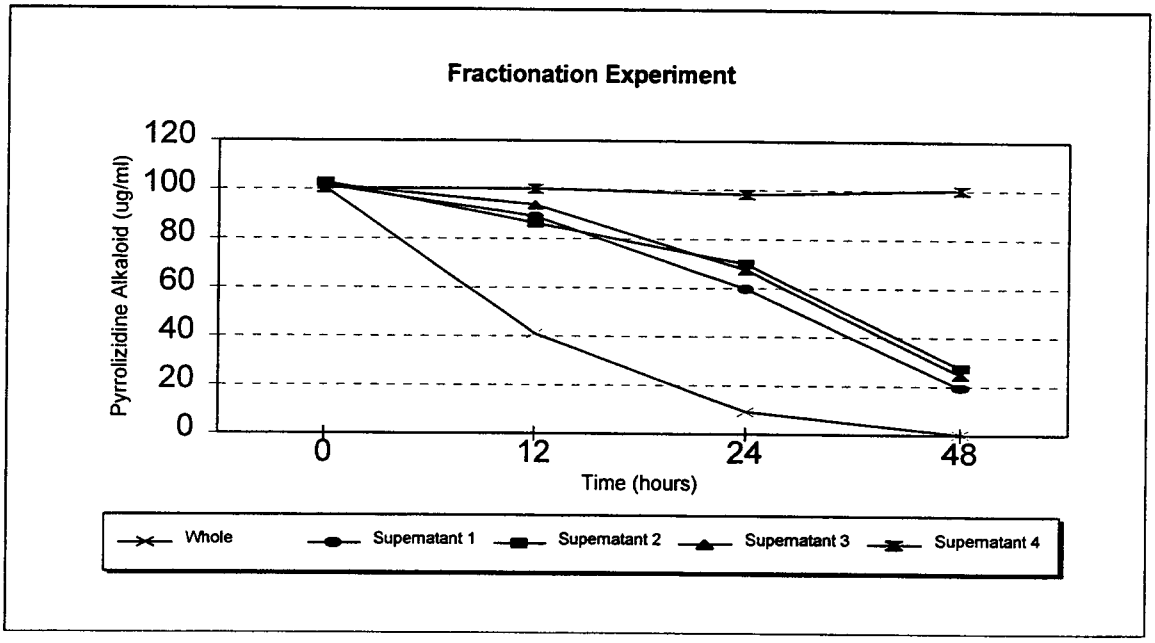

Figure 1.4. PA detoxification over time with various fractions of rumen fluid. (Craig et al., 1992). Supernatant 1 contained protozoa, large bacteria, and small bacteria; supernatant 2 contained large bacteria and small bacteria; supernatant 3 contained only small bacteria; supernatant 4 was organism free. Whole rumen fluid detoxified the PAs the fastest, but, by elimination, the organisms responsible for PA degradation are among the small bacteria, the common factor among fractions 1, 2, and 3.

Feeding trial. To further demonstrate bacterial action in the detoxification, a feeding trial was conducted in which sheep rumen fluid was placed directly into the rumen of treatment cattle. Both treatment and control cattle were fed alfalfa-tansy pellets at 365 mg PA/g food. Serum enzymes, bile acids, and biopsy samples were collected to monitor liver function (Craig et al., 1978, 1979, 1991). Treatment cattle demonstrated a greater resistance to PA toxicosis than did the control cattle (figure 1.5). This further demonstrates the activity of bacteria in detoxifying PAs in tansy ragwort, and supports the possibility of utilizing those bacteria as a probiotic to protect susceptible livestock.

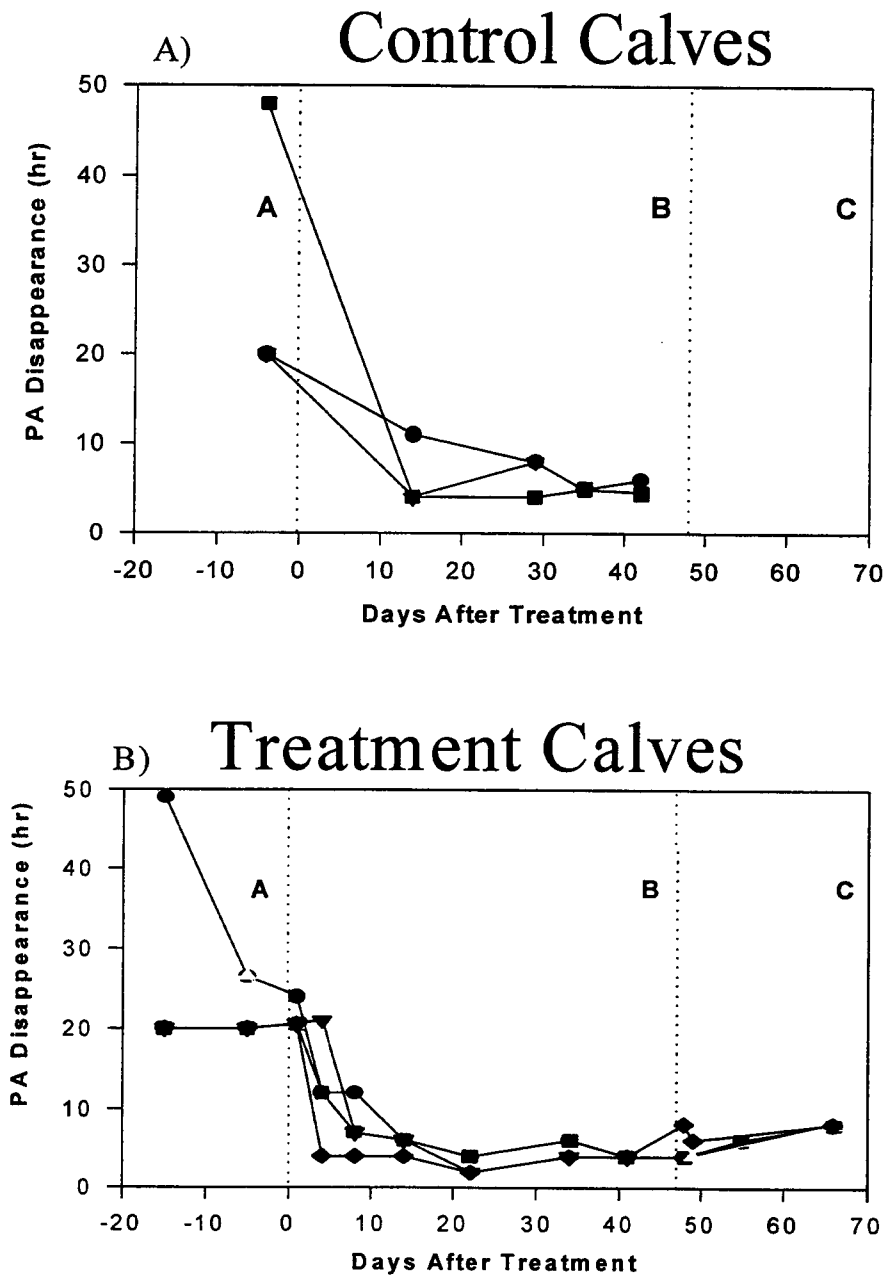

Figure 1.5. Feeding trial using whole ovine rumen fluid in treatment calves for protection against tansy toxicosis. (Johnston, 1998) Time periods A, B, and C represent the periods before, during, and after treatment, respectively. Whole ovine rumen fluid was placed in the rumen of fistulated treatment calves. Control calves did not receive whole rumen fluid, and were fed the same pelleted tansy diet as the treatment calves. Graph A represents the time in which rumen samples from control calves (n=3) degraded PA throughout the trial. Graph B represents the time in which rumen samples from treatment calves (n=4) degraded PA.

Background of PA Detoxifying Enrichment

Enrichment culture. The importance of culturing the organisms was not only potentially to describe new species, but to employ the degradation enzymes and/or bacteria as a probiotic to protect susceptible livestock from tansy toxicosis. As a result of the experiments described above, an enrichment culture was derived from whole rumen fluid and used in an *in vitro* TLC assay to determine the rate of PA degradation. Most probable number estimates were approximately 2.8 X 10^7 PA degrading bacteria/ml (Wachenheim et al., 1992a). Laboratory workers have had difficulty growing the members of the enrichment on solid media or obtaining the bacteria in pure culture using classical anaerobic culturing techniques (Johnston, personal communication).

Chemical inhibitors, antibiotics, heat treatments were applied to narrow down the numbers of species observed in Gram stains (figure 1.6). Chemical inhibitors and antibiotics against gram positive bacteria inhibited PA degradation most effectively (Wachenheim et al., 1992b). Spore formation was difficult to detect, therefore, heat treatments were applied (75, 85, 95, 100°C for 10-20 minutes). However, heat treatments were not extensive enough to stop PA degradation in cultures 40-60 hours old. The culture members were not isolated on solid media, but streaks taken from the first section of the isolation streak demonstrated PA degradation activity. Improved growth on plates occurred with low agar concentration (0.6%) and long incubation period (one week). Isolates recovered did not independently or in combination demonstrate degradation activity. Isolate descriptions included spindle shaped Gram positive rods, curved Gram negative rods, large Gram positive rods, small Gram positive diplococci,

and large Gram positive cocci. There was one organisms which seemed to be present consistently in working liquid and plate cultures. It was a large Gram positive, pleomorphic rod which trailed off into a chain of large cocci. In a dilution series, this organism would be present in the last tube that degraded PA. Despite many attempts, the organism(s) responsible for PA degradation have not been isolated using classical, anaerobic techniques, but the microbial activity is detected *in vitro* by the following TLC (thin layer chromatography) method.

TREATMENT	ACTIVITY	EFFECT
Sulfinsoxazol 20 μg/ml	Analog of P-aminobenzoic acid; Inhibits G-, gonococcal, shigella staphylococcal, streptococcal,	Did not affect PA degradation activity Degraded <20 hr
Nalidixic acid 20 μg/ml	Interferes with DNA gyrase; Active against most G- coliforms	Did not affect PA degradation activity Degraded <20 hr
Polymixin B 2 μg/ml	Cation detergent activity; Interferes with phospholipids of cell membranes; Selective against G- bacilli	Degraded <20 hr; Degradation inhibited at concentrations higher than 2 μg/ml
Chloroamphenocol 1μg/ml	Binds to 50S ribosome; Active against wide variety of G+ and G-	Inhibited degradation; Degradation at ≤0.5μg/ml
Phenyl Ethyl Alcohol 0.25%	Inhibits G- facultative anaerobes; Useful for isolating G+ cocci	Degraded in 40 hr
Metronodiazol 1, 2, 5, 10 μg/ml	Antiprotzoal; Active against anaerobic bacteria *(Bacteroides,* clostridia, some streptococci*)*	All concentrations stopped PA degradation
Heat treatments 75, 85, 95, 100°C	Kills non-heat resistant bacteria Selects for spore forming bacteria	In cultures 40-60 hrs old, degradation continued after all treatment temperatures

Figure 1.6. Treatments applied to narrow and characterize enrichment culture and their effects. A variety of chemical treatments were applied to the anaerobic ruminal enrichment culture to narrow the number of organisms. PA degradation was most effected by Gram positive chemical inhibitors. Samples other than 20 or 40 hr timepoints were not collected indicating degradation occurred before those sampling points.

TLC detection method. The TLC method employed in observing PA degradation was performed as follows (Wachenheim et al., 1992a): Collect a one ml sample, add 100µl of 5M NaOH and 500µl of methylene chloride, vortex the mixture one minute. Centrifuge the mixtures for 5-12 minutes at 14,000g. Transfer the lower methylene chloride layer with a Pasteur pipette and collect in a glass tube. Dry the tube contents under vacuum at 43°C for 15-20 minutes. After complete evaporation, add 20µl of methylene chloride and a glass bead to each tube, vortex for three seconds, and spot on a HPKF silica gel TLC plate. Develop the plate in chloroform:methanol:propionic acid (36:9:5) for 20 minutes. Spray the plate with Dragendorff's spray reagent (Sigma) followed by sodium nitrite (5%). If PAs from tansy ragwort are present, three colored spots will separate (figure 1.7). The limit of detection for the disappearance of PAs is 1 ppm.

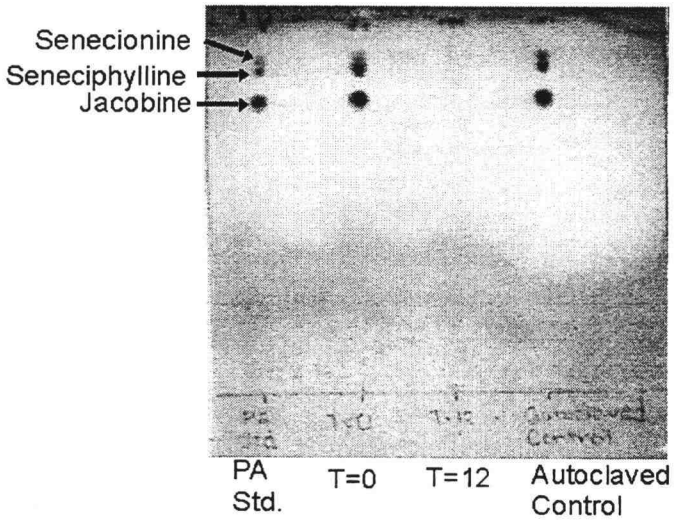

Figure 1.7. TLC assay for tansy ragwort PAs. Plate was spotted with sample and developed in (36:9:5) chloroform:methanol:propionic acid. T=0 sample was taken immediately after inoculation. T=12, sampled 12 hours later, showed the PAs were no longer present. Dragendorff's spray binds to any amines on the plate. Sodium nitrate is used as a color enhancer.

The Rumen Environment

The Rumen

Ruminants are cloven-hoofed mammals of the order *Artiodactyla* and include sheep, cattle, goats, camels, llamas, buffaloes, caribou, and reindeer. The rumen is the largest of the four compartments in ruminants' digestive systems, in addition to the reticulum, omasum, and abomasum. The conditions in the rumen provide favorable conditions for microbial life to thrive with a temperature range of 36-41°C. The rumen contents are buffered by saliva derived bicarbonate and microbially produced CO_2 resulting in a pH 5.7-7.3. The absence of oxygen, however, is a major selection factor, and provides an environment for fermentation to occur.

Fermentation

Cells obtain energy using two 'currencies', NADH and ATP. The highest number of NADH and ATP can be produced as carbon sources are broken down and oxygen is used as a final electron acceptor. In the rumen environment where oxygen is lacking, energy still must be produced. Fermentation occurs to obtain a portion of the available energy from the metabolism of organic sources producing organic acids and alcohols. Organic acid production is favored because more energy is produced than from alcohol production. Fermentation products are a function of individual microbial physiology, and products may include acetate, butyrate, propionate, lactate, formate, succinate, valerate, 2,3-butanediol, ethanol, and/or isopropanol (figure 1.8).

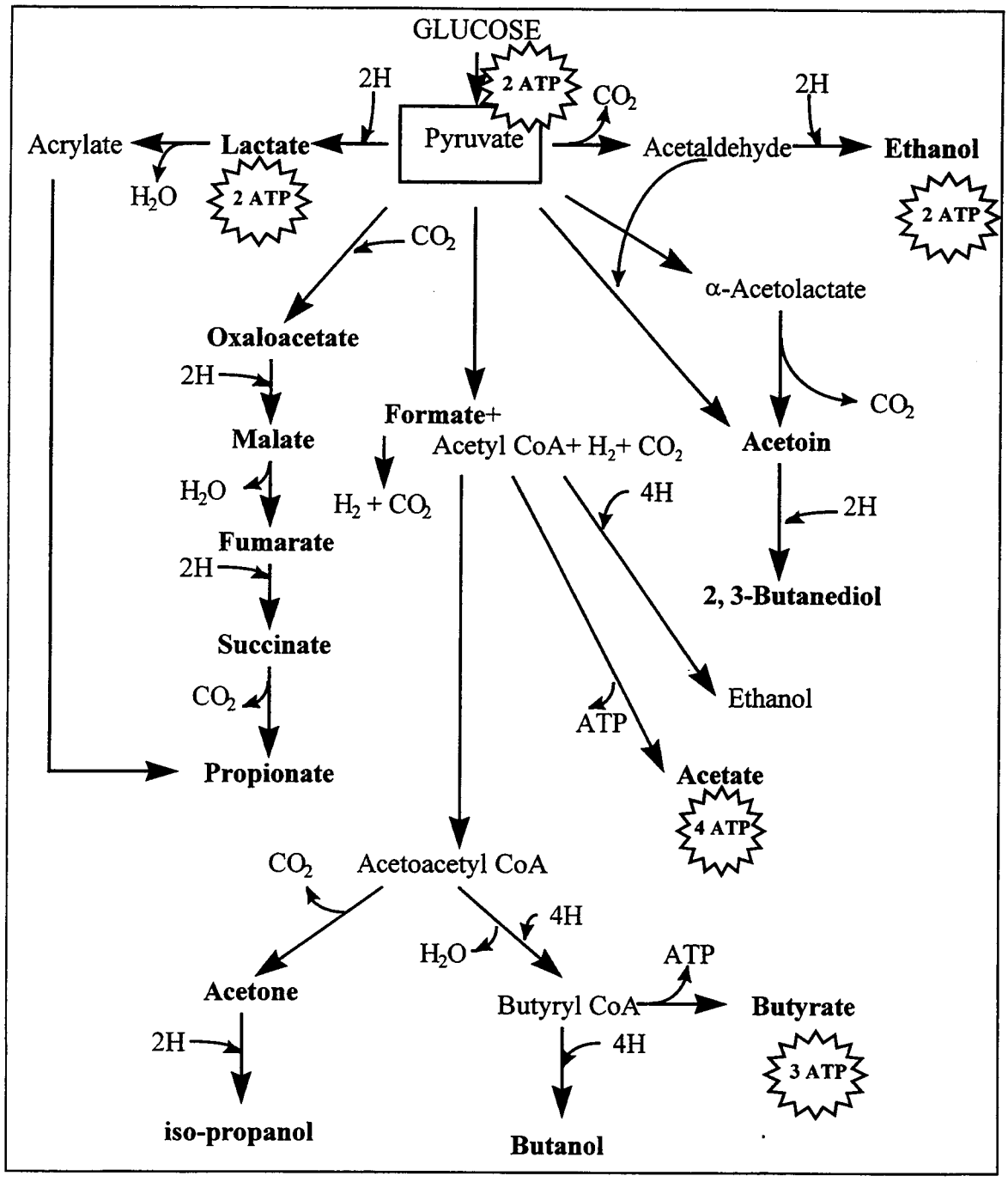

Figure 1.8. Diagram of selected fermentation pathways. (Hamilton, 1979) This flow diagram shows selected fermentation end-products and the moles of ATP/mole glucose produced. Acetate ⇒ 4 ATP; Butyrate ⇒ 3 ATP; Lactate ⇒ 2 ATP; Ethanol ⇒ 2 ATP.

Ruminal Microflora

Microorganisms in the rumen play major roles in the digestion and nutrition of animals. Microorganisms in the rumen include bacteria, protozoa, and anaerobic fungi. Microorganisms produce fermented acids that the hosts use as energy sources, and the organisms themselves serve as a protein source. The organisms are diverse, but those able to extract the most energy from the available substrates survive the best.

Total prokaryotic population is estimated around 10^9-10^{11}/ml depending upon counting method. Numbers of various populations performing specific functions can range among 10^5-10^9/ml depending upon counting method and feed source (Hobson, 1988).

Changes in populations are observed under a variety of conditions. Diurnal changes have been sited, showing the lowest direct and viable cell counts on cattle fed high forage or high grain diets, were two to four hours after feeding (Leedle et al., 1982). This observation may be due to a dilution effect. Alternatively, a few hours before feeding is the optimal time to obtain the most uniform sample. Bacterial concentration may also vary upon sampling site within the rumen. Bryant reported bacterial numbers were highest in samples taken from the dorsal rumen, whereas the numbers were lowest in the ventral or reticulum samples (Bryant and Robinson, 1961). Diet has more of an effect on the minor subpopulations than the major digesters or the total cell number (Hespell et al., 1997). The most numerous bacterial types perform functions such as cellulose, starch, and hemicellulose digestion, sugar fermentation, acid utilization, methanogenesis, proteolysis, and lipolysis. The predominant culturable celluloytic

bacteria include *Fibrobacter succinogenes, Ruminococcus albus, R. flavefacians,* and *Butyrivibrio fibrosolvens.* The bacteria identified as hemicellulose digesters include *Ruminococcus, Prevotella ruminicola,* and *B. fibrosolvens.* Pectin degraders include *Lachnospira multiparus, B. fibrosolvens, Succinivibrio dextrinosolvens,* and *Treponema bryantii.* Major starch degraders include *Ruminobacter amylophilus, Streptococcus bovis,* and *B. fibrosolvens.* Proteolytic bacteria comprise 12-43% of the total bacteria population and include *P. ruminicola, B. fibrosolvens, R. amylophilus, Selenomonas, Eubacterium, Lachnospira, and Streptococcus* (Hespell et al., 1997). Many other species present in the rumen have not yet been cultured. Much of the lack of success in culturing rumen bacteria arises from failure to supply important nutrient factors and substrates, lack of anaerobiosis, and confusion from a lack of success (Hungate, 1966). Figures 1.9 and 1.10 list major and minor bacterial species that have been cultured from the rumen.

Organism	Gram	Morph.	Motility	[a]Physiology	[b]Fermentation	Special Features
Megasphaera elsdenii	-	coccus	-	SS, FBA, NS	P, B, B, C, H	Present in low numbers; poor survival
Veillonella parvula	-	coccus	-	NS	A, P	
Methanomicrobium mobile	-	coccus	+	NS	M	
Anaeroplasma abactoclastium	-	coccus	-	A, LS	F, A, E, L, S, C	
A. bactoclastium	-	coccus	-	SP, PR, LS	F, A, L, C, H	Lyses bacterial cells
Syntrophococcus sucromutans	-	coccus	+	SS, SP	A, C	Reduces aromatics; uses sugars as e- donor
Magnoovum eadii	-	coccus	+	SS		
Quinella ovalis	-	coccus	+	SS	A, P, L, C	Sugar fermenter
Ruminococcus flavefaciens	+	coccus	-	C, X,	A, S	Major cellulolytic species
R. albus	+	coccus	-	C, X	F, A, E, C, H	Major cellulolytic species
R. bromii	+	coccus	-	A	F, A, E, C, H	Not cellulolytic
Streptococcus bovis	+	coccus	-	A, SS	F, A, L, E	Capable of fast growth rates
Methanosarcina barkerii	+	coccus	-	SP	M, C	One of most ubiquitous methanogenic species
Treponema bryantii	-	spiral	+	SS	F, A, S	
T. saccharophilum	-	spiral	+	P, A, SS	F, A, E	
Lampropedia sp.	-	spiral	-	LS, NS, ?	?	
Oscillospira guillermondii	-	spiral	+	?	?	

Figure 1.9. Characteristics of culturable, coccal-shaped and spiral-shaped ruminal bacteria. (Hespell et al., 1997)
[a]A=amylolytic; C=cellulolytic; P=pectinolytic; L=lipolytic; X=xylanolytic; PR=proteolytic; LS=limited sugar usage; SS=soluble sugar usage; SP=specialized substrates; NS=non-sugar usage; BFA=branched-chain fatty acid formation substrate. [b]F=formate; A=acetate; E=ethanol; P=propionate; L=lactate; B=butyrate; S=succinate; V=valerate; H=hydrogen gas; C=carbon dioxide; M=methane; N=ammonia.

Organism	Gram	Morph.	Motility	[a]Physiology	[b]Fermentation	Special Features
Butyrivibrio fibrosolvens	-	curved rod	+	A, P, X, SS, PR, L, C	F, A, B, H, C	Common organism; physiologically diverse
Fibrobacter succinogenes	-	rod	-	C, LS	A, S	Major cellulolytic
F. intestinalis	-	rod	-			
Prevotella ruminicola	-	rod	-	A, P, X, PR, SS,BFA	F,A,P,S	Most commonly isolated; main proteolytics
Selenomonas ruminantium	-	rod	+	SS, SP	A, P, L, C, H	
Ruminobacter amylophilus	-	rod	-	A, LS	F, A, S	Only ferments starch or maltose
Succinivibrio dextrinosolvens	-	rod	-	LS	A, S	
Succinomonas amylolytica	-	rod	+	A, LS	A, S	
Anaerovibrio lipolytica	-	rod	+	LS	A, P, S,C	Hydrolyzes triglycerides
Wolinella succinogenes	-	rod	+	SP, NS	S,C	Narrow niche;
Oxalobacter formigenes	-	rod	-	SP, NS	F, C	Narrow niche; oxalate degrader
Fusobacterium necrophorum	-	rod	-	SP, NS	A, P, B	Can cause liver abcesses
Methanobrevibacter ruminatium	+	rod	-	SP, NS	M	Major ruminal methanogen; 10^8-10^9/mL
Methanobacterium formicicum	+	rod	-	SP, NS	M	
Lachnospira multiparus	+	rod	+	P, LS	F, A, E, L, C	Pectinolytic
Lactobacillus vitulinus	+	rod	-	SS	L	Predominant in young animals
L. ruminis	+	rod	+	SS	L	Predominant in young animals
Eubacterium ruminatium	+	rod	-	SS, X, A	F, A, L, B	
E. cellulosolvens	+	rod	+	C, SS	A, L, B	High numbers in ruminants fed hay
E. limosum	+	rod	-	SS, SP, BFA	A, B, C	Numerous in diets high in molassas
E. xylanophilum	+	rod	+	X, SS	F, A, B	
E. uniforme	+	rod	-	X, LS	F, A, E, L	
E. oxidoreducans	+	rod	-	SP, NS		Reduces aromatics; narrow niche
Clostridium pfennigii	+	rod	+	SP, NS	A, B, C	Reduces aromatics
C. aminophilium	+	rod	-	SP, NS	A, B, N	Not proteolytic

Figure 1.10. Characteristics of culturable, rod-shaped ruminal bacteria. (Hespell et al., 1997) See Figure 1.9 for abbreviations used.

Observations of special enrichments. Microbial population distributions can change depending upon the substrates entering the rumen. Ruminants are often more resistant to toxins than monogastric organisms due to the metabolism of these compounds by ruminal microbes (Hobson, 1988). Microbial adaptation to toxic compounds occurs either by 1) induction of specific enzymes followed by growth of specific subpopulations able to take up and metabolize the substrate, or 2) selection of mutants with altered or novel metabolic activities not present at induction, requiring longer adaptation time. Several ruminal microbes have demonstrated the ability to detoxify toxins including mimosine, pyrrolizidine alkaloids, oxalates, nitrates, fungal toxins, and other plant byproducts.

Mimosine is a nonprotein amino acid found in tropical leguminous shrubs. This compound is toxic to sheep, goats, cattle, pigs, horses, and poultry. Mimosine is degraded in the rumen to 3-hydroxy-4-(1H)-pyridone (3,4DHP), which is considered the primary toxic compound (Jones, 1985). *Synergistes jonesii* was isolated and shown to degrade 3,4DHP to non-toxic metabolites (Allison et al., 1992b; McSweeney et al., 1993). The detoxification may occur by a hydrogenase in a reductive ring cleavage (Allison et al., 1994).

Oxalate is found in many plants. Oxalate degraders are widespread among gastrointestinal tracts of many warm-blooded animals. *Oxalobacter formingenes* has been isolated, and several energy and enzymatic studies have been performed using this organism (Dawson et al., 1980; Allison et al., 1983).

Pyrrolizidine alkaloids (PAs) are found in over eight thousand plants species around the world. PA (heliotrine) from the plant *Heliotropium europaeum* were shown to be converted to 1-methyl derivatives by the cytochrome-producing ruminal anaerobe

Peptostreptococcus heliotrinreducans (Lanigan, 1976). Inhibition of methanogens increases the rate of ruminal metabolism of heliotrine to nontoxic metabolites (Lanigan, 1971, 1972). This may supply more hydrogen for the reduction of the noncyclic PA, or, in the absence of methanogens and increased partial pressure of hydrogen in the rumen, the organisms are forced to dispose of electrons rather than produce hydrogen (McSweeney et al., 1997). *P. heliotrinreducans* can metabolize the noncyclic PAs heliotrine and lasocarpine but not the cyclic PAs seneciphylline or jacobine from tansy ragwort. The enrichment grown by Craig and coworkers can degrade both open and closed ringed PAs (Hovermale, 1998).

Nitrocompounds present in the rumen either as metabolic byproducts or in feed substrates can be toxic to ruminants. Nitrate reduced to nitrite can be absorbed through the ruminal wall, enter the blood stream, and oxidize oxyhemoglobin to methemaglobin, reducing the blood's oxygen carrying capacity. The compound 3-nitropropanol (NPOH) is found in many leguminous plants and can be converted to the toxic 3-nitropropionic acid. Microbial enhanced degradation of NPOH can prevent its transformation to toxic 3NPA (NPA). Majak described ten strains that degrade NPOH and/or NPA including *Coprococcus, Megasphaera,* and *Selenomonas ruminantium* (Majak, 1981). Several recent reviews describe additional examples and processes of microbial detoxification of plant and fungal toxins (McSweeney et al., 1997; Craig, 1987).

Identification techniques. Several taxonomic systems have been used to identify and classify bacteria including the binomial, conventional, and Bergey's systems. The binomial system groups bacteria by genera and species names e.g., *Escherichia coli*. The

conventional system uses morphology, gram reaction, nutritional requirements, cell wall chemistry, capsule chemistry, pigments, carbon, nitrogen, sulfur sources, fermentation products, gaseous needs, temperature, pH, antibiotic sensitivities, pathogenicity, habitat, and symbiotic relationships to group organisms. API and BIOLOG test kits are used to identify bacteria based upon patterns of carbohydrate utilization. Lipid profiles of bacteria are also being collected and used to identify unknowns. Finally, Bergey's system uses Gram reaction, morphology and physiology. Unfortunately, these characteristics can be transient and unreliable upon which to base organism relatedness.

Classically, scientists also have used biochemical and phenotypic characteristics to identify rumen bacteria. Organisms in pure culture are subjected to a variety of tests to demonstrate carbohydrate utilization, nitrogenous compound utilization, fermentation product production, Gram reaction, and oxygen sensitivity. These chemotaxonomic features, however, can vary depending on growth media, if organisms are in pure culture, and on overall growth conditions, making identification based on these observations inconsistent. Mole percent guanine+cytosine data was introduced as a more objective measure of identity. Microbial identification based upon genetic data are more suitable because genomics of organisms are consistent despite environmental conditions. The following section is an overview of current genetic techniques.

Genetics Techniques

Introduction

Genetics are now being used as a taxonomic tool. The nucleotide sequence of an entire bacterial chromosome is passed on to daughter cells generation after generation remaining relatively constant. The genetic codes of important cellular components and molecules need to remain constant in order for organisms to survive. These consistent characters provide reliable information with which to compare organisms and infer relationships. Methods that have been applied to the gastrointestinal tracts include quantification of mRNA to monitor gene expression, development of bioluminescent gene reporter systems, development of DNA probes, and utilization of probes for rRNA hybridization. Often techniques used in these systems employ selective amplification, cloning, screening and sequencing. One would use these techniques to study viable, but unculturable organisms, or to study bacteria from an environment without culture condition bias. The following sections describe some basics of nucleic acid techniques.

Extraction

Nucleic acids are extracted from cells by mechanical and/or chemical means. Mechanically, nucleic acids can be released from cells by a bead-beating method in which tiny glass beads puncture cells releasing cellular contents. Cells can also undergo chemical lysis. Nucleic acids may be mechanically, electrically, or chemically separated from proteins, other nucleic acids and cellular debris. Ultracentrifugation in a chemical

gradient or separation by electrical charge are common methods. Chemically, phenol, chloroform, salts, and alcohols are typically used to purify nucleic acids. Aseptic technique is critical at this step to prevent contaminating nucleic acids from other sources (countertop, equipment, reagents, handling) to be collected along with the desired sample.

Amplification

The method of amplification is known as Polymerase Chain Reaction, or PCR. Overall, the process entails two key features: reagents and varying temperature cycles. The reagents include buffer, magnesium chloride, dinucleoside triphosphates, (dNTPs), primers, template, and a DNA polymerase. The primers provide free 3 prime -OH ends onto which DNA polymerases then add dNTPs. *Taq* polymerase, the thermostable DNA polymerase from the bacterium *Thermus aquaticus* is commonly used and connects the dNTP building blocks as they are complemented to the template. The varying temperature stages include a denaturing (95°C), a reannealing (40-60°C), and an elongation temperature (72°). The denaturing step occurs at a high temperature during which the double strands of the DNA template are "broken apart". As they are broken apart and the temperature decreases, the primers are joined (reannealed) at the appropriate positions. Elongation occurs at approximately 72°C with the thermostable DNA polymerase matching free dNTPs to the template until a complete double strand is formed. This cycle is repeated a designated number of times, thereby making many copies of the template. Ideally, the ultimate number of products is related to N_o2^n, N_o=starting number of template molecules, n= number of cycles (figure 1.11). Thus

many copies of the gene can be produced to be used for sequencing, analyzing, or cloning. The largest assumption with this method is that the product produced is the product desired.

A concern, especially when applying this method in environmental studies, involves selective amplification, or the process of selecting and copying one DNA type more efficiently over another, skewing the final proportion of copies as compared to the proportion existing in an environment. Adjusting conditions in the PCR (number of cycles, length of cycles, temperatures, enzyme fidelity) may correct the instances in which bias may be occurring. Another concern is over the statistical validity of a population analysis based on one extraction and amplification sample. However, much useful information can come from only one sample which may be applied to the direct study of an environment (hybridization probes) (Stahl et al., 1988). Another concern about using PCR methods include the formation of chimeric amplification products and questions about gene clustering. The significance or normality of these phenomena are still unknown and under investigation. However, the information derived from genetic studies can provide useful information and should be used in conjunction with physiological data and culture-based studies.

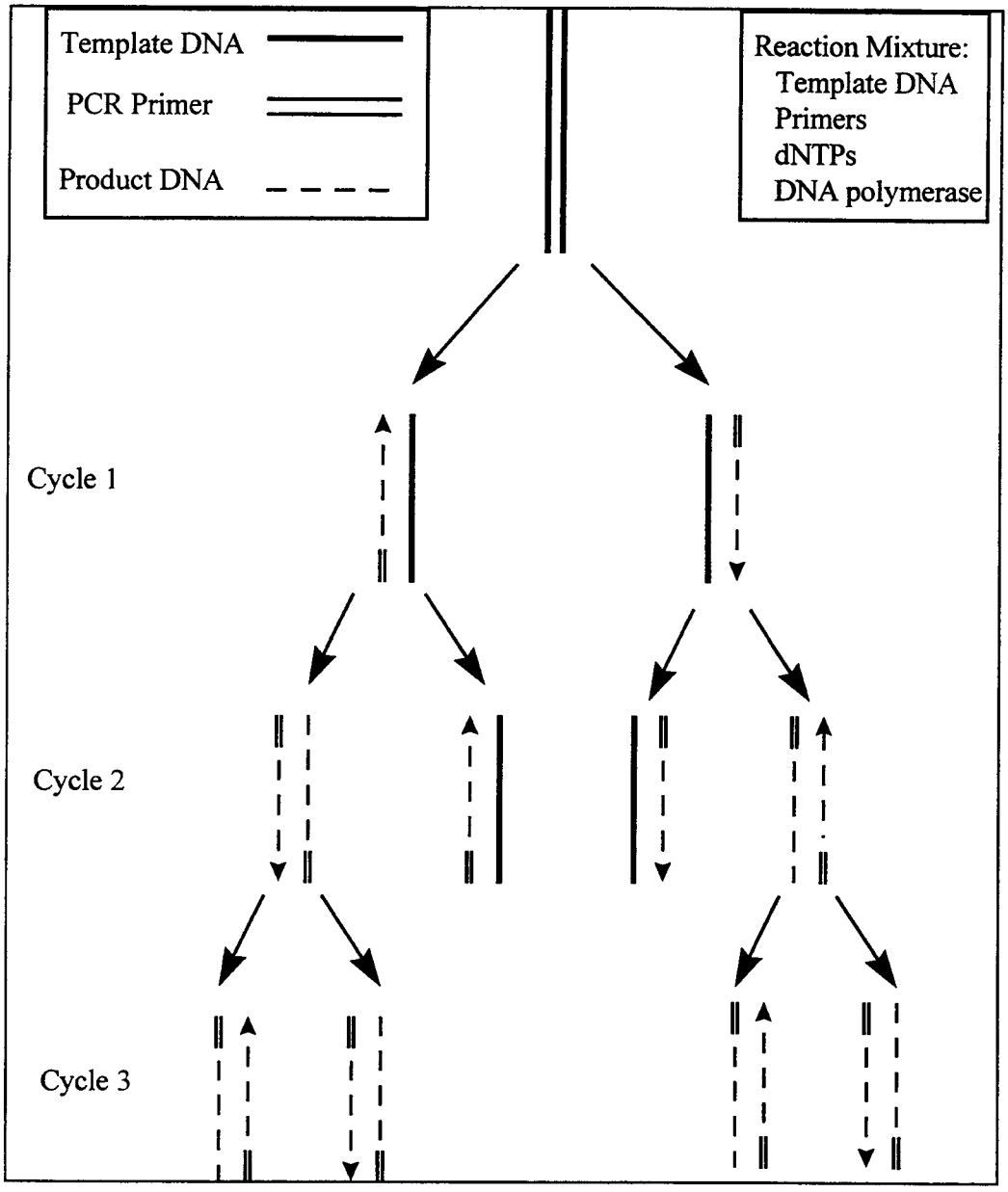

Figure 1.11. Schematic outline of the polymerase chain reaction. Cycle 1: Template DNA is denatured during a high temperature stage (95°C). During the next stage, PCR primers anneal to complementary positions on the template. During the elongation stage, DNA polymerase adds dNTPs to the ends of the primers, complementing the template DNA. At the end of cycle one, there are twice as many copies of the gene of interest. As the cycles continue, the number of PCR products increase exponentially.

Gene Cloning

Gene cloning is the process of placing a gene in a vector which self replicates when placed in a cell. Gene fragments may come from PCR product or restriction fragments. Vectors may be plasmids or phages, and placed in prokaryotic or eukaryotic cells. Then, many copies of the gene or its product are produced by the organism and its progeny. A collection of organisms with cloned genes from a single source, called a library, may be screened for the presence of the insert and vector using colormetric and/or antibiotic resistance, and/or PCR (figure 1.12).

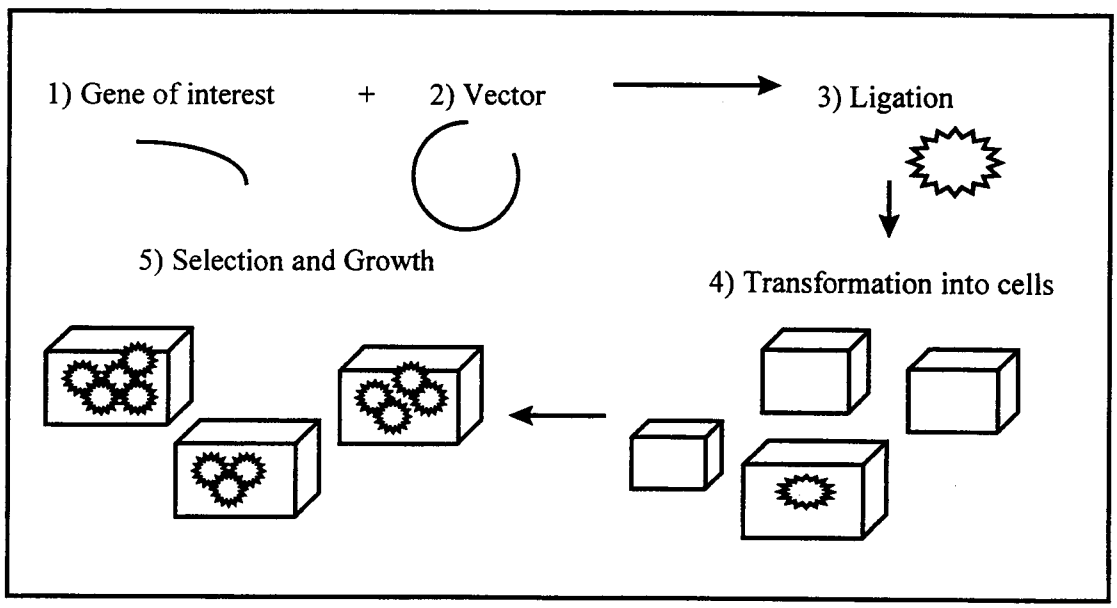

Figure 1.12. Schematic diagram of gene cloning. In gene cloning, a gene of interest is 'pasted' into a self-replicating extra chromosomal unit and moved into viable cells. Vectors with inserts enable cells to survive selective growth conditions (by producing antibiotic resistance and blocking β-lactamase activity), thereby producing many copies of the gene.

Screening may also include 'fingerprint' analysis, for example, restriction fragment length polymorphism (RFLP). These techniques involve observing patterns (basepair combinations) in the nucleic acid sequences by employing the activity of restriction endonucleases in cleaving strands of DNA at sites specific for each nuclease. For example, *HaeIII* is an restriction endonuclease isolated from *Haemophilus aegyptus* and cleaves double stranded DNA at 5'-GG↓CC-3' sites in a sequence. The resulting fragments may be of varying sizes and are separated out by gel electrophoresis (figure 1.13). Sequences that are the same will have similar banding patterns. This method can help differentiate among cloned gene types.

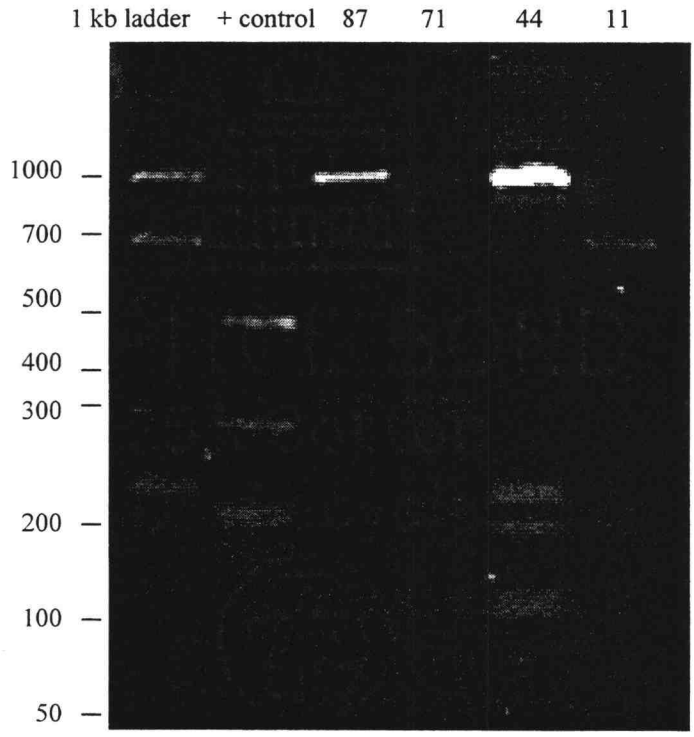

Figure 1.13. RFLP. Restriction digest of PCR products from library screen in project (Chapter 2). Lane 1 = 1 kb ladder (basepair standard); Lane 2 = positive control; Lane 3 = clone 87; Lane 4 = clone 71; Lane 5 = clone 44; Lane 6 = clone 11.

Hybridization Techniques

Genomic. Nucleic acid hybridization techniques are useful by using the binding and pairing properties of guanine, cytosine, thymine, adenine, and uracil. In DNA-DNA hybridization methods, genomic DNA is extracted and fixed to a membrane. The DNA for comparison is labeled for detection (radioactive or fluorescently labeled). Under determined conditions, if the two samples are similar in sequence, they bind together giving a signal. This method may be approached using a direct binding method or a competition method. Twenty percent similarity may indicate genus and seventy percent, species level. RNA may also be used to hybridize to the genomic DNA using similar approaches.

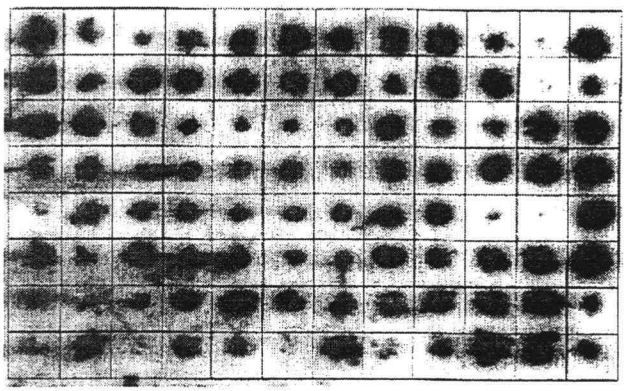

Figure 1.14. Hybridization blot. Colony hybridization blot of E Medium library with universal 338R probe (chapter 2).

Oligonucleotide hybridization. Oligonucleotides are short fragments of RNA or DNA (18-30 basepairs) that may be used in detecting similar nucleic acid contents among organisms. Oligonucleotides, or probes, are employed in membrane hybridization studies

with extracted DNA or whole cells, and in samples or *in situ* hybridizations for detecting types and distribution of organisms.

Nucleic Acid Sequencing

The method of determining the nucleotide-by-nucleotide sequence has been an extremely useful tool. However, chromosomes are large molecules and can be difficult to manipulate. Therefore, smaller sections of a chromosome may be used for easier management. Sequenced genes have provided vast quantities of information regarding the historicity and functionality of catabolic and structural genes.

Specific genes. Some examples of genes common in all bacteria include ATPases, DNA polymerases, RNA polymerases, ribosomal RNAs, elongation factors. Molecules that have been used in bacterial genetic comparisons include ferredoxins, globins, cytochromes, RNA polymerase, transfer RNA, and ribosomal RNA. The part of the chromosome used must be present in all bacteria, must be within the population and functional for a long time, must not be laterally transferred, and must be of a size to contain sufficient information.

16S ribosomal RNA genes. 16S rRNA gene fits the criteria before mentioned for genetic comparison. The gene contains regions that are universally conserved, i.e., regions that are common among all bacteria, and also contains regions that are unique to genera and to species.

Bacterial 70S ribosomes can be dissociated into two subunits: 50S and 30S. The unit S (sedimentation coefficient) is the Svedberg unit (10^{-13} sec), and is derived from the ratio velocity/field strength. The values are not liner (i.e., 30S + 50S ≠ 70S) largely because of the frictional coefficient from centrifugation. The 50S subunit is composed of 23S rRNA, 5S rRNA and 31 proteins. The 30S subunit is composed of 16S rRNA and 21 proteins. The 16S rRNA gene, the gene that codes for the subunit structure, has been the most widely used molecule for the development of a genetic taxonomic system.

The 16S rRNA molecule or gene can be sequenced and cataloged as an organism's code. These codes may be viewed in alignment programs to identify regions of similarity or variability, applied to algorithmic programs to calculate relatedness, and viewed in the form of dendrograms or phylogenetic trees. Unknown organisms can have their 16S rRNA genes sequences be compared to known genes

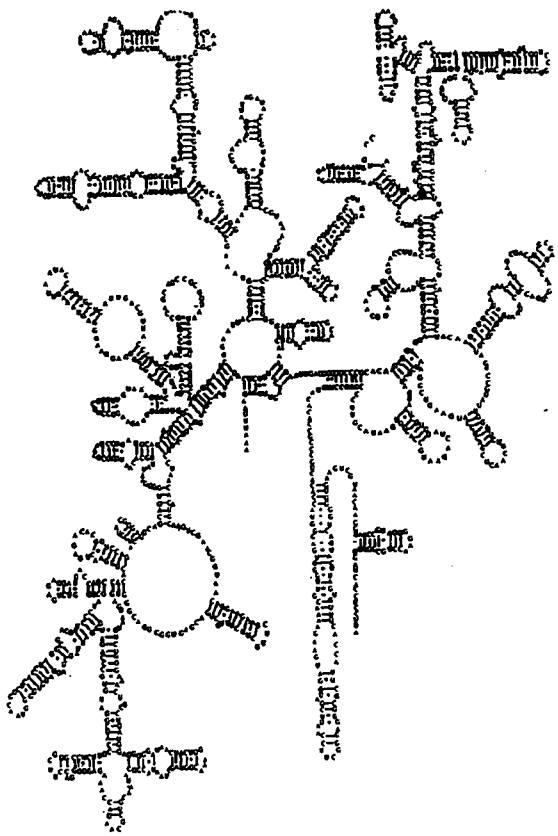

Figure 1.15. 16S rRNA secondary structure. General shape of the *E. coli* ribosomal RNA secondary structure. Stem and loop regions tend to be variable regions specific to species or genera.

and be placed in the trees according to their similarity or dissimilarity. Unknown 16S rRNA gene sequences that match known sequences by 95% are considered to be within the same species, whereby matches 60% are considered to be kingdom specific. This is in contrast to hybridization techniques that are not as specific.

Phylogenetic analyses of organisms using 16S rRNA oligonucleotide analysis was introduced by Carl Woese in the 1970's. Sequence data are applied to complex algorithms producing models used to visualize relationships among organisms. In applying these techniques to bacteria and eukaryotes, Woese discovered a branch of organisms very different from either of the two previous groups: the Archaea (figure 1.16). Organisms in this group were often found in extreme environments, and the search for more unique organisms and novel genes increased rapidly as the ability to find organisms without culturing them became widespread.

Sequence Analysis

Sequences to be analyzed are imported into programs like Genetic Data Environment (GDE) or Genetic Computer Group (GCG) to align regions of similarity among each other. Two general approaches to analyzing the data are using distance-based methods or character-based methods. Distance based methods mathematically determine similarity between sequences. Character based methods statistically infer evolutionary relationships.

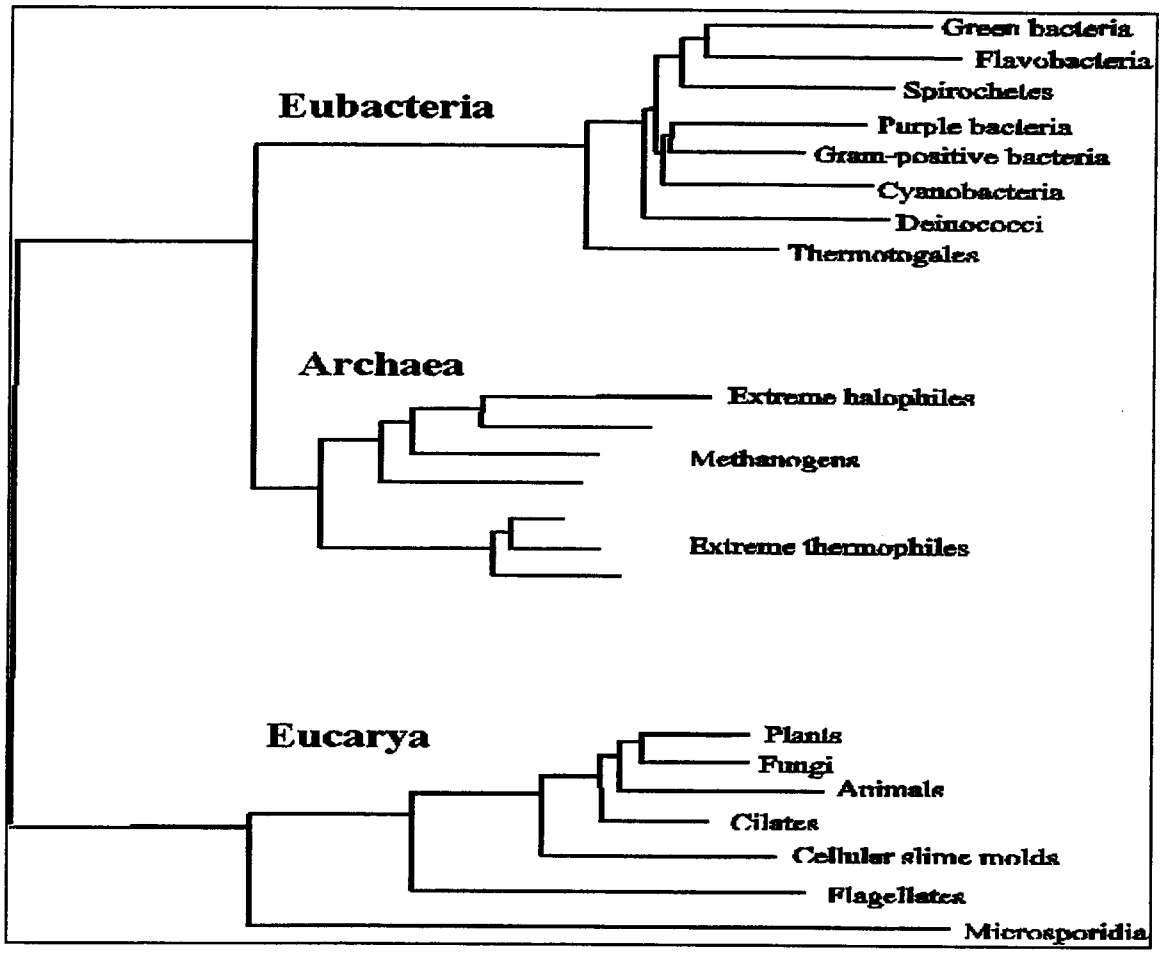

Figure 1.16. Universal Phylogenetic Tree. (Mills, 1997) (Taken from Brock) Tree is based on comparative sequencing of 16S and 18S rRNA and shows the three main classification domains: Eubacteria, Archaea, and Eucarya.

Distance-based methods. Distance methods all begin with the calculation of pairwise sequence comparisons, reducing the data to a 2-D matrix. The matrix links the most similar taxa, calculates lengths of segments, and represents relationships in a dendrogram (figure 1.17). Different programs vary in calculating weight of transitions, transversions, insertions, or deletions (Jukes and Cantor, Fitch and Margoliash, Neighbor Joining). The advantages of these methods are their simplicity, speed, and performance using a variety

of data types, but these methods are more sensitive to systematic error than character-based methods.

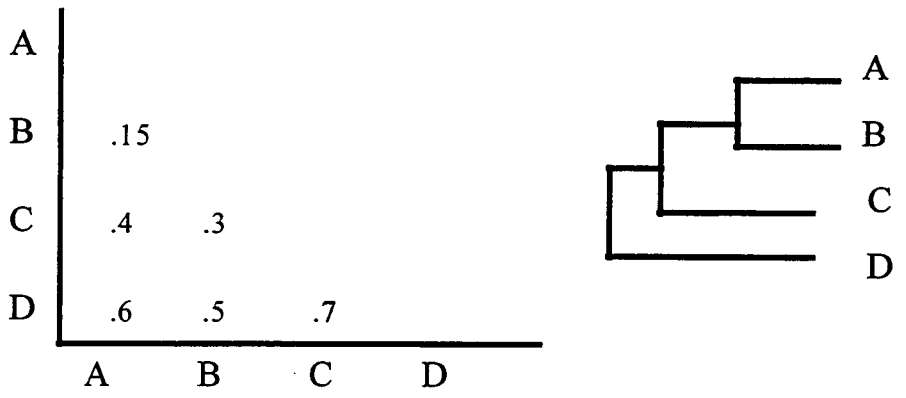

Figure 1.17. Distance matrix with corresponding dendrogram. Values in the matrix are derived from similarity values (similarity between two sequences, i.e., 90%). Distance values are calculated: Distance=1-similarity. The distances between values in matrix are proportional to distances on tree. (Distances on this dendrogram not to scale). This matrix and dendrogram infer sequences A and B are more alike than A to C or D.

Character-based methods. Character based methods include parsimony and maximum likelihood. These methods take into account the individual characters, their positions, and their likelihood of developing. These are statistical models to generate phylogenetic trees which infer evolutionary relationships. With parsimony, the program identifies informative sites, calculates the minimum number of substitutions required to give each informative site, sums the changes and chooses the tree requiring the fewest number of changes to explain the data (figure 1.18). Maximum likelihood generates the tree most likely to have occurred given the observed data and assumed model of evolution. These methods yield relationship estimates with lower variance, and are more robust to

assumptions about the evolutionary model. However, these methods require fast, super-computers and quantities of time to process the data. The method chosen for sequence analysis depends upon the desired information. Distance generated dendrograms represent current similarities, while character generated phylogenetic trees represent hypothetical evolutionary relationships.

	Site								
Sequence	1	2	3	4	5	6	7	8	9
1	G	G	A	G	A	C	A	T	G
2	G	A	T	T	A	C	A	T	A
3	G	A	G	C	G	C	T	T	G
4	G	A	G	A	G	C	T	T	A
					*		*		*

Figure 1.18. Informative site identification for maximum parsimony tree. A nucleotide site is informative only if it favors some trees over the others. In this example, there are three possible unrooted trees. A site is informative only if there are at least two different kinds of nucleotides at the site (Li and Graur, 1991).

Genetics Techniques Applied to Rumen Microbes

Introduction

In studying the rumen environment, investigators have lacked the appropriate methods to identify and enumerate community members. This is because investigators have used indirect microbiological techniques like selective enrichments, pure culture isolation, and MPN estimates to describe ruminal flora. Ruminal studies have concentrated on the measurement of degradation rates and end product fermentation.

Culture-based technique biases have given inaccurate understanding of population dynamics (Stahl et al., 1988; Odenyo et al., 1994; Amann et al., 1990). Phenotypic characteristics often mask genetic similarites. Nucleic acid techniques can provide community descriptions without cultivating the organisms. A more complete understanding of ruminal systems will come from combining genetic and physiological techniques. The following sections describe how nucleic acid techniques have been applied to ruminal environments.

Examples

Bacteroides. *Bacteroides* were a group of ruminal bacteria described as anaerobic, Gram negative, nonmotile or peritrichous, non-spore forming rods that produced butyric acid (Cato and Salmon, 1976). Characterizing isolates using these criteria caused much confusion, and considering genetic relationships have resolved many of the problems associated with the original taxonomic description. Other groups that are now described that were originally grouped in *Bacteroides* include *Fibrobacter, Prevotella, Lachnospira* and *Clostridium* (Avgustin et al., 1994; Cato and Salmon, 1976; Montgomery et al., 1988).

Fibrobacter. Stahl and coworkers studied the effect of diet on *Fibrobacter* in the rumina of steers. *Fibrobacter* were used as model organisms to develop rRNA based techniques (Stahl et al., 1988). Group, species, and sub-species probes were designed to describe the

population of *Fibrobacter* in ruminal samples. Using selective amplification, cloning and sequencing, two new *F. succinogenes* subspecies were discovered.

Prevotella. *Prevotella,* also grouped with *Bacteroides,* are believed to have synergistic relationships with cellulolytic bacteria, and may play a role in the breakdown of plant cell wall material. High genetic diversity has been detected among isolated strains of *P. ruminicola* based upon G+C content, DNA-DNA hybridization, RFLP analyses of 16S rRNA genes, and analysis of total cell proteins (Avgustin et al., 1994).

Ruminococcus. Ribosomal RNA probes designed from *Ruminococcus* enabled investigators to study bacterial interactions during fermentations of cellobiose, cellulose and alkaline hydrogen peroxide treated wheat straw (Odenyo et al., 1994). This study demonstrated 16S rRNA probes were beneficial in the substrate degradation studies. Separately, sequence analysis established the relationship between *R. hansenii* and *R. productus* (Ezaki et al., 1994).

Butyrivibrio. *Butyrivibrio* have been the subject of genetic engineering efforts. Cloning systems have been used to develop a strain that detoxifies the plant toxin fluoroacetate. Thorough knowledge of the genetically modified rumen bacteria to other rumen bacteria is essential before release. *Butyrivibrio* are another group that has undergone internal reclassification based upon genetic data. Forester and coworkers described *Butyrivibrio* isolated from rumina of white-tailed deer that were found to have less than 89 percent sequence similarity as compared to an ATCC strain (Forester et al., 1996). Recent

studies have grouped *Butyrivibrio* into three distinct phylogenetic groups (Forester et al., 1997). In 1996, van Gylswyk and coworkers described *Pseudobutyrivibrio* which phenotypically appeared to be similar to *Butyrivibrio*, but was placed into a separate genus based upon 16S rRNA gene sequence analysis.

Other examples. As ecological investigations of ruminal microflora continue, bacterial genes are being detected that show low similarity to organisms described in pure culture. Most new discoveries are 16S rRNA gene novelties, for example: *Schwartzia succinivorans,* fermenters of the metabolic byproduct succinate (van Gylswyk et al., 1997); *Streptococcus caprinus*, a tannin-resistant ruminal bacterium from feral goats (Brooker et al., 1994); *Ruminococcus schinkii*, a hydrogen-oxidizing, carbon dioxide-reducing acetogenic bacterium isolated from the rumen contents of lambs, llamas, and bison (Rieu-Lesme et al., 1996). These examples are paled by the collection of 16S rRNA molecule and gene sequences present in major databases like Genbank and Ribosomal Database Project (RDP). Overall, the future direction of using nucleic acid-based techniques to study the rumen environment will be focused on reclassifying knowns, evaluating the biodiversity, and determining the link between diversity and metabolic function.

Summary

Pyrrolizidine alkaloids are toxic compounds found in many plant species worldwide. Seneciphylline and jacobine are two predominant PAs found in tansy

ragwort which have been shown to be toxic to many livestock animals with the exception of sheep and goats. Bacteria present in the rumen of sheep have been shown to detoxify the PAs, and when transferred to cattle, can offer protection. Fastidious growth conditions and requirements have made members of this PA detoxifying enrichment culture difficult to isolate and identify using classical, anaerobic culturing techniques. Genetic techniques are being used to classify viable, nonculturable bacteria including those from ruminal contents. The importance of culturing the PA detoxifying organism(s) is not only potentially to describe new species, but to employ the degradation enzymes and/or bacteria as a probiotic to protect susceptible livestock from tansy toxicosis.

Chapter 2

BACTERIAL 16S RIBOSOMAL DNA ANALYSIS OF PYRROLIZIDINE ALKALOID DETOXIFYING ENRICHMENTS FROM THE OVINE RUMEN

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Introduction

Pyrrolizidine alkaloids (PAs) are principle toxins found in many plants around the world including the common weed, tansy ragwort (*Senecio jacobaea).* Seneciphylline and jacobine are the two predominant PAs in tansy ragwort and are characterized by macrocyclic heterocyles with one unsaturated bond (Figure 2.1).

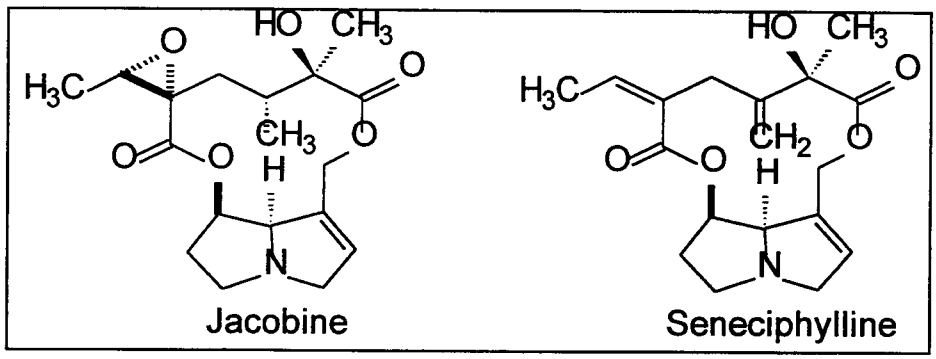

Figure 2.1. Structures of the pyrrolizidine alkaloids jacobine and seneciphylline. The structures of the pyrrolizidine alkaloids jacobine and seneciphylline are composed of retronocine bases and macrocyclic necic acid deriviatives.

Although PAs themselves are not toxic, their metabolic pyrrole derivatives are damaging to the liver. Hepatotoxicity is most often observed with PA toxicosis, but effects in pulmonary and renal tissues have also been observed. Feeding studies indicate cattle and horses are susceptible to hepatic disease after consuming 5-10% of their body weight in tansy plant material (Craig et al., 1991). However, sheep are not susceptible to toxicosis suggesting animal differences in liver metabolism, ruminal absorption, or ruminal flora activity. Craig and coworkers demonstrated the difference between animals

is due to microbial detoxification in the rumen (Craig et al., 1979, 1986, 1992). Attempts to isolate and identify the organism(s) responsible for detoxification have proven unfruitful.

The rumen is an expansive environment supporting the existence of a variety of organisms and metabolic processes. Microbes in the rumen play major roles in animals' digestion and nutrition. Ruminants are often more resistant to toxins than monogastric animals due to the metabolism of these toxic compounds by ruminal microbes (Craig, 1987). Ruminal microbes have been shown to detoxify mimosine (McSweeney et al., 1993), pyrrolizidine alkaloids (Lanigan, 1976), oxalates (Dawson et al., 1980), nitrotoxins (Majak and Cheng, 1981), fungal toxins (Westlake et al., 1987; Craig et al., 1985), and other plant byproducts (Olsen, 1987; Rasmussen et al., 1993; King and McQueen, 1981).

Study of ruminal microbes has proven challenging because many of the organisms are difficult to cultivate and isolate on solid media, even using established anaerobic techniques. Classical identification techniques require organisms to be isolated in pure culture for phenotypic observation and characterization. Furthermore, it has been demonstrated that organisms cultured in growth media do not adequately represent the population of organisms from an environment (Pace et al., 1986; Ward et al., 1990; Bond et al., 1995; Wagner et al., 1993). The organisms that grow are those capable of surviving media's selective growth conditions. Therefore, molecular techniques are now being used to study the microbial diversity of the rumen environment.

The molecular phylogenetic methods use informational macromolecular sequences (RNA, DNA, proteins) to provide comparative information among organisms.

The 16S ribosomal RNA gene is commonly used in genetic studies because of its universal presence among organisms and its informational content. This approach has been used to study the population dynamics of hot springs (Pace et al., 1986), marine environments (Giovannoni et al., 1990; Fuhrman et al., 1994), and the rumen environment (Stahl et al., 1988; Amann et al., 1990; Krumholtz et al., 1993). Gene sequences that have been determined are stored in public access databases and can be used for identifying unknown genes.

The purpose of this study was to identify the members the PA detoxifying enrichments using their 16S ribosomal RNA genes, and to compare the compositions of two different enrichments to find microbes they have in common, which may identify the organisms with the detoxifying capabilities.

Materials and Methods

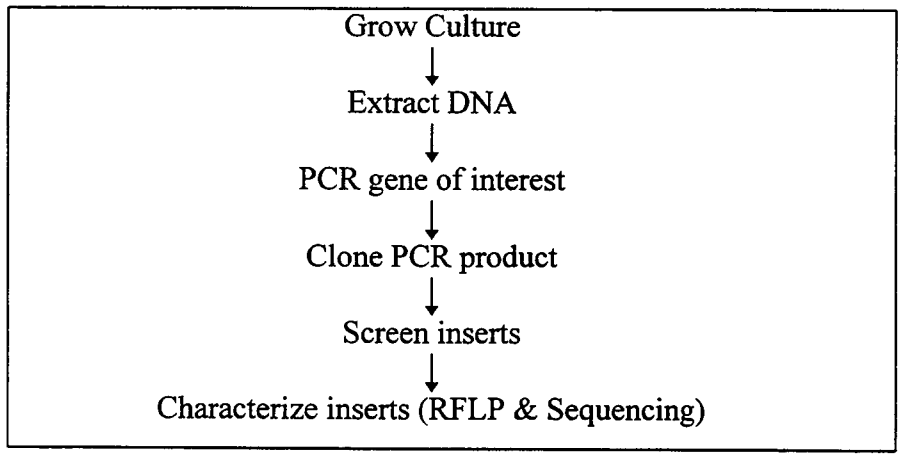

Figure 2.2. Flow chart of methods.

Enrichment Culture

Ovine rumen fluid was collected from sheep fed on alfalfa pellets, blended into McDougalls buffer, and anaerobically inoculated into both a rich (TYM) and semi-minimal (E medium) containing 50μg/ml PA (figure 2.3). TYM medium is the same as Lanigan's Medium #4 (Lanigan, 1976). E medium is a mixture of trace minerals, salts, volatile fatty acids, hemin and clarified rumen fluid. The inoculated tubes were incubated at 37°C and transferred at least 20 times. The enrichment cultures were assayed for the presence of PAs via TLC (Wachenheim et al., 1992a). Cultures from both media showing the absence of PAs were collected. The TYM culture used in the analysis did not grow in the presence of PA, but the subsequent transfer incubated with and degraded PA.

DNA extraction and PCR

DNA were extracted via a guanidium thiocyanate method (Pitcher, 1989). Bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3')
and 1525R (5'-AAGGAGGTGATCCANCCRCA-3') were used for PCR amplification of the 16S rRNA genes. PCRs were performed using a Perkin-Elmer thermocycler. In a final volume of 100μl, the amplification reactions contained 1X *Taq* polymerase reaction buffer and 1.5mM $MgCl_2$ (Promega), 0.2μM of each amplification primer, 200μM of each dNTP (Stratagene, La Jolla, California), and 2.5 U *Taq* polymerase (Promega). Temperature cycle conditions were 93°C for 1 min, 55°C for 1 min, and 72°C for 2 min, extended 5 s per cycle for 35 cycles. Following the final cycle, the reaction was extended

Components	TYM (%)	E Med (%)
Tryptone	1.0	-
Yeast Extract	1.0	-
$NaHCO_3$	0.6	-
K_2HPO_4	0.05	0.025
KH_2PO_4	0.05	0.025
NaCl	0.1	0.05
$MgSO_4$	0.01	0.004
$CaCl_2$	0.01	0.004
Resazurin	0.0001	0.0001
Cysteine	0.03	0.03
Na_2CO_3	-	0.4
$(NH_4)_2SO_4$	-	0.05
Hemin	-	0.00001
Sodium sulfide	-	0.0125
Rumen Fluid	-	10.0
Major VFA:		
Sodium acetate		0.05
Sodium propionate	-	0.01
Sodium butyrate	-	0.006
Supplemental VFA:		
Isobutyric acid	-	0.01
2-Methylbutyric acid	-	0.01
Isovaleric acid	-	0.01
Valeric acid	-	0.01
Trace Metals:		
Na_2EDTA	-	0.0005
$FeSO_4*7H_2O$	-	0.0002
$MnSO_4*H_2O$	-	0.0002
H_3BO_3	-	0.00003
$CoCl_2*6H_2O$	-	0.00001
$ZnSO_4*7H_2O$	-	0.00001
$NaMoO_4*2H_2O$	-	0.000003
$NiCl_2*6H_2O$	-	0.000002
$CuCl_2*2H_2O$	-	0.000001

Figure 2.3. Components of TYM and E media used to grow enrichment cultures. TYM medium is the same as Lanigan's Medium #4. E medium contains trace minerals, salts, volatile fatty acids, hemin, and clarified rumen fluid. Components are combined in water, serum vials are gassed with CO_2, the media is dispensed and vials are capped with butyl stoppers. After autoclaving and cooling, sterile sodium sulfide, cysteine, sodium bicarbonate, and PAs are injected through the butyl stoppers.

at 72°C for 10 min. The amplification product was resolved through a 1.0% agarose gel in 1X TAE (40 mM Tris-acetate, 1 mM EDTA) containing 0.4μg/ml ethidium bromide. Products were purified using QIAquick-spin PCR purification columns (Qiagen, Chatsworth, California) following manufacturer's instructions. Purified product was quantified using a Shimadzu UV160U or Beckman DU-64 spectrophotometer.

Clone Library Construction

Two clone libraries were constructed from the rich and the minimal media cultures' PCR products. The purified PCR products were ligated and transformed using reagents and competent cells from a TA cloning kit (Invitrogen Corporation, San Diego, CA). The ligation reaction mixture contained 2μl 10X ligation buffer, 4μl pCRII vector (25ng/μl), 2μl T4 DNA ligase, 12μl PCR product for a total volume of 20μl. The reaction tubes were placed on a Perkin-Elmer thermocycler at 14°C overnight. Seven vials (one ligation control, one transformation control and five ligation reactions) were transformed according to manufacturer's instructions and plated onto LB agar plates (1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar) containing 50μg/ml ampicillin and spread with 40μl of X-gal (40 mg/ml). Plates were incubated and developed according to TA cloning instructions. White colonies from the transformations were picked and restreaked onto LB agar plates containing 200μg/ml ampicillin and X-gal. The plates incubated at 37°C overnight and developed at 4°C for at least two hours. Confirmed white colonies were picked and inoculated into microtiter plate wells containing 200μl LB broth+200μg/ml ampicillin in each well. These titer plates incubated at 37°C overnight.

For long term storage, sterile Sarstedt tubes (Sarstedt, Inc. Newton, N. Carolina) and 96-well titer plates were inoculated with the same LB broth and incubated overnight. LB broth with 200μg/ml ampicillin + 28% glycerol was added and placed in -70°C storage. Clones in the TYM library were numbered 1-90 and the clones in the E medium library were numbered 142-309.

RFLP Analysis

Both libraries were screened for inserts using PCR (same conditions as above) with primers M13F (5'-TGTAAAACGACGGCCAGT-3') and M13R (5'-CAGGAAACAGCTATGACC-3') followed by Restriction Fragment Length Polymorphism (RFLP) analysis. The restriction digest was 1μl *HaeIII* (Gibco BRL Cat.# 15205-016), 1μl 85mM MgSO4, and 8μl of PCR product for each reaction. The tubes were centrifuged six seconds, placed in a 37°C waterbath for 1 hr, and visualized by electrophoresis on a 3% NuSieve (FMC BioProducts) gel with 0.6μg/ml ethidium bromide. Groups were verified using *MspI* (Gibco #15419-013) using the same reaction conditions.

rDNA Sequencing

Selected transformants were grown in a 37°C shaker overnight in LB broth with 200μl/ml ampicillin. Plasmids were extracted using Qiagen quickspin plasmid preparation (Qiagen) or standard alkaline lysis (Sambrook, 1989). DNA was quantified

by spectrophotometry and submitted for sequencing. The plasmid DNAs containing full length inserts were partially, bidirectionally sequenced on an ABI model 373A or 377 automated sequencer (Applied Biosystems, Foster City, California) with dye-terminator chemistry using sequencing primers 27F and 519R (5'GWATTACCGCGGCKGCTG-3'). DNA sequence data from cloned 16S rRNA genes were manually aligned to bacterial sequences using Genetic Data Environment (GDE) v2.0 (Steve Smith, Millipore Corporation, Marlborough, MA).

Sequence Analysis

Sequences representing each RFLP type were submitted to Similarity_Rank at the Ribosomal Database Project (RDP). Sequences were analyzed using Phylip distance methods, dendrograms were constructed using Neighbor Joining UPGMA v. 3.53c, visualized using treetool, and bootstrapped with 100 replicate samples. The organisms with the highest SAB matches were included in analyses and are listed in figure 2.4.

Results

Culture Characterization

Two clone libraries were constructed from an enrichment culture grown in two different media. The culture grown in TYM contained mostly gram positive cocci and gram negative rods. The culture grown in E medium contained a mix of gram negative

rods, ovoids, and a few gram positive cocci. Both cultures degraded PA within 12-16 hr (Johnston, personal communication).

Accession #	Organism	Gram	Morph.	Characteristics
AB002481	*Streptococcus bovis*	+	cocci	Low G+C, Streptococcaceae
Y10868	*Streptococcus caprinus*	+	cocci	Low G+C, Streptococcaceae
X81137	*Succinioclasticum ruminis*	+	cocci	Low G+C, Sporomusa
M62702	*Selenomonas ruminantium*	+	cocci	Low G+C, Sporomusa
M26493	*Megasphaera elsdenii*	+	cocci	Low G+C, Sporomusa
M62701	*Quinella ovalis*	-	cocci	Low G+C, Sporomusa
X73437	*Clostridium bifermentans*	+	rod	Low G+C, Clostridiaceae
X73450	*C. difficile*	+	rod	Low G+C, Clostridiaceae
D14139	*Peptostreptococcus prevotii*	+	cocci	Low G+C, Clostridiaceae
D14150	*P. anaerobius*	+	cocci	Low G+C, Clostridiaceae
X89971	*Butyrivibrio fibrosolvens*	+	rod	Low G+C, Clostridiaceae
M59089	*C. clostridiiformes*	+	rod	Low G+C, Clostridiaceae
L04165	*C. aminophilum*	+	rod	Low G+C, Clostridiaceae
M34116	*Chloroflexus aurantiacus*	-	rod	Green non-sulfur/Deinococcus
M34117	*Herpetosiphon aurantiacus*	-	rod	Green non-sulfur/Deinococcus
X96965	*Shigella boydii*	-	rod	Gamma proteobacteria
J01695	*Escherichia coli*	-	rod	Gamma proteobacteria
X80680	*Sh. dysenteriae*	-	rod	Gamma proteobacteria
L16469	*Prevotella melaninogenica*	-	rod	Bacteroides
L16473	*Prevotella veroralis*	-	rod	Bacteroides
L16482	*P. ruminicola*	-	rod	Bacteroides
Y09434	*Schwartzia succinivorans*	-	rod	Unclassified Firmicutes

Figure 2.4. 16S rDNA GENBANK sequences used in alignments.

Clone Library Analysis

The single bands of amplification product were observed by electrophoresis. PCR products ligated and transformed resulted in the production of 90 and 167 white colonies for the TYM and E medium libraries, respectively. Both libraries contained 80 clones with full length inserts. Over half of the E medium library colonies picked either did not

amplify or contained inserts of a size less than 1700 base pairs. RFLP analysis of TYM library gave nine different patterns, and the E medium library gave 23 patterns (figure 2.5). Similar patterns were placed into groups and verified using *MspI* showing further diversity within some groups. If additional patterns were detected using the second enzyme, and new group was formed.

DNA Sequencing and Sequence Analysis

DNA sequences of clones representing each RFLP type consolidated many of the patterns into a few clusters. These sequences were aligned with one another and sequences in the databases, indicating variable RFLP patterns represented gene variations or possibly gene families. Clones 27, 23, 71, 160, 193, 196, and 294 were chosen to represent gene families; 27 showed associations with members of the clostridia, clone 71, with *Prevotella*, clone 23, with *E. coli*, clone 196, with *Butyrivibrio*, and clone 294, with *Streptococcus*. Figure 2.6 shows the distribution of the clone types between the two libraries, and figure 2.7 shows associations with known rDNA sequences in the databases.

Discussion

The members of the PA detoxifying enrichment culture have not been isolated into pure culture and hence, have remained unidentified. Historically, chemical inhibitors, antibiotics, heat treatments were applied to narrow down the numbers of species observed by Gram staining. Chemical inhibitors and antibiotics against gram

Clone	Sequence Type	RDP Match	SAB/Similarity	# Found
158	EubB	*Clostridium aminophilum*	0.278	4
193	**EubB**	***Clostridium aminophilum***	**0.90/99%**	**1**
272	EubB	*Clostridium aminophilum*	0.682	1
241	EubB	*E. cellulosolvens (C. aminophilum* 0.453)	0.461	3
226	GDE consensus	*Clostridium coccoides (C. aminophilum* 0.410)	0.412	1
11	EubB	*Clostridium bifermentans*	0.815	3
5	EubB	*Clostridium bifermentans*	0.76	1
87	EubB	*Clostridium bifermentans*	0.81	7
63	GDE consensus	*Clostridium bifermentans*	0.893	18
27	**STADEN**	***Clostridium bifermentans***	**0.87/99%**	**28**
200	EubB	*Clostridium clostridiiforme*	0.583	1
214	EubB	*Clostridium clostridiiforme*	0.612	1
149	GDE consensus	*Clostridium clostridiiforme*	0.734	5
160	**STADEN**	***Clostridium clostridiiforme***	**0.541/88%**	**7**
156	STADEN	*E. ramulus (C. clostridiiforme* 0.597)	0.603/89%	1
260	EubB	*Eubacterium hadrum (C. clostridiiforme* 0.524)	0.546	1
283	EubB	*Eubacterium ramulus (C. clostridiiforme* 0.534)	0.567	1
42	EubB	*Escherichia coli*	0.829	1
44	EubB	*Escherichia coli*	0.578	1
76	EubB	*Escherichia coli*	0.602	1
23	**STADEN**	***Escherichia coli***	**0.883/99%**	**2**
196	EubB	*Eub. cellulosolvens (Butyrivibrio* by BLAST 96%)	0.542	3
212	EubB/EubA	*Azoarcus (P. melaninogenica* 0.128)/*Bacillus*	0.134/0.164	1
221	EubB	*Prevotella corporis*	0.586	1
169	EubB/EubA	*Prevotella ruminicola/ Streptococcus*	0.506/0.82	3
33	EubB	*Prevotella ruminicola*	0.687	3
71	**GDE consensus**	***Prevotella ruminicola***	**0.615/95%**	**15**
173	EubB	*Selenomonas lacticifex*	0.275	1
180	GDE consensus	*Selenomonas ruminantium*	0.790	1
280	GDE consensus	*Acidaminococcus ruminis*	0.506	1
151	EubB	*Succiniclasticum ruminis*	0.138	1
155	EubB	*Streptococcus bovis*	0.580	1
176	EubB	*Streptococcus bovis*	0.891	2
178	EubB	*Streptococcus bovis*	0.492	4
233	EubB	*Streptococcus bovis*	0.836	2
291	EubB	*Streptococcus bovis*	0.846	
171	GDE consensus	*Streptococcus bovis*	0.753	2
286	EubB	*Streptococcus bovis*	0.935	1
294	**STADEN**	***Streptococcus bovis***	**0.814/98%**	**28**

Figure 2.5. Clones representing RFLP types and corresponding RDP similarity matches. All genes represented by unique RFLP patterns were sequenced and submitted to RDP: type EubB- only bases 51-600 from single EubB read submitted; type GDE-consensus sequence of EubB and 519R reads prepared in GDE and submitted; type STADEN- used STADEN to prepare consensus sequence. Selections in boldface represent clones chosen to represent gene clusters in figure 2.6. Similarities were calculated from number of mismatches in the first 500 bases of the 16S rRNA gene. Clones 169, 212, 173, and 151 were determined to be chimeras by one or more of the following criteria: poor quality sequence data, CHIMERA_CHECK indicated high probability of these being chimeras, and EubB and EubA sequence reads were matched to different families of organisms.

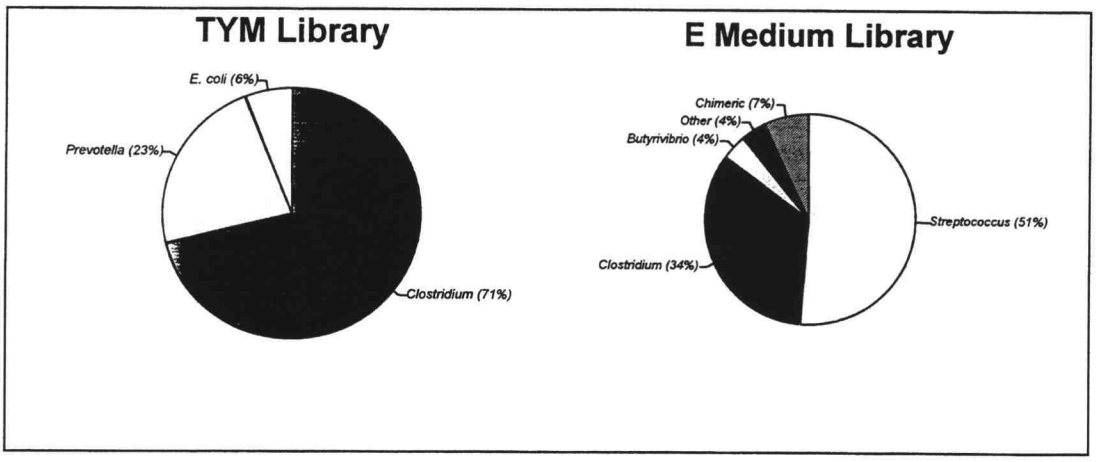

Figure 2.6. Distribution of bacterial 16S rRNA genes between the TYM and E medium libraries. The TYM library (80 clones total) comprises one *Clostridium* species, *Prevotella,* and *E. coli.* The E medium (80 clones total) comprises *Streptococcus,* several *Clostridium* species, *Butyrivibrio*, and a single *Selenomonas, Acidaminococcus,* and *Prevotella.* No common species were found between the two libraries, only the common genus *Clostridium.* The one *Prevotella* in the E medium library may have been an artifact from PCR or from the rumen fluid used to make the medium. Its SAB value was below 0.25, indicating this identification was unreliable.

positive bacteria inhibited PA degradation most effectively (Wachenheim et al., 1992b). Spore formation was difficult to detect, therefore, heat treatments were applied (75, 85, 95, 100°C for 10-20 minutes). However, heat treatments were not extensive enough to stop PA degradation in cultures 40-60 hours old. The culture members were not isolated on solid media, but streaks taken from the first section of the isolation streak demonstrated PA degradation activity. Improved growth on plates occurred with low agar concentration (0.6%) and long incubation period (one week). Isolate descriptions included spindle shaped Gram positive rods, curved Gram negative rods, large Gram positive rods, small Gram positive diplococci, and very large Gram positive cocci.

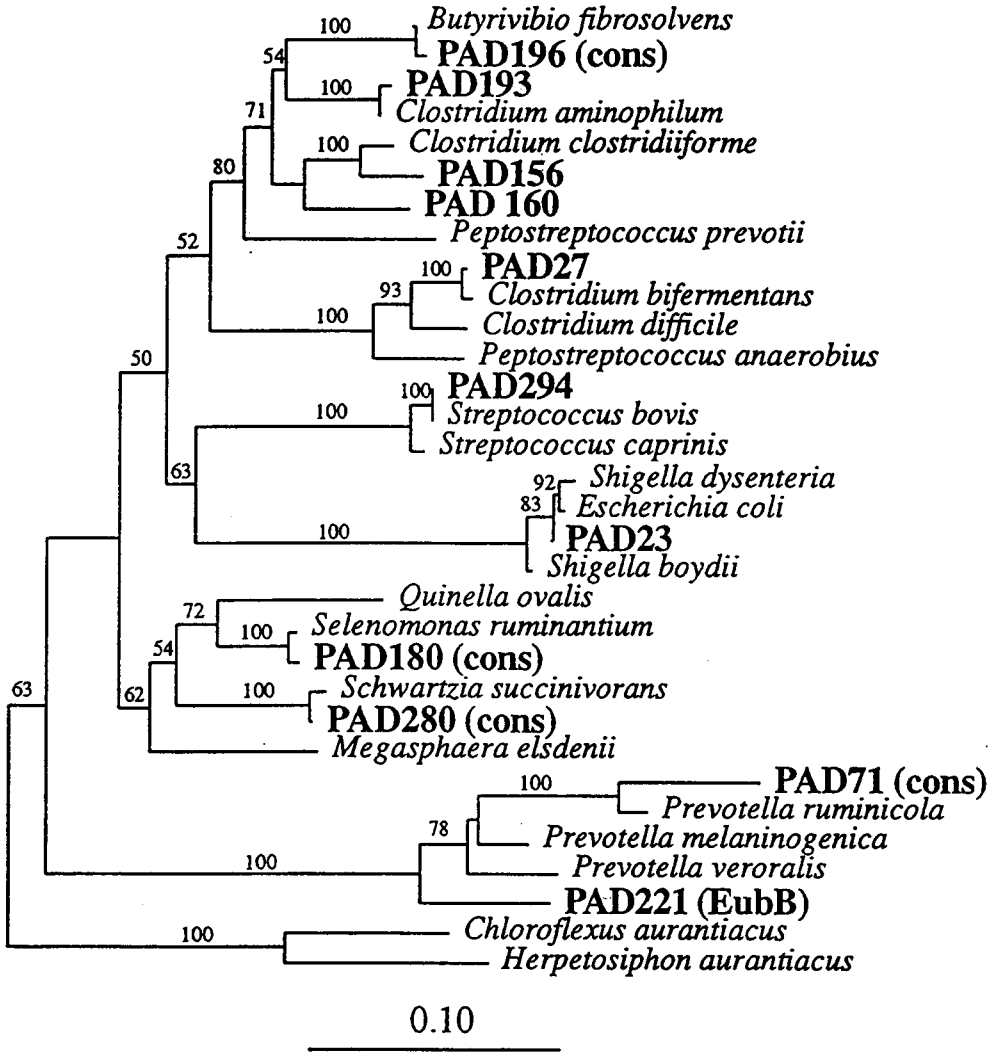

Figure 2.7. Dendrogram showing relationship of partial sequences from TYM and E medium libraries to their closest neighbors. TYM PA detoxifying (PAD) clones were numbered 1-90, and E medium PAD clones were numbered 142-309. PAD 193, 156, 160, 27, 294, 23 were bidirectionally sequenced with EubB and 519R, and consensus sequences were produced by STADEN. Those labeled PAD 'cons' were bidirectionally sequenced, but consensus sequences were produced in GDE. PAD221 was included in tree using only its single directional EubB sequence. This is an unrooted tree inferred by neighbor joining from positions 30 to 600 *(E. coli* numbering) with 100 bootstrap replicates supporting branching order (values below 50 are not shown). The scale bar represents the number of nucleotide substitutions per sequence position.

There was one organism that seemed to be present consistently in working liquid and plate cultures. Isolates recovered did not independently or in combination demonstrate degradation activity. It was a large Gram positive, pleomorphic rod that trailed off into a chain of large cocci. In a dilution series, this organism would be present in the last tube that degraded PA.

The morphological and gram character of the bacteria grown in the enrichments vary with growth media. It is possible that bacteria accompanying the detoxifying bacteria vary from one enrichment to another. Because of the presence of Gram positive bacteria in the cultures, the guanidium thiocyanate DNA extraction method was used to ensure the gram positive bacteria were well represented (Pitcher et al., 1989).

Newer molecular approaches to studying the diversity of environmental samples have been applied to classify the bacteria in this specialized enrichment culture. Archaeal primers were used in a PCR to test for the presence of methanogen 16S rRNA genes. Finding none, eubacterial specific primers were used to amplify the 16S rRNA genes. Choosing the number of clones for the libraries depended upon the number of types of bacteria thought to be present in the cultures as indicated by gram stains. Five types and two types were estimated in the TYM and the E medium cultures, respectively. Eighty white colonies from each should have represented the diversity in the cultures. Two separate libraries were constructed for the purpose of comparison. The clone type common between the libraries may then be the organism responsible for PA detoxification activity.

RFLP analysis was used to screen the libraries to ensure the diversity was detected. Specific probe construction and colony hybridization was attempted, but the

probes were not specific enough to detect or differentiate the diversity (data not shown). The unknowns were only partially sequenced because the 5' end of the 16S rRNA gene was sufficient in differentiating the groups present in the culture. However, more sequence data may be necessary to better match the unknowns' to their nearest known neighbor. The bidirectional sequence data of the 5' end was sufficient to differentiate among the genera of both libraries and to provide templates from which species/type specific oligonucleotide probes may be designed. The sequence data representing the unique RFLP patterns consolidated the diversity for each library into three main groups (Figure 2.6). The single gene types found in the E medium (*Selenomonas, Succiniclasticum*, *Prevotella*) were disregarded because of poor sequence data, conflicting sequence identifications between the 5' and 3' ends of the molecule, and CHIMERA_CHECK results. The six genes may be chimeras, or they may have come from nonviable bacteria in the autoclaved rumen fluid used to make the E medium, contributing to the high number of RFLP patterns, but not representing the active PA degrader(s).

An observation common among libraries constructed from environmental samples is the clustering of closely related sequences branching closer with one another than with a known gene. This phenomenon has been observed in many environmental studies including those of marine environments, peat bogs, termite hindguts, in libraries prepared and not prepared using PCR. Field et al. discussed this genetic variability and its possible origins, but the significance of gene clustering is unknown (Field et al., 1997). Gene clustering was also observed in this study in both libraries. Therefore, clones 27, 23, 71, 160, 193, 196, and 294 were chosen to represent those gene clusters.

RFLP analysis and subsequent sequence data showed only one bacterial type, the *Clostridia,* were common to both libraries. The sequence data and SAB values show they were only common to the genera level, but percent similarities indicate close relationships to those in the database. The TYM library had one *Clostridium* type from Collins' cluster XI (*C. bifermentans*), and E medium had several types of Clostridia, but all from cluster XIVa (*C. clostridiiforme, C. aminophilum*). Collins, in his paper showing the phylogeny of the genus Clostridia, supports figure 2.7 and the distance between the two clusters (Collins et al., 1994; Lawson et al., 1993).

The other bacterial types observed in the TYM library included *Prevotella* and a gamma proteobacteria, most likely E. coli, and in the E medium library, *Streptococcus.* The other single gene types from the E medium library may be residual bacteria from the whole rumen fluid, and perhaps have not been present in other PA degrading enrichment cultures grown previously. Some of the types of bacteria detected in these libraries are commonly found the rumen (*Prevotella, Streptococcus, Butyrivibrio)* (Hespell et al., 1997). Clostridia and *E. coli* are often detected in the rumen, but in lower numbers, and are thought to be transient, entering the rumen via feed or water, because *Clostridium* spp. are more common in soil, and *E. coli*, in the lower gastrointestinal tract (Hespell et al., 1997). The TYM medium with 1% tryptone and yeast extract strongly selected for a proteolytic organism, perhaps something similar to *C. bifermentans*, the most predominant gene type in the library.

There are two explanations why no identical organisms were found between the two libraries: either the organism responsible for PA detoxification was not detected, or more than one species of *Clostridium* degrade PAs. Noting that the clostridia from both

libraries branched separately, the detoxification activity may be indigenous in groups from the *Clostridiaceae*. The latter hypothesis is based on observations that other members of the *Clostridiaceae* have demonstrated the capability of degrading PAs or similar compounds. *Peptostreptococcus heliotrinreducans,* a cytochrome-producing ruminal anaerobe metabolizes the PAs heliotrine and lasocarpine to methylene derivatives (Lanigan, 1976). McSweeny et al. demonstrated *Synergistes jonesii* as capable of degrading toxic pyridinediols.

Future studies should concentrate on growing and isolating the detoxifying organism in a continuous culture and on solid media, as well as developing species specific probes to identify and enumerate those species in PA detoxifying cultures. Upon isolation and identification, the organism may be developed into a probiotic to protect animals that come into contact with the toxic pyrrolizidine alkaloids in tansy ragwort.

Summary

Many pyrrolizidine alkaloid metabolic derivatives are toxic to animals that eat plants containing the parent compounds. Many bacteria have demonstrated the ability to degrade toxic compounds including bacteria from enrichments from sheep rumen fluid. The bacteria in this enrichment have been difficult to grow on solid media using classical anaerobic techniques. Therefore, the 16S rRNA genes were amplified, cloned, and sequenced from DNA extracts in order to identify the organisms in the enrichment culture. These sequence data were compared to known sequences in major databases. This procedure was applied to two different enrichments demonstrating the detoxification

ability to find an organism common to both cultures which may indicate the organism performing the degradation. The organisms detected were from the genera *Clostridium, Prevotella,* and *E. coli* from the rich medium (TYM) culture, and *Streptococcus, Clostridium, Butyrivibrio,* and *Selenomonas* from the minimal-plus-rumen fluid (E medium) culture. *Clostridium* spp. were detected in both libraries, but they were not from the same species.

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