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LLNL-JRNL-420969

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December 3, 2009

Infection, Genetics and Evolution

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Amino acid and structural variability of *Yersinia pestis* LcrV protein

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Running title: LcrV polymorphism in *Yersinia pestis*

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Abstract

The LcrV protein is a multifunctional virulence factor and protective antigen of the plague bacterium which is generally conserved between the epidemic strains of *Yersinia pestis*. We investigated the diversity in the LcrV sequences among non-epidemic *Y. pestis* strains which have a limited virulence in selected animal models and for humans. Sequencing of *lcrV* genes from ten *Y. pestis* strains belonging to different phylogenetic groups ("subspecies") showed that the LcrV proteins possess four major variable hotspots at positions 18, 72, 273, and 324-326. These major variations, together with other minor substitutions in amino acid sequences, allowed us to classify the LcrV alleles into five sequence types (A-E). We observed that the strains of different *Y. pestis* "subspecies" can have the same type of LcrV, and different types of LcrV can exist within the same natural plague focus. The LcrV polymorphisms were structurally analyzed by comparing the modeled structures of LcrV from all available strains. All changes except one occurred either in flexible regions or on the surface of the protein, but local chemical properties (i.e. those of a hydrophobic, hydrophilic, amphipathic, or charged nature) were conserved the across all of the strains. Polymorphisms in flexible and surface regions are likely subject to less selective pressure, and have a limited impact on the structure. In contrast, the substitution of tryptophan at position 113 with either glutamic acid or glycine likely has a serious influence on the regional structure of the protein, and these mutations might have an effect on the function of LcrV. The polymorphisms at positions 18, 72 and 273 were accountable for differences in oligomerization of LcrV. The importance of the latter property in emergence of epidemic strains of *Y. pestis* during evolution of this pathogen will need to be further investigated.

***Keywords*:** LcrV, *Yersinia pestis*, polymorphism, 3D structure, modeling, oligomerization

Introduction

Plague is an acute and highly lethal disease caused by *Yersinia pestis*. Based on the ability to ferment glycerol and to reduce nitrate, *Y. pestis* strains can be assigned to the biovars antiqua, medievalis and orientalis, which are thought to be responsible for three major plague pandemics [Devignat, 1951 #124][Perry, 1997 #594]. In addition, there is a group of atypical isolates of *Y. pestis*, circulating in populations of different species of voles (*Microtus* spp.), which have not been associated with epidemics of human plague thus far. These non-epidemic strains which currently include eight "subspecies" [Li, 2009 #1469] that were originally named Pestoides [Martinevsky, 1969 #916], and in contrast to the most of epidemic-causing isolates, are capable of fermenting rhamnose and melibiose [Anisimov, 2004 #917]; however, five of "subspecies" have lost the ability to ferment arabinose [Zhou, 2004 #1201][Zhou, 2004 #1116][Li, 2009 #1469]. In addition, some of these strains are sensitive to pesticin 1 of *Y. pestis*, and, generally have significantly reduced virulence in guinea pigs as well as in rabbits, rhesus monkeys, sheep, and humans [Anisimov, 2004 #917][Zhou, 2004 #1116]. Soviet Union researches recommended that strains of this group, specifically *altaica*, *caucasica, hissarica*, *ulegeica*, and *talassica*, were to be classified into a "subspecies" of *Y. pestis* and named according to the areas in which they were isolated [Anisimov, 2004 #917]. Recently, Chinese researchers described similar strains isolated in China and proposed to assign

rhamnose-positive and arabinose-negative isolates into the biovar microtus, because the main rodent host for these strains in the natural plague reservoirs is a vole (*Microtus* genus) [Zhou, 2004 #1116][Zhou, 2004 #1145]. More recently it has been proposed to include all non-epidemic rhamnose-positive variants into biovar microtus as *Y. pestis* subspecies *altaica*, *caucasica, hissarica*, *ulegeica*, *talassica*, *xilingolensis, qinghaiensis* and *angola* [Li, 2009 #1469]. In this paper we use the term "microtus" as a synonym for "Pestoides". The microtus strains form a separate phylogenetic group when compared with the epidemic strains of other biovars by different methods of genotyping [Motin, 2002 #653][Achtman, 2004 #1094][Bobrov, 2006 #1200][Vergnaud, 2007 #1160][Li, 2009 #1469]. Two strains of this biovar have been sequenced and shown to have a unique genomic profile of gene loss and pseudogene distribution, which most likely accounts for the human attenuation of this new biovar [Song, 2004 #924][Zhou, 2004 #1145][Garcia, 2007 #1134].

LcrV (V antigen) is an essential virulence factor and protective antigen of *Yersinia* spp., which was discovered more than 50 years ago [Burrows, 1956 #4]. This protein is attributed to have a variety of immunomodulatory effects on the host [Nakajima, 1995 #27][Nedialkov, 1997 #187][Motin, 1997 #86][Welkos, 1998 #465][Sing, 2002 #649][Sing, 2002 #735][Sing, 2003 #867][Brubaker, 2003 #814][Reithmeier-Rost, 2004 #1097][Sing, 2005 #1005][Overheim, 2005 #1034][Sharma, 2005 #1190][Heesemann, 2006 #1187][Abramov, 2007 #1098][Foligne, 2007 #1154][Reithmeier-Rost, 2007 #1173][Depaolo, 2008 #1403], and indirectly regulates expression and secretion of type 3 secretion system (T3SS) virulence factors Yops [Bergman, 1991 #3][Price, 1991 #10][Skrzypek, 1995 #11][Nilles, 1997 #185][Nilles, 1998 #369][Matson, 2001 #611], as

well as being involved in their translocation to eukaryotic cells [Sarker, 1998 #321][Pettersson, 1999 #527][Roggenkamp, 1999 #1159][Lee, 2000 #612][DeBord, 2001 #1161][Holmstrom, 2001 #1162][Weeks, 2002 #1169][Broms, 2003 #1164][Marenne, 2003 #833][Mueller, 2005 #1155][Cowan, 2005 #1156][Philipovskiy, 2005 #1058][Goure, 2005 #1166][Mota, 2006 #1163][Broms, 2007 #1150]. Recently, the crystal structure of this multifunctional protein has been resolved, and it was revealed that LcrV is a dumbbell-like molecule with two globular domains on either end separated by a coiled-coil motif that is uncommon in bacterial proteins [Derewenda, 2004 #1093].

The sequence of the *lcrV* gene is generally conserved between the epidemic strains of *Y. pestis*; however, sequencing of a few representatives of the microtus biovar revealed a surprising polymorphism in this virulence factor [Motin, 1992 #177][Adair, 2000 #1115] [Song, 2004 #924][Garcia, 2007 #1134]. In this study we sequenced *lcrV* genes from ten strains of *Y. pestis*, eight of each belonged to the microtus strains of *Y. pestis* isolated in the territory of the Former Soviet Union (FSU) and Mongolia. The polymorphism in amino acid sequences of LcrV was used to evaluate the possible influence of these variations on the three-dimensional structure of the protein and functional activity of this major virulence factor of *Y. pestis*.

(A part of this work was presented at the 9th International Symposium on *Yersinia*, October 10-14, 2006, in Lexington, Kentucky, USA) [Anisimov, 2007 #1152].

Materials and methods

Bacterial strains

The strains of *Y. pestis* used in this study as a source of *lcrV* genes represented five "subspecies" circulating in the Eurasian natural plague foci and differed in their epidemiological significance [Anisimov, 2004 #917]. Two isolates (I-1996 and I-2638) were the epidemic-type strains of the biovar antiqua, while other isolates belonged to the group of atypical strains (Table 1).

Determination of nucleotide sequence of *lcrV* genes

The nucleotide sequence of each *lcrV* gene was determined by the direct sequencing of the PCR fragment obtained after amplification of the part of the *lcrGVH* operon of the corresponding strain. The primers LcrVF (5'-CAGCCTCAACATCCCTACGA-3') and LcrVR (5'-TGTCTGTCGTCTCTTGTTGC-3'), both flanking the *lcrV* gene, were located within the *lcrG* and *lcrH* genes, respectively. The additional primer LcrVFI (5'-GCAAAATGGCATCAAGCGAG-3') resided inside the *lcrV* gene. Determined sequences of the *lcrV* genes were deposited to the GenBank (see accession numbers in the Table 1) and compared with the available sequences of this gene from other *Y. pestis* strains (strain KIM, medievalis biovar, accession no. M26405; strain CO92, orientalis biovar, accession no. AL117189, strain Pestoides F, likely subspecies *caucasica* [Motin, 2002 #653], accession no. AF167309; atypical strain Angola (subspecies *angola*) [Motin,

2002 #653][Li, 2009 #1469], accession no. AF167310; strain 91001, microtus biovar (subspecies *xilingolensis*) [Li, 2009 #1469], accession no. AE017043; strain Antiqua, antiqua biovar, accession no. CP000311). *Y. pestis* strains and DNA isolates are from the State Research Center for Applied Microbiology and Biotechnology, Obolensk (Moscow Region, Russia) or were kindly provided by Prof. S. V. Balakhonov (Antiplague Research Institute of Siberia and Far East, Irkutsk, Russia).

LcrV expression and purification

The *lcrV* gene of the strains Angola, KIM and Pestoides F were amplified by using a forward primer, GCGGGATCCATTAGAGCCTACGAACAAAACCCAC, and reverse primer, CGGAATTCTCATTTACCAGACGTGTCATCTAGCA, containing BamHI and EcoRI sites, respectively. The PCR fragment was cloned into expression vector pRSET A (Invitrogen, Carlsbad, CA) digested by BamHI and EcoRI that resulted in a construct containing a fusion of the *lcrV* with the vector-encoded N-terminal His-Tag and the leader sequence of T7 gene 10 under control of T7 promoter. This N-terminal tail increased the molecular weight of the native LcrV protein by 3.9 kDa. After sequence of *lcrV* was verified, the constructs were transformed to *E. coli* BL21 (DE3) host. Expression and purification of recombinant protein was performed essentially as described by us previously [Motin, 1996 #1]. Purified proteins were analyzed by using SDS-PAGE followed by silver stain or immunoblot with the monoclonal antibody to LcrV [Brubaker, 1987 #24][Motin, 1994 #32]. Protein sample buffer contained a reducing agent dithiothreitol (DTT) at concentration of 100 mM.

Modeling of LcrV

The crystal structure of LcrV deposited in the Protein Data Bank (PDB) under the code 1R6F [Derewenda, 2004 #1093] was used as a structural template for modeling the LcrV sequence from *Y. pestis* Angola strain. This crystal structure is of the truncated (Δ1-22) triple mutant variant (K40A/D41A/K42A) from the KIM strain LcrV protein. In the deposited triple mutant structure of LcrV, pdb entry 1R6F, there are several disordered regions, these included the N- and C- termini (M1-H27 and T323-K326, respectively), Y90 and two large loops (Y50-A60 and N263-S273). Several atoms are also missing in the following amino acids: K49, N61, R62, D91, S274, and K276. The structural elements missing from the crystal structure were modeled by "grafting" suitable fragments from structures in the PDB using a Local-Global Alignment method (LGA) [Zemla, 2003 #893]. The secondary structure prediction from the PSIPRED method [Jones, 1999 #1102] was used to aid in the modeling of two long loops (50-60, and 263-273). The N-terminus was not modeled for lack of sequence identity to any known structural elements from the PDB. Finally, the 3D model of LcrV from the Angola strain was used as a structural template to generate models for all of the *Y. pestis* LcrV protein sequences analyzed in this study using the amino acid sequence to tertiary structure system (AS2TS) [Zemla, 2005 #1104]. In all models, coordinates of side chain atoms were left unchanged for residues that are identical between the sequence of the modeled structures and the sequence of the protein in the crystal structure (1R6F). Side chain atom positions for the remaining residues were calculated using SCWRL [Canutescu, 2003 #1103].

Results

Comparative analysis of the V antigen sequence heterogeneity

The alignment of all LcrV sequences from *Y. pestis* known to date with those determined in this study resulted in the selection of the Angola strain of *Y. pestis* as a consensus sequence (Fig. 1). Four major "hot points" of the amino acid polymorphism were found at positions 18, 72, 273, and 324-326, which allowed us to classify the LcrV alleles into several types (Table 1). The type A (N18, R72, S273, and S324-G325-K326) group consisted of the Angola strain, as well as both strains of subspecies *altaica* (I-3455 and I-2359). All three strains of this type differed at a single position 113. In addition strain I-2359 contains two changes at the N-terminus. The type B (N18, R72, C273, and S324-G325-K326) was restricted to a single strain of the subspecies *ulegeica* (I-2422). In comparison with the LcrV of the type A, the LcrV of this strain contained the substitution C273 in the "hot point" area, as well as three additional changes at positions 3, 7 and 84. The type C group (N18, R72, S273 and R324) included the strains A-1728 and C-582 of the subspecies *hissarica* and *caucasica*, respectively, which had a deletion of two amino acid residues at the C-terminus. The LcrV sequences of these two strains differed from each other at position 103. The type D (K18, K72, C273 and S324-G325-K326) group contained the LcrV from the epidemic strains of all three known biovars, such as antiqua (I-1996, I-2638, Antiqua), medievalis (KIM) and orientalis (CO92), as well as the strain of the subspecies *ulegeica* (I-2836). The LcrV sequences of the type D strains were 100%

homologous at both nucleotide and amino acid levels. Finally, type E (K18, K72, C273 and R324) was represented by strains of the subspecies *caucasica* (1146, C-585, Pestoides F) and the subspecies *xilingolensis* strain 91001 isolated in China [Song, 2004 #924]. The LcrV sequence of type E had a deletion at the C-terminus identical to that of the strains of type C. Otherwise, the LcrV sequence of type E was the same as that of type D.

Although the number of investigated isolates for each subspecies was limited, it is worth mentioning that the LcrV sequences of both *ulegeica* strains belonged to different types (B and D), which suggested an increased LcrV variability among these groups. The LcrV of the *caucasica* isolates also could be divided into two types (C and E). In contrast, the type D sequences characteristic of the epidemic strains of *Y. pestis* were conserved between different biovars (including microtus strain I-2836) and this finding is in good agreement with the clonal nature of the origin of the plague bacterium and its spread over the globe during the major pandemics [Devignat, 1951 #124][Achtman, 1999 #493][Achtman, 2004 #1094].

Location of sequence polymorphisms on 3D model of LcrV

Since LcrV of the Angola strain of *Y. pestis* corresponded to the consensus sequence (Fig. 1), the 3D model of LcrV from this strain was constructed by using the crystal structure of this protein from *Y. pestis* KIM [Derewenda, 2004 #1093]. According to the Structural Classification of Proteins (SCOP; release 1.71) [Murzin, 1995 #1106] the fold

of the LcrV protein consists of an "all-alpha" domain, made mostly from the N-terminal region, and an "alpha+beta" domain. The domains are connected by an antiparallel coiled coil. Most of the LcrV amino acid polymorphisms, including those located in the regions that were not modeled, are found within the N-terminal region, residues 1-146. Inspection of 3D molecular models of LcrV showed that, with the exception of residue 273, all polymorphisms (72, 84, 103, 113, and 324) are located within the “all-alpha” domain (Fig. 2).

Effect of amino acid residue substitutions on the structure of LcrV

Sequence polymorphisms at positions 273 and 324 in the LcrV protein sequence occur in regions that are disordered in the crystal structure. Alignment of the LcrV of *Y. pestis* with its homologs from other bacterial species revealed that there are no conserved residues in the C-terminus region, 321-326, suggesting a limited structural importance of this area (Fig. 1). Although the residue at position 273 is not highly conserved, the internal loop (261-281) contained ten identical residues between all species, indicating a possible structural and functional significance of this region of LcrV. The sequence variations at positions 72, 84 and 103 probably have minimal structural consequences and lead to minor local perturbations of the structure.

The substitution of lysine for arginine at position 72 occurs on the surface of the protein and preserves the local inter-residue interaction. The substitution of leucine for isoleucine at position 84 does not occur at the protein surface; moreover, the side chain is

mostly buried, but does preserve the local chemical properties and, likely, the inter-residue interactions. The substitution of glutamic acid with glycine at position 103 in the *Y. pestis* C-582 strain does not preserve the local chemical properties, though the mutation does occur at the surface of the protein in the middle of a helix. This mutation might destabilize the helix, but would not likely significantly disrupt the structure. Of the sequence substitutions we were able to model, the one most likely to have a serious impact on the local structure of the LcrV protein is the tryptophan to glutamic acid (strain I-2359) or glycine (strain I-3455) at position 113 (Fig. 3). Tryptophan is bulky and mostly hydrophobic, though it can contribute one hydrogen bond from the ε nitrogen. Tryptophan 113 of LcrV is mostly buried in a large hydrophobic region near the core of the N-terminal domain. The ε nitrogen stabilizes the local loop structure by hydrogen bonding to the backbone carbonyl of asparagine 110 (Fig. 3, panel A). When tryptophan is replaced by glutamic acid, the favorable hydrophobic interactions are mostly lost, though glutamate is somewhat ampipathic, and E113 can no longer hydrogen bond to the backbone carbonyl of N110. E113 might hydrogen bond with N144, which might, in turn hydrogen bond with the carbonyl of N110 to stabilize the local loop structure. When W113 is replaced with glycine, G113, all of the hydrophobic and hydrogen bonding interactions are lost, likely destabilizing the 108-112 loop and leading to a disordering of this loop, and possibly to some lesser stability of the whole protein (Fig. 3, panel C).

Sequence polymorphism and oligomerization of LcrV

We cloned, expressed in *E. coli* and purified LcrV of types A (Angola), E (Pestoides F) and D (CO92) of LcrV. When non-boiled samples were analyzed by SDS-PAGE, silver staining revealed a major band around 40 kDa that corresponded to a monomeric form of LcrV with deduced molecular weight of 41.1 kDa (Fig. 4 A). All three V antigens displayed a similar intensity of the band corresponding to their monomeric form; however, we noticed that in contrast to the LcrV of Angola strain, the LcrV proteins from CO92 and Pestoides F contained additional minor bands of higher molecular weight. Immunoblot analysis using anti-LcrV monoclonal antibody confirmed that these bands belong to LcrV and likely represent multimeric forms of this protein (Fig. 4B). Since immunoblot assay is generally more sensitive than the silver staining, we could detect multimers of LcrV from Angola as well, although the intensity of these bands was significantly lower than that of the LcrV from other two strains. Nevertheless, only LcrV of CO92 and Pestoides F could form a detectable band of approximately 160 kDa, likely corresponding to a tetrameric form of this protein. The LcrV proteins from CO92 and Pestoides F capable forming a large amount of multimers are identical except three terminal amino acids, while this part of low multimer-forming LcrV from Angola is identical to that of CO92 (Fig. 1). Thus, these three last amino acids of C-terminus are not involved in multimerization of LcrV, but other variables located at positions 18, 72 and 273 are likely responsible for the observed phenomenon.

Discussion

Y. pestis, a highly lethal pathogen, is a recently evolved clone of enteropathogenic *Y. pseudotuberculosis* which has spread throughout the world [Achtman, 1999 #493][Achtman, 2004 #1094][Li, 2009 #1469]. The LcrV protein is a multifunctional virulence factor and protective antigen of the plague bacterium [Brubaker, 2003 #814] which is generally conserved among the epidemic strains of *Y. pestis* [Motin, 1992 #177][Adair, 2000 #1115]. In contrast, LcrV sequences of enteropathogenic *Yersinia* spp. displayed a greater diversity [Motin, 1992 #177][Roggenkamp, 1997 #116], which can affect the functional activity of this protein [Sing, 2005 #1005]. In addition to the epidemic strains of *Y. pestis* of the biovars antiqua, medievalis, orientalis and intermedium [Li, 2009 #1469], there are non-epidemic *Y. pestis* strains which have a limited virulence in selected animal models and for humans. These atypical isolates are referred to as the strains of microtus biovar [Li, 2009 #1469]. Prior to this study, nucleotide sequences of only three *lcrV* genes that originated from this type of strains were available, such as the strains Angola and Pestoides F [Adair, 2000 #1115] as well as the strain 91001 [Song, 2004 #924]. The *lcrV* sequence of all of these isolates differed from the conserved *lcrV* characteristic of the epidemic strains. The Angola strain was different at three single nucleotide positions, while the Pestoides F and 91001 diversity comprised a single deletion involving two direct repeats ATGACACG at the C terminus of the protein [Adair, 2000 #1115][Song, 2004 #924]. Therefore, we investigated the polymorphism of the *lcrV* among the strains of the different *Y. pestis* "subspecies" by sequencing this gene from the representative strains isolated in natural plague reservoirs located in the territory of the FSU and Mongolia.

We found that there are four major variable hotspots at positions 18, 72, 273, and 324-326, which allowed us to classify the LcrV alleles into five types (A-E). This classification of the types of LcrV accounts for the polymorphism from the consensus sequence, and is now different from that assigned by us previously [Anisimov, 2007 #1152]. We found that both *altaica* strains (I-3455 and I-2359) belonged to the same type A allele as the previously sequenced Angola strain. In contrast, two *caucasica* strains fell in different types of LcrV, one of which (C-582) was grouped with the *hissarica* strain (A-1728) of type C while the other two (C-585 and 1146) belonged to the Pestoides F type E. Similarly, two *ulegeica* strains could be distinguished between type B (strain I-2422) and type D (strain I-2836). Thus, the strains of different *Y. pestis* "subspecies" can have the same type of LcrV and different types of LcrV can exist within the same natural plague reservoir. Moreover, the phenomenon of "selective virulence" characteristic of the strains of the microtus biovar is unlikely to be the result of polymorphism of the V antigen, since the LcrV protein of the *ulegeica* strain I-2836 (type D) was identical to that of the epidemic strains of *Y. pestis*. None of the LcrV proteins of other "subspecies" corresponded to this type D. One can speculate that this kind of *ulegeica* strain reduced the virulence following the adaptation of the typical epidemic strain to the population of *Microtus gregalis, Alticola strelzovi* and *Ochotona pallasi pricei*, the main rodent hosts of these strains (Balakhonov, S.V., personal communication). Alternatively, strains like *ulegeica* isolate I-2836 may represent a population of ancient, less virulent strains that gave rise to the epidemic variant of *Y. pestis* which caused plague pandemics. The latter suggestion is in line with the hypothesis that the Black Death of the 14th century

originated in the Gobi Desert of Mongolia (place of isolation of I-2836) and then reached Europe by the caravans of the Silk Road [Orent, 2004 #1117].

We tried to predict whether the sequence polymorphism of LcrV detected in the strains of *Y. pestis* of different origin resulted in a significant disturbance of the 3D structure. The crystal structure of LcrV from *Y. pestis* KIM has several missing regions not visible in the electron density maps [Derewenda, 2004 #1093], and thus a more complete model was generated using LGA [Zemla, 2003 #893]. Unfortunately, it was not possible to model the missing N-terminus (residues 1-27) due to the lack of similar structural elements in the PDB, so the sequence polymorphisms occurring in this region were not analyzed. This region is essential for LcrV secretion and virulence [Skrzypek, 1995 #11][Broms, 2007 #1150] as well as for protection [Pullen, 1998 #322]. The significance of the variation of the LcrV observed in this region for strains I-2359 and I-2422 remains to be determined experimentally. The alignment of all V antigens of *Y. pestis* produced the consensus sequence that was identical to that of the Angola strain, which belongs to the group of atypical, evolutionary ancient strains of the plague bacteria [Motin, 2002 #653][Radnedge, 2002 #1096][Anisimov, 2004 #917][Achtman, 2004 #1094][Li, 2009 #1469]. Therefore, a 3D model of LcrV was made for the primary sequence of this strain using homology-based modeling system AS2TS [Zemla, 2005 #1104], and a possible structural difference due to each individual amino acid residue substitution was evaluated.

A total of six LcrV polymorphisms were structurally analyzed by comparing the modeled structures of LcrV from all available *Y. pestis* strains. All changes except one occurred either in flexible regions or on the surface of the protein and conserved the local chemical properties (i.e. those of a hydrophobic, hydrophilic, amphipathic, charged nature) across all the strains. These variations would likely have a limited impact on the structure. Polymorphisms in flexible and surface regions are likely subject to less selective pressure. In contrast, the substitution of tryptophan at position 113 with either glutamic acid or glycine likely has a serious impact on the regional structure of the protein, and the mutations found in *Y. pestis* I-2359 and I-3455 might have an effect on the function of LcrV. In fact, the recombinant LcrV from I-3455 produced in *Escherichia coli* cells demonstrated a dramatically induced ability to agglomeration when compared with other LcrV variants which have a tryptophan residue at position 113 (Kopylov, P.Kh., Kiseleva, N.V., personal communication). Moreover, LcrV contains major murine H-2^d-, H-2^k- and H-2^b- restricted T-cell epitopes in the area of 102-121 [Parent, 2005 #1172][von Delwig, 2005 #1177][Shim, 2006 #1170], suggesting that the mutations at positions 104 and 113 may affect the immunogenicity of this antigen.

In addition to the structural comparisons, we analyzed whether the variations in the LcrV sequence might be located in the areas known to be important for established properties of this antigen. A major protective epitope(s) of LcrV resides internally between amino acids 168 and 275 [Motin, 1994 #32], the major antigenic region is located between the residues 135 and 245 [Hill, 1997 #554] and the smallest protective fragment is comprised amino acids 135-262 [Vernazza, 2009 #1481]. The combined

region (residues 135-275) is conserved between the LcrV proteins of *Y. pestis*, except for the variable position 273. Since the epitope mapping described in these studies was not precise, the significance of the variation of cysteine/serine at this position in terms of its effects on the immunogenic properties of V antigen could not be addressed at that time. However, a detailed peptide mapping of LcrV conducted later [Pullen, 1998 #322][Parent, 2005 #1172][Shim, 2006 #1170][Khan, 2007 #1171] has revealed that the cysteine-containing internal loop (261-281) does not possess immunodominant linear T- and B- cell epitopes. Nevertheless, the region 271-300, which includes the polymorphic position 273 is crucial for the immunomodulatory property of LcrV [DeBord, 2006 #1189] which depends on the suppression of proinflammatory cytokines by inducing Interleukin 10 (IL-10) [Nedialkov, 1997 #187][Sing, 2002 #649][Brubaker, 2003 #814][Reithmeier-Rost, 2004 #1097]. The N-terminal region of LcrV (residues 31-57), shown to be important for toll-like receptor 2 (TLR2)- and IL-10-stimulating activities in *Yersinia enterocolitica* O:8 [Sing, 2002 #735][Sing, 2005 #1005] was conserved in LcrV of *Y. pestis* determined by us. Also conserved were two sites of interaction of LcrV with human TLR2 (residues 32-35 and 203-205) and putative sites of binding with CD14 receptor (residues 41-43 and 95-97) [Abramov, 2007 #1098]. Interestingly, there were no polymorphic changes in a hypervariable region (residues 225-232) found between the LcrV sequences of *Yersinia* spp. [Motin, 1992 #177][Roggenkamp, 1997 #116]. Finally, none of the variations occurred at positions that are conserved between LcrV protein sequences from *Y. pestis* and LcrV homologs found in other bacterial species (Fig. 1).

The LcrV is a key component of the translocatory machine providing injection of Yop effectors into the host-cell target which forms a pentamer at the tip of the T3SS needle [Mueller, 2005 #1155][Broz, 2007 #1446]. Therefore, the ability of LcrV to form multimers is an essential property of this protein. It is a well established fact that recombinant LcrV may exist in solution as a dimer and higher order oligomers [Hamad, 2007 #1130][Lawton, 2002 #1100][Derewenda, 2004 #1093]. Moreover, a controlled refolding of this protein led to the assembly of LcrV into oligomeric doughnut-like complexes and the C-terminal helix α12 (a.a. 279-317) was crucial for this *in vitro* oligomerization [Caroline, 2008 #1438]. Previously, it was established that the helix α7 (a.a. 148-182) is involved in oligomerization as well [Lawton, 2002 #1100]. We observed that recombinant LcrV from CO92 and Pestoides F had a significantly better capacity for ologomerization than that from the Angola strain. However, the α7 and α12 helixes of all three strains are identical. The polymorphism of the three C-terminal residues also can not explain the differences in the level of multimer formation, since high-oligomeric LcrV of CO92 and low-oligomeric LcrV of Angola have the identical C-terminus which is different from that of the high-oligomeric LcrV of Pestoides F. Therefore, the rest of polymorphic residues located at positions 18, 72 and 273 should account for the differences in oligomerization. Further studies using site-directed mutagenesis will be necessary to determine which of this residues (of combination of thereof) is essential for the observed phenomena. The oligomers of LcrV were stable at denaturing conditions of SDS-PAGE and could withstand 10 min thermal denaturation at 95°C in sample buffer in the presence of the reducing agent DTT at concentration of 100 mM (data not shown). Similar properties of LcrV dimers and tetramers were observed for LcrV expressed in

DNA vaccine constructs. Interestingly, the ability of LcrV to form oligomers correlated with the efficiency of protection of mice from *Y. pestis* challenge followed by the DNA vaccination [Wang, 2004 #1184]. Therefore, understanding of the mechanism underlying a formation of LcrV multimers might be important for plague subunit vaccine optimization, especially, when truncated protective fragments of this antigen are used [Vernazza, 2009 #1481]. It was shown recently, that aggregates and high-molecular-weight multimers (larger than dimer and tetramer forms) of LcrV possessed TLR2-stimulating activity [Pouliot, 2007 #1131], which perhaps is involved in the virulence-associated immunomodulating property of LcrV via induction of IL-10 signalling through TLR2/TLR6/CD14 complex [Brubaker, 2003 #814][Depaolo, 2008 #1403]. Although the formation of large LcrV multimers has to be demonstrated *in vivo*, one can speculate that the observed polymorphism of LcrV provided a basis for the natural selection of more virulent variants of *Y. pestis* during evolution of this pathogen.

In summary, this study demonstrated that the LcrV proteins from the strains representing a different "subspecies" of *Y. pestis* displayed the size, sequence and 3D structure polymorphism. These variations in the LcrV apparently did not alter the lethality of these strains in mice and their natural hosts, since these atypical *Y. pestis* isolates were reported to be highly virulent for these animal species [Anisimov, 2004 #917][Zhou, 2004 #1116]. Nevertheless, we showed for the first time that LcrV derived from different isolates of *Y. pestis* can vary in their ability to form multimers *in vitro*, and the polymorphic changes responsible for this property are located outside of the α7 and α12 helixes previously assigned as essential for oligomerization [Caroline, 2008 #1438].

The impact of the variations of LcrV antigen on multimerization and their influence on virulence and protective properties will need to be further investigated.

Acknowledgments

This work was supported by the International Science and Technology Center (project #2426), Russian Foundation for Basic Research (project 08-04-00405-a), Sealy Center for Vaccine Development of the University of Texas Medical Branch at Galveston, and the NIH/NIAID grant 1R43 AI71634-01A2. This work was implemented under the auspices of the U.S. Department of Energy under contract no. W-7405-Eng-48. Work at LLNL was performed under the auspices of the U.S. Department of Energy under contract DE-AC52-07NA27344.

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Figures:

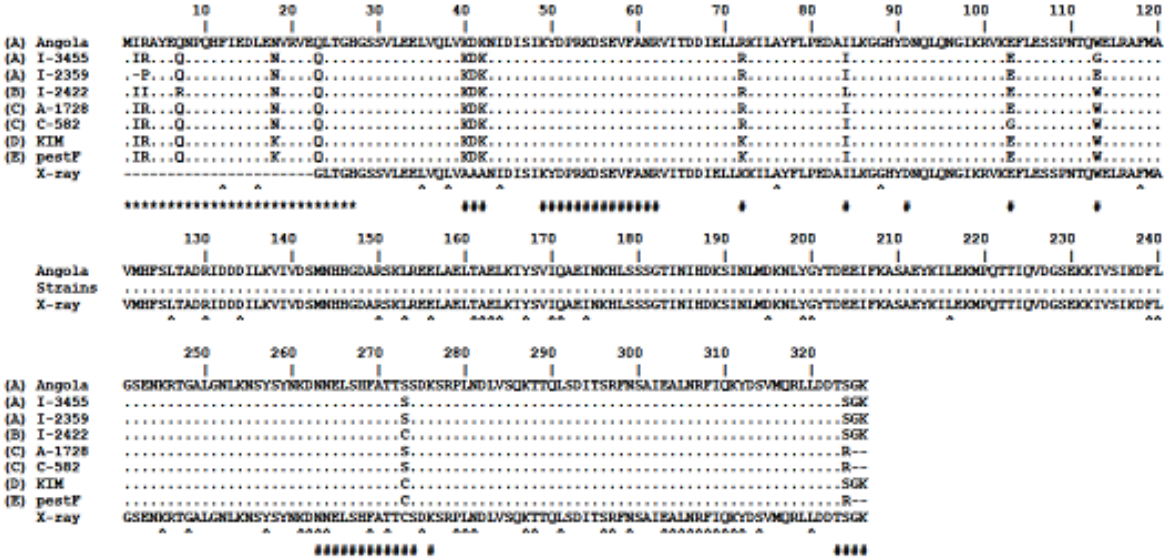

Figure 1. Alignment of known sequences of LcrV for different strains of *Y. pestis*, as well as a sequence from the crystal structure, which lacks the first 23 amino acids. The residues which were replaced or missed in the crystal structure are labeled with #, and the residues that are not modeled are labeled with * (N-termini:1-27). The residues which are absolutely conserved between the LcrV of *Y. pestis* and its homologs from *Pseudomonas aeruginosa*, *Photorhabdus luminescens*, *Aeromonas hydrophila* and *Aeromonas salmonicida* are marked with ^. The type of LcrV of *Y. pestis* is designated by the letters

A-E in parentheses: (A)- Angola, I-3455, I-2359; (B)- I-2422; (C)- A-1728, C-582; (D)- CO92, KIM, Antiqua, I-1996, I-2638, I-2836; (E)- Pestoides F, 91001, 1146, C-585.

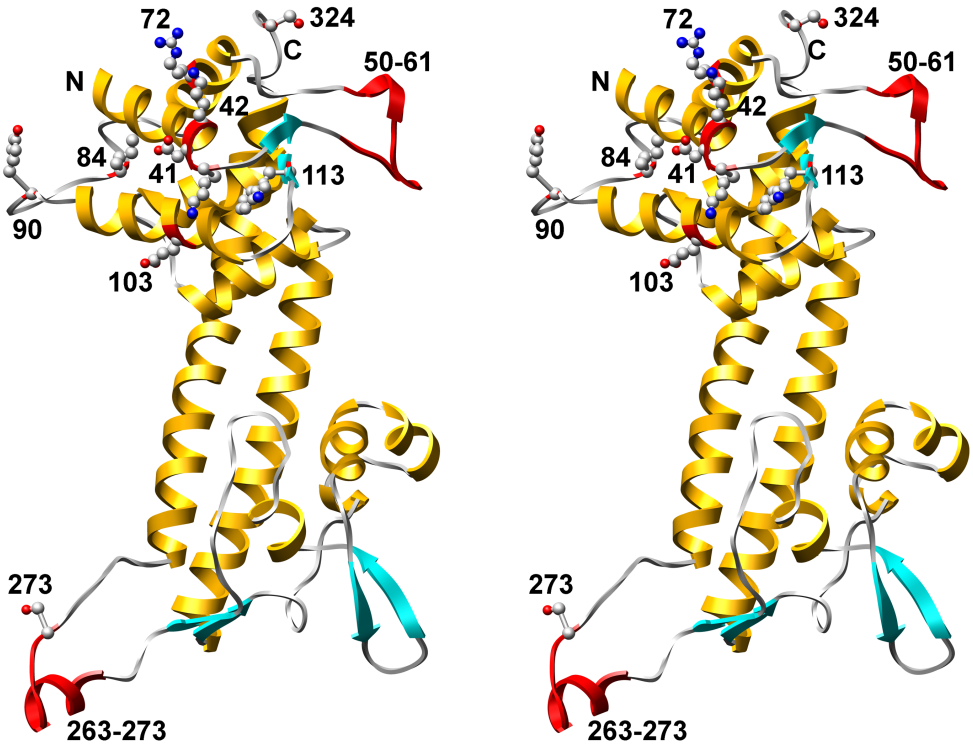

Figure 2. The modeled structure of LcrV from the Angola strain of *Y. pestis* is shown as a ribbon diagram in stereo. The differences between the modeled structure and the available crystal structure from the KIM strain are highlighted in red on the backbone. The site of point variations between the eight strains described here are shown with the side chain of each residue represented in the ball-and-stick. The three engineered mutations (K40A/D41A/K42A) in the crystal structure of the LcrV of *Y. pestis* KIM are also highlighted.

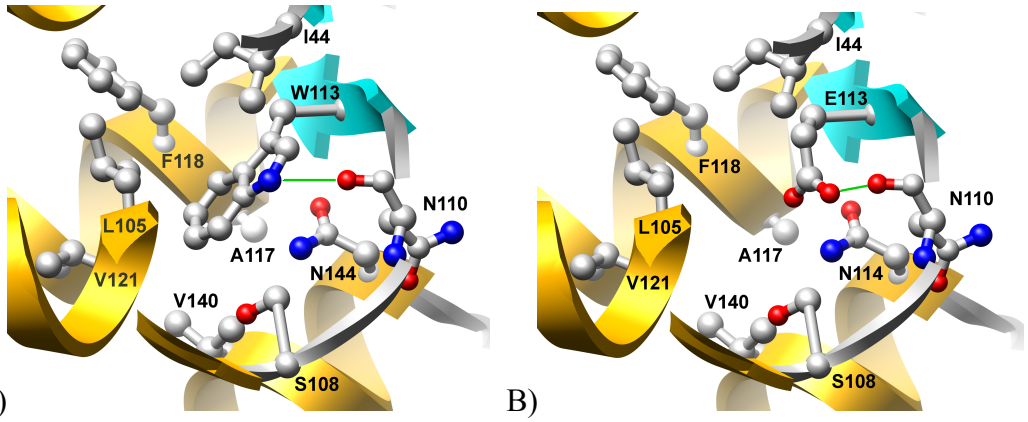

Figure 3. The structures of three variants of *Y. pestis* LcrV are shown highlighting the region surrounding position 113. The consensus sequence of LcrV contains a tryptophan residue at this position (panel A), but in strains I-2359 and I-3455 this residue is substituted with glutamic acid (panel B) and glycine (panel C), respectively.

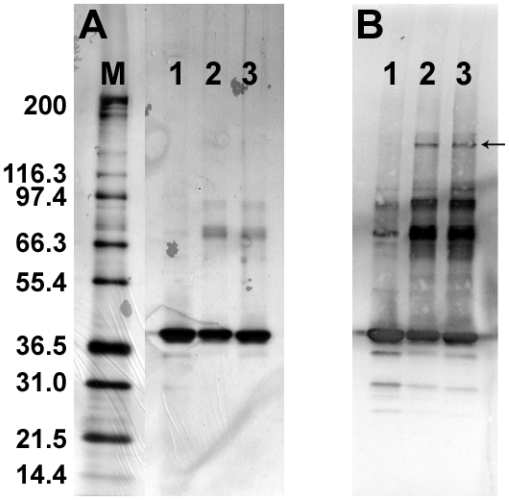

Figure 4. Results of SDS-PAGE followed by silver stain (A) and immunoblot with anti-LcrV monoclonal antibody (B) of non-boiled samples of purified LcrV derived from different strains of *Y. pestis*. Lane 1- Angola, 2- CO92, 3- Pestoides F. M- protein

standards Mark 12 (Invitrogen). The arrow on panel B indicates a position of possible tetramer of LcrV.

Table 1. *Yersinia pestis* strains used as a source for determination of the *lcrV* sequence

Strain	Geographical origin [a]	Biovar/ subspecies [b]	Main host	*lcrV* accession number	Type of LcrV (this study)
1146	Zanzegur-Karabakh focus #6, Armenia	antiqua/ *caucasica*	*Microtus arvalis*	EF645809	E
C-582	Transcaucasian highland (foci #4-6), Armenia	antiqua/ *caucasica*	*M. arvalis*	DQ489557	C
C-585	Transcaucasian highland (foci #5-6), Azerbaijan	antiqua/ *caucasica*	*M. arvalis*	EF645806	E
A-1728	Gissar focus #34, Tadjikistan, Uzbekistan	medievalis/ *hissarica*	*Microtus carruthersi*	DQ489552	C
I-1996	Trans-Baikal focus #38	antiqua/ *pestis*	*Citellus dauricus*	EF645807	D
I-2638	Mongun-Taigin focus #37	antiqua/ *pestis*	*Citellus undulatus*	EF645808	D
I-	Mountain-Altai focus	medievalis/	*Ochotona*	DQ489556	A

2359	#36	*altaica*	*pricei*		
I-3455	Mountain-Altai focus #36	medievalis/ *altaica*	*O. pricei*	DQ489555	A
I-2422	Northeast Mongolia, Gobi Desert	medievalis/ *ulegeica*	*O. pricei*	DQ489554	B
I-2836	Northeast Mongolia, Gobi Desert	medievalis/ *ulegeica*	*O. pricei*	DQ489553	D

For information on [a] geographical location of plague natural foci and [b] biovar-subspecies interrelations see ref. [Anisimov, 2004 #917].