GENETIC DIVERSITY OF *BRUCELLA ABORTUS* ISOLATES AS DETERMINED BY AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP) ANALYSIS

A Senior Honors Thesis

by

KATHERINE ANN BLISS

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
in partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

April 2003

Group: Life Sciences 1

GENETIC DIVERSITY OF *BRUCELLA ABORTUS* ISOLATES AS DETERMINED BY AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP) ANALYSIS

A Senior Honors Thesis

by

KATHERINE ANN BLISS

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
in partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

Approved as to style and content by:

Allison Rice-Ficht
(Fellows Advisor)

Edward A. Funkhouser
(Executive Director)

April 2003

Group: Life Sciences 1

ABSTRACT

Genetic Diversity of *Brucella Abortus* Isolates as

Determined by Amplified Fragment Length

Polymorphism (AFLP) Analysis. (April 2003)

Katherine Ann Bliss
Department of Biochemistry & Biophysics
Texas A&M University

Fellows Advisor: Dr. Allison Rice-Ficht
Department of Medical Biochemistry & Genetics
Texas A&M University Health Science Center

Although *Brucella abortus*, the causative agent of brucellosis, is mainly under control in the United States, outbreaks do occur, particularly in wild animal herds. This study coupled amplified fragment length polymorphism (AFLP) analysis with the powerful Bionumerics software package to determine the genetic relationships between *B. abortus* field isolates, collected from infections in wild herds of elk and bison to achieve a better understanding of the molecular diversity and evolution of *B. abortus*. The field isolates were also compared to classic strains of *B. abortus*. AFLP has been shown to be an excellent method, being both rapid and accurate, for identifying genetic polymorphisms in bacterial isolates. Unfortunately, this study did not produce meaningful resolution between the field isolates but supports AFLP as a potentially influential procedure for molecular diversity research.

ACKNOWLEDGEMENTS

Dr. Allison Rice-Ficht
Dr. Thomas Ficht
Dr. Patricia Klein
Brian O'Shea
Virginia Cox

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Brucella species cause brucellosis in many animals, including humans and marine animals (1). In most areas of the world, animal brucellosis is endemic and human brucellosis is not unusual (1). The prevalence of brucellosis in these areas is responsible for huge economic losses (1). *Brucella* is also classified as a category B biological threat agent (2). Although *Brucella* species are closely related, with greater than 90% homology in DNA-DNA hybridization experiments (1,2), there are detectable mutations, or polymorphisms. Variability in genome organization, polymorphisms in known and unknown DNA sequences, and insertion sequence polymorphisms have all been observed in the *Brucella* genus (3). The most frequent polymorphism in Brucella is small indels (1-34 kb) localized to certain regions, or polymorphic hotspots (4).

Brucella abortus's preferred host is cattle (1, 2). However, *B. abortus* can be transmitted to humans through direct or indirect contact with infected animals (2). Although *B. abortus* is primarily under control in domesticated and commercial cattle, natural *B. abortus* outbreaks do occur among cattle herds, and more often, among wild elk and bison herds in the United States. These natural genetic variations in the *B. abortus* genome can be used to determine genetic relationships, or the phylogeny, between isolates to better understand the host-pathogen interaction, genetic evolution and diversity of *B. abortus*.

Journal Model: *Applied and Environmental Microbiology*

A convenient comprehensive method for identifying genetic differences between isolates is AFLP analysis. AFLP generates a population of random amplified fragments in a pattern unique to the individual isolate. AFLP samples a greater proportion of the genome directly (5,6) than other polymorphism identification methods such as insertion element typing (5), giving a better resolution of genetic relationships. It also produces highly reproducible results (7,8).

For this project, 17 *B. abortus* isolates and 6 closely related bacterial isolates were analyzed using AFLP methodology. Thirteen of the *B. abortus* isolates were field isolates, collected from wild elk and bison herds with natural outbreaks of *B. abortus* in Idaho and Yellowstone National Park. These infections are of concern to the neighboring ranchers, whose herds may be infected by these wild herds, with devastating economic consequences. Therefore, a better understanding of the host-pathogen mechanisms and genomic evolution is required to better control *B. abortus* outbreaks. The field isolates were compared to type strain *B. abortus* isolates: S19, the classic vaccine strain since 1928; S2308, the model virulent strain; and RB51, the new vaccine strain derived from S2308. The profiles generated by AFLP were then analyzed with Bionumerics (Applied Maths, Inc.) software to generate a dendrogram, graphically representing the genetic relationships between the samples. This information was coupled with other data associated with the samples and analyzed to better understand the relationships between the samples.

LITERATURE REVIEW

Brucella is a small gram-negative cocci and the causative agent of brucellosis. Each of the six different species of the *Brucella* genus is capable affecting a small range of hosts, but shows distinct host preferences (see Table 1) (3). Recently, strains have been isolated (9) from marine mammals that are clearly *Brucella*, but do not fall into any of the defined species. *B. abortus* prefers rudiment hosts, in which it also causes spontaneous abortions, but can infect humans (2). Animal brucellosis is mainly controlled in developed countries, but remains endemic in many areas of the world (1). No vaccine is available for brucellosis because any vaccine causes the symptoms of the disease (2). The ability of *Brucella* to survive and multiple in macrophages, by preventing the formation of the phagosome, is key to its virulence (1). Due to the high degree of infectivity of *B. abortus*, the lack of a vaccine and the huge economic impact of *Brucella* infections, *B. abortus* is classified as a category B biological threat agent (2).

Brucella is a member of the α-2 subclass of the class *Proteobacteria.* Its closest relatives are *Ochrobactrum*, an opportunistic human pathogen, and *Rhizobium*, which thrive in symbiotic relationships with plants (3). In 1998, Julián Velasco determined a new species of *Ochrobactrum* that is most closely related to *Brucella* and named it *Ochrobactrium intermedium* (10).

It has been theorized that *Brucella* and its closest relatives, each of which survives through close interaction with respective eukaryotic host cells, each differentiated from a common ancestor when a suitable eukaryotic host appeared.

TABLE 1. *Brucella* Host Preferences

***Brucella* species**	**Host Preferences**
B. abortus	Mainly cattle. Can infect buffaloes, camels, deer, dogs, horses, sheep and humans.
B. melitensis	Mainly sheep and goats. Can infect cattle and humans. Most common human isolate.
B. suis	Biovars 1 and 3 - Swine. Biovar 2 - European wild hares. Biovar 4 - Reindeer and wild caribou. Biovar 5 – Rodents. All biovars can infect humans.
B. canis	Canines.
B. ovis	Sheep.
B. neotomae	Desert wood rat

differentiated from a common ancestor when a suitable eukaryotic host appeared. A better understanding of the differentiation and adaptation of each species to its host would help support or disprove this theory and perhaps lead to a better understanding of pathogen-host interactions and the development of host preference (3).

Perhaps the most comprehensive article on this topic was published in *Microbes and Infection* in 2000 by Nieves Vizcaino, et al (3), and was entitled "DNA Polymorphism in the genus *Brucella*." The author relates the various attempts made to identify polymorphisms and clarify the genetic relationships among the *Brucella* genome over the last few decades. In 1985, restriction fragment length polymorphism (RFLP) analysis and agarose gel electrophoresis were completed on *Brucella* isolates to characterize species within the genus *Brucella*. However, the limited resolving power of agarose gel electrophoresis made this technique difficult to analyze. Progress was made in 1988, through the utilization of pulsed-field gel electrophoresis to distinguish bands

generated by genomic restriction digests. Low-cleavage-frequency restriction enzymes were selected to create more meaningful restriction patterns. This technique was not able to distinguish between biovars within species. Recently, the discovery of the presence of insertion elements (IS) in the *Brucella* genome enabled more novel differentiation techniques. One of these techniques, based on IS711 RNA mismatch-cleavage, shows a great deal of promise, as it was able to distinguish between all of the *Brucella* species and sub-species biovars. However, more research is necessary for this technique, because only one strain of each *Brucella* species and biovar was analyzed and there were some confusion due to the presence of a background smear. Other repetitive elements, such as extragenic palindromic (REP) sequence and enterobacterial repetititive intergenic consensus (ERIC) have also been used through PCR to distinguish isolates. A combination of PCR patterns generated from both REP and ERIC sequences was successful at distinguishing between every *Brucella* isolate tested. Finally, random amplified polymorphic (RAPD) DNA methodology has been completed on *Brucella* samples, showing a great deal of potential for being able to resolve complex relationships between isolates. However, it was recognized that high levels of reproducibility are required for a meaningful process.

The need for more research into the genetic diversity of the *Brucella* genus isolates is demonstrated by another study done by Nieves Vizcaíno, et al. (11), in which a 25-kilobase deletion in the *B. abortus* genome is shown to be a gene cluster involved in the synthesis of a novel polysaccharide. The absence of this polysaccharide may have important implications for the host-preference exhibited by *B. abortus*.

AFLP has been shown to be an excellent method, being both rapid and accurate, for identifying genetic polymorphisms in bacterial isolates. Two studies are briefly described below to highlight the current bacterial AFLP analysis. In 1997, a study was published by Paul Keim, et al. (8), which surveyed 78 *Bacillus anthracis* isolates and six closely related *Bacillus* species with AFLP, determining 357 AFLP-generated polymorphic fragments. This AFLP approach directly examined approximately 6.3% of the *Bacillus* genome for length mutations, or mutations in the length of the unknown sequence between the restriction enzyme sites, and 0.36% of the genome for point mutations, or mutations within the restriction enzyme recognition sites. The experimentally determined genetic relationships between the *Bacillus* isolates were consistent with geographic distribution and the current theoretical origin of *B. anthracis*.

A study of the molecular diversity of *Vibrio cholerae* was published in 2000 by Sunny C. Jiang, et al. (7), analyzed sixty-seven *V. cholerae* isolates, from four different sampling sites over the course of a year, by AFLP to determine temporal and spatial genetic relationships. After similarity analysis, the isolates clustered into three groups – each representing a different time of the year. The authors suggest that the *V. cholerae* population has a shift in predominant genotypes during the year, perhaps in response to environmental cues.

METHODOLOGY

Due to the restrictions concerning handling of *Brucella abortus*, as a Biosafety Level 3 organism, Dr. Thomas Ficht provided me with genomic DNA preparations of all of the isolates. Thirteen unique isolates were obtained: one was obtained from National Animal Disease Center (NADC), one from the Colorado serum collection, two from South Dakota State University (SDSU), and the remaining from United States Department of Agriculture- Animal and Public Health Investigative Services (USDA-APHIS). Five of these samples were provided twice with different labels as a reproducibility control. The six outgroup samples, species *Ochrobactrum* and *Rhizobium*, were obtained from the Pasteur Institute. Outgroup samples, closely related but distinctly different to the samples of interest, are necessary to root the phylogenetic analysis. Small aliquots of the genomic DNA were run on a 1% agarose gel to confirm the quality of the samples and to determine the concentration of each isolate. Samples were then diluted to approximately a 100ng/ul concentration. Tables 1 and 2 contain more detailed information about each sample.

AFLP PROCESS

This protocol is based on "AFLP: A New Technique for DNA Fingerprinting" by P. Vos, et al., (14) and modified by Virginia Cox by Brian O'Shea. A schematic representation of the process is shown in Figure 1.

A sequential digestion of genomic DNA with MseI and PstI created three distinct populations of fragments: fragments with both ends cut with MseI, fragments with both

TABLE 2. *B. abortus* Isolate Information

Sample	Isolate Designation	O-antigen	Requires CO2	Host	Source	Origin
1	000-1100-1	R	N		USDA-APHIS	*Field*
2	000-1198-1	R	N		USDA-APHIS	*Field*
3	000-1198-2	R	N		USDA-APHIS	*Field*
4	000-0201-1	R	N		USDA-APHIS	*Field*
5	000-0201-2	R	N		USDA-APHIS	*Field*
6	000-0202-1	R	N		USDA-APHIS	*Field*
7	000-0202-2	R	N		USDA-APHIS	*Field*
8	2308-2	A	N	bovine	NADC[a]	*Field*
9	2308-1	A	N	bovine	NADC	*Field*
10	S19m3	I	N		USDA-APHIS	*Vaccine*
11	D1d5-60	A	N		USDA-APHIS	*Field*
12	02-4804-2	A	Y	bison	SDSU[b]	*Field*
13	02-683-1	A	Y	bison	SDSU	*Field*
14	02-683-2	A	Y	bison	SDSU	*Field*
15	S19	A	N		Colorado Serum Co.	*Vaccine*
16	m180	R	N		USDA-APHIS	*S2308*
17	RB51	R	N		USDA-APHIS	*S2308*

USDA-APHIS = United States Department of Agriculture, Animal Public Health
NADC = National Animal Disease Center
SDSU = South Dakota State University

TABLE 3. Outgroup Isolate Information.

Sample	Species
82-115	*Ochrobactrum anthropi*
105838	*Ochrobactrum intermedium*
105839	*Ochrobactrum intermedium*
105840	*Ochrobactrum intermedium*
105841	*Ochrobactrum intermedium*
Rm2011	*Rhizobium meliloti*

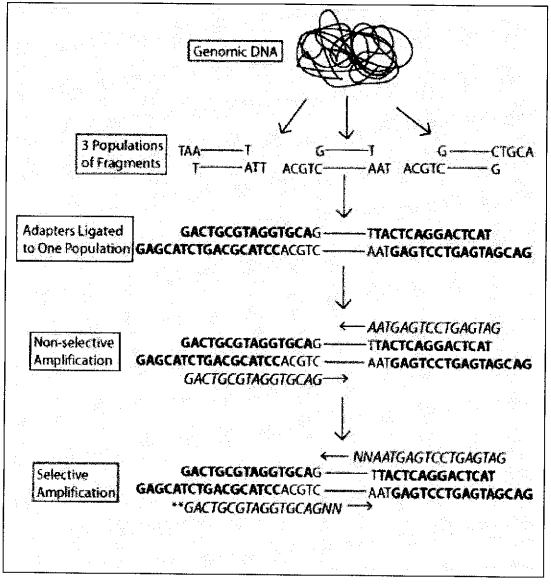

FIG. 1. AFLP Schematic. Please note the selective nucleotides (indicated by N) which reduce the number of fragments to create a meaningful profile. Also, the ** represents the IRD-labeling of the P-selective primer.

ends cut by PstI, and fragments with one end cut with MseI and one end cut with PstI. Five microliters of diluted genomic DNA (100ng/ul) were aliquoted into 0.5ul eppendorf tubes. A master mix was made (see Table 4) and 42.4ul of this master mix was added to each tube containing the aliquotted DNA. Each tube was incubated for 2 hours in a 37°C water bath and then the MseI was heat-inactivated by placing each tube in a 65°C water bath for 20 minutes.

TABLE 4. Components of Master Mix for MseI Digestion of Genomic DNA

Amount (1X)	Component
5ul	10X NE Buffer 2 (New England Biolabs)
0.5ul	Bovine Serum Albumin (BSA) (10mg/ml)
0.625ul	MseI (New England Biolabs) (4U/ul)
36.275ul	Sterile distilled water

Another master mix was prepared (see Table 5) for the PstI restriction digest, of which 2.625ul was added to each tube. Like the MseI digestion, each tube was incubated for 2 hours in a 37°C water bath and then the PstI was heat-inactivated by placing each tube in a 65°C water bath for 20 minutes.

TABLE 5. Components of Master Mix for PstI Digestion of MseI-Digested DNA

Amount (1X)	Component
0.5ul	5M NaCl solution
2ul	1M Tris-HCl solution, pH 7.9
0.125ul	PstI (New England Biolabs) (20U/ul)

Next, adapters are ligated to the cut ends of DNA fragments to generate short regions of known sequences on each end of the fragments. A master mix is prepared (see Table 6) and 10ul of the master mix is added to each tube. The adapter ligation reactions are then incubated overnight in a 37°C water bath. The samples are then diluted to a final concentration of 1ng/ul by adding 440ul of 1X TE.

TABLE 6. Components of Master Mix for Ligation of Adapters

Amount (1X)	Component
1ul	10X ligation buffer (Boehringer Mannheim)
1ul	MseI adapter (50 pmol/ul)
1ul	PstI adapter (5 pmol/ul)
1ul	T4 DNA ligase (Boehringer Mannheim) (1U/ul)
6ul	Sterile distilled water

Next, the large number of fragments is reduced by only amplifying one population of fragments: those with one end cut by MseI and one end cut by PstI. This is achieved through using PCR with primers engineered to match the desired adapter and remaining restriction enzyme cut-site. Five microliters of each dilute digested and ligated isolate are placed in a 0.2ul PCR tubes. A master mix is prepared (see Table 7) and 15ul of this master mix is added to each tube. The PCR reactions were amplified with 20 cycles of the following program: 94°C for 30 seconds, 56°C for 1 minute, 72°C for 1 minute. The amplified samples are diluted by adding 180ul of 0.5XTE buffer to each tube, creating a final concentration of the original DNA of 25 pg/ul.

TABLE 7. Components of Non-selective Preamplification of Digested DNA

Amount (1X)	Component
2ul	10X PCR buffer (Promega)
2ul	MgCl2 (Promega) (25mM)
0.4ul	dNTP mix (10mM)
1ul	PstI Preamplification Primer (30g/ul)
1ul	MseI Preamplification Primer (30ng/ul)
0.08ul	Taq Polymerase (Promega)
8.52ul	Sterile distilled water

The number of analyzable fragments is further reduced to create a meaningful pattern of fragments for each isolate, or fingerprint. This is achieved through using PCR with primers engineered to match the desired adapter, the remaining restriction enzyme cut-site, and two additional nucleotides in the unknown intervening sequence between the restriction enzyme cut-sites, effectively reducing the number of fragments by a factor of sixteen (see exact primer sequences in Figure 1). Also during this step, each fragment of interest is labeled with a fluorescent label through the IRD-labeled PstI-selective primer, obtained from Licor. Two microliters of each dilute pre-amplified DNA are placed in a 0.2ul PCR tube. A master mix is prepared (see Table 8) and 8ul of this master mix is added to each tube. The PCR reactions were amplified with a touch-down PCR consisting of a hot start (94°C for 2 minutes), 13 cycles of the following cycle with the annealing temperature reduced each cycle by 0.7°C: 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute, 23 cycles of the following parameters: 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute, and a 5 minute hold at 72°C. Due

to the light-sensitive nature of the fluorescent label, the reactions must be protected from the light, such as being stored in aluminum foil.

One benefit of the Licor electrophoresis system is that two samples can be run simultaneously in each lane. This is achieved through IRD-labeling with different fluorescent compounds that respond to different wavelengths of light: 700nm or 800nm. Therefore, 5ul of a 700nm-IRD-labeled reaction, 5ul of a 800nm-IRD-labeled reaction and 2ul of Licor basic fusion loading dye were mixed together in one well of a 32-well PCR plate, in preparation for loading into a Licor polyacrylamide gel. A 0.2mm thick,

TABLE 8. Components of Master Mix for Selective Amplification of Pre-amplified DNA.

Amount (1X)	Component
1 ul	10X PCR buffer (Promega)
1 ul	MgCl2 (Promega) (25mM)
0.2ul	dNTP mix (10mM)
2 ul	MseI Selective Primer (7.5ng/ul)
0.3 ul	PstI IRD-labeled Selective Primer (1 pmole/ul)
0.04 ul	Taq Polymerase (Promega)
3.46 ul	Sterile distilled water

7% LongRanger/7M Urea/1.2X TBE polyacrylamide gel was prepared for the Licor system. Molecular weight markers (Licor) were positioned for loading on each end of the gel and after every 4-8 samples, to later aide in normalizing and standardizing the gel.

The AFLP reactions were run on the prepared polyacrylamide gel for approximately three hours at 1500 vols on a Licor Base ImagIR 4200 System. This

system creates two digitized images of each gel (16-bit TIFF file), one for 700nm and one for 800nm, with each fragment represented by a white band on a dark background. Two primer combinations were used and each primer combination was run twice to minimize variations between individual gels.

DATA ANALYSIS

Each Licor image was inverted (creating dark bands on a white background) and processed by Bionumerics software (Applied Maths, Inc.). This process includes four steps: definition of the lanes, adjustments to the data (spot and noise removal), standardization of the gel, and assignment of bands. Next, bands were assigned to band-classes; that is, each band was either considered to be the same band as in other isolates or not. This generated a binary, or simple presence or absence pattern, for each isolate – the AFLP fingerprint. Similarity coefficients were then calculated between each pair of fingerprints by using the Dice equation, which double-weights the shared presence of a band while ignoring the shared-absence of a band. Using this similarity matrix, a dendrogram was generated, based on the Unweighted Pair Group Mathematical Average (UPGMA) technique which searches for the two most similar fingerprints and then, for the generation of the dendrogram, combines them into one new composite isolate, averaging their similarity coefficients.

Finally, after each individual primer combination and gel run were analyzed, a composite dataset was created which allowed all four fingerprint patterns (two runs each of two primer combinations) to be analyzed together to generate a complex and more

accurate dendrogram. For this final analysis, each individual fingerprint was weighted based on the number of fragments present in its fingerprint in comparison to the total number of fragments present in all four fingerprints for that isolate.

RESULTS

Four digitized images of the AFLP reactions were created by the Licor system for this experiment. Figure 2 shows a representative example of these images. Despite the close relationship between many of the samples, variation is evident between the lanes. Even a cursory visual analysis determines two primary patterns. As evident by Figure 2, there is considerable variability between the intensity of bands, which made it difficult in some cases to consistently determine the absence or presence of a band. This was particularly true in situations where a faint band appeared in one lane, which corresponded to more intense bands in many other lanes. Also, lane leakage appears to be problem. This is particularly visible in the lanes next to the molecular weight standards, but is probably manifested to some degree across the entire gel, necessitating different run orders for multiple runs of the same reaction for the best data.

Figure 3 shows the final composite dendrogram generated by Bionumerics from all four fingerprints (two each of two primer combinations). The metric along the top of the figure represents the degree of similarity between samples, or groups of samples, with 100 being identical patterns. The similarity value corresponding to each node indicates the highest degree of similarity that all of the samples beyond that node share.

There are two distinct subgroups: the upper group that contains six samples and the lower group that contains 16 samples. The upper group contains the out-group samples consisting of *Ochrobactrum anthropi, Ochrobactrum intermedium,* and *Rhizobium meliloti*. Oddly, the one *O. anthropi* (82-115) isolate clustered more closely with the one *R. meliloti* sample than with the *O. intermedium* samples. There is a

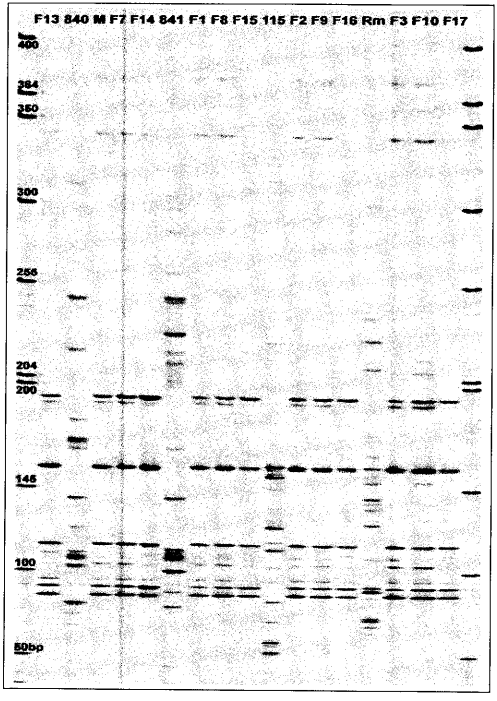

FIG. 2. Gel limage of AFLP Patterns. The reactions shown above were created using *B. abortus* DNA isolates, with the P-CC selective primer and M12 selective primer. Isolates are identified along the top of the gel. Molecular weight marker (outside lanes) standards are labeled in the far left lane.

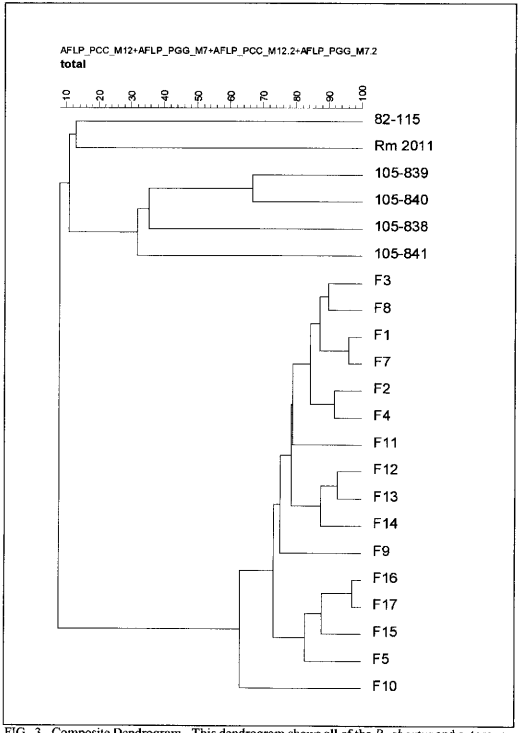

FIG. 3. Composite Dendrogram. This dendrogram shows all of the *B. abortus* and outgroup isolates, as derived from AFLP Fingerprint Patterns analyzed by Bionumerics software (Dice, UPGMA)

surprising degree of dissimilarity (11.01%) between these closely related samples, particularly between the *O. anthropi* (82-115) and the *R. meliloti* (Rm2011) isolates. Among the *O. intermedium* samples, similarity values range from about 31.84% to 66.67%. These samples were handled separately from the *B. abortus* samples as they were not available until late in the experimental process. The high degree of dissimilarity suggests either contamination of the samples at any stage of the experimental process or some inconsistency in the procedure that occurred from treating these samples in a different reaction batch, resulting in increased variability. Isolate F10 was also included in the second AFLP process set, with the highly-variable out-group samples, because of poor results (very few bands) from F10 from the first AFLP process experiment process or some inconsistency in the procedure that occurred from treating these samples in a different reaction batch, resulting in increased variability. Isolate F10 was also included in the second AFLP process set, with the highly-variable out-group samples, because of poor results (very few bands) from F10 from the first AFLP process set. Isolate F10 is the most diverse of the *B. abortus* samples, and therefore seems to support that an inconsistency in the AFLP procedure is most likely to be the cause of the increased variability among the out-group samples.

The lower group consists of the *B. abortus* isolates and contains several, less clearly defined, clusters. Many of these clusters include relatively similar pairs of isolates (F3 & F8, F1 & F7, F2 & F4, F12 & F13, and F16 & F17). Surprisingly, none of these pairs correspond to samples that were repeated in the sample group (i.e. two copies of the sample were present in the sample group). However, all but one of the

repeated pairs were represented in nodal groups with at least 85% similarity. Other patterns also emerged. All of the samples obtained from South Dakota State University, which were also the only samples requiring carbon dioxide to grow, were originally isolated from bison, clustered with 87.93% similarity, with the two most dissimilar isolates of that cluster being the same, repeated isolate (F13 and F14). With two exceptions, all of the R-type O-antigen samples clustered together at the top of the lower group (F3, F1, F7, F2, F4) with a shared similarity value of 84.67%. One exception, F8, was a repeat of F9, which clustered below with the A-type R-antigens, while the other exception, F5, clustered farther below with the vaccine strains. These deviations to the pattern suggested that F8 and F5 were perhaps subject to lane leakage or miscalling of faint bands. The samples with A-type O-antigen (F11, F21, F13, F14, F9) clustered less neatly, but still clustered in the middle of the lower group. Again, there was an exception: F8, as was already discussed, clustered above in the R-type samples. Finally, the bottom-most cluster among the lower group of *B. abortus* isolates contains all of the vaccine strains. Most interestingly, two vaccines strains derived from the current model virulent, S2308, clustered with the highest similarity of any pair of isolates with 97.44% similarity.

CONCLUSIONS

Several ideas emerge from the results of the AFLP analysis of these 17 *B. abortus* isolates and six outgroup isolates. The relationship between *O. anthropi* and *R.. meliloti* appears to be closer than expected, as both species distinctly clustered separate from the *O. intermedium* isolates. The relationship between all of these species, *O. anthropi*, *O. intermedium*, and *R. meliloti*, should be more thoroughly evaluated. Also, all of the vaccines clustered markedly together, containing the two most similar isolates. This is expected, as vaccine strains are cultivated to minimize genetic diversity, and have been altered from the natural B. *abortus* isolates.

It is clear that AFLP has the capability of characterizing bacterial isolates to a high degree of specificity, given proper standardization. More precise relationships were difficult to determine in this study due to the lack of reproducibility, primer combinations and more complete information about the isolates origin.

B. abortus remains an important research interest because of its economic and agricultural evolution of host preferences and pathogenicity. AFLP provides a promising tool for further investigations, as it allows for a rapid and remarkably thorough analysis. However, AFLP also requires a very high degree of standardization. Bionumerics software is an excellent way to analyze AFLP because it allows the researcher to create composite data sets, allowing for multiple primer set patterns to be analyzed simultaneously, which provides the most accurate genetic relationship between the samples.

REFERENCES

1. Boschiroli, Maria-Laura., et al. Brucellosis: a worldwide zoonosis. Current Opinion in Microbiology. 4:58-64. 2001.
2. Klietmann, Wolfgang G., and Ruoff, Kathryn L. Bioterrorism: Implications for the clinical microbiologist. Clinical Microbiology Reviews. 14(2):364-381. 2001.
3. Vizcaino, Nieves., et al. DNA polymorphism in the genus *Brucella*. Microbes and Infection. 2:1089-1100. 2000.
4. Michaus-Charachon, Sylvie., et al. Genome structure and phylogeny in the genus Brucella. Journal of Bacteriology. 179(10):3244-3249. 1997.
5. Goulding, Jonathan N., et al. Genome-sequence-based fluorescent amplified-fragment length polymorphism analysis of *Mycobacterium tuberculosis*. Jour of Clin Microbiol. 38(3): 1121-1126. 2000.
6. Keim, Paul., et al. Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers. Jour of Bacteriol. 179(3): 818-824. 1997.
7. Jiang, Sunny C., et al. Genetic diversity of clinical and environmental isolates of *Vibrio cholerae* determined by amplified fragment length polymorphism fingerprinting. Appl Environ. Microbiol. 66(1): 148-153. 2000.
8. Ticknor, Lawrence O., et al. Fluorescent amplified fragment length polymorphism analysis of Norwegian *Bacillus cereus* and *Bacillus thuringiensis* soil isolates. Appl Environ Microbiol. 76(10): 4863-4873. 2001.
9. Jensen A.E., et al. Genomic fingerprinting and development of a dendrogram for *Brucella* spp. isolated from seals, porpoises, and dolphins. J. Vet. Diagn. Invest. 11: 152-157. 1999.
10. Velasco, Julian., et al. Evaluation of the relatedness of *Brucella* spp. and *Ochrobactrum anthropi* and description of *Ochrobactrum intermedium* sp. nov., a new species with a closer relationship to *Brucella* spp. Internatl. J. Syst. Bact. 48: 759-768. 1998.
11. Vizcaino, Nieves., et al. Molecular characterization of a *Brucella* species large DNA fragment deleted in *Brucella abortus* strains: evidence for a locus involved in the synthesis of a polysaccharide. Infect. Immun. 67(6): 2700-2712. 1999.
12. 1. Vos, P. Hogers, et al. AFLP: a new technique for DNA fingerprinting. Nucl. Acids Res. 23: (21) 4407-4414. 1995.

VITA

KATHERINE ANN BLISS

74 La Paloma Dr
Los Alamos, NM 87544

I will receive my Bachelor of Science in Genetics from Texas A&M University in May 2003. While attending Texas A&M University, I received a Lechner Fellowship. I will attend graduate school at the University of Texas – Southwestern in Dallas, Texas. I have extensive research laboratory experience, most of which was gained while working at the Bioscience Division of Los Alamos National Laboratory. The research for this document was completed in Dr. Allison Rice-Ficht's Lab at Texas A&M University Health Science Center with the support of Dr. Thomas Ficht of the Department of Veterinary Pathobiology at Texas A&M University.

Publications:

Evolution of virulence in Bacillus anthracis. TAMU Undergraduate Journal of Science. Spring 2002. Pages 45-47.

Fluorescent Amplified Fragment Length Polymorphism (AFLP) Analysis of *Bacillus Anthracis, Cereus* and *Thuringiensis* Isolates. Applied and Environmental Microbiology. Submitted.