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Sequence Diversity and Molecular Evolution of the Leukotoxin (*lktA*) Gene in Bovine and Ovine Strains of *Mannheimia* (*Pasteurella*) *haemolytica*

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The molecular evolution of the leukotoxin structural gene (*lktA*) of *Mannheimia* (*Pasteurella*) *haemolytica* was investigated by nucleotide sequence comparison of *lktA* in 31 bovine and ovine strains representing the various evolutionary lineages and serotypes of the species. Eight major allelic variants (1.4 to 15.7% nucleotide divergence) were identified; these have mosaic structures of varying degrees of complexity reflecting a history of horizontal gene transfer and extensive intragenic recombination. The presence of identical alleles in strains of different genetic backgrounds suggests that assortative (entire gene) recombination has also contributed to strain diversification in *M. haemolytica*. Five allelic variants occur only in ovine strains and consist of recombinant segments derived from as many as four different sources. Four of these alleles consist of DNA (52.8 to 96.7%) derived from the *lktA* gene of the two related species *Mannheimia glucosida* and *Pasteurella trehalosi*, and four contain recombinant segments derived from an allele that is associated exclusively with bovine or bovine-like serotype A2 strains. The two major lineages of ovine serotype A2 strains possess *lktA* alleles that have very different evolutionary histories and encode divergent leukotoxins (5.3% amino acid divergence), but both contain segments derived from the bovine allele. Homologous segments of donor and recipient alleles are identical or nearly identical, indicating that the recombination events are relatively recent and probably postdate the domestication of cattle and sheep. Our findings suggest that host switching of bovine strains from cattle to sheep, together with inter- and intraspecies recombinational exchanges, has played an important role in generating leukotoxin diversity in ovine strains. In contrast, there is limited allelic diversity of *lktA* in bovine strains, suggesting that transmission of strains from sheep to cattle has been less important in leukotoxin evolution.

Mannheimia haemolytica is the etiological agent of bovine and ovine pneumonic pasteurellosis, a disease that causes considerable economic losses to the cattle and sheep industries (17, 20). Capsular serotyping provides the primary basis for the classification of strains and epidemiological typing of *M. haemolytica*, which has traditionally been subdivided into 13 serotypes (1, 49). The association of different serotypes with infections of cattle and sheep (17, 20) suggests that serotype-related strain differences occur in host specificity and virulence. For example, serotype A1 and A6 strains account for almost all cases of bovine pneumonic pasteurellosis, whereas serotype A2 and A7 isolates are the major causes of disease in sheep. It has also been shown that bovine and ovine isolates of the same serotype, e.g., A1, A2, or A6, can be distinguished by differences in chromosomal genotype (13) or outer membrane protein (OMP) profiles (10). The inference is that natural populations of *M. haemolytica* consist of distinct evolutionary lineages that are differentially adapted to either cattle or sheep (13).

Leukotoxin is a key virulence factor in the pathogenesis of pneumonic pasteurellosis (6, 32, 35, 46, 47). It is a member of the RTX (repeats in toxin) family of gram-negative bacterial cytotoxins, which includes the alpha-hemolysin of *Escherichia coli* (30, 45). Most RTX toxins interact with different cell types from a variety of species, but the cytotoxins produced by *M. haemolytica*, as well as those from *Actinobacillus actinomycetemcomitans* and *Actinobacillus pleuropneumoniae* (ApxIIIA), have both cell type- and species-specific effects. The leukotoxin of *M. haemolytica* interacts only with the alveolar macrophages, neutrophils, and lymphocytes of ruminants and is believed to promote bacterial proliferation by killing or incapacitating these cells (4, 7, 23, 40). It has been postulated that leukotoxin target cell specificity underlies the host specificity of *M. haemolytica* infections, and it has recently been demonstrated that β_2 integrins are the putative leukotoxin receptors (2, 22, 28). Restriction endonuclease analysis (5), together with studies on the neutralizing activity of monoclonal antibodies (19), has demonstrated interserotypic variation of the *M. haemolytica* leukotoxin determinants, but the significance of allelic diversity for the pathogenesis of pneumonic pasteurellosis and for host specificity is not known.

The purpose of the present study was to investigate nucleotide sequence variation in the *M. haemolytica* leukotoxins and to determine how this variation relates to differences in virulence and host specialization. Horizontal DNA transfer and recombination are now recognized as important evolutionary mechanisms, complementing mutation, in the diversification of molecules involved in virulence, such as those encoding cell surface structures and other macromolecules for which there is

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an adaptive advantage in structural diversity (8, 15, 24, 29, 36, 44). Here we used an established framework of evolutionary relationships among strains of *M. haemolytica* (13) to study the molecular evolution of the leukotoxin (*lktA*) gene and to determine the role of horizontal DNA transfer and recombination in leukotoxin evolution.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The complete *lktA* gene was sequenced in 31 *M. haemolytica*, 6 *Mannheimia glucosida*, and 4 *Pasteurella trehalosi* isolates. *M. glucosida* represents serotype A11 strains of *P. haemolytica*, which have recently been reclassified as a separate species (3), and *P. trehalosi* was recognized as the T biotype of *P. haemolytica* until its reclassification (43). Partial *lktA* sequences were obtained for a further 13 serotype A2 *M. haemolytica* isolates of bovine and ovine origin. The 54 isolates have been well characterized in previous studies (10–14) and were chosen to represent selected evolutionary lineages, serotypes, and hosts of origin. Properties of these isolates are presented in Table 1.

Bacteria that had been stored at −70°C in 50% (vol/vol) glycerol in brain heart infusion broth (BHIB) were subcultured on blood agar (brain heart infusion agar containing 5% [vol/vol] sheep's blood) and incubated aerobically overnight at 37°C. For preparation of DNA, a few colonies were inoculated into 5-ml volumes of BHIB and grown overnight at 37°C at 120 rpm.

Preparation of DNA. Cells from 0.5 ml of overnight cultures were harvested by centrifugation for 1 min at 13,000 × *g* and washed once in sterile distilled H_2O. DNA was prepared with the InstaGene Matrix (Bio-Rad) according to the manufacturer's instructions and stored at −20°C.

PCR amplification and DNA sequence analysis. *M. haemolytica* strains have been shown to possess only one *lktA* gene (5), and a direct PCR approach was adopted. The complete coding and flanking regions of the *lktA* gene were amplified with a *Taq* DNA polymerase kit (Boehringer Mannheim) according to the manufacturer's instructions. PCR error rates were shown to be insignificant by the complete sequence identity of duplicate amplifications of the *lktA* gene in isolate PH278. The *lktA* gene was amplified from the chromosomal DNA with the 5′ primer lktA9 (5′-TCAAGAAGAGCTGGCAAC-3′) and the 3′ primer lktA7 (5′-AGTGAGGGCAACTAAACC-3′). The primers were designed from the published sequences for the *lktA* genes of serotype A1 (21, 30) and A11 (5) isolates. Primer lktA9 corresponds to residues 53 to 70 upstream of the *lktA* initiation codon; primer lktA7 corresponds to residues 105 to 122 downstream of the *lktA* termination codon. Primers were designed using the Primer Designer (version 2.0) computer program and synthesized with a Beckman Oligo 1000 DNA synthesizer. PCRs were carried out in a Perkin-Elmer 480 DNA thermal cycler using the following amplification parameters: denaturation at 94°C for 45 s, annealing at 62°C for 45 s, and extension at 72°C for 2 min. Thirty cycles were performed, and a final extension step of 72°C for 10 min was used. Production of a PCR amplicon of the expected size (~3 kbp) was confirmed by agarose gel electrophoresis, and the DNA was purified with a QIAquick PCR purification kit (Qiagen, Chatsworth, Calif.). The DNA was finally eluted in 30 μl of sterile distilled H_2O and stored at −20°C. Sequence reactions were performed with the ABI Prism Dye Terminator Cycle Sequencing Kit (Perkin-Elmer), and sequence analysis was performed with an Applied Biosystems 373A DNA Sequencer. Both strands of the *lktA* gene were sequenced with seven internal pairs of primers designed as sequence data became available.

Analysis of nucleotide and protein sequence data. Nucleotide sequence data were analyzed and edited with the SEQED (version 1.0.3) computer program (Perkin-Elmer Applied Biosystems). Statistical and phylogenetic analyses were carried out with MEGA (25) in conjunction with alignment programs written by T.S.W. Statistical analyses for clustering of polymorphic sites were carried out by the maximum chi-square method (42) with a program written by T.S.W. Predictions of hydrophilicity, hydrophobicity, antigenic index, and surface probability of protein sequences were performed with the PROTEAN program (DNASTAR Inc.).

Nucleotide sequence accession numbers. The GenBank accession numbers for the *lktA* gene sequences obtained in this study are given in Table 1.

RESULTS

Nucleotide and amino acid sequence variation. The complete nucleotide sequence of the *lktA* gene was determined for 31 isolates of *M. haemolytica* representing 12 capsular serotypes and the 22 electrophoretic types (ETs) previously defined by multilocus enzyme electrophoresis (MLEE) (13). The *lktA* gene was also sequenced for six isolates representing different ETs of *M. glucosida* (13) and for four isolates that each represent one of the capsular serotypes (T3, T4, T10, and T15) of *P. trehalosi* (14). Part of the *lktA* gene was also sequenced for an additional 13 serotype A2 isolates of *M. haemolytica* recovered from cattle and sheep in order to identify the allele type. In this case, the first 700 nucleotides at both the 5′ and 3′ ends of the gene were analyzed. Properties of these isolates are shown in Table 1.

With the exception of four strains, the *lktA* genes of the *M. haemolytica* and *M. glucosida* isolates were 2,859 bp in length (953 amino acids); the *lktA* genes of *M. haemolytica* isolates PH202, PH494, and PH550 were 2,862 bp in length due to an additional amino acid (lysine) at position 885, whereas that of the *M. glucosida* isolate PH274 was 2,838 bp in length due to the deletion of 7 amino acids at positions 29 to 35. The *lktA* genes of the four *P. trehalosi* isolates were 2,865 bp in length (955 amino acids) due to two amino acid insertions between positions 7 and 23. The total aligned length (including gaps) of the 43 sequences was 2,868 nucleotides.

Twenty-four unique *lktA* sequences, representing distinct alleles, were identified among the 41 isolates for which complete sequences were obtained, but, based on overall sequence similarity, their characteristic mosaic structures (see below), and species of origin, these were assigned to one of 10 groups of allelic variants designated *lktA1* to *lktA10*. *M. haemolytica* was represented by allelic groups *lktA1* to *lktA3* and *lktA6* to *lktA10*, *M. glucosida* by *lktA4*, and *P. trehalosi* by *lktA5*. There were 740 (25.8%) polymorphic nucleotide sites and 177 (18.5%) variable inferred amino acid positions among the 24 sequences. Pairwise differences in nucleotide and inferred amino acid sequences between representative pairs of the 10 *lktA* allele types of *M. haemolytica*, *M. glucosida*, and *P. trehalosi* ranged from 39 to 485 nucleotide sites (1.4 to 17.0%) and 3 to 122 amino acid positions (0.3 to 12.7%) (Table 2).

Whereas there was a relatively high degree of nucleotide variation between most of the allelic groups *lktA1* to *lktA10* (Table 2), there was, in contrast, a low degree of variation among alleles representing each group, particularly the *M. haemolytica* groups. For example, *lktA1* was represented by 14 sequences that could be divided into five subgroups, alleles *lktA1.1* to *lktA1.5*, on the basis of variation at just two synonymous and two nonsynonymous sites (nucleotides 993, 1263, 1967, and 2521); *lktA2* was represented by alleles *lktA2.1* and *lktA2.2*, which differed at a single synonymous site (nucleotide 729); and *lktA8* was represented by alleles *lktA8.1* and *lktA8.2*, which differed at a single nonsynonymous site (nucleotide 425). The *lktA* gene of the six *M. glucosida* isolates was represented by alleles *lktA4.1* to *lktA4.6*, which had 58 polymorphic nucleotide sites, and the *lktA* gene of the four *P. trehalosi* isolates was represented by alleles *lktA5.1* to *lktA5.4*, which had 20 polymorphic nucleotide sites. Most of the variation in the *M. glucosida* and *P. trehalosi* isolates occurred in alleles *lktA4.3*, *lktA4.4*, and *lktA5.4*.

Association of *lktA* alleles with evolutionary lineages and serotypes of *M. haemolytica* and the host species of origin. The association of *lktA* alleles with evolutionary lineages (represented by ETs) and serotypes of *M. haemolytica*, together with

TABLE 1. Properties of 44 *M. haemolytica*, 6 *M. glucosida*, and 4 *P. trehalosi* isolates

Isolate	ET[a]	Capsular serotype	Host species	*lktA* allele[b]	GenBank accession no.
M. haemolytica					
PH2	1	A1	Bovine	*lktA1.1*	AF314503
PH30	1	A1	Bovine	*lktA1.1*	
PH376	1	A6	Bovine	*lktA1.1*	
PH346	1	A12	Ovine	*lktA1.2*	
PH540	2	A1	Bovine	*lktA1.1*	
PH338	3	A9	Ovine	*lktA1.2*	
PH388	4	A7	Ovine	*lktA1.3*	AF314504
PH50	5	A5	Ovine	*lktA1.2*	
PH56	5	A8	Ovine	*lktA1.4*	AF314505
PH238	5	A9	Ovine	*lktA1.4*	
PH8	6	A1	Ovine	*lktA1.5*	AF314506
PH398	7	A1	Ovine	*lktA1.5*	
PH284	8	A6	Ovine	*lktA1.2*	AF314507
PH232	9	A6	Ovine	*lktA1.5*	
PH66	10	A14	Ovine	*lktA9*	AF314508
PH706	11	A16	Ovine	*lktA7*	AF314509
PH296	12	A7	Ovine	*lktA8.1*	
PH396	13	A7	Ovine	*lktA8.1*	
PH484	14	A7	Ovine	*lktA8.1*	
PH588	15	A13	Ovine	*lktA6*	AF314510
PH494	16	A2	Ovine	*lktA2.1*	AF314511
PH672	16	A2	Ovine	*lktA2**	
PH550	17	A2	Bovine	*lktA2.1*	
PH294	17	A2	Bovine	*lktA2**	
PH758	17	A2	Bovine	*lktA2**	
PH196	18	A2	Bovine	*lktA3*	AF314512
PH786	18	A2	Bovine	*lktA3*	
PH526	19	A2	Ovine	*lktA8.1*	
PH598	20	A2	Ovine	*lktA8.1*	
PH202	21	A2	Bovine	*lktA2.2*	AF314513
PH210	21	A2	Bovine	*lktA2**	
PH470	21	A2	Bovine	*lktA2**	
PH546	21	A2	Bovine	*lktA2**	
PH278	21	A2	Ovine	*lktA10.1*	AF314514
PH372	21	A2	Ovine	*lktA10.1*	
PH380	21	A2	Ovine	*lktA10**	
PH486	21	A2	Ovine	*lktA10**	
PH536	21	A2	Ovine	*lktA10**	
PH576	21	A2	Ovine	*lktA10**	
PH714	21	A2	Ovine	*lktA10**	
PH292	22	A2	Ovine	*lktA8.1*	AF314515
PH392	22	A2	Ovine	*lktA8.2*	AF314516
PH358	22	A2	Ovine	*lktA8**	
PH384	22	A2	Ovine	*lktA8**	
M. glucosida					
PH344	1	A11	Ovine	*lktA4.1*	AF314517
PH498	3	A11	Ovine	*lktA4.2*	AF314518
PH240	5	A11	Ovine	*lktA4.3*	AF314519
PH496	7	UG3	Ovine	*lktA4.4*	AF314520
PH574	10	UG3	Ovine	*lktA4.5*	AF314521
PH290	16	UG3	Ovine	*lktA4.6*	AF314522
P. trehalosi					
PH246	2	T4	Ovine	*lktA5.1*	AF314523
PH252	4	T10	Ovine	*lktA5.2*	AF314524
PH254	15	T15	Ovine	*lktA5.3*	AF314525
PH68	19	T3	Ovine	*lktA5.4*	AF314526

[a] See references 13 and 14.
[b] Asterisks indicate partially sequenced *lktA* genes.

the host species of origin, is shown in Fig. 1. There were three principal findings, which are summarized below. First, *lktA1* type alleles were associated exclusively with strains representing ETs of lineage A. However, allele *lktA1.1* occurred only in bovine serotype A1 and A6 strains of ETs 1 and 2, whereas alleles *lktA1.2* to *lktA1.5* were present in ovine isolates of seven serotypes representing eight ETs. Second, *lktA2* type alleles occurred in serotype A2 strains of ETs 16, 17, and 21. With the

TABLE 2. Percent differences in nucleotide and amino acid sequences between representative pairs of the 10 *lktA* allele types of *M. haemolytica*, *M. glucosida*, and *P. trehalosi*

Allele	% Pairwise differences in nucleotide and amino acid sequences[a]									
	lktA1.1	*lktA2.1*	*lktA3*	*lktA4.1*	*lktA5.1*	*lktA6*	*lktA7*	*lktA8.1*	*lktA9*	*lktA10.1*
lktA1.1		14.5	7.5	7.9	15.4	13.9	12.7	13.4	12.7	1.5
lktA2.1	11.6		14.6	10.9	17.0	11.8	11.4	7.0	10.1	13.0
lktA3	3.4	7.8		13.0	16.8	15.7	15.4	15.4	15.4	9.0
lktA4.1	6.9	6.9	9.5		16.0	8.1	6.7	8.8	6.6	7.0
lktA5.1	11.1	12.0	11.6	11.4		9.7	10.6	11.1	11.2	15.9
lktA6	11.4	7.1	12.0	5.7	7.1		1.8	6.0	3.1	12.5
lktA7	10.5	6.7	12.2	4.5	8.0	1.5		4.5	1.4	11.8
lktA8.1	10.8	5.4	12.7	5.4	8.1	2.8	1.4		3.1	12.4
lktA9	10.5	6.4	12.4	4.5	8.1	1.8	0.3	1.0		11.8
lktA10.1	1.7	10.1	5.5	5.7	11.8	9.9	9.4	9.8	9.4	

[a] Values on the upper right represent pairwise differences in nucleotide sequences (percent polymorphic nucleotide sites), and values on the lower left represent pairwise differences in inferred amino acid sequences (percent variable amino acids).

exception of two strains of ET 16, all isolates possessing this allele type were of bovine origin. The uncommon allele *lktA3* was similarly associated only with bovine strains of ET 18. Third, the recombinant alleles *lktA6* to *lktA10* (see below) were associated with ovine strains having a wide range of genetic diversity. Alleles *lktA6*, *lktA7*, and *lktA9* occurred in serotype A13, A16, and A14 strains representing ETs 15, 11, and 10, respectively; *lktA8* type alleles were more widely distributed among serotype A2 isolates of ETs 19, 20, and 22 and among serotype A7 isolates of ETs 12 to 14; and allele *lktA10* was associated with serotype A2 isolates representing ET 21.

Intragenic recombination. Comparison of the distribution of polymorphic nucleotide sites among *lktA* alleles representing each of the 10 allelic groups revealed the presence of mosaic structures of varying degrees of complexity. Thus, for pairs of alleles, certain regions of the gene were identical, or nearly so, in sequence whereas adjacent regions of the same alleles were very different. The maximum chi-square method (42) was used

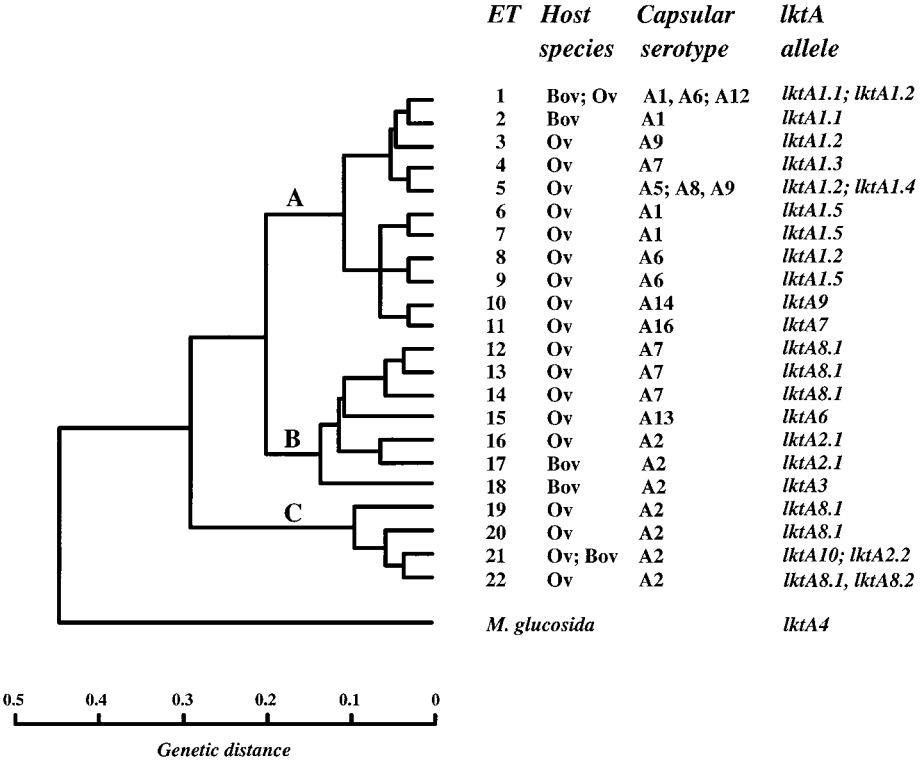

FIG. 1. Association of *lktA* alleles with evolutionary lineage, capsular serotype, and host species of origin. The dendrogram shows the genetic relationships of ETs of *M. haemolytica* and was generated by the UPGMA method of clustering from a matrix of coefficients of pairwise genetic distances based on 18 enzyme loci (11). For MLEE data, genetic distance is defined as the number of detectable codon changes per locus (39). Three lineages, identified at a genetic distance of 0.28, are indicated by the letters A, B, and C. Bov, bovine; Ov, ovine.

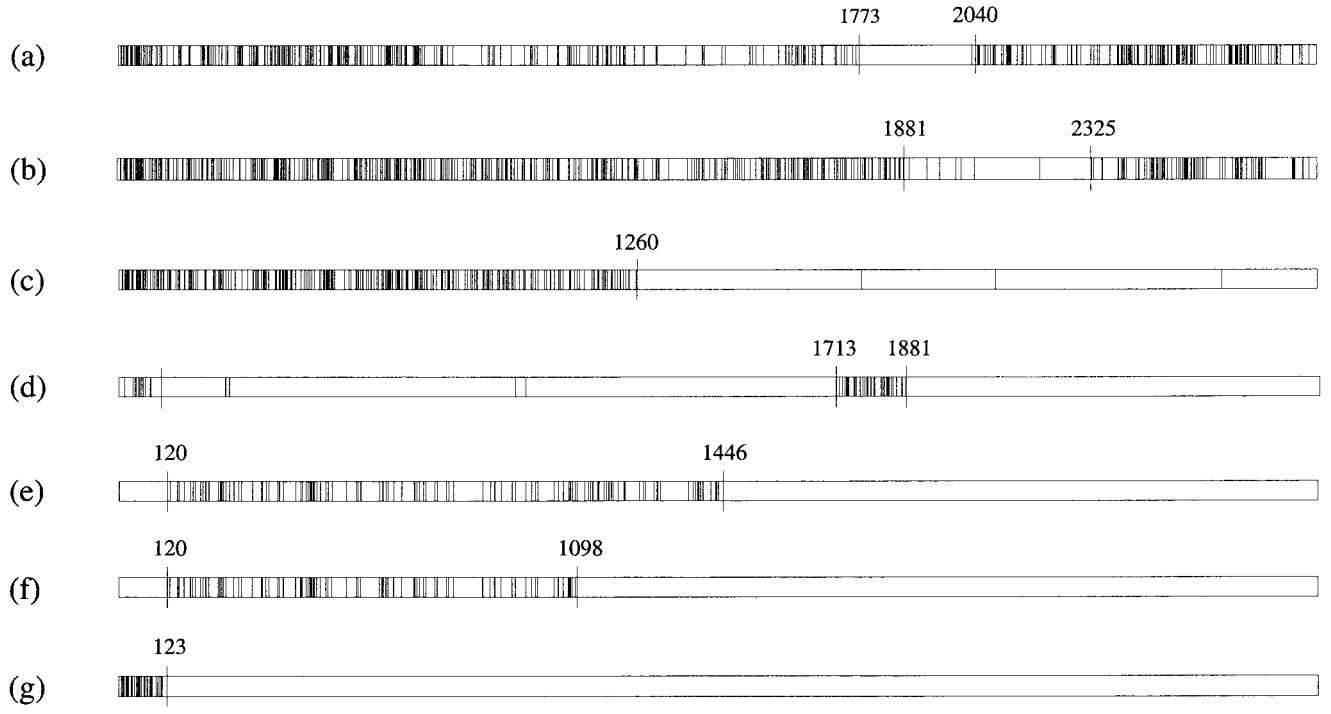

FIG. 2. Maximum chi-square comparisons for the following pairs of alleles: (a) *lktA1.1* versus *lktA2.1*, (b) *lktA4.1* versus *lktA5.1*, (c) *lktA5.1* versus *lktA6*, (d) *lktA6* versus *lktA7*, (e) *lktA7* versus *lktA8.1*, (f) *lktA8.1* versus *lktA9*, and (g) *lktA1.3* versus *lktA10*. Vertical lines represent polymorphic nucleotide sites, and numbered nucleotides indicate positions where the chi-square value was a maximum (i.e., k_{max}) with respect to the partitions on the left and right. These positions mark the endpoints of partitions that represent recombinant segments. The probability that the expected maximum chi-square value in 1,000 randomly generated data sets was greater than the observed value for the real data was <0.05.

to make pairwise comparisons of sequences representing each allelic group, identify statistically significant clusters of polymorphic nucleotide sites, and determine the endpoints of recombinant segments (Fig. 2). On the basis of these analyses, the mosaic structures of single alleles representative of each group are illustrated schematically in Fig. 3.

lktA1 and *lktA2* type alleles are 14.4 to 14.5% divergent but have a common 267-bp recombinant segment (nucleotides 1774 to 2040 [Fig. 2]) that is identical in sequence in all alleles (except *lktA1.5*, which differs at one nucleotide site). Allele *lktA3* consists of an upstream 1,392-bp segment (nucleotides 1 to 1392) that is most similar to the corresponding region of *lktA1* type alleles (2.8 to 2.9% divergence) and a downstream segment (nucleotides 1393 to 2868) that differs from the corresponding region of *lktA1* type alleles at ~12.0% of the nucleotides. The downstream section also differs substantially from the corresponding regions of *lktA2* (13.7% divergence), *lktA4* (11.6 to 12.5%), and *lktA5* (16.1 to 16.5%) alleles.

lktA4 and *lktA5* type alleles are 15.2 to 15.9% divergent overall but have a common 444-bp segment (nucleotides 1882 to 2325 [Fig. 2]) that differs at only ~1.0 to 2.0% of the sites; because it is present in all alleles, this segment presumably represents an early recombinational exchange (see Discussion). The *M. glucosida lktA4.3* allele is 15.4% divergent with respect to the *P. trehalosi* alleles *lktA5.1* to *lktA5.3* but has a 111-bp segment (nucleotides 2599 to 2709 [data not shown]) that exhibits 100% identity (*lktA5.3* differs at one site) with the corresponding region of the *P. trehalosi* alleles. This segment accounts for most of the diversity of the *M. glucosida lktA4.3* allele and probably represents a recombinant segment derived from *P. trehalosi*. The maximum chi-square analysis also identified a significant partition between *lktA1* and *lktA4* alleles at nucleotide position 732 (data not shown). The upstream segments vary at 168 nucleotide sites (23.0% divergence), whereas the downstream regions differ at only 51 to 69 sites (2.4 to 3.2% divergence), between these two groups of alleles. These data clearly indicate different evolutionary origins for the upstream and downstream segments of one or both allele types.

Alleles *lktA6* to *lktA10* have mosaic structures and consist of two to four distinct segments that have complete, or almost complete, identity with the corresponding regions of *lktA1*, *lktA2*, *lktA4*, and *lktA5* type alleles (Fig. 3). The most probable explanation for the structures of alleles *lktA6* to *lktA10* is that they have been derived from alleles of types *lktA1*, *lktA2*, *lktA4*, and *lktA5* in a series of sequential intragenic recombinational exchanges. A model for the sequence of events leading to the formation of these alleles is proposed and discussed in further detail below. Comparison of pairs of alleles by the maximum chi-square method (Fig. 2) clearly demonstrates the complete, or almost complete, identity of homologous regions of donor and recipient alleles as well as the endpoints of recombinant segments.

Synonymous and nonsynonymous substitutions. Visual inspection of the aligned amino acid sequences representing alleles *lktA1*, *lktA2*, *lktA4*, and *lktA5* indicated that the distribution of polymorphic amino acid sites is nonrandom, because

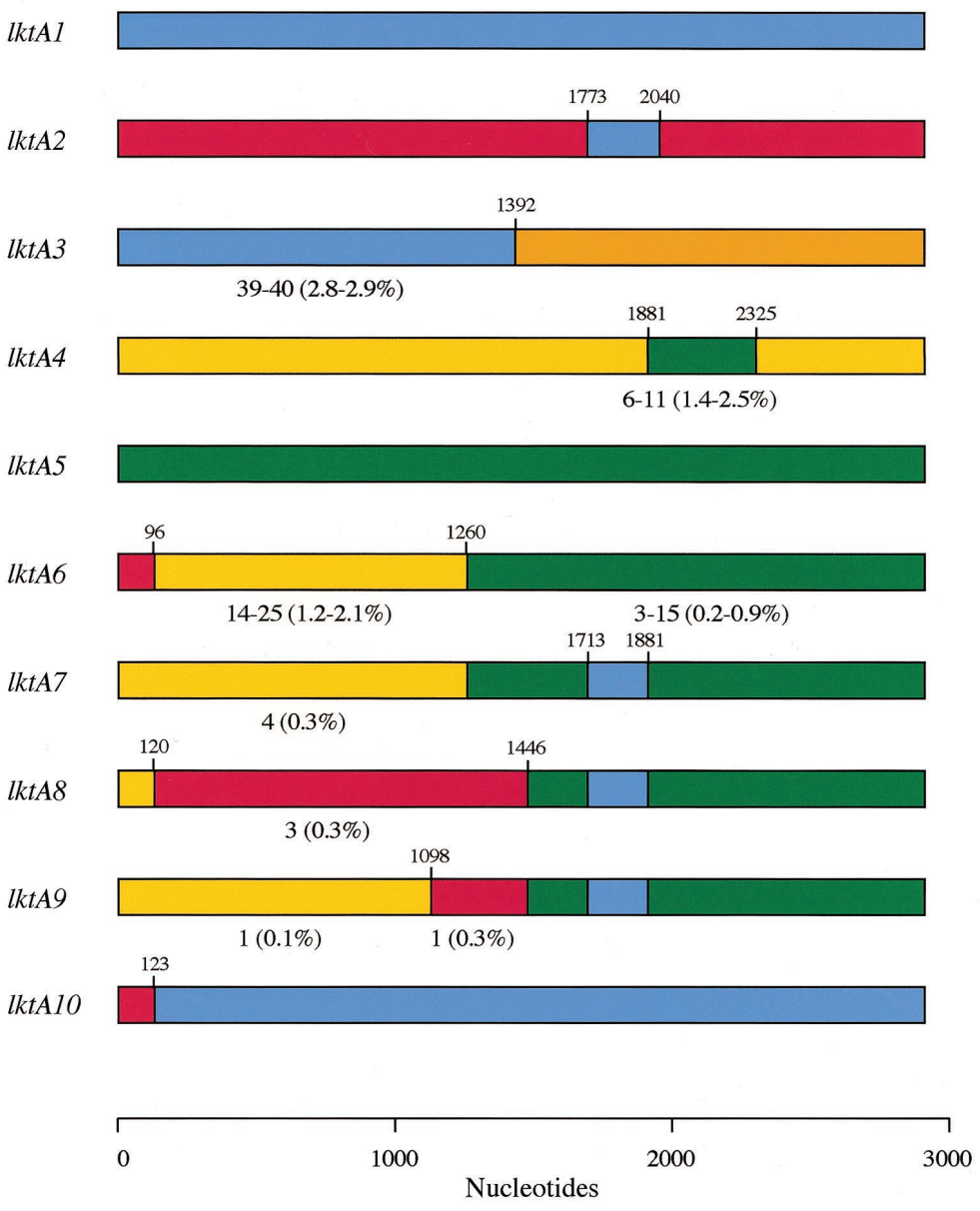

FIG. 3. Schematic representation of the mosaic structures of alleles representative of the major allelic groups *lktA1* to *lktA10*. The different colors indicate sequence identity and the likely origins of recombinant segments. The number of sites different from those in the corresponding region of the likely donor allele(s) (and the degree of divergence) are indicated below certain recombinant segments (see the text). All other segments exhibited 100% sequence identity to the corresponding regions of the donor alleles. Numbers above the proposed recombination sites indicate the position of the last nucleotide at the downstream end of the recombinant segment.

well-defined regions of amino acid conservation and heterogeneity occur throughout the leukotoxin molecule (Table 3). The numbers of synonymous substitutions per synonymous site (d_S) and nonsynonymous substitutions per nonsynonymous site (d_N) were estimated for the combined variable and conserved regions, and the d_S/d_N ratios were calculated. The d_S values for the variable (0.8269 ± 0.0800) and conserved (0.5724 ± 0.0322) regions were not significantly different, but the d_N value for the variable regions (0.1887 ± 0.0150) was an order of magnitude larger than that for the conserved regions (0.0214 ± 0.0026). The d_S/d_N ratios for the variable and conserved regions were 4.4 and 26.7, respectively, and provide evidence of strong selective constraint against amino acid replacement in the conserved regions of the gene and relaxed constraint in the variable regions.

To examine in further detail how the level of selective constraint varies along the leukotoxin molecule, the proportions of synonymous substitutions per synonymous site (p_S) and nonsynonymous substitutions per nonsynonymous site (p_N) were calculated for subsets of 30 codons in a sliding window for the length of the gene (Fig. 4). The synonymous substitution rate was, overall, much higher than the nonsynonymous substitution rate, indicating evolutionary constraint of amino acid replacement. However, synonymous substitution rates were very

TABLE 3. Amino acid variation in variable and conserved domains of leukotoxins encoded by alleles *lktA1.1*, *lktA2.1*, *lktA4.1*, and *lktA5.1*

Variable domains		Conserved domains	
Codons	No. of variable sites/total no. of sites (%)	Codons	No. of variable sites/total no. of sites (%)
1–38	21/38 (55.3)	39–51	1/13 (7.7)
52–60	6/9 (66.7)	61–79	0/19 (0)
80–100	8/21 (38.1)	101–120	1/20 (5.0)
121–139	12/19 (63.2)	140–165	1/26 (3.8)
166–172	6/7 (85.7)	173–196	1/24 (4.2)
197–238	17/42 (40.5)	239–791	43/553 (7.8)
792–807	7/16 (43.8)	808–823	0/16 (0)
824–861	14/38 (36.8)	862–880	0/19 (0)
881–913	18/33 (54.5)	914–956	6/43 (14.0)
Total	109/223 (48.9)	Total	53/733 (7.2)

low in the regions representing codons 433 to 452 and 628 to 668. In the case of codons 628 to 668, this represents the overlapping region (nucleotides 1882 to 2040) of the recombinant segments of alleles *lktA1* and *lktA2* (nucleotides 1774 to 2040) and *lktA4* and *lktA5* (nucleotides 1882 to 2325) (see Fig. 3). Six distinct peaks in p_N towards the N- and C-terminal ends of the molecule, corresponding to codons 1 to 38, 121 to 139, 197 to 238, 792 to 807, 824 to 861, and 881 to 913 (Table 3), indicate regions of relaxed constraint of amino acid replacement.

Relationship between degree of amino acid variation and hydrophilicity-hydrophobicity. Kyte-Doolittle hydrophilicity-hydrophobicity profiles for leukotoxins encoded by *lktA1*, *lktA2*, *lktA4*, and *lktA5* type alleles were remarkably similar (data not shown). The N-terminal half of the molecule (amino acids 1 to 400) consists of a series of hydrophobic and hydrophilic domains, and comparison of the plots with the data given in Table 3 revealed that hydrophobic domains representing codons 41 to 48, 65 to 72, 141 to 158, 172 to 190, 227 to 251, 262 to 318, and 355 to 401 correspond to regions of conserved amino acid sequence, whereas hydrophilic domains representing codons 1 to 40, 49 to 64, 73 to 86, 113 to 140, 159 to 171, and 191 to 226 correspond to regions of variable amino acid sequence. The hydrophobic domains also have low predicted antigenic indices and surface probabilities (data not shown).

DISCUSSION

The *lktA* gene of *M. haemolytica* is highly diverse and is represented by at least eight major allelic variants with a complex evolutionary history. These allelic variants have mosaic structures of varying degrees of complexity that reflect a history of extensive intragenic recombination. The frequency and sites of the recombinational exchanges are possibly related to the presence of chi sequences represented by "hot spots" of recombination at nucleotide positions 96 to 123 (alleles *lktA6*, *lktA8*, and *lktA10*), 1446 (*lktA8* and *lktA9*), and 1881 (*lktA4* and *lktA7*). The complete, or nearly complete, identity of homolo-

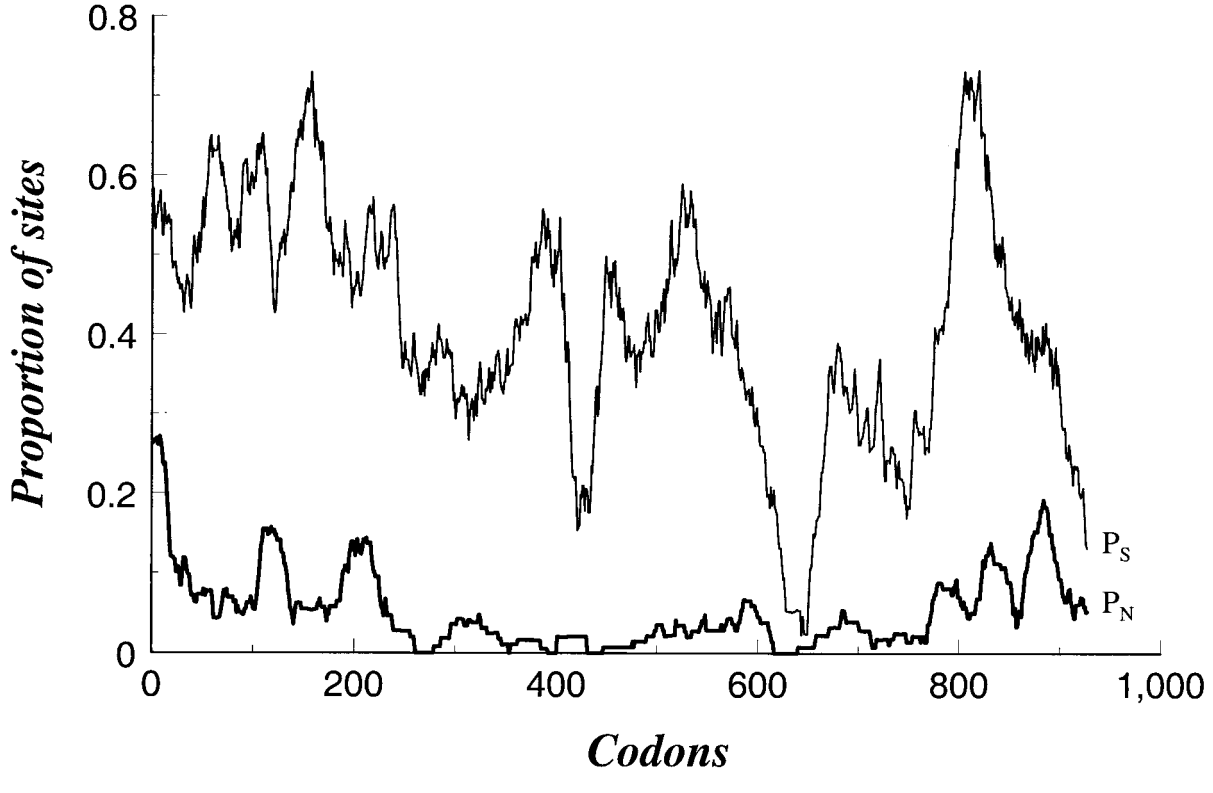

FIG. 4. Variation in frequency of synonymous and nonsynonymous nucleotide substitutions along the length of the *lktA* gene among alleles *lktA1*, *lktA2*, *lktA4*, and *lktA5*. p_S and p_N were calculated for subsets of 30 codons in a sliding window for the length of the gene.

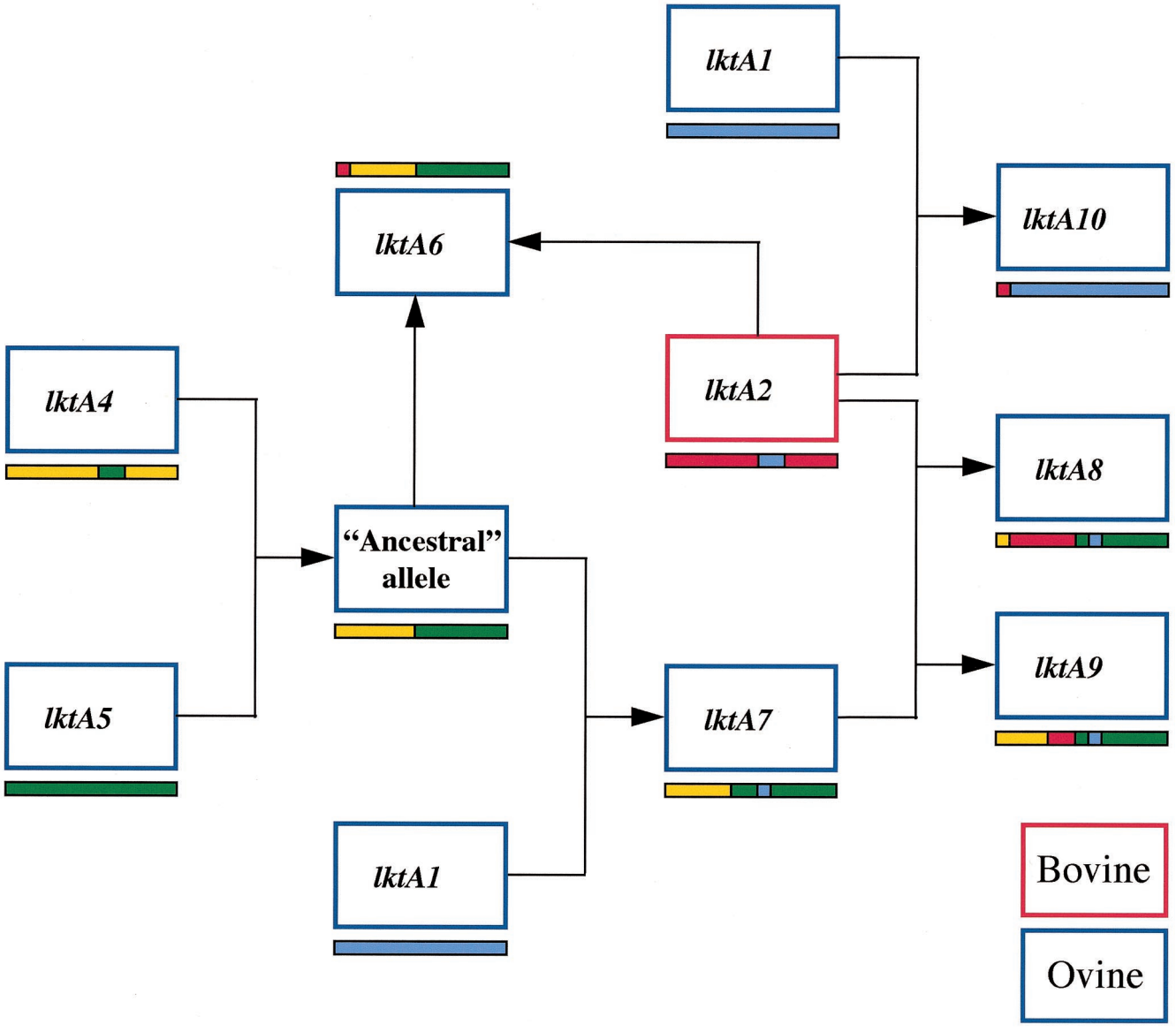

FIG. 5. Proposed sequence of recombination events in the evolution of *lktA* leading to the formation of *lktA8* and *lktA10* type alleles in the ovine-specific lineages represented by ETs 12 to 14 and 19 to 22. The central role of the bovine *lktA2* allele in the evolution of ovine alleles *lktA6*, *lktA8*, *lktA9*, and *lktA10* is clearly seen. The mosaic structures of the alleles are as shown in Fig. 3.

gous segments in donor and recipient alleles suggests that most of these recombination events occurred relatively recently and probably postdate the domestication of cattle and sheep; this applies to alleles *lktA6* to *lktA10* in particular. Additional evidence for a recent origin is provided by the fact that each allelic variant is represented by only a small number of alleles which exhibit very little interallelic diversity.

In addition to intragenic recombination, assortative (entire-gene) recombination has also contributed to genetic diversity in *M. haemolytica*. For example, the presence of *lktA8* alleles in genetically diverse strains representing six ETs (Fig. 1) is clearly due to horizontal gene transfer, because the formation of identical recombinant alleles by convergent evolution in different lineages is highly unlikely. The pattern of sequence diversity described here clearly accounts for the seven variants of the *lktA* gene previously identified by restriction endonuclease analysis (5).

Origin of the mosaic alleles *lktA6* to *lktA10*. The majority (5 of 8) of the *M. haemolytica* alleles, namely *lktA6* to *lktA10*, consist of two to four distinct segments that are identical or nearly identical to segments from *lktA1*, *lktA2*, *lktA4*, and *lktA5* type alleles (Fig. 3). To account for the complex mosaic structure of *lktA* alleles, we propose an evolutionary model that posits that new alleles have been created by a series of gene transfers and recombination events (Fig. 5). We hypothesize that these alleles have been formed by a sequential series of intragenic recombinational exchanges culminating in the formation of alleles *lktA8* and *lktA9*. A model representing the proposed sequence of events leading to the formation of alleles *lktA6* to *lktA10* is shown in Fig. 5. High percentages of the

DNA contents of alleles *lktA6* to *lktA9* (96.7, 94.1, 52.8, and 87.8%, respectively) originate from *M. glucosida* and *P. trehalosi* alleles of types *lktA4* and *lktA5* (Fig. 3). Strong support for a common origin of alleles *lktA6* to *lktA9* is provided by the fact that homologous segments have identical, or nearly identical, nucleotide sequences. The simplest explanation for this finding is that alleles *lktA6* to *lktA9* are derived from an ancestral allele that itself was formed by a recombination event involving *lktA4* and *lktA5* type alleles (Fig. 5). Such recombination events between these two species are not uncommon, because two examples of intragenic recombinational exchanges were discovered in the present study.

Allele *lktA6* could subsequently have been formed by the incorporation of a 96-bp segment (nucleotides 1 to 96) from a *lktA2* type allele into the ancestral allele, and allele *lktA7* could have been formed by the independent incorporation of a 168-bp segment (nucleotides 1714 to 1881) from a *lktA1* type allele (Fig. 5). *lktA8* could have been formed by the incorporation of a 1,326-bp segment (nucleotides 121 to 1446) from a *lktA2* type allele into *lktA7* (Fig. 5); in *lktA8.1* this segment differs from the corresponding region of *lktA2.1* at only three nucleotide sites, whereas the rest of *lktA8.1* is identical to *lktA7*. There are two possible explanations for the formation of *lktA9*. Either the 348-bp segment (nucleotides 1099 to 1446) could have been incorporated into allele *lktA7* from a *lktA2* type allele, or the 1,098-bp segment (nucleotides 1 to 1098) could have been incorporated into a *lktA8* type allele from allele *lktA7* or a *lktA4* type allele. We favor the first event (Fig. 5) because the genetic backgrounds of strains carrying *lktA7* and *lktA9* alleles are very similar, whereas the genetic backgrounds of strains carrying *lktA8* and *lktA9* alleles are different (Fig. 1). The 348-bp segment (nucleotides 1099 to 1446) of allele *lktA9* differs from the corresponding region of *lktA2.1* at a single nucleotide position; the remainder of *lktA9* differs from allele *lktA7* also at only a single nucleotide position. Finally, *lktA10* consists of a 123-bp upstream segment derived from a *lktA2* type allele and a 2,745-bp downstream segment (nucleotides 124 to 2868) that is identical to the corresponding region of allele *lktA1.3*. Since the genetic background of strains containing *lktA10* is similar to that of strains possessing allele *lktA2.2* (Fig. 1), it is most likely that the donor strain was a serotype A7 strain containing allele *lktA1.3* and that the recipient was a serotype A2 strain containing *lktA2.2*.

The finding that large proportions of *M. haemolytica* alleles have been derived from *M. glucosida* and *P. trehalosi* alleles was unexpected because, as well as being different species, *M. glucosida* and *P. trehalosi* differ from *M. haemolytica* in their pathobiology. *M. glucosida* represents a genetically diverse group of opportunistic sheep pathogens of low virulence (3, 13), whereas *P. trehalosi* is responsible for a systemic infection of sheep that is pathologically distinct from pneumonic pasteurellosis (20). It has also been shown that both of these species have low leukotoxic activities (37), suggesting that leukotoxin is likely to be a less important virulence determinant in *M. glucosida* and *P. trehalosi* than it is in *M. haemolytica*. Thus, recombinant leukotoxins have evolved in pathogenic ovine lineages of *M. haemolytica* from the *lktA* genes of two species in which leukotoxin probably has a less important role in infection.

It is clear that *lktA2*-type alleles have played a central role in leukotoxin evolution (Fig. 5). However, comparison of the nucleotide sequence of *lktA2* alleles with those of *lktA1*, *lktA4*, and *lktA5* alleles (Table 2) indicates that *lktA2* alleles are more divergent from *M. haemolytica lktA1* alleles (14.5% divergence) than are *M. glucosida lktA4* alleles (7.9%) and are almost as divergent as *P. trehalosi lktA5* alleles (15.4%). These data suggest that the bovine *lktA2* alleles were originally acquired by horizontal DNA transfer from a species more distantly related to *M. haemolytica* than is *M. glucosida*. Therefore, the recombinant alleles *lktA6* to *lktA10* have been derived from as many as three, and possibly four, different species, namely, *M. haemolytica* (*lktA1*), *M. glucosida* (*lktA4*), *P. trehalosi* (*lktA5*), and an unknown species (*lktA2*).

Host switching of bovine serotype A2 strains to sheep has led to the evolution of new recombinant alleles in ovine strains. Recombinational exchanges involving *lktA2* alleles have played a significant role in leukotoxin evolution, as evidenced by the fact that *lktA2*-derived segments are present in *lktA6*, *lktA8*, *lktA9*, and *lktA10* alleles (Fig. 5). The following evidence suggests that the latter four alleles have evolved as a consequence of host switching of serotype A2 strains from cattle to sheep. First, *lktA2* alleles are associated only with bovine or bovine-like serotype A2 strains (Fig. 1). Partial sequence analysis of the *lktA* gene from a wider range of serotype A2 strains confirmed that, with two exceptions, *lktA2* type alleles occur only in bovine strains of ETs 17 and 21 (Table 1). Although two exceptional *lktA2*-possessing serotype A2 isolates (ET 16 [Fig. 1]) were isolated from sheep, these strains possess OMP profiles characteristic of bovine isolates (10). Furthermore, recent sequence analysis of the OMP *pomA* gene (50) from bovine and ovine isolates (R. L. Davies, unpublished data) has confirmed that these two isolates are related to bovine and not ovine serotype A2 strains. Therefore, we suspect that the two ET 16 isolates are not true ovine-adapted strains but instead represent a bovine-adapted clone that recently spread to sheep. Second, *lktA4* and *lktA5* type alleles are present in the species *M. glucosida* and *P. trehalosi*, respectively, bacteria that occur only in sheep (3, 13, 20). In support of this, recombinant segments derived from *lktA4* and *lktA5* type alleles were not identified in any of the alleles associated with bovine strains. Third, the recombinant alleles *lktA6* to *lktA10* are associated only with ovine strains of serotypes A2, A7, A13, A14, and A16; with the exception of A2, none of these serotypes are known to occur in cattle (17). Partial sequence analysis of the *lktA* gene from additional serotype A2 strains confirmed that *lktA8* and *lktA10* alleles occur only in ovine isolates of ETs 19 to 22 (Table 1).

The distribution of alleles among bovine and ovine strains suggests that the recombinational exchanges leading to the formation of *lktA6* to *lktA10* alleles could not have occurred in cattle. It follows that the formation of *lktA6*, *lktA8*, *lktA9*, and *lktA10* alleles can be satisfactorily explained only by host switching of *lktA2*-containing serotype A2 strains from cattle to sheep and subsequent recombinational exchanges involving ovine strains. If sheep, rather than cattle, were the ancestral hosts of *lktA2*-containing serotype A2 strains, we would expect to isolate a higher number of such strains from sheep and, assuming random transmission, to recover *lktA8*- and *lktA10*-containing serotype A2 strains from cattle—but this is not the case. Therefore, transmission of strains from cattle to sheep,

together with horizontal DNA transfer and recombination, has resulted in the evolution of new ovine *lktA* alleles and an increase in leukotoxin diversity. In contrast, leukotoxin diversity is much lower in bovine strains than in ovine isolates, a finding that parallels the greater overall diversity of ovine strains and suggests limited transmission of strains from sheep to cattle.

Molecular evolution and leukotoxin structure. The leukotoxin molecule of *M. haemolytica* consists of well-defined regions of conservation and heterogeneity which correspond to different functional domains (9, 16, 26, 31, 33, 45, 48). In particular, there are three highly conserved regions that are common to all RTX toxins (45, 48). The N-terminal half of the molecule consists of a series of hydrophobic putative membrane-spanning domains, separated by hydrophilic regions, that are involved in pore formation; the hydrophobic domains are followed by a second region of approximately 200 amino acids, rich in β-turns, that is involved in cell binding and LktC-mediated toxin activation; the third conserved region consists of amino acids 733 to 786 in *M. haemolytica* and forms six glycine-rich tandem repeat domains that are involved in Ca^{2+} binding.

The synonymous substitution rate of the *lktA* gene is, overall, much higher than the nonsynonymous substitution rate, suggesting that amino acid replacement is subject to selective constraint. However, the patterns of synonymous and nonsynonymous substitution rates vary throughout the gene, indicating that differing selective pressures are operating on different regions of the protein. For example, amino acid replacement is highly constrained within the conserved regions, but amino acid constraint is more relaxed in the variable regions, particularly in the six domains located towards the C- and N-terminal ends of the molecule (Fig. 4). In the pore-forming N-terminal half of the molecule (amino acids 1 to 400), the degree of evolutionary constraint on amino acid replacement correlates with leukotoxin structure inasmuch as hydrophobic domains are generally conserved in amino acid sequence whereas hydrophilic domains exhibit heterogeneity in amino acid sequence. The conserved, hydrophobic domains have a low surface probability and, most likely, represent membrane-spanning regions involved in pore formation (16, 31, 45), whereas the variable, hydrophilic domains probably represent surface-exposed regions of lesser structural importance. Similarly, in the central part of the molecule that corresponds to the cell binding, toxin activation, and Ca^{2+}-binding domains (9, 33, 48), there is a high degree of evolutionary constraint on amino acid replacement that is reflected in a low nonsynonymous substitution rate compared to the synonymous substitution rate (Fig. 4). Despite the presence of substantial allelic diversity and extensive amino acid variation (Table 2) among different leukotoxins, the remarkable similarity in hydrophilicity and hydrophobicity profiles suggests that selective pressure is operating to maintain overall leukotoxin structure.

Although single-site mutational changes are uncommon among *M. haemolytica* alleles, indirect evidence suggests that a single amino acid substitution could be involved in leukotoxin adaptation to the bovine host. Replacement of G at nucleotide position 2521 in alleles *lktA1.2* to *lktA1.5* with A in allele *lktA1.1* has resulted in an amino acid change from aspartic acid to asparagine at position 841. *lktA1.1*-encoded leukotoxin is associated exclusively with genetically related bovine serotype A1 and A6 strains of ETs 1 and 2, whereas alleles *lktA1.2* to *lktA1.5* are present only in ovine strains (Fig. 1). In addition, asparagine is present at position 841 in the *lktA2*-encoded leukotoxin of bovine A2 strains. These findings suggest that asparagine at position 841 provides a selective advantage to leukotoxin function in the bovine host and that *lktA1.1* emerged in bovine strains by mutation and selection for this amino acid. Although this amino acid substitution occurs in that part of the molecule known to be involved in receptor binding and specificity, i.e., flanking the glycine-rich repeat region (27), the precise effect and significance of the change remain to be determined.

Recombination has been shown to play a role in the generation of antigenic variation in different surface antigens of a number of pathogens (8, 15, 29, 38) and is thought to be an adaptation to the host immune response. Since leukotoxin is an important virulence determinant and is involved in host immunity (18, 34, 41), it is likely that recombination of the *lktA* gene provides an adaptive advantage against the host antibody response by generating antigenic variation. Therefore, recombination produces new variants that may have an advantage in fitness either within hosts (cattle or sheep), with an enhanced ability to avoid the immune response, or between hosts, with an increased chance of spreading against the effects of herd immunity. The most antigenically diverse leukotoxins are those encoded by alleles *lktA6* and *lktA8* in that they contain variable domains from three different sources, including segments from the bovine *lktA2* alleles. It is reasonable to assume that the occurrence of *lktA8* type alleles in different lineages and their association with a high proportion of ovine disease isolates might be due to a selective advantage resulting from the antigenic diversity of the encoded leukotoxin.

lktA8 and *lktA10* type alleles are representative of the two major lineages of ovine serotype A2 strains, ETs 22 and 21, respectively, which are responsible for a high proportion of ovine disease (13). These alleles have very different evolutionary histories and encode divergent leukotoxins (5.3% amino acid divergence), but both contain segments derived from bovine *lktA2* alleles. The occurrence of two different leukotoxin types in A2 strains has important implications for vaccine design and disease prevention in sheep because leukotoxin is a key component of some vaccines (26). Furthermore, the recent evolutionary origin of these two leukotoxins suggests that new, immunologically distinct molecules could evolve in the future. Finally, the presence of *lktA10* alleles in ovine A2 strains of the same genetic background as *lktA2*-containing bovine A2 strains provides a clue about the possible origins of the ovine A2 lineages. It is interesting to speculate that these evolved from bovine A2 strains as a consequence of host switching and acquisition of specific genes necessary for adaptation to the ovine environment. Our findings for the *lktA* gene have wider implications for our understanding of the role of host switching in the evolution of virulence genes and in the emergence of new pathogens not only in *M. haemolytica* but also in other members of the *Pasteurellaceae*.

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