Attenuated enzootic (pestoides) isolates of *Yersinia pestis* express active aspartase

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It is established that *Yersinia pestis*, the causative agent of bubonic plague, recently evolved from enteropathogenic *Yersinia pseudotuberculosis* by undergoing chromosomal degeneration while acquiring two unique plasmids that facilitate tissue invasion (pPCP) and dissemination by fleabite (pMT). Thereafter, plague bacilli spread from central Asia to sylvatic foci throughout the world. These epidemic isolates exhibit a broad host range including man as opposed to enzootic (pestoides) variants that remain in ancient reservoirs where infection is limited to muroid rodents. Cells of *Y. pseudotuberculosis* are known to express glucose-6-phosphate dehydrogenase (Zwf) and aspartase (AspA); these activities are not detectable in epidemic *Y. pestis* due to missense mutations (substitution of proline for serine at amino position 155 of Zwf and leucine for valine at position 363 of AspA). In this study, functional Zwf was found in pestoides strains E, F and G but not seven other enzootic isolates; enzymic activity was associated with retention of serine at amino acid position 155. Essentially, full AspA activity occurred in pestoides isolates where valine (pestoides A, B, C and D) or serine (pestoides E, F, G and I) occupied position 363. Reduced activity occurred in strains Angola and A16, which contained phenylalanine at this position. The k_{cat} but not K_{m} of purified AspA from strain Angola was significantly reduced. In this context, *aspA* of the recently described attenuated enzootic microtus biovar encodes active valine at position 363, further indicating that functional AspA is a biomarker for avirulence of *Y. pestis* in man.

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INTRODUCTION

Yersinia pseudotuberculosis diverged from *Yersinia enterocolitica* about one million years ago (Achtman *et al.*, 1999). During subsequent evolution, the organism acquired a tropism for lymphatic tissue and gained unique LPS O-groups (Davies, 1961) and new enzymes including KatY (Garcia *et al.*, 1999). Nevertheless, *Y. pseudotuberculosis* remains an enteropathogenic species although its incidence of soil-borne zoonotic infection is generally greater than that of *Y. enterocolitica*, which typically causes food-borne gastrointestinal disease in humans. Both of these patterns are in stark contrast to the epidemic habit of *Yersinia pestis*. Probably less than 20 000 years have elapsed since *Y. pestis*

diverged from *Y. pseudotuberculosis* (Achtman *et al.*, 1999), yet plague bacilli cause the most devastating acute infectious disease known to man as judged by its rapid onset, systemic pathology and extensive mortality. The brief existence of *Y. pestis* as a distinct species suggests that only a limited number of genetic changes have occurred that promote acute disease. This possibility was supported by comparative annotation of the *Y. pestis* and *Y. pseudotuberculosis* genomes (Chain *et al.*, 2004, 2006; Deng *et al.*, 2002; Parkhill *et al.*, 2001). Results of these studies detected only a few *Y. pestis*-specific genes whereas about 13 % of the *Y. pseudotuberculosis* chromosome is now inactive in *Y. pestis* (Chain *et al.*, 2004). These losses accrued as a consequence of chromosomal duplication, deletion, inversion, transposition, and the insertion of over 70 copies of IS elements (Chain *et al.*, 2004, 2006; Deng *et al.*, 2002; Parkhill *et al.*, 2001). In addition, cells of typical

Abbreviations: Caf1, capsular antigen fraction 1; CRIM, cross-reacting immunological material; LCR, low calcium response; MT, murine toxin; Pla, plasminogen activator; T3SS, type III secretion system.

Y. pestis as compared to *Y. pseudotuberculosis* possess an assumed missense mutation in glucose-6-phosphate dehydrogenase characterized by a single base transversion (T·A→C·G), causing replacement of active serine by proline at amino acid position 155 (Chain *et al.*, 2004), and a proven missense mutation (Viola *et al.*, 2008) in aspartate ammonia-lyase or aspartase (AspA) characterized by a single base transversion (G·C→T·A), causing replacement of active valine by leucine (Chain *et al.*, 2004). The existence of these various types of loss mutation accounts for most of the classic phenotypic differences used to distinguish *Y. pestis* from *Y. pseudotuberculosis* (Brubaker, 1991).

The few important *Y. pestis*-specific genes were acquired by lateral transfer as evidenced by carriage of two plasmids not shared with the enteropathogenic yersiniae (Ferber & Brubaker, 1981). These consist of ~10 kb pPCP encoding plasminogen activator (Pla), the bacteriocin pesticin, and its immunity protein (Ferber & Brubaker, 1981), and ~100 kb pMT encoding capsular antigen fraction 1 (CafI) and murine toxin (MT) (Kutyrev *et al.*, 1986; Protsenko *et al.*, 1983). Plague bacilli share a third ~70 kb plasmid with the enteropathogenic yersiniae (termed pCD in *Y. pestis* and pYV in *Y. pseudotuberculosis* and *Y. enterocolitica*). The latter mediates a low calcium response (LCR) causing a temperature-dependent nutritional requirement *in vitro* for ~2.5 mM Ca²⁺ (Kupferberg & Higuchi, 1958). If present, Ca²⁺ promotes vegetative growth at 37 °C while down-regulating a host temperature-dependent pCD/pYV-encoded type III secretion system (T3SS) required for translocation of attendant virulence effectors. These determinants consist of yersiniae outer proteins (Yops) and LcrV, which collectively disrupt host cell scaffolding, cause apoptosis and repress inflammation (Heesemann *et al.*, 2006). If Ca²⁺ is omitted from the environment *in vitro*, the organisms upregulate this T3SS and cease multiplication (Lcr⁺), although full-scale growth can still occur at mildly acidic pH provided that Na⁺ is also eliminated from the medium (Brubaker, 2007). The LCR is particularly stringent in Lcr⁺ cells of *Y. pestis* as judged by abrupt shutoff of growth following shift from 26 °C to 37 °C associated with secretion of L-aspartic acid (Brubaker, 2005, 2007). This phenomenon reflects loss of AspA activity (Dreyfus & Brubaker, 1978), where exit of metabolic L-aspartate likely contributes to the abrupt shutoff in growth of Lcr⁺ cells of *Y. pestis*. Since bacteriostasis as well as release of L-aspartate in Ca²⁺-deficient medium is dependent on the presence of Na⁺ (Brubaker, 2005, 2007; Fowler & Brubaker, 1994), the possibility exists that the latter cation causes both phenomena via its established role as porter for the translocation of L-aspartate via GltS (Deguchi *et al.*, 1990). As noted below, the possibility also exists that missing Zwf activity (Mortlock, 1962; Mortlock & Brubaker, 1962) may also influence the LCR of *Y. pestis* during growth *in vitro*.

Cells of *Y. pseudotuberculosis* actively invade host intestinal epithelial cells and typically downregulate the formation of

a biofilm required by *Y. pestis* for blockage of fleas. Both of these properties have been lost by mutation in *Y. pestis* (Brubaker, 2004; Sun *et al.*, 2008). It is also established that acquisition of MT via pMT facilitated colonization of the flea vector (Hinnebusch *et al.*, 2002), thereby enabling the organisms to bypass natural environments by undergoing direct passage from one host to another. Little is known, however, about the sequence of these and other genetic changes that culminated in the ability of *Y. pestis* to express acute disease. For successful reliance upon transfer by fleabite, the organisms must kill their current rodent host in order to ensure that resident fleas disseminate in search of new hosts. Accordingly, mortality became an essential part of the life cycle of *Y. pestis* and selective pressure caused by the necessity for lethality has undoubtedly favoured subsequent increases in virulence. As a consequence, plague bacilli became efficient pathogens and have recently disseminated from ancient foci in central Asia to reservoirs throughout the world, where they now cause acute disease in a large number of animal species including man (Suntsov & Suntsova, 2008). The virulence of these potential 'epidemic' isolates contrasts sharply with 'enzootic' strains (including pestoides variants) of *Y. pestis* that remain contained within the original reservoirs where the species evolved (Anisimov *et al.*, 2004; Martinevskii, 1969). Enzootic isolates are now classified according to subspecies designation (*altaica*, *caucasica*, *hissarica*, *ulegeica* and *talassica*; Anisimov *et al.*, 2004) and commonly retain the normal ability of *Y. pseudotuberculosis* to ferment the sugars rhamnose and melibiose. They may also possess all known virulence factors of epidemic isolates. Nevertheless, enzootic strains are attenuated in many mammalian species, including guinea pigs and primates, but virulent in the rodent Superfamily Muroidea (Anisimov *et al.*, 2004). Although pestoides isolates possess polymorphism in LcrV (Anisimov *et al.*, 2007), a major pCD/pYV-encoded regulator, T3SS effector, and virulence factor, it seems unlikely that these differences influence biological activity (Abramov *et al.*, 2007). The purposes of this report are to provide a description of ten typical enzootic isolates and to demonstrate that only three produce functional Zwf while, in stark contrast to epidemic isolates of *Y. pestis*, all express biologically active AspA.

METHODS

Bacteria and cultivation. Bacterial strains were obtained from stock culture collections of the Centers for Disease Control and Prevention, Fort Collins, CO, or Michigan State University. Both epidemic and enzootic strains of *Y. pestis* were initially screened for the deletable Pgm locus by ability to form pigmented colonies at 26 °C on Congo red agar (Surgalla & Beesley, 1969). Plasmid profiles were characterized by electrophoresis in agarose (Kado & Liu, 1981). If plasmids or the Pgm locus were detected in original stock cultures, routine cultural precautions were undertaken to enrich their content in populations used for later experiments. Avirulent Lcr⁻ derivatives (lacking pCD/pYV) were selected by plating and incubation on magnesium oxalate agar at 37 °C (Higuchi & Smith, 1961) to facilitate subsequent biochemical testing and enzymic analyses,

although isolates containing pCD, if present, were used whenever possible to determine plasmid profiles (Fig. 1). Assurance that missing plasmids had not undergone integration into the chromosome was obtained by query of salient genes (*pla*, *cafI* and *lcrV*) via PCR; retention of the temperature-dependent nutritional requirement for Ca^{2+} by strains containing pCD was verified by use of magnesium oxalate agar. *Yersiniae* grown to late-exponential phase at 37 °C in brain heart infusion broth (Beckton Dickinson) were used to express *Cafl* and MT, which were determined by diffusion against monospecific polyclonal antisera in gels and by immunoblotting. Most of the pestoides strains used in this study were also described by Achtman *et al.* (2004).

Determinative assays. Ability of near-homogeneous populations of Pgm^+ cells to generate Pgm^- mutants at high frequency (Brubaker, 1969) was estimated after incubation for 2 days at 26 °C on Congo red agar (Surgalla & Beesley, 1969). Pesticin was purified to near-homogeneity by the method of Hu & Brubaker (1974) and assayed at 37 °C for antibacterial activity on pesticin agar against the indicator organism under investigation (Brubaker & Surgalla, 1962). Sensitivity to pesticin was expressed in units, where one unit is defined as the reciprocal of the highest dilution providing detectable inhibition of growth. Production of pesticin was estimated qualitatively as ability of colonies to form zones of inhibition in overlays of pesticin agar after seeding with cells of *Y. pseudotuberculosis* and incubation for 1 day at 37 °C (Brubaker & Surgalla, 1962). Pla was determined semiquantitatively by testing dilutions of bacteria washed in 0.033 M potassium phosphate buffer, pH 7.0, on fibrin plates (Astrup & Mullertz, 1952) as described previously (Beesley *et al.*, 1967). Units of Pla were defined as the reciprocal of the highest dilution yielding fibrinolytic activity. These results and the original geographical origins of individual *yersiniae* are listed in Table 1.

All biochemical assays, including expression of motility in 0.5% Tryptose phosphate agar (Beckton Dickinson), were undertaken at 26 °C. Abilities to reduce nitrate and hydrolyse urea were determined in urea and nitrate broth (Beckton Dickinson), respectively, and fermentation of carbohydrates was assayed in Purple broth (Beckton Dickinson) containing 1% (w/v) glycerol, L-rhamnose or melibiose (Sigma). Nutritional requirements were initially screened on solid minimal medium (Brubaker & Sulen, 1971) containing L-threonine, L-methionine, L-phenylalanine, L-isoleucine and L-valine and modified by omission of glycine and D-lactate; this medium is sufficient for growth of typical epidemic isolates of *Y. pestis* at 26 °C.

Cell-free extracts. Unless stated otherwise, bacteria used for preparation of cell-free extracts for enzyme analysis were always grown at 26 °C. The organisms were transferred from buffered glycerol stock cultures maintained at -20 °C (Beesley *et al.*, 1967) to slopes of blood agar base (Becton Dickinson). After incubation for 48 h, the organisms were removed from the agar surface in sterile 0.033 M potassium phosphate buffer (pH 7.0) and inoculated at an optical density (620 nm) of 0.1 into 100 ml brain heart infusion broth (Beckton Dickinson) contained in a stoppered 1 l Erlenmeyer flask. This subculture was aerated overnight at 200 r.p.m. on a model G76 gyrotary water bath shaker (New Brunswick Scientific) and then used to inoculate second cultures of the same medium (200 ml per 2 l Erlenmeyer flasks), which were similarly aerated until the cultures entered the late-exponential growth phase. The bacteria were then harvested by centrifugation (10 000 g for 30 min), washed twice in 0.033 M potassium phosphate buffer (pH 7.0), suspended in 0.05 M Tris/HCl buffer (pH 8.0), and disrupted by passage through a French pressure cell (model FA030, Spectronic Unicam). Cell debris was then removed by centrifugation (10 000 g for 30 min) and the resulting extracts were dialysed overnight at 4 °C against 0.05 M Tris/HCl buffer (pH 7.5) before immediate use in enzyme assays.

Enzyme assays. Zwf was determined by monitoring glucose-6-phosphate-dependent generation of NADPH by a minor modification of the assay described previously (Mortlock & Brubaker, 1962). The reaction mixture consisted of 100 µmol Tris/HCl (pH 8.0), 2.0 µmol MgCl_2 , 0.3 µmol NADP^+ and 100 µmol glucose 6-phosphate (Sigma) in a volume of 2.9 ml contained within a quartz cuvette. The reaction was started by addition of 0.1 ml dialysed cell-free extract and increase of absorbance at 340 nm was monitored with a Beckman DU spectrophotometer (Beckman Coulter).

AspA was estimated by determining the L-aspartic acid-dependent release of NH_4^+ with Nessler's reagent as described previously (Dreyfus & Brubaker, 1978). The reaction mixture consisted of 250 µmol Tris/HCl (pH 7.0), 5.0 µmol MgCl_2 and dialysed cell-free extract in a total volume of 4.5 ml. The assay was started by addition of 250 µmol sodium L-aspartate in a volume of 0.5 ml and samples of 0.5 ml were removed at intervals and added to Eppendorf tubes containing 0.1 ml 1.5 M trichloroacetic acid. The tubes were then centrifuged at highest speed for 1 min in a model II microfuge (Beckman Coulter) and then 0.5 ml of clear supernatant fluid was carefully removed and added to a tube containing 8.5 ml distilled water. These samples received 1.0 ml Nessler's reagent and, after

Fig. 1. Agarose gel illustrating the presence or absence of pPCP (~10 kb), pCD or pYV (~70 kb), and pMT (~100 kb) in DNA purified from epidemic *Y. pestis* CO92 (lanes 1, 13); enzootic *Y. pestis* pestoides A (lane 2), B (lane 3), C (lane 4), D (lane 5), E (lane 6), F (lane 7), G (lane 8), I (lane 9), J (lane 10), and Angola (lane 11) and *Y. pseudotuberculosis* TX83-0489 (lane 12).

Table 1. Source and characteristics of the strains of yersiniae used in this study

Strain	Species	Epidemiological habit*	Determinative property†					Geographical origin or source	Reference
			Pgm	LCR	CafI	MT	Pla‡		
1	<i>pseudotuberculosis</i>	Enteropathogenic	–	–	–	–	–	Sweden	Thal & Knapp (1971)
7	<i>pseudotuberculosis</i>	Enteropathogenic	–	–	–	–	–	Sweden	Thal & Knapp (1971)
A16	<i>pestis</i>	Enzootic	–	+	+	+	+	Africa	Devignat (1954)
Angola	<i>pestis</i>	Enzootic	+	+	–	+	+	Africa	Achtman <i>et al.</i> (2004)
Camel	<i>pestis</i>	Epidemic	+	+	+	+	+	Saudi Arabia	Bin Saeed <i>et al.</i> (2005)
CO92	<i>pestis</i>	Epidemic	+	+	+	+	+	Colorado, USA	Parkhill <i>et al.</i> (2001)
E705	<i>enterocolitica</i>	Enteropathogenic	–	+	–	–	–	Canada	Schiemann <i>et al.</i> (1981)
KIM	<i>pestis</i>	Epidemic	+	+	+	+	+	Kurdistan, Iran	Finegold <i>et al.</i> (1968)
Kuma	<i>pestis</i>	Epidemic	+	–	+	+	+	Manchuria	Wu <i>et al.</i> (1936)
PB1	<i>pseudotuberculosis</i>	Enteropathogenic	–	+	–	–	–	England	Burrows & Bacon (1960)
Pestoides A	<i>pestis</i>	Enzootic	+	+	+	+	+	Russia (CDC§)	Achtman <i>et al.</i> (2004)
Pestoides B	<i>pestis</i>	Enzootic	+	+	+	+	+	Russia (CDC)	Achtman <i>et al.</i> (2004)
Pestoides C	<i>pestis</i>	Enzootic	–	+	+	+	+	Russia (CDC)	Achtman <i>et al.</i> (2004)
Pestoides D	<i>pestis</i>	Enzootic	+	+	+	+	+	Russia (CDC)	Achtman <i>et al.</i> (2004)
Pestoides E	<i>pestis</i>	Enzootic	+	+	+	+	–	Russia (CDC)	Achtman <i>et al.</i> (2004)
Pestoides F	<i>pestis</i>	Enzootic	+	+	+	+	–	Russia (CDC)	Achtman <i>et al.</i> (2004)
Pestoides G	<i>pestis</i>	Enzootic	+	+	+	+	–	Russia (CDC)	Achtman <i>et al.</i> (2004)
Pestoides I	<i>pestis</i>	Enzootic	+	–	+	+	+	Russia (CDC)	–
Pestoides J	<i>pestis</i>	Epidemic	–	+	–	–	+	Unknown	Achtman <i>et al.</i> (2004)
TX83-0489	<i>pseudotuberculosis</i>	Enteropathogenic	–	+	–	–	–	CDC	–
UG05-0454	<i>pestis</i>	Epidemic	+	+	+	+	+	CDC	–
WA	<i>enterocolitica</i>	Enteropathogenic	–	+	–	–	–	New York, USA	Carter & Collins (1974)
Winblad	<i>enterocolitica</i>	Enteropathogenic	–	–	–	–	–	Sweden	Winblad <i>et al.</i> (1966)

*The enzootic habit was initially assigned to all pestoides strains according to Martinevskii (1969).

†Pgm, pigmentation; LCR, low calcium response; CafI, capsular antigen fraction I; MT, murine toxin; Pla, plasminogen activator; +, positive; –, negative.

‡Pla was determined semiquantitatively (Brubaker & Surgalla, 1962): –, <100 U; +, >10 000 U.

§CDC, from the stock culture collection of the CDC laboratory, Fort Collins, CO.

incubation for 10 min, were assayed for absorbance at 480 nm as described for determination of asparaginase (Yellin & Wriston, 1966). The resulting values were then compared against a standard curve prepared immediately before each determination composed of known concentrations of NH_4Cl in samples of 10 ml containing the same concentrations of trichloroacetic acid and Nessler's reagent that were used to prepare samples for spectrophotometric analysis. In all cases, protein was determined by the Lowry method.

Pyrosequencing. PCR was performed using an iCycler real-time thermocycler (Bio-Rad). DNA templates consisted of 1 μl boiled lysis preparations or QIAamp DNA mini-preparations (Qiagen) of pure cultures grown on blood agar plates. Reactions consisted of 10 μl SYBR Green SuperMix (Bio-Rad), 5 mM MgCl_2 and 10 pmol primers. The reaction volume was adjusted to 20 μl with sterile, nuclease-free sequencing buffer (Amresco). Primers (Table 2) were designed using the PSQ Assay Design program (Biotage) and synthesized by the Scientific Resources Program (CDC, Atlanta, GA). The reverse primer in each primer pair was biotinylated at its 5-prime end to facilitate streptavidin-mediated recovery of templates for subsequent pyrosequencing (Table 2). Amplification reactions were undertaken at 95 °C for 3 min followed by 45 cycles at 95 °C for 15 s and 60 °C for 30 s. PCR analysis and crossing threshold (C_t) value assignment was determined automatically by the iQ software (data not shown). Sequencing was performed on a PSQ96MA pyrosequencer (Biotage) according to the manufacturer's recommendations. Briefly, PCRs were adjusted to 40 μl with sterile MilliQ water (Millipore); a 40 μl slurry of streptavidin-coated Sepharose beads (Amersham Biosciences) in binding buffer (Biotage) was added to each sample. Reactions were mixed at 1400 r.p.m. for 10 min then bound to filter probes (Biotage) by vacuum aspiration. The probes were washed in 70 % ethanol, 0.2 M NaOH and wash buffer (Biotage) consecutively for 5 s each. The reaction mixes were eluted into a 96-well sequencing plate containing 40 μl aliquots of annealing buffer (Biotage) and sequencing primer (20 pmol). Sequencing reactions were heated at 80 °C for 2 min, and then allowed to cool to room temperature. The nucleotide dispensation orders were: zwf472-TA-

GCGATCTCAGTATACGATCAGTAGC, e-zwf472-GCGATCTCAC-GTGATACGATCAGTGCGATATCAC, aspA436-CACAGTATGCTA-CACGTCGCATGCGTCTAGC, e-aspA436-ACGTATGTACACTG-TCGCA, aspA1087-TACAGTCGATGAGCGTGTATGTCAGCATGTG-AGTCTATC, and e-aspA1087-CATCGATCGTCACTGAGCTGAC-TGCTCGCAGCATAG. All reactions were run in triplicate and pyrograms were analysed using the PSQ software (Biotage).

RESULTS

General properties

Previously designated species, associated epidemiological habit, major virulence functions, geographical origin or source, and salient references of all yersiniae used in this study are shown in Table 1. This information, as well as plasmid profiles, biochemical determinations and enzyme analyses, was used to combine organisms of similar habit in subsequent tables. Evidence is provided below in this context to reassign the pestoides J strain to the group of epidemic isolates and to retain the pestoides I strain within the group of enzootic isolates even though it failed to ferment rhamnose or melibiose.

Plasmid profiles

The plasmid profiles of enzootic pestoides variants were compared by agarose gel electrophoresis with those of a control isolate of epidemic *Y. pestis* CO92 and enteropathogenic *Y. pseudotuberculosis* TX83-0489 (Fig. 1). pMT appeared slightly larger in the pestoides A, B, C, D and Angola isolates as compared to the CO92 control (110 kb) due, at least in strain Angola, to two integrated copies of

Table 2. Primers used for the amplification (-f and -r) and pyrosequencing (-s) of codons for amino acid position 155 of Zwf and amino acid positions 146 (1) and 363 (2) of AspA from yersiniae

Gene target	Primer	Sequence (5'-3')	Amplicon size (bp)
zwf	zwf-f	TGGGAGAAGCCGGGTAA	105
	zwf-r	Biotin-ACTCGGCTACCTGATCGTTAATT	
	zwf-s	CATTGGGAACCGATT	
e-zwf	ezwf-f	CGCGGGTGGTAATGGAAAA	86
	ezwf-r	Biotin-CTCGTTGAAATATTTCGGCAA	
	ezwf-s	CATTGGGGAAAAGATTTA	
aspA (1)	aspA1-f	CCTTAACCCTAACGACCATCTG	90
	aspA1-r	Biotin-AGGATGGAGGCGTAGACCG	
	aspA1-s	CAAATGCCAGTCCAC	
e-aspA (1)	easpA1-f	ACCTAAACCCTAATGACCATCTGA	72
	easpA1-r	Biotin-CAATGCGGAACCCAGTTG	
	easpA1-s	AAATGCCAATCCACC	
aspA (2)	aspA2-f	TTTTAAGGTGATCGGCAACG	127
	aspA2-r	Biotin-CGCATTAGTCAGGATATGGATAGA	
	aspA2-s	AAGCGGGTCAGCTTCAGT	
e-aspA (2)	easpA2-f	Biotin-TACTATGGCTGCCGAAGCAGG	97
	easpA2-r	CGCATTTGTGTCAGGATGTGGAT	
	easpA2-s	CCCAATCACTGGCTC	

pPCP (NCBI accession NC_010158). As shown by separate measurements, pMT was further increased in size to 134 kb (pestoides E), 126 kb (pestoides F), and 118 kb (pestoides G). These three isolates entirely lacked pPCP whereas pCD and pMT were absent in the pestoides I and J isolates, respectively.

Nutritional requirements

A chemically defined solid medium containing the minimal nutritional requirements of epidemic *Y. pestis* was used to identify compounds necessary for growth of the pestoides isolates. Pestoides strains A, B, C, E and G

required L-arginine (Table 3). L-Leucine was essential for growth of the pestoides A, B, C and D isolates and a need for other amino acids was occasionally observed; requirements for purines, pyrimidines, or vitamins were not detected.

Expression of virulence factors

None of the Pgm⁺ pestoides strains that were available for study (Table 3) yielded Pgm⁻ mutants at high frequency as occurs in epidemic isolates of *Y. pestis* (Brubaker, 1969). Isolates lacking given plasmids also failed to express attendant virulence factors (Table 3) or encode salient

Table 3. Comparison of determinative properties of *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*

+, Positive; -, negative (where applicable).

Strain	Glycerol fermenta- tion	Nitrate reduction	Biotype or serotype*	Rhamnose fermenta- tion	Melibiose fermenta- tion	Urease	Motility at 26 °C	Nutritional require- ments†	High-fre- quency ΔPgm‡	Sensitivity to pesticin (U ml ⁻¹)§
Epidemic <i>Y. pestis</i>										
Camel	+	-	Medievalis	-	-	-	-	None	+	9
CO92	-	+	Orientalis	-	-	-	-	None	+	0
KIM-10	+	-	Medievalis	-	-	-	-	None	+	9
Kuma	+	+	Antiqua	-	-	-	-	None	+	1
Pestoides J	-	-	(atypical)	-	-	-	-	None	NA	0
UG05-0454	+	+	Antiqua	-	-	-	-	None	+	3
Enzootic <i>Y. pestis</i>										
A16	+	+	Antiqua	+	+	-	-	Pro	NA	0
Angola	+	+	Antiqua	+	+	-	-	His	-	27
Pestoides A	+	-	Medievalis	+	+	-	-	Arg, Leu	-	27
Pestoides B	+	-	Medievalis	+	+	-	-	Arg, Leu	-	243
Pestoides C	+	-	Medievalis	+	+	-	-	Arg, Leu	NA	243
Pestoides D	+	-	Medievalis	+	+	-	-	Leu	-	81
Pestoides E	+	+	Antiqua	+	+	-	-	Arg	-	5877
Pestoides F	+	+	Antiqua	+	+	-	-	Tyr	-	5877
Pestoides G	+	+	Antiqua	+	+	-	-	Pro, Arg	-	1959
Pestoides I	+	+	Antiqua	-	-	-	-	None	-	27
Enteropathogenic <i>Y. pseudotuber- culosis</i>										
1	+	+	O: Ia	+	+	+	+	None	NA	2187
PB1	+	+	O: Ib	+	+	+	+	None	NA	2187
7	+	+	O: IIa	+	+	+	+	None	NA	0
TX83-0489	+	+	O: Ib	+	+	+	+	None	NA	2187
Enteropathogenic <i>Y. enterocolitica</i>										
Winblad	+	+	O: 3	-	-	+	+	None	NA	0
WA	+	+	O: 8	-	-	+	+	None	NA	81
E705	+	+	O: 9	-	-	+	+	None	NA	0

*Biotypes of *Y. pestis* as defined by Devignat (1951) and serotypes of *Y. pseudotuberculosis* and *Y. enterocolitica* according to Thal & Knapp (1971) and Winblad (1968), respectively.

†Determined with added L-methionine, L-phenylalanine, L-cysteine and L-threonine typically required by cells of epidemic strains of *Y. pestis* (Brubaker & Sulen, 1971); 'none' indicates growth on this medium without supplementation.

‡Capability of undergoing high-frequency (10^{-5}) mutation to Pgm⁻ (Brubaker, 1969); NA, not applicable (due to the isolate already being Pgm⁻).

§Reciprocal of the highest dilution exhibiting visible inhibition of growth on pesticin agar.

genes detectable by PCR, emphasizing that these missing plasmids were indeed cured rather than integrated into the chromosome. Similarly, carriage of plasmids was associated with expression of known encoded virulence factors with the exception of the Angola isolate, which possessed pMT and produced MT (as detected by immunoassay) but not linked CafI. In this context, it is significant that there were no matches against the completed Angola genome using *cafI* from *Y. pestis* KIM as a query.

Pesticin

All pestoides strains harbouring pPCP produced similar amounts of the bacteriocin pesticin as judged by uniform zones of inhibition (8–10 mm) surrounding individual colonies overlaid with indicator cells of *Y. pseudotuberculosis* PB1. Isolates lacking pPCP (pestoides E, F and G) did not produce pesticin and were acutely sensitive to this bacteriocin as compared to pesticinogenic strains able to produce pPCP-encoded pesticin immunity protein (Table 3). Nevertheless, as reported by others (Anisimov *et al.*,

2004), a few pesticinogenic pestoides variants exhibited modest sensitivity to pesticin.

Determinative properties

All tested pestoides isolates as well as epidemic strains of *Y. pestis* failed to hydrolyse urea or exhibit motility at 26 °C. In contrast, all of the pestoides strains except I resembled *Y. pseudotuberculosis* in that they fermented rhamnose and melibiose.

Glucose-6-phosphate dehydrogenase

Dialysed cell-free extracts of all tested epidemic *Y. pestis* controls lacked detectable Zwf activity as judged by direct enzymic assay whereas control extracts of enteropathogenic yersiniae were positive (Table 4). Only the pestoides E, F and G strains exhibited detectable Zwf. As reported previously, the only known difference between the enzymically active enzyme in *Y. pseudotuberculosis* and the inactive form in epidemic *Y. pestis* is the presence of

Table 4. Codons for amino acids at position 155 of glucose-6-phosphate dehydrogenase (Zwf) and position 363 of aspartase (AspA) and corresponding specific activities in dialysed cell-free extracts prepared from yersiniae expressing these enzymes

Strain	Glucose-6-phosphate dehydrogenase (Zwf)			L-Aspartate ammonia-lyase or aspartase (AspA)		
	Codon	Amino acid	Specific activity*	Codon	Amino acid	Specific activity*
Epidemic <i>Y. pestis</i>						
Camel	CCC	Pro	≤0.001	UUG	Leu	≤0.001
CO92	CCC	Pro	≤0.001	UUG	Leu	≤0.001
KIM10	CCC	Pro	≤0.001	UUG	Leu	≤0.001
Kuma	CCC	Pro	≤0.001	UUG	Leu	≤0.001
Pestoides J	CCC	Pro	≤0.001	UUG	Leu	≤0.001
UG05-0454	CCC	Pro	≤0.001	UUG	Leu	≤0.001
Enzootic <i>Y. pestis</i>						
A16	CCC	Pro	≤0.001	UUU	Phe	0.020
Angola	CCC	Pro	≤0.001	UUU	Phe	0.008
Pestoides A	CCC	Pro	≤0.001	GUG	Val	0.064
Pestoides B	CCC	Pro	≤0.001	GUG	Val	0.042
Pestoides C	CCC	Pro	≤0.001	GUG	Val	0.057
Pestoides D	CCC	Pro	≤0.001	GUG	Val	0.028
Pestoides E	UCC	Ser	0.093	UCG	Ser	0.052
Pestoides F	UCC	Ser	0.084	UCG	Ser	0.039
Pestoides G	UCC	Ser	0.089	UCG	Ser	0.050
Pestoides I	CCC	Pro	≤0.001	UCG	Ser	0.050
<i>Y. pseudotuberculosis</i>						
1	UCC	Ser	0.102	GUG	Val	0.172
PB1	UCC	Ser	0.083	GUG	Val	0.137
7	UCC	Ser	0.101	GUG	Val	0.059
TX83-0489	UCC	Ser	0.080	GUG	Val	0.072
<i>Y. enterocolitica</i>						
Winblad	UCC	Ser	0.119	GUC	Val	0.202
WA	UCC	Ser	0.104	GUC	Val	0.202
E705	UCC	Ser	0.097	GUC	Val	0.207

*Values are expressed as $\mu\text{mol product produced min}^{-1} (\text{mg protein})^{-1}$ and are averaged from at least two independent determinations; variation was <15%.

serine instead of proline at amino acid position 155 (Chain *et al.*, 2004). Results obtained by pyrosequencing (Table 4) demonstrated that all active enzymes, including those of the pestoides E, F and G isolates, contained serine (encoded by UCC) at position 155 whereas all inactive enzymes contained proline (encoded by CCC).

Aspartase

As expected, dialysed cell-free extracts of all tested epidemic *Y. pestis* controls lacked detectable AspA activity as shown by direct enzymic assay whereas extracts of all control enteropathogenic yersiniae were positive (Table 4). All of the enzootic variants of *Y. pestis* also expressed biologically functional AspA, although variation was observed in specific activity. The results of DNA sequencing verified that the codon for valine at amino acid position 363 of the active enzymes of *Y. pseudotuberculosis* was GUG and that this similarly encoded amino acid was present at the same position of the active enzymes from the pestoides A, B, C and D isolates. In contrast, position 363 of the enzymes from the pestoides E, F, G and I strains was occupied by serine (encoded by UCG) while that of the less active enzymes from the Angola and A16 isolates was phenylalanine (encoded by UUU). The distantly related *Y. enterocolitica* also contained valine at amino acid position 363 of fully active AspA but its codon was GUC.

AspA from strain Angola was purified to near homogeneity as described for AspA of *Y. pseudotuberculosis* and the corresponding CRIM (cross-reacting immunological material) of *Y. pestis* and subjected to similar kinetic analysis (Viola *et al.*, 2008). The specific activity of the purified Angola AspA was 7.6 units mg^{-1} , a 30-fold higher estimate than the nearly inactive *Y. pestis* CRIM but still more than 10-fold lower than the AspA of *Y. pseudotuberculosis*. The Angola enzyme had a comparable K_m of 4.4 ± 1.2 mM for L-aspartate but, similar to the *Y. pestis* CRIM, exhibited a reduced k_{cat} (7.8 ± 1.0 s^{-1}). As a consequence, the k_{cat}/K_m of the Angola AspA was 1800 ± 200 $\text{M}^{-1} \text{s}^{-1}$, a value of only 4.2 % of the *Y. pseudotuberculosis* enzyme but still significantly more active than the *Y. pestis* CRIM (0.2 %) (Viola *et al.*, 2008).

Amino acid position 146 of AspA consists of aspartic acid in *Y. pseudotuberculosis* (Chain *et al.*, 2004) and *Y. pestis* strain CO92 (Parkhill *et al.*, 2001) but is occupied by tyrosine in *Y. pestis* strain KIM (Deng *et al.*, 2002). As judged by pyrosequencing, the codons of the aspartic acid and tyrosine residues at this location are GAU and UAU, respectively. Only the pestoides J and epidemic Camel isolates resembled *Y. pestis* KIM in encoding tyrosine at amino acid position 146; all remaining yersiniae that were evaluated expressed aspartic acid at this location. These results are consistent with the hypothesis that the transversion (G·C→T·A) at amino acid 146 of AspA occurs only in a limited (biovar *medievalis*) clone of Middle Eastern origin.

DISCUSSION

The pestoides phenotype

The pestoides collection used in this study comprised strains that likely possessed all virulence factors known to exist at the time of their isolation, but which nevertheless failed to cause disease in guinea pigs. It is well known that the Lcr^+ and Pgm^+ phenotypes of *Y. pestis* are lost at the high rates of 10^{-4} (Higuchi & Smith, 1961) and 10^{-5} (Brubaker, 1969), respectively, and that *CafI* is also lost at high frequency (Burrows, 1957; Burrows & Bacon, 1958). The possibility therefore exists that these determinants were initially present upon primary isolation but lost during subsequent storage. In this regard, pCD is absent in pestoides I, and pestoides J lacked pMT, which encodes MT and *CafI*. Cells of the Angola strain carried pMT but failed to express detectable *CafI* as judged by immunoassay, real-time PCR and query versus *cafI* of *Y. pestis* KIM. Deficiency of *CafI* is sufficient to cause reduced virulence in guinea pigs (Burrows, 1957); these phenomena alone may have prompted inclusion of the Angola and pestoides J strains among the enzootic variants. The pestoides E, F and G isolates lacked pPCP encoding plasminogen activator, a known invasive virulence factor of epidemic *Y. pestis* (Brubaker *et al.*, 1965; Lhtenmki *et al.*, 1998; Sodeinde *et al.*, 1992). However, this deficiency in the pestoides F isolate did not influence virulence in mice by peripheral routes of injection (Worsham & Hunter, 1998; Worsham & Roy, 2003) although the absence of plasminogen activator might account for attenuation in hosts other than *Muridae*. The pestoides E, F and G isolates also carried oversize pMT plasmids similar to a pestoides strain from the Caucasus (Golubov *et al.*, 2004).

The mutation frequency to Pgm^- provided another criterion that distinguished tested enzootic and epidemic isolates of *Y. pestis*. The high mutation rate to pesticin resistance in epidemic Pgm^+ yersiniae lacking pPCP (Brubaker, 1969) reflects the fact that the ~100 kb deletable *Pgm* region (Lucier & Brubaker, 1992) is flanked by polar IS elements that undergo reciprocal recombination, thereby favouring deletion of the entire sequence (Fetherston *et al.*, 1992; Fetherston & Perry, 1994). This event eliminates the Hms^+ locus responsible for pigment binding and biofilm production (Kirillina *et al.*, 2004; Perry *et al.*, 2001), the yersiniabactin operon including the pesticin/yersiniabactin receptor (Fetherston *et al.*, 1995), and *ripABC* required for growth in macrophages activated post-infection (Pujol *et al.*, 2005). Yersiniae that undergo this high-frequency deletion therefore lose the linked pesticin receptor (Fetherston *et al.*, 1992; Fetherston & Perry, 1994) and are thus resistant to pesticin. Similarly, yersiniae and other enterobacteriaceae that otherwise lack this receptor typically exhibit little or no sensitivity to pesticin (Buchrieser *et al.*, 1999). Typical pestoides isolates, however, were found by Zudina (2000) to possess only one flanking IS element and are therefore incapable of yielding *Pgm* isolates at high frequency. Mutation to *Pgm* in our

pestoides strains also occurred at low frequency and this event was ostensibly a point mutation as judged by retention of other elements of the Pgm locus.

Results of further study showed that some of the pestoides isolates possessed nutritional requirements not shared by typical epidemic strains of *Y. pestis*. Further effort will be required to determine the missing enzymes accounting for these requirements but it may be significant that certain arginine auxotrophs of *Y. pestis* are known to be attenuated in guinea pigs but not mice (Brubaker, 1972). Most isolates considered here were typical of those described in the literature in that they fermented rhamnose and melibiose but lacked other obvious properties of *Y. pseudotuberculosis*. A major exception to this rule was the pestoides I isolate, which did not ferment either one of these carbohydrates. Considered together, our characterization of this collection as it presently exists is generally consistent with previous reports of enzootic variants (Achtman *et al.*, 2004; Anisimov *et al.*, 2004; Garcia *et al.*, 2007; Golubov *et al.*, 2004) and supports consideration of the pestoides A, B and C isolates as *Y. pestis* subspecies *altaici*, D as *hissarica*, and E, F and G as *caucasica* (Anisimov *et al.*, 2004).

The pestoides J isolate was an authentic epidemic isolate as judged by typical loss of *zwf* and *aspA*. This strain was also atypical in that it is negative for both glycerol fermentation and nitrate reduction and therefore does not correspond to any of Devignat's biovars (Devignat, 1951). However, like the Middle Eastern epidemic KIM and Camel strains (biovar *medievalis*), pestoides J encodes a tyrosine residue rather than aspartic acid at amino acid position 146 of AspA, suggesting the same clonal origin.

The Zwf determinant

The pestoides E, F and G strains contained serine at amino acid position 155 of Zwf and thus expressed full biological activity. These isolates are therefore more closely related to their progenitor (*Y. pseudotuberculosis*) than are the remaining pestoides strains which, like epidemic *Y. pestis*, contained inactive proline at this location. The position of these three strains in the initial phase of the evolutionary pathway leading to epidemic *Y. pestis* is also supported by the absence of pPCP and carriage of atypically large and probably more primitive pMT (Golubov *et al.*, 2004).

Zwf⁻ mutants of *Escherichia coli* exhibit a silent phenotype that requires concomitant loss of transketolase or transaldolase in the absence of pentose before growth becomes limited (Fraenkel, 1968). Transketolase and transaldolase are functional in both *Y. pseudotuberculosis* (Brubaker, 1968) and *Y. pestis* (Mortlock, 1962); furthermore, exogenous pentose is probably always available to *Y. pestis* during its closed life cycle. In this context, catabolism of gluconate, arabinose and ribose undergoes significant upregulation at 37 °C in cells of the epidemic strain KIM (Motin *et al.*, 2004), further emphasizing the dispensable nature of biosynthetic Zwf. On the other hand, Zwf is a

major supplier of NADPH to the bacterial cell and a deficiency of this intermediate could have serious consequences during expression of the LCR. It would therefore be interesting to determine if cells of the Zwf⁺ pestoides isolates are resistant to the D-glucose-dependent lysis in synthetic medium reported for epidemic strains of *Y. pestis* (Brownlow & Wessman, 1960). These findings emphasize that loss of Zwf is an obvious early milestone in the emergence of *Y. pestis* from *Y. pseudotuberculosis*.

The AspA determinant

It is remarkable that all of the tested pestoides isolates expressed at least partially active AspA. This phenomenon resulted from either retention of valine at amino acid number 363 (pestoides A, B, C and D) or intragenic suppressor mutations causing introduction at that position of serine (pestoides E, F, G and I) or phenylalanine (Angola and A16). The specific activity of crude AspA from strain Angola was unusually low, due perhaps to the presence of a second missense mutation in this enzyme at amino acid position 378 causing replacement of isoleucine with threonine (NCBI accession no. NC_010159.1). Further study will be necessary to determine if the marginal activity of AspA in strain Angola reflects this additional amino acid substitution. In any event, the reduced activity detected for the crude extract of the Angola enzyme remained proportional to that observed for the purified cloned enzyme. The basis of this decrease, as in the case of the essentially inert AspA of *Y. pestis* caused by replacement of leucine for valine at position 363 (Viola *et al.*, 2008), was a markedly reduced k_{cat} but little change in the K_m for aspartic acid. This finding is in accord with the previous suggestion that mutations at position 363 indirectly inhibit enzyme activity by altering the position of catalytic residues Glu334 and Gln191, thereby disrupting their contributions to turnover (Viola *et al.*, 2008).

Elimination of AspA, unlike Zwf, may have untoward consequences for the host. As already noted, this mutation accentuates the pCD/pYV-dependent nutritional requirement for Ca²⁺ at 37 °C and promotes excretion of L-aspartic acid at the expense of metabolic L-glutamate (Brubaker, 2005; Dreyfus & Brubaker, 1978). This event, should it occur *in vivo*, could be deleterious by altering the equilibrium of amino acid pools, thereby reducing host adenylate energy charge. It is significant that the *aspA* of the attenuated microtub biovar encodes valine at amino acid position 363, indicating that AspA is active in these organisms; cells of this enzootic isolate are also attenuated in man (Fan *et al.*, 1995) but are fully virulent in mice (Zhou *et al.*, 2004). AspA is widespread among saprophytic bacteria but was neither required for virulence of salmonellae (Tchawa Yimga *et al.*, 2006) nor detected in the genomes of other facultative intracellular parasites including *Mycobacterium tuberculosis* (Fleischmann *et al.*, 2002) and *Francisella tularensis* (Larsson *et al.*, 2005) or obligate intracellular rickettsiae (Andersson *et al.*, 1998).

The latter, like epidemic isolates of *Y. pestis*, also convert exogenous L-glutamate to L-aspartate, which is released into the culture medium (Bovarnick & Miller, 1950).

Some of the enzootic strains used in this study possessed mutations that would cause attenuation of even epidemic isolates in guinea pigs. Formal proof demonstrating that mutational loss of AspA activity in epidemic isolates of *Y. pestis* and the consequences of this loss are directly associated with lethality in guinea pigs (and humans) will require additional effort involving exchange of *aspA*. The present study, however, provides convincing evidence that functional AspA is at least a biomarker for attenuation.

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REFERENCES

- Abramov, V. M., Khlebnikov, V. S., Vasiliev, A. M., Kosarev, I. V., Vasilenko, R. N., Kulikova, N. L., Khodyakova, A. V., Evstigneev, V. I., Uversky, V. N. & other authors (2007). Attachment of LcrV from *Yersinia pestis* at dual binding sites to human TLR-2 and human IFN- γ receptor. *J Proteome Res* **6**, 2222–2231.
- Achtman, M., Zurth, K., Morelli, G., Torrea, G., Guiyoule, A. & Carniel, E. (1999). *Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. *Proc Natl Acad Sci U S A* **96**, 14043–14048.
- Achtman, M., Morelli, G., Zhu, P., Wirth, T., Diehl, I., Kusecek, B., Vogler, A. J., Wagner, D. M., Allender, C. J. & other authors (2004). Microevolution and history of the plague bacillus, *Yersinia pestis*. *Proc Natl Acad Sci U S A* **101**, 17837–17842.
- Andersson, S. G., Zomorodipour, A., Andersson, J. O., Sicheritz-Pontén, T., Alsmark, U. C., Podowski, R. M., Näslund, A. K., Eriksson, A. S., Winkler, H. H. & Kurland, C. G. (1998). The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. *Nature* **396**, 133–140.
- Anisimov, A. P., Lindler, L. E. & Pier, G. B. (2004). Intraspecific diversity of *Yersinia pestis*. *Clin Microbiol Rev* **17**, 434–464.
- Anisimov, A. P., Panfertsev, E. A., Svetoch, T. E. & Dentovskaya, S. V. (2007). Variability of the protein sequences of LcrV between epidemic and atypical rhamnase-positive strains of *Yersinia pestis*. *Adv Exp Med Biol* **603**, 23–27.
- Astrup, T. & Mullertz, S. (1952). The fibrin plate method for estimating fibrinolytic activity. *Arch Biochem Biophys* **40**, 346–351.
- Beesley, E. D., Brubaker, R. R., Janssen, W. A. & Surgalla, M. J. (1967). Pesticins. III. Expression of coagulase and mechanism of fibrinolysis. *J Bacteriol* **94**, 19–26.
- Bin Saeed, A. A., Al-Hamdan, N. A. & Fontaine, R. E. (2005). Plague from eating raw camel liver. *Emerg Infect Dis* **11**, 1456–1457.
- Bovarnick, M. R. & Miller, J. C. (1950). Oxidation and transamination of glutamate by typhus rickettsiae. *J Biol Chem* **184**, 661–676.
- Brownlow, W. J. & Wessman, G. E. (1960). Nutrition of *Pasteurella pestis* in chemically defined media at temperatures of 36 to 38 C. *J Bacteriol* **79**, 299–304.
- Brubaker, R. R. (1968). Metabolism of carbohydrates by *Pasteurella pseudotuberculosis*. *J Bacteriol* **95**, 1698–1705.
- Brubaker, R. R. (1969). Mutation rate to nonpigmentation in *Pasteurella pestis*. *J Bacteriol* **98**, 1404–1406.
- Brubaker, R. R. (1972). *Yersinia*: biochemistry and genetics of virulence. *Curr Top Microbiol Immunol* **57**, 111–158.
- Brubaker, R. R. (1991). Factors promoting acute and chronic diseases caused by yersiniae. *Clin Microbiol Rev* **4**, 309–324.
- Brubaker, R. R. (2004). The recent emergence of plague: a process of felonious evolution. *Microb Ecol* **47**, 293–299.
- Brubaker, R. R. (2005). Influence of Na⁺, dicarboxylic amino acids, and pH in modulating the low-calcium response of *Yersinia pestis*. *Infect Immun* **73**, 4743–4752.
- Brubaker, R. R. (2007). Intermediary metabolism, Na⁺, the low calcium-response, and acute disease. In *The Genus Yersinia: From Genomics to Function*, pp. 116–129. Edited by R. D. Perry. New York: Springer.
- Brubaker, R. R. & Sulen, A. J. (1971). Mutations influencing the assimilation of nitrogen by *Yersinia pestis*. *Infect Immun* **3**, 580–588.
- Brubaker, R. R. & Surgalla, M. J. (1962). Pesticins II. Production of pesticin I and II. *J Bacteriol* **84**, 539–545.
- Brubaker, R. R., Beesley, E. D. & Surgalla, M. J. (1965). *Pasteurella pestis*: role of pesticin I and iron in experimental plague. *Science* **149**, 422–424.
- Buchrieser, C., Rusniok, C., Frangeul, L., Couve, E., Billault, A., Kunst, F., Carniel, E. & Glaser, P. (1999). The 102-kilobase *pgm* locus of *Yersinia pestis*: sequence analysis and comparison of selected regions among different *Yersinia pestis* and *Yersinia pseudotuberculosis* strains. *Infect Immun* **67**, 4851–4861.
- Burrows, T. W. (1957). Virulence of *Pasteurella pestis*. *Nature* **179**, 1246–1247.
- Burrows, T. W. & Bacon, G. A. (1958). The effects of loss of different virulence determinants on the virulence and immunogenicity of strains of *Pasteurella pestis*. *Br J Exp Pathol* **39**, 278–291.
- Burrows, T. W. & Bacon, G. W. (1960). V and W antigens in strains of *Pasteurella pseudotuberculosis*. *Br J Exp Pathol* **41**, 38–44.
- Carter, P. B. & Collins, F. M. (1974). Experimental *Yersinia enterocolitica* infection in mice: kinetics of growth. *Infect Immun* **9**, 851–857.
- Chain, P. S., Carniel, E., Larimer, F. W., Lamerdin, J., Stoutland, P. O., Regala, W. M., Georgescu, A. M., Vergez, L. M., Land, M. L. & other authors (2004). Insights into the evolution of *Yersinia pestis* through whole-genome comparison with *Yersinia pseudotuberculosis*. *Proc Natl Acad Sci U S A* **101**, 13826–13831.
- Chain, P. S. G., Hu, P., Malfatti, S. A., Radnedge, L., Larimer, F., Vergez, L. M., Worsham, P., Chu, M. C. & Andersen, G. L. (2006). Complete genome sequence of *Yersinia pestis* strains Antiqua and Nepal516: evidence of gene reduction in an emerging pathogen. *J Bacteriol* **188**, 4453–4463.
- Davies, D. A. L. (1961). Dideoxysugars of *Pasteurella pseudotuberculosis*-specific polysaccharides, and the occurrence of ascarylose. *Nature* **191**, 43–44.
- Deguchi, Y., Yamato, I. & Anraku, U. (1990). Nucleotide sequence of *gltS*, the Na⁺/glutamate symport carrier gene of *Escherichia coli*. *J Biol Chem* **265**, 21704–21708.
- Deng, W., Burland, V., Plunkett, G. I., 3rd, Boutin, A., Mayhew, G. F., Liss, P., Perna, N. T., Rose, D. J., Mau, B. & other authors (2002). Genome sequence of *Y. pestis* KIM. *J Bacteriol* **184**, 4601–4611.
- Devignat, R. (1951). Variétés de l'espèce *Pasteurella pestis*. Nouvelle hypothèse. *Bull World Health Organ* **4**, 247–263.

- Devignat, R. (1954).** Comportement biologique et biochimique de *P. pestis* et de *P. pseudotuberculosis*. *Bull World Health Organ* **10**, 463–494.
- Dreyfus, L. A. & Brubaker, R. R. (1978).** Consequences of aspartase deficiency in *Yersinia pestis*. *J Bacteriol* **136**, 757–764.
- Fan, Z., Luo, Y., Wang, S., Jin, L., Zhou, X., Liu, J., Zhang, Y. & Li, F. (1995).** *Microtus brandti* plague in the Xilin Gol Grassland was inoffensive to humans. *Chin J Control Endemic Dis* **10**, 56–57 (in Chinese).
- Ferber, D. M. & Brubaker, R. R. (1981).** Plasmids in *Yersinia pestis*. *Infect Immun* **31**, 839–841.
- Fetherston, J. D. & Perry, R. D. (1994).** The pigmentation locus of *Yersinia pestis* KIM6⁺ is flanked by an insertion sequence and includes the structural genes for pesticin sensitivity and HMWP2. *Mol Microbiol* **13**, 697–708.
- Fetherston, J. D., Schuetze, P. & Perry, R. D. (1992).** Loss of the pigmentation phenotype in *Yersinia pestis* is due to the spontaneous deletion of 102 kb of chromosomal DNA which is flanked by a repetitive element. *Mol Microbiol* **6**, 2693–2704.
- Fetherston, J. D., Lillard, J. W., Jr & Perry, R. D. (1995).** Analysis of the pesticin receptor from *Yersinia pestis*: role in iron deficient growth and possible regulation by its siderophore. *J Bacteriol* **177**, 1824–1833.
- Finegold, M. J., Petery, R. F., Berendt, R. F. & Adams, H. R. (1968).** Studies on the pathogenesis of plague. Blood coagulation and tissue responses of *Macaca mulatta* following exposure to aerosols of *Pasteurella pestis*. *Am J Pathol* **53**, 99–114.
- Fleischmann, R. D., Alland, D., Eisen, J. A., Carpenter, L., White, O., Peterson, J., DeBoy, R., Dodson, R., Gwinn, M. & other authors (2002).** Whole-genome comparison of *Mycobacterium tuberculosis* clinical and laboratory strains. *J Bacteriol* **184**, 5479–5490.
- Fowler, J. M. & Brubaker, R. R. (1994).** Physiological basis of the low calcium response in *Yersinia pestis*. *Infect Immun* **62**, 5234–5241.
- Fraenkel, D. G. (1968).** Selection of *Escherichia coli* mutants lacking glucose-6-phosphate dehydrogenase or gluconate-6-phosphate dehydrogenase. *J Bacteriol* **95**, 1267–1271.
- Garcia, E., Nedialkov, Y. A., Elliott, J., Motin, V. L. & Brubaker, R. R. (1999).** Molecular characterization of KatY (antigen 5), a thermo-regulated chromosomally encoded catalase-peroxidase of *Yersinia pestis*. *J Bacteriol* **181**, 3114–3122.
- Garcia, E., Worsham, P., Bearden, S., Malfatti, S., Lang, D., Larimer, F., Lindler, L. & Chain, P. (2007).** Pestoides F, an atypical *Yersinia pestis* strain from the former Soviet Union. *Adv Exp Med Biol* **603**, 17–22.
- Golubov, A., Neubauer, H., Nölting, C., Heesemann, J. & Rakin, A. (2004).** Structural organization of the pFra virulence-associated plasmid of rhamnose-positive *Yersinia pestis*. *Infect Immun* **72**, 5613–5621.
- Heesemann, J., Sing, A. & Trülsch, K. (2006).** *Yersinia*'s stratagem: targeting innate and adaptive immune defense. *Curr Opin Microbiol* **9**, 55–61.
- Higuchi, K. & Smith, J. L. (1961).** Studies on the nutrition and physiology of *Pasteurella pestis*. VI. A differential plating medium for the estimation of the mutation rate to avirulence. *J Bacteriol* **81**, 605–608.
- Hinnebusch, B. J., Rudolph, A. E., Cherepanov, P., Dixon, J. D., Schwan, T. G. & Forsberg, A. (2002).** Role of *Yersinia* murine toxin in survival of *Yersinia pestis* in the midgut of the flea vector. *Science* **296**, 733–735.
- Hu, P. C. & Brubaker, R. R. (1974).** Characterization of pesticin: separation of antibacterial activities. *J Biol Chem* **249**, 4749–4753.
- Kado, C. I. & Liu, S. T. (1981).** Rapid procedure for detection of and isolation of large and small plasmids. *J Bacteriol* **145**, 1365–1373.
- Kirillina, O., Fetherston, J. D., Bobrov, A. G., Abney, J. & Perry, R. D. (2004).** HmsP, a putative phosphodiesterase, and HmsT, a putative diguanylate cyclase, control Hms-dependent biofilm formation in *Yersinia pestis*. *Mol Microbiol* **54**, 75–88.
- Kupferberg, L. L. & Higuchi, K. (1958).** Role of calcium ions in the stimulation of growth of virulent strains of *Pasteurella pestis*. *J Bacteriol* **76**, 120–121.
- Kutyrev, V. V., Popov, Y. A. & Protsenko, O. A. (1986).** Pathogenicity plasmids of the plague microbe (*Yersinia pestis*). *Mol Gen Mikrobiol Virusol* **6**, 3–11.
- Lähteenmäki, K., Virkola, R., Sarén, A., Emödy, E. & Korhonen, T. K. (1998).** Expression of the plasminogen activator Pla of *Yersinia pestis* enhances bacterial attachment to the mammalian extracellular matrix. *Infect Immun* **66**, 5755–5762.
- Larsson, P., Oyston, P. C., Chain, P., Chu, M. C., Duffield, M., Fuxelius, H. H., Garcia, E., Hålltorp, G., Johansson, D. & other authors (2005).** The complete genome sequence of *Francisella tularensis*, the causative agent of tularemia. *Nat Genet* **37**, 153–159.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951).** Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**, 265–275.
- Lucier, T. S. & Brubaker, R. R. (1992).** Determination of genome size, macrorestriction pattern polymorphism, and nonpigmentation-specific deletion in *Yersinia pestis* by pulsed-field gel electrophoresis. *J Bacteriol* **174**, 2078–2086.
- Martinevskii, I. L. (1969).** *Biology and Genetic Features of Plague and Plague-Related Microbes*. Moscow, USSR: Meditsina Press.
- Mortlock, R. P. (1962).** Gluconate metabolism of *Pasteurella pestis*. *J Bacteriol* **84**, 53–59.
- Mortlock, R. P. & Brubaker, R. R. (1962).** Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities of *Pasteurella pestis* and *Pasteurella pseudotuberculosis*. *J Bacteriol* **84**, 1122–1123.
- Motin, V. L., Georgescu, A. M., Fitch, J. P., Gu, P. P., Nelson, D. O., Mabery, S. L., Garnham, J. B., Sokhansanj, B. A., Ott, L. L. & other authors (2004).** Temporal global changes in gene expression during temperature transition in *Yersinia pestis*. *J Bacteriol* **186**, 6298–6305.
- Parkhill, J., Wren, B. W., Thomson, N. R., Titball, R. W., Holden, M. T., Prentice, M. B., Sebaihia, M., James, K. D., Churcher, C. & other authors (2001).** Genome sequence of *Yersinia pestis*, the causative agent of plague. *Nature* **413**, 523–527.
- Perry, R. D., Bearden, S. W. & Fetherston, J. D. (2001).** Iron and heme acquisition and storage systems of *Yersinia pestis*. *Recent Res Dev Microbiol* **5**, 13–27.
- Protsenko, O. A., Anisimov, P. I., Mosarov, O. T., Donnov, N. P., Popov, Y. A. & Kokushkin, A. M. (1983).** Detection and characterization of *Yersinia pestis* plasmids determining pesticin I, fraction I antigen and mouse toxin synthesis. *Genetika* **19**, 1081–1090.
- Pujol, C., Grabenstein, J. P., Perry, R. D. & Bliska, J. B. (2005).** Replication of *Yersinia pestis* in interferon gamma-activated macrophages requires *ripA*, a gene encoded in the pigmentation locus. *Proc Natl Acad Sci U S A* **102**, 12909–12914.
- Schiemann, D. A., Devenish, J. A. & Toma, S. (1981).** Characteristics of virulence in human isolates of *Yersinia enterocolitica*. *Infect Immun* **32**, 400–403.
- Sodeinde, O. A., Subrahmanyam, Y. V. B. K., Stark, K., Quan, T., Bao, Y. & Goguen, J. D. (1992).** A surface protease and the invasive character of plague. *Science* **258**, 1004–1007.

- Sun, Y. C., Hinnebusch, B. J. & Darby, C. (2008). Experimental evidence for negative selection in the evolution of a *Yersinia pestis* pseudogene. *Proc Natl Acad Sci U S A* **105**, 8097–8101.
- Suntsov, V. & Suntsova, N. (2008). Concepts of macro- and microevolution as related to the problem of origin and global expansion of the plague pathogen *Yersinia pestis*. *Biol Bull* **35**, 333–338.
- Surgalla, M. J. & Beesley, E. D. (1969). Congo red-agar plating medium for detecting pigmentation in *Pasteurella pestis*. *Appl Microbiol* **18**, 834–837.
- Tchawa Yimga, M., Leatham, M. P., Allen, J. H., Laux, D. C., Conway, T. & Cohen, P. S. (2006). Role of gluconeogenesis and the tricarboxylic acid cycle in the virulence of *Salmonella enterica* serovar Typhimurium in BALB/c mice. *Infect Immun* **74**, 1130–1140.
- Thal, E. & Knapp, W. (1971). A revised antigenic scheme of *Yersinia pseudotuberculosis*. *Symp Series Immunobiol Stand* **15**, 219–222.
- Viola, R. E., Yerman, L., Fowler, J. M., Arvidson, C. G. & Brubaker, R. R. (2008). A missense mutation causes aspartase-deficiency in *Yersinia pestis*. *Microbiology* **154**, 1271–1280.
- Winblad, S. (1968). Studies on O-antigen factors of “*Yersinia enterocolitica*”. *Prog Immunobiol Stand* **9**, 337–342.
- Winblad, S., Nilehn, B. & Jonsson, M. (1966). Two further cases, bacteriologically verified, of human infection with “*Pasteurella X*” (syn. *Yersinia enterocolitica*). *Acta Pathol Microbiol Scand* **67**, 537–541.
- Worsham, P. & Hunter, M. (1998). Characterization of pestoides F, an atypical strain of *Y. pestis*. *Medische Microbiologie* **6**, S34–S35.
- Worsham, P. L. & Roy, C. (2003). Pestoides F, a *Yersinia pestis* strain lacking plasminogen activator, is virulent by the aerosol route. *Adv Exp Med Biol* **529**, 129–131.
- Wu, L.-T., Chun, J. W. H. & Pollitzer, R. & Wu, C. Y. (1936). *Plague: a Manual for Medical and Public Health Workers*. Shanghai: China.
- Yellin, T. O. & Wriston, J. C. (1966). Purification and properties of guinea pig serum asparaginase. *Biochemistry* **5**, 1605.
- Zhou, D., Tong, Z., Song, Y., Han, Y., Pei, D., Pang, X., Zhai, J., Li, M., Cui, B. & other authors (2004). Genetics of metabolic variations between *Yersinia pestis* biovars and the proposal of a new biovar, microtus. *J Bacteriol* **186**, 5147–5152.
- Zudina, I. V. (2000). *Genetic characterization of the chromosomal pigmentation region of five Yersinia pestis subspecies*. PhD dissertation, Russian Research Anti-Plague Institute ‘Microbe’, Saratov, Russia.

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