Recent Findings Regarding Maintenance of Enzootic Variants of *Yersinia pestis* in Sylvatic Reservoirs and Their Significance in the Evolution of Epidemic Plague

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Abstract

Despite the widespread presence of bubonic plague in sylvatic reservoirs throughout the world, the causative agent (*Yersinia pestis*) evolved in its present form within the last 20,000 years from enteropathogenic *Yersinia pseudotuberculosis*. Comparison of the genomes from the two species revealed that *Y. pestis* possesses only a few unique plasmid-encoded genes that contribute to acute disease, whereas this organism has lost about 13% of the chromosomal genes that remain active in *Y. pseudotuberculosis*. These losses reflect readily detectable additions, deletions, transpositions, inversions, and acquisition of about 70 insertion sequence (IS) inserts, none of which are likely to promote increased virulence. In contrast, major enzymes of intermediary metabolism, including glucose 6-phosphate dehydrogenase (Zwf) and aspartase, are present but not catalytically functional due to the presence of missense mutations. The latter are generally not detectable by the technology of bioinformatics and, in the case of *Y. pestis*, result in radical changes in the metabolic flow of carbon. As an important consequence, plague bacilli exhibit a stringent low-calcium response characterized by conversion of L-glutamate (and metabolically related amino acids) to L-aspartate with secretion of the latter into supernatant fluid at 37°C in culture media containing Na⁺ but lacking added Ca²⁺. This phenomenon also occurs *in vivo* and likely adversely affects the bioenergetics of host amino acid pools. Curiously, aspartase is functional in all tested enzootic (pestoides) strains of *Y. pestis*. These isolates are typically restricted to the ancient plague reservoirs of Central Asia and Africa and are fully virulent in members of the rodent Superfamily Muroidea but avirulent in guinea pigs and man. The implications of these findings for the distribution and ecology of *Y. pestis* could be significant.

Key Words: Aspartase—Glucose 6-phosphate dehydrogenase—Intermediary metabolism—Pestoides.

Introduction

APPROXIMATELY 200 MILLION YEARS AGO the yersinia line carrying the progenitor low Ca²⁺-response (LCR) virulence plasmid, now termed pCD (calcium dependence) in *Yersinia pestis* (Ferber and Brubaker 1981), diverged, leading to the emergence of modern *Yersinia enterocolitica* and *Y. pseudotuberculosis* (Achtman et al. 1999). These organisms retain slightly different functional forms of this ~70-kb plasmid (Hu et al. 1998), where it is designated pYV (*Yersinia* Virulence) (Zink et al. 1980), and exhibit both modest gains and losses of chromosomal genes (Chain et al. 2004, 2006, Thomson et al. 2006). Important additions to *Y. pseudotuberculosis* that occurred during this long period of evolution include the

ability to synthesize and incorporate 2,6-dideoxy-hexoses (not present in *Y. enterocolitica*) into lipopolysaccharide O-groups (Davies 1961), express a catalase-peroxidase termed KatY (Garcia et al. 1999), and colonize regional lymphatic tissue (Avtsyn et al. 1990) as opposed to *Y. enterocolitica*, which more commonly promotes direct gastrointestinal disease (Bottone 1997). Nevertheless, both of these species remain enteropathogens and, except for the new world serogroup O:8 isolates of *Y. enterocolitica* (Carter and Collins 1974), seldom cause systemic disease in noncompromised human hosts.

In contrast, *Y. pestis* (the etiological agent of bubonic plague), which evolved from a serotype O:1b strain of *Y. pseudotuberculosis* (Skurnik et al. 2000) within the last 5000–15,000 years (Achtman et al. 1999), arguably causes the most severe

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epidemic disease known to human (Pollitzer 1954, Orient 2004, Kelly 2005). This eyeblink of geological time is far too short to accommodate *de novo* gene evolution or significant modification and regulation of preexisting genes capable of causing acute infection. However, salient new genetic information was acquired during this period by lateral transfer of two unique plasmids of ~10 and ~110 kb termed pPCP (pesticin coagulase plasminogen activator) and pMT (murine toxin), respectively (Ferber and Brubaker 1981). The plasminogen activator of the plague bacillus (Beesley et al. 1967, Sodeinde et al. 1992, Lhtenmki et al. 1998, Kukkonen et al. 2004) is encoded by pPCP (Ferber and Brubaker 1981) and fraction 1 antigen (F1) of typically encapsulated *Y. pestis* (Baker et al. 1952) and murine toxin, a phospholipase D (Rudolph et al. 1999), are encoded by pMT (Protsenko et al. 1983, Kuttyrev et al. 1986). Nevertheless, these *Y. pestis*-specific determinants are not always essential for acute disease as judged by the observation that transformation of pPCP into *Y. pseudotuberculosis* did not significantly increase virulence in mice (Kuttyrev et al. 1999), and the absence of this plasmid in certain enzootic isolates of *Y. pestis* (described below) did not hinder tissue invasiveness (Worsham and Roy 2003). Similarly, F1 is not required for virulence in mice (Burrows 1957), and a fatal human case of plague was caused by an F1-deficient mutant (Winter et al. 1960). These findings strongly suggest that other factors contribute to the acute nature of plague. This paper discusses recent data regarding the importance of the absence of an active aspartase enzyme for the virulence of *Y. pestis* in mammalian hosts and its potential implications for the distribution and ecology of plague.

Discussion

Chromosomal degeneration

An attractive alternative explanation for the severity of plague is the possibility that invasiveness is a function of genetic loss. The major phenotypic differences between *Y. pestis* and *Y. pseudotuberculosis* are summarized in Table 1. These include recognition that plague bacilli typically exhibit a longer doubling time than cells of the enteropathogenic *Yersinia*, especially at host temperature (Brubaker 1972, Perry and Fetherston 1997). This reduced rate of growth is correlated with a requirement for CO₂ that is not essential but, if supplied, shortens the division time of *Y. pestis* to that of *Y. pseudotuberculosis* provided that other essential nutrients are present (Delwiche et al. 1959, Surgalla et al. 1964). Early studies demonstrated that CO₂ is normally fixed by *Y. pestis* into phosphoenolpyruvate (via phosphoenolpyruvate carboxykinase and an irreversible phosphoenolpyruvate carboxylase) to form oxaloacetate (Baugh et al. 1964) as is typical of many bacterial species. Although this approach did not resolve the basis for the stimulatory role of CO₂ for *Y. pestis*, it did implicate oxaloacetate, the immediate precursor of L-aspartic acid, as a critical intermediate. Results of subsequent work (Dreyfus and Brubaker 1978) showed that classical strains of *Y. pestis* lacked the common prokaryotic enzyme aspartate ammonia-lyase or aspartase (AspA) that converts L-aspartate into fumarate, an intermediate of the tricarboxylic acid cycle. Unrelated parallel work (Mortlock 1962, Mortlock and Brubaker 1962) undertaken on hexose metabolism also demonstrated the absence in typical plague bacilli of glucose 6-phosphate dehydrogenase (Zwf), the initial enzyme of

the pentose phosphate pathway (required for production of pentose for nucleic acid synthesis). These two genetic lesions promote a significant lack of fitness, at least *in vitro*, and likely account in full for the fastidious nature of *Y. pestis* as opposed to *Y. pseudotuberculosis* and *Y. enterocolitica* (Fowler and Brubaker 1994, Brubaker 2005, 2007, Viola et al. 2008).

Comparative annotation of the genomes of *Y. pestis* (Parkhill et al. 2001, Deng et al. 2002, Zhou et al. 2004a) and *Y. pseudotuberculosis* (Chain et al. 2004, 2006) revealed that plague bacilli have lost about 13% of all genes that remain functional in *Y. pseudotuberculosis*. This gene reduction reflects the consequences of chromosomal inversion, transposition, deletion, insertion of ~70 IS elements, and various mechanisms causing altered reading frames. These events account for known alterations between pCD and pYV (Hu et al. 1998), loss mutations promoting a reduced inflammatory response (Kukkonen et al. 2001, Kawahara et al. 2002), and the differences accounting for phenotypic distinctions in nutritional requirements, carbohydrate fermentation, and other biochemical properties (Brubaker 2004). The comparison did not, however, accommodate the critical absence of functional *aspA* or *zwf*, and subsequent effort demonstrated that these losses were caused by altered single DNA base pairs causing missense mutations.

Loss of *AspA* and *Zwf* in *Y. pestis* via missense mutation

A single base transition (T · A → C · G) at amino acid position 155 causes replacement of serine in the active Zwf of *Y. pseudotuberculosis* with proline in the inactive enzyme of typical *Y. pestis* (Chain et al. 2004, 2006). It is well established that proline causes distortion of secondary structure, and thus loss of enzyme activity after its introduction into a given enzyme is not surprising. In contrast, inactivity of AspA reflects a single base transversion (G · C → T · A) at amino acid position 363, where valine is replaced in the active enzyme of *Y. pseudotuberculosis* by the structurally similar, but inactive, leucine of typical isolates of *Y. pestis* (Chain et al. 2004, 2006). Skepticism regarding the possibility that substitution of one aliphatic amino acid by another solely accounts for loss of catalytic ability was eliminated by demonstrating that restoration of the active valine at position 363 of the AspA of *Y. pestis* restored full enzymatic activity. This seemingly innocuous change, which significantly reduces turnover (*k*_{cat}) but not substrate binding (*K*_m), may cause alteration in positioning of the adjacent catalytic residues Glu334 and Gln191 (Viola et al. 2008).

Influence of *AspA* and *Zwf* on the low calcium response

As noted above, the LCR is mediated by pCD/pYV. This phenotype is defined as the ability to either initiate vegetative growth at 37°C *in vitro* in the presence of ~2.5 mM Ca²⁺ without upregulation of a pCD/pYV-encoded type 3 secretion system (T3SS) or to undergo bacteriostasis with full induction of this T3SS. The LCR is especially stringent in typical strains of *Y. pestis*, and restricted growth in Ca²⁺-deficient medium is characterized by accumulation of L-aspartic acid in spent culture medium (Dreyfus and Brubaker 1978, Brubaker 2005). It is highly likely that this lost aspartic acid accounts for the prompt shutoff of growth observed for cells of *Y. pestis*

TABLE 1. PHENOTYPIC DISTINCTIONS BETWEEN TYPICAL STRAINS OF *YERSINIA PESTIS* AND *YERSINIA PSEUDOTUBERCULOSIS* (BRUBAKER 2004)

Determinant	<i>Y. pestis</i>	<i>Y. pseudotuberculosis</i>	Mutational mechanism or event	Consequence in <i>Y. pestis</i>	Reference
pPCP	+	0	Lateral transfer	Acquisition of plasminogen activator (accelerated dissemination in host tissues and posttranslational degradation of undelivered Yops). Acquisition of pesticin (assures retention of pPCP)	Ben-Gurion and Shafferman 1981, Ferber and Brubaker 1981, Protsenko et al. 1983
pMT	+	0	Lateral transfer	Acquisition of capsular fraction 1 antigen (resistance to phagocytosis). Acquisition of murine toxin (survival in the flea vector and prompt death of murine hosts)	Protsenko et al. 1983, Kutyrev et al. 1986, Protsenko et al. 1991, Hinnebusch et al. 2002
Hms ⁺	+	0	Constitutive expression of <i>hmsT</i>	Ability to colonize and block the flea proventriculus	Jones et al. 1999, Hare and McDonough 1999
Lipopolysaccharide O group structure at 26°C	0	+	Cryptic R genes	Constitutive resistance to complement (alternative pathway)	Skurnik et al. 2000
YadA	0	+	Frame-shift	Minimized association with host cell surfaces	Rosqvist et al. 1988
Host cell invasins (Inv)	0	+	IS1541 insert	Minimized association with host cell surfaces	Simonet et al. 1996
Host cell invasins (Ail)	0	+	IS285 insert	Minimized association with host cell surfaces	Torosian and Zsigray 1966
Glucose 6-phosphate dehydrogenase	0	+	Missense mutation	Loss of hexose monophosphate pathway dictating reversal of transketolase and transaldolase function or reliance on host/flea pentose for synthesis of RNA	Mortlock and Brubaker 1962
Aspartase	0	+	Missense mutation	Stoichiometric conversion of exogenous L-glutamate to L-aspartate with loss of the oxalacetate pool and enhanced requirement for CO ₂	Dreyfus and Brubaker 1978
Biosynthesis of glycine (or L-threonine)	0 ^a	+	Loss of serine hydroxymethyltransferase activity by regulatory mutation	Elimination of unnecessary function	Burrows and Gillett 1966, Brubaker and Sulen 1971
Biosynthesis of L-phenylalanine	0 ^a	+	Large deletion	Elimination of unnecessary function	Burrows and Gillett 1966
Biosynthesis of L-methionine	0 ^a	+	IS insert	Elimination of unnecessary function	Englesberg and Ingraham 1957, Burrows and Gillett 1966
Fermentation of rhamnose	0 ^a	+	Small deletion	Elimination of unnecessary function	Englesberg 1957
Fermentation of melibiose	0 ^a	+	Single base addition	Elimination of unnecessary function	Pollitzer 1954
Urease	0 ^a	+	Frame-shift (single base addition)	Elimination of unnecessary function	Sebbane et al. 2001
Assimilation of low levels of NH ₄ ⁺ into organic linkage	0 ^a	+	Unknown mechanism	Loss of primary NH ₄ ⁺ pick-up.	Brubaker and Sulen 1971
Toxicity of lipid A	0	+	Lack of acetylation	Elimination of unnecessary function Minimizes inflammation	Kawahara et al. 2002

^aKnown to undergo meiotrophic reversion to the *Y. pseudotuberculosis* phenotype.

after shift from 26°C to 37°C in Ca^{2+} -deficient medium (Brubaker 2007) since this form of bacteriostasis occurs as a consequence of reduced adenylate energy charge (Zahorchak et al. 1979). Another variable that influences the nutritional response of typical plague bacilli to Ca^{2+} is the presence of Na^+ , which may serve as a porter for the exit of aspartic acid at 37°C. Indeed, at slightly acidic pH the organisms exhibit normal full-scale growth if both Na^+ and Ca^{2+} are omitted from the culture medium. Na^+ did not, however, significantly modify the parallel upregulation of the attendant T3SS known to be functional in Ca^{2+} -starved cells grown at 37°C (Brubaker 2007). In summary, these findings suggest that the abrupt bacteriostasis observed for typical Ca^{2+} -starved cells of *Y. pestis* is caused by loss of metabolic carbon released in the form of aspartic acid by at least one Na^+ -driven export reaction.

As already noted, Zwf initiates the synthesis of ribose from hexose via the pentose phosphate pathway. This reaction probably has little importance in cells of *Y. pestis*, which typically reside within a closed flea-host cycle where exogenous pentose is readily available; the organisms can also generate ribose from 3-carbon fragments via transketolase-transaldolase rearrangement (Mortlock 1962). However, nicotinamide adenine dinucleotide phosphate (NADPH) is an

important secondary product of Zwf that must be generated by alternative means by *Y. pestis* grown on hexose. Plague bacilli cultivated at 37°C in a Ca^{2+} -deficient chemically defined medium containing glucose undergo marked lysis (Brownlow and Wessman 1960); thus, Zwf-deficiency may also modulate the LCR in these organisms. This phenomenon, of course, is distinct from the typical bacteriostasis with retention of viability that occurs in Ca^{2+} -deficient medium containing Na^+ , at neutral pH.

Enzootic strains of *Y. pestis*

Available evidence indicates that the transmission of plague in sylvatic reservoirs is almost exclusively undertaken by fleabite. Reliance upon this method of transfer depends upon a sufficient host bacteremia to ensure infection of the flea vector (Engelthaler et al. 2000, Eisen et al. 2006) and the ability of *Y. pestis* to kill their current host, thereby assuring that infectious fleas disseminate in search of new hosts. Mortality therefore became an essential part of the life cycle of *Y. pestis*, and selective pressure caused by this necessity has obviously directed the further evolution of acute disease. As a consequence of this process, plague bacilli became efficient patho-

TABLE 2. CODONS FOR AMINO ACIDS AT POSITION 155 OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE (ZWF) AND POSITION 363 OF ASPARTASE AND CORRESPONDING SPECIFIC ACTIVITIES^a IN DIALYZED CELL-FREE EXTRACTS PREPARED FROM YERSINIAE^b EXPRESSING THESE ENZYMES (BEARDEN ET AL. 2008)

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

^aValues are expressed as μmol of product produced $\text{min}^{-1} \text{mg protein}^{-1}$ and are averaged from at least two independent determinations; variation was <15%.

^bAll enzootic isolates are attenuated in guinea pigs ($\text{LD}_{50} > 10^6$ bacteria) (unpublished observations).

^cInitially considered to be an enzootic isolate based evidently on lack of pMT but now recognized as an epidemic strain.

gens and have now disseminated from ancestral foci in Central Asia and perhaps Africa to reservoirs throughout the world where they cause lethal disease in many animal species, including humans (Suntsov and Suntsova 2008). The virulence of these potential "epidemic" isolates contrasts markedly with "enzootic" strains of *Y. pestis* (also termed "pestoides" variants) that remain contained within their original reservoirs (Martinevskii 1969, Anisimov et al. 2004). Enzootic isolates are now classified according to subspecies designation *altaica*, *caucasica*, *hissarica*, *ulegeica*, and *talassica* (Anisimov et al. 2004). With the exception of *caucasica* isolates (which lack pPCP) these enzootic strains possess all known virulence factors of epidemic isolates, and commonly retain the normal ability of *Y. pseudotuberculosis* to ferment the sugars rhamnose and melibiose. Curiously, they are attenuated in many mammalian species, including guinea pigs and primates, but remain virulent in members of the rodent Superfamily Murioidea and in nature have only been recovered from these rodents or their fleas (Martinevskii 1969, Anisimov et al. 2004). Further study of enzootic variants has recently shown that they sometimes express catalytically active Zwf and always exhibit functional AspA (Bearden et al. 2008).

AspA and Zwf in enzootic *Y. pestis*

A comparison of 10 enzootic isolates of *Y. pestis* with their epidemic counterparts (Bearden et al. 2008) demonstrated that three of the former expressed functional Zwf associated with retention of serine at amino acid position 155 (Table 2). These strains were the Russian pestoides E, F, and G strains, all of subspecies *caucasica*, that also exhibit other primitive traits, including the absence of pPCP and attendant plas-

minogen activator. All tested enzootic isolates produced biologically active AspA although its specific activity exhibited significant variation depending upon the amino acid present at amino acid position 363. The value of aspartase activity was essentially identical to that of *Y. pseudotuberculosis* when valine was retained at this position (the Russian pestoides A, B, C, and D strains) or when serine was substituted for valine (the Russian pestoides E, F, G, and I strains). Curiously, the enzootic Angola and A16 strains from Africa contained phenylalanine at this position and exhibited significantly reduced but nevertheless significant activity (Table 2). Further, cells of the attenuated enzootic microtus biovar isolate from China (Fan et al. 1995) encode valine at amino acid position 363 of AspA (Zhou et al. 2004b) and should therefore exhibit enzymatic activity. These findings indicate that synthesis of enzymatically active AspA is a biomarker of attenuation in nonmuroid hosts. Further, the observations have obvious significance with respect to expression of acute disease as well as the evolution and potential spread of epidemic isolates.

Acute disease and deficiency of AspA

Formal proof that active AspA in *Y. pestis* promotes attenuation will be, of course, dependent upon experiments involving salient gene exchange. Nevertheless, this correlation is presently absolute; thus, it is appropriate to consider possible mechanisms where genetic loss of AspA could enhance the ability to cause acute disease. The physiological role of AspA is to assure return of the four carbons comprising the backbone of aspartic acid (generated via catabolic transamination of L-glutamate with oxalacetate) into the tricarboxylic acid cycle in the form of fumarate (Halpern and Umbarger 1960). This

TABLE 3. ENZYMATIC REACTIONS INVOLVED IN THE CATABOLISM OF L-GLUTAMATE BY ASPARTASE-POSITIVE *YERSINIA PSEUDOTUBERCULOSIS* (AND ENZOOTIC STRAINS OF *YERSINIA PESTIS*) AND EPIDEMIC ASPARTASE-NEGATIVE ISOLATES OF *Y. PESTIS*

Yersinia pseudotuberculosis

L-Glutamate + Oxalacetate → 2-Oxyglutarate + L-Aspartate
^aL-Aspartate → Fumarate + NH₃
 Fumarate + H₂O → Malate
 Malate + NAD⁺ → Oxalacetate + NADH
 2-Oxyglutarate + CoASH + NAD⁺ → Succinyl-CoA + CO₂ + NADH
 Succinyl-CoA + H₂O → Succinate + CoASH
 Succinate + FAD⁺ → Fumarate + FADH
 Fumarate + H₂O → Malate
 Malate + NAD⁺ → Oxalacetate + NADH

Sum: L-Glutamate + 2NAD⁺ + FAD⁺ + 3H₂O → Oxalacetate + 2NADH + FADH + NH₃ + CO₂

Epidemic Yersinia pestis

L-Glutamate + Oxalacetate → 2-Oxyglutarate + L-Aspartate
 2-Oxyglutarate + CoASH + NAD⁺ → Succinyl-CoA + CO₂ + NADH
 Succinyl-CoA + H₂O → Succinate + CoASH
 Succinate + FAD⁺ → Fumarate + FADH
 Fumarate + H₂O → Malate
 Malate + NAD⁺ → Oxalacetate + NADH

Sum: L-Glutamate + 2NAD⁺ + FAD⁺ + 2H₂O → L-Aspartate + 2NADH + FADH + CO₂

^aReaction catalyzed by aspartase.

mechanism assures the efficient flow of carbon by preventing accumulation of catabolic end-products other than CO₂ and NH₄⁺ (Table 3). As such, nearly ubiquitous AspA assures that a large number of saprophytic bacteria capable of its expression remain capable of efficient metabolism during growth in austere environments. Plague bacilli, however, typically reside in the closed, highly enriched, and protected environments of the host and flea vector where efficient processes of catabolism are unnecessary. Indeed, examination of mRNA transcribed during steady-state growth at 37°C showed that the catabolic process used by *Y. pestis* in the host is inefficiently regulated and largely wasteful if not prodigal (Motin et al. 2004). Accordingly, plague bacilli may have no need for the tempering influence of AspA on catabolism but rather proceed to consume and destroy host nutrient pools quickly and with abandon.

The ability of epidemic strains of *Y. pestis* to accumulate metabolic aspartate may have an additional sinister effect. Amino acids other than L-glutamate also serve as a source of carbon for synthesis of aspartate during starvation for Ca²⁺. These include metabolically linked L-proline as well as L-asparagine, which is in equilibrium with L-glutamine in the host (Soupart 1962) and rapidly and stoichiometrically undergoes conversion to L-aspartate *in vitro* (Dreyfus and Brubaker 1978). This event would be highly deleterious to the host should it occur *in vivo* because the largest amino acid components of mammalian serum and cytoplasm are L-glutamate, L-glutamine, and L-asparagine, whereas L-aspartate comprises the smallest (Soupart 1962). Reversal of this ratio by secretion of metabolic aspartate would seriously affect the metabolic equilibrium and bioenergetics of these pools by the host, an event that might be especially deleterious during the terminal stages of infection. In this context, it may be significant that only a few other aerobic bacteria also lack AspA, but this group includes *Mycobacterium tuberculosis* (Fleischmann et al. 2002), *Francisella tularensis* (Larsson et al. 2005), and 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 and Miller 1950).

Expression of AspA by and attenuation of enzootic isolates of *Y. pestis* still renders these strains fully capable of killing muroid rodents, suggesting that they possess some factor (other than that associated with AspA-deficiency) that contributes to this process. A likely possibility is pMT-encoded murine toxin, which is highly lethal in muroids but not essential for virulence of epidemic strains of *Y. pestis* in mice, although it does appear to be important for survival of plague bacteria in the midgut of the flea vector (Hinnebusch et al. 2000, Hinnebusch et al. 2002). It seems possible that the *raison d'être* for AspA in enzootic strains of *Y. pestis* is no more than that in the enteropathogenic yersiniae where it facilitates efficient amino acid catabolism in minimal environments. These strains are often, but not always limited, to grasslands and similar habitats containing burrowing muroid rodents (e.g., gerbils and voles), and their maintenance may depend in part on survival in soils including those surrounding decomposed host carcasses (Ayyadurai et al. 2008, Eisen et al. 2008).

Finally, the paucity of available enzootic isolates underscores the importance of further studies examining the mechanisms for the ecological persistence of these genetically and phenotypically diverse strains in sylvatic foci. Moreover,

the behavior of these strains in fleas, particularly as it relates to plague virulence, pathogenesis, host immunity, and focal distribution, warrants further investigation. Needless to say, construction and virulence testing of AspA-positive epidemic isolates and murine toxin-deficient enzootic isolates should resolve many of the issues raised in this review.

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