Published in final edited form as:

Infect Genet Evol. 2010 January ; 10(1): 137. doi:10.1016/j.meegid.2009.10.003.

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

Andrey P. Anisimov^{a,*}, Svetlana V. Dentovskaya^a, Evgeniy A. Panfertsev^a, Tat'yana E. Svetoch^a, Pavel Kh. Kopylov^a, Brent W. Segelke^b, Adam Zemla^c, Maxim V. Telepnev^d, and Vladimir L. Motin^{d,e,*}

^aState Research Center for Applied Microbiology and Biotechnology, 142279, Obolensk, Moscow Region, Russia

^bChemistry, Materials and Life Sciences Directorates, BACE, Lawrence Livermore National Laboratory, Livermore, California 94550, USA

^cComputing Applications & Research, BACE, Lawrence Livermore National Laboratory, Livermore, California 94550, USA

^dDepartment of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas 77555, USA

^eDepartment of Pathology, University of Texas Medical Branch, Galveston, Texas 77555, USA

Abstract

The LcrV protein is a multifunctional virulence factor and protective antigen of the plague bacterium and 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 19 *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, including that conserved in epidemic strains, and different types of LcrV can exist within the same natural plague focus. Therefore, 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. 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 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

© 2009 Elsevier B.V. All rights reserved.

*Corresponding authors. Tel.: +7 (4967) 360117; FAX: +7 (4967) 360061; anisimov@obolensk.org (A. P. Anisimov) Tel.: 1 (409) 772-3155; FAX: 1 (409) 747-2437; vlmotin@utmb.edu (V.L. Motin).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

(A part of this work was presented at the 9th International Symposium on *Yersinia*, October 10–14, 2006, in Lexington, Kentucky, USA) (Anisimov *et al.*, 2007).

might have an effect on the function of LcrV. The polymorphisms at positions 18, 72 and 273 were accountable for differences in the oligomerization of LcrV.

Keywords

LcrV; *Yersinia pestis*; polymorphism; 3D structure; modeling; oligomerization

1. 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; Perry & Fetherston, 1997). 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 *et al.*, 2009) that were originally named Pestoides (Martinevsky, 1969), and in contrast to the most of epidemic-causing isolates, are capable of fermenting rhamnose and melibiose (Anisimov *et al.*, 2004); however, five of “subspecies” have lost the ability to ferment arabinose (Li *et al.*, 2009; Zhou *et al.*, 2004a; Zhou *et al.*, 2004b). 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 *et al.*, 2004; Zhou *et al.*, 2004b). 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 *et al.*, 2004). 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 *et al.*, 2004b; Zhou *et al.*, 2004c). More recently it has been proposed to include all non-epidemic, rhamnose-positive variants into biovar microtus as the *Y. pestis* subspecies *altaica*, *caucasica*, *hissarica*, *ulegeica*, *talassica*, *xilingolensis*, *qinghaiensis* and *angola* (Li *et al.*, 2009). 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 (Achtman *et al.*, 2004; Cui *et al.*, 2008; Li *et al.*, 2009; Motin *et al.*, 2002; Vergnaud *et al.*, 2007). 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 (Garcia *et al.*, 2007; Song *et al.*, 2004; Zhou *et al.*, 2004c).

LcrV (V antigen) is an essential virulence factor and protective antigen of *Yersinia* spp., which was discovered more than 50 years ago (Burrows, 1956). This protein is attributed to have a variety of immunomodulatory effects on the host (Abramov *et al.*, 2007; Brubaker, 2003; Depaolo *et al.*, 2008; Foligne *et al.*, 2007; Heesemann *et al.*, 2006; Motin *et al.*, 1997; Nakajima *et al.*, 1995; Nedialkov *et al.*, 1997; Overheim *et al.*, 2005; Reithmeier-Rost *et al.*, 2004; Reithmeier-Rost *et al.*, 2007; Sharma *et al.*, 2005; Sing *et al.*, 2002a; Sing *et al.*, 2002b; Sing *et al.*, 2003; Sing *et al.*, 2005; Welkos *et al.*, 1998), and indirectly regulates expression and secretion of type 3 secretion system (T3SS) virulence factors Yops (Bergman *et al.*, 1991; Matson & Nilles, 2001; Nilles *et al.*, 1998; Nilles *et al.*, 1997; Price *et al.*, 1991; Skrzypek & Straley, 1995), as well as being involved in their translocation to eukaryotic cells (Broms *et al.*, 2007; Broms *et al.*, 2003; Cowan *et al.*, 2005; DeBord *et al.*, 2001; Goure *et al.*, 2005; Holmstrom *et al.*, 2001; Lee *et al.*, 2000; Marenne *et al.*, 2003; Mota, 2006; Mueller *et al.*, 2005; Pettersson *et al.*, 1999; Philipovski *et al.*, 2005; Roggenkamp *et al.*, 1999; Sarker *et*

al., 1998; Weeks *et al.*, 2002). 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 *et al.*, 2004).

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 (Adair *et al.*, 2000; Motin *et al.*, 1992) (Garcia *et al.*, 2007; Song *et al.*, 2004). In this study we sequenced *lcrV* genes from 19 strains of *Y. pestis*, 17 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*.

2. Materials and methods

2.1. 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 *et al.*, 2004). 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).

2.2. 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'-TGTCTGTCGTCTCTTGTGTC-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 *et al.*, 2002), accession no. AF167309; atypical strain Angola (subspecies *angola*) (Li *et al.*, 2009; Motin *et al.*, 2002), accession no. AF167310; strain 91001, microtus biovar (subspecies *xilingolensis*) (Li *et al.*, 2009), 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).

2.3. LcrV expression and purification

We amplified the *lcrV* gene of the strains Angola, KIM and Pestoides F 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 the T7 gene 10 under control of the T7 promoter. This N-terminal tail increased the molecular weight of the native LcrV protein by 3.9 kDa. After the sequence of *lcrV* was verified, the constructs were transformed to an *E. coli* BL21 (DE3) host. Expression and purification of recombinant protein was performed essentially as described by us previously (Motin *et al.*, 1996). We analyzed purified proteins by using SDS-PAGE followed by silver stain or immunoblot with

the monoclonal antibody to LcrV (Brubaker *et al.*, 1987; Motin *et al.*, 1994). Protein sample buffer contained a reducing agent dithiothreitol (DTT) at a concentration of 100 mM. Protein concentration was determined by using a BCA kit (Pierce, Rockford, IL) and prepared to equalize the amounts of protein loaded to SDS-PAGE.

2.4. Modeling of LcrV

The crystal structure of LcrV deposited in the Protein Data Bank (PDB) under the code 1R6F (Derewenda *et al.*, 2004) was used as a structural template for modeling the LcrV sequence from *Y. pestis* Angola strain. This crystal structure is of the truncated ($\Delta 1-22$) 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). The secondary structure prediction from the PSIPRED method (Jones, 1999) 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 *et al.*, 2005). 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 *et al.*, 2003).

3. Results

3.1. 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 reason that these positions were chosen as the major variable hotspots is that at least one of such substitutions will be repeated in two or more LcrV types. The type A (N18, R72, S273, and S324-G325-K326) group consisted of the Angola strain, as well as two 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 (positions 2 and 3). 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 strains of the subspecies *ulegeica* (I-2836, I-2487, I-2457), *caucasica* (C-715, C-540) and *altaica* (I-3519, I-2131). Therefore, both epidemic and non-epidemic strains (except subspecies *hissarica*) contained this type of LcrV. 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), *xilingolensis*

strain 91001 isolated in China (Li *et al.*, 2009; Song *et al.*, 2004), altaica (I-3132, I-3088) and hissarica (A-1249). This LcrV type was not found in epidemic strains and in the isolates of the subspecies ulegeica. 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.

3.2. 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 *et al.*, 2004). According to the Structural Classification of Proteins (SCOP; release 1.71) (Murzin *et al.*, 1995) 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).

3.3. 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 amphipathic, 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).

3.4. 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). When non-boiled samples were analyzed by SDS-PAGE, silver staining revealed a major band of around 40 kDa that corresponded to a monomeric form of LcrV with a deduced molecular weight of 41.1 kDa (Fig. 4A); however, we noticed that, in contrast to the LcrV of the Angola strain, the LcrV proteins from CO92 and Pestoides F contained additional minor bands of higher molecular weight. Immunoblot analysis performed by using an anti-LcrV monoclonal antibody confirmed that these bands belong to LcrV and likely represent multimeric forms of this protein (Fig. 4B). The diffused band of about 70 kDa was a possible product of interaction of LcrV monomer with the degraded forms of this protein that arose during LcrV purification. Since the immunoblot assay is generally more sensitive than 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 the 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 of forming a large amount of multimers are identical except for three terminal amino acids, while this part of the low multimer-forming LcrV from Angola is identical to that of CO92 (Fig. 1). Thus, these three last amino acids of the 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.

4. Discussion

Y. pestis, a highly lethal pathogen, is a recently evolved clone of enteropathogenic *Y. pseudotuberculosis* which has spread throughout the world (Achtman *et al.*, 1999; Achtman *et al.*, 2004; Li *et al.*, 2009). The LcrV protein is a multifunctional virulence factor and protective antigen of the plague bacterium (Brubaker, 2003) which is generally conserved among the epidemic strains of *Y. pestis* (Adair *et al.*, 2000; Motin *et al.*, 1992). In contrast, LcrV sequences of enteropathogenic *Yersinia* spp. displayed a greater diversity (Motin *et al.*, 1992; Roggenkamp *et al.*, 1997), which can affect the functional activity of this protein (Sing *et al.*, 2005). In addition to the epidemic strains of *Y. pestis* of the biovars *antiqua*, *medievalis*, *orientalis* (Devignat, 1951) and *intermedium* (Li *et al.*, 2009), 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 *et al.*, 2009). 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 *et al.*, 2000) as well as the strain 91001 (Song *et al.*, 2004). The *lcrV* sequence of all of these isolates differed from the conserved *lcrV* characteristic of the epidemic strains. 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, a finding which allowed us to classify the LcrV alleles into five types (A–E). This classification of the LcrV types accounted for the polymorphism from the consensus sequence and is now different from that assigned by us previously (Anisimov *et al.*, 2007). We found that two of the *altaica* strains (I-3455 and I-2359) possessed the same type A allele as did the previously sequenced Angola strain, but this subspecies also possessed LcrV of two other types, D (I-2131, I-3519) and E (I-3088, I-3132). Similarly, the *caucasica* strains fell into 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 type E, and strains C-540 and C-715 had the D type of LcrV. The *ulegeica* strains also could be distinguished between type B (strain I-2422) and type

D (strains I-2836, I-2487, I-2457). 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.

The major result of the observed V antigen sequence heterogeneity was a finding that type D sequences characteristic of the epidemic strains of *Y. pestis* were widely distributed among microtus biovar isolates. This conclusion was confirmed by the sequencing of *lcrV* from additional isolates of subspecies *ulegeica* (2 strains), *caucasica* (10 strains) and *altaica* (12 strains) (data not shown). The strains of subspecies *hissarica* did not reveal this type of LcrV; however, only two isolates of this subspecies were available in our collection. We expect that the population of *Y. pestis* ssp. *hissarica* also contains the LcrV of type D, because the isolate A-1249 of this subspecies possessed LcrV of type E which is a truncated version of the type D sequence (see below). The existence of the LcrV of the type D in non-epidemic strains of *Y. pestis* was somewhat unexpected, since both previously sequenced strains of *Y. pestis* microtus biovar 91001 (Song *et al.*, 2004) and Pestoides F (Adair *et al.*, 2000; Garcia *et al.*, 2007) had the type E sequence. The latter type of LcrV is a derivative of the type D sequence as a result of a single deletion involving two direct repeats ATGACACG at the C terminus of the protein (Adair *et al.*, 2000; Song *et al.*, 2004). The *Y. pestis* subspecies strain Angola, which does not have this C-terminal deletion, possessed a significantly distant sequence of LcrV different from the type D variant in three out of four major variable hotspots (positions 18, 72, 273). Thus, all *lcrV* genes sequenced to the present from epidemic strains of *Y. pestis*, including those from ancient Siberian foci in Russia and Mongolia, such as I-1996, I-2636 and eight more isolates (data not shown), were restricted to type D. This finding is in good agreement with the clonal nature of the origin of the plague bacterium and its spread over the globe during major pandemics (Achtman *et al.*, 1999; Achtman *et al.*, 2004; Devignat, 1951; Li *et al.*, 2009). On the other hand, the LcrV from *Y. pestis* subspecies exhibited all of the varieties of the sequences (types A-E), which indicated these strains were the earliest in origin of *Y. pestis* pathogen and had more time for still continuing microevolution or such mutations were neutral in the course of adaptation to the specific host, which is a vole. Therefore, 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.

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 *et al.*, 2004), and thus a more complete model was generated using LGA (Zemla, 2003). 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 (Broms *et al.*, 2007; Skrzypek & Straley, 1995) and contains protective epitope(s) that are sufficient for partial protection (Pullen *et al.*, 1998). 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 (Achtman *et al.*, 2004; Anisimov *et al.*, 2004; Li *et al.*, 2009; Motin *et al.*, 2002; Radnedge *et al.*, 2002). Therefore, a 3D model of LcrV was made for the primary sequence of this strain using homology-based modeling system AS2TS (Zemla *et al.*, 2005), 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 *et al.*, 2005; Shim *et al.*, 2006; von Delwig *et al.*, 2005), 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 *et al.*, 1994), the major antigenic region is located between the residues 135 and 245 (Hill *et al.*, 1997) and the smallest protective fragment is comprised of amino acids 135–262 (Vernazza *et al.*, 2009). 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 (Khan *et al.*, 2008; Parent *et al.*, 2005; Pullen *et al.*, 1998; Shim *et al.*, 2006) 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 *et al.*, 2006) which depends on the suppression of proinflammatory cytokines by inducing Interleukin 10 (IL-10) (Brubaker, 2003; Nedialkov *et al.*, 1997; Reithmeier-Rost *et al.*, 2004; Sing *et al.*, 2002a). 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 *et al.*, 2002b; Sing *et al.*, 2005) was conserved in LcrV of *Y. pestis* as 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 *et al.*, 2007). Interestingly, there were no polymorphic changes in a hypervariable region (residues 225–232) found between the LcrV sequences of *Yersinia* spp. (Motin *et al.*, 1992; Roggenkamp *et al.*, 1997). 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 (Broz *et al.*, 2007; Mueller *et al.*, 2005). 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 (Derewenda *et al.*, 2004; Hamad & Nilles, 2007; Lawton *et al.*, 2002). Moreover, a controlled refolding of this protein led to the assembly of LcrV into oligomeric doughnut-like complexes, and the C-terminal helix α 12 (a.a. 279–317) was crucial for this *in vitro* oligomerization (Caroline *et al.*, 2008). Previously, it was established that the helix α 7 (a.a. 148–182) is involved in oligomerization as well (Lawton *et al.*, 2002). We observed that recombinant LcrV from CO92 and Pestoides F had a significantly better capacity for oligomerization than did that from the Angola strain. However, the α 7 and α 12 helices of all three strains are identical. The polymorphism of the three C-terminal residues also cannot 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 these residues (or 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 a 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 *et al.*, 2004). Therefore, an 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 *et al.*, 2009). It was shown recently, that aggregates and high-molecular-weight multimers (larger than the dimer and tetramer forms) of LcrV possessed TLR2-stimulating activity (Pouliot *et al.*, 2007), which perhaps is involved in the virulence-associated, immunomodulating property of LcrV via induction of IL-10 signaling through the TLR2/TLR6/CD14 complex (Brubaker, 2003; Depaolo *et al.*, 2008). 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 the 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 *et al.*, 2004; Zhou *et al.*, 2004b). 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 $\alpha 7$ and $\alpha 12$ helices previously assigned as essential for oligomerization (Caroline *et al.*, 2008). 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.

References

- Abramov VM, Khlebnikov VS, Vasiliev AM, Kosarev IV, Vasilenko RN, Kulikova NL, Khodyakova AV, Evstigneev VI, Uversky VN, Motin VL, Smirnov GB, Brubaker RR. Attachment of LcrV from *Yersinia pestis* at Dual Binding Sites to Human TLR-2 and Human IFN-gamma Receptor. *J Proteome Res* 2007;6:2222–2231. [PubMed: 17441749]
- Achtman M, Zurth K, Morelli G, Torrea G, Guiyoule A, Carniel E. *Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. *Proc. Natl. Acad. Sci. USA* 1999;96:14043–14048. [PubMed: 10570195]
- Achtman M, Morelli G, Zhu P, Wirth T, Diehl I, Kusecek B, Vogler AJ, Wagner DM, Allender CJ, Easterday WR, Chenal-Francisque V, Worsham P, Thomson NR, Parkhill J, Lindler LE, Carniel E, Keim P. Microevolution and history of the plague bacillus, *Yersinia pestis*. *Proc Natl Acad Sci U S A* 2004;101:17837–17842. [PubMed: 15598742]

- Adair DM, Worsham PL, Hill KK, Klevytska AM, Jackson PJ, Friedlander AM, Keim P. Diversity in a variable-number tandem repeat from *Yersinia pestis*. *J Clin Microbiol* 2000;38:1516–1519. [PubMed: 10747136]
- Anisimov AP, Lindler LE, Pier GB. Intraspecific diversity of *Yersinia pestis*. *Clin Microbiol Rev* 2004;17:434–464. [PubMed: 15084509]
- Anisimov AP, Panfertsev EA, Svetoch TE, Dentovskaya SV. Variability of the protein sequences of LcrV between epidemic and atypical rhamnose-positive strains of *Yersinia pestis*. *Adv Exp Med Biol* 2007;603:23–27. [PubMed: 17966402]
- Bergman T, Håkansson S, Forsberg Å, Norlander L, Macellaro A, Backman A, Wolf-Watz H. Analysis of the V antigen lcrGVH-yopBD operon of *Yersinia pseudotuberculosis*: evidence for a regulatory role of LcrH and LcrV. *J. Bacteriol* 1991;173:1607–1616. [PubMed: 1705541]
- Broms JE, Francis MS, Forsberg A. Diminished LcrV secretion attenuates *Yersinia pseudotuberculosis* virulence. *J Bacteriol* 2007;189:8417–8429. [PubMed: 17873031]
- Broms JE, Sundin C, Francis MS, Forsberg A. Comparative analysis of type III effector translocation by *Yersinia pseudotuberculosis* expressing native LcrV or PcrV from *Pseudomonas aeruginosa*. *J Infect Dis* 2003;188:239–249. [PubMed: 12854079]
- Broz P, Mueller CA, Muller SA, Philippsen A, Sorg I, Engel A, Cornelis GR. Function and molecular architecture of the *Yersinia* injectisome tip complex. *Mol Microbiol* 2007;65:1311–1320. [PubMed: 17697254]
- Brubaker RR. Interleukin-10 and inhibition of innate immunity to *Yersiniae*: roles of Yops and LcrV (V antigen). *Infect Immun* 2003;71:3673–3681. [PubMed: 12819047]
- Brubaker RR, Sample AK, Yu D-Z, Zahorchak RJ, Hu PC, Fowler JM. Proteolysis of V antigen from *Yersinia pestis*. *Microb. Pathogen* 1987;2:49–62. [PubMed: 2853272]
- Burrows TW. An antigen determining virulence in *Pasteurella pestis*. *Nature* 1956;177:426–427. [PubMed: 13309325]
- Canutescu AA, Shelenkov AA, Dunbrack RL Jr. A graph-theory algorithm for rapid protein side-chain prediction. *Protein Sci* 2003;12:2001–2014. [PubMed: 12930999]
- Caroline G, Eric F, Bohn YS, Sylvie E, Attree I. Oligomerization of PcrV and LcrV, protective antigens of *Pseudomonas aeruginosa* and *Yersinia pestis*. *J Biol Chem* 2008;283:23940–23949. [PubMed: 18583342]
- Cowan C, Philipovski AV, Wulff-Strobel CR, Ye Z, Straley SC. Anti-LcrV antibody inhibits delivery of Yops by *Yersinia pestis* KIM5 by directly promoting phagocytosis. *Infect Immun* 2005;73:6127–6137. [PubMed: 16113334]
- Cui Y, Li Y, Gorge O, Platonov ME, Yan Y, Guo Z, Pourcel C, Dentovskaya SV, Balakhonov SV, Wang X, Song Y, Anisimov AP, Vergnaud G, Yang R. Insight into microevolution of *Yersinia pestis* by clustered regularly interspaced short palindromic repeats. *PLoS One* 2008;3:e2652. [PubMed: 18612419]
- DeBord KL, Lee VT, Schneewind O. Roles of LcrG and LcrV during type III targeting of effector Yops by *Yersinia enterocolitica*. *J Bacteriol* 2001;183:4588–4598. [PubMed: 11443094]
- DeBord KL, Anderson DM, Marketon MM, Overheim KA, DePaolo RW, Ciletti NA, Jabri B, Schneewind O. Immunogenicity and protective immunity against bubonic plague and pneumonic plague by immunization of mice with the recombinant V10 antigen, a variant of LcrV. *Infect Immun* 2006;74:4910–4914. [PubMed: 16861680]
- Depaolo RW, Tang F, Kim I, Han M, Levin N, Ciletti N, Lin A, Anderson D, Schneewind O, Jabri B. Toll-like receptor 6 drives differentiation of tolerogenic dendritic cells and contributes to LcrV-mediated plague pathogenesis. *Cell Host Microbe* 2008;4:350–361. [PubMed: 18854239]
- Derewenda U, Mateja A, Devedjiev Y, Routzahn KM, Evdokimov AG, Derewenda ZS, Waugh DS. The structure of *Yersinia pestis* V-antigen, an essential virulence factor and mediator of immunity against plague. *Structure* 2004;12:301–316. [PubMed: 14962390]
- Devignat, R. Bull. W.H.O.; 1951. Varietes de l'espece *Pasteurella pestis* Nouvelle hypothese. p. 247-263.
- Foligne B, Dessein R, Marceau M, Poiret S, Chamaillard M, Pot B, Simonet M, Daniel C. Prevention and treatment of colitis with *Lactococcus lactis* secreting the immunomodulatory *Yersinia* LcrV protein. *Gastroenterology* 2007;133:862–874. [PubMed: 17678918]

- Garcia E, Worsham P, Bearden S, Malfatti S, Lang D, Larimer F, Lindler L, Chain P. Pestoides F, an atypical *Yersinia pestis* strain from the former Soviet Union. *Adv Exp Med Biol* 2007;603:17–22. [PubMed: 17966401]
- Goure J, Broz P, Attree O, Cornelis GR, Attree I. Protective anti-V antibodies inhibit *Pseudomonas* and *Yersinia* translocon assembly within host membranes. *J Infect Dis* 2005;192:218–225. [PubMed: 15962216]
- Hamad MA, Nilles ML. Structure-function analysis of the C-terminal domain of LcrV from *Yersinia pestis*. *J Bacteriol* 2007;189:6734–6739. [PubMed: 17644582]
- Heesemann J, Sing A, Trulzsch K. *Yersinia's* stratagem: targeting innate and adaptive immune defense. *Curr Opin Microbiol* 2006;9:55–61. [PubMed: 16413818]
- Hill J, Leary SE, Griffin KF, Williamson ED, Titball RW. Regions of *Yersinia pestis* V antigen that contribute to protection against plague identified by passive and active immunization. *Infect. Immun* 1997;65:4476–4482. [PubMed: 9353022]
- Holmstrom A, Olsson J, Cherepanov P, Maier E, Nordfelth R, Pettersson J, Benz R, Wolf-Watz H, Forsberg A. LcrV is a channel size-determining component of the Yop effector translocon of *Yersinia*. *Mol Microbiol* 2001;39:620–632. [PubMed: 11169103]
- Jones DT. Protein secondary structure prediction based on position-specific scoring matrices. *J Mol Biol* 1999;292:195–202. [PubMed: 10493868]
- Khan AA, Babu JP, Gupta G, Rao DN. Identifying B and T cell epitopes and studying humoral, mucosal and cellular immune response of V antigen of *Yersinia pestis*. *Vaccine* 2008;26:316–332. [PubMed: 18096277]
- Lawton DG, Longstaff C, Wallace BA, Hill J, Leary SE, Titball RW, Brown KA. Interactions of the type III secretion pathway proteins LcrV and LcrG from *Yersinia pestis* are mediated by coiled-coil domains. *J Biol Chem* 2002;277:38714–38722. [PubMed: 12107165]
- Lee VT, Tam C, Schneewind O. LcrV, a substrate for *Yersinia enterocolitica* type III secretion, is required for toxin targeting into the cytosol of HeLa cells. *J. Biol. Chem* 2000;275:36869–36875. [PubMed: 10930402]
- Li Y, Cui Y, Hauck Y, Platonov ME, Dai E, Song Y, Guo Z, Pourcel C, Dentovskaya SV, Anisimov AP, Yang R, Vergnaud G. Genotyping and phylogenetic analysis of *Yersinia pestis* by MLVA: insights into the worldwide expansion of Central Asia plague foci. *PLoS One* 2009;4:e6000. [PubMed: 19543392]
- Marenne MN, Journet L, Mota LJ, Cornelis GR. Genetic analysis of the formation of the Ysc-Yop translocation pore in macrophages by *Yersinia enterocolitica*: role of LcrV, YscF and YopN. *Microb Pathog* 2003;35:243–258. [PubMed: 14580388]
- Martinevsky, IL. Biology and genetic features of plague and plague-related microbes. Moscow, USSR: Meditsina Press; 1969.
- Matson JS, Nilles ML. LcrG-LcrV interaction is required for control of Yops secretion in *Yersinia pestis*. *J. Bacteriol* 2001;183:5082–5091. [PubMed: 11489861]
- Mota LJ. Type III secretion gets an LcrV tip. *Trends Microbiol* 2006;14:197–200. [PubMed: 16564172]
- Motin VL, Nedialkov YA, Brubaker RR. V antigen-polyhistidine fusion peptide: binding to LcrH and active immunity against plague. *Infect. Immun* 1996;64:4313–4318. [PubMed: 8926104]
- Motin VL, Kutas SM, Brubaker RR. Suppression of mouse skin allograft rejection by Protein A-yersinia V antigen fusion peptide. *Transplantation* 1997;63:1040–1042. [PubMed: 9112365]
- Motin VL, Nakajima R, Smirnov GB, Brubaker RR. Passive immunity to yersiniae mediated by anti-recombinant V antigen and Protein A-V antigen fusion peptide. *Infect. Immun* 1994;62:4192–4201. [PubMed: 7927675]
- Motin VL, Pokrovskaya MS, Telepnev MV, Kutyrev VV, Vidyaeva NA, Fillippov AA, Smirnov GB. The difference in the lcrV sequences between *Yersinia pestis* and *Yersinia pseudotuberculosis* and its application for characterization of *Y. pseudotuberculosis* strains. *Microb. Pathog* 1992;12:165–175. [PubMed: 1614327]
- Motin VL, Georgescu AM, Elliott JM, Ping H, Worsham PL, Ott LL, Slezak TR, Sokhansanj BA, Regala WM, Brubaker RR, Garcia E. Genetic variability of *Yersinia pestis* isolates as predicted by PCR-based IS100 genotyping and analysis of structural genes encoding glycerol-3-phosphate dehydrogenase (*glpD*). *J. Bacteriol* 2002;184:1019–1027. [PubMed: 11807062]

- Mueller CA, Broz P, Muller SA, Ringler P, Erne-Brand F, Sorg I, Kuhn M, Engel A, Cornelis GR. The V-antigen of *Yersinia* forms a distinct structure at the tip of injectosome needles. *Science* 2005;310:674–676. [PubMed: 16254184]
- Murzin AG, Brenner SE, Hubbard T, Chothia C. SCOP: a structural classification of proteins database for the investigation of sequences and structures. *J Mol Biol* 1995;247:536–540. [PubMed: 7723011]
- Nakajima R, Motin VL, Brubaker RR. Suppression of cytokines in mice by Protein A-V antigen fusion peptide and restoration of synthesis by active immunization. *Infect. Immun* 1995;63:3021–3029. [PubMed: 7622225]
- Nedialkov YA, Motin VL, Brubaker RR. Resistance to lipopolysaccharide mediated by the *Yersinia pestis* V antigen-polyhistidine fusion peptide: amplification of interleukin-10. *Infect. Immun* 1997;65:1196–1203. [PubMed: 9119451]
- Nilles ML, Fields KA, Straley SC. The V antigen of *Yersinia pestis* regulates Yop vectorial targeting as well as Yop secretion through effects on YopB and LcrG. *J. Bacteriol* 1998;180:3410–3420. [PubMed: 9642196]
- Nilles ML, Williams AW, Skrzypek E, Straley SC. *Yersinia pestis* LcrV forms a stable complex with LcrG and may have a secretion-related regulatory role in the low- Ca^{2+} response. *J. Bacteriol* 1997;179:1307–1316. [PubMed: 9023216]
- Overheim KA, Depaolo RW, Debord KL, Morrin EM, Anderson DM, Green NM, Brubaker RR, Jabri B, Schneewind O. LcrV plague vaccine with altered immunomodulatory properties. *Infect Immun* 2005;73:5152–5159. [PubMed: 16041032]
- Parent MA, Berggren KN, Mullarky IK, Szaba FM, Kummer LW, Adamovicz JJ, Smiley ST. *Yersinia pestis* V protein epitopes recognized by CD4 T cells. *Infect Immun* 2005;73:2197–2204. [PubMed: 15784563]
- Perry RD, Fetherston JD. *Yersinia pestis* - etiologic agent of plague. *Clin. Microbiol. Rev* 1997;10:35–66. [PubMed: 8993858]
- Petersson J, Holmstrom A, Hill J, Leary S, Frithz-Lindsten E, von-Euler-Matell A, Carlsson E, Titball R, Forsberg Å, Wolf-Watz H. The V antigen of *Yersinia* is surface exposed before target cell contact and involved in virulence protein translocation. *Mol. Microbiol* 1999;32:961–976. [PubMed: 10361299]
- Philipovskiy AV, Cowan C, Wulff-Strobel CR, Burnett SH, Kerschen EJ, Cohen DA, Kaplan AM, Straley SC. Antibody against V antigen prevents Yop-dependent growth of *Yersinia pestis*. *Infect Immun* 2005;73:1532–1542. [PubMed: 15731051]
- Pouliot K, Pan N, Wang S, Lu S, Lien E, Goguen JD. Evaluation of the role of LcrV-Toll-like receptor 2-mediated immunomodulation in the virulence of *Yersinia pestis*. *Infect Immun* 2007;75:3571–3580. [PubMed: 17438030]
- Price SB, Cowan C, Perry RD, Straley SC. The V antigen is a regulator protein necessary for the Ca^{2+} -dependent growth and the maximal expression of low Ca^{2+} -response virulence genes in *Yersinia pestis*. *J. Bacteriol* 1991;173:2649–2657. [PubMed: 1901573]
- Pullen JK, Anderson GW Jr, Welkos SL, Friedlander AM. Analysis of the *Yersinia pestis* V protein for the presence of linear antibody epitopes. *Infect. Immun* 1998;66:521–527. [PubMed: 9453605]
- Radnedge L, Agron PG, Worsham PL, Andersen GL. Genome plasticity in *Yersinia pestis*. *Microbiology* 2002;148:1687–1698. [PubMed: 12055289]
- Reithmeier-Rost D, Bierschenk S, Filippova N, Schroder-Braunstein J, Sing A. *Yersinia* V antigen induces both TLR homo- and heterotolerance in an IL-10-involving manner. *Cell Immunol* 2004;231:63–74. [PubMed: 15919371]
- Reithmeier-Rost D, Hill J, Elvin SJ, Williamson D, Dittmann S, Schmid A, Wilharm G, Sing A. The weak interaction of LcrV and TLR2 does not contribute to the virulence of *Yersinia pestis*. *Microbes Infect* 2007;9:997–1002. [PubMed: 17556003]
- Roggkamp A, Geiger AM, Leitritz L, Kessler A, Heeseman J. Passive immunity to infection with *Yersinia* spp. mediated by anti-recombinant V antigen is dependent on polymorphism of V antigen. *Infect. Immun* 1997;65:446–451. [PubMed: 9009295]
- Roggkamp A, Leitritz L, Sing A, Kempf VA, Baus K, Heesemann J. Anti-recombinant V antigen serum promotes uptake of *Yersinia enterocolitica* serotype 08 by macrophages. *Med Microbiol Immunol* 1999;188:151–159. [PubMed: 10776846]

- Sarker MR, Neyt C, Stanier E, Cornelis G. The *Yersinia* Yop virulon: LcrV is required for extrusion of the translocators YopB and YopD. *J. Bacteriol* 1998;180:1207–1214. [PubMed: 9495760]
- Sharma RK, Sodhi A, Batra HV. Involvement of TLR6/1 in rLcrV-mediated immunomodulation of murine peritoneal macrophages in vitro. *Mol Immunol* 2005;42:695–701. [PubMed: 15781113]
- Shim HK, Musson JA, Harper HM, McNeill HV, Walker N, Flick-Smith H, von Delwig A, Williamson ED, Robinson JH. Mechanisms of major histocompatibility complex class II-restricted processing and presentation of the V antigen of *Yersinia pestis*. *Immunology* 2006;119:385–392. [PubMed: 16919002]
- Sing A, Roggenkamp A, Geiger AM, Heesemann J. *Yersinia enterocolitica* evasion of the host immune response by V antigen-induced IL-10 production of macrophages is abrogated in IL-10-deficient mice. *J. Immunol* 2002a;168:1315–1321. [PubMed: 11801671]
- Sing A, Rost D, Tvardovskaia N, Roggenkamp A, Wiedemann A, Kirschning CJ, Aepfelbacher M, Heesemann J. *Yersinia* V-antigen exploits Toll-like receptor 2 and CD14 for interleukin 10-mediated immunosuppression. *J. Exp. Med* 2002b;196:1017–1024. [PubMed: 12391013]
- Sing A, Tvardovskaia N, Rost D, Kirschning CJ, Wagner H, Heesemann J. Contribution of toll-like receptors 2 and 4 in an oral *Yersinia enterocolitica* mouse infection model. *Int J Med Microbiol* 2003;293:341–348. [PubMed: 14695062]
- Sing A, Reithmeier-Rost D, Granfors K, Hill J, Roggenkamp A, Heesemann J. A hypervariable N-terminal region of *Yersinia* LcrV determines Toll-like receptor 2-mediated IL-10 induction and mouse virulence. *Proc Natl Acad Sci U S A* 2005;102:16049–16054. [PubMed: 16239347]
- Skrzypek E, Straley SC. Differential effects of deletions in *lcrV* on secretion of V antigen, regulation of the low- Ca^{2+} response, and virulence of *Yersinia pestis*. *J. Bacteriol* 1995;177:2530–2542. [PubMed: 7730287]
- Song Y, Tong Z, Wang J, Wang L, Guo Z, Han Y, Zhang J, Pei D, Zhou D, Qin H, Pang X, Zhai J, Li M, Cui B, Qi Z, Jin L, Dai R, Chen F, Li S, Ye C, Du Z, Lin W, Yu J, Yang H, Huang P, Yang R. Complete genome sequence of *Yersinia pestis* strain 91001, an isolate avirulent to humans. *DNA Res* 2004;11:179–197. [PubMed: 15368893]
- Vergnaud G, Li Y, Gorge O, Cui Y, Song Y, Zhou D, Grissa I, Dentovskaya SV, Platonov ME, Rakin A, Balakhonov SV, Neubauer H, Pourcel C, Anisimov AP, Yang R. Analysis of the three *Yersinia pestis* CRISPR loci provides new tools for phylogenetic studies and possibly for the investigation of ancient DNA. *Adv Exp Med Biol* 2007;603:327–338. [PubMed: 17966429]
- Vernazza C, Lingard B, Flick-Smith HC, Baillie LW, Hill J, Atkins HS. Small protective fragments of the *Yersinia pestis* V antigen. *Vaccine* 2009;27:2775–2780. [PubMed: 19366573]
- von Delwig A, Musson JA, Shim HK, Lee JJ, Walker N, Harding CV, Williamson ED, Robinson JH. Distribution of productive antigen-processing activity for MHC class II presentation in macrophages. *Scand J Immunol* 2005;62:243–250. [PubMed: 16179011]
- Wang S, Heilman D, Liu F, Giehl T, Joshi S, Huang X, Chou TH, Goguen J, Lu S. A DNA vaccine producing LcrV antigen in oligomers is effective in protecting mice from lethal mucosal challenge of plague. *Vaccine* 2004;22:3348–3357. [PubMed: 15308359]
- Weeks S, Hill J, Friedlander A, Welkos S. Anti-V antigen antibody protects macrophages from *Yersinia pestis* -induced cell death and promotes phagocytosis. *Microb Pathog* 2002;32:227–237. [PubMed: 12071679]
- Welkos S, Friedlander A, McDowell D, Weeks J, Tobery S. V antigen of *Yersinia pestis* inhibits neutrophil chemotaxis. *Microb. Pathog* 1998;24:185–196. [PubMed: 9514641]
- Zemla A. LGA: A method for finding 3D similarities in protein structures. *Nucleic Acids Res* 2003;31:3370–3374. [PubMed: 12824330]
- Zemla A, Zhou CE, Slezak T, Kuczmarski T, Rama D, Torres C, Sawicka D, Barsky D. AS2TS system for protein structure modeling and analysis. *Nucleic Acids Res* 2005;33:W111–W115. [PubMed: 15980437]
- Zhou D, Han Y, Song Y, Huang P, Yang R. Comparative and evolutionary genomics of *Yersinia pestis*. *Microbes Infect* 2004a;6:1226–1234. [PubMed: 15488743]
- Zhou D, Tong Z, Song Y, Han Y, Pei D, Pang X, Zhai J, Li M, Cui B, Qi Z, Jin L, Dai R, Du Z, Wang J, Guo Z, Wang J, Huang P, Yang R. Genetics of metabolic variations between *Yersinia pestis* biovars and the proposal of a new biovar, microtus. *J Bacteriol* 2004b;186:5147–5152. [PubMed: 15262951]

Zhou D, Han Y, Song Y, Tong Z, Wang J, Guo Z, Pei D, Pang X, Zhai J, Li M, Cui B, Qi Z, Jin L, Dai R, Du Z, Bao J, Zhang X, Yu J, Wang J, Huang P, Yang R. DNA microarray analysis of genome dynamics in *Yersinia pestis*: insights into bacterial genome microevolution and niche adaptation. J Bacteriol 2004c;186:5138–5146. [PubMed: 15262950]

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* *Phototrhabus 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, I-2457, I-2487, C-715, C-540, I-3519, I-2131; (E)- Pestoides F, 91001, 1146, C-585, I-3088, I-3132.

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 *lerV* sequence

Strain	Geographical origin ^a	Biovar/ subspecies ^b	Main host	<i>lerV</i> accession number	Type of <i>LcrV</i> (this study)
I-146	Zanzhur-Karabakh focus #6, Armenia	microtus/ <i>caucasicus</i>	<i>Microtus arvalis</i>	EF645809	E
C-582	Transcaucasian highland (foci #4-6), Armenia	microtus/ <i>caucasicus</i>	<i>M. arvalis</i>	DQ489557	C
C-585	Transcaucasian highland (foci #5-6), Azerbaijan	microtus/ <i>caucasicus</i>	<i>M. arvalis</i>	EF645806	E
C-540	Dagestan-highland (focus #39), Russia	microtus/ <i>caucasicus</i>	<i>M. arvalis</i>	GQ468418	D
C-715	Dagestan-highland (focus #39), Russia	microtus/ <i>caucasicus</i>	<i>M. arvalis</i>	GQ468417	D
A-1728	Gissar focus #34, Tadjikistan, Uzbekistan	microtus/ <i>hissarica</i>	<i>Microtus carruthersi</i>	DQ489552	C
A-1249	Gissar focus #34, Tadjikistan, Uzbekistan	microtus/ <i>hissarica</i>	<i>M. carruthersi</i>	GQ468423	E
I-1996	Trans-Baikal focus #38	antiqua/ <i>pestis</i>	<i>Citellus dauricus</i>	EF645807	D
I-2638	Mongun-Taigin focus #37	antiqua/ <i>pestis</i>	<i>Citellus undulatus</i>	EF645808	D
I-2359	Mountain-Altai focus #36	microtus/ <i>altaica</i>	<i>Microtus gregalis</i> , <i>Alticola strelzovi</i> and <i>Ochotona pricei</i>	DQ489556	A
I-3455	Mountain-Altai focus #36	microtus/ <i>altaica</i>	<i>M. gregalis</i> , <i>A. strelzovi</i> and <i>O. pricei</i>	DQ489555	A
I-3088	Mountain-Altai focus #36	microtus/ <i>altaica</i>	<i>M. gregalis</i> , <i>A. strelzovi</i> and <i>O. pricei</i>	GQ468422	E
I-3132	Mountain-Altai focus #36	microtus/ <i>altaica</i>	<i>M. gregalis</i> <i>A. strelzovi</i> and <i>O. pricei</i>	GQ468421	E
I-2131	Mountain-Altai focus #36	microtus/ <i>altaica</i>	<i>M. gregalis</i> <i>A. strelzovi</i> and <i>O. pricei</i>	GQ468420	D
I-3519	Mountain-Altai focus #36	microtus/ <i>altaica</i>	<i>M. gregalis</i> <i>A. strelzovi</i> and <i>O. pricei</i>	GQ468419	D

Strain	Geographical origin ^a	Biovar/ subspecies ^b	Main host	lerV accession number	Type of LerV (this study)
I-2422	Northeast Mongolia, Gobi Desert	microtus/ <i>ulegeica</i>	<i>M. gregalis</i> , <i>A. strelzovi</i> and <i>O. pricei</i>	DQ489554	B
I-2836	Northeast Mongolia, Gobi Desert	microtus/ <i>ulegeica</i>	<i>M. gregalis</i> , <i>A. strelzovi</i> and <i>O. pricei</i>	DQ489553	D
I-2457	Northeast Mongolia, Gobi Desert	microtus/ <i>ulegeica</i>	<i>M. gregalis</i> , <i>A. strelzovi</i> and <i>O. pricei</i>	GQ468416	D
I-2487	Northeast Mongolia, Gobi Desert	microtus/ <i>ulegeica</i>	<i>M. gregalis</i> , <i>A. strelzovi</i> and <i>O. pricei</i>	GQ468415	D

^aFor information on ^a geographical location of plague natural foci and ^b biovar-subspecies interrelations see ref. [Anisimov, 2004 #917] and [Li, 2009 #1469], respectively.