Pseudogene accumulation might promote the adaptive microevolution of *Yersinia pestis*

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Plague is a natural focus-based disease, and for better understanding of this disease it is crucial to determine the molecular mechanisms of its pathogen, *Yersinia pestis*, for adapting to different foci. Gene inactivation, loss and acquisition are the main mechanisms that contribute to a pathogen's fitness. Determination of the whole-genome sequences of three *Y. pestis* strains, CO92, KIM and 91001, provided a good opportunity to probe into its genome in minute detail. Many genetic variations were found between the three strains. The present work focused on adaptive microevolutionary analysis of *Y. pestis* from different natural plague foci in China based on pseudogene profiles. Twenty-four mutations that led to inactivation in the corresponding genes were analysed, and a PCR-based screening method was employed to investigate the distribution of these mutations among *Y. pestis* isolates from different foci and also among seven strains of *Yersinia pseudotuberculosis*. It was found that *Y. pestis* isolates from the same focus had identical mutation profiles, and 260 isolates of *Y. pestis* were divided into eight genotypes, while *Y. pseudotuberculosis* harboured wild-type alleles for all the mutations. The isolates of three known biovars were grouped into distinct branches in the phylogenetic tree, which supports the proposition that biovars *mediaevalis* and *orientalis* directly arose from biovar *antiqua* individually. The constructed phylogenetic tree suggests that the isolates from focus B should be the oldest lineage of *Y. pestis* in China except for isolates from foci L and M, which might be a special lineage of *Y. pestis* and originated differently to the others.

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INTRODUCTION

The causative agent of plague, *Yersinia pestis*, is a Gram-negative bacterium evolved from *Yersinia pseudotuberculosis* 1500–20 000 years ago, shortly before the first known pandemics of human plague (Achtman *et al.*, 1999; Skurnik *et al.*, 2000). There have been three historical human plague pandemics, which have killed about 200 million people (Perry & Fetherston, 1997). Plague is circulating in most foci, although the human plague pandemic has been con-

trolled. The recent outbreak of human plague and appearance of multidrug-resistant strains suggest that plague still poses a significant threat to public health (Galimand *et al.*, 1997; Ratsitorahina *et al.*, 2000).

The environments, host and vector of each plague focus differ markedly, and these are the driving forces that contribute to the final shape of bacterial genomes. To adapt to a different niche, *Y. pestis* has to develop different mechanisms to change its genome, such as gene acquisition or loss and point mutation (Hinchliffe *et al.*, 2003; Radnedge *et al.*, 2002). The accumulation of these changes allowed *Y. pestis* to diversify into different genotypes. The epidemiological, aetiological and virulence features are different for each genotype. It will be very useful to the control and prevention of plague if the genotype of an isolate can be

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Abbreviations: DFR, difference region; IS, insertion sequence.

Supplementary tables giving isolate numbers and PCR results in full, and a supplementary figure illustrating the geographical distribution of genotypes and biovars are available in JMM Online.

quickly determined and used to track down the origin of pathogens.

The whole-genome sequences of two fully virulent *Y. pestis*, CO92 and KIM, were decoded in 2001 and 2002 (Deng *et al.*, 2002; Parkhill *et al.*, 2001). The complete genome sequence of another strain of *Y. pestis*, 91001, which is avirulent to humans, has been finished recently in our laboratory (Song *et al.*, 2004). These genome data provide a good opportunity to study the polymorphisms of the *Y. pestis* genome. A large number of pseudogenes were identified in the genomes of the three *Y. pestis* strains. Gene inactivation is one of the mechanisms for *Y. pestis* to adapt to new niches (Kukkonen *et al.*, 2004; Oyston *et al.*, 2003; Perry *et al.*, 1998; Simonet *et al.*, 1996; Skurnik *et al.*, 2000).

METHODS

Bacterial strains. There are 11 plague foci in China and many *Y. pestis* have been isolated from these foci in the past years. Two hundred and sixty isolates of *Y. pestis*, isolated between 1943 and 2001 from 10 natural plague foci in China, were included in this study (Table 1 and Supplementary Table S1, available in JMM Online). Four subfoci of the *Marmota himalayana* plague focus of the Qinghai-Tibet plateau were investigated separately and referred to as foci C, D, G and K. All of these isolates were collected by the Qinghai Center for Disease Prevention and Control, China. In addition, seven strains of *Y. pseudotuberculosis* (CMCC53518, CMCC53519, CMCC53520, CMCC53521, CMCC53522, ATCC29833 and CMCC53502) were included, the first five belonging to biotypes I to V, respectively. These strains were originally from the China Medical Culture Collection except for ATCC29833, which was purchased from the American Type Culture Collection, and their dates of isolation and origins are unknown. All the strains were grown in Luria-Bertani broth. The extraction of genomic DNA was performed using a conventional phenol/chloroform extraction method (Adair *et al.*, 2000).

Comparative analysis of pseudogenes and primer design. The genome sequence data for *Y. pestis* CO92 (AL590842), KIM (AE009952) and 91001 (AE017042) were downloaded from GenBank. The nucleotide sequences of all predicted pseudogenes on the chromosomes of the three *Y. pestis* strains were extracted and compared with their alleles using BLAST and CLUSTAL_X to find detailed variations between different strains. (There are 149, 54 and 141 pseudogenes predicted in CO92, KIM and 91001, respectively). Primers were designed to discriminate between wild-type and mutant in each mutation site using Primer 5.0 (Tables 2 and 3).

Allele-specific PCR. The genomic DNAs of 260 *Y. pestis* isolates and seven *Y. pseudotuberculosis* strains were pre-arrayed in 96-well plates with 10 ng per well. Each primer pair was pre-tested to ensure the specificity of amplification with CO92 and 91001 genomic DNA as template. The 30 µl PCR reaction mixture contained 50 mmol l⁻¹ KCl, 10 mmol l⁻¹ Tris/HCl (pH 8.0), 2.0 mmol l⁻¹ MgCl₂, 0.001 % gelatin, 0.1 % BSA, 100 µmol l⁻¹ each of dATP, dCTP, dGTP and dTTP, 0.3 µmol l⁻¹ of each primer, 1 unit of *Taq* DNA polymerase (MBI) and 10 ng of template DNA. The parameters for amplification were as follows: pre-denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 1 min, and a final extension at 72 °C for 5 min to ensure the complete extension of the amplicons. Minor changes of cycling parameters were made for different primer pairs. After amplification, 15 µl of each PCR product was subjected to electrophoresis on a 1.2 % agarose gel and visualized by ethidium bromide staining and UV

irradiation. There were positive, negative and blank controls in each 96-well plate.

Data analysis. For every primer pair, each isolate was scored as '+' for positive amplification or '-' for negative amplification (Supplementary Table S2, available in JMM Online). Then the amplification results of all the primer pairs that were used to study the same mutation site were integrated to determine the wild-type or mutant status for each isolate. For every mutation site that was analysed, a score of '1' was given for wild-type and '0' for mutant (Table 4). The similarity of mutation profiles was analysed using PHYLIP. The phylogenetic tree was reconstructed with *Y. pseudotuberculosis* as an outgroup (Fig. 3).

RESULTS AND DISCUSSION

Mechanisms of gene inactivation

There are three kinds of mutation that lead to gene inactivation: nonsense mutation, frameshift and disruption by insertion of insertion sequence (IS) elements. Fig. 1(a) shows the primers designed to discriminate wild-type and non-sense-mutation alleles. Primer pairs LW/R and LM/R should give positive and negative amplification results, respectively, for the wild-type, and the opposite results for the mutant. If both primer pairs give no product it is very likely that the target gene is absent from the genome, for gene acquisition and loss are common in *Y. pestis* (Hinchliffe *et al.*, 2003; Radnedge *et al.*, 2002).

Fig. 1(b) shows an example of primers designed to discriminate wild-type and frameshift mutation alleles, in which a change was caused by a 19 bp fragment insertion. The 3' end of primer LM is located on the inserted fragment, while the primer LW flanks the two sides of the insertion site. The primer pairs LW/R and LM/R should give negative and positive amplification results, respectively, in the mutant allele, but they should give the reverse results in the wild-type one.

The two disrupted parts of a gene that was interrupted by an IS element insertion either flank the two sides of the inserted IS element (mutant 1) or are located remotely due to genome rearrangement (mutant 2) (Fig. 1c). The primer pairs L1/R2 and L2/R1 should give identical amplicons in mutants 1 and 2, but no product in the wild-type. Primer pair L1/R1 should produce an amplicon for mutant 1 that is much longer than that for the wild-type, while giving no product in mutant 2.

Pseudogene distribution in *Y. pestis* strains CO92, KIM and 91001

Different annotators have taken different gene prediction rules, so there are many differences in definition and numbers of pseudogenes between the three *Y. pestis* strains. For example, YPO3775 was predicted as a pseudogene in CO92 due to IS100 element insertion and its allele (YP3274) was interrupted by IS100 and predicted as a pseudogene in 91001 too, but the homologous sequences of the two disrupted parts were predicted as two intact coding sequences in KIM (y0455, y0458). To employ more information, we took the longest sequence to be the whole length of a gene if they were

Table 1. Isolates of *Y. pestis* used in this study

Focus code	Biovar	Main host	Geographic region (province)	No. of isolates*	Focus name
A	<i>Antiqua</i>	<i>Marmota caudata</i>	Xinjiang	11	<i>Marmota caudata</i> plague focus of the Pamirs plateau
B	<i>Antiqua</i>	<i>Marmota baibacina</i> and <i>Spermophilus undulatus</i>	Xinjiang	30	<i>Marmota baibacina</i> - <i>Spermophilus undulatus</i> plague focus of the Tianshan mountains
C	<i>Antiqua</i>	<i>Marmota himalayana</i>	Qinghai, Gansu and Tibet	41	<i>Marmota himalayana</i> plague focus of the Qinghai-Gansu-Tibet grassland
D	<i>Antiqua</i>	<i>Marmota himalayana</i>	Qinghai and Gansu	16	<i>Marmota himalayana</i> plague focus of the Qilian mountain
E	<i>Antiqua</i>	<i>Apodemus chevrieri</i> and <i>Eothenomys miletus</i>	Yunnan	10	<i>Apodemus chevrieri</i> - <i>Eothenomys miletus</i> plague focus of the highland of Northwestern Yunnan province
F	<i>Orientalis</i>	<i>Rattus flavipectus</i>	Yunnan, Guangdong, Fujian, Guizhou and Guangxi	22	<i>Rattus flavipectus</i> plague focus of the Yunnan-Guangdong-Fujian provinces
G	<i>Antiqua</i>	<i>Marmota himalayana</i>	Tibet	11	<i>Marmota himalayana</i> plague focus of the Gangdisi mountains
H	<i>Antiqua</i>	<i>Spermophilus dauricus</i>	Heilongjiang, Jilin and Liaoning	30	<i>Spermophilus dauricus</i> plague focus of the Song-Liao plain
I	<i>Mediaevalis</i>	<i>Meriones unguiculatus</i>	Inner Mongolia, Ningxia and Hebei	21	<i>Meriones unguiculatus</i> plague focus of the Inner Mongolian plateau
J	<i>Mediaevalis</i>	<i>Spermophilus dauricus</i> <i>alaschanicus</i>	Gansu and Ningxia	20	<i>Spermophilus dauricus alaschanicus</i> plague focus of the Loess plateau in Gansu and Ningxia provinces
K	<i>Mediaevalis</i>	<i>Marmota himalayana</i>	Xinjiang	9	<i>Marmota himalayana</i> plague focus of the Kunlun mountains
L	<i>Mediaevalis</i>	<i>Microtus brandti</i>	Inner Mongolia	10	<i>Microtus brandti</i> plague focus of the Xilin Gol grassland
M	<i>Mediaevalis</i>	<i>Microtus fuscus</i>	Qinghai and Sichuan	29	<i>Microtus fuscus</i> plague focus of the Qinghai-Tibet plateau

*For the year of isolation and isolate numbers see Supplementary Table S1 (available in JMM Online).

Table 2. Oligonucleotide primers used

Primer	Sequence (5'→3')
IS100-c-L	actcattccctgctgtg
IS100-c-R	cactgacctcagcgatgc
IS285-c-L	atctcaatacattcttcggc
IS285-c-U	aacgctgtttcgacggtaagc
YPO0089-c-L	ccagcgggttattgagtg
YPO0090-c-R	gatgccaccgattctgag
YPO0098L	gatcgtggacatcgctgg
YPO0098R	gcaaggcgcaggatcatg
YPO0170L	gtatccgcattgcatcag
YPO0170R	cttcggtcgtagcatcg
YPO1337L	gccgttatctggtatg
YPO1337R	gctttcaccgcatcaatg
YPO1380L	ctccacacaactgctcag
YPO1380R	ctgaactgaccagcaacg
YPO1562R	gccgaatgagtaagacca
YPO1582F	aacatcatcatagccattaacg
YPO1582R	gttgacgggtgtagagtattcc
YPO1676LM	aagtcgtaatttagcat
YPO1676LW	aagtcgtaatttagcac
YPO1676R	gcatgctggcctgctctt
YPO1728L	cgtagccatttacccaag
YPO1728R	gccagaggacctgtagtt
YPO1752L	gtccttactgccagctcg
YPO1752R	atgctgagcagaggatg
YPO1923L1M	caggttttctttacgtac
YPO1923L1W	caggttttctttacgtag
YPO1923R1	atggcattatcatcgcg
YPO1967L	aggcgcatatcatcatc
YPO1967R	ataacgcttagatctac
YPO1923LM	tgaagccccagacaccag
YPO1923LW	tgaagccccagacaccat
YPO1923R	ctccactcactggttagg
YPO2034L	aatcgttcaacagcctac
YPO2034R	tgctccacattacctg
YPO2142L	tattattctcgccacctc
YPO2142RM	ggcgaccgccaacaatta
YPO2142RW	ggcgaccgccaacaattc
YPO2309L	tgagtataaactgaccg
YPO2309R	atcaagttcgatctcacg
YPO2398-L	aactgcgactcaacactg
YPO2398-R	tgccgttgaaactgatgg
YPO2493L	caatatgaaccagacgc
YPO2493R	gcacattgaacattgagc
YPO2493RW	aagccgtggaagaggac
YPO2572L	gaactgagctattccaag
YPO2572R	gagcgtttggcattggtg
YPO2943L	gcgataggtaacgcgttc
YPO2943R	ctgggtcgatcgactc
YPO3049F	tgctggcgacctaactctatc
YPO3049RM2	gagccgttcgcatcagcatgc
YPO3049RW2	gagccgttcgcatcagcatgt
YPO3250L	acgtgaatgcgcagagt

Table 2. cont.

Primer	Sequence (5'→3')
YPO3250R	atgaccacgcccataacg
YPO3775L	tggcaagatagtgctcag
YPO3775R	taactcaaccagccgga
YPO4008L	gtaccgttctgtttggcg
YPO4008R	gccgtgtatgctcctcag

different in the three strains. For example, we took y0455 and y0458 as a pseudogene in KIM, and so the total number of pseudogenes present in Fig. 2 is greater than the predicted number of 54.

It was found that pseudogene profiles were different in the three *Y. pestis* strains (Fig. 2). Ninety-one genes were inactivated in all the three strains, while 22, 10 and 39 pseudogenes were specific to CO92, KIM and 91001, respectively. It is very interesting that 23 pseudogenes are shared by CO92 and KIM although they belong to different biovars, but only two pseudogenes are shared by 91001 and KIM while they belong to the same biovar. The distribution of pseudogenes suggests that 91001 and KIM might have originated differently.

All of these pseudogenes are non-essential for the survival of *Y. pestis*, but their inactivation may relate to the adaptation of *Y. pestis* to different niches (Kukkonen *et al.*, 2004; Oyston *et al.*, 2003; Perry *et al.*, 1998; Simonet *et al.*, 1996; Skurnik *et al.*, 2000), and so it can be hypothesized that pseudogene profile should have some correlation with an isolate's geographic origin and could disclose the adaptive micro-evolution of *Y. pestis*. Therefore we focused on the gene inactivation mutations and tried to use them as genetic markers for genotyping and analysis of the adaptive micro-evolution of *Y. pestis* in different natural plague foci.

In total, 24 mutations in 24 genes were screened in this study (Table 3), including 17 IS element insertions, two frame-shifts, one in-frame deletion, one fragment deletion, two nonsense mutations and one nonsynonymous mutation, which represent all mechanisms of mutation found in *Y. pestis*. The pseudogenes involved in this study were classified into six groups according to the strains in which they were present (Fig. 2). The first three groups contain genes only inactivated in one strain, the fourth contains genes inactivated in all three strains, the fifth contains those inactivated in both CO92 and KIM, and the sixth those inactivated in both CO92 and 91001. For two genes, YPO3367 and YPO3720, which are inactivated both in 91001 and in KIM due to frameshifts, allele-specific primers that discriminate the wild-type and mutant cannot be designed because the two frameshifts occurred within a homopolymeric tract of 11G and 7C, respectively.

Table 3. Genetic variations analysed in this study and allele-specific PCR-screening resultsGene codes and descriptions of product are according to *Y. pestis* CO92.

Gene	Variation (91001/CO92/KIM)	Product	Amplification result
YPO0089-YPO0090	Intact/940 bp deletion/intact	GlpX protein glycerol kinase	No isolate had this 940 bp deletion
YPO0098	Intact/disrupted by IS100/intact	ABC-transport protein, ATP-binding component	The allele was disrupted by IS100 in isolates from focus F
YPO0170	Intact/disrupted by IS100/intact	Acetylornithine aminotransferase	The allele was intact in all <i>Y. pestis</i> isolates
YPO1337	Intact/disrupted by IS100/intact	Arginine-binding periplasmic protein 2 precursor	The allele was disrupted by IS100 in isolates from focus F
YPO1380	Intact/intact/disrupted by IS100	Putative membrane protein	The allele was disrupted by IS100 in isolates from foci G, H, I, J and K
YPO1562	Intact/disrupted by IS285/disrupted by IS285	Putative intimin	The allele was intact in isolates from foci L, M
YPO1582	Intact/disrupted by IS285/disrupted by IS285	Lactose permease	The allele was intact in isolates from foci L, M and B
YPO1676	Intact/nonsense mutation/intact	Methyl-accepting chemotaxis protein	The nonsense mutation was specific to isolates of biovar <i>orientalis</i>
YPO1728	Intact/disrupted by IS100/disrupted by IS100	Putative transcriptional regulator of sugar transport	The allele was intact in isolates from foci L, M and B
YPO1752	Intact/disrupted by IS100/intact	Hypothetical protein	The allele was intact in all <i>Y. pestis</i> isolates
YPO1923-1	Intact/18 bp in-frame deletion/18 bp in-frame deletion	Putative sensor protein	The allele was intact in isolates from foci L and M
YPO1923-2	Intact/frameshift/intact	Putative sensor protein	The thymidine deletion was specific to isolates of biovar <i>orientalis</i>
YPO1967	Intact/disrupted by IS100/disrupted by IS100	Porin	The allele was intact in isolates from foci L, M and B
YPO2034	All were disrupted by IS1541	Putative ABC transporter ATP-binding protein	The allele was disrupted by IS1541 insertion in all <i>Y. pestis</i> isolates
YPO2142	Intact/intact/nonsense mutation	Na ⁺ /H ⁺ antiporter	The nonsense mutation was specific to isolates from focus K
YPO2309	Disrupted by IS100/intact/intact	Two-component regulatory system, sensor kinase protein	The allele was disrupted by IS100 in isolates from foci L and M
YPO2398	Disrupted by IS100/disrupted by IS100/intact	Putative exported protein	The allele was intact in isolates from foci G, H, I, J and K
YPO2493	Intact/intact/frameshift	Putative dioxygenase alpha subunit	The frameshift was specific to isolates from foci G, H, I, J and K
YPO2572	Intact/disrupted by IS285/intact	Bifunctional penicillin-binding protein 1C	The allele was disrupted by IS285 in isolates from foci C, D, E and F
YPO2943	All were disrupted by IS285	Outer membrane usher protein	The allele was disrupted by IS285 in all <i>Y. pestis</i> isolates
YPO3049	Nonsynonymous mutation: CGC/CAC/CAC	Putative binding protein-dependent transport system, inner-membrane component	The allele was CGC in isolates from foci L, M and B
YPO3250	All were disrupted by IS100	Putative amino acid transaminase	The allele was intact in isolates from foci G and H
YPO3775	All were disrupted by IS100	Deoxyribonuclease TatD	The allele was intact in isolates from focus B
YPO4008	Intact/disrupted by IS100/disrupted by IS100	Two-component system, sensor kinase	The allele was intact in isolates from foci L, M and B

Table 4. Digitized mutation profile of eight genotypes and comparison with typing result based on DFR profiles

Focus or strain*	Genotype	Genomovar†	Gene score‡																		
			A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
<i>Y. pseudotuberculosis</i>			1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
A	2	04	1	1	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1
B	1	01, 02, 03	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
C	4	05, 06, 08	1	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1
D	4	08	1	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1
E	4	07	1	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1
F	7	09	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
<i>Y. pestis</i> CO92	7		1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
G	3	10	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	1	0	0	1
H	3	10	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	1	0	0	1
I	5	11, 12	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	1
J	5	11, 13	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	1
K	6	11	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0
<i>Y. pestis</i> KIM	6		1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0
L	8	14	0	0	0	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
M	8	14	0	0	0	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1

*The codes for the foci are as in Table 1.

†Genomovars were typed based on 22 DFR profiles (Zhou *et al.*, 2004b).

‡Genes are as follows: A, YPO2309; B, YPO3049; C, YPO2398; D, YPO3250; E, YPO1562; F, YPO1923-1; G, YPO1582; H, YPO1728; I, YPO1967; J, YPO4008; K, YPO3775; L, YPO2572; M, YPO0098; N, YPO1337; O, YPO1676; P, YPO1923-2; Q, YPO1380; R, YPO2493; S, YPO2142.

Fig. 1. (a) Schematic diagram of primers designed for identifying a nonsense mutation. The gene was inactivated in the mutant due to a nonsense mutation (tac/taa). (b) Schematic diagram of primers designed for identifying a frameshift. The gene was inactivated in the mutant due to a 19 bp fragment insertion (cgatgctggtttctttg). (c) Schematic diagram of primers designed for identifying genes disrupted by an insertion sequence. The gene was disrupted by an IS element insertion in mutants 1 and 2, and in mutant 2 the two disrupted parts are located remotely on the chromosome due to a genome rearrangement.

Fig. 2. Distribution of pseudogenes in three strains of *Y. pestis*. The number of pseudogenes harboured by each strain or shared with the neighbouring strains and (in parentheses) the number of pseudogenes analysed in this study are shown.

Genotyping of homologous isolates of *Y. pestis* based on mutation profile

Five of the 24 mutations analysed showed identical alleles in the 260 *Y. pestis* isolates. Three of these five mutations (YPO1752, YPO0170 and YPO0089-YPO0090) may be specific to *Y. pestis* CO92, while inactivation of the other two genes (YPO2943 and YPO2034) may have accelerated the speciation of *Y. pestis* because both of them are intact in *Y. pseudotuberculosis*. These five mutations were discarded from further genotyping and evolutionary analysis, because they have no discriminatory power among the *Y. pestis* isolates from different geographic areas (Table 3). A 0/1 matrix for the 260 *Y. pestis* isolates and the seven *Y. pseudotuberculosis* strains was constructed (Table 4) by using the remaining mutation profiles. From the results of this PCR-based genotyping method, it was found that isolates from the same focus had identical mutation profiles, and there were several mutations specific to isolates from the same focus.

The 260 *Y. pestis* isolates were grouped into eight genotypes based on their mutation profiles (Table 4). *Y. pestis* CO92, 91001 and KIM showed identical mutation profiles to genotypes 7, 8 and 6, respectively, and so they were assigned into the corresponding genotype. The seven *Y. pseudotuberculosis* strains harboured the wild-type alleles for all the mutations analysed, and they were assigned into a single genotype and used as an outgroup in the following phylogenetic analysis. The phylogenetic tree of the nine genotypes was reconstructed using PHYLIP MIX algorithm (Fig. 3).

Based on whole-genome DNA microarray hybridization, Zhou *et al.* (2004b) have identified 22 difference regions (DFRs) in the *Y. pestis* genome. The 260 *Y. pestis* isolates were divided into 14 genomovars based on DFR profile typing, and a paradigm of plague transmission, colonization and expansion in China was deduced (Zhou *et al.*, 2004b). The results of the two typing methods that were based on different molecular genetic markers agreed with each other (Table 4).

Fig. 3. Reconstructed phylogenetic tree based on mutation profile using PHYLIP MIX algorithm. The upper-case letters in parentheses indicate the focus code as listed in Table 1.

However, DFR typing had more discriminatory power than the mutation profile, since some isolates of one genotype were divided into two or more genomovars.

Many genotyping methods that are based on different molecular biological techniques have been applied to typing *Y. pestis*, such as ribotyping, RFLP and PFGE (Guiyoule *et al.*, 1994, 1997; Huang *et al.*, 2002). Ribotyping and RFLP analysis provide information about the local genome environments of specific gene sequences on the basis of the probe used. PFGE separates DNA fragments upon digestion of the chromosome with a restriction endonuclease that cleaves infrequently. All of these methods rely on the separation of different length DNA fragments using electrophoresis, which cannot resolve the co-migration of DNA bands and presence of too small or too large bands. Recently, a variable-number tandem repeat (VNTR) technique was used to type *Y. pestis* (Adair *et al.*, 2000), and a typing method based on divergence of IS100 locations has also been reported (Motin *et al.*, 2002).

The genotyping method described here is based on the mutations that led to gene inactivation, which may relate to *Y. pestis* phenotype and niche adaptation. These mutations not only include IS element insertions, but also include frameshift and nonsense mutations, which represent all possible mechanisms of pseudogene evolution that were employed by *Y. pestis* to reshape its genome to adapt to a new niche.

Different lifestyles determine different genotypes

Plague is a typical natural focus-based disease. Long-term survival of *Y. pestis* and periodic epidemics of animal plague both occur in natural plague foci. Plague primarily affects rodent reservoirs, and disease transmission among rodents is accomplished by the bites of flea vectors.

The lifestyle of *Y. pestis* can be viewed as its surrounding environment and the relationships that it establishes with rodent reservoirs and flea vectors. The lifestyles in different foci vary markedly, although the rodent reservoirs or flea vectors may be the same in different foci, which determine the final shape of the *Y. pestis* genome.

Y. pestis isolates from the same focus showed identical mutation profiles and were always grouped into one genotype (Table 4). We did not find that isolates from one focus were split into different genotypes that were shared by isolates from different foci, which suggests that adaptation of *Y. pestis* to a particular lifestyle contributes to the reshaping of its genome and leads to the accumulation of mutations within functional genes. However, isolates from adjacent foci (foci C, D and E; foci I and J) or geographically distant foci (foci L and M; foci H and G) did have identical mutation profiles and belonged to the same genotype (Supplementary Fig. S1, available in JMM Online). This may be due to the lifecycle of adjacent foci having some common feature in the rodent reservoir or environment. They might be grouped into different genotypes with more molecular markers. These mutated genes are non-essential

for survival of *Y. pestis* under the selective pressure exerted by its specific lifestyle. The different selective pressures correlated with the different lifestyles account for the discrete segregation of genotypes of *Y. pestis*.

Gene inactivation accelerates the formation of different biovars

Y. pestis have been historically divided into three biovars, *antiqua*, *mediaevalis* and *orientalis*, according to their ability to ferment glycerol and to reduce nitrate (Achtman *et al.*, 1999). This kind of biovar assignment is based on their metabolic variations, but does not seem to correlate with virulence. In this study, the isolates of the three biovars were clustered into four distinct branches in the phylogenetic tree (Fig. 3), which strongly supports the notion that biovars *mediaevalis* and *orientalis* directly arose from biovar *antiqua* individually (Gonzalez *et al.*, 2002).

Focus F covers a large area in the southeast of China and has been thought of as the third human plague pandemic origin (Supplementary Fig. S1, available in JMM Online). *Y. pestis* isolates from this focus are of biovar *orientalis*. All the tested isolates showed identical mutation profiles (genotype 7) and had accumulated some specific mutations. YPO0098, YPO1337 and YPO1676 were found to have become pseudogenes only in biovar *orientalis* isolates. The inactivation of these genes, leading to the unique mutation profile (genotype) of biovar *orientalis* strains, might have correlated with the emergence of biovar *orientalis* from *antiqua* and promoted its adaptation to the newly expanded niche.

The specific origin of isolates from foci L and M

All of the *Y. pestis* isolates from foci L and M had an identical mutation profile and were grouped into genotype 8, although these two plague foci are geographically distant (Supplementary Fig. S1, available in JMM Online). The isolates from focus B showed an identical mutation profile and were grouped in genotype 1. A nonsynonymous mutation within YPO3049 was found only in the *Y. pestis* isolates from foci L, M and B, whereas the interruptions of YPO1582, YPO1728, YPO1967 and YPO4008 by the IS element were found in all *Y. pestis* isolates except for those from foci L, M and B. The clade formed by *Y. pestis* isolates from foci L and M (genotype 8) and the clade of the isolates from focus B (genotype 1) diverged next to *Y. pseudotuberculosis* in the phylogenetic tree (Fig. 3). Given the fact that *Y. pestis* is a clone recently evolved from *Y. pseudotuberculosis*, it can be speculated that isolates of genotypes 1 and 8 are the oldest lineages of *Y. pestis* isolates used in this study. The isolates from focus B are of biovar *antiqua*, and so this is consistent with the conclusion that biovars *mediaevalis* and *orientalis* originated directly from biovar *antiqua*. However, it is striking that isolates of genotype 8 are the oldest lineages, because the isolates of genotype 8 are of biovar *mediaevalis* according to the conventional biotyping method.

The isolates from foci L and M (genotype 8) have accumulated mutations that are unique except for some shared with

genotype 1, such as YPO2309, which encodes a two-component regulatory system sensor kinase protein interrupted by IS100 that is intact in the isolates from other foci, and another seven unique mutations identified in our previous study (Zhou *et al.*, 2004a). The isolates from these two foci showed identical biochemical features (they do not ferment arabinose) and almost identical genomic content as suggested by DNA microarray hybridization, besides the above unique mutation profile (Zhou *et al.*, 2004b). Another interesting fact is that human plague has never been reported in these two foci. Isolates from these foci are thought to be of low pathogenicity to humans and *Y. pestis* strain 91001 isolated from focus L has even been verified as avirulent to human beings by volunteer trial through subcutaneous inoculation (Fan *et al.*, 1994, 1995). Based on these unique features, we propose that the isolates from these two foci are a special old lineage of *Y. pestis* and speciated differently to others. The main host is *Microtus brandti* in focus L and *Microtus fuscus* in focus M, so these strains were proposed as a new biovar *microtus* based on three biochemical features (*microtus*, negative for reducing nitrate, ferment glycerol and negative for fermenting arabinose; *antiqua*, reduce nitrate, and ferment glycerol and arabinose; *mediaevalis*, negative for reducing nitrate, ferment glycerol and arabinose; *antiqua*, reduce nitrate, negative for fermenting glycerol but positive for fermenting arabinose) (Zhou *et al.*, 2004a).

The accumulation of pseudogenes promotes speciation of *Y. pestis*

For all analysed mutations, the wild-type alleles were found in all of the seven *Y. pseudotuberculosis* strains used in this study, which is in accordance with the proposal that *Y. pseudotuberculosis* is the 'young mother' of *Y. pestis* and they cannot be discriminated at the DNA level except for point mutations (Achtman *et al.*, 1999). There is the accumulation of pseudogenes in *Y. pestis* during the course of speciation, such as the O-antigen gene cluster, *yadA* and *inv*; all of these genes are intact in *Y. pseudotuberculosis* but inactivated in *Y. pestis*, which is the outcome of the switch of *Y. pestis* from an enteric lifestyle to a mammalian blood-borne lifestyle (Kukkonen *et al.*, 2004; Oyston *et al.*, 2003; Perry *et al.*, 1998; Simonet *et al.*, 1996; Skurnik *et al.*, 2000). It was revealed in this study that YPO2034 and YPO2943, which encode a putative ABC transporter ATP-binding protein and an outer membrane usher protein, are disrupted by IS1541 and IS285 insertions, respectively, in all *Y. pestis*, but are intact in the seven *Y. pseudotuberculosis* strains. These two genes may be non-essential or even harmful to *Y. pestis* and so gene inactivation would be an adaptive mutation for *Y. pestis* to evolve from *Y. pseudotuberculosis* and adapt to a new niche.

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