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Extensive Homology between the Leukotoxin of *Pasteurella haemolytica* A1 and the Alpha-Hemolysin of *Escherichia coli*

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The 19.8- and 101.9-kilodalton leukotoxin proteins of *Pasteurella haemolytica* (LKTC and LKTA, respectively) share extensive homology with the HLYC and HLYA alpha-hemolysin proteins of *Escherichia coli*. The leukotoxin LKTA protein cross-reacts with hemolysin-specific antisera in Western blot (immunoblot) analysis, indicating that it shares epitopes with the alpha-hemolysin HLYA protein. Both LKTA and HLYA contain a conserved hydrophobic region, as well as a set of tandemly repeated domains. These features have been implicated in the lytic function of the alpha-hemolysin.

Bovine pneumonic pasteurellosis, also known as shipping fever, is the major cause of economic loss in the feedlot cattle industry in North America (16, 24). The principal microorganism associated with the disease is the bacterium *Pasteurella haemolytica* A1. Live *P. haemolytica* secretes a cytotoxin specific for ruminant leukocytes (10, 20). This leukotoxin may contribute to the pathogenesis of the disease by impairing the primary lung defenses and the subsequent immune response or by inducing inflammation as a result of leukocyte lysis (1, 9, 20). The leukotoxin has been studied extensively in recent years in an attempt to resolve its role as a potential virulence factor. However, efforts to physically purify and analyze the toxin have not provided clear results (1, 9, 17). Through the successful application of recombinant DNA technology, the toxin was cloned into *Escherichia coli* (13) and the nucleotide sequence of its coding regions was determined; two proteins with molecular masses of 19.8 and 101.9 kilodaltons (kDa) were found to be essential for leukotoxin activity (14). By using the nucleotide sequence of these coding regions as a probe, a search of the GenBank genetic database (by using the IBI/Pustell programs [19]) identified extensive homology with the *E. coli* chromosomal alpha-hemolysin genes *hlyC* and *hlyA*, which code for the 20-kDa HLYC protein and the 107-kDa HLYA protein, respectively (5). By using their deduced amino acid sequences, extensive homology with the leukotoxin proteins was observed. As a reflection of these homologies, we proposed that the 19.8-kDa leukotoxin protein and the 101.9-kDa leukotoxin protein be designated LKTC and LKTA, respectively (14).

The homologies between the leukotoxin and the hemolysin can be demonstrated by cross-reaction with specific antisera. A Western blot (immunoblot) analysis of the cloned leukotoxin as expressed in *E. coli* and resolved through sodium dodecyl sulfate-polyacrylamide gel electrophoresis is shown in Fig. 1. Briefly, *E. coli* HB101 containing either the plasmid vector pBR322 (lanes a) or pLKT5 (a recombinant plasmid expressing the leukotoxin genes [14]) (lanes b) was grown to late logarithmic phase in LT broth. Cells from a 1-ml portion were collected by centrifugation and lysed by boiling for 5 min in 0.1 ml of sodium dodecyl sulfate sample buffer (13). After electrophoresis through a sodium dodecyl sulfate–7.5% polyacrylamide gel and transfer to nitrocellulose (13), the proteins were probed with antisera raised against the culture supernatants of either *P. haemolytica* (13) or *E. coli* (R. Welch, personal communication), designated anti-LKT and anti-AH antisera, respectively. The 100-kDa LKTA leukotoxin protein can be detected with either antiserum, indicating that LKTA and HLYA must share some common epitopes. The differences in intensity between the two blots is a reflection of the number of epitopes not shared by the proteins. Interestingly, we have not been able to demonstrate leukotoxin neutralization with the hemolysin antisera, suggesting that the shared epitopes either are not involved directly in cell lysis or, alternatively, are not accessible on the surface of the protein. The hydropathy profiles of each of the proteins show that both pairs of proteins are remarkably similar throughout all regions (Fig. 2). Two hydrophobic regions present in both LKTA and HLYA (indicated by the bars in Fig. 2) are features which have been implicated in the toxic activities of the proteins. In the case of the hemolysin, amino acid substitutions which disrupt the integrity of these regions give rise to a nonfunctional toxin (15).

A direct comparison of the amino acid sequences of the leukotoxin and the chromosomal hemolysin proteins is shown in Fig. 3. We introduced a limited number of breaks into each sequence to optimize existing homologies. LKTC and HLYC differ in length by only three residues, share 84 identical amino acids (50.3% homology), and are continuously homologous relative to their lengths. In contrast to this pattern, LKTA and HLYA are striking examples of discontinuously homologous proteins. Although differing in length by 70 residues and sharing only 347 amino acids (36.4% homology), the proteins are highly homologous over a number of distinct regions, as indicated by the vertical segmented lines in Fig. 3. In particular, the region centered around amino acid residue 750 shows a distinct pattern of tandemly repeated amino acid domains (marked by horizontal unbroken lines for both LKTA and HLYA) (23). There are 6 of these domains in LKTA and 11 in HLYA. Each domain contains nine amino acid residues and is a variation of the following sequence: -1-Gly-Gly-2-Gly-(Asp/Asn)-Asp-3-(Ile/Leu)-, where 1, 2, and 3 represent amino acid residues which vary among the domains. A detailed analysis of the occurrence of the amino acid residues throughout the domains for both LKTA and HLYA is shown in Tables 1 and 2. Although not indicated here, the HLYA protein from the pHly152-encoded hemolysin determinant contains a series of domains essentially identical to those described for the

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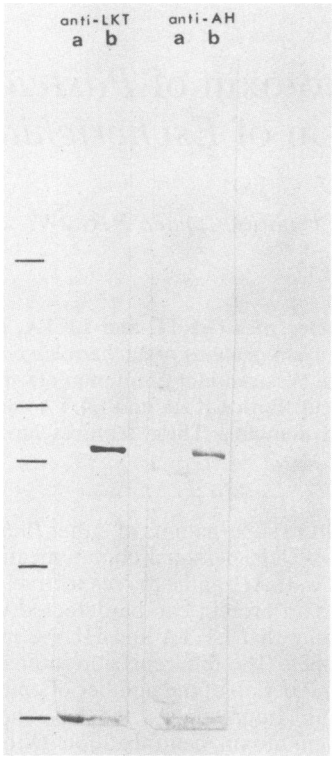

FIG. 1. Western blot of the cloned *P. haemolytica* leukotoxin as expressed in *E. coli*. Duplicate blots were probed with leukotoxin-specific antisera (anti-LKT) or alpha-hemolysin-specific antisera (anti-AH), as indicated. Lanes: a, *E. coli*(pBR322); b, *E. coli*(pLKT5) (14). Molecular mass markers are from Bio-Rad Laboratories and are 200, 116, 92.5, 66, and 45 kDa, respectively. The LKTA protein is clearly visible at ca. 100 kDa; some minor cross-reacting *E. coli* proteins not relevant to this analysis were also detected by using the anti-AH antiserum.

chromosomal hemolysin (8). Frameshift mutations in the *hlyA* gene which lead to a loss of these repeated domains give rise to a nonsecreted and nonfunctional toxin (T. A. Felmlee, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, D92, p. 87). It is apparent that these conserved domains must play some significant but as yet unidentified role in the activity of the proteins.

TABLE 1. Occurrence of amino acid residues within the repeated domains of LKTA and HLYA[a]

Domain	Residues	
	LKTA[b]	HLYA
1	NGG DGV DT I	HG ADG DDH I
2	DGN DGN DR L	EG NDG NDR L
3	FGG KGD D I L	YG DKG NDT L
4	DGG NGD DF I	SG GNG DDQ L
5	DGG KGN DL L	YG GDG NDK L
6	HGG KGD D I F	I GGAG NNY L
7		NG GDG DDE L
8		SG GKG NDK L
9		YG S EG ADL L
10		DG GEG NDL L
11		KG GYG ND I Y

[a] The domains are aligned to show the occurrence of identical amino acids at particular positions. Six of the positions are highly conserved, whereas the other three are variable.

[b] Residues given correspond to positions 733 to 786 for LKTA and to positions 740 to 850 for HLYA, as shown in Fig. 3.

A model of the potential secondary structure adopted by the domains as they occur in LKTA is presented in Fig. 4. There is no evidence of any alpha-helix- or beta-pleated-sheet-forming elements in the region, although there are amino acid residues with high turn potentials present. The high glycine content (31.5% in LKTA and 35.1% in HLYA) must impart considerable flexibility to the region, perhaps allowing for a porelike structure to be formed in target cell membranes (2). Interestingly, there are almost twice as many domains in HLYA as there are in LKTA. There is one "gap" in HLYA in which a twelfth domain could be located. This suggests that a duplication event could account for the difference in the number of repeated domains between LKTA and HLYA. If a break is introduced into LKTA to account for such a duplication, then the carboxy termini of the two proteins nearly coincide (as aligned in Fig. 3).

This datum lends strong support to the hypothesis that the toxins have homologous activities and share similar functional domains. It has been suggested that the hemolysin acts by forming a single transmembrane pore in its target cells (2), which include leukocytes and renal tubular cells, as well as erythrocytes (6, 21). The leukotoxin is more limited in its

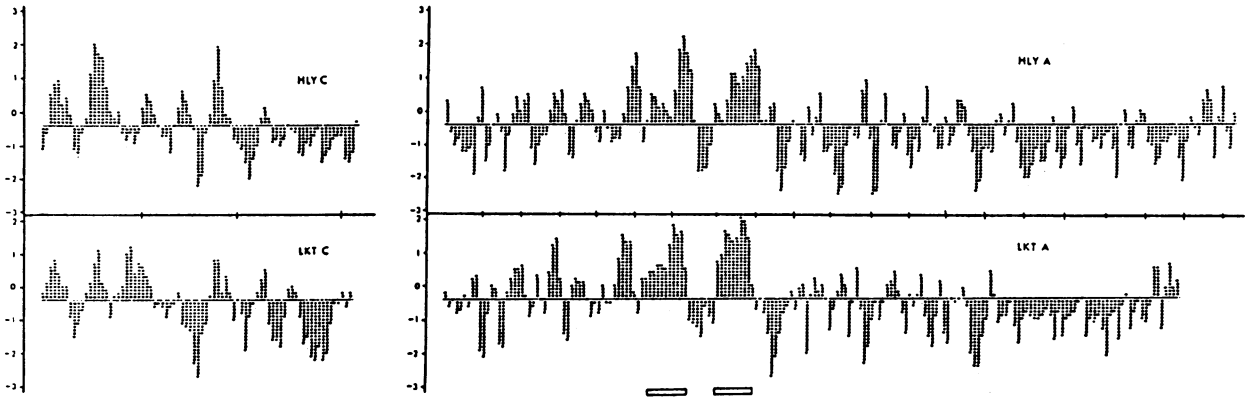

FIG. 2. Hydropathy profiles of the leukotoxin and alpha-hemolysin proteins. The method used is that of Kyte and Doolittle (12). The vertical axes give the scale of the hydrophobic (positive) and hydrophilic (negative) values established for each window of nine residues. The horizontal axes give the scale for each pair of proteins and are delineated at intervals of 50 residues. The open boxes indicate large hydrophobic domains in LKTA and HLYA.

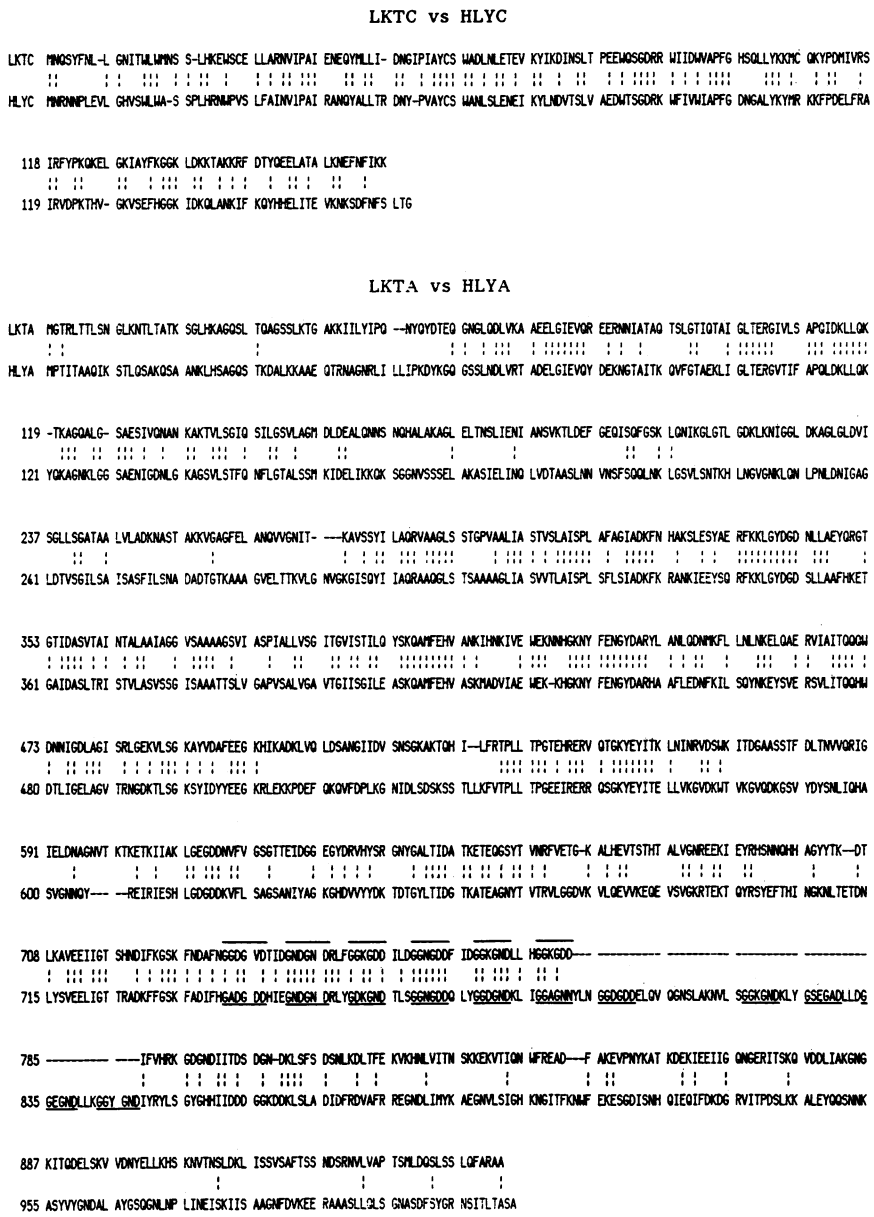

FIG. 3. Homology between the leukotoxin and the alpha-hemolysin. The amino acid sequences of the LKTC, HLYC, LKTA, and HLYA proteins as inferred from the DNA sequences of their respective coding regions are presented (5, 14). The sequences are numbered along the left margin. The segmented vertical lines show the locations of identical residues. The segmented horizontal lines in each sequence represent breaks introduced to maximize homology. The horizontal unbroken lines indicate the repeated domains described in the text.

TABLE 2. Consensus sequence of the domains shown in Table 1 and frequency of amino acid residues present at each of the conserved positions[a]

Protein	Amino acid frequency at position:								
	1	G	G	2	G	(N/D)	D	3	(I/L)
LKTA		6/6	5/6		6/6	5/6	6/6		5/6
HLYA		11/11	7/11		11/11	10/11	10/11		10/11

[a] The variable positions are designated 1, 2, and 3. At position 1, three of the six amino acid residues in LKTA are aspartic acid, whereas the majority are polar in HLYA (5). Amino acid residues at position 2 tend to be charged (either positive or negative), whereas those at position 3 have a low turn potential.

range of target cells, which are restricted to ruminant lymphocytes (9, 20). Both toxins have been implicated as major virulence factors (3, 20–22).

The significant homologies between the toxins also suggest a common origin for the genes involved. Because the hemolysin determinant has been found in chromosomal as well as in extrachromosomal locations among various virulent *E. coli* isolates, it has been suggested that the genes involved are part of a genetically mobile element (7). Indeed, insertionlike sequences flanking the genes have been found to account for this phenomenon (25). On the basis of codon preference plots and base composition studies, it has been further postulated that the hemolysin genes were fortuitously acquired by *E. coli* from an as yet unidentified donor

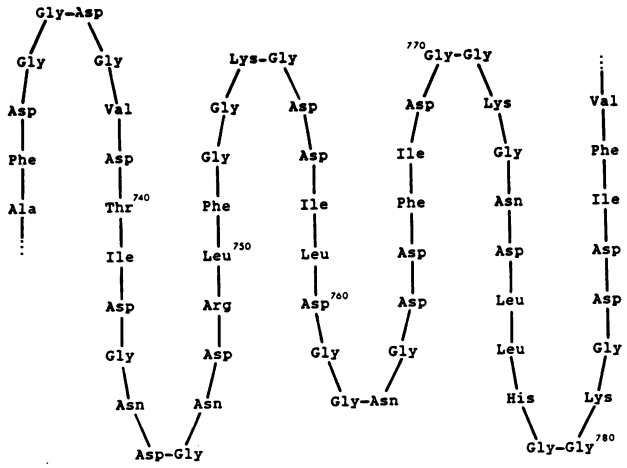

FIG. 4. Predicted secondary structure adopted by the repeated domains of LKTA. Shown is a 57-residue sequence spanning residues 731 through 787. The model is numbered on the basis of the protein sequence given in Fig. 3. Predictions are based on the conformational rules established by Chou and Fasman (4).

(5). Recently, related hemolysin determinants were identified in the genomes of *Proteus mirabilis*, *Proteus vulgaris*, and *Morganella morganii*, and a close genetic relationship with the *E. coli* determinant was confirmed (11, 21). The genomic G + C contents of *Proteus mirabilis* and *Proteus vulgaris* (11), as well as that of *P. haemolytica* (18), are close to that of the leukotoxin and hemolysin determinants at 39% (5, 14), indicating that the determinant could have originated in and spread from any of these species. However, the origin of these determinants cannot be ascertained until a more-extensive analysis of other pathogenic species has been completed to discern the extent to which this determinant has been disseminated.

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