The Evolution of Flea-borne Transmission in *Yersinia pestis*

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Abstract

Transmission by fleabite is a recent evolutionary adaptation that distinguishes *Yersinia pestis*, the agent of plague, from *Yersinia pseudotuberculosis* and all other enteric bacteria. The very close genetic relationship between *Y. pestis* and *Y. pseudotuberculosis* indicates that just a few discrete genetic changes were sufficient to give rise to flea-borne transmission. *Y. pestis* exhibits a distinct infection phenotype in its flea vector, and a transmissible infection depends on genes that are specifically required in the flea, but not the mammal. Transmission factors identified to date suggest that the rapid evolutionary transition of *Y. pestis* to flea-borne transmission within the last 1,500 to 20,000 years involved at least three steps: acquisition of the two *Y. pestis*-specific plasmids by horizontal gene transfer; and recruitment of endogenous chromosomal genes for new functions. Perhaps reflective of the recent adaptation, transmission of *Y. pestis* by fleas is inefficient, and this likely imposed selective pressure favoring the evolution of increased virulence in this pathogen.

Introduction

Pathogenic bacteria must overcome several physiological and immunological challenges to successfully infect even a single type of host, such as a mammal. It is remarkable, then, that bacteria transmitted by blood-feeding arthropods are capable of infecting two very different hosts during their life cycle: an invertebrate (usually an insect or tick) and a mammal. As if this were not enough of a challenge, it is not sufficient that an arthropod-borne bacterium successfully infect both vector and host. It must establish a transmissible infection in both; that is, it must infect the vector in such a way as to be transmitted during a blood meal, and it must infect the mammal in a way that allows uptake by a blood-feeding arthropod. This feat of evolution has occurred relatively rarely, but nonetheless arthropod-borne transmission has developed independently in a phylogenetically diverse group of microorganisms, including the rickettsiae, spirochetes in the genus *Borrelia*, and the Gram-negative bacteria.

Compared to the ancient relationship of rickettsiae and spirochetes with arthropods, the vector relationship between *Y. pestis* and fleas is new. Population genetics and comparative genomics analyses indicate that *Y. pestis* is a clonal variant of *Y. pseudotuberculosis* that diverged only within the last 1,500 to 20,000 years (Achtman *et al*., 1999; Hinchcliffe *et al*., 2003; Chain *et al*., 2004). Presumably, the change from the food- and water-borne transmission of the *Y. pseudotuberculosis* ancestor to the flea-borne transmission of *Y. pestis* occurred during this evolutionarily short period of time. The monophyletic relationship of these two sister-species implies that the genetic changes that underlie the ability of *Y. pestis* to use the flea for its transmission vector are relatively few and discrete. Therefore, the *Y. pseudotuberculosis* –*Y. pestis* species complex provides an interesting case study in the evolution of arthropod-borne transmission. Some of the genetic changes that led to flea-borne transmission have been identified using the rat flea *Xenopsylla cheopis* as model organism, and an evolutionary pathway can now be surmised. Reliance on the flea for transmission also imposed new selective pressures on *Y. pestis* that help explain the evolution of increased virulence in this pathogen.

***Y. pestis*–flea interactions**

There are an estimated 2,500 species and subspecies of fleas that constitute 220 genera and 15 families in the insect order Siphonaptera (Lewis, 1998). Of these, approximately 80 species, associated with some 200 species of wild rodents, have been found to be infected with *Y. pestis* in nature, or to be susceptible to experimental infection (Pollitzer, 1954). Accordingly, the ecology of plague is extremely complex, involving many different rodent-flea cycles.

Different species of fleas vary greatly in their ability to transmit *Y. pestis*, at least under laboratory conditions; some species, such as the common cat flea *Ctenocephalides felis*, are incapable of transmission. Wheeler and Douglas (1945) and Burroughs (1947) developed a mathematical model to estimate the vector efficiency of different flea species that took into account i) infection potential (the percentage of fleas becoming infected after feeding on a septicemic animal); ii) vector potential (the percentage of infected fleas which become infective or blocked, i.e., develop a transmissible infection as described below); and iii) the transmission potential (the average number of successful transmissions per flea). Kartman (1957) later added two more factors: iv) the life span of infective (blocked) fleas; and v) the field prevalence index (the average number of fleas per species per rodent or rodent nest). By these measures an experimental vector efficiency could be calculated for different fleas (Wheeler and Douglas, 1945; Burroughs, 1947; Kartman, 1957; Kartman and Prince, 1956). These comparisons have sometimes been intriguing and enigmatic. For example, the rat flea *Xenopsylla cheopis* has most frequently been identified as the most efficient vector, yet the closely related *Xenopsylla astia* is a poor vector (Hirst, 1923). The physiological mechanisms that account for differences in vector efficiency among different

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Table 1. Flea-specific factors which might affect the ability of *Y. pestis* to produce a transmissible infection
Flea anatomy and physiology
Midgut
pH, redox potential, osmolarity, etc.
Biochemical composition of host blood
Digestive enzymes, digestive byproducts
Endogenous microbial flora
Frequency of feeding and defecation
Insect immunity components
Proventriculus
Size (volume)
Number, density, length, and shape of spines
Rate of opening and closing during feeding
Hydrodynamic forces generated during feeding
Insect immunity components
Ecology
Ambient temperature
Flea life span after infection

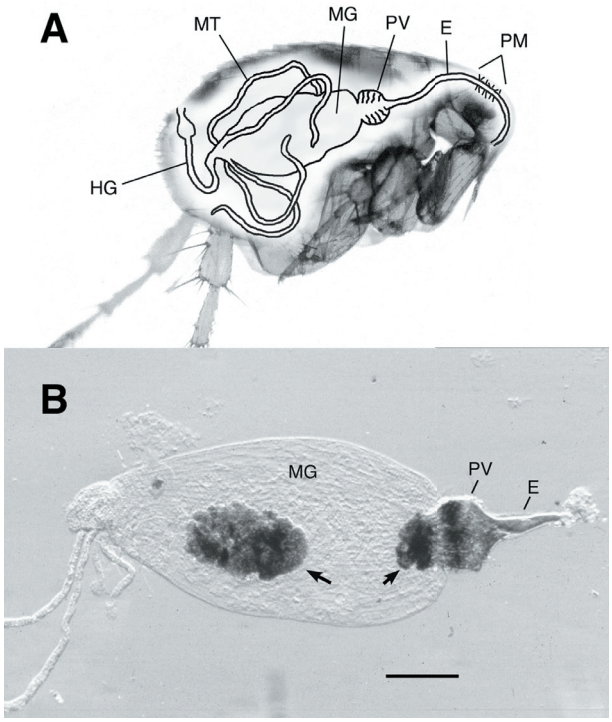

Fig. 1. (A) Digestive tract anatomy of fleas. E = esophagus; PV = proventriculus; MG = midgut; HG = hindgut. The muscles that pump blood into the midgut (PM) and the malpighian tubules (MT) are also indicated. (B) Digestive tract dissected from a blocked *X. cheopis* flea. Arrows indicate the large aggregate of *Y. pestis* that fills and blocks the proventriculus and an independent bacterial aggregate in the midgut. The *Y. pestis* aggregates are surrounded by a dark colored extracellular matrix. Bar = 0.25 mm.

flea species are not known, but some possible factors are described in the following sections and listed in Table 1.

A high degree of vector specificity is characteristic of many arthropod-borne agents. For example, human malaria is transmitted by anopheline but not culicine mosquitoes, different subspecies of *Leishmania* are transmitted by different sandfly species, and the closely related North American species of *Borrelia* spirochetes that cause relapsing fever are each transmitted by a different species of *Ornithodoros* tick (Sacks and Kamhawi, 2001; Barbour and Hayes, 1986). Whether the same co-evolutionary process is occurring in *Y. pestis* remains to be demonstrated, but Russian researchers have proposed that, at least for some natural plague cycles, discrete triads of flea species, rodent, and subspecies or strain of *Y. pestis* have co-evolved (Anisimov *et al*., 2004).

The flea gut environment

Y. pestis infection of the flea is confined to the digestive tract, which is depicted in Fig. 1. Storage, digestion, and absorption of the blood meal all occur in the simple midgut made of a single layer of columnar epithelial cells and associated basement membrane. The proventriculus, a valve at the base of the esophagus that guards the entrance to the midgut, is central to the transmission mechanism. The interior of the proventriculus is arrayed with densely packed rows of inward-directing spines, which are coated with an acellular layer of cuticle, the same material that makes up the insect exoskeleton (Fig. 2). In *X. cheopis*, there are a total of 264 proventricular spines in the male and 450 in the female (Munshi, 1960). The proventricular valve is normally tightly closed by layers of surrounding muscle. During feeding periods, however, the proventricular muscles rhythmically open and close the valve in concert with a series of three sets of pump muscles located in the flea's head to propel blood into the midgut and to keep it from leaking back out. Fleas usually live on or in close association with their hosts and take small but frequent (every few days) blood meals. Digestion of the blood meal begins quickly, resulting in hemolysis and liquefaction of ingested blood cells by six hours (Vaughan and Azad, 1993). During the next two to three days, the blood meal digest is brown-colored, viscous, and contains many large and small lipid droplets, but is eventually processed to a compact dark residue. Fleas defecate partially digested portions of their blood meals, which are used as a food source by flea larvae. Unlike other blood-feeding arthropods, fleas do not secrete a chitinous peritrophic membrane around the blood meal.

Few details are known about flea gut physiology and associated environmental conditions in the digestive tract. A probable midgut pH of 6 to 7 has been cited (Wigglesworth, 1972), but other basic parameters such as osmotic pressure and redox potential are unknown. The biochemical composition may initially reflect that of hemolyzed blood, but is subject to rapid change due to selective absorption of certain nutrients, ions, and water. Insect midgut epithelium secretes a variety of digestive enzymes that are similar to those of vertebrates, including trypsin, chymotrypsin, amino- and carboxypeptidases, cathepsins, lysozymes, glycosidases, and lipases (Terra and Ferreira, 1994). Mammalian blood is composed

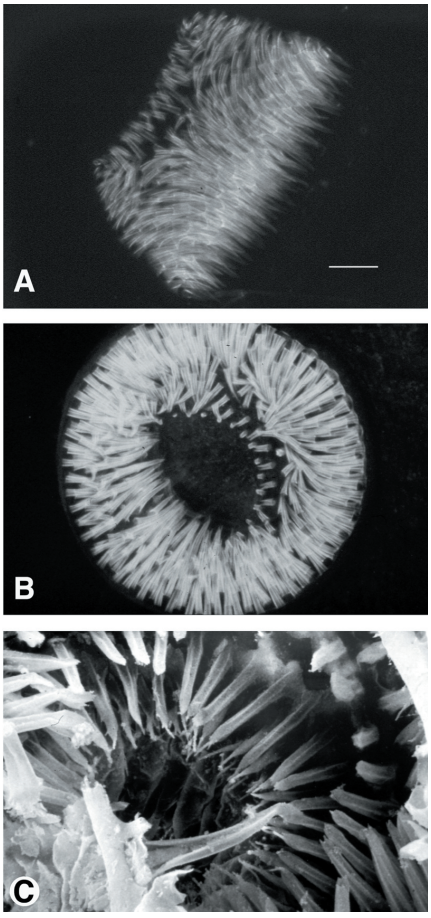

Fig. 2. Proventricular spines of *Xenopsylla cheopis*. Side (A) and front (B) views of an uninfected proventriculus viewed by fluorescence microscopy. The proventricular spines are covered with cuticle, which is autofluorescent. (C) Scanning electron microscopy of the interior of an uninfected proventriculus. Bar = 5 μm.

principally of protein and lipid, and lipids are a major energy source for hematophagous arthropods. Lipids are relatively insoluble in water, and the mechanism of their solubilization and absorption in fleas is unknown.

It is in this active digestive milieu that *Y. pestis* lives in the flea. These conditions must be relatively hostile and refractory to colonization, because fleas have rather limited normal digestive tract flora, and few pathogens are transmitted by fleas (Savalev *et al.*, 1978; Beard *et al.*, 1990). Besides *Y. pestis*, flea-borne pathogens include *Bartonella henselae*, the agent of cat-scratch disease and bacillary angiomatosis, and *Rickettsia typhi* and *Rickettsia felis*, members of the typhus group (Chomel *et al.*, 1996; Azad *et al.*, 1997). The Gram-negative bacterium *Francisella tularensis* is also associated with fleas, although the importance of flea vectors in the overall ecology of tularemia is unclear (Hopla, 1974). Fleas have also been implicated in transmission of the poxvirus that causes myxomatosis in rabbits (Chapple and Lewis, 1965). Vaughan and Azad (1993) have hypothesized that the rapid digestive process of fleas and lice is not conducive to the development of eukaryotic parasites, but that it can be better tolerated by prokaryotes.

The midgut epithelium of mosquitoes and the blood sucking fly *Stomoxys calcitrans* has been shown to be an immune-competent tissue, and the presence of bacteria in the blood meal of these insects induces the secretion of antimicrobial peptides into the gut lumen (Dimopoulos *et al.*, 1997; Lehane *et al.*, 1997). Whether this occurs in fleas is unknown. At any rate, *Y. pestis* appears to be inherently resistant to the flea immune response (Hinnebusch *et al.*, 1996; and unpublished data).

Biological transmission of Y. pestis *by fleas: the proventricular blockage model*

The general mechanism of *Y. pestis* transmission by fleas was described by Bacot and Martin (1914). They observed that in infected *X. cheopis* and *Ceratophyllus fasciatus* fleas, solid masses of *Y. pestis* could fill the lumen of the proventriculus and obstruct the flow of blood (Fig. 1B). These fleas made persistent, vigorous attempts to feed, but could not pump blood past the blocked proventriculus. Instead, the esophagus became distended with blood which was then partially refluxed back into the bite site due to contraction and relaxation of the cibarial and pharyngeal pumps. This phenomenon correlated with efficient transmission. Based on their observations, Bacot and Martin proposed the proventricular blockage-regurgitation mechanism for plague transmission. According to this model, blockage of the proventriculus by a mass of *Y. pestis* precedes transmission, and *Y. pestis* is conveyed to the bite site by regurgitation when blocked fleas attempted to feed. Bacot (1915) later amended this model by proposing that fleas with only partial obstruction of the proventriculus were actually better transmitters. Partially blocked fleas can still pump blood into the midgut through an open channel in the proventriculus. The bacterial growth prevents complete closing of the valve, however, so that blood mixed with *Y. pestis* from the midgut is able to flow back out the proventriculus into the bite site. This can happen because during flea feeding the pumping action is not continuous, but stops for short intervals. Transmission as a result of partial blockage was an important addendum to the model because complete proventricular blockage does not readily develop in some flea species that are good vectors of plague (Burroughs, 1947; Pollitzer, 1954). According to the Bacot model, complete blockage of the proventriculus is not necessary for efficient transmission; partial interference with its valvular function is sufficient.

Mechanical transmission of Y. pestis *by fleas.* Although several investigators have established that biological transmission (requiring *Y. pestis* growth in the digestive tract to produce a proventricular infection) is the only reliable means of transmission (Burroughs, 1947; Pollitzer, 1954), there is evidence that mechanical transmission may also play a role in the ecology of plague. For mechanical transmission, infection of the vector is not necessary. It is only necessary that septicemia levels are high and that *Y. pestis* survive on the blood-stained mouthparts of fleas between consecutive feedings. For example, *X. cheopis* and the wild rodent flea *Malaraeus telchinum* allowed to feed en masse on uninfected mice one day after feeding on a highly septicemic mouse

consistently transmitted the disease (Burroughs, 1947). Since that time interval is too short for proventricular infection to develop, transmission presumably occurred by mechanical transference of bacteria on contaminated mouthparts. The phenomenon of mechanical or mass transmission provides a potential mechanism for fleas that do not develop proventricular blockage readily, such as *M. telchinum* and the human flea *Pulex irritans*, to transmit *Y. pestis* during epidemics. Human to human transmission via *P. irritans* has been hypothesized to have contributed to the plague pandemics of medieval Europe (Beaucournu, 1999). Because mechanical transmission does not rely on specific interactions with the vector, it will not be considered further here.

Y. pestis *transmission factors*

A central hypothesis, now substantiated by experimental evidence, is that bacteria that cycle between a mammal and an arthropod express distinct subsets of genes in their two hosts. Genes specifically required to infect the vertebrate host are referred to as virulence factors, and the analogous genes required to produce a transmissible infection in the arthropod vector have been termed transmission factors (Hinnebusch *et al.*, 1996; Paskewitz, 1997). Many virulence factor genes of *Y. pestis* that are required to infect and cause disease in the mammal have been identified and studied. In contrast, the genetic factors required in the insect host have been relatively neglected. Nevertheless, some of the genetic factors of *Y. pestis* that are specifically involved in flea-borne transmission have been identified (Table 2).

The Yersinia *murine toxin: a phospholipase D required for flea gut colonization*

The *Yersinia* murine toxin (Ymt) was described in the 1950s as a protein fraction of *Y. pestis* that was toxic to mice and rats (Ajl *et al.*, 1955), so it has universally been considered to be a virulence factor. Brown and Montie (1977) presented evidence that Ymt is a β-adrenergic receptor antagonist, blocking epinephrine-induced mobilization of glucose and fatty acids. In mice, Ymt causes circulatory failure due to vascular collapse, resulting in death in ten hours with an LD_{50} of 0.2 to 3.7 μg (Schär and Meyer, 1956). Ymt is not toxic to guinea pigs, rabbits, dogs, or primates even in enormous doses, however (Montie and Ajl, 1970). Murine toxin was described as a cell-associated protein that is only released upon bacterial death, and, correspondingly, its effects are seen only in the late stages of septicemic murine plague, when the animal is already succumbing to the disease (Montie and Ajl, 1970). Interestingly, recombinant Ymt protein produced in and purified from *Escherichia coli* is nontoxic for mice, whereas native Ymt purified from *Y. pestis* is toxic (Hinnebusch *et al.*, 2000; and unpublished data). The explanation for this is not known, but Walker (1967) suggested that synergism between Ymt, endotoxin, and possibly other *Y. pestis* factors was responsible for murine toxicity.

Sequence analysis showed that the *Y. pestis ymt* gene mapped to the 100-kb pFra plasmid and encodes a 61-kDa protein that is a member of a newly described family of phospholipase D enzymes found in all kingdoms of life: animals, plants, fungi, and eukaryotic viruses as well as bacteria (Cherepanov *et al.*, 1991; Ponting and Kerr, 1996). All members of this PLD family have two copies of a signature HKD ($HxKx_4Dx_6GG/S$) motif, which come together to form the catalytic site for binding and hydrolysis of the phosphodiester bond (Stuckey and Dixon, 1999). The *Y. pestis* Ymt has classic PLD activity, as shown by its ability to cleave the polar head group from phosphatidylcholine, phosphatidylethanol amine, and other phospholipids. Ymt is also capable of transphosphatidlyation of phospholipid with an alcohol acceptor, a second characteristic PLD reaction (Rudolph *et al.*, 1999). Because of this proven biochemistry, it has been proposed that the *Y. pestis ymt* gene should be renamed *pldA* (Carniel, 2003).

Despite the known toxic effects of murine toxin, a *ymt* deletion mutant of *Y. pestis* was essentially fully virulent for mice (Drozdov *et al.*, 1995; Du *et al.*, 1995; Hinnebusch *et al.*, 2000). Thus, Ymt is not required for morbidity or mortality, even in mice, but only adds insult to injury. This likely reflects the fact that Ymt is not a classic exotoxin, but is a cytoplasmic enzyme that is only released upon bacterial cell death and lysis. The full virulence of Ymt⁻ *Y. pestis*, and other results showing that *ymt* expression is downregulated at 37°C (Du *et al.*, 1995), suggested that the principle biological function of this PLD is not as a virulence factor.

A role for Ymt in transmission was first indicated by a study evaluating the fate of plasmid-cured *Y. pestis* strains in the flea. Whereas the 9.5-kb pPst and the 70-kb pYV virulence plasmid were not required for normal infection and blockage of *X. cheopis*, strains lacking the 100-kb pFra plasmid failed to block the fleas (Hinnebusch *et al.*, 1998a). Complementation of the pFra⁻ strains with the *ymt* gene alone fully restored normal ability to infect and block fleas. Specific Ymt⁻ *Y. pestis* mutants were used for further analysis (Hinnebusch *et al.*, 2002b). Within hours of being taken up in a blood meal by a flea, Ymt⁻ *Y. pestis* assumed an aberrant, spheroplast-like cell morphology, and then rapidly disappeared from the flea midgut within the first day after infection. Rarely, the Ymt⁻ bacteria established an initial foothold in the proventriculus, which is part of the foregut and physically separated from

Table 2. *Y. pestis* genes important for flea-borne transmission

Gene	Llocation	Present in: *Y. pestis* *Y. pstb*	Function in *Y. pestis* and role in transmission
ymt	pFra plasmid	+ –	Phospholipase D, survival in flea midgut
hmsHFSR, T	Chromosome	+ +	Extracellular matrix synthesis, biofilm formation, infection and blockage of the proventriculus
pla	pPst plasmid	+ –	Plasminogen activator, dissemination from fleabite site

the midgut by the stomodeal valve except during the few minutes per week that the flea is actively feeding. Secluded in the proventriculus, the mutants could grow normally and eventually cause blockage. Because the proventriculus is rarely the primary site of infection, but is usually seeded secondarily from a prior midgut infection, the Ymt$^-$ mutant infected < 5% and blocked < 0.5% of fleas, compared to the normal infection and blockage rates of 50–60% and 25–45%, respectively (Hinnebusch *et al.*, 2002b). Remarkably, introduction of the *ymt* gene into *Y. pseudotuberculosis* and *E. coli* significantly enhanced their ability to colonize the flea midgut also. Thus, Ymt may have a similar substrate and mechanism of action in both *Yersinia* and *E. coli*. Members of the PLD family of enzymes to which Ymt belongs are found in many different cell types and can have many different functions. Serendipitously, the PLD activity of Ymt enhances survival of Gram-negative bacteria in the flea midgut. Acquisition of this single gene by *Y. pestis* would have been a crucial step in the evolution of the flea-borne route of transmission.

Models for the protective mechanism of Ymt

How might an intracellular PLD protect *Y. pestis* in the flea midgut? The first option to consider is that Ymt might be secreted or released from lysed bacteria in the flea, and degrade an external cytotoxic agent in the midgut environment. Three types of experimental results argue against that: 1) Addition of exogenous Ymt protein to the infectious blood meal did not enhance the survival of Ymt$^-$ *Y. pestis* in the flea gut. 2) Coinfection of fleas with an equal mixture of Ymt$^+$ and Ymt$^-$ *Y. pestis* did not result in a coequal infection pattern. If active PLD were secreted, one might expect that enzyme from Ymt$^+$ bacteria would also protect Ymt$^-$ bacteria in the flea gut, resulting in infections consisting of an equal mixture of both strains. Instead, in these experiments the Ymt$^-$ mutant again survived primarily in the proventriculus. In the midgut, it persisted only in small clusters that were embedded within larger aggregates of wild-type bacteria. 3) In digestive tracts dissected from fleas infected with *Y. pestis* that synthesized a Ymt-GFP fusion protein, fluorescence localized only to the cytoplasm and was never detected extracellularly (Hinnebusch *et al.*, 2002b).

An intracellular PLD conceivably could protect *Y. pestis* in the flea gut either by modifying an endogenous membrane component to make the bacteria impervious to the cytotoxic agent (prophylaxis model), or by neutralizing the agent, directly or indirectly, after it interacts with the bacteria (antidote model). In the prophylaxis model, the outer membrane of Ymt$^-$ *Y. pestis* would be differentially affected in the flea gut. Loss of outer membrane integrity could lead to the observed spheroplasty, because lysozymes are commonly secreted by insect midgut epithelium (Terra and Ferreira, 1994). Evidence for this model was sought by analyzing the outer membrane composition of Ymt$^+$ and Ymt$^-$ *Y. pestis*. Quantitative comparisons of membrane phospholipids and phosphodiester-linked substitutions of lipid A revealed no differences. The mutant was also no more susceptible than the parent *Y. pestis* to polymixin B, SDS and EDTA, cationic detergents, and other agents that target the Gram-negative outer membrane. Attempts to mimic the flea gut environment by culturing the bacteria in triturated flea gut contents; in whole or sonicated mouse blood containing proteases, lipase, and lysozyme; or under osmotic and oncotic pressure, oxidative stress, or low pH likewise failed to reveal any difference between mutant and wild type strains (B. J. Hinnebusch, unpublished). In sum, no phenotypic difference between Ymt$^-$ and Ymt$^+$ *Y. pestis* has been detected outside of the flea gut.

Alternatively, according to the antidote model, the toxic agent in the flea gut would interact with both Ymt$^-$ and Ymt$^+$ *Y. pestis*, but its effects then be neutralized by Ymt. In many bacteria, exposure to harmful environments induces autolytic pathways that result in self-digestion of the bacterial cell wall by endogenous peptidoglycan hydrolases (Lewis, 2000). The environmental stimuli, signal transduction mechanisms, and gene expression pathways leading to this programmed cell death are incompletely understood in bacteria. If an agent in the flea gut stimulates *Y. pestis* autolysis and leads to the observed rapid spheroplast formation, intracellular Ymt activity may block or redirect a step in the autolytic pathway. Such a role would be analogous to that of mammalian PLD, which is an intracellular effector in multiple signal transduction cascades (Gomez-Cambronero and Keire, 1998).

Whichever model is correct, the agent in the flea gut that is harmful to Ymt$^-$ *Y. pestis* appears to derive from a digestive product of blood plasma. Elimination from the flea gut during the first 24 hours after infection occurred if either filtered mouse plasma or whole blood was the source of the infectious meal fed to the fleas. However, if fleas were infected by feeding on an artificial plasma substrate consisting of PBS, pH 7.4, containing 7% bovine serum albumin, 6 mM glucose, 12 mM sodium bicarbonate, 10 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 1 mM citric acid, the Ymt$^-$ *Y. pestis* survived as well as the Ymt$^+$ parent strain during the first 24 hours after infection. Identical results were obtained when washed mouse red blood cells were added to the artificial plasma substrate (Hinnebusch *et al.*, 2002b). The fact that the artificial plasma meals were digested by the fleas further suggests that flea digestive enzymes, or the digestive milieu *per se*, do not directly harm the mutant. The mutant also survived as well as wild type *Y. pestis* after injection into the flea hemocoel. These results implicate a blood plasma digestive product as the cytotoxic agent, but the native substrate of the *Y. pestis* PLD and its protective mechanism remain to be discovered.

The hms *genes and the biofilm model of proventricular blockage*

One of the first temperature-dependent phenotypes described for *Y. pestis* was the formation of densely pigmented colonies when incubated at 28°C or less on media containing hemin or the structurally analogous dye Congo red (Jackson and Burrows, 1956a; Surgalla and Beesley, 1969). The phenotype is not due to the production of a pigment by *Y. pestis*, but rather to the avid adsorption of the exogenous hemin or Congo red to the outer membrane (Perry *et al.*, 1993). Despite the fact that the phenotype was not expressed at 37°C, pigmentation correlated with virulence. Spontaneous nonpigmented

mutants had greatly reduced virulence for mice after peripheral routes of infection unless iron salts were injected simultaneously (Jackson and Burrows, 1956b). Further study of nonpigmented *Y. pestis* strains showed that they did not grow in iron-chelated media *in vitro*, failed to synthesize several iron-regulated proteins, and did not interact with pesticin, the bacteriocin encoded on the pPst plasmid (Brubaker, 1969; Sikkema and Brubaker, 1987, 1989).

The reason for the wide range of physiological effects associated with loss of pigmentation gradually emerged from a series of molecular genetics analyses. Robert Perry and colleagues used transposon mutagenesis to identify a 9.1-kb chromosomal locus required for the pigmentation phenotype (Lillard *et al.*, 1997). Nucleotide sequence of this region, termed the hemin storage (*hms*) locus, revealed a 4-gene operon, *hmsHFRS*. Two unlinked genes, *hmsT* and *hmsP*, were later found to be essential for the normal pigmentation phenotype (Hare and McDonough, 1999; Jones *et al.*, 1999; Kirillina *et al*., 2004). Concurrently, investigations by several groups into the iron-regulated proteins synthesized by pigmented *Y. pestis* culminated in the characterization of the *Yersinia* high-pathogenicity island (HPI), which encodes a siderophore-based iron acquisition system (for recent reviews see Lesic and Carniel, 2004; Perry and Fetherston, 2004). A key unifying discovery was reported by Fetherston *et al*. (1992) showing that most nonpigmented *Y. pestis* mutants resulted from spontaneous deletion of a 102-kb segment of the *Y. pestis* chromosome that was termed the pigmentation (Pgm) locus. The 102-kb Pgm locus contains not only the *hms* genes, but also the *Yersinia* HPI. It is flanked by IS*100* elements, and homologous recombination between these extensive direct repeat sequences likely accounts for the high spontaneous deletion rate (10^{-5} to 10^{-3}) of the entire 102-kb segment (Hare *et al.*, 1999; Fetherston and Perry, 1994). Thus, elimination of this large locus by a single deletion event results not only in the nonpigmented (Hms^-) phenotype, but also in decreased virulence due to concomitant loss of the HPI. Not all nonpigmented mutants result from the loss of the entire 102-kb segment, however. The existence of certain nonpigmented *hmsHFSR*-negative, HPI-positive *Y. pestis* strains indicated that the *hmsHFRS* locus could be autonomously deleted and that pigmentation and iron acquisition phenotypes are clearly separable (Iteman *et al.*, 1993; Buchrieser *et al.*, 1998). The incidental linkage of the *hmsHFRS* locus and the HPI within the same deletion-prone segment accounts for the long-held consideration of pigmentation as a virulence determinant, reinforced by referring to the entire 102-kb segment as the Pgm locus. However, the connection between pigmentation *per se* and virulence turns out to be merely "guilt by association" with the HPI. In retrospect, the temperature-dependence of the pigmentation phenotype provided an important clue as to its true biological role. As noted previously, pigmentation develops only at temperatures less than about 28°C, a temperature that matches the flea environment. It is not detected at 37°C, the mammalian body temperature. In fact, nonpigmented *Y. pestis* strains containing specific loss-of-function mutation of the *hms* genes are fully virulent, at least in mice, and the hypothesis that hemin storage is important nutritionally has also been disproven (Hinnebusch *et al.*, 1996; Lillard *et al.*, 1999). In contrast, nonpigmented *Y. pestis* strains lacking a functional *hmsHFRS* locus, or the entire 102-kb Pgm locus, were completely unable to produce proventricular blockage in *X. cheopis* fleas, although they survived in and established a chronic infection of the midgut at the same rate as the isogenic pigmented *Y. pestis*. The ability of Pgm^- *Y. pestis* to infect and block the proventriculus could be completely restored by reintroducing the *hmsHFRS* genes alone, indicating that the HPI and other genes in the 102-kb Pgm locus are not required in the flea (Hinnebusch *et al.*, 1996). Earlier, working with genetically undefined strains, Bibikova (1977) correlated the pigmentation phenotype with the ability to cause proventricular blockage in *X. cheopis*; and Kutyrev *et al.* (1992) reported that a nonpigmented but pesticin sensitive and virulent *Y. pestis* strain failed to survive in the vole flea *Nosopsyllus laeviceps*. Whether physiological differences between the two flea species account for the ability of nonpigmented *Y. pestis* to colonize *X. cheopis* but not *N. laeviceps* is unknown.

Role of the hms genes: Production of a Y. pestis *biofilm required for proventricular blockage.* Ironically, given its close phylogenetic relationship with enteric pathogens, *Y. pestis* does not penetrate or even adhere to the flea midgut epithelium, but remains confined to the lumen of the digestive tract. Because *Y. pestis* is not invasive in the flea, it is at constant risk of being eliminated by peristalsis and excretion in the feces. In fact, approximately half of *X. cheopis* fleas spontaneously rid themselves of infection in this way even if they feed on highly septicemic blood (Pollitzer, 1954; Hinnebusch et al., 1996). Success or failure in stable colonization of the flea gut depends on the ability of the bacteria to produce aggregates that are too large to be excreted (Fig. 1B). Both Hms^+ and Hms^- *Y. pestis* are able to do this, and so achieve comparable infection rates in *X. cheopis*. However, transmission to a new host further requires that *Y. pestis*, which is nonmotile, move against the direction of blood flow when the flea feeds. As described above, this is accomplished by infecting the proventriculus, interfering with its valvular action in such a way as to generate backflow of blood into the bite site. The *hms* genes are required for proventricular infection, and recent evidence suggests that they synthesize an extracellular matrix required for biofilm formation.

HmsH and HmsF were characterized as surface-exposed outer membrane proteins (HmsF also contains a lipid attachment site typical of a lipoprotein), and HmsR, HmsR, and HmsT contain transmembrane domains and appear to be inner membrane proteins (Parkhill *et al*., 2001; Pendrak and Perry, 1993; Perry *et al*., 2004), but the first predictive clue as to the function of the *hms* genes came from database searches showing that they are similar to glycosyl transferase and polysaccharide deacetylase genes in other bacteria that are required to produce extracellular polysaccharides (Fig. 3). Notably, the *E. coli* operon *pgaABCD* is homologous to *Y. pestis hmsHFRS*; *pgaC* and *pgaD* restore pigmentation to *Y. pestis hmsR* and *hmsS* mutants, respectively, and the adjacent *ycdT* gene is an *hmsT* homolog (Lillard *et al.*, 1997; Jones *et al.*, 1999; Wang *et al*., 2004). The four

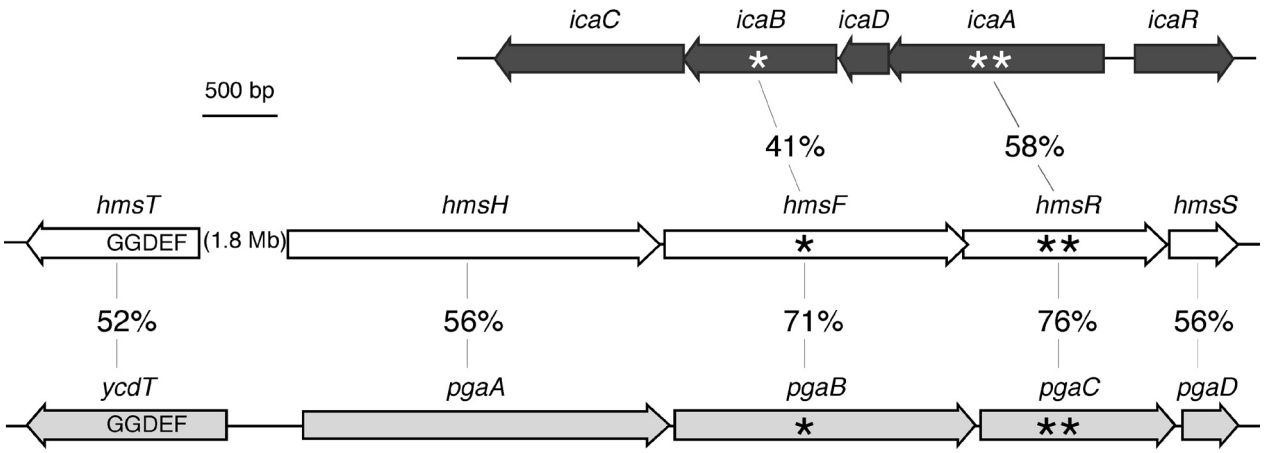

Fig. 3. Comparison of the *ica, hms*, and *pga* operons of *S. epidermidis*, *Y. pestis*, and *E. coli*, respectively. Numbers indicate the percent amino acid similarity of the predicted products of *Y. pestis hms* genes with *ica*, *pga*, and *ycd* gene products. Single asterisks indicate polysaccharide deacetylase domains, double asterisks indicate glycosyl transferase domains, and GGDEF indicates diguanylate cyclase domains.

pga gene products are predicted to be outer surface proteins that synthesize extracellular poly-β-1,6-*N*-acetyl-D-glucosamine (PGA) that is required for biofilm formation (Wang *et al*., 2004; Itoh *et al*., 2005). Similarity was also detected between *hmsR* and *hmsF* and two genes in the *ica* (intercellular adhesion) operon of *Staphylococcus epidermidis* that is required to synthesize a linear β-1,6-*N*-acetyl-D-glucosamine polymer called the polysaccharide intercellular adhesin (PIA) (Heilmann *et al*., 1996; Lillard *et al*., 1999;). PIA is an extracellular polysaccharide that leads to bacterial cell-cell aggregation and is required for the formation of staphylococcal biofilms. Interestingly, PIA as well as the extracellular polysaccharide associated with several other bacterial biofilms binds Congo red (Heilmann and Götz, 1998; Weiner *et al*., 1999). The *ica* operon consists of four genes (*icaADBC*). HmsR has 39% identity and 58% amino acid sequence similarity to IcaA, an N-acetylglucosamine transferase that functions to polymerize UDP-N-acetylglusosamine units; and HmsF has 23% identity and 41% similarity to IcaB, a poly (β-1, 6) N-acetylglocosamine deacetylase that removes N-acetyl groups from the extracellular PIA polymer (Götz, 2002). Thus, it is likely that the *Y. pestis hms* gene products also synthesize a PGA-like extracellular polysaccharide, but its chemical structure remains to be determined.

The chromosomal *hmsT* and *hmsP* genes are unlinked to the *hmsHFRS* operon and to each other but are required for normal expression of the pigmentation phenotype and biofilm formation (Hare and McDonough, 1999; Jones *et al*., 1999; Kirillina *et al*., 2004). Although the biochemistry remains to be demonstrated, the presence of specific domains within HmsT and HmsP indicate their probable function. HmsT belongs to the family of GGDEF domain proteins (Jones *et al*., 1999) and is predicted to synthesize cyclic-di-GMP, a known effector of extracellular polysaccharide production in other bacteria (Kirillina *et al*., 2004; Ross *et al*., 1987; Ryjenkov *et al*., 2005). HmsP belongs to the family of EAL domain proteins and is predicted to have phosphodiesterase activity that hydrolyzes cyclic-di-GMP (Kirillina *et al*., 2004). Based on the presence of these domains and their predicted catalytic activities, Kirillina *et al*. (2004) proposed that HmsT and HmsP regulate Hms-dependent extracellular polysaccharide production (and therefore biofilm formation) by coordinately controlling the level of the cyclic-di-GMP activator. Transcription of the *hmsT* and the *hmsHFRS* operons is not affected by growth temperature; however, protein levels of HmsT, HmsH, and HmsR are much lower in *Y. pestis* grown at 37° than at 26°C, which likely accounts for the temperature-dependence of the pigmentation phenotype (Perry *et al*., 2004).

The amino acid sequence comparisons indicate that the *hms* genes encode products that synthesize the extracellular matrix of a biofilm. A bacterial biofilm is a complex, compact community of cells enclosed in an extracellular matrix, often attached to a surface (Costerton *et al*., 1995). Biofilms can form in spite of high shear forces and rapid currents, and are produced *in vivo*, particularly on implanted medical devices, by many bacterial pathogens (Costerton *et al*., 1999). Previous investigations have shown that the dense aggregates of *Y. pestis* that develop in the flea midgut and block the proventriculus are surrounded by an extracellular matrix, fitting the operational definition of a biofilm (Hinnebusch *et al*., 1998a; 2002a; Jarrett *et al*., 2004). The ability of *Y. pestis* to produce an extracellular matrix in the flea, along with the ability to block the proventriculus, depends on the *hms* genes. The role of the individual *hms* genes in this *in vivo* phenotypes have not been systematically studied, but mutation of *hmsR* or *hmsT* eliminates or greatly reduces the ability of *Y. pestis* to block fleas (Hinnebusch *et al*., 1996; and unpublished data). The *hms* genes are also required for the ability of *Y. pestis* to produce an adherent biofilm on the surface of a glass flowcell, and to synthesize an extracellular material observed by scanning electron microscopy (Jarrett *et al*., 2004). Like pigmentation, the *in vitro* biofilm and extracellular material are only produced at low temperatures and not at 37°C. Darby *et al.* (2002) and Joshua *et al*. (2003) have also shown that *Y. pestis* and *Y. pseudotuberculosis* produce biofilm-like growth on agar plates that accumulates on the external mouthparts

of *Caenorhabditis elegans* nematodes placed on them, and that this phenotype is *hms*-dependent.

Taken together, the genetic, *in vitro*, and *in vivo* observations strongly suggest that *Y. pestis* forms an *hms*-dependent biofilm to infect the hydrophobic, acellular surface of the flea's proventricular spines, and in this way overcomes the rhythmic, pulsating action of the proventricular valve and the inward flow of blood during feeding that would otherwise counteract transmission by washing the bacteria backwards into the midgut. Given the homology between the *Y. pestis hms* genes and the staphylococcal *ica* genes, it seems likely that the function of the *hms* gene products is to synthesize an extracellular polysaccharide required for biofilm development. The composition of the extracellular matrix that surrounds the *Y. pestis* biofilm in the flea is unknown, but appears to contain flea midgut-derived lipid components as well as *hms*-dependent components (Jarrett *et al*., 2004). The *hms* genes do not appear to be required in the mammal; thus, their primary biological function is to enable flea-borne transmission (Hinnebusch *et al*., 1996; Lillard *et al*., 1999). Transmission of *Leishmania* parasites also depends on a foregut-blocking phenomenon in the sandfly vector (Stierhof *et al*., 1999), but *Y. pestis* is unique among bacteria characterized to date in using a biofilm mechanism to enable arthropod-borne transmission. In retrospect, the first hint that the *hms* genes pertained to biofilm formation was the observation in the original paper by Jackson and Burrows (1956a) that cells in pigmented colonies resist resuspension and remain bound together in densely packed masses. Surgalla (1960) also observed that another aspect of the pigmentation phenotype is the production of a substance in liquid cultures at room temperature that promotes autoaggregation and pellicle formation on the sides of the culture vessel – typical of biofilm formation.

The *Y. pestis* plasminogen activator and dissemination following flea-borne transmission

Like murine toxin and the Hms pigmentation phenotype, the biological functions attributed to the *Y. pestis* plasminogen activator (Pla) have undergone revision. The *pla* gene is on the 9.5-kb *Y. pestis* plasmid referred to as pPCP1, pPst or pPla (Sodeinde and Goguen, 1988). It encodes a surface protease associated with increased tissue invasiveness and systemic spread of the bacteria (Korhonen *et al*., 2004). Pla is considered to be an essential factor for the flea-borne route of transmission because it greatly enhances dissemination following subcutaneous injection, which is assumed to mimic transmission by fleas (Sodeinde *et al*., 1992). The requirement for Pla for dissemination from peripheral infection sites may not be universally true for all *Y. pestis* strains or for all animals, however (Samoilova *et al*., 1996; Welkos *et al*., 1997).

A prominent role for Pla in proventricular blockage of the flea has been proposed previously. The extracellular matrix that embeds the blocking masses of *Y. pestis* in the flea has often been assumed to be a fibrin clot derived from the flea's blood meal. It was also known that proventricular blockage does not develop normally in fleas kept at elevated temperatures, which helps explain striking epidemiological observations that flea-borne bubonic plague epidemics terminate abruptly with the onset of hot, dry weather (Cavanaugh and Marshall, 1972). Cavanaugh (1971) hypothesized that Pla activity could explain both phenomena. Pla synthesis is not temperature-dependent, but its plasminogen activator ability that leads to fibrinolysis is much greater at 37°C than at temperatures below 28°C (McDonough and Falkow, 1989). In fact, Pla has an opposite procoagulant activity at low temperatures, although this fibrin clot-forming ability is weak and is detected only in rabbit plasma and not in mouse, rat, guinea pig, squirrel, or human plasma (Jawetz and Meyer, 1944; Beesley *et al*., 1967). Nevertheless, it was hypothesized that this low-temperature activity of Pla formed what was presumed to be the fibrin matrix of the blocking mass of *Y. pestis* in the flea. The clot-dissolving plasminogen activator function was invoked to explain why blockage does not develop in fleas at higher temperatures. McDonough *et al.* (1993) later reported that Pla$^+$ *Y. pestis* caused greater mortality in fleas than an isogenic Pla$^-$ mutant, and attributed this to an increased blockage rate. Blockage was not directly monitored in that study, however, and the mortality occurred only four days after infection, well before blockage would be expected to occur.

When the Cavanaugh hypothesis was put to the test, it was found that Pla is not required for normal proventricular blockage to develop in the flea (Hinnebusch *et al*., 1998a). Hms$^+$ *Y. pestis* strains lacking pPst were able to infect and block *X. cheopis* fleas as well as the wild-type parent strain. Both Pla$^+$ and Pla$^-$ strains failed to block fleas kept at 30°C, even though midgut colonization rates were little affected by temperature; in other words, the identical *in vivo* phenotype as seen for Hms$^-$ *Y. pestis*. Thus, the inability of *Y. pestis* to block fleas kept at 30°C can be fully explained by temperature dependence of the Hms phenotype. Furthermore, the presumption that flea-blocking masses of *Y. pestis* are embedded in a fibrin matrix is inconsistent with the fact that the matrix is not degraded by proteases or the fibrinolytic enzyme plasmin. Therefore, the *hms*-dependent biofilm model of proventricular blockage better fits the available data than the Pla-based fibrin clot model.

Insect pathogen-related genes in *Y. pestis* and *Y. pseudotuberculosis*

Like most bacterial genomes, the *Y. pestis* genome contains several loci that appear to have been introduced by lateral transfer from unrelated organisms. Among these are several homologs of known insecticidal toxin complex (Tc) genes of bacterial pathogens of insects, and a homolog of a baculovirus enhancin protease gene required for insect pathogenesis, which conceivably could influence *Y. pestis* interaction with the flea (Parkhill *et al*., 2001). In beginning efforts to assess the role of these genes, fleas were infected with *Y. pestis* strains containing specific mutations in the baculovirus enhancin homolog and *tcaA*, a homolog of one of the Tc genes. Both mutants infected and blocked *X. cheopis* fleas normally, indicating that these two genes are not important for interaction with the flea (B. J. Hinnebusch and R. D. Perry, unpublished). Many of the insect pathogen-related genes are also present in *Y. pseudotuberculosis*; therefore, their acquisition

appears to predate the divergence of *Y. pestis*. When outside the host in soil and water, *Y. pseudotuberculosis* would be expected to come into contact with and even be ingested by insects and other invertebrates, and the insecticidal toxins may help the bacteria survive those encounters. Because *Y. pestis* transmission depends on chronic infection of the flea gut, overt toxicity would be counterproductive. Thus, it seems likely that insect toxicity would be lost or moderated in *Y. pestis*.

Y. pestis at the host–vector interface

Successful transmission of an arthropod-borne agent and subsequent infection depends on a complex co-evolved interaction between pathogen, vector, and host that has not been well-characterized for any arthropod-borne disease. Plague is initiated during the brief encounter between an infectious flea and a vertebrate host. For practical reasons, intradermal or subcutaneous inoculation by needle and syringe of *in vitro*-grown *Y. pestis* is routinely used for pathogenesis studies in animal models in lieu of flea-borne transmission. While this is a reasonable challenge method which may be adequate for most purposes, certain aspects of the flea-bacteria-host transmission interface are unique, and have unknown effects on the host-pathogen interaction and the initiation of disease.

Feeding mechanism of fleas and the microenvironment of the transmission site

Two basic strategies have been described for the manner in which blood-feeding arthropods acquire a blood meal (Lavoipierre, 1965). Vessel feeders, such as triatomine bugs, penetrate the skin and cannulate a superficial blood vessel with their mouthparts before they begin to feed. In contrast, pool feeders, such as ticks and tsetse flies, lacerate blood vessels with their mouthparts as they probe, and feed from the resulting extravascular hemorrhage. Fleas, like mosquitoes, were originally classified as vessel feeders, but this may be an oversimplification. The flea mouthparts include a pair of thin serrated laciniae that act as cutting blades to perforate the dermis. Alternating, rapid contractions and thrusts of the left and right laciniae pierce the skin, and this pneumatic drill-like cutting motion continues as the mouthparts move vertically and laterally in the dermal tissue during probing (Wenk, 1980), an activity that can cause hemorrhage. When blood is located, the laciniae and epipharynx come together to form a feeding channel, and feeding ensues. Whether the tip of the mouthparts is inserted into a vessel or in an extravascular pool of blood has obvious implications for plague pathogenesis. If intravascular feeding occurs, *Y. pestis* might be regurgitated directly into the blood stream (i.v. transmission). If extravascular feeding occurs, intradermal transmission is the appropriate model (the flea mouthparts are not long enough to penetrate into the subcutaneous tissue). The most careful observations of flea feeding (Deoras and Prasad, 1967; Lavoipierre and Hamachi, 1961) suggest that fleas can suck extravascular blood that leaks from a capillary, but prefer to feed directly from a blood vessel. Whether blocked fleas show the same discretion, however, is unknown. The usual progression of bubonic plague, in which *Y. pestis* can produce a primary lesion at the fleabite site and disseminates first to the local draining lymph node, seems to better fit an intradermal transmission model.

Flea saliva is also secreted into the bite site. The saliva of all blood-feeding arthropod vectors contains anticoagulants, and may contain other factors that influence the outcome of transmission. For example, a component of sandfly saliva greatly enhances the infectivity of *Leishmania* (Titus and Ribeiro, 1988). Flea saliva is known to contain the anticoagulant apyrase, an enzyme which acts to inhibit platelet and neutrophil aggregation (Ribeiro *et al*., 1990), but this is the only component that has been identified to date.

The transmission phenotype of *Y. pestis*

The phenotype of *Y. pestis* as it exits the flea and enters the mammal is clearly different from *in vitro* growth phenotypes. As described in previous sections, *Y. pestis* growth in the flea resembles a biofilm and is associated with an extracellular membrane. The infectious units transmitted by the flea may consist not only of individual *Y. pestis*, but small clumps of bacteria derived from the periphery of the proventriculus-blocking mass. If pieces of the biofilm are regurgitated by fleas, the bacteria within them may be protected from the initial encounter with the host innate immune response, because bacteria embedded in a biofilm have been shown to be more resistant to uptake or killing by phagocytes (Donlan and Costerton, 2002). Because known antiphagocytic factors such as the F1 capsule and the Type III secretion system are not produced by *Y. pestis* at the low temperature of the flea gut (Straley and Perry, 1995; Perry and Fetherston, 1997), the extracellular matrix associated with growth in the flea may provide initial protection until the known antiphagocytic virulence factors are synthesized. Secondarily, regurgitated aggregates that are larger than the diameter of the intradermal blood vessels would preclude direct intravenous transmission.

In nature, *Y. pestis* in a particular phenotype is transmitted along with flea saliva into an intradermal microenvironment. Details of the flea-bacteria-host interface during and after transmission have not been characterized, and cannot be satisfactorily mimicked by transmission using a needle and syringe. Consequently, aspects of host-parasite interactions specific to the unique context of the fleabite site are unknown and merit future investigation.

Evolution of arthropod-borne transmission

Y. pestis provides a fascinating case study of how a bacterial pathogen can evolve a vector-borne route of transmission. Given the short evolutionary timeframe in which it occurred, the change from an enteric, food- and water-borne pathogen to systemic, insect-borne pathogen was too abrupt to result from the slow evolutionary process of random mutation of individual genes leading to natural selection. Instead, more rapid evolutionary processes were responsible, such as horizontal gene transfer and the fine-tuning of existing genetic pathways to perform new functions.

Carniel (2003) has proposed a sequential evolutionary scenario for the switch to vector-borne transmission in the

yersiniae. Because the *ymt* (*pldA*) gene enhances survival in the flea digestive tract, a likely first step was acquisition of the 100-kb pFra plasmid by horizontal transfer to generate a *Y. pseudotuberculosis* (pFra) or pre-pestis 1 clone. *Y. pseudotuberculosis* can cause septicemia in rodents, so it probably was taken up by fleas periodically. This would have been a dead end, until the hypothetical pre-pestis 1 clone acquired the PLD activity encoded on the pFra replicon, which allowed it to survive in and colonize the flea midgut.

A second necessary step in the evolution of flea-borne transmission took advantage of a pre-existing biofilm-forming capacity, which involves the *hms* genes. *Y. pestis* makes an *hms*-mediated biofilm to produce an obstructing infection in the proventricular valve, which is required for efficient transmission. The *hms* genes are present in *Y. pseudotuberculosis*, but, curiously, most isolates that have been tested do not exhibit the pigmentation phenotype (Brubaker, 1991). Some *Y. pseudotuberculosis* strains do show the Congo red-binding pigmentation phenotype *in vitro*, but all *Y. pseudotuberculosis* that have been tested, whether pigmented or not, are unable to block the proventriculus of *X. cheopis* (B. J. Hinnebusch, unpublished). Thus, a separate, as yet undiscovered genetic change may have occurred in pre-pestis 1 to extend its biofilm-forming capacity to include the flea gut environment. The presumptive change likely affected the outer membrane in such a way as to enhance aggregate formation on the hydrophobic proventricular spines in the context of the flea digestive tract milieu.

A third important step in the evolution of flea-borne transmission occurred when the progenitor clone acquired the small plasmid containing the *pla* gene, which is thought to enable *Y. pestis* to disseminate from the fleabite site after transmission. The clone containing both of the new *Y. pestis*-specific plasmids has been referred to as pre-pestis 2 by Carniel (2003). Given the ecology of the *Y. pseudotuberculosis* ancestor, horizontal transfer of pFra and pPst could have occurred in a mammal, a flea, or the environment. Of course, it is likewise impossible to know with certainty the order in which the plasmids were transferred, or the plasmid donors, although molecular biology analyses may provide some clues. For example, the 100-kb pFra shares major sequence identity with a *Salmonella* Typhi plasmid, suggesting that the *Y. pseudotuberculosis* ancestor acquired what became pFra from a *Salmonella* donor (Prentice *et al.*, 2001). This horizontal transfer to generate the *Y. pseudotuberculosis* (pFra) clone may have occurred in the digestive tract of a rodent, since both bacteria are enteric pathogens. On the other hand, plasmid transfer by conjugation occurs readily in mixed bacterial biofilms, both in the environment and in the flea gut (Hinnebusch *et al.*, 2002a).

Coevolution of flea-borne transmission and increased virulence in *Y. pestis*

The evolutionary path that led to flea-borne transmission also led to *Y. pestis* becoming one of the most virulent and feared pathogens of human history. It is probably no accident that increased virulence coevolved with vector-borne transmission. In fact, reliance on the flea for transmission imposed new selective pressures that would have strongly favored this. Some consideration of the dynamics of the *Y. pestis*-flea relationship serve to reinforce this point (Fig. 4). First, flea-borne transmission is actually quite inefficient, which may reflect the fact that *Y. pestis* has only recently adapted to its insect host. The number of *Y. pestis* needed to infect 50% of susceptible mammals (the ID_{50}, often referred to as the minimum infectious dose) is the same as the 50% lethal dose (LD_{50}) – less than 10 (Perry and Fetherston, 1997). In contrast, the ID_{50} of *Y. pestis* for *X. cheopis* is about 5,000 bacteria (Lorange *et al.*, 2005). Fleas take small blood meals (0.1–0.3 μl), so *Y. pestis* must achieve a level of $>10^7$ per milliliter in the peripheral blood in order to have a 50% chance of infecting its vector. Bacteremias of 10^8 to 10^9 per milliliter are routinely present in moribund white laboratory mice (Douglas and Wheeler, 1943). The concept of a very high threshold level of bacteremia, below which infection of feeding fleas does not occur or is rare, is supported by the observations of several investigators (Douglas and Wheeler, 1943; Pollitzer, 1954; Kartman and Quan, 1964). Thus, *Y. pestis* does not infect the flea very efficiently in the first place, and this would have been strong selective pressure favoring more invasive, and consequently, more virulent strains able to produce the severe bacteremia that typifies plague.

A second weak link in the *Y. pestis* life cycle is that, even after successful infection of the vector, subsequent transmission is not very efficient. Not all infected fleas develop transmissible proventricular infections – for *X. cheopis*, the rate is only about 50%, and this rate can be much lower for other flea species (Wheeler and Douglas, 1945; Burroughs, 1947; Pollitzer, 1954). Furthermore, it is well established that the bite of a blocked flea does not always result in disease. Past studies report that individual blocked *X. cheopis* fleas that feed on a susceptible host transmit plague only about 50% of the time (Burroughs, 1947). Very few studies have addressed the number of *Y. pestis* transmitted by a single infectious fleabite. Burroughs (1947) triturated and plated mouse-skin biopsies taken from the site where a blocked flea had been allowed to

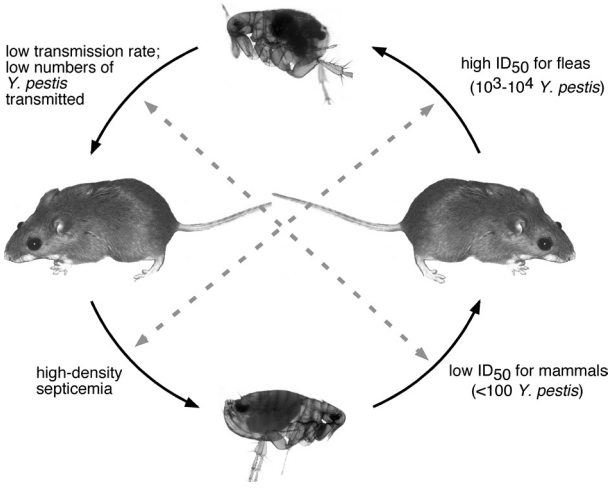

Fig. 4. Dynamics of flea-borne transmission of plague. The high ID_{50} of *Y. pestis* for fleas is compensated for by the ability to produce a high-density septicemia in the rodent. The inefficient transmission by blocked fleas is compensated for by a low ID_{50} (LD_{50}) from a peripheral inoculation site.

attempt to feed. Only one of thirty samples was positive, which contained 88 *Y. pestis* colony-forming units (CFU). This number was multiplied by a factor to compensate for the low plating efficiency from other skin samples into which known numbers of *Y. pestis* had been injected, to calculate an estimate of 11,000 to 24,000 bacteria transmitted by the flea. However, this estimate was derived indirectly from a single positive sample, which seems less than satisfactory. We recently reexamined this topic by allowing individual blocked *X. cheopis* fleas to feed on a mouse or an artificial feeding device and quantifying the number of *Y. pestis* transmitted. Only 45% of the fleabites resulted in transmission, and the median number of *Y. pestis* transmitted was less than 100 (Lorange *et al*., 2005). This rather erratic transmission, frequently involving only a small number of bacteria, would have favored the selection of more invasive clones (such as the ancestral $pPla^+$ clone), able to successfully disseminate from the fleabite site even from a small initial dose.

Because reliance on the flea vector to complete the *Y. pestis* life cycle is contingent upon the ability to produce a high-density bacteremia and to invade from a small infectious dose in the skin, the evolution of vector-borne transmission and of increased virulence would have been mutually reinforcing. Parasite evolution is often assumed to evolve towards commensalism, or a state of peaceful coexistence with the host. Actually, the degree of virulence that is evolved by a successful parasite is functionally coupled with transmissibility (Ewald, 1983). That is, pathogens will tend to evolve to a level of virulence that optimizes their chance of successful transmission. Paul Ewald (1983) has argued that parasites transmitted by blood-feeding arthropods pay little cost for harming their hosts, and may actually benefit by being virulent. The extensive reproduction and hematogenous spread associated with severe disease increases the probability that a blood-feeding vector would acquire an infectious dose; and host immobilization and morbidity would not hinder, and may even enhance, the ability of a vector to find the host and feed to repletion.

The risk for a pathogen as virulent as *Y. pestis* is killing the host too quickly for transmission to occur. *Y. pestis* may have gotten away with the risky strategy of being rapidly fatal because most mammals maintain a more or less permanent flea population in their fur and in their burrows. It is not unusual for a single rodent to carry 5 to 80 fleas, with more than 100 fleas present in the nest or burrow, and it is simply a fact of life for most rodents to experience daily fleabites throughout their lives (Traub, 1972). Thus, *Y. pestis* does not have to rely on a chance encounter with a flying vector like a mosquito. Even if there are only a few hours between the development of high-density septicemia and death, there is good probability that one or more resident fleas will take an infectious blood meal during that short window of time. Because fleas do not readily leave their host mammal, killing the host may actually be important to *Y. pestis* transmission. Brubaker (2000) has made the point that death of the host compels the resident infected fleas to seek a new, healthy host – a necessary step to complete the transmission cycle.

Arthropod-transmitted pathogens may be indifferent to or even benefit from producing morbidity and mortality in their mammalian hosts, but infections in the arthropod vector are rarely symptomatic. As a rule it would be counterproductive to impair the health of the vector they rely on for transmission. For example, malaria parasites do not cause morbidity in the mosquitoes that transmit them to new hosts. *Y. pestis* breaks this rule – it causes an overwhelming, fatal infection in the flea as well as the mammal. A single blocked flea contains greater than 10^6 *Y. pestis* on average, and proventricular blockage leads to dehydration and death by starvation within a few days (Hinnebusch *et al*., 1998b; Kartman and Prince, 1956; Pollitzer, 1954)). During this time, however, a blocked flea will make persistent, frequent attempts to feed. A normal flea probes the skin and feeds to repletion within a few minutes. A blocked flea, in contrast, will try to take a blood meal in one location for a period of several minutes, then withdraw, move to another location, and try again. As it gradually starves, this process is repeated many times. Finally, the dehydrated blocked flea often remains with its mouthparts embedded in the skin for an hour or more, expending its last bit of energy in a futile attempt to satisfy its appetite. This anomalous feeding behavior provides multiple opportunities for transmission to occur before the flea dies, somewhat analogous to the manner in which the altered behavior of a rabid dog enhances the transmission of rabies virus.

Conclusions

Y. pestis exhibits a distinct life stage in its flea vector. The barriers to infection and the environmental conditions it faces in the flea digestive tract are quite different from those encountered in the mammalian host. Consequently, the development of a transmissible infection requires a distinct subset of genes. None of the known mammalian virulence factors that have been tested (the F1 capsule, *pla*, the type III secretion system encoded on the pYV virulence plasmid, and the HPI) are required in *X. cheopis* (Hinnebusch *et al*., 1996; 1998). Conversely, the two genetic loci (*ymt* and *hms*) that have been shown to be required for the flea-specific phenotype are not required for virulence in the mammal. Thus, a distinction can be made between *Y. pestis* genes required for pathogenesis in the mammal (virulence factors) and the genes required to produce a transmissible infection in the flea (transmission factors).

When a flea takes up *Y. pestis* in a blood meal, the bacteria experience a drop in temperature from 37°C to the ambient temperature of the flea. This temperature shift appears to be an important environmental signal for the bacteria to regulate gene expression appropriate to the invertebrate or vertebrate host. The expression of many *Y. pestis* virulence factors is upregulated at 37°C compared to room temperature (Straley and Perry, 1995; Perry and Fetherston, 1997), whereas the upregulation of the Hms phenotype and the *ymt* gene at room temperature compared to 37°C was a predictive initial clue that their biological role might occur in the flea. With the advent of DNA microarray and proteomics technologies, the global effect of the temperature shift from mammal to flea on *Y. pestis* gene expression can be analyzed, which may identify new candidate transmission factors (Han *et al*., 2004; Motin *et al*., 2004).

In this review, I have focused on recent work using *X. cheopis* as the animal model and genetically defined strains of *Y. pestis* KIM (biovar Medievalis) and 195/P (biovar Orientalis). Earlier work, particularly by Russian investigators, led to often contradictory conclusions, with some studies suggesting a role in flea infection or blockage for the F1 capsule and Pla virulence factors, and others finding no role (Kutyrev *et al.*, 1992; McDonough *et al.*, 1993; Anisimov, 1999). Such reported differences in host–parasite relationships have yet to be resolved, but if nothing else serve as a reminder of the ecological complexity of plague, which can involve over 200 species of mammals and their fleas (Pollitzer, 1954). Factors that could explain contradictory data include differences in: 1) the digestive tract physiology and proventricular anatomy of the various flea species investigated; 2) the biochemical composition of the blood of different rodent species; 3) *Y. pestis* strains used; and 4) the temperature at which the fleas were maintained (Table 1).

Three discrete genetic steps that led to the recent evolutionary transition of *Y. pestis* from an enteric to a flea-borne route of transmission can now be identified. Two of them involved horizontal transfer of the *ymt*- and *pla*-harboring plasmids that are unique to *Y. pestis*. The third step involved adapting the pre-existing *hms* chromosomal genes to a new function – biofouling of the proventriculus to interfere with its normal valvular operation. Several unanswered questions remain. The biochemical mechanisms of action of the PLD and the Hms proteins in the flea have yet to be fully characterized. Additional transmission factors probably remain to be discovered before a complete recounting of the adaptation to the flea vector can be told. The *Y. pestis* genome also contains many pseudogenes, and the consequence, if any, of this large-scale gene loss on the interaction with the flea remains to be explored. Careful comparison of *Y. pseudotuberculosis* and *Y. pestis* genomes should provide important insights into these and other questions. Identifying and characterizing the molecular mechanisms that ensued from the specific genetic changes responsible for flea-borne transmission will ultimately provide an instructive case study in the evolution of bacterial pathogenesis.

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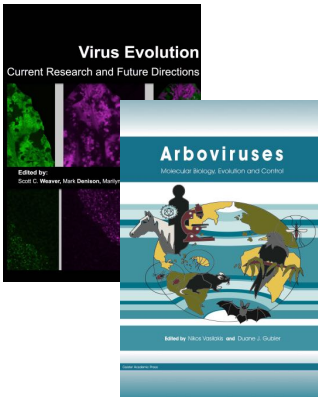

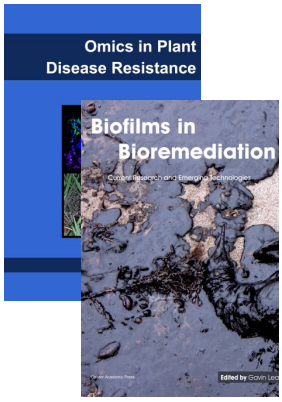

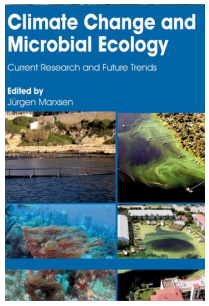

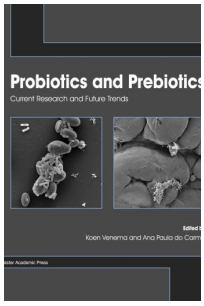

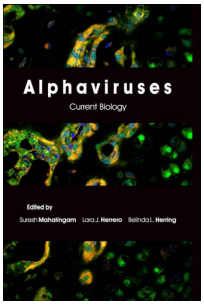

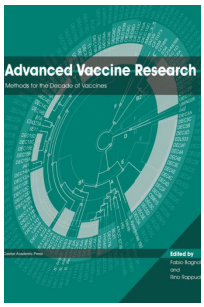

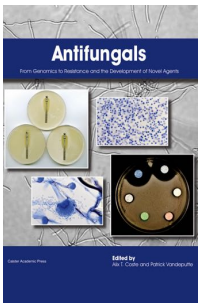

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