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Characterisation of *Yersinia pestis* isolates from natural foci of plague in the Republic of Georgia, and their relationship to *Y. pestis* isolates from other countries

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ABSTRACT

Forty *Yersinia pestis* isolates from endemic foci of plague in the Republic of Georgia, and six *Y. pestis* isolates from neighbouring former Soviet Union countries, were analysed for their biochemical and phenotypic properties, and their genetic relatedness was compared with *Y. pestis* strains KIM and CO92 by pulsed-field gel electrophoresis (PFGE). In addition, 11 *Y. pestis* isolates from the USA, together with published nucleotide sequences from *Y. pestis* strains KIM, CO92 and 91001, were compared with the 46 isolates in the present collection using multilocus sequence typing (MLST), based on sequence data for the 16S rRNA, *hsp60*, *glnA*, *gyrB*, *recA*, *manB*, *thrA* and *tmk* loci. Four virulence gene loci (*caf1*, *lcrV*, *psaA* and *pla*) were also sequenced and analysed. Two sequence types (ST1 and ST2), which differed by a single nucleotide, were identified by MLST. With the exception of a single isolate (771G), all of the Georgian *Y. pestis* isolates belonged to ST2. PFGE also grouped the Georgian *Y. pestis* isolates separately from the non-Georgian isolates. Overall, PFGE discriminated the *Y. pestis* isolates more effectively than MLST. The sequences of three of the four virulence genes (*lcrV*, *psaA* and *pla*) were identical in all Georgian and non-Georgian isolates, but the *caf1* locus was represented by two allele types, with *caf1* NT1 being associated with the non-Georgian isolates and *caf1* NT2 being associated with the Georgian isolates. These results suggest that Georgian *Y. pestis* isolates are of clonal origin.

Keywords Clonal relatedness, Georgia, multilocus sequence typing, pulsed-field gel electrophoresis, typing, *Yersinia pestis*

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INTRODUCTION

Yersinia pestis, the aetiological agent of plague, is a fairly young species that is thought to have evolved from *Yersinia pseudotuberculosis* (a cause of mesenteric lymphadenitis in humans, but considered primarily to be an animal pathogen) within the last 1500–20 000 years, shortly before the first known pandemics [1]. *Y. pestis* has been endemic in many countries of Europe and Asia for several centuries, but was not introduced into the USA until the turn of the 20th century, when it entered through the port of San Francisco. Because of the relatively recent introduction of *Y. pestis* into the USA, the genetic and phenotypic diversity of American *Y. pestis* isolates is relatively restricted compared to *Y. pestis* isolates from central and eastern Asia [2], and most of the American isolates that have been characterised to date are fairly homogeneous [1,3,4].

In the USA and much of the developed world, the genetic organisation, virulence mechanisms

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and life-cycle of *Y. pestis* have been extensively studied using a few *Y. pestis* isolates [5], some of which have been fully sequenced [6,7]. However, there is a striking paucity of data concerning *Y. pestis* isolates from some other parts of the world, including the former Soviet Union (FSU) [2,8]. In this context, although natural foci of plague have existed in the FSU for several centuries (possibly since the first pandemic), human cases of plague in the FSU were not reported to the WHO until 1989 [9]. At the present time, 43 natural foci of plague are recognised in the southern and south-eastern regions of the FSU, covering *c.* 2×10^8 ha [2]. Twelve of these foci are located in the Caucasian region, with two being located in the Republic of Georgia (Georgia), i.e., the Transcaucasian highland (encompassing the Ninotsminda and Akhalkalaki regions on the Javakheti plateau) and the Iori region (encompassing the eastern part of Georgia, including the border between Georgia and Azerbaijan).

Reports of plague epizootics in Georgia date back to at least the 18th century. Several outbreaks of plague occurred during the 19th century, including an outbreak that started in the village of Larsi in 1807, spread rapidly throughout the Caucasus region, and affected 1596 people (1144 of whom died) over a 6-month period [10]. During the 20th century, plague epizootics in Georgia occurred in the Iori focus (Eldari Valley) during 1966, in the Karayazi Valley during 1968–1971, and in the Transcaucasian highland focus during 1979–1983 and 1992–1997. During the Soviet era, plague surveillance was pursued actively in the FSU (including Georgia), and a large network of anti-plague stations, centres and research institutions throughout the FSU was involved in *Y. pestis* surveillance and research. However, because of difficult economic conditions, surveillance for plague epizootics in Georgia and surrounding regions has recently been reduced drastically. Nevertheless, analysis of several rodent and flea samples obtained from five natural foci of plague in the North Caucasus revealed that all contained *Y. pestis* [11], thus suggesting that the natural foci remain active. However, with a few exceptions [2,12,13], detailed data concerning the Georgian *Y. pestis* population are currently unavailable, and *Y. pestis* isolates from natural foci of plague in Georgia have not been readily available for studies outside the FSU.

The aims of the present study were therefore to elucidate the basic biochemical and phenotypic properties of *Y. pestis* isolates from Georgia, and to characterise and compare the genetic background of the Georgian isolates with their better-characterised non-FSU counterparts. This study is the first report to describe the biochemical and genetic characteristics of *Y. pestis* isolates from natural foci of plague in Georgia and the neighbouring regions during the past 35 years.

MATERIALS AND METHODS

Bacteria

Forty-six *Y. pestis* isolates were obtained from the bacterial strain collection of the National Centre for Disease Control and Public Health (NCDCPH), Tbilisi, Georgia. Of these, 40 isolates were from various natural foci of plague in Georgia during 1966–1997. The remaining six isolates were from neighbouring countries (Azerbaijan, Armenia, etc.), and were collected during 1961–1976 (Table S1; see Supplementary material). The *Y. pestis* collection also included the well-characterised *Y. pestis* strains KIM and CO92 (Table S1). In addition, DNA from 11 *Y. pestis* isolates, obtained from the US CDC and from Brigham Young University (Provo, UT, USA), was compared by multilocus sequence typing (MLST) with the 46 NCDCPH isolates. Finally, sequence data for the appropriate loci from *Y. pestis* strains KIM (biovar Medievalis) [7], CO92 (biovar Orientalis) [6] and 91001 (biovar Medievalis) [14] were obtained from TIGR (http://www.tigr.org) and were analysed *in silico* with the other isolates (Table S1).

Storage and growth conditions, biovar determination and biochemical characterisation

Multiple small aliquots of the isolates were prepared in Luria–Bertani (LB) broth/glycerol (70%/30% v/v) after arrival at the US collaborating laboratories, and stored at −80°C. Each vial was opened once and discarded after use. The isolates were grown (26–30°C for 24–48 h) in LB broth or brain–heart infusion (BHI) broth, and on LB or BHI agar. The biochemical properties of the isolates were determined using the API 20E System (bioMérieux, Durham, NC, USA). Biovars were determined on the basis of glycerol fermentation and nitrate reduction [5]. The substrates used to determine the biochemical properties of isolates and their biovars are listed in Table S2 (see Supplementary material).

Congo red (CR)-binding

The ability of the isolates to bind CR was determined as described previously [15]. In brief, the isolates were grown (28°C for 24 h, without agitation) in BHI broth, and were then inoculated at various dilutions on CR agar (BHI 1% v/v, agar 2% w/v, CR 0.01% w/v) in Petri dishes. Binding of CR was recorded after incubation at 26°C for 4 days. Isolates that formed white/pale colonies were classified as CR-negative, while isolates that formed red colonies were classified as CR-positive.

Antibiotic susceptibility testing

Susceptibility to spectinomycin, streptomycin, gentamicin, doxycycline and ciprofloxacin was determined using BBL Sensi-Disc Antimicrobial Susceptibility Test Discs (Becton Dickinson, Sparks, MD, USA), according to the manufacturer's instructions. For these antibiotics, isolates with zones of inhibition of <14, <11, <12, <12 and <27 mm, respectively, were considered to be resistant, isolates with inhibition zones of >18, >15, >15, >16 and >41 mm, respectively, were considered to be susceptible, and isolates with inhibition zones between the above two cut-off values were considered to be intermediately resistant.

Pulsed-field gel electrophoresis (PFGE)

PFGE was performed essentially as described by the CDC PulseNet protocol for PFGE typing of *Y. pestis* (http://www.cdc.gov/pulsenet/protocols.htm). The DNA in the plugs was digested (37°C for 4–24 h) with *Asc*I or *Xba*I, and PFGE was performed on agarose 1% w/v gels in 0.5× Tris–borate–EDTA buffer (1× Tris–borate–EDTA buffer is 89 mM Tris, 89 mM boric acid, 2 mM EDTA), using a CHEF Mapper apparatus (Bio-Rad Laboratories, Hercules, CA, USA). For the *Asc*I-digested plugs, a low molecular mass standard of 25 kb, a high molecular mass standard of 215 kb, an initial switch time of 1.79 s, a final switch time of 18.66 s and a run time of 18 h were used. For the *Xba*I-digested plugs, a low molecular mass standard of 25 kb, a high molecular mass standard of 290 kb, an initial switch time of 1.79 s, a final switch time of 18.66 s and a run time of 18 h were used.

Virulence genes and genes used for MLST

Eight loci were selected for MLST analysis: 16S rRNA, *hsp60* (encodes a 60-kDa heat-shock protein), *glnA* (glutamine synthetase), *gyrB* (DNA gyrase B subunit), *recA* (DNA repair and recombination), *manB* (phosphomannomutase), *thrA* (bifunctional aspartokinase/homoserine dehydrogenase I) and *tmk* (thymidylate kinase). In addition, loci from the following four virulence genes were analysed: *caf1* (F1 capsule antigen), *lcrV* (V antigen, anti-host protein), *psaA* (antigen fimbrial subunit) and *pla* (coagulase/fibrinolysin precursor).

Primer design and DNA sequencing

The primers used for PCR amplification and sequencing are listed in Table S3 (see Supplementary material). The PCR amplification conditions for all gene fragments comprised 94°C for 5 min, followed by 35 cycles of 94°C for 45 s, 51°C for 45 s and 72°C for 1 min, followed by a final extension at 72°C for 5 min. The amplified fragments were sequenced in both directions using a BigDye Terminator Cycle Sequencing Kit and an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

PFGE and MLST data analysis

PFGE patterns were compared using the Dice coefficient and Molecular Analyst/Fingerprinting Plus Software (Bio-Rad). Clustering of strains was based on the unweighted pair-group method using arithmetic averages (UPGMA) with 1% position tolerance and 1% optimisation; bands <40 kb in size were excluded from the analyses. For sequence-based analyses, reading of trace files and assembly of contigs were performed using the Phred [16,17] and Phrap (available at http://www.washington.edu) programs, respectively. The contigs were viewed with Consed [18], and the resulting DNA sequences were trimmed by removing low-quality nucleotide sequences from the ends; this was followed by sequence alignment using CLUSTALX [19]. The START (sequence type analysis and recombinational tests) program [20] was used to determine the number of polymorphic sites and the guanine + cytosine (G + C) content. Sequence types (STs) were assigned manually, based on the data obtained by the START analysis.

RESULTS AND DISCUSSION

Biochemical identification

The biochemical properties of the 46 FSU isolates were determined with the API 20E system, which has been used previously to identify *Y. pestis* [21] (Table S2). All isolates were glycerol-, glucose- and mannitol-positive, and urease-, inositol- and oxidase-negative. Sixteen (35%) and 22 (48%) of the isolates were rhamnose- and arabinose-positive, respectively. Among the 22 arabinose-positive isolates, 12 were rhamnose-negative. The API 20E system correctly identified 28 (61%) of the 46 isolates as *Y. pestis*; the remaining isolates were identified as other species, and often as members of another genus. However, further analysis, including sequence data for the 16S rRNA gene and the seven housekeeping genes, unambiguously identified all 46 isolates as *Y. pestis*.

Several additional problems were encountered when using the API 20E system. For example, eight isolates (1392G, 1412G, 1413G, C14735, C1522, C2944, C1045 and 8787G) failed repeatedly to demonstrate any positive biochemical reactions when processed strictly according to the manufacturer's instructions, and three or four colonies of these isolates had to be used as the inoculum in order to elicit positive biochemical reactions. In addition, the reproducibility of the biochemical reactions was poor for several isolates, and these required multiple confirmatory analyses. The inability of the API 20E system to identify *Y. pestis* consistently has been reported previously [21]. The percentage (61%) of isolates identified correctly in the present study was in agreement with the previous report. These observations suggest that many *Y. pestis* isolates, including Georgian *Y. pestis* isolates, have biochemical profiles distinct from those of the reference

Y. pestis strains used to develop the API 20E system. Thus, the API 20E system may be of limited value as a diagnostic tool for *Y. pestis*, as the biochemical properties of a large number of isolates have not been rigorously characterised. The BBL Crystal Enteric/Nonfermenter Identification system (Becton Dickinson) has been reported to identify *Y. pestis* more accurately than the API 20E system [21], but has not, to date, been tested using the Georgian isolates.

Biovars

Y. pestis isolates are commonly grouped in three intra-species groups (Antiqua, Medievalis and Orientalis), termed biovars, based on their ability to ferment arabinose and glycerol, and to reduce nitrate [5]. Two additional biovars (Microtus and Pestoides) have been used less frequently to characterise *Y. pestis* isolates, but have been described more frequently in recent reports [22,23]. Biovar characteristics are often unstable, and isolates may undergo spontaneous phenotypic variation that may cause them to be classified in a different biovar [2]. Based on the results of the glycerol fermentation and nitrate reduction tests, the majority (n = 36) of the Georgian *Y. pestis* isolates were classified as Antiqua (glycerol- and nitrate-positive), with six isolates being classified as Medievalis (glycerol-positive, but nitrate-negative) (Table S2). The potassium nitrate reaction (nitrate reduction) of the API 20E system continuously yielded variable results; thus, the nitrate reduction test was performed independently of the API 20E system; nevertheless, three isolates (C2944, C1045 and 8787G) could not be allocated to a biovar because of the poor reproducibility of this test (Table S2).

CR-binding

The ability to bind CR has commonly been used for estimating the virulence potential of *Y. pestis* isolates [15]. This ability (i.e., the CR^+ phenotype) is associated with an ability to block the flea's proventriculus/gut, which is essential for regurgitation of the bacterium into the bite site and its transmission from fleas to the mammalian/human host [24], and for increased uptake by eukaryotic cells and survival in the host's phagocytic cells [5]. Thirty-five (76%) of the FSU isolates examined were CR^+ (Table S2), which suggests that they would be of increased concern with respect to disease spread and transmission.

Antibiotic sensitivity

All 46 *Y. pestis* isolates studied from the FSU were susceptible to spectinomycin, streptomycin, gentamicin and doxycycline; 43 were ciprofloxacin-susceptible, with the three remaining isolates being borderline intermediate–resistant to ciprofloxacin (inhibition zone of 40 mm). The WHO's Expert Committee on Plague recommends using streptomycin (the drug of choice), tetracycline and chloramphenicol to treat *Y. pestis* infections [2]. This list has recently been expanded by the US Working Group on Civilian Biodefense [25] to include gentamicin, doxycycline and ciprofloxacin. The present data suggest that these recommendations are appropriate for treating possible plague outbreaks caused by *Y. pestis* strains originating from natural foci of plague in Georgia. In general, antibiotic-resistant strains of *Y. pestis* are rare throughout the world [5], and the present data suggest that antibiotic resistance has not yet emerged in the Georgian *Y. pestis* population. However, an increased emergence of multiresistance among *Y. pestis* strains has recently been suggested [26]. Therefore, in view of the epidemic nature of *Y. pestis* infections and their high virulence, the possible emergence of antibiotic-resistant mutants should be monitored in natural foci of plague in Georgia, the Caucasus region, and elsewhere in the world.

PFGE

PFGE analysis was performed for all FSU *Y. pestis* isolates, as well as the KIM and CO92 strains, after separately digesting the plugs with *Asc*I and *Xba*I. The *Xba*I-based analysis yielded more PFGE types than did the *Asc*I-based analysis (ten and eight PFGE types, respectively) (Table S1). The PFGE types were designated $P1_{AscI}$, $P2_{AscI}$, etc., and $P1_{XbaI}$, $P2_{XbaI}$, etc., respectively. In order to streamline the data analysis, 'final' PFGE types were assigned for isolates that had a distinct PFGE pattern after digestion with either of the two restriction endonucleases. In total, 12 'final' PFGE types were identified using this strategy (Table S1 and Fig. 1).

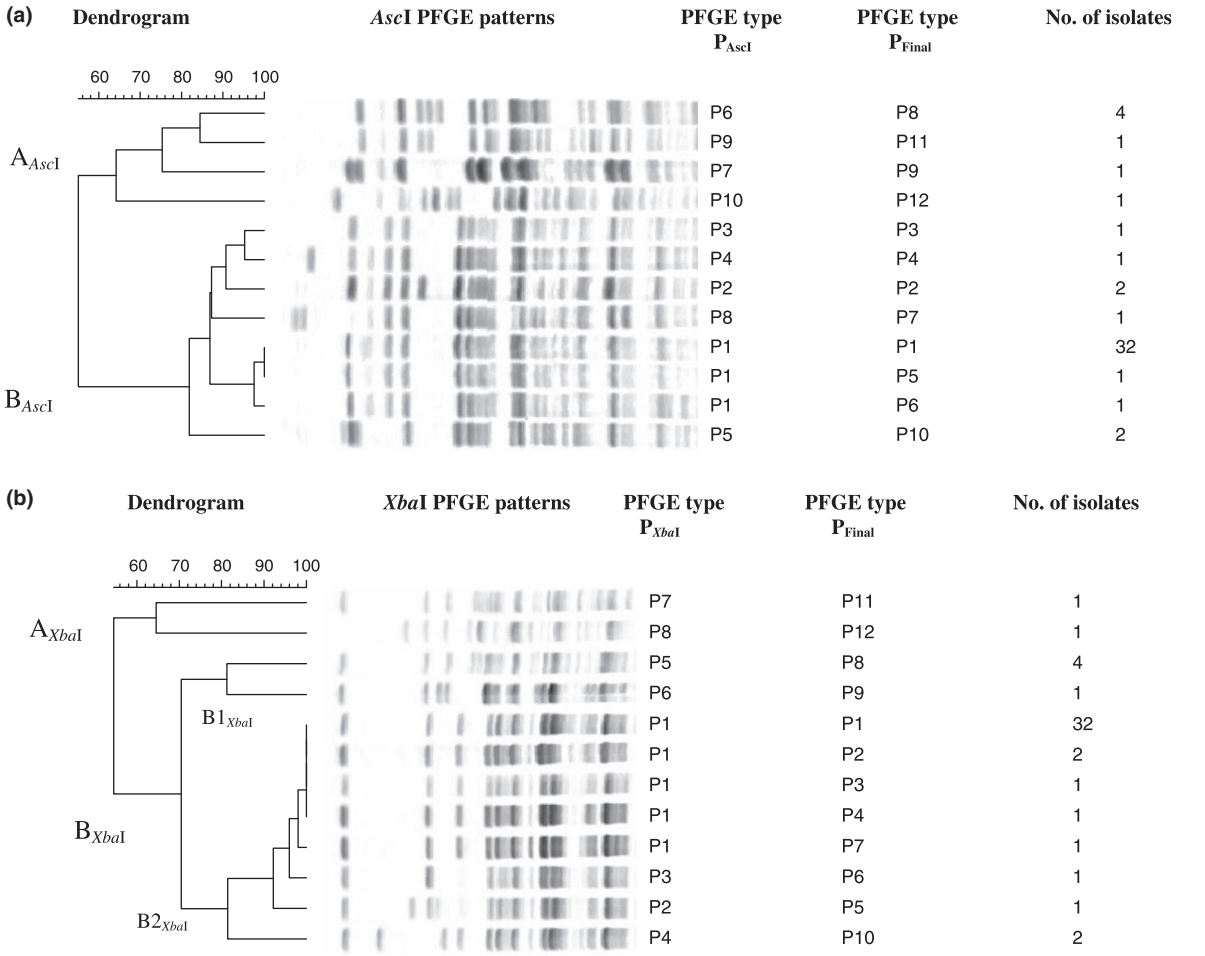

Fig. 1. Dendogram based on pulsed-field gel electrophoresis (PFGE) using (a) *Asc*I and (b) *Xba*I analysis of the *Yersinia pestis* isolates. The identities of the isolates in each PFGE type are given in Table S1.

PFGE patterns for *Y. pestis* isolates have been reported [27] to differ among colonies of the same strain. In order to verify the stability and reproducibility of the PFGE patterns observed during the present study, seven randomly selected *Y. pestis* isolates were grown at 28°C for 48 h, and three distinct colonies originating from each isolate (a total of 21 colonies) were used to prepare plugs for PFGE analysis. In all instances, the patterns obtained for each of the three colonies were identical with those of the original isolates from which the colonies were obtained (data not shown). Nevertheless, it is possible that unstable patterns would have been observed if more samples were analysed. However, it seemed that PFGE analysis provided stable and reproducible results for the isolates examined in the present study, possibly because of the standardised storage conditions used for these isolates. After being received at the US collaborating laboratories, all isolates were tested for purity, and single-use aliquots were then prepared from a single colony. This approach avoided multiple freeze–thaw cycles and multiple subculturing, during which mutations may accumulate. It is therefore recommended that the above procedure should be followed to minimise possible genetic alterations associated with serial passaging of *Y. pestis* isolates.

Dendrograms constructed using PFGE patterns generated separately with each of the two enzymes revealed that the Georgian *Y. pestis* isolates generally grouped separately from the non-Georgian isolates (Fig. 1, Table S1). Among the Georgian isolates, one notable exception was isolate 771G, which grouped consistently with the non-Georgian isolates in clusters A_{AscI} and $B1_{XbaI}$ (Fig. 1, Table S1). Among the non-Georgian isolates, two

Armenian isolates (C1522 and C14735) grouped consistently with the Georgian isolates in clusters B_{AscI} and $B2_{XbaI}$ (Fig. 1, Table S1). There was no clear-cut association between the PFGE type of an isolate and its ability to bind CR.

MLST

Achtman *et al.* [1] recently reported the results of an MLST analysis of *Y. pestis* isolates, based on the use of six loci (*dmsA*, *trpE*, *glnA*, *manB*, *thrA* and *tmk*). However, it was difficult to amplify the *dmsA* and *trpE* loci from many of the Georgian isolates; therefore, the MLST analysis used in the present study employed four loci (*glnA*, *manB*, *tmk* and *thrA*) used previously [1], and four additional loci (16S rRNA, *hsp60*, *gyrB* and *recA*). The 16S rRNA locus and six (*glnA*, *gyrB*, *manB*, *thrA*, *tmk* and *hsp60*) of the seven housekeeping genes examined were identical for all of the Georgian and non-Georgian isolates (Table S1). The only exception was the *recA* gene, which differentiated the isolates into two nucleotide types (NTs) on the basis of a single nucleotide change, i.e., *recA* NT1 and *recA* NT2. Thirty-nine of the 40 *Y. pestis* isolates from Georgia carried *recA* NT2, as compared with only two (C1522 and C14735) of the 20 non-Georgian isolates/DNA samples examined, which mostly carried *recA* NT1. Eighteen of the 20 non-Georgian isolates, but only one Georgian isolate (771G), had *recA* NT1 (Table S1).

START analysis of the concatenated sequences of the 16S rRNA and housekeeping genes identified two STs (ST1 and ST2; Table S1) that differed by a single nucleotide. With the exception of one isolate (771G), all of the Georgian *Y. pestis* isolates belonged to ST2. The MLST analysis of Achtman *et al.* [1] identified more STs among *Y. pestis* isolates than did the present MLST data. This difference in discriminatory power probably relates to a difference in the genetic composition of the isolates examined rather than to differences in the MLST schemes used. For example, Achtman *et al.* [1] examined isolates collected from throughout the world (Vietnam, Brazil, Madagascar, Germany, etc.), whereas the present study focused on isolates primarily from the Republic of Georgia and its neighbouring countries (Table S1).

Sequence data for the four virulence genes were analysed at the nucleotide level and by translating nucleotide sequences into amino-acid data. All nucleotide substitutions were synonymous. The NTs of three virulence genes (*lcrV*, *psaA* and *pla*) were identical in all Georgian and non-Georgian isolates (Table S1). In contrast, most (39 of 40) of the Georgian isolates carried *caf1* NT2 (the one exception was strain 771G), and most (18 of 20) of the non-Georgian isolates carried *caf1* NT1 (the two exceptions were isolates C1522 and C14735) (Table S1).

Comparison of MLST and PFGE data

PFGE discriminated the isolates examined in the present study better than did MLST, and differentiated a number of isolates that were identical by MLST (Table S1). A superior discriminating ability for PFGE, compared to MLST, has also been reported for *Escherichia coli* O157:H7 [28], which is a highly pathogenic serotype with a genetic structure that is also generally recognised as being homogeneous. These observations support the view that MLST is not well-suited for subtle differentiation or epidemiological investigations of bacterial species with highly conserved genomes. The superior differentiating ability of PFGE revealed during the present study also suggests that the loci responsible for differentiating these isolates by PFGE are outside the 16S rRNA locus and the seven housekeeping genes that were examined. Such differences may be caused by spontaneous rearrangements (e.g., insertions or deletions) in the *Y. pestis* genome. In this context, it has been suggested [29] that a large chromosomal deletion of *c.* 100 kb triggers a change in the PFGE patterns of *Y. pestis* strains. This deletion is also associated with loss of the pigmentation phenotype [29]. At the present time, it is not clear whether a similar phenomenon is responsible for the variation in PFGE patterns observed for the isolates in the present study. However, because of the potential impact of such rearrangements on the physiology and virulence of the bacterium, it would be of interest to further characterise the genetic background of the Georgian *Y. pestis* isolates belonging to various PFGE types.

Clonal origin of Georgian strains

Despite the difference in their discriminatory power, both PFGE and MLST grouped the

Y. pestis isolates unambiguously into two major groups; one group contained predominantly Georgian isolates, and the other contained predominantly non-Georgian isolates, which suggests that the population structure of *Y. pestis* in Georgia is clonal. This suggestion is supported by several observations, including: (i) the distinct clustering of the Georgian *Y. pestis* isolates separately from non-Georgian isolates according to MLST and PFGE analyses; (ii) the tight grouping of all Georgian isolates according to MLST and PFGE analyses; and (iii) the strong association between the Georgian isolates and *caf1* NT2, and a similarly strong association between the non-Georgian isolates and *caf1* NT1, based on sequence analysis of virulence genes. The Georgian isolate 771G and the Armenian isolates C1522 and C14735 were clear outliers according to most of the criteria used (Table S1). Characterisation of more isolates from Azerbaijan, Armenia and other FSU countries bordering Georgia (and, ideally, of 'ancient' *Y. pestis* DNA from burial sites in Georgia and surrounding countries) [30] using the approaches described in this study, together with additional methodologies (e.g., ribotyping, multilocus molecular methods targeting genome-wide synonymous single nucleotide polymorphisms, variations in tandem repeats numbers, and insertion sites of IS*100* insertion elements), as used in other studies of *Y. pestis* [8,22,27,31], are likely to provide new insights into the evolutionary history of *Y. pestis* and the emergence and spread of plague pandemics in the Caucasus region.

Nucleotide sequence accession numbers

The DNA sequences of the gene loci described in this study have been deposited in GenBank under accession numbers EF165975 (16S rRNA), EF165976 (*caf1* NT1), EF165977 (*caf1* NT2), EF165978 (*glnA*), EF165979 (*gyrB*), EF165980 (*hsp60*), EF165981 (*lcrV*), EF165982 (*manB*), EF165983 (*pla*), EF165984 (*psaA* NT1), EF165985 (*psaA* NT2), EF165986 (*recA* NT1), EF165987 (*recA* NT2), EF165988 (*thrA*) and EF165989 (*tmk*).

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SUPPLEMENTARY MATERIAL

The following Supplementary material is available for this article online at http://www.blackwell-synergy.com.

Table S1. Characteristics of *Yersinia pestis* isolates included in the study

Table S2. Phenotypic properties of the 46 *Yersinia pestis* isolates included in the study

Table S3. PCR primers used to analyse the *Yersinia pestis* isolates included in the study

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