Letter to the Editor

Screening of Genes with Unique Mutations of Microcus

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Yersinia pestis is the causative agent of bubonic and pneumonic plagues. Strains of *Y. pestis* are classified into four biovars: antiqua, mediaevalis, orientalis, and microtus[1]. There are two microtus-related plague foci in China: the *Microtus brandti* plague focus in the Xilin Gol Grassland (focus L) and the *Microtus fuscus* plague focus in the Qinghai-Tibet Plateau (focus M).

Microtus strains are avirulent to humans, and experiments have shown that strains isolated from *Microtus brandti* in Inner Mongolia are virulent to small rodents but avirulent to larger mammals . In a previous human volunteer study, the subcutaneous injection of 1.5×10^7 cells of strain 91001 was found to cause neither bubonic nor pneumonic plague in the volunteers who participated in the trial[2]. Previous experiments have revealed that the growth rate of the microtus biovar in human serum is significantly slow than that of other *Y. pestis* biovars[3].

The complete genome sequence of *Y. pestis* strain 91001 has been determined[4]. Comparison of the genomes of strains 91001, CO92, and KIM revealed that strain 91001 lacks a 33 Kb fragment related to a phage. However, the absence of this fragment does not result in any obvious alterations in the life cycle and virulence of *Y. pestis*[5].

In this study, the unique genes of strain 91001 were screened by comparing the genome sequence of this strain with those of other *Y. pestis* biovars. The complete coding sequences (CDSs) of *Y. pestis*, including the strains sequenced by our laboratory (D182038, D106004, and Z176003)[6] and six international strains (CO92, KIM, 91001, Antique, Nepal516, and Pestoides F), were downloaded from the NCBI database. Pairwise comparisons of the CDSs of each strain were carried out using BLAST. The gene characteristics were extracted and compared between the identified unique sequences of strain 91001 and the sequence of strain CO92 at the mutation sites.

For further screening of genetic mutations, the following conditions were applied in order to exclude genes that exhibited less similarity: (1) genes with the same amino acid sequence in bacterial strains such as CO92 and *Y. pseudotuberculosis* IP32953; (2) identical mutations existing in strains 91001 and IP32953; and (3) genes with no mutations compared with the whole genome sequence of 91001.

Furthermore, genomic and PCR analyses were used to identify the *Y. pestis* strains isolated from different plague foci in China. In this study, we screened 129 strains including 22 and 28 *Y. pestis* strains isolated from the L and M foci, respectively. Supplementary Table 1 presents the background information of all the strains investigated in this study. The primer sequences are shown in supplementary Table 2. According to the gene mutation characteristics, all the PCR products of point mutation and short deletion genes were sequenced. The PCR products of long deletion mutation genes were randomly selected for sequencing.

Initial comparison of the CDSs among nine *Y. pestis* strains revealed 69 mutant genes, in strain 91001 on nucleic acid sequences. Further analysis as described above, a total of 21 unique mutated genes were identified in strain 91001. Table 1 presents the characteristics of the 21 mutations and the range of detection. As shown in the table, there were 15 point mutations and six insertion/deletion mutations. As classified by COG function, seven of these genes are involved in metabolism; five in cellular processes and signaling; two in information storage and processing; and the remaining seven genes remain poorly characterized or are unclassified.

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Table 1. Unique Mutant Genes of Strain 91001

ID	91001	CO92	Site of Mutation	Features	Mutation rate in Microtus	Primer Name	Expected Length	Gene Annotation
1	*YP_0950*	*YPO1186*	352th mutated site	C-A	22/50	S1-1	535	Hypothetical protein
2	*YP_1081*	*YPO2634*	62th mutated site	T-G	0/50	T2-5	443	Ferric uptake regulator
3	*YP_1259*	*YPO1334*	261–302th intercalary deletion	42 bp more	22/50	T3-2	457/415	Putrescine transport system permease protein
4	*YP_1382*	*YPO1492*	mutation deletion after 632th site	31 bp less	50/50	T4-1	336/367	Hypothetical protein
5	*YP_0191*	*YPO0193*	intercalary deletion of 6 sites	6 bp less	50/50	Q5	859	Peptidyl-prolyl cis-trans isomerase
6	*YP_1914*	*YPO2071*	intercalary deletion of later stage at 1043th site	510 bp less	50/50	T6-1	920/410	Putative DEAD box family helicase
7	*YP_1972*	*YPO2173*	82th mutated site	G-T	50/50	T7-1	480	Probable response regulator
8	*YP_2041*	*YPO2243*	284th-295th deletion	12 bp more	50/50	T8-1	441	Putative AraC-family transcriptional regulatory protein
9	*YP_2637*	*YPO3013*	647th mutated site	G-A	49/50	T13-3	586	Sulfate transport system permease protein CysW
10	*YP_3190*	*YPO3855*	563th mutated site	T-G	0/50	T18-3	465	Putative UDP-*N*-acetyl-D-mannosaminuronic acid transferase
11	*YP_3244*	*YPO3805*	502th mutated site	T-A	50/50	T19-1	447	High-affinity transport ATP-binding protein
12	*YP_0355*	*YPO3332*	433th mutated site	G-A	50/50	T21-1	548	Putative sugar ABC transporter, permease protein
13	*YP_3528*	*YPO0832*	302th mutated site	C-T	50/50	T22-1	444	Putative tagatose 6-phosphate kinase
14	*YP_3626*	*YPO0558*	428th mutated site	C-A	50/50	T23-2	605	Cell division protein FtsQ
15	*YP_3713*	*YPO0467*	T-base deletion at the 552th site	T-base deletion	0/50	T24-1	851	Hypothetical protein
16	*YP_3782*	*YPO0399*	230th mutated site	G-T	50/50	T25-2	535	Hypothetical protein
17	*YP_3784*	*YPO0397*	454th mutated site	A-C	50/50	T26-1	676	Hypothetical protein
18	*YP_3989*	*YPO4080*	1814th mutated site	G-A	22/50	T29-1	406	Periplasmic alpha-amylase precursor
19	*YP_0459*	*YPO0302*	569th mutated site	G-T	50/50	T30-1	636	Putative outer membrane fimbrial usher protein
20	*YP_0499*	*YPO0345*	275th mutated site	C-A	50/50	T31-1	548	Thiol:disulfide interchange protein precursor
21	*YP_0501*	*YPO0347*	10th mutated site	G-A	50/50	T32-2	546	Anaerobic C4-dicarboxylate transporter

Inconsisten mutations the published sequence data at the mutation sites in genes *YP_1081*, *YP_3190,* and *YP_3713* revealed the presence of TG, TG, and T deletion mutations; however, the 91001 strain and the strains isolated from the L and M foci did not show the presence of these mutations. This inconsistency is speculated to be a result of errors in the complete genome sequencing procedures used to sequence these strains.

The *YP_2637* gene mutation (49/50) was observed in the 49 strains isolated from foci L and M. However, this mutation did not occur in one strain isolated from focus L of Inner Mongolia in 1988 (strain b18). The epidemic during the strain b18 separation was normal; therefore, the mutation may be unrelated to the virulence of this strain.

Two incompletely consistent gene mutations (*YP_0191* and *YP_0459*) were observed in the strains isolated from other foci (except the L and M foci) in China. The mutation was located in gene *YP_0191* of *slyD* showing a tandem repeat sequence. The length of the repeat unit was 6 bp. Strains isolated from other foci had 1-2 repeat units more than the *Y. pestis* strain isolated from focus L. The mutation site was in the *YP_0459* gene, whereas other *Y. pestis* strains mostly demonstrated GT mutations. Strains collected from focus B showed interruption in the IS285 element, although this may be unrelated to the virulence characteristics of the *Y. pestis* strains isolated from focus L.

Three genes (*YP_0950*, *YP_1259,* and *YP_3989*) were found to be mutated (22/50) in the microtus strains isolated from focus L but not in the strains isolated from focus M. These three mutations may be associated with differences in the virulence factors of *Y. pestis* between the strains isolated from focus L and focus M. Therefore, these three mutations can be used to distinguish between the strains of these two foci.

Y. pestis strains exhibit a highly conserved genetic sequence. The declined virulence of microtus strains cannot be attributed to the occurrence of different mutations in different *Y. pestis* strains. The mutations that occurred in all *Y. pestis* strains isolated from both plague foci could be responsible for the low level of virulence of the microtus strains in humans. The remaining 12 mutations were unique and present in the microtus strains isolated from both foci investigated in this study. Table 2 lists the nature and type of mutations in the genes.

YP_3244, *YP_0355,* and *YP_3528* are metabolism-related genes that play important roles in the basic growth and metabolism of bacteria. These relatively conserved genes have been found to be mutated in microtus strains. The hydrophobic and hydrophilic nature of the amino acids remained unchanged at the mutated sites. Hence, the functions of the proteins are probably unaffected by these mutations.

The functions of five genes (*YP_1972*, *YP_1382*, *YP_3782*, *YP_3784,* and *YP_0501*), commonly known as “pseudogenes”, are yet to be clarified in the protein cluster analysis. However, the quantity of these pseudogenes in *Y. pestis* is remarkably large. The hydrophobic/hydrophilic nature of the amino acids in these proteins was altered by the mutations. The *YP_1382* gene underwent a deletion in microtus strain 91001, and its role in the altered virulence of this strain should be highlighted.

Table 2. Unique Mutant Genes of Microtus that Caused Amino Acid Mutations

ID	91001	Mutation Type	Amino Acid Change	Nature of Mutation
1	*YP_3244*	point mutation	C-S	hydrophilic—hydrophilic
2	*YP_0355*	point mutation	G-S	hydrophilic—hydrophilic
3	*YP_3528*	point mutation	P-L	hydrophobic—hydrophobic
4	*YP_1972*	point mutation	G-C	hydrophilic—hydrophilic
5	*YP_1382*	gene deletion	deletion	Deletion of 14 amino acids
6	*YP_3782*	point mutation	R-L	hydrophilic—hydrophobic
7	*YP_3784*	point mutation	T-P	hydrophilic—hydrophobic
8	*YP_0501*	point mutation	V-I	hydrophobic—hydrophobic
9	*YP_3626*	point mutation	R-L	hydrophilic—hydrophobic
10	*YP_0499*	point mutation	P-Q	hydrophobic—hydrophilic
11	*YP_2041*	Increase	increase	4 amino acids more
12	*YP_1914*	gene deletion	deletion	Deletion of 170 amino acids

FtsQ is the cell division protein expressed by *YP_3626*. It is a crucial protein in the bacterial cell division protein family, which contains at least 12 proteins. The *FtsQAZ* complex plays a key role in bacterial cell division[7]. *FtsQ* affects cell division by reacting with other proteins. The *FtsQ* gene mutation occurs at nucleotide 428, which results in the expression of leucine instead of arginine; this changes the nature of the amino acid from hydrophilic to hydrophobic.

The *YP_0499* gene expresses a protein precursor for thiol disulfide interchange. The hydrophobic/hydrophilic nature of the amino acid changed at the mutation site in microtus strain 91001. The *YP_2041* gene encodes a transcriptional regulatory protein of the *AraC* protein family, which is involved in signal storage and processing. *YP_2041* may affect the acid resistance of the bacteria and consequently play a positive regulatory role in bacterial virulence[8].

The *YP_1914* gene encodes an RNA helicase of the *DEAD* box protein family. Compared to other strains, microtus strain 91001 lacks a 510 bp fragment of the *YP_1914* gene. RNA helicase is involved in all intracellular RNA metabolic processes, and plays numerous indispensable roles in the growth and development of cells. RNA helicase is also involved in the formation and degradation of mRNA and plays a crucial role in the growth rate of the entire cell[9].

In addition, as we compared the point mutations with the 933 SNPs discovered by Morelli[10], we found that 7 SNPs are the same, one of them is *YP_0950* mutated in the microtus strains isolated from focus L but not in the strains isolated from focus M. Other 6 SNPs were unique and present in the microtus strains isolated from both foci investigated in this study. The 7 SNPs were all in branch III-0.PE4 in the minimal spanning tree. These 7 SNPs play an important role in the minimal spanning tree and can be used in genotyping in microtus and other biovars isolates.

In conclusion, the 12 identified gene mutations can be used in genotyping in microtus and other biovars and they may have affected the novel virulence determinants of *Y. pestis* and may also be responsible for the low level of virulence of microtus strain 91001 in humans.

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REFERENCES

1. Zhou D, Tong Z, Song Y, et al. Genetics of metabolic variations between Yersinia pestis biovars and the proposal of a new biovar, microtus. J Bacteriol, 2004; 186, 5147-52.
2. Fan Z, Luo Y, Wang S, et al. Microtus brandti plague in the Xilin Gol Grassland was inoffensive to human, Chin J Control Endemic Dis, 1995; 10, 56-7.
3. Wang S, Chen J, Mao F, et al. A study on growth curves of different ecotypes of Yersinia pestis in human sera. Monograph of Pestis, Chinese Academy of Medical Science, 1983; 12, 52-8.
4. Song Y, Tong Z, Wang J, et al. Complete genome sequence of Yersinia pestis strain 91001, an isolate avirulent to humans. DNA Res, 2004; 11, 179-97.
5. Derbise A, Chenal-Francisque V, Huon C, et al. Delineation and analysis of chromosomal regions specifying Yersinia pestis. Infect Immun, 2010; 78, 3930-41.
6. Shen X, Wang Q, Xia L, et al. Complete genome sequences of Yersinia pestis from natural foci in China. J Bacteriol, 2010; 192, 3551-2.
7. Villanelo F, Ordenes A, Brunet J, et al. A model for the Escherichia coli FtsB/FtsL/FtsQ cell division complex. BMC Struct Biol, 2011; 11, 28.
8. Frota CC, Papavinasasundaram KG, Davis EO, et al. The AraC family transcriptional regulator Rv1931c plays a role in the virulence of Mycobacterium tuberculosis. Infect Immun, 2004; 72, 5483-6.
9. Fuller-Pace FV. DExD/H box RNA helicases: multifunctional proteins with important roles in transcriptional regulation. Nucleic Acids Res, 2006; 34, 4206-15.
10. Morelli1 G, Song Y Mazzoni CJ, et al. Phylogenetic diversity and historical patterns of pandemic spread of Yersinia pestis. Nat Gene, 2010 ; 42, 1140-3.