

# A missense mutation causes aspartase deficiency in *Yersinia pestis*

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It is established that cells of *Yersinia pestis*, the causative agent of bubonic plague, excrete L-aspartic acid at the expense of exogenous L-glutamic acid during expression of the low-calcium response. Results of enzymic analysis provided here suggest that a previously defined deficiency of aspartase (AspA) accounts for this phenomenon rather than an elevated oxaloacetate pool. The only known distinction between most sequenced isolates of *aspA* from *Y. pestis* and the active gene in *Yersinia pseudotuberculosis* (the immediate progenitor of *Y. pestis*) is a single base transversion (G·C→T·A) causing replacement of leucine (encoded by UUG) for valine (encoded by GUG) at amino acid position 363. The gene from *Y. pestis* KIM possesses a unique second transversion (G·C→T·A) at amino acid 146 causing substitution of aspartic acid (encoded by GAU) with tyrosine (encoded by UAU). We show in this study that *Y. pestis* expresses *aspA* as cross-reacting immunological material (CRIM). Functional and inactive *aspA* of *Y. pseudotuberculosis* PB1 and *Y. pestis* KIM, respectively, were then cloned and expressed in AspA-deficient *Escherichia coli*. After purification to near homogeneity, the products were subjected to biochemical analysis and found to exhibit similar secondary, tertiary and quaternary (tetrameric) structures as well as comparable Michaelis constants for L-aspartic acid. However, the  $k_{\text{cat}}$  of the *Y. pestis* CRIM of strain KIM is only about 0.1 % of that determined for the active AspA of *Y. pseudotuberculosis*. Return of valine for leucine at position 363 of the *Y. pestis* enzyme restored normal turnover ( $k_{\text{cat}}$   $86 \pm 2 \text{ s}^{-1}$ ) provided that the amino acid substitution at position 146 was also reversed. These observations have important implications for understanding the nature of the stringent low-calcium response of *Y. pestis* and its role in promoting acute disease.

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## INTRODUCTION

Bubonic plague caused by *Yersinia pestis* has killed over 200 million people during the course of history and is thus the most devastating acute infectious disease known to man. Nevertheless, the molecular basis of its extraordinary virulence and broad host range remains uncertain. It is established, however, that the plague bacillus diverged from the enteropathogenic *Yersinia pseudotuberculosis* within the last 20 000 years (Achtman *et al.*, 1999), implying that high lethality reflects only a few genetic changes. Comparison of the annotated genomes of *Y. pestis* (Chain *et al.*, 2006; Deng *et al.*, 2002; Parkhill *et al.*, 2001; Zhou *et al.*, 2004) and *Y. pseudotuberculosis* (Chain *et al.*, 2004) verified and

extended earlier studies showing that cells of *Y. pestis* have both gained new functions and lost important features of *Y. pseudotuberculosis* (Brubaker, 1991; Perry & Fetherston, 1997). Salient new genes were acquired by lateral transfer of two unique plasmids (Ferber & Brubaker, 1981). These are the ~10 kb pPCP encoding a plasminogen activator accounting for the low subcutaneous LD<sub>50</sub> (<10 cells) of *Y. pestis* in mice (Brubaker *et al.*, 1965; Sodeinde *et al.*, 1992) and the ~100 kb pMT, which carries structural genes for capsular antigen fraction 1 and murine toxin (Kutyrev *et al.*, 1986). Loss mutations include conversion of *yadA* and *ylpA* encoded by the shared ~70 kb low-calcium response (LCR) plasmid (termed pCD in *Y. pestis* and pYV in *Y. pseudotuberculosis*) into pseudogenes in *Y. pestis* (Hu *et al.*, 1998; Rosqvist *et al.*, 1988). The LCR phenotype of *Y. pestis* is more stringent than that of *Y. pseudotuberculosis* (Brubaker, 2007) and is defined as the ability to either promote abrupt bacteriostasis in Ca<sup>2+</sup>-deficient medium

**Abbreviations:** CD, circular dichroism; CRIM, cross-reacting immunological material; DLS, dynamic light scattering; ESI-MS, electrospray ionization mass spectrometry; LCR, low-calcium response; TCA, tricarboxylic acid cycle; T3SS, type III secretion system.

at 37 °C accompanied by upregulation of a pCD/pYV-encoded type 3 secretion system (T3SS) or maintain vegetative growth with added  $\text{Ca}^{2+}$  while downregulating the T3SS ( $\text{Lct}^+$ ) (Brubaker, 2007). The chromosome of *Y. pestis* contains large inversions, transpositions, and numerous small deletions and additions relative to its *Y. pseudotuberculosis* progenitor. These changes, as well as acquisition by *Y. pestis* of over 70 IS insertions (Chain *et al.*, 2006; Deng *et al.*, 2002; Parkhill *et al.*, 2001; Zhou *et al.*, 2004), have resulted in loss of about 13% of all functional genes of *Y. pseudotuberculosis* (Chain *et al.*, 2004). A few of these mutations, especially those associated with down-regulation of inflammation, are almost certainly associated with acute disease (Kawahara *et al.*, 2002; Kukkonen *et al.*, 2004). The remaining missing genes are largely regulatory in nature or encode metabolic functions; their elimination accounts for the established nutritional requirements of *Y. pestis* (Chain *et al.*, 2004).

Chromosomal annotation did not provide obvious explanations for the loss in *Y. pestis* of detectable glucose-6-phosphate dehydrogenase (Zwf) (Mortlock, 1962; Mortlock & Brubaker, 1962) and aspartate ammonia-lyase or aspartase (AspA) (Brubaker, 2005, 2007; Dreyfus & Brubaker, 1978). Zwf is required for synthesis of pentose from hexose and its absence may account for a temperature-dependent toxic effect of glucose on  $\text{Lcr}^+$  *Y. pestis* (Brownlow & Wessman, 1960). AspA activity catalyses the deamination of L-aspartate to form fumarate, a component of the tricarboxylic acid (TCA) cycle; thus its absence prevents catabolism of L-aspartate (and metabolically related amino acids) via this efficient bioenergetic mechanism. Indeed, the LCR of *Y. pestis* is accompanied by excretion of L-aspartic acid at the expense of exogenous L-glutamate, causing a loss of metabolic carbon that otherwise would be conserved as oxaloacetate (Brubaker, 2005, 2007). Regeneration of lost oxaloacetate via phosphoenolpyruvate carboxykinase and phosphoenolpyruvate carboxylase (Baugh *et al.*, 1964) accounts for the stimulatory effect of  $\text{CO}_2$  on growth of *Y. pestis* (Delwiche *et al.*, 1959; Surgalla *et al.*, 1964).

Further comparison of *zwf* and *aspA* of *Y. pseudotuberculosis* and *Y. pestis* revealed putative missense mutations in the latter (Chain *et al.*, 2004). For example, proline replaces serine at amino acid position 158 in the inactive Zwf of *Y. pestis*; this change is caused by a single base transition ( $\text{T}\cdot\text{A}\rightarrow\text{C}\cdot\text{G}$ ). Similarly, a single base transversion ( $\text{G}\cdot\text{C}\rightarrow\text{T}\cdot\text{A}$ ) at amino acid position 363 of AspA causes exchange of valine in the active enzyme of *Y. pseudotuberculosis* for leucine in *Y. pestis* (Chain *et al.*, 2004, 2006; Deng *et al.*, 2002; Parkhill *et al.*, 2001; Zhou *et al.*, 2004). While no other substitutions occur in Zwf, *Y. pestis* strain KIM (used in this study) also possesses a unique second base transversion ( $\text{G}\cdot\text{C}\rightarrow\text{T}\cdot\text{A}$ ) at position 146 of AspA, causing replacement of aspartate by tyrosine (Deng *et al.*, 2002) (Fig. 1). The substitution at position 146 was not reported for other yersiniae, including strains of *Y. pseudotuberculosis*.

Substitution of proline for other amino acids significantly alters the tertiary structure of many proteins and this effect may account for loss of Zwf activity in *Y. pestis*. However, loss of AspA accompanied by secretion of L-aspartate by conservative replacement of leucine for valine seemed unlikely. Alternative explanations for accumulation of L-aspartate include the possibility of an increase in the oxaloacetate pool, favouring its transamination and release. It is significant in this context that the major chaperone GroEL, required for T3SS activity, is the only chromosomally encoded protein known to be produced during expression of the LCR by *Y. pestis* (Mehigh & Brubaker, 1993). This heat-shock protein is also essential for the biological activity of citrate synthase (Grallert *et al.*, 1998); thus T3SS-dependent depletion of GroEL could cause accumulation of oxaloacetate and its subsequent transamination and excretion as L-aspartate. Other obvious alternatives exist, although a block in transcription of *aspA* seems unlikely as judged by its evident normal expression as determined by microarray analysis (Motin *et al.*, 2004). The aims of this study were to demonstrate that AspA is expressed as cross-reacting immunological material (CRIM) in *Y. pestis* and to determine if the conserved single  $\text{G}\cdot\text{C}\rightarrow\text{T}\cdot\text{A}$  transversion at amino acid position 363 of AspA accounts for loss of enzymic activity.

## METHODS

**Bacterial strains and cultivation.** Yersiniae used in this study were the type strains *Y. pseudotuberculosis* PB1 (Burrows & Bacon, 1960) of serogroup 1b (Thal & Knapp, 1971) and *Y. pestis* KIM (Finegold *et al.*, 1968) of the Medievalis biovar (Devignat, 1951). Avirulent  $\text{Lcr}^-$  mutants of both species were selected on magnesium oxalate agar (Higuchi & Smith, 1961) and a mutant of *Y. pestis* KIM lacking the ~100 kb pigmentation sequence was isolated on Congo red agar (Surgalla & Beesley, 1969). Transformed cells of *Escherichia coli* JRG1476 (*aspA*) were used to prepare the *aspA* products of *Y. pestis* and *Y. pseudotuberculosis*. The chemically defined medium employed previously for microarray analysis (Motin *et al.*, 2004) and definition of the LCR (Brubaker, 2005; Fowler & Brubaker, 1994) was used to cultivate yersiniae for enzyme analysis. Conditions of growth, modifications of the medium that induce bacteriostasis versus steady-state growth, and preparation of the variations used in this study have been described in detail (Brubaker, 2007). The medium used in these experiments contained L-glutamate (25 mM) and  $\text{Na}^+$  (100 mM) in the presence (4.0 mM) and absence of added  $\text{Ca}^{2+}$ . *E. coli* JRG1476 was grown in Luria broth with kanamycin (100  $\mu\text{g ml}^{-1}$ ) for preparation of cloned *aspA* products as defined below.

**Enzyme analysis.**  $\text{Lcr}^+$  and  $\text{Lcr}^-$  yersiniae were cultivated in chemically defined medium (Brubaker, 2007), harvested by centrifugation at 4 °C (10 000 g for 30 min), suspended and washed twice by similar centrifugation in cold 0.033 M potassium phosphate buffer, pH 7.0, brought to an  $\text{OD}_{620}$  of ~400 in 0.05 M Tris/HCl buffer, pH 7.8, and disrupted by passage through a French pressure cell. The resulting preparation was again centrifuged as described and the cell-free extract carefully removed with a pipette, passed through a 0.22  $\mu\text{m}$  low-protein-binding filter (Millipore), and dialysed overnight against 0.01 M Tris/HCl buffer, pH 7.8, containing DTT (1.0 mM). The resulting cell-free extracts were used immediately for enzymic analysis. In all cases, protein concentration was determined by the Lowry method.

1	MSNNRIEED	LLGTREVPAE	AYYGVHTLRA	IENFYISNSK	ISDVPEFVRG	MVMVKKAAAM	ANKELHTIPR	KIADIIIQAC	DEVLDKGGKCM	DQFPVDVFGQ
	MSNNRIEED	LLGTREVPAE	AYYGVHTLRA	IENFYISNSK	ISDVPEFVRG	MVMVKKAAAM	ANKELHTIPR	KIADIIIQAC	DEVLDKGGKCM	DQFPVDVFGQ
	MSNNRIEED	LLGTREVPAD	AYYGVHTLRA	IENFYISNNK	ISDIPEFVRG	MVMVKKAAAM	ANKELQTIPTK	SVANAIIAAC	DEVLNNGKCM	DQFPVDVYQG
	MSNNRIEED	LLGTREVPAD	AYYGVHTLRA	IENFYISNNK	ISDIPEFVRG	MVMVKKAAAM	ANKELQTIPTK	SVANAIIAAC	DEVLNNGKCM	DQFPVDVYQG
							↑	↑	↑	↑↑
101	GAGTSLNMNT	NEVLANIGLE	LMGHQKGEYQ	YLNPNDDLK	CQSTNDAYPT	GFR IAVYASI	LKLIDAINQL	GEGFGRKSKE	FEKILKMGRT	QLQDAVPMTL
	GAGTSLNMNT	NEVLANIGLE	LMGHQKGEYQ	YLNPNDDLK	CQSTN <u>Y</u> AYPT	GFR IAVYASI	LKLIDAINQL	GEGFGRKSKE	FEKILKMGRT	QLQDAVPMTL
	GAGTSLNMNT	NEVLANIGLE	LMGHQKGEYQ	YLNPNDHVNK	CQSTNDAYPT	GFR IAVYSSI	LKLIDAINQL	GEGFGRKSKE	FEKILKMGRT	QLQDAVPMTL
	GAGTSLNMNT	NEVLANIGLE	LMGHQKGEYQ	YLNPNDHVNK	CQSTNDAYPT	GFR IAVYSSI	LKLIDAINQL	REGFERKAVE	FQDILKMGRT	QLQDAVPMTL
								▲	▲	↑
									↑	↑
201	GQEFRAFQVL	LNEETKNLQR	TAEELLEVLN	GATAIGTALN	TPEGYSQLAV	QKLAEISGLA	CVPAEDLIEA	TSDCGAYVMV	HSALKRLAVK	MSKICNDLRL
	GQEFRAFQVL	LNEETKNLQR	TAEELLEVLN	GATAIGTALN	TPEGYSQLAV	QKLAEISGLA	CVPAEDLIEA	TSDCGAYVMV	HSALKRLAVK	MSKICNDLRL
	GQEFRAFQVL	LNEETKNLQR	TAEELLEVLN	GATAIGTALN	TPEGYSQLAV	QKLAEISGLA	CVPAEDLIEA	TSDCGAYVMV	HSALKRLAVK	MSKICNDLRL
	GQEFRAFQVL	LKEEVKNLQR	TAEELLEVLN	GATAIGTGLN	TPKEYSPLAV	KKLAIEVTFGP	CVPAEDLIEA	TSDCGAYVMV	HGALKRLAVK	MSKICNDLRL
	GQEFRAFQVL	LKEEVKNLQR	TAEELLEVLN	GATAIGTGLN	TPKEYSPLAV	KKLAIEVTFGP	CVPAEDLIEA	TSDCGAYVMV	HGALKRLAVK	MSKICNDLRL
		▲		◆	◆	▲				
301	LSSGPRTGLN	EINLPELQAG	SSIMPAKVNP	VIPEVVNQVC	FKVIGNDTCI	TMAAEAGQLQ	LNVMPEVIGQ	AMFESIHILT	NACYNLLEKC	INGITANKEV
	LSSGPRTGLN	EINLPELQAG	SSIMPAKVNP	VIPEVVNQVC	FKVIGNDTCI	TMAAEAGQLQ	LNLMPEVIGQ	AMFESIHILT	NACYNLLEKC	INGITANKEV
	LSSGPRTGLN	EINLPELQAG	SSIMPAKVNP	VIPEVVNQVC	FKVIGNDTCI	TMAAEAGQLQ	LNLMPEVIGQ	AMFESIHILT	NACYNLLEKC	INGITANKEV
	LSSGPRTGLN	EINLPELQAG	SSIMPAKVNP	VIPEVVNQVC	FKVIGNDTTV	TMAAEAGQLQ	LNVMPEVIGQ	AMFESIHILT	NACYNLLEKC	INGITANKEV
	LSSGPRTGLN	EINLPELQAG	SSIMPAKVNP	VIPEVVNQVC	FKVIGNDTTV	TMAAEAGQLQ	LNVMPEVIGQ	AMFESIHILT	NACYNLLEKC	INGITANKEV
401	CERYVFNSIG	IVTYLNPFIG	HHNGDIVGKI	CAETGKNVRE	VVLERGLLTE	AELDDIFSVE	NLMHPAYKAK	RYTDENEQ	<i>Y. pseudotuberculosis</i> IP32953	
	CERYVFNSIG	IVTYLNPFIG	HHNGDIVGKI	CAETGKNVRE	VVLERGLLTE	AELDDIFSVE	NLMHPAYKAK	RYTDENEQ	<i>Y. pestis</i> KIM	
	CERYVFNSIG	IVTYLNPFIG	HHNGDIVGKI	CAETGKNVRE	VVLERGLLTE	AELDDIFSVE	NLMHPAYKAK	RYTDENEQ	<i>Y. pestis</i> CO92	
	CEGYVYNSIG	IVTYLNPFIG	HHNGDIVGKI	CAETGKSVRE	VVLERGLLTE	AELDDIFSVE	NLMHPAYKAK	RYTDESEQ	<i>E. coli</i> K12	
	CEGYVYNSIG	IVTYLNPFIG	HHNGDIVGKI	CAETGKSVRE	VVLERGLLTE	AELDDIFSVE	NLMHPAYKAK	RYTDESEQ	<i>E. coli</i> O157:H7	
		↑					↑			

**Fig. 1.** Sequence alignment of AspA from *Y. pestis* (Deng *et al.*, 2002; Parkhill *et al.*, 2001), *Y. pseudotuberculosis* (Chain *et al.*, 2004) and *E. coli* (Blattner *et al.*, 1997; Hayashi *et al.*, 2001). Mutations at positions 146 and 363 (underlined) are the only differences found between the yersinia aspartases. In contrast, there are numerous conservative and non-conservative changes between the *E. coli* and yersinia enzymes, including non-polar or polar amino acids replaced by charged residues (▲), charged amino acids replaced by neutral residues (▲), and reversals of positively and negatively charged groups (◆).

AspA activity in these preparations was estimated by determining the L-aspartic acid-dependent release of  $\text{NH}_4^+$  with Nessler's reagent as undertaken previously (Dreyfus & Brubaker, 1978). The reaction mixture consisted of 250  $\mu\text{mol}$  Tris/HCl buffer (pH 7.0), 5.0  $\mu\text{mol}$   $\text{MgCl}_2$ , and dialysed cell-free extract in a total volume of 4.5 ml. The assay was started by addition of 250  $\mu\text{mol}$  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 Beckman II microfuge and 0.5 ml 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 incubation for 10 min, were assayed for asparaginase activity by measuring  $A_{480}$  (Yellin & Wrison, 1966). The resulting values were then evaluated against a standard curve, prepared immediately before each determination by assays using known concentrations of  $\text{NH}_4\text{Cl}$  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. AspA activity in purified preparations was measured by monitoring the appearance of fumarate at 240 nm ( $\epsilon=2.53 \text{ mM}^{-1} \text{ cm}^{-1}$ ) (Karsten *et al.*, 1985) in an assay buffer consisting of HEPES, pH 8.0 (50 mM), magnesium acetate (10 mM) and L-aspartate (20 mM). Enzyme units were defined as  $\mu\text{mol}$  product generated per minute. The kinetic parameters of the yersinia aspartases were measured by varying the concentration of L-aspartate and fitting the data to the Michaelis-Menten equation. Kinetic assays were performed on either a Perkin-Elmer Lambda-1 or a Cary 50 UV/visible spectrophotometer at a constant temperature of 20 °C. Citrate synthase in crude extracts was

measured by determining the oxaloacetate-dependent release of CoA from acetyl-CoA at 412 nm (Srere, 1969). L-Glutamate:oxaloacetate transaminase and L-glutamate dehydrogenase in these samples were determined as described previously (Dreyfus & Brubaker, 1978).

**Cloning, expression and purification of AspA.** The *aspA* genes of yersinia were PCR-amplified using the conserved primers CGA214 (5'-GAAGGTTTCATATGTCAAATAACATTCG-3') and CGA215 (5'-CCGAGAAAATGAACAATAAGCTTAATATTC-3'). CGA214 incorporates a *Nde*I site at the start codon and CGA215 incorporates a *Hind*III site at the stop codon of *aspA* (underlined). These enzymes were used to digest the products, which were ligated into the expression vector pET24a digested with the same enzymes. The resulting plasmids were transformed into the *aspA*-deficient *E. coli* JRG1476 (Guest *et al.*, 1984). Mutagenic primers were obtained from Integrated DNA Technologies. AspA production was initiated during cultivation at 37 °C in a shaker bath by addition of 1 mM IPTG, followed by incubation for an additional 5 h at 32 °C. Approximately 2.5 g of wet cell paste was harvested from each litre of growth medium. This material was then suspended in the purification buffer (50 mM potassium phosphate, pH 6.5, with 1 mM EDTA and 1 mM DTT) and the organisms were disrupted by sonication. Soluble proteins were fractionated in purification buffer with a 0 to 1.0 M sodium chloride gradient on successive anion-exchange Sepharose XL high-capacity and Source 30Q high-resolution chromatography columns (Amersham Bioscience). The same purification protocol was used for preparation of the cloned *aspA* products of both yersinia. The overall yield of highly purified (>95%) enzyme was about 50 mg protein from each litre of growth medium.

**Analysis for cross-reacting immunological material (CRIM).**

Biologically active AspA from *E. coli* K-12 and *Y. pseudotuberculosis* PB1 and the inactive protein from *Y. pestis* were isolated essentially as outlined above and used to prepare polyclonal antiserum in rabbits. This process entailed use of the immunization regimen used previously for LcrV (Une & Brubaker, 1984) except that adjuvant was omitted; generation of precipitating antibodies was verified by diffusion in agar against the purified proteins. Immunoblots were undertaken as described previously (Motin *et al.*, 1994).

**Site-directed mutagenesis.** Mutations of *aspA* from *Y. pseudotuberculosis* and *Y. pestis* were constructed using the Quik-Change II Site Directed Mutagenesis kit (Stratagene) and confirmed by DNA sequencing (MWG). A single base change is needed to alter the GUG codon (valine) to UUG (leucine) in order to obtain the V363L *Y. pseudotuberculosis* mutant. The *Y. pestis* L363V mutation also requires only this single base change and, in order to make the Y146D mutant of *Y. pestis* KIM, the UAU codon of tyrosine was changed to the GAU codon of aspartate. The Y146D/L363V *Y. pestis* double mutant incorporated both of these base changes. These mutants were expressed and purified by using the same protocol as described above for the native enzymes.

**Quaternary structure determination.** The purified yersiniae aspartases (0.5–1.0 mg ml<sup>-1</sup> in the phosphate purification buffer) were studied by dynamic light scattering (DLS) (DynaPro Titan, Wyatt Technologies) at 20 °C and 4 °C to determine the average particle size in solution. Native gel electrophoresis was carried out in Tris/glycine buffer (25 mM Tris, pH 8, 200 mM glycine) using 4–12% Bistris polyacrylamide gels (Invitrogen). Gel filtration studies were conducted on a Superdex 200 column in 50 mM potassium phosphate, pH 6.5, with 1 mM EDTA and 1 mM DTT.

**Circular dichroism (CD) spectroscopy.** To compare the tertiary structure of *Y. pestis*, *Y. pseudotuberculosis* and the mutant V363L aspartases, the purified enzymes were examined by CD spectroscopy (Cary 62DS spectrometer) over the range 190–260 nm at room temperature. The enzyme concentrations in these solutions were varied from 6 to 20 µM, and the spectra reported represent an average of five scans.

**Mass spectrometry.** The purified aspartases from *Y. pestis* and *Y. pseudotuberculosis* were examined on a Q-TOF2 electrospray ionization mass spectrometer (ESI-MS) to determine accurate molecular

masses. Each enzyme form was dialysed into 10 mM ammonium acetate buffer, pH 6.5, and about 0.5 mg of each protein was used for mass analysis.

**RESULTS****Comparative catabolism of L-glutamate**

As noted above, cells of Lcr<sup>+</sup> *Y. pestis* exhibit a stringent LCR characterized by prompt bacteriostasis and secretion of L-aspartate arising from exogenous L-glutamate. Results of enzymic analysis were consistent with normal transamination by *Y. pestis* and *Y. pseudotuberculosis* of L-glutamate by L-glutamate:oxaloacetate transaminase with a catalytic amount of oxaloacetate (derived from 2-oxoglutarate via the TCA) to form L-aspartate (Table 1). The latter, however, was evidently unable to regenerate oxaloacetate in *Y. pestis* by recycling into the TCA as judged by the observed loss of AspA activity. The presence of similar levels of citrate synthase activity in both yersiniae suggests that a limitation of this enzyme in *Y. pestis* does not promote accumulation of oxaloacetate and its attendant conversion to L-aspartate. Finally, no significant differences were detected between the yersiniae in the activity of (primarily anabolic) L-glutamate dehydrogenase. The specific activity of these enzymes was not significantly influenced by Ca<sup>2+</sup>. Considered together, these findings indicate that the reported secretion of L-aspartate during the LCR is consistent with the observed deficiency of AspA activity in *Y. pestis*.

**Translation of *aspA* in *Y. pestis***

Results of prior microarray analysis indicated that *aspA* undergoes normal transcription in *Y. pestis* (Motin *et al.*, 2004); thus we considered the possibility that inactive AspA in this species reflects a lesion in translation. To examine this prospect, polyclonal rabbit antiserum was raised

**Table 1.** Specific activities of selected enzymes in Lcr<sup>+</sup> and Lcr<sup>-</sup> cells of *Y. pestis* KIM and *Y. pseudotuberculosis* PB1

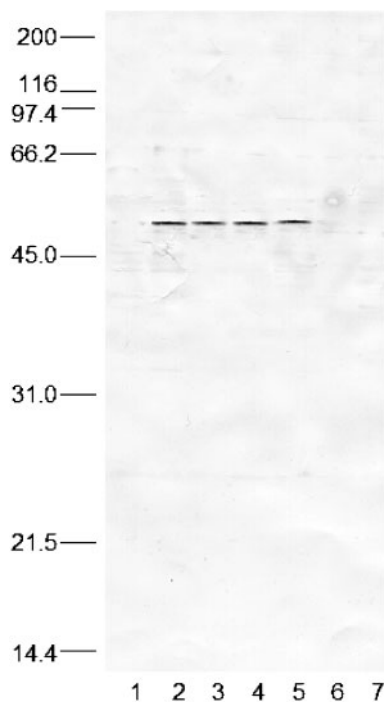
The bacteria were cultivated at 37 °C in chemically defined medium with L-glutamate (25 mM) and Na<sup>+</sup> (100 mM) in the presence (4.0 mM) and absence of added Ca<sup>2+</sup>. See Methods for details of enzyme assays and units.

Enzyme	Added Ca <sup>2+</sup>	<i>Y. pestis</i>		<i>Y. pseudotuberculosis</i>	
		pCD <sup>+</sup>	pCD <sup>-</sup>	pYV <sup>+</sup>	pYV <sup>-</sup>
L-Glutamate:oxaloacetate transaminase	0	0.074	0.089	0.085	0.087
	+	0.116	0.079	0.086	0.072
L-Glutamate dehydrogenase	0	0.033	0.030	0.027	0.026
	+	0.033	0.028	0.024	0.020
Citrate synthase	0	0.039	0.049	0.059	0.033
	+	0.045	0.043	0.038	0.031
Aspartase (L-aspartate ammonia-lyase)	0	<0.001	<0.001	0.075	0.050
	+	<0.001	<0.001	0.060	0.044

against highly purified AspA activity from Lcr<sup>-</sup> cells of *Y. pseudotuberculosis*. The resulting antiserum reacted in immunoblots with the homologous ~50 kDa peptide of *Y. pseudotuberculosis*, the corresponding material purified from *Y. pestis*, and crude extracts of *Y. pestis*, *Y. pseudotuberculosis* and *E. coli* (Fig. 2). A slight excess of the antiserum also eliminated >99% of the enzymic activity of AspA from *Y. pseudotuberculosis*. These findings indicate that *Y. pestis* normally expresses defective AspA as a CRIM and suggest that one or both of the known changes in primary structure of the inactive enzyme (Y146D and L363V) accounts for loss of enzymic activity.

### Activities of AspA from *Y. pseudotuberculosis* versus the *Y. pestis* CRIM

The specific activity of the purified cloned AspA of *Y. pseudotuberculosis* was 80 units mg<sup>-1</sup> (Table 2), which is comparable to that of AspA from *E. coli* (Karsten *et al.*, 1985). In contrast, the specific activity of the similarly



**Fig. 2.** Immunoblot prepared with rabbit polyclonal antiserum raised against highly purified AspA of *Y. pseudotuberculosis* PB1 after SDS-PAGE directed against phosphate-buffered saline (lanes 1, 7), AspA of *Y. pseudotuberculosis* (1.0 µg ml<sup>-1</sup>) used for immunization (lane 2), similarly prepared AspA (CRIM) of *Y. pestis* KIM (lane 3), crude cell-free extract (10 µg ml<sup>-1</sup>) of *Y. pseudotuberculosis* PB1 (lane 4), crude cell-free extract (10 µg ml<sup>-1</sup>) of *Y. pestis* KIM (lane 5), and crude cell-free extract (10 µg ml<sup>-1</sup>) of *Y. pestis* KIM after absorption with excess anti-AspA serum (1 mg γ-globulin) (lane 6). Positions of size standards (kDa) are shown on the left.

prepared CRIM of *Y. pestis* was more than 300-fold lower (0.2 units mg<sup>-1</sup>). The catalytic rates of the *Y. pestis* and *Y. pseudotuberculosis* enzymes were measured at varying concentrations of L-aspartate to assess whether this reduction was a consequence of changes in substrate recognition. The  $K_m$  for L-aspartate was comparable between these two enzyme forms, but the  $k_{cat}$  of the *Y. pestis* CRIM was significantly reduced (Table 2). To establish the importance of position 363, a mutant of the *Y. pseudotuberculosis* AspA was constructed where valine was replaced by leucine to mimic the nearly inactive CRIM of *Y. pestis*. The latter was purified, kinetically characterized, and found to have a low specific activity of 0.2 units mg<sup>-1</sup> similar to that of the *Y. pestis* AspA. Accordingly, the single conservative replacement of one aliphatic amino acid by another at position 363 was sufficient to dramatically attenuate catalytic activity without inducing gross structural changes.

### Consequences of missense mutations in AspA on specific activity

Since conservative replacement of leucine for valine in the *Y. pseudotuberculosis* enzyme caused dramatic loss of catalytic activity, full recovery was anticipated upon substitution of valine for leucine in the CRIM of *Y. pestis*. This L363V variant was constructed, expressed and purified but, surprisingly, showed the same low catalytic activity as the native *Y. pestis* AspA (Table 2). As previously noted, the only other difference between the enzyme from *Y. pseudotuberculosis* PB1 and *Y. pestis* KIM is a non-conservative replacement in the latter of tyrosine for aspartic acid at position 146, although aspartic acid occupies this position in other yersiniae, including *Y. pestis* CO92 (Parkhill *et al.*, 2001). A Y146D variant of the *Y. pestis* KIM AspA was prepared and characterized but this replacement similarly failed to enhance catalysis (Table 2). Since neither of these single reversions alone resulted in significant recovery of enzymic activity, either one or the other forward mutation is evidently sufficient to cause the observed inhibition. To verify this assumption, a double Y146D/L363V mutant possessing an amino acid sequence identical to that of the fully active *Y. pseudotuberculosis* enzyme was prepared and subjected to enzymic analysis. These two changes resulted in an enzyme form with high specific activity (72 units mg<sup>-1</sup>) and kinetic parameters that were indistinguishable from the native *Y. pseudotuberculosis* AspA (Table 2).

### Structural comparison of AspA from *Y. pseudotuberculosis* and the *Y. pestis* CRIM

The similar  $K_m$  for L-aspartic acid suggested that the primary structure responsible for substrate recognition and binding was not affected by the Y146D and L363V substitutions but that these mutations alter the positioning of catalytic residues at the active site, thereby causing a loss of activity. By analogy with the well-established tetrameric

**Table 2.** Kinetic parameters of L-aspartases from *Y. pestis* KIM and *Y. pseudotuberculosis* PB1

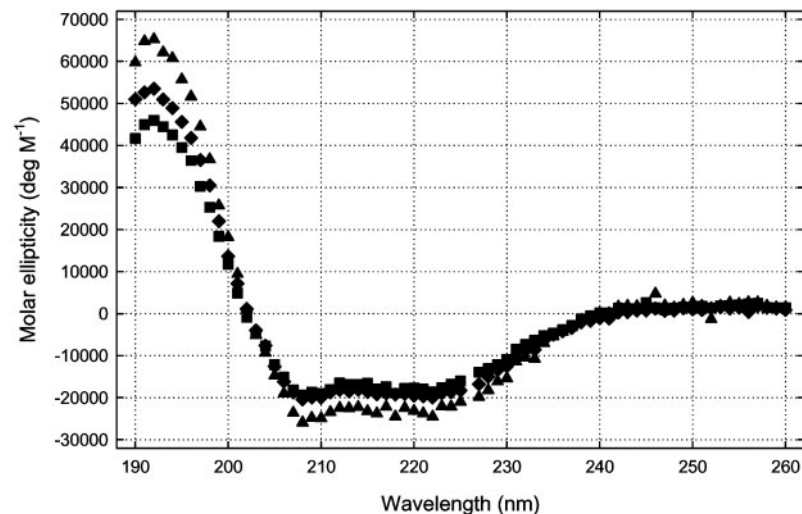
Source	Specific activity (U mg <sup>-1</sup> )	$k_{\text{cat}}$ (s <sup>-1</sup> )	$K_{\text{m}}$ (mM)	$k_{\text{cat}}/K_{\text{m}}$ (M <sup>-1</sup> s <sup>-1</sup> )	$k_{\text{cat}}/K_{\text{m}}$ (%)
<i>Y. pseudotuberculosis</i>	80	87 ± 4	2.1 ± 0.2	41 700 ± 2600	100
<i>Y. pestis</i>	0.2	0.10 ± 0.06	1.3 ± 0.2	80 ± 7	0.2
L363V <i>Y. pestis</i>	0.2	0.12 ± 0.01	1.5 ± 0.2	80 ± 8	0.2
Y146D <i>Y. pestis</i>	0.2	0.13 ± 0.01	1.3 ± 0.2	100 ± 10	0.25
Y146D/L363V <i>Y. pestis</i>	72	86 ± 2	1.2 ± 0.1	74 000 ± 3000	180

structure of *E. coli* AspA (Watanabe *et al.*, 1981), the enzymes from yersiniae are also expected to exist as tetramers. However, because the active site of AspA is formed at the interface between subunits (Shi *et al.*, 1997), any changes in subunit association as a consequence of these point mutations might alter the active site structure and therefore affect catalytic activity.

This possibility was investigated by analysis of the quaternary structure and subunit conformation of these enzyme forms. The average particle size of the AspA species was determined in solution at 20 °C and 4 °C by DLS. Both enzymes showed a similar peak via DLS at both temperatures, with a particle size predicting a molecular mass of 130 ± 20 kDa. The actual molecular mass of the AspA subunit was 52 kDa, so the expected mass of the tetramer should be about 200 kDa. Mass prediction by DLS assumes that the protein has a spherical shape, whereas the structure of tetrameric *E. coli* AspA is quite elongated (Shi *et al.*, 1997), so the value predicted by DLS deviates from the actual mass. To compensate for the errors in mass estimation due to this non-spherical shape, the well-characterized *E. coli* enzyme was compared and found to yield a predicted value of 135 kDa. Since the yersiniae enzymes have essentially the same particle size as the *E. coli* AspA, they likely also exist predominantly as tetramers in solution. This assumption was supported by gel filtration studies, in which a mixture of purified *E. coli* and yersiniae enzymes co-eluted from a Superdex 200 size-

exclusion column. Native gel electrophoresis also showed bands at the same position as the *E. coli* AspA for each of the yersiniae enzymes, although some smearing was observed for the *Y. pestis* AspA (data not shown), suggesting partial equilibrium with a lower molecular mass component. These findings argue against the possibility that loss of activity reflects significant changes in subunit association.

AspA from *Y. pseudotuberculosis* and *Y. pestis* was also examined by CD spectroscopy over the wavelength range 190–260 nm to assess possible changes in secondary structure that could be responsible for the observed catalytic differences. The two native enzymes exhibited virtually identical spectra (Fig. 3), indicating similar overall folding and secondary structure. A nearly identical CD spectrum was also measured for the V363L mutant of *Y. pseudotuberculosis* aspartase. Therefore the mutations in primary structure found to cause dramatic differences in catalytic activity evidently do not promote significant changes in secondary structure. Samples of *Y. pseudotuberculosis* and *Y. pestis* AspA were also examined by mass spectrometry to identify potential covalent protein modifications at specific amino acids that could be capable of causing the observed changes in activity. The molecular mass of the CRIM from *Y. pestis* as determined by ESI-MS was 52 526 Da compared to a calculated mass of 52 536 Da; the corresponding value estimated for the AspA of *Y. pseudotuberculosis* was within 2 Da of the calculated



**Fig. 3.** CD spectra of the purified aspartases from *Y. pseudotuberculosis* PB1 (◆), *Y. pestis* KIM (■) and the V363L mutant of *Y. pseudotuberculosis* (▲).

molecular mass of 52 474 Da. These results indicate that covalent modifications do not account for the dramatic differences in catalytic activity observed between the two proteins.

## DISCUSSION

### Sequence comparison of aspartases

Although most mutational events causing loss of enzymic activity are readily identified by bioinformatics analysis, missense mutations are often overlooked because the investigator cannot distinguish between amino acid substitutions in silent regions and those occurring at critical positions such as the catalytic centre. Prior knowledge that a given function is defective may warrant the suggestion that a markedly dissimilar amino acid accounts for inactivity (as occurs upon replacement of proline for serine in *Zwf* of *Y. pestis*). However, conservative amino acid changes cannot, at present, be correlated with altered enzymic activity. The fully active *AspA* of *Y. pseudotuberculosis* emphasizes this phenomenon: its sequence differs from that of the *E. coli* enzyme at about 50 amino acid positions (Fig. 1). While many of these differences are conservative replacements, there are nine positions in which polar or non-polar amino acids are replaced by charged residues, an additional five positions where charged residues have been replaced by uncharged amino acids, and two positions where positively and negatively charged amino acids have been swapped. Despite these extensive changes, the kinetic parameters of the *E. coli* and *Y. pseudotuberculosis* aspartases are indistinguishable. As previously defined, the sequences of the L-aspartases from *Y. pseudotuberculosis* and *Y. pestis* are virtually identical (differing by only one or two amino acids) yet the *Y. pestis* enzyme is essentially inactive. This relationship suggests that the identity of the amino acids at these positions, Asp146 and Val363, must play significant roles in maintaining catalytic activity.

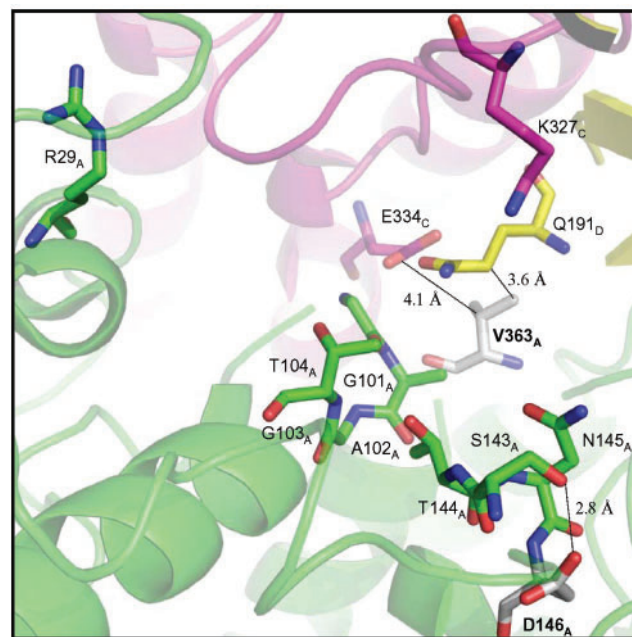
### Structural comparison of aspartases

Several members within this ammonia-lyase family catalyse a deamination reaction that is related to that of aspartase, and they do so by a mechanism involving a dehydroalanine that is generated through modification of an active site serine (Langer *et al.*, 1994; Schuster & Rétey, 1995). However, no such modifications were observed by mass spectrometry in the yersiniae aspartases that could account for the observed differences in activity. While changes in the identity of the amino acids at these positions were shown to have a dramatic impact on the catalytic activity of aspartase, this effect is not caused by significant changes in the higher-order structure of the *Y. pestis* protein. CD spectra of the active and inactive forms of aspartase are nearly identical, indicating no substantial changes in secondary structure.

DLS and size-exclusion chromatography data are consistent with tetrameric structures for each enzyme form.

### Active site structure of L-aspartases

We have examined the location of Asp146 and Val363 within the aspartase structure to gain a better understanding of their potential roles. The location of the active site of aspartase was previously determined by comparing the apo-aspartase structure (Shi *et al.*, 1997) with *E. coli* fumarase C (Weaver *et al.*, 1995; Weaver & Banaszak, 1996), a well-studied enzyme from the same family that catalyses a similar reaction. There are four active sites in the tetramer of aspartase, with each active site found at the junction of sets of three out of the four subunits. For example, the putative active site residues of one such site in *E. coli* aspartase includes the proposed substrate-binding groups Lys327 from subunit C and Arg29 from subunit A, along with the catalytic Ser143 from subunit A and an additional loop from subunit D (Fig. 4). Each of these active site amino acids is fully conserved throughout the entire aspartase family. Ser143 was proposed to function as a catalytic acid (Jayasekera *et al.*, 1997), and a conservative substitution of Ser143 by threonine results in a dramatic loss of catalytic activity to <2 % that of native aspartase (Jayasekera & Viola, 1999). However, the amino acid at position 363 is more than 10 Å (1 nm) away from the putative substrate-binding residues. Thus, there is a low possibility for changes at this



**Fig. 4.** Putative active site of aspartase from *E. coli* at the junction of subunits A (green), C (pink) and D (yellow) (Shi *et al.*, 1997), with the mutation positions of amino acids from the *Y. pestis* KIM enzyme (in grey) and their proximity to functionally important amino acids in two adjacent subunits.

position to affect substrate binding. This is consistent with the unaltered Michaelis constant for L-aspartate within the tested family of yersinia enzymes (Table 2). The amino acid at the other varied position, Asp146, is situated adjacent to the putative catalytic amino acid, Ser143, with the aspartate carboxyl oxygen about 2.8 Å (0.28 nm) from the serine hydroxyl group (Fig. 4). This carboxylate group is involved in helping to position this serine for catalysis, along with Asn145. A non-conservative replacement of Asp146 would certainly disrupt the orientation of the serine side chain and diminish catalytic efficiency. However, as noted, a mutation leading to a tyrosine in position 146 is only observed in the *Y. pestis* KIM strain, while the other low-activity yersinia isolates retain an aspartate at this position. Replacement of this tyrosine with an aspartic acid was not sufficient to recover enzymic activity, so other changes must also disrupt catalysis in the *Y. pestis* CRIM.

### Effect of mutations on catalytic activity

Mutations at position 363 are found in all *Y. pestis* aspartases, and seem to be more directly correlated with diminished enzyme performance. Val363, present in the active *E. coli* and *Y. pseudotuberculosis* aspartases, is positioned at the bottom of the active site cleft (Fig. 4) and is 3.6–4.0 Å (0.36–0.4 nm) away from the closest contacts with Glu334 of subunit C and Gln191 of subunit D. The comparable amino acids in fumarase (Glu331 and His188) form a charge-relay pair that is proposed to activate a water molecule for proton abstraction from the L-malate substrate (Weaver & Banaszak, 1996). However, this putative catalytic histidine of fumarase is a glutamine in the aspartases, a functional group that could not be involved in the proposed charge-relay system. Thus, the roles of these residues are not as well established for aspartases. The side chain of Val363 in the catalytically active *E. coli* and *Y. pseudotuberculosis* aspartases is small enough to avoid steric clashes with the side chains of Glu334 and Gln191. However, the enzymes with low catalytic activity each have larger residues at this position. Even the conservative mutation to a leucine with a slightly larger side chain volume in *Y. pestis* aspartase was found to have a great impact on catalytic activity. These observations suggest that mutations at position 363 indirectly inhibit enzyme activity, perhaps by causing the catalytic residues Glu334 and Gln191 to alter their positions, thus disrupting their contributions to catalysis.

### Excretion of L-aspartate

The species-specific deficiency of AspA activity (Dreyfus & Brubaker, 1978) and attendant secretion of L-aspartate by Ca<sup>2+</sup>-starved *Y. pestis* is well established (Brubaker, 2005, 2007). Nevertheless, only a casual relationship existed between these phenomena prior to this study, which detected no significant differences in major enzymes that influence the aspartate pool other than the AspA of *Y. pseudotuberculosis* and corresponding CRIM of *Y. pestis*. Additional work will be

required to determine the reasons why secretion of L-aspartate is limited to Ca<sup>2+</sup>-starved plague bacilli and why Na<sup>+</sup> promotes the associated nutritional requirement for Ca<sup>2+</sup> (Brubaker, 2007). Structural studies have begun, with the aim of verifying the proposed alterations in side chain positioning at the active site of aspartase. We are also examining the possibility that the loss of enzymic activity caused by these changes may promote acute disease. This prospect is in accord with the finding that the attenuated *Microtis* biovar of *Y. pestis*, known to remain virulent for the Muridae but not humans (Fan *et al.*, 1995), possesses the same functional *aspA* as does *Y. pseudotuberculosis* (Zhou *et al.*, 2004).

### Concluding remarks

In summary, these results indicate that the observed replacement of one aliphatic amino acid (leucine) for another (valine) in AspA of *Y. pestis* is hardly conservative and does indeed contribute to the loss of enzymic activity, resulting in major phenotypic changes that define the plague bacillus. Taken together, these findings strongly indicate that the missense mutation at amino acid position 363 accounts for loss of enzymic activity in *Y. pestis* AspA. Further study will be necessary to determine if the similar event at position 146 is limited to the KIM strain. In this context, isolates possessing only the missense mutation at position 363 could readily revert or suppress this mutation in one step, whereas two such events would be required to restore AspA activity in strain KIM.

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