APPLICATION OF MOLECULAR TECHNIQUES TO IDENTIFY BACTERIA ISOLATED FROM THE LEATHER INDUSTRY*

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ABSTRACT

DNA-based methods are known to be accurate, precise and rapid in identifying bacteria but there is little information about the use of these techniques in the leather industry. A study was undertaken to investigate and characterize the bacterial contaminants of fresh hides using 16S ribosomal RNA sequencing techniques. The proteolytic activities of these isolates were also studied using different substrates. Organisms belonging to 16 different genera of bacteria were isolated, and many of the species found have not been previously reported for this environment. Some of the species of bacteria being reported for the first time in the tanning environment included *Janibacter terrae, Acidovorax sp., Dietzia maris, Arthrobacter pr tophormiae, Comamonas sp., Brevibacterium lutescens, Jeotgalicoccus psychrophilus, Nocardiopsis sp., Delftia acidovorans, Pseudomonas cannabina, Pseudomonas fulgida* and certain species of *Aeromonas* and *Acinetobacter*. Most species of bacteria found in the incoming process water were non-proteolytic. Analysis of fresh hides revealed a large number of both proteolytic and non-proteolytic bacteria. The proteolytic bacteria included several species of *Aeromonas, Proteus vulgaris*, and *Shewanella putrefaciens. Pseudomonas sp.* were common but not all of them showed proteolytic activity. Knowledge about the identities and proteolytic activities of these organisms that occur in this environment would provide a better way to design programs to control theirundesirable activities.

ABSTRACTO

Métodos basados en ADN se conocen por rápidos, precisos y exactos en la identificación de bacteria, pero hay poca información a cerca del uso de tales técnicas en la industria del cuero. Se emprendió un estudio para investigar y caracterizar la contaminación por bacteria en pieles frescas por la utilización de técnicas de secuenciamiento por medio de 16S ARN ribosómico. La actividad proteolítica de estos componentes así aislados, también se examinó sobre diferentes substratos. Organismos pertenecientes a 16 géneros de bacteria fueron aislados, y muchas de las especies encontradas no habían sido reportadas previamente en este medio-ambiente. Algunas de las especies de bacterias siendo reportadas por primera vez en el medio-ambiente de la curtición incluyen *Janibacter terrea, Acidovorax sp., Dietzia marls, Arthrobacter protophormiae, Comamonas sp., Brevibacterium lutescens, Jeotgalicoccus psychrophilus, Nocardiopsis sp., Delftia acidovorans, Pseudomonas cannabina, Pseudomonas fulgida* y ciertas especies de *Aeromonas* y *Acinetobacter*. La mayoría de las especies bacterianas encontradas en el agua de suministro para el proceso no demostraron actividad proteolítica. Análisis de las pieles frescas revelaron grandes números de bacterias tanto proteolíticas como no proteolíticas. Las bacterias proteolíticas incluían varias especies de *Aeromonas, Proteus vulgaris* y *Shewanella putrefaciens*. *Pseudomonas sp.* fueron comunes pero no todas demostraron actividad proteolítica. Los conocimientos a cerca de las identidades y actividad proteolítica de los organismos que ocurren en este medio-ambiente conducirían a un mejor camino para el diseño de programas de control de sus actividades indeseables.

INTRODUCTION

Bacterial growth and degradation of fresh hides and skins is a significant economic problem in the leather industry but the identities of the causative organisms are poorly known. When an animal is killed and the skin is removed, microbial degradation of the hide begins if steps are not taken to control microbial growth. The bacteria that contaminate the hides come from many sources including the pasture, slaughterhouse, feces, the animal skins themselves, and the tannery. The organisms grow rapidly because of the immediate availability of nutrients, high moisture content, favorable pH of the hide, and also the favorable temperature of the tanning environment.[1]

The undesirable effects of uncontrolled growth of bacteria on fresh hides have long been recognized.[1] These effects include odor and hairslip problems. Many of the microorganisms are known to produce proteolytic enzymes[1,2] such as collagenase[3,4] which can adversely affect collagen and hence the quality of the finished leather.

There have been several attempts over the years to classify bacteria found on fresh hides.[1,2,5-7] Much of the efforts in this direction have relied mainly on traditional microbiological methods such as the morphological, cultural, physiological, and biochemical characteristics of the bacteria isolated. Traditional methods have in many cases helped to advance our knowledge of the microbiology of fresh hides but in recent times, such methods are increasingly being replaced by newer and better detection and identification methods based on nucleic acid technologies.

Genetic methods of identification take many forms. In the MicroSeq (a proprietary microbial identification system developed by Applied Biosystems, Foster City, CA. USA) DNA is first extracted from a pure culture of microorganisms isolated from a particular sample. A phylogenetic gene marker, such as 16S ribosomal RNA, is then amplified by a method called polymerase chain reaction (PCR).[8] PCR uses the enzyme, DNA polymerase, primers, and deoxynucleotide triphospates in a buffered medium to make millions of copies of the gene of interest in a few hours. The reaction is done in a thermal cycler. The amplified gene can either be sequenced directly or cloned and then sequenced. Gene sequencing helps to determine the order in which the various bases occur in the DNA. The gene sequence is unique to a particular organism and using this information, the organisms can be identified in the MicroSeq database or a public database such as the GenBank.[9]

Genetic methods of identification tend to be rapid, specific and accurate, and have been used in the medical field,[10] pharmaceutical industry,[11] the food industry,[12] pulp and paper,[13-15] soil,[16] and wastewater.[17] Using genetic-based methods, a number of previously unknown microorganisms have been found in many environments. It has also made it possible to gain information about many microorganisms which exist in natural environments but would not be cultured on traditional laboratory media.

While the use of genetic-based methods has become popular in many industries, there is little published information about their use in detecting or identifying bacteria in the leather industry. The objectives of this work were to isolate and identify bacteria on fresh hides using genetic methods and also to study the proteolytic properties of these organisms.

EXPERIMENTAL

Isolation of bacteria

Samples of fresh hides and incoming process water were obtained from a tannery located in mid-western United States. Samples were collected in February and in June of 2004 and sent on ice to the laboratory for microbiological analyses. The samples were processed immediately or kept refrigerated and used within 1-2 days after arrival in the laboratory. The hide samples were aseptically cut into small pieces of approximately 1/4x1/4 sq. in. A 3g amount of these sample pieces was randomly selected and added to 30ml saline (0.85% NaCl). For the water samples, 1 ml was added to 9ml saline (0.85% NáCl). The hide and water samples were serially diluted and plated on plate count agar (Difco), plate count agar plus 7% NaCl (w/v), and plate count agar plus 25% NaCl (w/v). Samples were incubated at 30 C for 2-7 days and different colonies that appeared were picked and re-streaked to obtain pure cultures of bacteria.

DNA Isolation and Amplification

DNA was extracted from the cultures using the instructions provided in the PrepMan Ultra Sample Preparation Reagent Protocol (Applied Biosystems, Foster City, CA. USA). The DNA was amplified using the protocol in the MicroSeq 500 16S rDNA Bacterial Identification PCR Kit (Applied Biosystems, Foster City, CA, USA).

16S rRNA sequence and data analysis

16S rRNA gene sequences of the isolates were generated using the MicroSeq 500 16S rDNA Bacterial Identification Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using Applied Biosystems' MicroSeq microbial analysis and database. In the cases where there was no match, a BLAST[9] search of the GenBank[9] was conducted for a possible match.

Proteolytic activity

Three tests namely, collagenase, gelatinase, and caseinase, were used to study the proteolytic activities of the isolates.

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TABLE I
Bacterial Isolates and their Proteolytic Activities

Isolate[d]	Identified as	Proteolytic activity[a]	Isolated	Identified as	Proteolytic activity[a]
AW1	*Acinetobacter junii*	-	EW6	*Aeromonas salmonicida*	+
AW3	*Acidovorax sp.*	-	EW7	*Acinetobacter johnsonii*	-
BW3	*Delftia acidovorans*	-	EW8	*Aeromonas veronii*	+
BW4	*Staphylococcus epidermis*	-	EZ4	*Klebsiella sp.*	-
BX3	*Janibacter terrae*	+[b]	FW2	*Comamonas acquatica*	-
CW1	*Aeromonas hydrophila*	+	FW5	*Klebsiella sp.*	-
CW2	*Shewanella putrefaciens*	+	FW6	*Shewanella putrefaciens*	+
CW3	*Pseudomonas fulgida*	+	FW7	*Citrobacter freundii*	-
CW4	*Staphylococcus homonis*	-	FW8	*Proteus vulgaris*	+
CW5	*Aeromonas veronii*	+	FW9	*Pseudomonas sp.*	+
CX1	*Proteus vulgaris*	+	FW12	*Acinetobacter radioresistens*	-
CX4	*Brevibacterium lutescens*	+	FZ3	*Acinetobacter calcoaceticus*	-
CX6	*Dietzia maris*	-	FC1	*Pseudomonas sp.*	+
CX8	*Janibacter terrae*	+	FC4	*Pseudomonas stutzeri*	-
CZ2	*Pseudomonas cannabina*	-	FH1	*Pseudomonas sp.*	-
CZ5B	*Pseudomonas sp.*	-	FH3	*Aeromonas media*	+[c]
DW1	*Pseudomonas sp.*	-	FH7	*Brevibacterium lutescens*	+
DX2	*Arthrobacter protophormiae*	-	FH8	*Jeotgalicoccus psychrophilus*	-
DZIA	*Pseudomonas putida*	-	FH9	*Nocardiopsis sp.*	+
EW4	*Aeromonas hydrophila*	+			

[a] + means a positive reaction. i.e. isolate was positive for collagenase, caseinase and gelatinase tests.

[a] - means a negative reaction. i.e. isolate was negative for collagenase, caseinase and gelatinase tests.

[b]This culture was positive for both collagenase and caseinase but not gelatinase.

[c]This culture was positive for gelatinase and collagenase but not caseinase.

[d]Isolates AW1, AW3, BW3, BW4, and BX3 were isolated from incoming process water. Isolates with coded name beginning with C or D e.g. CW1 were isolated from fresh hides before soaking. Isolates with coded names beginning with E or F e.g. EW4 were isolated from fresh hides after soaking.

Proteolytic activity of bacteria is an indication of the potential of the organisms to cause putrefaction and hence damage to hides and skins. The collagenase test was based on Azocoll-nutrient agar medium as described previously.[18] The methods used to determine the ability of the isolates to liquefy gelatin and also to hydrolyze casein have been published.[19]

Nucleotide sequence accession number

The gene sequences of the identified organisms have been submitted to the GenBank under the Accession numbers AY880180 to AY880219.

Results and Discussion

Samples of fresh hides, soaked hides, and process water were obtained from a tannery and analyzed for their bacterial content. All samples studied contained relatively high numbers of bacteria (data not shown). The bacterial counts found on PCA were not significantly different from counts on PCA containing 7% NaCl. The bacterial counts for the hide samples were approximately 10^8 cfu/g and those for the process water were approximately 10^6 cfu/ml. There was no bacterial growth when the samples were plated on PCA containing 25% NaCl.

Over 100 pure bacterial cultures were obtained and studied. With the exception of two isolates, all the three methods used to detect proteolytic activity compared well with each other. Organisms identified as non-proteolytic were negative for all three proteolytic tests. All organisms that were proteolytic were positive for all three tests apart from the strains of *Aeromonas media* and *Janibacter terrae*. *A. media* was positive for gelatinase and collagenase but not caseinase. *J. terrae* was positive for both collagenase and caseinase but not gelatinase.

To identify the isolates, their DNA was extracted. Then the 16S ribosomal RNA gene was amplified and sequenced. Based on their DNA sequences, 35 different species of bacteria were identified (Table I). Many different species of bacteria were identified that have not been described previously in the leather environment.

Some species of *Pseudomonas* found, including *P. cannabina, P. fulgida*, and *P. stutzeri*, have not been reported before as contaminants of fresh hides. *Pseudomonas aeruginosa* has been described before for this environment[2,7] but this organism was not found in this study. Pseudomonas species are known to be versatile in their ability to utilize a number of compounds for growth. The ability of *P. aeruginosa* to damage collagen has been recognized.[20] It is interesting that with the exception of *P. fulgida, Pseudomonas sp. C1* and *Pseudomonas sp. FW9*, the other species of *Pseudomonas* identified were not proteolytic.

Shewanella putrefaciens,[21] *Acidovorax sp.*,[22] *Comamonas aquatica*,[23] and *Delftia acidovorans*[24] were once classified under the genus, *Pseudomonas*, but modern DNA techniques have shown that they are different and they have now been reclassified and placed into their respective genera. *Shewanella putrefaciens* was proteolytic and this organism has been identified before as *P. putrefaciens*.[7]

Four different species of *Aeromonas* were found and all were proteolytic. *A. veronii, A. hydrophila,* and *A. salmonicida*, were positive for all three tests used to determine proteolytic activities. The strain of *A. media* studied was positive for collagenase and gelatinase but negative for caseinase. *Aeromonas* species are widely distributed in the aquatic environment[25] and they have been isolated from grocery store produce, meats, and seafood[26] but none of the four species of *Aeromonas* found has been described before as a contaminant of fresh hides.

Four species of *Acinetobacter* were found (Table1). These species have not been described previously in the leather industry. These were all non-proteolytic with respect to the three substrates tested and their importance in terms of direct deterioration of fresh hides is not known.

Brevibacterium lutescens was found to be a bright yellow, Gram positive bacterium. It was positive for the collagenase, gelatinase and caseinase tests. There is no information about the isolation of this particular species from fresh hides. However, such species as *B. lipolyticum* and *B. insectiphylum* have been described previously in the literature.[6]

Four different actinobacteria, namely, *Nocardiopsis sp., Dietzia maris, Janibacter terrae*, and *Arthrobacter protophormiae* were isolated from PCA containing 7% NaCl. *Nocardiopsis* was positive for all three proteolytic tests. *J. terrae* was positive for collagenase and caseinase tests but did not liquefy gelatin. *D. maris* and *A. protophormiae* were not proteolytic. The occurrence of any of these organisms in the leather industry has not been published.

Jeotgalicoccus psychrophilus was isolated from plates containing PCA plus 7% salt. It is a Gram positive, halotolerant or moderately halophilic coccus which was first isolated from a traditional Korean fermented seafood, geotgal.[27] Its occurrence in the leather industry has never been described.

Three bacteria, *Proteus vulgaris, Citrobacter freundii, Klebsiella,* in the Enterobacteriaceae family, were found. These organisms are found in diverse areas such as water, soil, plants and bowels of humans and animals. *Proteus vulgaris* was proteolytic but the other two were not. *Proteus sp.* have been described before in the leather industry.[5,28]

Two staphylococci were identified as *S. hominis* and *S. epidermis*. Both species were found to be non-proteolytic.

Summary/Conclusions

Genetic-based methods are increasingly being used in environmental samples to identify and understand the nature of microorganisms that inhabit these areas. In this study, DNA-based techniques were applied to identify bacteria from fresh hides and process water. The method was relatively rapid, less cumbersome than traditional methods, and allowed over 100 bacterial isolates to be identified in a relatively short time. Many bacteria in this environment not previously described were found. A significant number of the isolates were proteolytic and thus are of significance in terms of their potential to cause putrefaction of fresh hides and hence deterioration of the quality of the finished leather. Many non- proteolytic bacteria were also isolated. The significance of the later group in terms of their ability to degrade fresh hides is not known. It is possible many of them are contaminants thriving on other non-proteinaceous materials such as fat, manure and other extraneous organic matter. It is also possible that these organisms exist and contribute to degradation by utilizing some of the metabolic by-products of the primary proteolytic organisms. Some of the organisms such as *Acinetobacter, Aeromonas*, and *Pseudomonas* species have been isolated in the hospital

environment and some species have been implicated in certain diseases. Information gained in this study including the identities and the proteolytic activities will help in designing better programs to control these organisms.

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CONVENTION DISCUSSION

David G. Bailey, Bailey Consulting - I want to thank you for a fine paper. Some of the work is very near and dear to me because while I was at the USDA we did work with the University of Wyoming which unfortunately the University would not permit us to publish. We identified quite a number of organisms on fresh hides and we did it the old fashioned way. This would be a great technique to repeat some of that work. What we found was the organisms that are present on a fresh hide change almost 100% after a number of hours after the hide is removed from the carcass. The entire population seems to be entirely different. I would suggest that this would be a great thing for you pursue because those are the organisms that you have to take care of with bactericides or in some way because they are the ones that are actually destroying the hide. The original organisms, at least the population, is apparently not doing a whole lot of damage. So it would be interesting to look at that. The question that I have is how much quantitative information can you get from this technique? It is very, very good qualitative. That is, can you determine the relative populations of the different bacteria.

You can use what is called "Real Time PCR" to get quantitative information.

Robert Dudley, ERRC, USDA - Did you find any pathogenic organisms?

For pathogenic organisms like *E. Coli and Campylobacter,* you will need special techniques to detect them. We used general techniques to detect the bacteria in our work. Most of the time, these pathogens occur in small numbers so are out competed by other organisms.

Robert Dudley, ERRC, USDA - The reason I asked is that the packing industry is very concerned about E. Coli 015787 and our sister lab has shown that cross contamination from the hide to the carcass is a very important path- way for contaminating the carcass. Packers are very interested in looking at the hide ecology as it comes off the carcass and the pathogens that are present on the hide. I was just wondering if your technique was very applicable?

You don't even need to isolate the organisms. In a sample, it is now very easy to design an assay to pinpoint specific organisms using probes or primers that are specific for those organisms.

Robert Dudley, ERRC, USDA - They were working with plate counts - the old fashioned way. The bacteria that produces the gelatinase. Did you just do a quick survey against a medium enriched with gelatin or did you actually go in and try to figure out whether it was gelatinase A or B?

Our interest was to find the general trend. To see the potential organisms that would produce these enzymes. We used basic tests like Azocoll (collagen), gelatin, and casein. We did not do specific tests to distinguish various forms of gelatinases.

David Rabinovich, Jos. Lowenstein & Sons, Inc. - Do you think perhaps you might eventually develop a method for when someone gives you a wet blue and you could say that it is likely or not likely to have bacterial damage?

This will be possible with some of the methods that are available. I think the sky is the limit. This is the beginning. I just returned from a conference American Society for Microbiology. There are tests based on computer chips that you can put into your pocket that can be used to detect bacterial genes and a whole lot of things. So this is the beginning and I am sure that what you are proposing will be done one day.