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MICROBIAL DIVERSITY IN BIGHORN SHEEP REVEALED BY CULTURE-INDEPENDENT METHODS

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ABSTRACT: We investigated the effectiveness of culture-independent molecular methods for determining host-associated microbial diversity in bighorn sheep (*Ovis canadensis*). Results from bacterial culture attempts have been the primary source of information on host-associated bacteria, but studies have shown that culture-based results significantly underestimate bacterial diversity in biological samples. To test the effectiveness of culture-independent methods, we extracted DNA from nasal and oropharyngeal swab samples collected from bighorn sheep in four different populations. From these samples, we amplified, cloned, and sequenced small subunit (16S) ribosomal DNA (rDNA) to identify the scope of microbial diversity in bighorn respiratory tracts. Phylogenetic analysis of these rDNA gene sequences revealed organismal diversity an order of magnitude higher than was determined by culture methods. Pasteurellaceae bacteria were the most diverse phylogenetic group in live bighorn sheep, and members of bacterial genera often associated with respiratory disease were found in all the samples. Culture-independent methods were also able to directly detect leukotoxin (*lktA*) gene sequences in swab and lung tissue samples. Overall, our results show the power of culture-independent molecular methods for identifying microbial diversity in bighorn sheep and the potential for these methods to detect the presence of virulence genes in biological samples.

Key words: 16S rDNA, bighorn sheep, leukotoxin, *Mannheimia*, *Pasteurella*, polymerase chain reaction.

INTRODUCTION

Bighorn sheep (*Ovis canadensis*) populations in North America have been profoundly affected by disease for over 100 years, principally through recurrent pneumonia die-offs, often following contact with domestic sheep (*O. aries*) (Miller, 2001). Members of the Pasteurellaceae–Proteobacteria, such as *Mannheimia haemolytica* and *Pasteurella trehalosi*, have been implicated as important contributory agents of these epidemics (Silflow and Foreyt, 1994). Strains of these organisms that are virulent in bighorn sheep can be transferred from domestic sheep and goats (*Capra hircus*) (Foreyt and Jessup, 1982; Rudolph et al., 2003); however, Pasteurellaceae are also commensal organisms in healthy bighorn sheep, and the presence of particular strains is not always associated with disease (Aune et al., 1998; Jaworski et al., 1998). Moreover, little is understood concerning the influence of other factors on disease outbreaks, such as human disturbance, weather extremes, population density, nutritional deficiencies, and competition for resources with domestic animals (Miller, 2001).

One of the principal difficulties in identifying the cause of outbreaks might relate to the failure of culture methods to isolate all, or even the majority, of the bacteria species present in biologic samples (Hugenholtz and Pace, 1996; Pace, 1997). Less than 1% of the existing microbial diversity on the planet has been cultured, and a growing number of molecular studies have revealed that standard culturing methods significantly underestimate the microbial diversity of environmental communities (Amann et al., 1995; Hugenholtz et al., 1998a). The development of culture-free techniques based on the amplification of small-sub-

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unit ribosomal DNA (16S rDNA) genes from biologic samples has revolutionized our understanding of microbial diversity (Pace et al., 1985). Using molecular methods, researchers have uncovered astounding microbial diversity in everything from hot springs to soils to shower curtains (Borneman et al., 1996; Hugenholtz et al., 1998b; McCaig et al., 1999; Kelley et al., 2004).

These methods have also been very effective for examining microbial diversity associated with animals. For instance, studies of corals (Casas et al., 2004), termites (Graber et al., 2004), squids (Barbieri et al., 2001), and humans (Dunne, 2001) all have revealed an astonishing diversity of uncultured microorganisms (Pace, 1997). Culture-independent molecular approaches appear to be especially promising for studying complex diseases. For example, a rDNA study of diseased human prostates uncovered a complex and diverse array of corynebacterial 16S rRNA sequences in sick patients that were missed by culturing and, apparently, had no cultured representatives (Tanner et al., 1999). If these methods prove successful with bighorn sheep, we expect the resulting analysis of microbial diversity to significantly enhance our understanding of microbial communities associated with bighorn sheep respiratory diseases.

Although the direction of transfer is difficult to ascertain, identical Pasteurellaceae bacteria have been isolated in bighorn sheep, and domestic goats and sheep occupying the same ranges (Ward et al., 1997; Rudolph et al., 2003). However, the potential presence of multiple pathogens, the taxonomic complexity of identifying virulent versus commensal variants of the same organism, and the difficulty of collecting and preserving microorganisms for culture in field conditions present challenges to traditional diagnostic technology (Wild and Miller, 1991; Foreyt et al., 1994).The purpose of this study was to determine the utility of culture-independent molecular methods for studying host-associated microbial communities in bighorn sheep, and to determine if these methods could overcome some of the limitations of traditional approaches. We used rDNA methods to determine the microbial diversity in nasal and oropharyngeal swab samples collected from bighorn sheep in four isolated populations. Three of the populations, one in California and two in Colorado, included only healthy animals with no recorded illness. Pneumonia had occurred in the Oregon metapopulation we examined. In this metapopulation, we collected samples from live, apparently healthy animals, and from individuals that had died from pneumonia. Using swabs collected from individuals in these four populations, we tested whether culture-independent methods were effective tools for studying microbial diversity. Because leukotoxin is thought to be one of the most important virulence factors of *Pasteurella* and *Mannheimia* bacteria, we also attempted to detect the leukotoxin A (*lktA*) gene using *lktA* polymerase chain reaction (PCR) primers specific for the Pasteurellaceae (Davies et al., 2002; Fisher et al., 1999). Detection of virulence genes directly from swab or tissue samples could prove an effective means of correlating the presence of these genes with disease severity.

Using rDNA molecular methods, we asked the following basic questions: 1) Do rDNA methods detect a greater range of microbial diversity than culture methods as they have in other studies? 2) Can we find evidence of novel, not yet cultured, bacteria? 3) Can molecular methods detect genes coding for toxins directly from DNA isolated from swabs? and 4) Can rDNA methods detect microbial diversity in cases where cells become unviable for culturing? After obtaining rDNA sequence data from swab samples, we used phylogenetic and population genetic methods to address two additional questions: 1) How phylogenetically diverse are the microbial communities of healthy

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bighorn sheep? and 2) What phylogenetic groups of bacteria are abundant in the upper-respiratory tract of live bighorn sheep? The results from this study confirmed that culture-independent rDNA methods provide a powerful tool for studying microbial diversity and disease patterns in wildlife. We show that the respiratory tracts of wild bighorn sheep harbor an extraordinary array of bacteria, including many that have no cultured representatives.

MATERIALS AND METHODS

Sample collection

Nasal and oropharyngeal samples were collected using sterile cotton or rayon-tipped swabs from bighorn sheep in Colorado; California; and Hells Canyon, Oregon in 2004. The Colorado samples came from a population near Georgetown, Colorado (39°71′N, 105°70′W) and the St. Vrain population (40°24′N, 105°39′W) near Estes Park, and the California samples were obtained from live sheep in the Tejon Hills population (37°41′N, 118°69′W) near Bishop, California. In Oregon, samples were collected from live sheep in the Lostine subpopulation (45°43′N, 116°48′W) and from dead sheep in the Imnaha subpopulation (45°24′ N, 117°23′W). Double swabs were used, one for culturing and one for molecular analysis. Samples from live sheep were taken during capture for research purposes. Nasal and oral swab samples from one dead sheep (samples HC6-0 and HC7-N) were taken at necropsy.

All swabs were placed immediately in BBL "Port-a-Cul" tubes (Becton, Dickinson and Company, Sparks, Maryland, USA) according to the manufacturer's directions. Port-A-Cul tubes contain a transport medium that maintains viability of numerous anaerobic, facultative, aerobic, and microaerophilic organisms for 72 hr at 20 to 25 C. The tubes were insulated in heavy paper, placed on ice, and delivered to the Caine Veterinary Teaching Center laboratory in Caldwell, Idaho within 72 hr. Following removal of the swab for culture, the remaining samples were shipped on dry ice to San Diego State University where they were stored at −80 C until DNA extractions were performed. Samples were also taken from the lungs of two dead animals (HC-8 and HC-9) during necropsy. The samples from dead sheep were collected during February 2004 within one week of death. The animals were necropsied in the field and lungs and other tissue were submitted to the Washington Animal Disease and Diagnostic Laboratory (WADDL) Pullman, Washington for diagnostic services and culturing. During necropsy, a 7×7 cm sample was removed from the lungs and these samples were then sampled with double swabs. One of the swabs was used for culturing at WADDL and the other was shipped on ice to San Diego State University.

Culturing methods

Swabs for culture were inoculated onto Pasteurellaceae nonselective Columbia blood agar, containing 5% sheep blood or Columbia blood agar with selective antibiotics containing 5% bovine blood (Slee and Stephens, 1985). Plates were incubated for 18 to 24 hr at 37 C in a 10% CO_2 atmosphere. Following incubation, a representative of each colony type was propagated on fresh Columbia blood agar for species identification and biovariant typing. Isolates were determined to be *M. haemolytica* and *P. trehalosi* by testing whether they were MacConkey's-, urea-, and indole-negative; oxidase-, nitrate-, glucose-, and sucrose-positive; and xylose- or trehalose-positive. Additional biochemical tests were then applied to identify biovariants (Bisgaard and Mutters, 1986; Jaworski et al., 1998).

DNA extraction

The DNA was extracted using Ultra Clean Soil DNA Kits (MO BIO Corporation, Solana Beach, California, USA) following the manufacturer's protocol. This kit works especially well for extracting DNA from environmental microbial samples. Samples were taken from the −80 C freezer and placed on dry ice during the extraction process. Scissors were sterilized and swab tips were clipped and dropped directly into sterile, DNA-free tubes provided with the kit. Precipitated DNA was dissolved in 50 µl ultra clean water and stored at −20 C until PCR reactions were performed.

Polymerase chain reaction

Bacterial specific universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 805R (5′-GACTACCAGGGTATCTAATCC-3′) were used to amplify specific 16S rDNA gene fragments. Leukotoxin primers specific for the Pasteurellaceae, *lktA*1 (5′-TGTGGATGCGTTTGAAGAAGG-3′) and *lktA*2 (5′-ACTTGCTTTGAGGTGATCCG-3′), were used to amplify *lktA* (Fisher et al., 1999). M13 primers were used to check for clone

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inserts. For all primer combinations, PCR was carried out in a total reaction volume of 50 μl volume, including 1 μl of sample DNA as template, 1× PCR buffer II (Perkin-Elmer, Foster City, CA), 2.5 mM $MgCl_2$, 4×200 μM deoxynucleoside triphosphates, 200 nM each forward and reverse primer, and 0.025 U of AmpliTaq Gold (Perkin-Elmer) per μl. The PCR thermal cycling conditions for 16S rDNA amplification reaction began with a one-time denaturing step at 94 C for 2 min, followed by 35 cycles of amplification. The cycling parameters included an initial denaturing step at 94 C for 1 min, an annealing step at 50 C for 45 sec, and extension step at 72 C for 1.5 min. To insure complete extension for efficient cloning, a final 72 C extension step for 20 min was added after the 35th cycle. The PCR thermal cycling conditions for the leukotoxin gene were the same except the annealing step was 55 C for 1 min.

Cloning and sequencing

The PCR products were purified using the QIA Quick PCR Purification Kit (QIAGEN Corporation, Valencia, CA) and cloned using the pGEM commercial cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Transformants produced white colonies on LB agar plates containing ampicillin and X-gal. All clones were grown overnight in 150 μl LB with 6% glycerol and 100 mg/ml ampicillin at 37 C prior to sequencing. All clones were sequenced in one direction using the 8F primer. Clones were sequenced by the San Diego State University Microchemical Core Facility.

16S rDNA sequence analysis

We used the Fastgroup II program (http://coral.sdsu.edu/project1/fastgroup.html, accessed November 2005) to dereplicate the 16S rDNA libraries. Using Fastgroup II, we trimmed the 3′ end of the rDNA sequences at a conserved site found in all known bacterial sequences (*E. coli* position 534). We then dereplicated the sequence by grouping all the sequences that were 99% similar to one another. For each grouping, one sequence was selected as a representative sequence and those representative sequences were lumped into an Operational Taxonomic Unit (OTU). We used a 99% cutoff because 16S rDNA sequences differing more than 1% within the Pasteurellaceae typically represent different strains or species (Angen et al., 1999). This allowed us to distinguish all truly unique sequences and also avoid sequence differences attributable to PCR-induced mutation (very low with Amplitaq Gold) and sequencing errors; we only sequenced the clones in one direction due to cost constraints. The trimmed and dereplicated 16S rRNA sequences were aligned using the Arb software program (http://www.arb-home.de, accessed March 2006). The Arb package includes tools for creating rigorous secondary structure-based alignment and for visualizing phylogenetic trees.

Phylogenetic and population genetic analyses

We initially used BLAST and Arb to identify the bacterial family or genus of the 16S rDNA clone library sequences. We then used the PAUP* phylogenetic software package program to perform Maximum Parsimony (MP), Maximum Likelihood (ML), and Neighbor-joining (NJ) phylogenetic estimation and bootstrap analysis. A random addition heuritic search strategy with Transverse Bisection–Reconnection (TBR) branch swapping was used to find the optimal MP and ML trees. One hundred heuristic searches were used to find the best tree with MP and 10 heuristic searches with ML. One hundred bootstrap replicates were performed under both MP and ML criterion, and one thousand replicates for NJ, all using the PAUP* default search options for each replicate. The ML analysis used the HKY85 model of sequence evolution with estimated transition/transversion rates and base frequencies.

The PAUP* was also used to perform randomization tests called P tests. The P tests determine whether there are significant differences in the phylogenetic composition of any two microbial communities (Martin, 2002). For example, we applied the P tests to check whether the oropharyngeal and nasal communities within the same sheep contained bacteria from different phylogenetic groups. Additionally, we used the Arlequin population analysis software package to estimate Φ_{ST} between populations. Φ_{ST} measures the overlap in genetic diversity of two communities or populations by comparing the genetic diversity within two populations to the total genetic diversity in both populations (Martin, 2002). Martin applied Φ_{ST} to measure the overlap of genetic diversity between microbial communities. Φ_{ST} values range from zero to one. A Φ_{ST} value close to zero means there is complete overlap in the genetic diversity, whereas values close to one indicate no overlap of genetic diversity.

Nucleotide data

Sequences obtain in this study have been deposited in GenBank under accession numbers AY938537 to AY939524.

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TABLE 1. Culturing results for the thirteen samples selected for cloning.

Individual	Sample ID	Population	Sex	Age	Bacteria isolated	Biovariant[††]
1	SV1-O	St. Vrain, CO	F	N/A	Not cultured	
	SV1-N	St. Vrain, CO	F	N/A	Not cultured	
2	G8-O	Georgetown, CO	M	2	*Mannheimia haemolytica*	$1^{\alpha E}$ (h)
					Pasteurella trehalosi	$3^{\alpha BCDE}$ (nh)
	G8-N	Georgetown, CO	M	2	Not cultured	
3	B3-O	Bishop, CA	M	N/A	Not cultured	
4	HC3-O	Lostine, OR Hells Canyon	F	2.5	*Mannheimia haemolytica*	$1^{\alpha E}$ (h)
					Pasteurella trehalosi	2^{B} (nh)
	HC3-N	Lostine, OR Hells Canyon	F	2.5	*Mannheimia haemolytica*	$1^{\alpha E}$ (h)
5	HC5-O	Lostine, OR Hells Canyon	F	3.5	*Pasteurella trehalosi*	2^{B} (nh)
	HC5-N	Lostine, OR Hells Canyon	F	3.5	*Pasteurella trehalosi*	2^{B} (nh)
6	HC6[†]	Imnaha, OR Hells Canyon	F	9.5+	Mixed bacterial growth	
7	HC7[†]	Imnaha, OR Hells Canyon	F	9.5+	Mixed bacterial growth	
8	HC8[†]	Imnaha, OR Hells Canyon	F	7.5	*Pasteurella multocida* Mixed bacterial growth	
9	HC9[†]	Imnaha, OR Hells Canyon	M	1.5	*Arcanobacterium pyogenes*, Mixed bacterial growth	

[†] Samples collected from lung tissue of sheep that died from pneumonia. Samples were collected within one week of death during February 2004 (ambient temperature range from −1 to 9 C).

[††] Biovariants are identified by the numbers and superscript letters; ability of isolate to produce hemolysis is indicated by (h) and (nh) indicates the isolates were non-hemolytic.

RESULTS

The DNA extractions and 16S rDNA PCR amplifications were successful for all 42 swab and tissue samples analyzed. The PCR amplification of *lktA* produced bands in 10 out of the 42 samples tested. Clone libraries (rDNA) were successfully made from 13 of the 15 swabs that were attempted. Nine of these rDNA libraries were derived from live, apparently healthy individuals in California, Colorado, and Oregon. The samples selected for cloning included oropharyngeal and nasal swabs (only oropharyngeal samples were available from the California population) from a randomly selected individual representing each of the five populations: two populations in Colorado, one in California, and two subpopulations in Oregon. Cloning and sequencing costs prohibited a wider sampling of the population, but we felt this number was sufficient for our study because its main purpose was to demonstrate the utility and effectiveness of the molecular methods. We also successfully cloned bacterial sequences from DNA extracted from the lung tissues of bighorn sheep that had been dead for up to one week (Table 1). Culturing results are reported in Table 1.

Sequence libraries obtained from live sheep were highly diverse. Figure 1 shows the phylogenetic diversity of the 16S rDNA bacterial sequences found in a healthy bighorn sheep from Bishop, California. The number of unique OTUs detected in healthy bighorn sheep ranged from 62 to 160. Based on 16S rDNA identification, Pasteurellaceae species of-

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TABLE 2. The number of 16S rDNA OTUs (>1% divergence) identified from the most consistently abundant bacterial species or genera in swabs of live bighorn sheep. Percentages indicate the relative proportion of these sequences in the clone libraries obtained from each sample.

Sample ID[a]	*Mannheimia haemolytica*	*Pasteurella trehalosi*	*Haemophilus* spp.	*Pseudomonas* spp.	*Moraxella* spp.	*Neisseria* spp.	*Streptococcus* spp.	Total[b]
SV1	– (0%)	– (0%)	– (0%)	41 (66%)	10 (16%)	– (0%)	3 (5%)	62
G8	5 (3%)	7 (4%)	57 (35%)	3 (2%)	2 (1%)	8 (5%)	7 (4%)	160
B3-O	5 (6%)	19 (22%)	4 (5%)	– (0%)	4 (5%)	25 (30%)	15 (18%)	83
HC3	– (0%)	– (0%)	7 (7%)	– (0%)	2 (2%)	5 (5%)	23 (23%)	100
HC5	– (0%)	5 (4%)	31 (26%)	9 (8%)	3 (3%)	1 (>1%)	30 (25%)	119

[a] Numbers have been combined from oropharyngeal and nasal swabs for all individuals except B3 where no nasal swabs were available for analysis.

[b] Total includes OTUs from other bacterial groups not in the table.

ten associated with disease, such as *Pasteurella trehalosi* and *Mannheimia haemolytica*, were found in samples from the healthy individuals (Table 2). Other bacteria related to *Pseudomonas* spp., *Moraxella* spp., *Streptococcus* spp., and *Neisseria* spp. also were common in both healthy and dead animal samples (Fig. 1). We also were able to amplify and clone rDNA bacterial sequences from dead animals even when bacteria could not be cultured (Table 1).

Phylogenetic and population genetic analyses

Phylogenetic analysis of 16S rDNA samples uncovered a diverse array of bacterial species from a number of different phylogenetic groups; and the results are summarized in Table 2. Figure 1 presents the results of a representative phylogenetic analysis for 16S rDNA sequences from a swab clone library. The library was constructed for one individual (B3-O) from the Bishop, California population. (Results from other individuals are available from the corresponding author.) The P tests found no significant difference in the phylogenetic diversity of bacterial communities between nasal and oropharyngeal samples within individuals with the exception of the HC5 population (Table 3). The P tests were highly significant when comparing bacterial communities in individuals within and among populations.

DISCUSSION

Our results demonstrate the effectiveness of molecular methods for determining host-associated microbial diversity of bighorn sheep respiratory tracts. We were able to PCR amplify bacterial 16S rDNA from all the swab samples taken from live animals. These samples included swabs that did not provide viable cells for culturing because of mishandling, improper storage, or field conditions that precluded rapid sample collection and culturing. We also found that the same molecular techniques could work for primers designed from toxin genes, specifically the leukotoxin A gene (*lktA*). This result was especially promising because detection of toxin genes directly from swab or tissue samples could be used to correlate the presence of toxin genes with disease. For example, we detected *lktA* in approximately 18% (seven of 38) of the live bighorn swab samples and in 67% (two of three) of the dead bighorn samples. More animals need to be tested to determine if this is a consistent pattern, but our results show the potential for this approach. In future studies, we plan to use the PCR-based molecular approaches to detect other virulence-associated genes and we also plan to examine the levels of mRNA expression of the *lktA* genes in bighorn tissue and swab samples.

Phylogenetic analysis of clone library sequences obtained from the swab sam-

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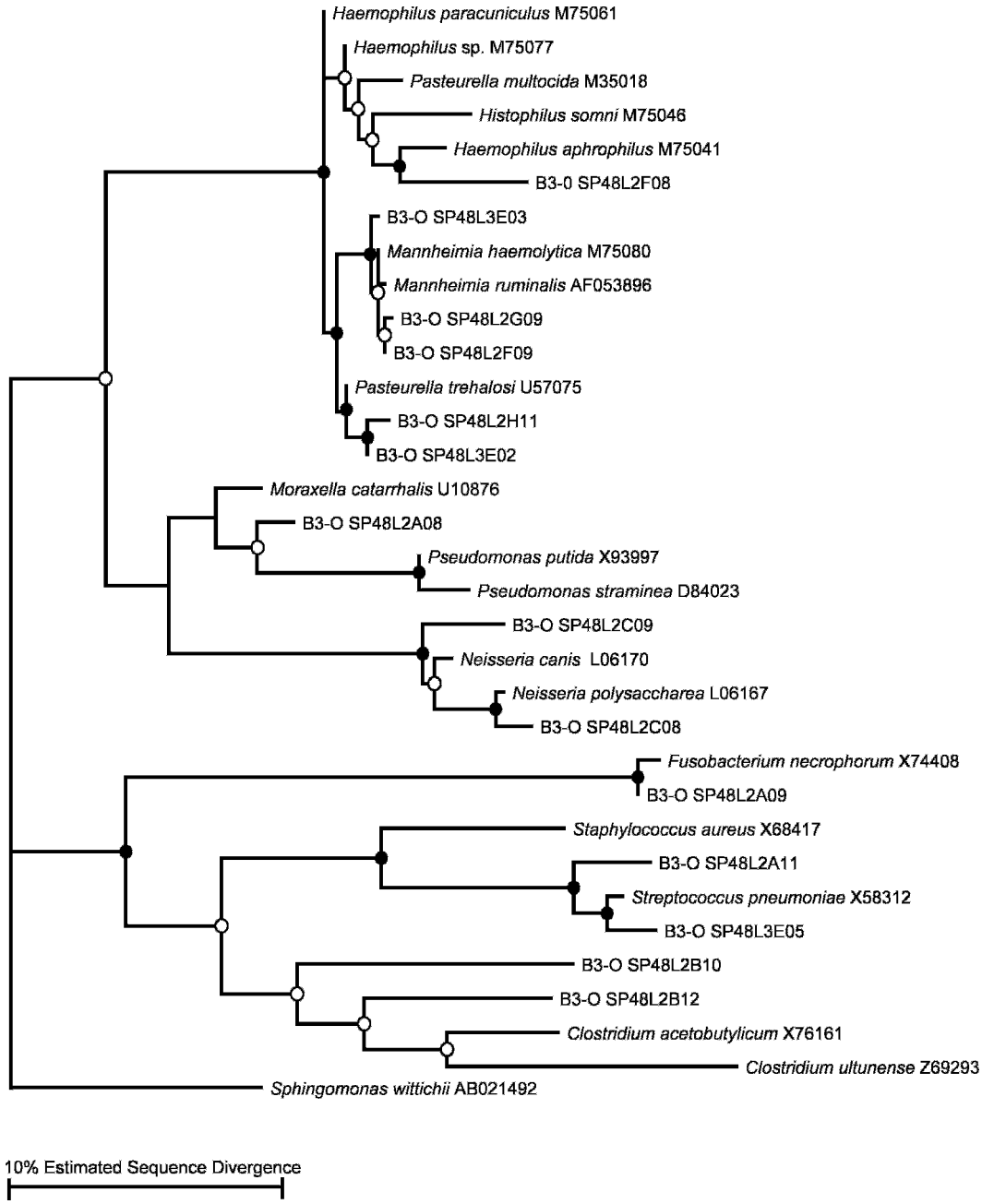


FIGURE 1. Results of a maximum likelihood phylogenetic analysis (ln = −3500.3) of bacterial 16S rDNA sequences isolated from the upper respiratory tract of a live bighorn sheep (B3-0) captured near Bishop, California. The tree is based on a 405-nucleotide alignment of 14 cloned sequences from the B3-O library and rDNA sequences of 19 cultured organisms related to the cloned sequences based on BLAST and Arb analyses. (Genbank accession numbers are placed next to the names of the cultured organisms.) These 14 sequences represented the greatest breath of phylogenetic diversity in the library. The sequence names starting with "B3-O" indicate cloned bacterial sequences, and the codes after the B3-O designation are internal references to glycerol stocks of the cloned sequences. Open circles indicate bootstrap support between 50% and 70%, and filled circle indicate bootstrap support ≥70%.

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TABLE 3. Phylogenetic-based statistical comparisons of microbial diversity among clone library samples.

Samples	P TEST[a]	Φ_{ST}
Oropharyngeal vs. Nasal, same individual		
SV1-O vs. SV1-N	0.1855	0.02441
G8-O vs. G8-N	0.2877	0.03789
HC3-O vs. HC3-N	0.0559	0.03351
HC5-O vs. HC5-N	0.0001	0.08992
Pair-wise comparisons between individuals		
SV1-O vs. G8-O	0.0050	0.08043[b]
SV1-O vs. B3-O	0.0001	0.07667[b]
SV1-O vs. HC3-O	0.0001	0.04897[b]
G8-O vs. B3-O	0.0001	0.08507[b]
G8-O vs. HC3-O	0.0001	0.07397[b]
B3-O vs. HC3-O	0.0001	0.06347[b]

[a] Number of replicates = 10,000. (*P*-value)

[b] Comparison of microbial diversity in two oropharyngeal swabs.

ples found a highly diverse suite of bacteria in the upper-respiratory tracts of live bighorn sheep. At the >1% divergence level, we identified between 62 and 160 different bacterial OTUs in oropharyngeal and nasal samples from live bighorn sheep. This diversity included bacteria from a number of different genera such as *Pseudomonas* spp., *Moraxella* spp., *Streptococcus* spp., and *Neisseria* spp. (Fig. 1 and Table 2). Members of the Pasteurellaceae were particularly abundant in the healthy animals with the exception of one animal from Colorado (Table 2). In every library, we found numerous 16S rDNA sequences that were more than 3% divergent from any sequence in the database, suggesting that a significant fraction of Pasteurellaceae diversity still remains to be characterized through culturing. The diverse array of Pasteurellaceae found in the live bighorns, many from populations with no history of disease, supports the premise that these bacteria commonly live in the bighorn sheep upper respiratory tract as commensals.

Using a sequence divergence cutoff of 1% for 16S rDNA, we detected almost two orders of magnitude more bacterial species with the culture-independent methods than we did with culturing approaches. At the more conservative 3% divergence cutoff used by some authors, we still detected approximately 10 times as many species with culture-independent methods as with culture-based approaches. Admittedly, classifying bacterial species based on percentage sequence divergence presents difficulties. However, 16S rDNA has been regularly used to identify the relationships among many bacterial groups, including the Pasteurellaceae, and based on these studies we feel confident that 16S rDNA genes with 3% or greater sequence divergence represent different bacterial species.

Although some of the samples were mishandled or stored improperly for culturing, the five samples that did produce bacterial cultures were handled with great care. Moreover, the culture results in terms of species numbers were consistent with previous studies of bighorn respiratory tracts (Jaworski et al., 1998). A wider array of culture media might have resulted in the culture of more species of bacteria, but many studies have shown that culture-independent methods consistently detect higher microbial diversity than culture-based methods alone (Amann et al., 1995; Hugenholtz et al., 1998a). The culture-independent molecular methods also worked effectively with dead animal lung tissue that was up to one week old (data not shown). Naturally, one must be

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cautious when interpreting culture or culture-independent results with tissue obtained from dead animals due to colonization of necrotic tissue unrelated to pathogenesis. However, if samples can be obtained quickly after death, our results demonstrate that the culture-independent methods could potentially serve as useful diagnostic tools. Culture-independent techniques might also be able to detect primary pathogens that have been overgrown by opportunistic organisms. For example, we were able to detect *Mannheimia* and *Pasteurella* spp. in swab samples from a dead individual with molecular analysis; these were not detected in cultures.

The current cost of culture-independent methods limited our ability to sample many individuals in each population. However, we were able to determine the repeatability of our sampling by statistically comparing the diversity of oropharyngeal and nasal microbial diversity within individual sheep. With one exception, P tests found no significant differences between oropharyngeal and nasal communities within individuals (Table 3; $P>0.05$). This suggests that culture-independent diversity measures are repeatable, but additional work is certainly necessary to confirm this result. In HC-5, the bighorn sheep that died from pneumonia, there were significant differences between microbial communities in the oropharyngeal and nasal regions. This might reflect actual differences in bacterial flora due to postmortem colonization. A comparison of the microbial community diversity among individuals found highly significant differences in the phylogenetic composition of the bacterial communities (Table 3). These preliminary results need to be bolstered by the study of many more individuals, but we believe they show the potential for statistically analyzing the patterns of this diversity within and among populations.

Considerable further research is needed to establish how complex bighorn respiratory communities form and how they change over time. More culture-independent studies clearly need to be done to understand the baseline microbial diversity of healthy bighorn sheep populations. This information will be vital for determining whether pathogens have arisen from within isolated populations as environmental conditions change, or if the bighorn sheep are picking up microbes through contact with domestic animals. In the future, we plan to undertake a more comprehensive analysis of microbial diversity associated with the respiratory tracts of isolated bighorn populations. As sequencing costs continue to fall, and as the process of creating clone libraries becomes more automated, we foresee culture-independent methods becoming increasingly useful as diagnostic tools. Also, if culture-independent methods detect new pathogens, these organisms could then be targets of focused culturing efforts vital for understanding and preventing disease.

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LITERATURE CITED

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