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Yersinia-flea interactions and the evolution of the arthropod-borne transmission route of plague

Iman Chouikha and **B. Joseph Hinnebusch***
Laboratory of Zoonotic Pathogens, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana 59840 USA

Abstract

Yersinia pestis, the causative agent of plague, is unique among the enteric group of Gram-negative bacteria in relying on a blood-feeding insect for transmission. The *Yersinia*-flea interactions that enable plague transmission cycles have had profound historical consequences as manifested by human plague pandemics. The arthropod-borne transmission route was a radical ecologic change from the food- and water-borne transmission route of *Yersinia pseudotuberculosis*, from which *Y. pestis* diverged only within the last 20,000 years. Thus, the interactions of *Y. pestis* with its flea vector that lead to colonization and successful transmission are the result of a recent evolutionary adaptation that required relatively few genetic changes. These changes from the *Y. pseudotuberculosis* progenitor included loss of insecticidal activity, increased resistance to antibacterial factors in the flea midgut, and extending *Yersinia* biofilm-forming ability to the flea host environment.

Introduction

The genus *Yersinia* consists of seventeen species of Gram-negative rod-shaped bacteria in the family Enterobacteriaceae of the gammaproteobacteria. Most of them are widely distributed in the environment and two of them, *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*, cause relatively mild food- and water-borne enteric diseases. A third pathogenic species, *Yersinia pestis*, stands out in several respects. *Y. pestis*, the cause of plague, is one of the most virulent bacterial pathogens responsible for three devastating pandemics in human history. Plague is primarily a disease of rodents, however, and *Y. pestis* is transmitted between them primarily via flea vectors. Maintenance of *Y. pestis* in nature depends on these rodent-flea transmission cycles, and the ecology of plague is complex, involving many different rodent and flea species. Today, *Y. pestis* exists in permanently entrenched enzootic foci in many parts of the world. Thus, unlike all other *Yersinia* species, *Y. pestis* no longer needs to survive in the environment but instead alternates between life in two different eukaryotic hosts-insects and mammals. Remarkably, this radical change in ecology is a recent evolutionary adaptation, because *Y. pestis* is essentially a clonal variant that diverged from the closely related *Y. pseudotuberculosis* only within the last 1,500 to 20,000 years [1,2].

*Corresponding author: Tel: 406-363-9260, Fax: 406-375-9681 .
iman.chouikha@nih.gov, jhinnebusch@niaid.nih.gov

As with most bacteria of public health importance, *Yersinia* research has focused on bacterial interactions with the mammalian host that lead to disease. The interactions of *Y. pestis* with fleas that lead to transmission have received comparatively little attention. In this review we examine the mechanisms by which *Y. pestis* is able to colonize the flea digestive tract and to produce a transmissible infection, and the evolutionary pathway that led to arthropod-borne transmission in the genus *Yersinia*.

The flea host environment

Fleas are wingless insects of the order Siphonaptera, most closely related to the Diptera and Mecoptera orders. Over 2,000 species have been described, and adult fleas are obligate blood-feeding ectoparasites. Most live in close association with their hosts and depend on small but frequent blood meals for survival. Unlike mosquitoes, blood meal storage, digestion, and adsorption all take place in a simple, non-compartmentalized midgut (Fig. 1); hemolysis and complete liquefaction of the blood meal occur within a few hours and digestion is fully completed within a few days.

Little is known about the biochemical and physiological environment of the flea digestive tract. The intense digestive milieu of the flea midgut and continual disturbance due to peristalsis and excretion would appear to be a somewhat hostile environment, because fleas have limited (but as yet poorly defined) normal midgut flora, and few pathogens are transmitted by fleas. Nevertheless, rickettsia-like endosymbionts are common in flea tissue, protists occur in the hindgut and Malpighian tubules, and Gram-positive and Gram-negative bacteria can also be detected in fleas [3-6]. How this normal flora might influence the flea gut environment or flea-*Yersinia* interactions and vector competency is unknown. The flea immune response to infection with *Y. pestis* has not been characterized either, but likely will resemble the immune response of mosquitoes and other insects to ingested foreign bacteria. Both *Y. pestis* and *Y. pseudotuberculosis* are constitutively resistant to cationic antimicrobial peptides, common elements of insect immunity, especially at low growth temperatures that match the flea environment [7-9].

Yersinia infection of the flea digestive tract

Y. pestis infection of the flea is confined to the digestive tract, entering the midgut as individual, planktonic bacteria when a flea feeds on an infected rodent. The bacteria do not adhere to or invade the midgut epithelium and are initially quite susceptible to elimination in flea feces-up to half of fleas completely clear themselves of infection in this way even after feeding on blood containing $>10^8$ *Y. pestis* per milliliter [10,11]. Thus, plague transmission cycles depend on the ability of *Y. pestis* to produce a high-density bacteremia in the mammalian host. *Y. pestis* can achieve levels of 10^9 per ml in the peripheral blood of susceptible rodents, and this overwhelming sepsis accounts for the extreme virulence and high mortality rate of plague.

Persistence in the flea gut depends on the ability of *Y. pestis* to form multicellular aggregates that are too large to be passed in the feces. Clumps of bacteria begin to form within the first few days after infection and grow and consolidate as the bacteria multiply during the first week (Fig. 2). *Y. pestis* numbers in a successfully colonized flea reach 10^5 to $>10^6$ by the second week after infection, and then plateau [12].

Survival of *Y. pestis* in the flea midgut depends on the activity of a phospholipase D (PLD) enzyme, originally characterized as a toxin for mice and rats, that is encoded by the *ymt* (*Yersinia* murine toxin) gene carried on a *Y. pestis*-specific plasmid [13]. Ymt is a member of a large, phylogenetically widespread family of PLDs characterized by signature HKD motifs in the catalytic site of the enzyme. *Y. pestis* mutants lacking functional Ymt assume

an aberrant spherical shape, indicative of loss of outer membrane integrity, and rapidly disappear from the flea midgut within a day after infection. Presumably, Ymt protects the bacteria against a bacteriolytic agent that is generated in the flea midgut during digestion of the blood meal, but the agent and mechanism by which PLD activity protects against it remain to be identified. Interestingly, transformation of *Y. pseudotuberculosis* and *E. coli* with the *Y. pestis ymt* gene greatly increase their ability to infect the flea midgut also [13].

Role of bacterial biofilm in producing a transmissible infection of fleas

Colonization of the flea midgut is not sufficient to produce a transmissible infection. Unlike many other arthropod-borne pathogens, which are transmitted via vector saliva after dissemination to the salivary glands, *Y. pestis* is transmitted by regurgitation from the digestive tract. Regurgitative transmission is possible when the bacteria infect the proventriculus, a valve in the flea foregut that connects the esophagus and the midgut (Fig. 1, 2). The proventriculus opens and closes rhythmically during feeding, but otherwise is tightly closed to seal the blood meal in the midgut. The interior of the proventriculus is arrayed with stiff spines that are coated with cuticle, the same hard acellular material that makes up the exoskeleton.

Y. pestis infection of the proventriculus depends on its ability to grow in the form of a biofilm on the surface of the proventricular spines [14,15]. As the biofilm grows and consolidates, it can impede and eventually completely block normal blood feeding, a process that typically requires one to two weeks after the infectious blood meal. Complete blockage of the proventriculus results in gradual starvation, persistent feeding attempts, and eventual death of the flea; but infected, unblocked fleas do not experience increased morbidity or mortality. Interference with proventricular valve function by the *Y. pestis* biofilm (which does not require complete blockage) potentiates regurgitation of *Y. pestis* from the biofilm into the flea bite site [16]. The polysaccharide extracellular matrix (ECM) of the biofilm is synthesized and exported by the *Y. pestis hmsHFRS* genes and is similar or identical in composition to the poly-β-1,6-*N*-acetyl-D-glucosamine ECM of *E. coli* and staphylococcal biofilms [17,18]. *Y. pestis hmsHFRS* mutants are able to colonize the flea midgut in the form of multicellular aggregates nearly as well as wild-type bacteria, but are devoid of ECM and associated material and completely unable to colonize the proventriculus (Fig. 2) [12].

A second mechanism of regurgitative transmission can occur the first time a flea feeds again on a naïve animal in the week following an infectious blood meal. The mechanism of this early-phase transmission is not known, but it does not require biofilm formation [19]. We have proposed an ingestion-salivation mechanism for early-phase transmission, similar to the mechanism by which aphids transmit plant viruses [20]. According to this model, a residue of infected blood remains on the interior surface of the flea mouthparts and distal foregut following an infectious blood meal. Upon the next feeding, salivation washes residual bacteria present in the salivary groove into the bite site to effect transmission [21].

Most research on *Yersinia*-flea interactions has been with the rat flea *Xenopsylla cheopis*. Proventricular blockage and transmission rates are higher in this species than in others, but the physiological basis for these differences is unknown. Since bacterial adherence to insect cuticle appears to be critical for a transmissible infection, subtle differences in the biophysical properties of the proventricular or mouthpart epicuticle of different flea species could be important. Bacterial adhesion and biofilm formation are highly dependent on surface charge and other properties of the substrate [22].

Molecular mechanisms of *Yersinia*-flea interactions

Upon entering a flea in a blood meal, *Y. pestis* experiences a rapid drop in temperature from 37°C to < 26°C. This temperature switch is a major environmental cue that *Y. pestis* uses to regulate gene expression appropriately in order to adapt to the insect host. Many *Y. pestis* phenotypes, including biofilm formation, are temperature-dependent [23]. The transcriptomes of *Y. pestis* in infected fleas and in the lymph node of rats with bubonic plague, as well as the transcriptomes of *Y. pestis* grown in liquid culture media at 21° and 37°C, have been characterized and compared to identify adaptational gene expression responses associated with successful colonization and blockage of the flea [24,25].

Regulation of biofilm formation in the flea

The bacterial biofilm phenotype, critical to the regurgitative transmission mechanism of *Y. pestis*, involves a complex, multistage developmental process. Although this process has a common end point– a dense surface-attached multicellular bacterial aggregate embedded in a self-synthesized ECM– the responsible genetic pathways differ among different bacteria and environmental conditions [26]. A few common elements are evident, however. Synthesis and transport of the biofilm ECM is essential, and in many bacteria this is under the control of the bacterial second messenger c-di-GMP [27]. Intracellular levels of c-di-GMP depend on the opposing activities of GGDEF-domain diguanylate cyclase (DGC) enzymes that synthesize c-di-GMP and EAL-domain phosphodiesterase (PDE) enzymes that degrade it. *Y. pestis* has two functional DGC genes, *hmsT* and *y3730*; and a single functional PDE gene, *hmsP* [28,29]. Interestingly, the Y3730 DGC has the major role in biofilm formation in the flea but virtually no role in biofilm formation *in vitro*, which depends almost entirely on HmsT [29]. The two DGC genes are transcribed equally in both environments, suggesting that Y3730 activity is specifically modulated by an environmental signal detected only in the flea. Y3730, but not HmsT, has a molecular architecture common to many signal transduction proteins in which a HAMP signal converter domain separates an extracellular sensor domain and a cytoplasmic output domain. This pattern suggests that a flea-specific environmental signal sensed by Y3730 is transduced via the HAMP signaling domain to activate the GGDEF catalytic domain, resulting in c-di-GMP production and the appropriate physiological response– biofilm formation [29].

The genetic pathways and molecular mechanisms leading from c-di-GMP to ECM synthesis and biofilm formation in *Y. pestis* have yet to be delineated. Like the c-di-GMP metabolizing genes, expression of the *Y. pestis hmsHFRS* operon responsible for the synthesis and transport of the biofilm ECM is not significantly upregulated in the flea or during growth at low temperatures [24]; temperature-dependent ECM production is controlled post-transcriptionally [30]. The phosphoheptose isomerase gene *gmhA*, required for synthesis of the inner core of the bacterial lipopolysaccharide (LPS), is also required for biofilm formation and proventricular blockage in the flea, but is not required for midgut colonization [31] and is not differentially expressed in the flea gut environment [24].

Physiologic adaptations to the flea gut environment

In the flea gut, many *Y. pestis* genes involved in the uptake and catabolism of dipeptides, oligopeptides, and the L-glutamate group of amino acids (Gln, His, Arg, Pro) are significantly upregulated. In contrast, genes for hexose carbohydrate uptake, glycolysis, and fermentation are differentially underexpressed in the flea compared to the other environments. This would seem to match the available nutrients (mammalian blood and its digestion byproducts), which contains comparatively little carbohydrate. Blood also contains substantial lipid, but *Y. pestis* fatty acid uptake or catabolism genes were not upregulated in

the flea. Thus, *Y. pestis* appears to rely on oligopeptides and select amino acids as its major carbon and energy source during persistent infection of the flea gut.

LPS, the major component of the Gram-negative outer membrane, also varies with the temperature shift that accompanies infection of the flea gut. At 37°C, the lipid A moiety of *Y. pestis* LPS is tetra-acylated whereas at < 26°C hexa-acylated lipid A predominates [8,32]. However, a *Y. pestis* mutant able to synthesize only tetra-acylated lipid A was able to infect and block fleas normally [33]. Exposure to the flea antibacterial response may induce expression in *Y. pestis* of genes that protect against both the insect and the mammalian innate immune response. For example, the *Y. pestis* PhoP-PhoQ gene regulatory system, which modifies LPS and other outer membrane components to confer protection against cationic antimicrobial peptides, is upregulated in the flea [24].

Insecticidal-like toxin complex (Tc) genes of *Yersinia*

Most *Yersinia* species encode homologs of the toxin complex (Tc) family of insect toxins of *Photorhabdus* and other bacteria that are associated with insects and other invertebrates. The *Y. pestis* Tc genes are strikingly upregulated during infection of the flea [24,34], but their role, if any, in the *Yersinia*-flea interaction is not obvious. *Y. pestis* Tc mutants show no defect in their ability to infect and block fleas. Although the Tc proteins of several *Yersinia* species have some oral toxicity to *Manduca sexta* larvae, there is no evidence that they are orally toxic to fleas [34-36]. The recently described *Yersinia entomophaga*, the first true insect pathogen in the genus, encodes a more complex array of potent insecticidal Tc toxins [37].

Evolution of flea-borne transmission in *Yersinia*

Comparing the interactions of *Y. pestis* and *Y. pseudotuberculosis* with fleas identifies some of the important adaptive changes that were necessary. To begin with, *Y. pseudotuberculosis*, unlike *Y. pestis*, is orally toxic to fleas– when fleas take a blood meal containing *Y. pseudotuberculosis* they experience acute diarrhea and significant mortality [34]. The responsible enterotoxic factor(s) have not been identified but the Tc insecticidal-like proteins have been ruled out. Toxicity subsides within 24 hours after the infectious blood meal, and most serotypes of *Y. pseudotuberculosis* are able to persist in the flea gut, although their numbers decrease with time unless they are transformed with the *Y. pestis ymt* gene [13,38]. Notably, *Y. pseudotuberculosis* does not form biofilm in the flea or colonize the proventriculus, but exists in the flea gut in a planktonic, single-celled state [38].

Some of the key genetic changes in *Y. pestis* that enabled flea-borne transmission have now been identified, and both gene gain and gene loss were important (Table). *Y. pestis* acquired two new plasmids since it diverged from *Y. pseudotuberculosis*, and each contains a gene important for transmission. One (*ymt*) is highly similar to the *Photorhabdus luminscens ymt* gene, suggesting that it was acquired by horizontal gene transfer from that species recently. A plasminogen activator gene (*pla*) on the smaller *Y. pestis*-specific plasmid is irrelevant to the flea interaction but is highly expressed in the flea and greatly enhances *Y. pestis* dissemination from the flea bite site following transmission [39,40]. Pla is an omptin-family outer surface protease that is catalytically active at 37°C but not at the flea temperature. The combined activities of Pla result in fibrinolysis and damage to the basement membrane and extracellular matrix of host tissues at the site of infection, disrupting normal barrier functions and enabling systemic spread [41].

Although *Y. pseudotuberculosis* forms biofilm in some environments, it does not do so in fleas [38]. The *Y. pestis* orthologs of >200 *Y. pseudotuberculosis* genes are nonfunctional pseudogenes [42,43], and selective loss of gene function has been important in extending

biofilm-forming ability to the flea gut environment. For example, *rcsA*, a negative regulator of biofilms, is a functional gene in *Y. pseudotuberculosis* but not in *Y. pestis*. Replacement of the *Y. pestis* pseudogene with the functional *Y. pseudotuberculosis* version of *rcsA* results in greatly reduced proventricular biofilm and blockage of fleas [44]. Similarly, pseudogene degradation in *Y. pestis* of *nghA*, which in *Y. pseudotuberculosis* encodes a glycosyl hydrolase that degrades the ECM of *Yersinia* biofilms, may have led to enhanced stability of *Y. pestis* biofilms [18]. Two functional PDE genes of *Y. pseudotuberculosis* are pseudogenes in *Y. pestis* [28], and it is possible that this gene loss also contributed to biofilm-forming ability in the flea [29].

Conclusions

Like other environmental *Yersinia*, the *Y. pseudotuberculosis* progenitor of *Y. pestis* would have been subject to ingestion by invertebrates. Based on flea infections, the insect digestive tract does not appear to be a favored niche for *Y. pseudotuberculosis*– it induces diarrhea and does not form biofilm in fleas, both of which lead to rapid elimination from the insect back into the environment. In contrast, the new *Y. pestis* life cycle depended on establishing a stable infection in the flea. This ecological about-face evolved recently and required only a few key genetic changes, including some that served to extend the preexisting biofilm-forming ability to the flea gut. The *Y. pestis* ancestor probably had the advantage of already being constitutively resistant to insect antimicrobial defenses because the genus *Yersinia* appears to be more closely related to insect- and invertebrate-associated genera of the Enterobacteriaceae (*Photorhabdus, Serratia, Sodalis*) than to vertebrate-associated genera (*Escherichia, Salmonella*) [45,46]. Perhaps reflective of an as yet brief coevolutionary history of *Y. pestis*-flea interactions, vector infectivity and transmission efficiency are low compared to other arthropod-borne disease systems [11]. The rather poor vector competence of fleas would have imposed positive selection pressure for *Y. pestis* strains able to produce severe septicemia in the mammal. Thus, the evolution of flea-borne transmission and increased virulence of *Y. pestis* probably went hand-in-hand and were were mutually reinforcing.

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Highlights

- Flea-borne transmission of *Y. pestis* is a recent evolutionary phenomenon
- *Y. pestis* biofilm formation in the flea gut is important for transmission
- *Y. pestis* biofilm requires *hms* genes and enzymes controlling c-di-GMP levels
- *Y. pestis* regulates its gene expression to specifically adapt to the flea vector
- Gene gain and gene loss contributed to the evolution of flea-borne transmission

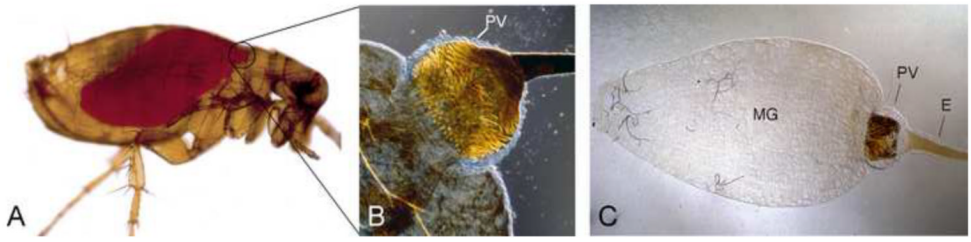

Figure 1.
The flea digestive tract. (A) Uninfected *X. cheopis* flea immediately after taking a blood meal, with the midgut filled with fresh blood. (B, C) Digestive tracts dissected from uninfected fleas showing the anatomy of the proventriculus and the simple midgut.

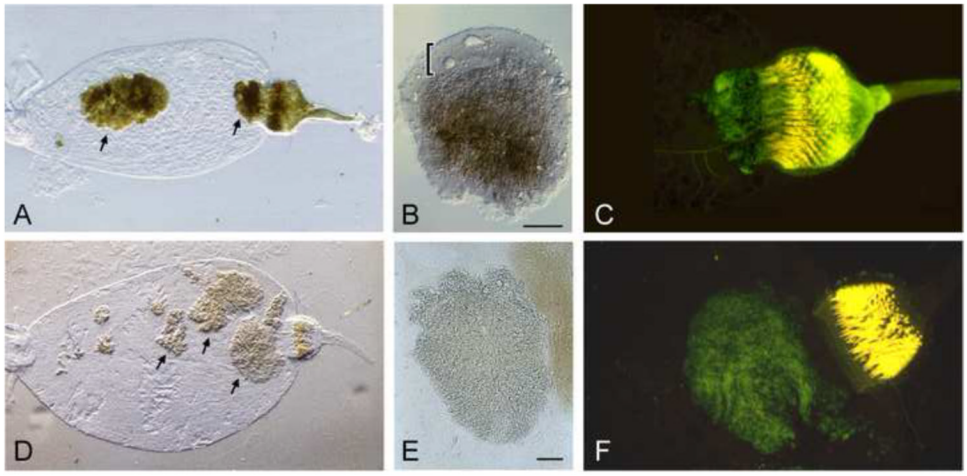

Figure 2.

The *Y. pestis* life stage in the flea. (A) Digestive tract from an *X. cheopis* flea dissected two weeks after infection with wild-type *Y. pestis*. Arrows indicate large aggregates of bacteria, one of which fills the proventriculus. (B) Wild-type *Y. pestis* aggregate dissected from the midgut. The dense mass of bacteria is brown-pigmented due to *hmsHFRS*-dependent sequestration of hemin derived from the flea blood meal and is enveloped by a viscous layer (indicated by the bracket) composed of biofilm ECM and lipid components, also derived from the blood meal [16]. (C) Fluorescence micrograph of the digestive tract from a flea blocked with wild-type *Y. pestis* expressing green fluorescent protein (GFP). The PV is swollen due to the large bacterial mass that fills it. (D) Digestive tract from a flea dissected two weeks after infection with *hmsHFRS*$^{-}$ *Y. pestis*. Multicellular aggregates of bacteria are present in the midgut but the proventriculus is uninfected. (E) Aggregate of *hmsHFRS*$^{-}$ *Y. pestis* dissected from the midgut. The bacterial aggregate is not pigmented and not surrounded by ECM. (F) Fluorescence micrograph of the digestive tract from a flea infected with *hmsHFRS*$^{-}$ *Y. pestis* expressing GFP. The infection is confined to the midgut and does not involve the proventriculus. Dissected digestive tracts in both Fig. 1 and Fig. 2 were mounted in H_20, which clears red blood cell material from previous blood meals but not the bacterial aggregates. MG, midgut; PV, proventriculus; E, esophagus; ECM, extracellular matrix. Scale bars = 0.05 mm.

Table 1

Yersinia genes important for interaction with fleas and for transmission

Role	Gene[a]	Function[b]	Location in the genome	Present in :		Differential expression in the flea[c]
				Y. pestis	*Y. pseudotuberculosis*	
Survival in the flea midgut	***ymt***	phospholipase D	Y. pestis-specific plasmid (pFra)	+	−	yes
Proventricular biofilm formation/regulation	*hmsHFRS*	ECM synthesis/transport	chromosome	+	+	no
	hmsT, y3730	DGC; c-di-GMP synthesis	chromosome	+	+	no
	hmsP	PDE; c-di-GMP degradation	chromosome	+	+	no
	rcsA	transcriptional regulation	chromosome	pseudogene	+	unknown
	nghA	glycosyl hydrolase; ECM degradation	chromosome	pseudogene	+	unknown
Dissemination from the flea bite site	***pla***	plasminogen activator (surface protease)	*Y. pestis*-specific plasmid (pPla)	+	−	no
Other	Toxin complex (Tc) genes	antiphagocytic	chromosome	+	+	yes
	unidentified	flea enterotoxin	unknown	−	+	unknown

[a] Gene differences between *Y. pseudotuberculosis* and *Y. pestis* are indicated in **bold** type.

[b] ECM, extracellular matrix of biofilm ; DGC, diguanylate cyclase ; PDE, phosphodiesterase

[c] See [24] for details