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Phylogenetic diversity of Pasteurellaceae and horizontal gene transfer of leukotoxin in wild and domestic sheep

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Abstract

Wild and domestic animal populations are known to be sources and reservoirs of emerging diseases. There is also a growing recognition that horizontal genetic transfer (HGT) plays an important role in bacterial pathogenesis. We used molecular phylogenetic methods to assess diversity and cross-transmission rates of Pasteurellaceae bacteria in populations of bighorn sheep, Dall's sheep, domestic sheep and domestic goats. Members of the Pasteurellaceae cause an array of deadly illnesses including bacterial pneumonia known as ''pasteurellosis'', a particularly devastating disease for bighorn sheep. A phylogenetic analysis of a combined dataset of two RNA genes (16S ribosomal RNA and RNAse P RNA) revealed remarkable evolutionary diversity among *Pasteurella trehalosi* and *Mannheimia* (*Pasteurella*) *haemolytica* bacteria isolated from sheep and goats. Several phylotypes appeared to associate with particular host species, though we found numerous instances of apparent cross-transmission among species and populations. Statistical analyses revealed that host species, geographic locale and biovariant classification, but not virulence, correlated strongly with Pasteurellaceae phylogeny. Sheep host species correlated with *P. trehalosi* isolates phylogeny (PTP test; $P = 0.002$), but not with the phylogeny of *M. haemolytica* isolates, suggesting that *P. trehalosi* bacteria may be more host specific. With regards to populations within species, we also discovered a strong correlation between geographic locale and isolate phylogeny in the Rocky Mountain bighorn sheep (PTP test; $P = 0.001$). We also investigated the potential for HGT of the leukotoxin A (*lktA*) gene, which produces a toxin that plays an integral role in causing disease. Comparative analysis of the combined RNA gene phylogeny and the *lktA* phylogenies revealed considerable incongruence between the phylogenies, suggestive of HGT. Furthermore, we found identical *lktA* alleles in unrelated bacterial species, some of which had been isolated from sheep in distantly removed populations. For example, *lktA* sequences from *P. trehalosi* isolated from remote Alaskan Dall's sheep were 100% identical over a 900-nucleotide stretch to sequences determined from *M. haemolytica* isolated from domestic sheep in the UK. This extremely high degree of sequence similarity of *lktA* sequences among distinct bacterial species suggests that HGT has played a role in the evolution of *lktA* in wild hosts.

Keywords: Horizontal gene transfer; Leukotoxin; Pasteurellaceae; Phylogeny

1. Introduction

Wild animal populations have long been recognized as reservoirs of emerging and re-emerging viral and bacterial diseases (McEvedy, 1988). More recently, domestic animals have been identified as sources of disease in wildlife (Woodroffe, 1999). Bacterial pathogens such as *Yersinia pestis* (Brubaker, 2004), *Borrelia burgdorferi* (Stanek and Strle, 2003), *Bartonella* spp. (Anderson and Neuman, 1997), *E. coli* O157:H7 (LeBlanc, 2003), and *Pasteurella* spp. (Donnio et al., 2004; Rudolph et al., 2003) are known to have persistent animal reservoirs. Numerous studies have documented the transmission of infectious bacteria from natural populations to humans and domesticated animals and vice versa (Brubaker, 2004; Gallagher-Smith et al., 2004; Rudolph et al., 2003; Stanek and Strle, 2003). Estimating the potential for the emergence of new diseases therefore requires examination of the existing microbial diversity in both wild and domestic animal populations.

The process of horizontal gene transfer (HGT) and the molecular evolutionary rate of genes involved in pathogenesis are increasingly being recognized as critical factors in the

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disease process. HGT appears to be a common theme in bacterial virulence (Dzidic and Bedekovic, 2003; Faruque and Mekalanos, 2003), and a number of genes directly involved in pathogenesis are known to have transferred between bacterial species (Bukhalid et al., 2002; Davies et al., 2002; Hacker and Kaper, 2000). For example, shiga toxin, cholera toxin, neurotoxins of *Clostridium botulinum*, and the cytotoxin of *Pseudomonas aeruginosa* are all encoded on bacteriophages that can transfer horizontally between bacterial species (Novick, 2003). HGT transmission mechanisms also include plasmid-mediated HGT, which may be followed by recombination, and transposition events (Bushman, 2002). Although many HGT events have apparently resulted from ancient evolutionary events (Ochman et al., 2000), studies of multi-drug resistance in hospital bacteria (Dzidic and Bedekovic, 2003) and controlled experimental studies (Kidambi et al., 1994; Shoemaker et al., 2001) have demonstrated that HGT can occur frequently under certain circumstances. Given the important role of particular genes (e.g., toxin genes) in pathogenicity, identifying the frequency of HGT and determining the rates of evolution of these genes is critical for understanding patterns of emerging diseases.

Few studies have investigated the evolutionary diversity of pathogens in wild animal populations, or levels of HGT between potentially virulent bacteria in natural and domesticated populations. In this study, we used molecular phylogenetic methods to investigate both bacterial evolutionary diversity and toxin gene HGT in Pasteurellaceae isolated from wild and domesticated sheep populations. Two members of the Pasteurellaceae, *Mannheimia* (*Pasteurella*) *haemolytica* and *P. trehalosi*, are globally distributed bacteria and infectious agents of cattle, sheep, and wild ruminants (Frank, 1989; Gilmour and Gilmour, 1989; Ward et al., 1999). Infections of *M. haemolytica* and *P. trehalosi* cause a respiratory type illness known as ''pasteurellosis'' that often leads to severe morbidity and death (Frank, 1989; Gilmour and Gilmour, 1989). Pasteurellosis has decimated numerous bighorn sheep (*Ovis canadensis*) populations in western North America. Bighorn sheep appear to contract the disease after contact with domestic sheep, although Pasteurellaceae are also endemic to many wild bighorn sheep populations (Fisher et al., 1999; Jaworski et al., 1998; Miller, 2001).

Understanding the phylogenetic diversity among naturally occurring strains of Pasteurellaceae is crucial for understanding the complex nature of pasteurellosis and determining the source of disease. Phylogenetic approaches can help identify which strains evolved in wild or domestic hosts and whether or not these strains are endemic. These methods can also assess how often strains transfer between wild and domestic animal populations. Culture-based studies have revealed a remarkable array of Pasteurellaceae strains in wild populations as well as evidence of cross-transmission between wild and domestic animals (Rudolph et al., 2003). However, these methods provide only limited phylogenetic information for determining evolutionary diversity. On the other hand, molecular phylogenetic studies of Pasteurellaceae isolates using small subunit ribosomal RNA (16S rRNA) gene sequences suggest that this gene and other housekeeping genes can provide sufficient information for determining Pasteurellaceae phylogenetic diversity (Angen et al., 1999; Davies et al., 1997a,b).

Studies of domestic sheep and cattle also indicate that virulence genes often experience HGT. Genes in the leukotoxin operon appear to transfer horizontally among strains of *M. haemolytica* (Davies et al., 2002) and even between *M. glucosida* and *P. trehalosi* (Davies et al., 2001). Leukotoxin is a pore-forming cytotoxin considered to be an important virulence factor, especially in *M. haemolytica* (Wang et al., 1998). The leukotoxin A (*lktA*) gene encodes the protoxin which, after modification and secretion, targets and destroys ruminant lymphoid cells by binding to $\beta(2)$ integrins (Leite et al., 2003; Li et al., 1999). High concentrations of *lktA* cause pore formation in lymphoid cell membranes, while low concentrations of *lktA* induce apoptosis (Clarke et al., 1998; Cudd et al., 2001; Sun et al., 2000). Previous sequencing based studies of the leukotoxin operon uncovered substantial levels of recombination and HGT of the four gene leukotoxin operon (lktCABD) among *M. haemolytica* and *P. trehalosi* isolated from domestic sheep and cattle (Davies et al., 2001, 2002).

The objective of this study was to determine the phylogenetic diversity and degree of cross-transmission of *M. haemolytica* and *P. trehalosi* strains isolated from wild and domestic sheep populations in North America. We also examined HGT of *lktA* among natural and domesticated populations in North America and Europe. To these ends, we amplified and sequenced genetic markers from 48 bacterial strains isolated from populations of wild Rocky Mountain and California bighorn sheep (*O. canadensis*), Dall's sheep (*O. dalli*) and from domestic sheep (*O. aries*) and goats (*Capra hircus*). Sequences from two housekeeping genes, the 16S rRNA gene and the RNAse P RNA gene, were used to identify the phylogenetic relationships among strains. We also PCR amplified and sequenced the *lktA* gene from these same isolates to determine their overall sequence diversity and rate of HGT.

2. Materials and methods

2.1. Sample collection and bacterial isolation

We sequenced 48 bacterial isolates collected from 13 populations of bighorn sheep in Idaho, Washington, Oregon, Colorado, Alberta, and British Columbia and 4 populations of Alaska Dall's sheep, all between 1994–2002. Isolates were selected to be a representative of the genetic diversity as possible. Specifically, we choose isolates to represent the broadest diversity of biovariant types. Although some of the isolates were collected from the same animal populations at the same time, most were not. Seven of these isolates were collected from bighorn sheep that had died from pneumonia in four populations in 1995, 1996, 1997, 1998, and 2000. We also sequenced seven isolates from domestic sheep collected 1997–2002, four of which were from animals that had died from pneumonia; and two samples collected in 1995 from healthy free-ranging domestic goats near bighorn sheep in Idaho and Washington. Samples from live animals were collected on pharyngeal swabs. Wild sheep pharyngeal swabs

were collected during routine health screenings. Samples from animals that had died from pneumonia consisted of lung or tonsil biopsies taken at necropsy. All samples were placed immediately in either BBL 'Port-a-Cul' or Amies (Becton, Dickinson & Company, Sparks, Maryland) without charcoal transport media. The samples were placed on ice and transported to the laboratory within 72 h, where they were inoculated onto nonselective Columbia blood agar, containing 5% sheep blood and selective Columbia blood agar with selective antibiotics, containing 5% bovine blood (Slee and Stephens, 1985), and incubated for 18–24 h at 37 °C in a 10% CO_2 atmosphere.

2.2. Species and biovariant classification

Following incubation, representatives of each colony type were propagated on fresh Columbia blood agar for species and biovariant classification. Isolates were determined to be *M. haemolytica* and *P. trehalosi* by testing whether they were MacConkey's-, urea- and indole-negative; oxidase-, nitrate-, glucose-, sucrose-positive; and xylose- or trehalose-positive. Additional biochemical tests were then applied to identify biovariants (Bisgaard and Mutters, 1986; Jaworski et al., 1998). Biovariant and serotype classification of wildlife isolates is unsuccessful using traditional methods developed to characterize isolates from domestic cows and sheep. Wildlife isolates typically exhibit deviations in biochemical tests and show more diversity than domestic isolates. Jaworski et al. (1998) refined and added to the biochemical testing scheme developed for isolates from domestic animals (Bisgaard and Mutters, 1986), and adapted the method for identifying isolates from wildlife. Briefly, the method uses the biochemical utilization reactions from 23 tests and compares these with the reactions of internationally accepted serotype standards 1–16. The method has identified over 150 biovariant types from wildlife.

The above method differs from that of Angen et al. (1999), which primarily uses DNA–DNA hybridization and 16S rRNA gene sequencing to identify clusters of isolates from domestic animals. The diversity identified by this method includes five named, and two unnamed, species along with an untypeable group. There appears to be more diversity present in isolates from wildlife than could be discerned by this method, although applicability of this method to characterize wildlife isolates has not been fully investigated. Table 1 presents detailed information on the isolates used in the study.

2.3. DNA extraction and PCR

DNA was extracted from colonies using a bead-beating isolation protocol (Tanner et al., 1999) and stored in 50 μL TE buffer at −20 °C until PCR reactions were performed. The following primers were used in the PCR reactions: (1) bacterial-specific universal 16S rRNA gene primers 8F (AGAGTTTGATCCTGGCTCAG) and 805R (GACTACCAGGGTATCTAATCC); (2) RNAse P RNA gene primers 59F (CGGGATCCGIIGAGGAAAGTCIIGC; I = inosine) and 347R (CGGAATTCRTAAGCCGGRTTCTGT; R = A or G) (Brown et al., 1996); (3) *lktA* forward (5′-TGTGGATGCGTTTGAAGAAGG-3′) and reverse (5′-ACTTGCTTTGAGGTGATCCG-3′) (Fisher et al., 1999). PCR was carried out in a total reaction volume of 50 μL volume, including 1 μL of sample DNA as template, 200 μM deoxynucleoside triphospates, 1.5 mM $MgCl_2$, 0.4 μM of each primer, 1 mg/mL Bovine Serum Albumin (BSA) and 5 units of Ampli*Taq* Gold (Applied-Biosystems, Foster City, CA). The PCR thermal cycling conditions for the RNAse P RNA gene and the 16S rRNA gene amplification reactions consisted of 30 cycles of amplification. Each cycle included one initial denaturing step at 94 °C for 1 min, followed by a 45 s annealing step at 55 °C and a 1.5 min extension step at 72 °C. The amplification cycles were preceded by a one-time denaturing step at 94 °C for 2 min prior to the first cycle, and included a final 72 °C-extension step for 20 min to ensure complete extension for efficient cloning. The PCR amplification conditions using the *lktA* primer set were identical, except that the annealing temperatures spanned a gradient from 40 to 60 °C.

2.4. Cloning and sequencing

PCR products of the RNAse P RNA and 16S rRNA genes, and cloned PCR products of the *lktA* gene, were sequenced directly on an ABI PRISM 377 sequencer (Applied Biosystems, Foster City, CA) following the manufacturer's protocol. RNAse P RNA and 16S rRNA genes were sequenced with the same primers used for PCR. *lktA* PCR products were cloned using a pGEM T-easy vector system cloning kit (Promega Corporation, Madison, WI) following the kit protocols, and the inserts were sequenced with pGEM T-easy vector specific primers flanking the insert region: Sp6 (5′-ATTTAGGTGACACTATAG-3′) and T7 (5′-TAATACGACTCACTATA-3′). The sequences obtained were compared to the published data in GenBank using a standard nucleotide BLAST (basic local alignment search tool) search to verify that the PCR product and clone was the correct sequence and not vector sequence or some other gene product.

2.5. Sequence analysis

16S rRNA gene and RNAse P RNA gene sequences were aligned and manually refined using the ARB software program (http://www.arb-home.de) with special consideration given to secondary structure. 16S rRNA has often been used for determining the relationships among microbes. The RNAse P RNA gene has been found in a single copy in all bacterial genomes so far analyzed. In bacteria, this RNA is part of a ribonucleic protein enzyme that includes a catalytic RNA subunit and an associated protein. The RNAse P RNA processes the 5′ prime precursor of tRNAs to make functional tRNA molecules, an essential function for all cells (Brown et al., 1996). *lktA* sequences were aligned to each other, and to published *lktA* sequences, using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The published *lktA* sequences used in phylogenetic analysis (Fig. 3) were obtained from GenBank using the accession numbers provided in the original publications (Davies et al., 2002).

Table 1
Details of isolates used in the study

Isolate	Host[a]	Biovariant	Herd location	Health[b]	Sample type	CVTC number
I01	R	8	Cadomin, Alberta	H	Pharyngeal	00-175-17
I03	R	2	Nevada	C	Pharyngeal	94-829-147
I04	O	1	Idaho	P	Lung	95-1221-1
I05	O	1	Idaho	P	Unknown	97-129-4
I06	R	4CDS	Colorado	P	Unknown	98-1109-1
I07	R	2B	Ram Mountain, Alberta	H	Pharyngeal	99-1110-1
I08	R	2	Ram Mountain, Alberta	H	Pharyngeal	99-1425-31
I09	R	2B	Cadomin, Alberta	H	Pharyngeal	99-175-1
I10	R	2B	Cadomin, Alberta	H	Pharyngeal	99-199-1
I11	D	2E	Dry Creek, Alaska	H	Pharyngeal	99-390-12
I12	D	2E	Dry Creek, Alaska	H	Pharyngeal	99-412-1
I13	D	8B	Anaktuvuk Pass, Alaska	H	Pharyngeal	00-381-24
I14	D	16aBE	Anaktuvuk Pass, Alaska	H	Pharyngeal	00-381-33
I15	D	16aBE	Tanana Hills, Alaska	H	Pharyngeal	00-443-45
I16	D	16aR	Tanana Hills, Alaska	H	Pharyngeal	00-443-103
I17	D	9aER	Dry Creek, Alaska	H	Pharyngeal	99-412-42
I18	D	10aEX	Hammond River, Alaska	H	Pharyngeal	00-391-24
I19	D	2E	Hammond River, Alaska	H	Pharyngeal	00-391-52
I21	D	1BE	Brooks range, Alaska	H	Pharyngeal	00-433-24
I22	D	UaER	Dry Creek, Alaska	H	Pharyngeal	00-363-5
I23	R	1aBE	Ram Mountain, Alberta	H	Pharyngeal	99-1425-13
I24	R	7B	Ram Mountain, Alberta	H	Pharyngeal	99-1110-21
I25	R	UabB	Ram Mountain, Alberta	H	Pharyngeal	99-1425-52
I26	R	1E	Cadomin, Alberta	H	Pharyngeal	99-199-11
I27	R	2	Cadomin, Alberta	H	Pharyngeal	00-217-352
I28	R	10	Cadomin, Alberta	H	Pharyngeal	00-217-82
I29	R	2	Lostine, Oregon	C	Pharyngeal	02-356-51
I30	R	3	Lostine, Oregon	C	Pharyngeal	02-356-1
I31	R	2B	Lostine, Oregon	C	Pharyngeal	02-356-33
I32	R	2B	Spences Bridge, British Columbia	C	Pharyngeal	97-1410-16
I33	R	4BLS	Redbird, Idaho	C	Pharyngeal	97-0377-084
I34	R	1	Redbird, Idaho	C	Pharyngeal	97-0377-054
I35	CA	4BS	Vaseux Lake, British Columbia	P	Unknown	00-1211-9
I36	CA	2	Vaseux Lake, British Columbia	P	Unknown	00-1211-4
I37	R	2B	Black Butte, Washington	P	Lung	96-42-1
I38	R	2B	Black Butte, Washington	P	Lung	95-1535-005
I39	R	2B	Black Butte, Washington	P	Lung	96-3-2
I40	R	2B	Wenaha, Oregon	P	Lung	97-797-1
I41	O	6aBGRX	Wenaha, Oregon	C	Pharyngeal	02-187-5
I42	O	2	Hells Canyon, Washington	C	Pharyngeal	95-1485-081
I43	O	1G	Hells Canyon, Washington	C	Pharyngeal	95-1485-084
I44	CP	2	Hells Canyon, Idaho	H	Pharyngeal	95-1516-027
I45	CP	1	Hells Canyon, Washington	H	Pharyngeal	95-1519-008
I46	O	2E	Captive, Idaho	P	Unknown	97-866-1
I47	O	1	Captive, Idaho	P	Unknown	97-866-2
I49	R	1G	Spences Bridge, British Columbia	C	Pharyngeal	97-1410-5
I50	R	1E	Spences Bridge, British Columbia	C	Pharyngeal	97-1410-24
I51	R	2	Wenaha, Oregon	P	Lung	97-758-1

[a] R: Rocky Mountain bighorn sheep; CA: California bighorn sheep; D: Dall's sheep; O: domestic sheep; CP: goat.
[b] P: pneumonia; C: potential contact with livestock or wild population; H: healthy.

Phylogenetic analyses, including maximum likelihood (ML), maximum parsimony (MP), neighbor-joining (NJ) and permutation tail probability (PTP) analyses were performed using the PAUP* phylogenetic software package (Swofford, 1998). The following description of phylogenetic analyses applies to both the RNA gene and *lktA* data sets. The RNAse P RNA gene and 16S rRNA gene sequence data sets were first analyzed separately, and later combined for the final analysis presented in Figs. 1 and 2. We used a heuristic search with 100 random addition sequence replicates to find the highest likelihood trees for all data sets under the ML criterion. ML searches were performed under an HKY85 substitution model using estimated nucleotide frequencies, gamma shape distributions and transition/transversion ratios for each data set (Hasegawa et al., 1985). We also performed NJ (GTR distance) and MP analyses for comparison (100 random addition sequence replicates were performed to determine the most parsimonious sets of trees). Bootstrap analyses were performed using the ML and MP criteria. For the ML bootstrap analysis we performed 100 bootstrap replicates with 10 random-addition sequence heuristic searches per

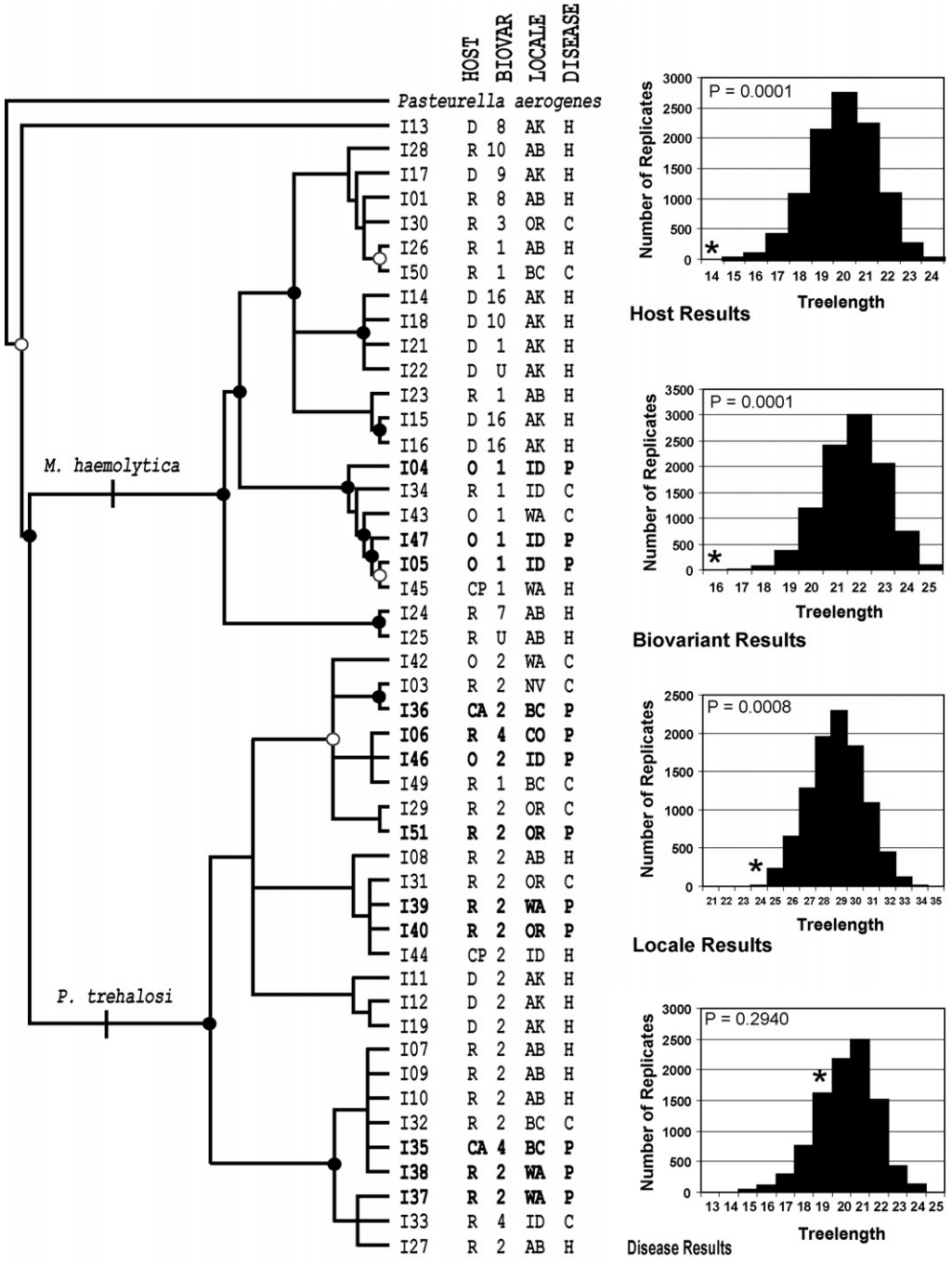

Fig. 1. Phylogenetic relationships of isolates collected in the study based on a combined analysis of the 16S rRNA gene and RNAse P RNA gene sequence data sets. The tree shown is the highest likelihood tree ($-\ln L = 3510.4$) from a ML analysis. MP and NJ analyses produced similar trees. Filled circle indicate both ML and MP bootstrap support exceeding 70% while open circle indicate bootstrap support exceeding 50%. Character states for the host type, and health status are as follows: D: Dall's sheep, R: Rocky Mountain bighorn sheep, CA: California bighorn sheep, O: domestic sheep and CP: goat; H: healthy, no known contact with livestock, C: healthy, possible contact with livestock, P: sick with pneumonia (boldface in figure). Biovariant types are indicated by numbers and locales are indicate by the US state abbreviations except for two Canadian provinces: BC: British Columbia, AB: Alberta. Graphical results of the PTP tests with 10,000 randomizations are presented for each of the four characteristics and the *P*-values for the tests are presented at the top left of the graphs. Asterisks indicate the observed number of steps for each character on the phylogeny.

replicate, and for the MP bootstrap we performed 500 bootstrap replicates with 10 random-addition sequence heuristic searches per bootstrap replicate. The RNA and *lktA* phylogenetic trees were rooted with sequences obtained from GenBank for *Pasteurella aerogenes*: U66492 (16S rRNA gene) and AF295983 (RNAse P RNA gene).

Permutation tail probability (PTP) tests, implemented in PAUP*, were used to determine whether there were associations between particular isolate characteristics and the phylogeny of the isolate strains (Martin, 2002). Specifically, the PTP approach determines whether the observed evolutionary changes for a particular character, such as host type, are fewer

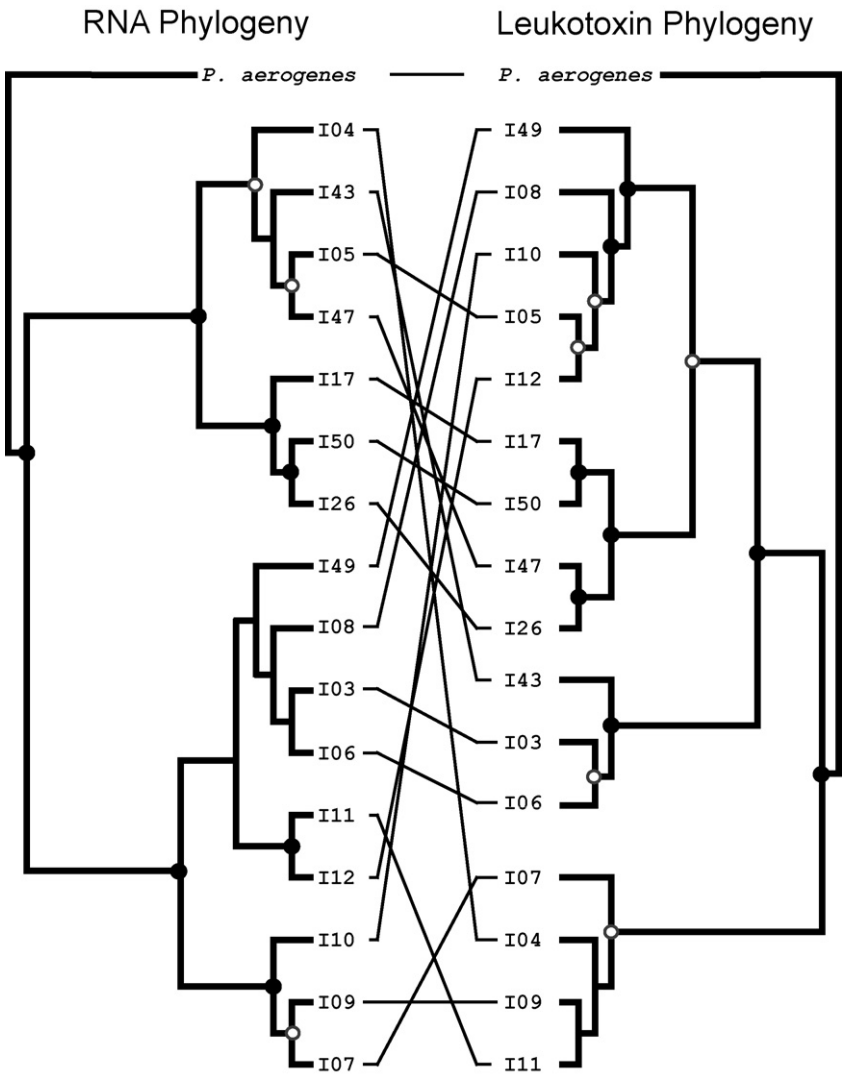

Fig. 2. Comparison of combined RNA based phylogenetic tree and leukotoxin A (*lktA*) gene tree for the 16 isolates that tested positive for the *lktA* gene with the PCR screen. The phylogenetic trees presented are the highest likelihood trees from the ML analysis ($-\ln L = 2753.34$). Filled circles indicate both ML bootstrap support exceeding 70% and open circles indicate bootstrap support exceeding 50%. The lines between the phylogenetic trees connect the same isolate on the two phylogenies and crossed lines indicate incongruence between the phylogenies. Because of recombination, the *lktA* gene tree probably does not accurately reflect the true evolutionary history of the gene (see text). Rather, the main purpose of this analysis was to show that the genes trees were very different, and that related isolates (based on the combined RNA dataset) often contained distantly related *lktA* alleles, indicating past occurrence of HGT.

than expected under a random model of character change (Martin, 2002). In order to determine this, the number of evolutionary changes on the best tree (also know as the ''number of steps'' or ''tree-length'') is determined using the parsimony criterion for a particular characteristic. The phylogenetic relationships are then fixed, and the character states are randomized on the tree with the number of steps calculated for each randomization. If the number of steps or the tree-length on the characteristic is significantly fewer ($P < 0.05$) than expected given the randomizations, then there is a correlation between the given characteristic and the phylogeny of the organisms. We used the TreeMap version 1.0 program (Page, 1995) to compare phylogenetic trees and detect potential inconguence between gene trees. Finally, we used the branch removal tool in MacClade ver. 3.01 to ''prune'' down the trees for more directed PTP analysis (Maddison and Maddison, 1992). Specifically, we performed separate PTP tests with phylogenetic sub-trees that included just the *P. trehalosi* isolates, the *M. haemolytica* isolates, and the isolates found on Rocky Mountain bighorn sheep.

3. Results

3.1. PCR, cloning and sequencing results

PCR amplifications and DNA sequencing of 16S rRNA gene and RNAse P RNA gene PCR products was successful for all of the isolates utilized in this study (Table 1). The PCR amplifications of 16S rRNA genes were robust and clean enough to allow direct sequencing of the PCR product. The PCR amplifications using the *lktA* primers amplified *lktA* in 20 of the 48 isolates used in the study. These results are similar to

those of earlier studies, in which the *lktA* gene was present in less than 50% of the isolates analyzed (Fisher et al., 1999). However, even when the amplifications of the *lktA* genes succeeded they sometimes produced multiple bands, which necessitated cloning of the PCR products. After cloning, we managed to successfully sequence the *lktA* gene from 16 of the 20 isolates that yielded positive PCR results. Although we managed to clone bands from all the 20 PCR products, four of them would not produce high quality sequence even after multiple attempts to sequence the clones. The 16 *lktA* sequences we obtained came from 3 of the 12 Dall's isolates, 4 of the 8 domestic sheep isolates, and 9 of the 26 isolates from bighorn sheep. Of the 12 isolates obtained from pneumonic animals, only 4 proved positive for *lktA* using the PCR test. Sequences have been deposited in GenBank under the following accession numbers: *lktA*: AY757693–AY757709; 16S rRNA: AY757710–757759; RNAse P RNA: AY757761–757809.

3.2. Phylogenetic analyses

The final 16S rRNA gene dataset included an alignment of 650 nucleotides corresponding to positions 64–712 of the *E. coli* 16S rRNA gene. This alignment included gaps in less than 1% of the alignment and they were treated as missing characters in the phylogenetis analyses. The RNAse P RNA gene dataset included an alignment of 263 nucleotides corresponding to positions 194–442 of the *E. coli* RNAse P RNA gene. This alignment included gaps in less than 1% of the alignment and they were treated as missing characters in the phylogenetis analyses. Sequence alignments are available upon request from the corresponding author.

Phylogenetic analysis and BLAST results with 16S rRNA gene sequences revealed that two of the isolates, I13 and I41 (Table 1), were not closely related to either *M. haemolytica* or *P. trehalosi* (data not shown). According to the 16S rRNA gene data, I13 belonged to the Pasteurellaceae but I41 was most closely related to *Neisseria meningitidis*. We, therefore, removed I41 and I13 from all the following phylogenetic analysis and the PTP tests.

Phylogenetic analyses of 16S rRNA gene and RNAse P RNA gene sequences found no substantial incongruence between the gene trees (data not shown) and the data from these two genes was combined into a single data set for further analysis. The summary of results for the combined RNA gene sequence data set of the isolates is shown in Fig. 1 along with the bootstrap values. Each of the methods produced slightly different tree topologies, but none of these differences were supported by the bootstrap results. Areas in which the methods disagreed were left unresolved in the final strict consensus tree of the combined dataset (Fig. 1).

The phylogeny revealed that related isolates are sometimes found on different host species or geographic locales, and that isolates within the same biovariant classification are not always closely related (Fig. 1). However, despite these occasional discrepancies, PTP tests revealed significant correlation between phylogeny and host species, locale and biovariant classification (Fig. 1). The phylogenetic analysis also revealed that isolates associated with pasteurellosis mortality were not necessarily related and the association with the isolate phylogeny was not significant (Fig. 1).

Because the outcome of the PTP test with the full isolate phylogeny might have been biased by mixing of isolates from the two Pasteurellaceae lineages (*P. trehalosi* and *M. haemolytica*), we re-ran the PTP analyses on each bacterial species separately. This involved using the MacClade phylogenetic program to prune down the tree so that we only used the *P. trehalosi* clade or the *M. haemolytica* clade (see Fig. 1). These sub-trees were then used in PTP tests (1000 randomizations per analysis). For the reanalysis, we tested for associations of host and phylogeny for both bacterial species. We also tested for associations between disease and phylogeny for the *P. trehalosi* clade but not the *M. haemolytica* clade because there were only three instances of Pasteurellosis associated with *M. haemolytica* isolates (Table 1, Fig. 1). We did not reanalyze the Biovariant classifications because these results were fairly obvious anyway.

The reanalysis uncovered a significant correlation between host and phylogeny amongst the *P. trehalosi* isolates (PTP test; $P = 0.002$) but not amongst the *M. haemolytica* isolates (PTP test; $P = 0.162$). Reanalysis of the correlation between disease severity and phylogeny found no correlation with either *P. trehalosi* isolate phylogeny (PTP test; $P = 0.991$) or with *M. haemolytica* isolates (PTP test; $P = 0.199$).

Since host and geography are confounded in our situation, we also reanalyzed the association between geography and phylogeny within a single host species with multiple locales. The Rocky Mountain bighorn sheep isolates were the only host set with enough different geographic locations for this test. Again, we used MacClade to prune the tree in Fig. 1 so that it only included isolates from RMBHS. For this follow-up analysis we did not analyze the bacterial species separately. Among the RMBHS, we found a highly significant association between isolate phylogeny and geographic locale (PTP test; $P = 0.001$).

Although the gene trees of the RNA genes were congruent with one another, they were completely at odds with the well-supported phylogeny of *lktA*. Fig. 2 shows the results of both the phylogenetic analyses of the *lktA* gene, including bootstrap support values, and the results of the TreeMap analysis. In the TreeMap analysis, the relationships among the *lktA* genes from 16 isolates were compared to a phylogenetic tree of the combined RNAse P RNA gene and 16S rRNA gene sequence data sets for those same isolates. The TreeMap algorithm found the gene trees to be highly incongruent (Fig. 2).

3.3. lktA diversity

We also performed a more expansive analysis of *lktA* genes with respect to sequences published by Davies et al. (2002). Alignment of our sequences to the previously published sequences proved trivial and there were no indels. The *lktA* genes obtained from 16 of the 48 isolates in the study were closely related, and in many cases identical to the *lktA* alleles

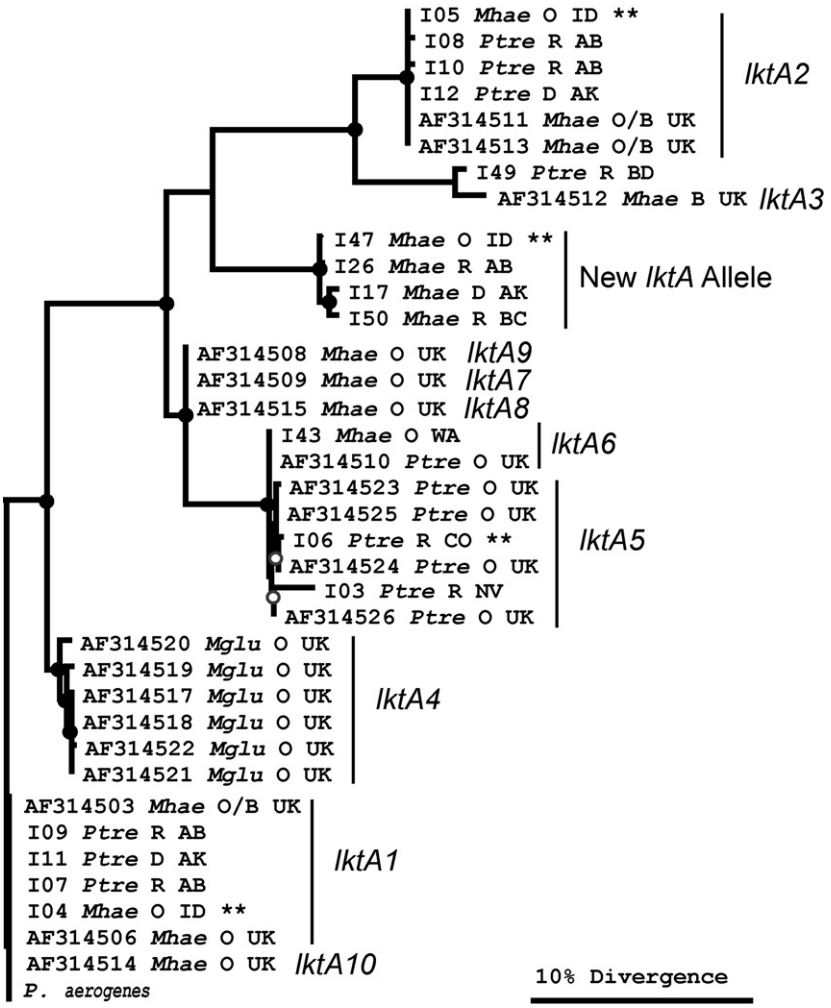

Fig. 3. Phylogenetic relationships of *lktA* sequences obtained in this study to those obtained by Davies et al. (2002). The tree was arbitrarily rooted with the A1 *lktA* allelic grouping for easier visualization of relative branch lengths. The phylogenetic tree presented is the highest likelihood tree from the ML analysis ($-\ln L = 3453.02$). Filled circles indicate ML bootstrap support exceeding 70% and open circles indicate bootstrap support exceeding 50%. NJ and MP results were identical in terms of tree topology. The branch lengths indicate the degree of nucleotide sequence divergence among sequences with respect to the scale bar. The vertical lines indicate allelic groupings designated by Davies et al. (2001). All the sequences we obtained in the study corresponded to known alleles with the exception of *lktA* sequences from four isolates (I17, I26, I47, and I50), which appear to be members of a different allelic recombination event (see Section 4). Isolate designations and GenBank accession numbers for the isolates obtained by Davies et al., are presented at the tips of the branches along with bacterial species, host species and locale information, respectively. Bacterial species: Mhae: *M. haemolytica*, Ptre: *P. trehalosi*, Mglu: *M. glucosida*. Host species: D: Dall's sheep, R: Rocky Mountain bighorn sheep, O: domestic sheep, O/B: domestic sheep and cattle. Locales are indicate by the US state abbreviations except for: BC: British Columbia, Canada; AB: Alberta, Canada; and UK: United Kingdom. Asterisks indicate *lktA* sequences isolated from pneumonic sheep.

obtained from domestic sheep and cattle in the UK. Fig. 3 shows the relationships of the new sequences to the previously published sequences, indicates the level of sequence divergence among the sequences and shows the allelic groupings identified by Davies et al. (2001). Sequence alignments are available upon request from the corresponding author.

4. Discussion

Phylogenetic analyses of *M. haemolytica* and *P. trehalosi* isolates uncovered substantial evolutionary diversity of these organisms in both wild and domesticated sheep populations (Fig. 1). Relationships among the isolates collected for this study detected a number of instances of inter-population and cross-species spread of Pasteurellaceae. This included occurrence of identical bacteria in bighorn and domestic sheep, bighorn and Dall's sheep, bighorn sheep and goats, and domestic sheep and goats (Fig. 1). Despite these exchanges among species and populations, statistical analyses indicated that isolates cultivated within a species or geographic locale tended to be more closely related to each other than to isolates cultivated from other species or locales (Fig. 1; PTP test; $P < 0.001$).

However, because we included isolates from two different bacterial species in the first PTP analysis, we may have introduced bias in the analysis. Therefore, we repeated the PTP analysis with the *P. trehalosi* and *M. haemolytica* isolates separately and found that only the phylogeny of *P. trehalosi* was correlated with phylogeny (PTP test; $P = 0.002$), and not the *M. haemolytica* phylogeny (PTP test; N.S.). These results suggest

that *P. trehalosi* bacteria tend to be more host-specific than *M. haemolytica*.

We also re-ran the PTP analysis with geographic locale using only the isolate collected from Rocky Mountain bighorn sheep. This avoided confounding the effect of host species and locale, since the other host species tended to be found only in one locale. For these sheep, we found a very strong correlation between isolate phylogeny and geographic locale (PTP test; $P = 0.001$). This suggests that bacterial spread tends to be restricted among locales, although the extent of restriction remains to be determined. From these results we conclude that wild sheep populations harbor a large diversity of endemic organisms, some of which may have co-evolved with particular sheep species before transmitting to other species and populations.

However, we did find instances of cross-transmission, and the fact that endemic strains of Pasteurellaceae can transfer with some frequency between wild and domestic sheep and goats, and likely other ruminants (Silflow and Foreyt, 1994), implies the existence of a large pool of mobile bacteria with the potential to cause disease in new hosts with naive immune systems. The evidence of cross-transmission is particularly remarkable for the Dall's sheep, which are not sympatric with bighorn sheep. This suggests either that the shared bacteria have been transmitted through an intermediate host, or that the similarity of shared bacteria is ancestral in nature. Given the additional evidence for HGT of the *lktA* alleles (see below), we tend to favor the intermediate host hypothesis, but much more sampling will be necessary to fully describe the spread of these bacteria.

The significant correlation observed between bacterial phylogeny and the geographic locale of the host population in Rocky Mountain bighorns also provides insight into the evolution of these potentially infectious bacteria. Apparently, spread of bacteria can be a localized phenomenon. Contrary to the hypothesis of ''ubiquitous dispersal'' (Finlay, 2002), the relationship between bacterial phylogeny and geography suggests there may be some phylogeographic patterning to Pasteurellaceae evolution.

Phylogenetic analysis using 16S rRNA gene and RNAse P RNA gene sequences proved extremely useful for classifying isolates. As would be expected, the biovariant classifications agreed with the RNA phylogenetic tree (PTP test; $P < 0.001$). *P. trehalosi* biovariant types 2 and 4 clustered as a monophyletic group as did most of the *M. haemolytica* isolates (all other types; Fig. 1). However, the correlation was far from perfect. For instance, phylogenetic analysis determined that I49, a biovariant type 1 isolate (*M. haemolytica* type), grouped within a clade of types 2 and 4 *P. trehalosi* biovariants (Fig. 1). Similarly, biovariant types within *M. haemolytica* and *P. trehalosi* (e.g., Biovariant types 4 and 16) did not always form strict monophyletic groups. These results concur with previous studies that found similar biovariants were not always closely related (Angen et al., 1999). However, the broad agreement between RNA gene phylogeny and biovariant classification indicates that the biovariant system can still be a useful tool for quickly identifying bacterial species.

We found no significant correlation between disease severity and isolate phylogeny, either in the full tree (Fig. 1; PTP test; $P = 0.294$), or within the *P. trehalosi* and *M. haemolytica* clades analyzed separately. Specifically, the isolates associated with pasteurellosis did not form a monophyletic group. Rather, they were scattered across the phylogenetic tree. The fact that many unrelated *lktA* sequences were found in pneumonic animals suggests that the presence of leukotoxin by itself is not enough to cause illness.

Comparisons between RNA-based and *lktA*-based phylogenetic trees revealed these trees to be highly incongruent, and suggested that there have been numerous instances of HGT of the *lktA* gene among *M. haemolytica* and *P. trehalosi* lineages (Fig. 2). Gene tree comparisons are often used to detect evidence of HGT. However, the high rates of recombination known to occur among *lktA* alleles poses serious problems for phylogeny reconstruction and, despite the strong bootstrap support of the *lktA* phylogeny in Fig. 2, this tree may not represent the true evolutionary history of the *lktA* gene.

In order to better understand the evolutionary history of these alleles, we undertook a more comprehensive phylogenetic analysis of *lktA* sequences that included the sequences obtained in our study as well as isolates obtained by Davies et al. (2001, 2002) from domestic sheep and cattle in the United Kingdom (Fig. 3). These authors had identified 10 allelic variants and proposed hypotheses concerning the series of events that may have given rise to all the allelic variants. A comparison of our expanded phylogeny (Fig. 3) with the previous work of Davies et al. (2001, 2002) found that phylogenies of Figs. 2 and 3 largely agree with the interpretations of the evolutionary history of the gene. All the major allelic groupings are the same as they present in figure 5 of Davies et al. (2002) and related alleles tend to have the same relationships. For example, A1 is close to A10 and this group is closely related to the A4 group. Also, the A7, A8, and A9 alleles group together next to the A5 alleles, and these four alleles are related to most closely to the A2 clade. Furthermore, these groupings correspond well with the sequence of recombination events proposed by Davies et al. (2001). For example, the A10 allele is thought to be derived from a recombination event between A1 and A2, but has the greatest overall similarity to the A1 allele.

The only major difference is the phylogenetic position of the A3 allele (accession number AF314512; Fig. 3), which is at a very different part of the phylogeny than was reported in Fig. 2 of Davies et al. (2002). Based on their Neighbor-joining analysis, the A3 allele was most closely related to the A1 and A10 alleles, although the sequence divergence indicated that the A3 allele was quite different from any of the other known recombinational variants. Interestingly, when re-ran the phylogenetic analysis excluding the I49 *lktA* sequence, the A3 allele grouped again most closely to the A1 and the A10 alleles (data not shown). This result suggests that difference between our results and past results is an effect of sampling bias, a common issue in phylogenetic analysis. The relationship of the A6 allele also differed somewhat from past results, but it was still part of a clade that included alleles A5 through A9.

Given that recombination appears to be happening very frequently among *lktA* sequences, there are probably many more alleles to be discovered, and the discovery of more alleles could further enhance our understanding of *lktA* molecular evolution. Indeed, our phylogenetic analysis uncovered another group of *lktA* variants from four isolates (I47, I26, I17 and I50; Fig. 3) that were not similar to any of the previously discovered alleles. A sliding-window analysis found that that this new allelic group is likely to be a mosaic recombinant of several different alleles. The middle portion of this allele has high similarity to the A3 allele (94% similarity) and the end portion has high similarity to the A7/A9 alleles (∼93–99%).

Although the frequency of *lktA* recombination makes reconstruction the evolutionary history of this gene difficult, it turns out that simple sequence comparisons make HGT inference for our data very straightforward. An analysis of pairwise divergences among *lktA* sequences based on the *lktA* alignment of 913 nucleotides found identical, or nearly identical, *lktA* sequences isolated from two very different bacterial species on two different continents (see Fig. 3 for tree-based divergences). For example, *lktA* sequences from I11, I09, and I07 *P. trehalosi* isolates from Alaskan Dall's sheep and Rocky Mountain bighorns in Alberta, are identical to the *lktA* sequences from two *M. haemolytica* isolates cultured in the UK and a *M. haemolytica* isolated from a domestic sheep in Idaho. Similarly, we found the *lktA* from M. *haemolytica* isolate I43 (isolated from a Washington domestic sheep) to be identical to a *P. trehalosi* isolate from a UK domestic (Fig. 3). Finally, we found *lktA* sequences from *P. trehalosi* isolates I08, I10 and I12 were between 0 and 0.3% divergent to three *lktA* sequence from *M. haemolytica* isolates, one from an Idaho domestic sheep and two from sheep or cattle in the UK (Fig. 3).

Given our results, we suggest that the same kind of recent HGT detected between cattle and sheep in the UK may extend to wild sheep populations on two continents. Since most of the *lktA* alleles we discovered in wild sheep were identical to alleles found in European domestic cattle and sheep, we presume that any HGT of *lktA* to (or from) bacterial species found in wild sheep happened after the introduction of domestic sheep to North America. However, further analysis of *lktA* diversity in wild sheep populations needs to be completed before firm conclusions about the rate and direction of HGT may be drawn.

If HGT has occurred very recently as we suspect, this rapid and recent HGT suggests the leukotoxin operon resides in the midst of a large mobile element, possibly carried on a bacteriophage. Davies et al. (2002) hypothesized that bacteriophage transduction, rather than conjugation and transformation, is the primary mechanism of DNA transfer, but this remains to be determined. The operon may also be part of a plasmid or be flanked by transposable elements (Bushman, 2002; Novick, 2003). Searches of the newly released *Mannheimia haemolytica* genome (strain PHL213, serotype ST1) found the entire *lkt* operon was present on the bacterial chromosome as a single unit (http://www.hgsc.bcm.tmc.edu/projects/microbial/Mhaemolytica/). We also used BLAST to search genomic sequences flanking the operon to detect evidence of phage genes, but did not find any phage-related sequence 40 KB upstream or downstream of the operon. This negative evidence does not rule out phage transduction as a mode of HGT, because the transducing phage may be of an undiscovered variety. At this point, we cannot rule out any possible mechanisms of HGT (transformation of plasmid DNA, phage induction or transposition; Bushman, 2002) and further experimentation will be needed to determine the precise mechanism of transfer.

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