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"Fleaing" the Plague: Adaptations of *Yersinia pestis* to Its Insect Vector That Lead to Transmission*

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Abstract

Interest in arthropod-borne pathogens focuses primarily on how they cause disease in humans. How they produce a transmissible infection in their arthropod host is just as critical to their life cycle, however. *Yersinia pestis* adopts a unique life stage in the digestive tract of its flea vector, characterized by rapid formation of a bacterial biofilm that is enveloped in a complex extracellular polymeric substance. Localization and adherence of the biofilm to the flea foregut is essential for transmission. Here, we review the molecular and genetic mechanisms of these processes and present a comparative evaluation and updated model of two related transmission mechanisms.

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THE INSECT HOST OF *Y. PESTIS*

Yersinia pestis, the bacterial agent of plague, is maintained in many areas of the world by a complex network of transmission cycles involving many different wild rodents and their fleas. Because the principal concern is plague in the mammalian host, the counterterm vector is understandable and conceptually intuitive. It is also rather dismissive. Far from being a simple, neutral transport device that conveys a microbial pathogen from one mammal to another, the arthropod is a true host in which half of an arthropod-borne pathogen's life cycle takes place. Life stages in the arthropod are quite distinct, and it is the transmission stage of *Y. pestis* that is first encountered by the mammalian immune system in an intradermal microenvironment that cannot be duplicated by injecting cultured bacteria.

Y. pestis remains confined to the digestive tract during the entire course of infection in the flea. Few details are known about this environment. Unlike most blood-feeding arthropods, fleas have a simple, undivided midgut (**Figure 1**), and storage, digestion, and absorption of the blood meal take place throughout its entire length and diameter (82). However, the proventriculus, a valve between the midgut and esophagus, is an elaborate structure compared to that of other insects. Its interior surface is arrayed with rows of backward-directed spines (**Figure 1**). Fleas take small (~0.1 to 0.4 μL), frequent blood meals. During feeding, muscles in the flea's head, acting like a peristaltic pump, propel blood down the esophagus. The proventriculus opens and closes rhythmically in coordination with the pump muscles to alternately allow blood to enter the midgut and to prevent backflow. After feeding, the midgut epithelium of blood-feeding insects secretes trypsin and a number of other proteases into the midgut lumen (71). Digestion of the flea blood meal begins immediately and is usually complete within 24 h (62, 97). Dangerous amounts of pro-oxidizing heme are produced from red blood cell digestion (37), and a dark, tarry liquid containing unabsorbed heme is excreted during digestion. The midgut contents of an unfed flea are slightly acidic (B.J. Hinnebusch, unpublished data; 72).

Blood is rich in protein and essential amino acids but limited in carbohydrate and fat and deficient in some vitamins. Many blood-feeding insects harbor bacterial symbionts that provide

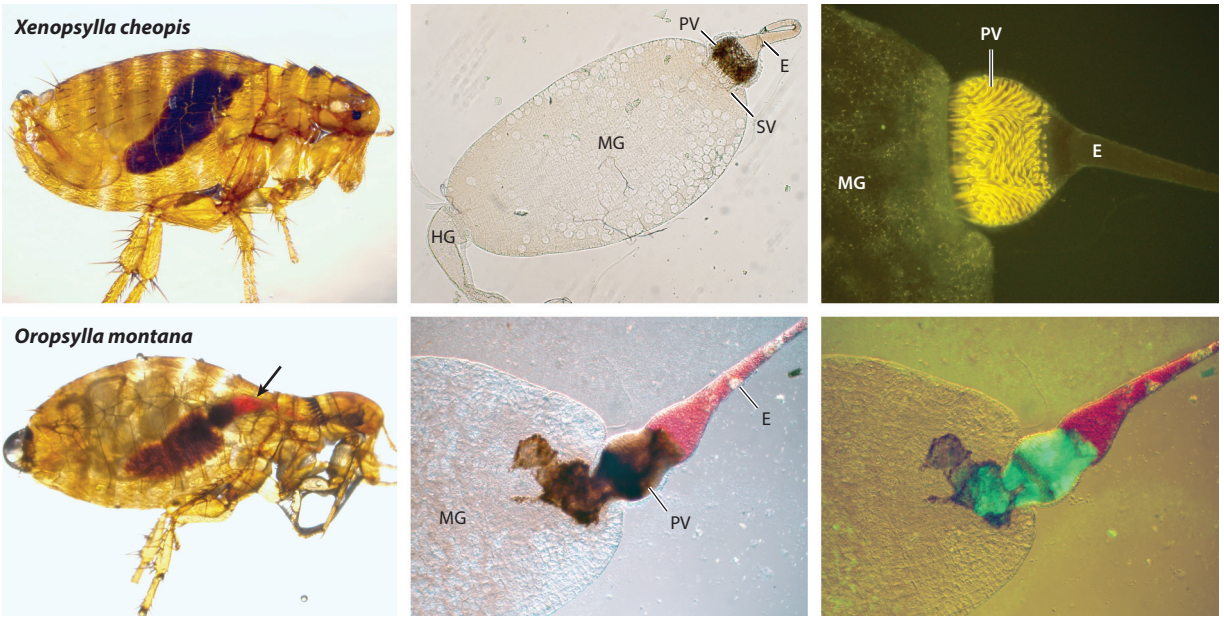

Figure 1

The flea digestive tract habitat of *Y. pestis*. Uninfected intact flea and dissected digestive tract samples of *Xenopsylla cheopis* are shown in the top panels. The bottom images are of a single *Oropsylla montana* infected with *Y. pestis* expressing GFP. The proventriculus of this flea is completely blocked with a *Y. pestis* biofilm that prevented blood from entering the midgut (*arrow*). Dissected midguts were visualized using DIC (*middle*) or DIC and fluorescent microscopy (*right*). The proventricular spines are coated with cuticle, the same material that coats the exoskeleton, and are autofluorescent. Abbreviations: DIC, differential interference contrast; E, esophagus; HG, hindgut; MG, midgut; PV, proventriculus; SV, stomodeal valve. Images of the blocked *O. montana* flea are from Reference 41.

B vitamins or convert protein to carbohydrate (86). The bacterial communities associated with fleas, as is true of those associated with blood-feeding insects in general, are of low diversity and differ among species and individuals across time and geographic location (16, 18, 53, 55, 56). Intracellular bacteria such as *Rickettsia*, *Wolbachia*, and *Bartonella* species are commonly detected. Most surveys of flea-associated bacteria are based on triturated whole-flea samples, so microbes specifically present in the lumen or epithelial layer of the digestive tract have not been well characterized; however, environmental bacteria, probably picked up from the skin of the host, can be at least transient residents (5, 16, 28, 54).

TWO CHALLENGES FACED BY *Y. PESTIS* IN THE FLEA

As for any parasite in any host, *Y. pestis* faces two challenges upon entering the flea in a blood meal: how to overcome host defenses and exploit host resources to reproduce and establish an infection, and how to be transmitted to a new host.

Establish a Foothold

Y. pestis adopts a peculiar life stage in the flea gut to meet the first challenge. Somewhat ironically for the recent descendent of an enteric pathogen, *Y. pestis* does not adhere to flea gut epithelial cells or appear to interact with them in any way. Because fleas process their blood meals rapidly,

Y. pestis is at continual risk of being eliminated by excretion; in fact, a significant percentage of fleas clear themselves of infection in this way (45, 76).

The temporal development of *Y. pestis* infection in the flea digestive tract was first described over 100 years ago (3). In the earliest stage depicted, *Y. pestis* was described as having coalesced into small clusters in the midgut that were darker in color and of firmer consistency than the rest of the midgut contents. In fact, dense bacterial aggregates associated with a rough, amorphous, brown, waxy-looking material form in the flea gut within a few hours after an infectious blood meal (**Figure 2**). The amorphous material does not include the *Y. pestis* extrapolysaccharide synthesized by products of the *hmsHFRS* operon, at least initially, because it is identical in fleas infected with *hmsHFRS* mutants (**Figure 2**). It was later hypothesized that the bacteria are embedded in a fibrin clot matrix. This has been ruled out (43, 51), but the exogenous matrix probably does derive from blood meal components, including lipids (15, 51). The brown color is undoubtedly due to adsorbed heme, a lipophilic molecule. Thus, the life stage of *Y. pestis* in the flea is a large, dense autoaggregate enveloped with an exogenous matrix (51). A stable, persistent infection is assured when these aggregates become too large to be excreted or when they adhere to the proventriculus (**Figure 2**).

Exit Strategy

Arthropod-borne microbes like *Y. pestis* that reside in the alimentary canal of their vectors can be transmitted in three ways: (*a*) residual organisms from a prior infectious feed that contaminate the mouthparts infect the bite site during a subsequent feed; (*b*) organisms excreted in vector feces infect the bite wound or other skin lesion; and (*c*) during a blood meal, organisms proceed forward through the esophagus and enter the bite site. Although the first two ways are possible for flea-borne transmission of *Y. pestis*, it has become apparent that the primary mechanism is the third. This is surprising at first, because it occurs against the direction of blood flow during feeding and *Y. pestis* is nonmotile, unable to swim upstream. *Y. pestis* meets this challenge by interfering with the normal fluid dynamics of blood feeding and the function of the proventricular valve.

TWO MODES OF TRANSMISSION

Early-Phase or Mass Transmission

Two modes of flea-borne transmission have been described for plague. During the first week after infection, fleas have the potential to transmit the very next time they feed. This transmission mode has historically been referred to as mass transmission, emphasizing the fact that disease very rarely results unless a group of fleas simultaneously feeds on a naïve animal (14, 78, 101). Recently, this phenomenon has been referred to as early-phase transmission, emphasizing the fact that peak transmission occurs at the very next feeding event within the first few days after an infectious blood meal (20). Although given a new name, early-phase transmission is not a newly discovered phenomenon—it was actually the first to be described. Between 1904 and 1947, the basic outlines of early-phase/mass transmission were defined: (*a*) Groups of fleas serially transferred on consecutive days to naive animals beginning immediately after they have fed on an animal with terminal plague remain infective for about a week, with the highest transmission rate on the first few days after their infectious blood meal (74, 75, 98). (*b*) Transmission rarely occurs unless five or more fleas are used in simultaneous challenge, and the transmission success rate increases with the number of fleas used per challenge (13, 14, 75, 98). (*c*) Flea species vary in their transmission efficiency during the first week after the infectious blood meal (14, 75, 98). For example, only 3 of 38 early-phase transmission experiments with groups of 20 *Pulex irritans* resulted in infections,

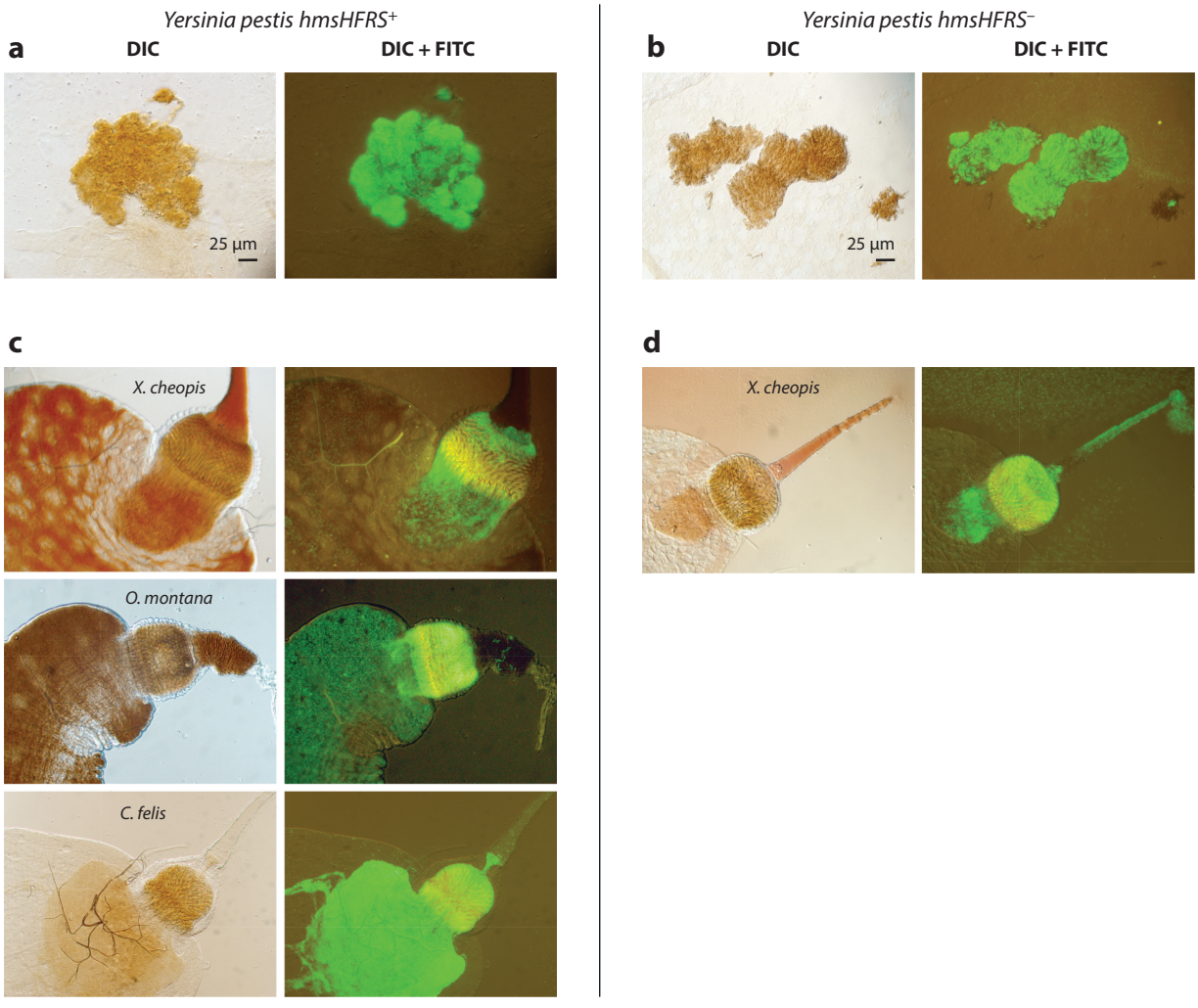

Figure 2

The *Y. pestis* life stage in the flea. *Y. pestis* ingested in a blood meal rapidly forms large aggregates in the flea digestive tract, in association with an amorphous, brown exogenous matrix. Typical examples recovered from the midgut of *Xenopsylla cheopis* fleas infected with (*a*) *hmsHFRS*$^{+}$ or (*b*) *hmsHFRS*$^{-}$ *Y. pestis* expressing GFP. These aggregates localize in the proventriculus within one day after infection. (*c*,*d*) Images of the anterior digestive tract of *X. cheopis*, *Oropsylla montana*, and *Ctenocephalides felis*: Fleas were dissected one day after feeding on blood containing ~5×10^{8} to 1×10^{9} *Y. pestis* KIM6^{+} (pAcGFP1)/mL. The foregut (stomodeal valve, proventriculus, and the esophagus) as well as the midgut are colonized. Abbreviations: DIC, differential interference contrast; FITC, fluorescein isothiocyanate.

compared to about half for *Xenopsylla cheopis* (75). After being neglected for decades, the early-phase/mass transmission mode has been reexamined during the last ten years (7, 11, 20, 21, 23, 24, 26, 52, 87, 99, 102, 103). In addition to reinforcing earlier descriptions in a more systematic way, these studies provided quantitative estimates of the early-phase transmission efficiency of six fleas species (~0–10%, depending on the bacteremia level in the infectious blood meal), as well as important new information that is discussed below.

Late-Stage Biofilm-Dependent Transmission

A second mode of transmission was described in 1914–1915 by the English medical entomologist Arthur W. Bacot (2, 3). This type of transmission is potentiated after *Y. pestis* forms a cohesive bacterial biofilm in the proventricular valve in the flea foregut (51). As the biofilm grows and consolidates, it gradually prevents the proventricular valve from closing completely and can eventually fill and block it, resulting in regurgitative transmission when such partially or completely blocked fleas attempt to feed (**Figures 1**, **3*c***). By sealing off the entrance to the midgut, *Y. pestis* dramatically alters flea feeding behavior and enhances its transmission. A blocked, starving flea will spend the last few days of its life trying to obtain a blood meal, making repeated probing attempts, each one potentially resulting in transmission (**Supplementary Video 1**); often the flea will die with its mouthparts still inserted, suggesting that even posthumous transmission is possible (B.J. Hinnebusch, unpublished data).

Supplemental Material

The blocked flea scenario has attained something of an iconic status, which probably accounts for the consistently oversimplified misinterpretation of the Bacot transmission model found in the literature. It is often stated or assumed that complete blockage is required for efficient transmission, and that the extrinsic incubation period (EIP; the time between the infectious blood meal and transmissibility by the biofilm-dependent mechanism) is the time for complete blockage to develop. In fact, Bacot stressed the importance of transmission by partially blocked fleas and considered them to be more efficient transmitters than completely blocked fleas (2). They also have a shorter EIP and live longer. Fleas that purportedly do not block could still transmit efficiently in this manner.

TRANSMISSION FACTORS: *Y. PESTIS* GENES AND MOLECULAR MECHANISMS INVOLVED IN PRODUCING A TRANSMISSIBLE INFECTION

As they alternate between their vertebrate and invertebrate hosts, arthropod-borne pathogens sense a temperature shift and other host-specific environmental cues that rapidly induce adaptive gene expression and phenotypic changes. Genes that contribute to successful infection of the arthropod vector and subsequent transfer have been termed transmission factors, in apposition to virulence factors (45, 70). **Table 1** contains a list of proven and potential *Y. pestis* transmission factors and their functions.

Figure 3

Flea-borne transmission mechanism models. The initial steps of biofilm development (the formation of bacterial aggregates in the flea gut and association with a matrix derived from the flea blood meal) are required for both mechanisms, which become sequentially operative as the *Y. pestis* biofilm matures. (*a*) Early-phase transmission results when incoming blood encounters an accretion of bacterial aggregates in the proventriculus. The impedance and resulting hydrodynamic turbulence is sometimes sufficient to reflux blood carrying a few bacteria back into the bite site. The partial blockage responsible for early-phase transmission is ephemeral and is cleared from the proventriculus after a few initial pulses of incoming blood, allowing unimpeded entrance to the midgut and normal blood feeding. (*b*,*c*) Late-stage biofilm-dependent transmission occurs after expression of the extracellular polysaccharide encoded by the *hmsHFRS* operon enables persistent colonization of the proventriculus. (*b*) The proventricular valve gradually becomes incompetent as the mature biofilm expands, with the result that there is nothing to restrict the normal peristalsis of the midgut from forcing contaminated digestive tract contents back out through the esophagus and into the bite site (2). These fleas can appear to be partially blocked after feeding, with fresh blood not only in the midgut but also in the esophagus. (*c*) In some fleas, the dense biofilm eventually fills the valve completely and prevents any blood from entering the midgut at all. Such completely blocked fleas are still able to suck blood, and persistent efforts to force it backward into the midgut result in a distension of the base of the esophagus and contact with the bacterial biofilm. Some of the bacteria are mixed into the currents of inflowing blood, and when pumping pressure is released, retrograde pressure from the swollen esophagus can result in regurgitation (3). Sometimes feeding pressure can force a channel through the proventricular biofilm, releasing complete blockage but still leaving an incompetent valve and a return to a partially blocked state.

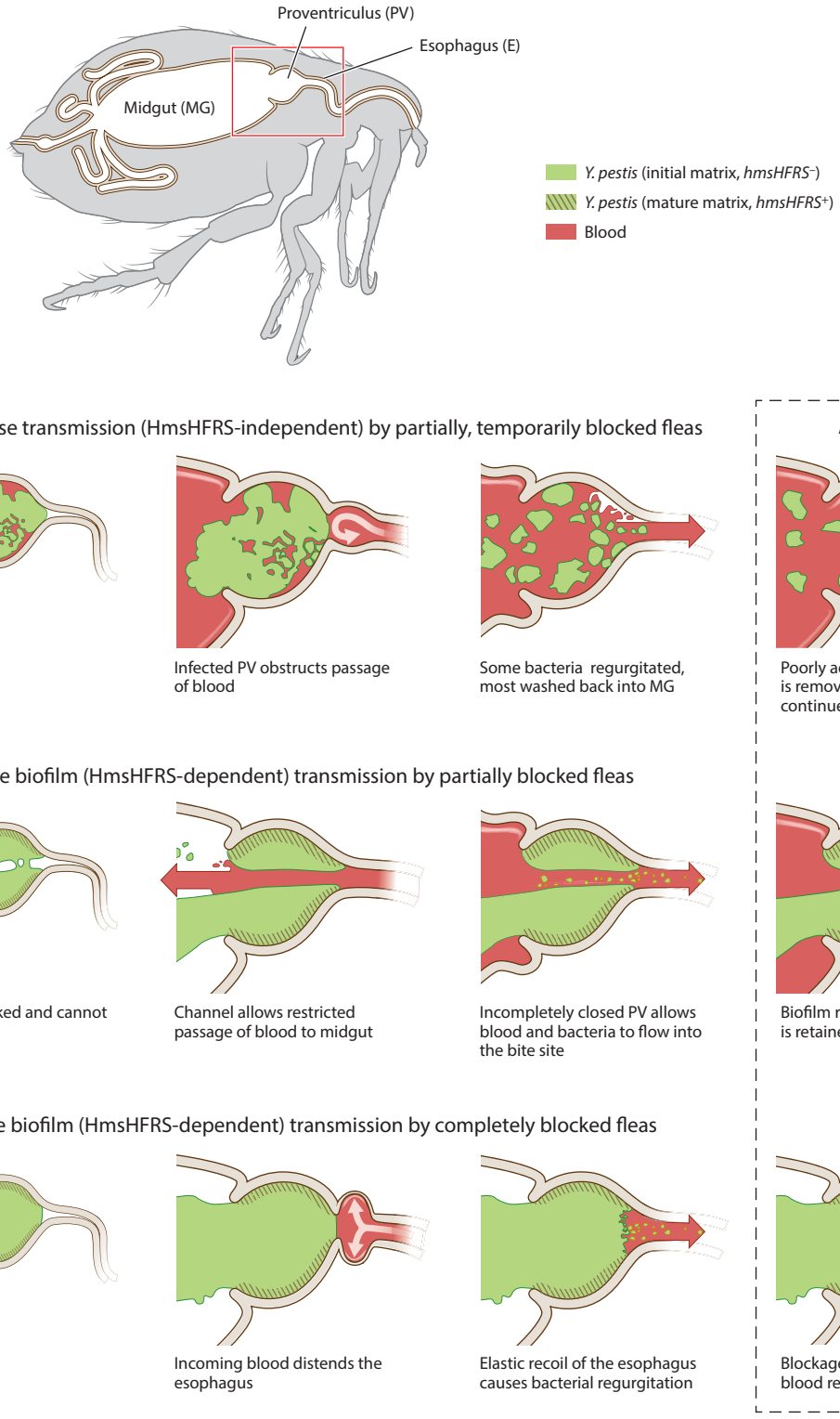

Table 1 Transmission factors: *Y. pestis* genes implicated in the ability to infect and be transmitted by fleas

Gene	Gene product	Relevant function or phenotype	Reference
Survival and growth in flea gut			
ymt	Phospholipase D	Protection against toxic by-product of blood digestion	46
rovM	Transcriptional regulator	Metabolic adaptation to the flea gut	95
oxyR	Transcriptional regulator	Protection against reactive oxygen species	19, 30, 108
galU, *arnB*	Lipid A modification enzymes	Protection against antimicrobial peptides	1
Biofilm development: initial aggregation and adherence			
ail[a]	Outer surface protein	Virulence factor	4, 44, 60, 61
hmsD + *hmsT*[a]	Diguanylate cyclases	c-di-GMP synthesis	91
pgmA[a]	Phosphoglucomutase	Synthesis of UDP-sugars; LPS and other outer surface modifications	34
hcp[a]	Pilus-like structure	Type VI secretion system component	77
Biofilm maturation			
hmsHFRS	Glycolsyl transferase, polysaccharide deacetylase, transport porin	Synthesis of extracellular β-1,6-GlcNAc polysaccharide biofilm matrix component	8, 45, 51
hmsCDE	Diguanylate cyclase and its regulators	c-di-GMP synthesis	9, 10, 84, 85, 91
hmsT	Diguanylate cyclase	c-di-GMP synthesis	9, 91
hmsP	Phosphodiesterase	c-di-GMP degradation	59
hmsA[a]	sRNA	Upregulates *hmsCDE*, *hmsT*, *hmsHFRS*; increases intracellular c-di-GMP	66
hmsB[a]	sRNA	Upregulates *hmsCDE*, *hmsT*, *hmsHFRS*; represses *hmsP*; increases intracellular c-di-GMP	32
rcsAB	Transcriptional regulator	Represses *hmsT*, *hmsCDE*, *hmsHFRS*; upregulates *hmsP*	33, 88
hfq	sRNA	Regulates *hmsT* and *hmsP*	6, 83
phoP	Transcriptional regulator	Enhances stability of biofilm; mechanism unknown	81, 90
rovM[a]	Transcriptional regulator	Upregulates *hmsCDE*, *hmsT*; increases intracellular c-di-GMP and biofilm production	65
crp	cAMP receptor protein	Carbon catabolite regulation required for biofilm production; mechanism unknown	64, 105; Hinnebusch, unpubl. data
csrA[a]	Carbon storage regulator protein	Carbon catabolite regulation; enhances biofilm production; mechanism unknown	105
yfbA	Transcriptional regulator	Enhances biofilm production; mechanism unknown	92
gmhA	Phosphoheptose isomerase	Heptose synthesis; LPS truncation	17
yrbH[a]	Arabinose 5-phosphate isomerase	Kdo synthesis; LPS modification	93

[a]Phenotypic effect demonstrated in vitro; not yet evaluated in the flea.

Survival of *Y. pestis* in the intensive digestive milieu of the flea midgut depends on the activity of a phospholipase D, Ymt, that protects enteric bacteria against a bacteriolytic by-product of blood digestion (46, 89). In keeping with their protein-rich diet, many *Y. pestis* genes involved in the uptake and metabolism of oligopeptides and amino acids, particularly the L-glutamate group (Gln, His, Arg, Pro) are upregulated, implicating amino acids as the primary carbon and energy source in the flea (47, 96). The gene for the *Y. pestis* transcriptional regulator RovM, which is induced in minimal media in *Yersinia pseudotuberculosis* (39), is highly induced in the flea gut (96). Further evidence that the flea gut is a nutrient-restricted environment is that the cAMP receptor protein (CRP), a global regulator of metabolic adaptation to alternate carbon sources when glucose is scarce, is required for normal growth and biofilm formation in the flea (B.J. Hinnebusch, unpublished data). CRP and the carbon storage regulator protein CsrA are also required for in vitro biofilm formation by *Y. pestis* (64, 105). Bacteria tend to form biofilms in low-nutrient conditions (73), and this may be one environmental stimulus for *Y. pestis* biofilm development in the flea.

Given the central importance of the biofilm life stage in the flea, much research has focused on identifying the *Y. pestis* genes required for this phenotype (**Table 1**). The hallmark of a mature biofilm is the matrix or extracellular polymeric substance (EPS) in which the bacteria are embedded, which forms a cohesive scaffold and is essential for stable adherence to a surface (35). A major component of the *Y. pestis* EPS is poly-β-1,6-*N*-acetyl-D-glucosamine, which is synthesized and exported by the products of the *hmsHFRS* operon (8, 29). The EPS of many bacterial biofilms also contains matrix-associated proteins and extracellular DNA (35, 36, 48). These components have not been identified yet in *Y. pestis* biofilms, but as described above, the biofilm matrix in the flea appears to incorporate components derived from the blood meal. The PhoP-PhoQ gene regulatory system of *Y. pestis*, which acts to modify the bacterial outer membrane in response to certain environmental stresses, is induced in the flea and is required for a fully cohesive EPS (81, 90, 96).

Biofilm development is regulated by the second messenger c-di-GMP (38). Intracellular c-di-GMP levels are determined by the opposing activities of diguanylate cyclase enzymes, which synthesize c-di-GMP, and phosphodiesterase enzymes, which degrade it. *Y. pestis* encodes two diguanylate cyclases, *hmsT*, of primary importance in vitro, and *hmsD*, of primary importance in the flea; and one phosphodiesterase, *hmsP* (9, 91). Several regulators that cumulatively control the relative expression of these enzymes, and therefore c-di-GMP flux, have been identified (**Table 1**). Another nucleotide second messenger, cAMP, is also important in regulation of biofilm development, via CRP and the global carbon catabolite repression system (B.J. Hinnebusch, unpublished data; 64, 105). Involvement of both the cAMP-CRP and the c-di-GMP global regulatory networks is evidence of a complex systems biology behind *Y. pestis* biofilm development.

Little is known about the molecular mechanisms important for the first step of *Y. pestis* biofilm development, the initial attachment of bacteria to a surface or to each other. In other bacteria, cell surface proteins, fimbriae, and autotransporter adhesins participate in this initial attachment (36, 48, 73, 94). A few factors that affect autoaggregation in vitro have been identified (**Table 1**); however, their role in the flea has not been determined.

A UNIFIED THEORY OF FLEA-BORNE TRANSMISSION VIA PARTIALLY OR COMPLETELY BLOCKED FLEAS

Early-phase/mass transmission was long assumed to be mechanical, via contaminated mouthparts (14, 78, 79). However, recent work indicates that the mechanism is not that simple. Early-phase transmission efficiency varies among flea species, even after feeding on blood with the same level of bacteremia (21, 23, 75, 98). In vitro mass transmission trials also show this, and infected fleas

did not transmit *Y. pseudotuberculosis* by the early-phase mechanism, even after feeding on highly bacteremic blood (7, 89). These results are not consistent with mechanical transmission, as fleas feeding on blood with a standardized level of bacteremia would be expected to contaminate their mouthparts equivalently. Egestion of bacteria from the digestive tract and transmission via saliva flowing through contaminated mouthparts have also been suggested as possible early-phase mechanisms (20, 40, 58, 80).

Our recent observations suggest a possible mechanism and anatomical source of the bacteria transmitted early. Colonization of the proventriculus has been reported to occur more rapidly and frequently in *X. cheopis* than in other fleas (14, 31). However, this difference is not apparent after feeding on blood with the very high bacteremia levels required to support early-phase transmission (**Figure 2**). In all three species examined, 67% to 100% of fleas dissected 1 to 3 days after infection contained bacterial aggregates in the proventriculus as well as in the midgut, and many showed moderate to heavy proventricular colonization, resembling the biofilm-dependent blockage phenotype (**Figures 1**, **2**).

Based on these results, we propose a unified theory of flea-borne transmission, in which transmission by both modes occurs by regurgitation from fleas in which the proventriculus is obstructed by masses of *Y. pestis* at different points of biofilm maturation (**Figure 3**). Biofilm development involves four stages: (*a*) initial attachment of individual cells to each other or to a surface, (*b*) formation of microcolonies, (*c*) maturation, and (*d*) detachment and dispersal (94). The rapid formation of autoaggregates of *Y. pestis* surrounded by the amorphous brown exogenous matrix (**Figure 2**) constitutes the initial stage of biofilm development in the flea, similar to the initial stages of in vivo biofilm formation described for other bacteria, which often begins with bacterial aggregation in association with a host-derived matrix or conditioning film (69). These aggregates likely concentrate in the proventriculus by means of the peristaltic waves of the midgut epithelium and rhythmic contractions of the proventriculus, which continue for several hours after feeding (49) (**Supplementary Video 2**). These contractions draw midgut contents into the valve, where blood cell clumps are disrupted by the milling action of the spines, and then forcibly eject the proventricular contents back into the midgut. In infected fleas, however, the bacterial clumps remain lodged in the proventriculus. This may be due to hydrophobic interactions between the waxy exogenous matrix of the bacterial aggregates and the cuticle that covers the proventricular spines. Notably, the initial autoaggregation stage of biofilm formation and early colonization of the proventriculus do not depend on the *hmsHFRS* genes (**Figure 2**). The initial colonization of the proventriculus is ephemeral, however. Fleas examined immediately after a maintenance blood meal 3 days after infection with *hmsHFRS*$^{-}$ *Y. pestis* have a clean proventriculus, the blood meal having effectively washed the bacterial aggregates back into the midgut. Permanent adherence to the proventriculus requires further maturation of the biofilm, particularly the expression of the *hmsHFRS*-encoded extracellular polysaccharide (45).

The proposed model is consistent with known characteristics of early-phase transmission: (*a*) Proventricular colonization occurs within one day after infection, coinciding with the EIP of early-phase transmission (22) (**Figure 2**), (*b*) the bacterial aggregation phenotype and initial colonization of the proventriculus do not require the *hmsHFRS* genes (99) (**Figure 2**), and (*c*) a *Y. pestis ymt* mutant, which is deficient in midgut colonization but able to infect the proventriculus, is transmissible during the early phase (46, 52).

DETERMINANTS OF VECTOR EFFICIENCY OF DIFFERENT FLEA SPECIES

The recent evolutionary adaptation of *Y. pestis* to flea-borne transmission involved extending biofilm-forming ability to the flea digestive tract (42, 89). This simple strategy is generally effective

in many different flea species, but transmission efficiency by one or both mechanisms can vary considerably among them (reviewed in 22). Based on our model (**Figure 3**), there are three major determinants of vector efficiency: the infection rate, the incidence and kinetics of at least partial blockage of the proventriculus in infected fleas, and the probability that a sufficient number of *Y. pestis* are regurgitated from a partially or fully blocked proventriculus to result in disease.

The infection rate is sensitive to the bacteremia level of the blood meal, the blood source, the temperature at which the fleas are maintained, and the normal feeding and excretion rate of a particular flea species (7, 25, 43). Fleas such as *P. irritans*, the cat flea *Ctenocephalides felis*, the gerbil flea *Xenopsylla skrjabini*, and the mouse fleas *Leptopsylla segnis* and *Aetheca wagneri*, which exhibit daily, sustained feeding behaviors, digest their blood meals rapidly, and defecate large amounts of blood during and shortly after feeding, have been found to clear themselves of infection at a high rate and to be poor vectors (7, 22, 23, 62, 106). Notably, however, forcing *C. felis* to adopt an intermittent, abbreviated feeding schedule greatly increases its infection rate and transmission potential (7). In general, important plague vector species feed intermittently and rapidly. Even among fleas with this type of feeding behavior, however, ID_{50} and infection rate can be variable, even when the same blood source is used (27, 31, 41, 106).

The EIP for later-phase transmission also correlates with the level of bacteremia in the infectious blood meal. *Oropsylla montana* and *X. skrjabini* fleas infected at high dose appear to be partially or even fully blocked by four days after feeding on highly bacteremic blood, suggesting that there could be temporal overlap between early-phase and later transmission (41, 106). Again, the EIP is not the time for complete blockage to develop, but the time for sufficient biofilm to interfere with blood flow and competence of the valve.

Whether regurgitative transmission occurs, and how many bacteria are regurgitated, likely depends on hydrodynamic forces determined by the strength of contractions of the pump muscles and the muscle layers surrounding the proventriculus; and the volume, diameter, and length of the proventriculus and the esophagus. These factors vary among flea species and may contribute to differences in transmission efficiency (**Figure 4**). For example, in *C. felis*, an inefficient vector by both transmission mechanisms, the proventricular muscle layer extends to the base of the esophagus and may act as a sphincter that restricts regurgitation of the proventricular contents (7). In *O. montana*, a highly efficient vector by the biofilm-dependent mechanism, the base of the esophagus is very wide, providing a large surface area of contact between incoming blood and the proventricular biofilm (41).

RELATIVE IMPORTANCE OF THE TWO MODES OF TRANSMISSION

Early-phase transmission has been hypothesized to account for the rapid spread of epizootics that afflict ground squirrels and prairie dogs, based in part on mathematical models that indicate that their fleas transmit too inefficiently by the late-stage biofilm mechanism (12, 20, 100, 102). However, results of studies comparing the efficiency at which different species transmit beyond the early phase are difficult to gauge and have sometimes been misleading, because of the variety of experimental conditions employed and the absence of important controls (22, 41). Most work has been done with the model species *X. cheopis*, the peridomestic rat flea that is an efficient vector. The comparative vector efficiencies of wild rodent fleas are in need of systematic reexamination, and standardized methods have been developed that have the potential to provide more reliable estimates (7, 22, 41).

Two aspects of early-phase transmission would appear to limit its ecological significance. First, transmission by this mode is rare unless the infectious blood meal contains at least 10^8 *Y. pestis*/mL, and reported early-phase transmission efficiency values are based on blood bacteremia levels of

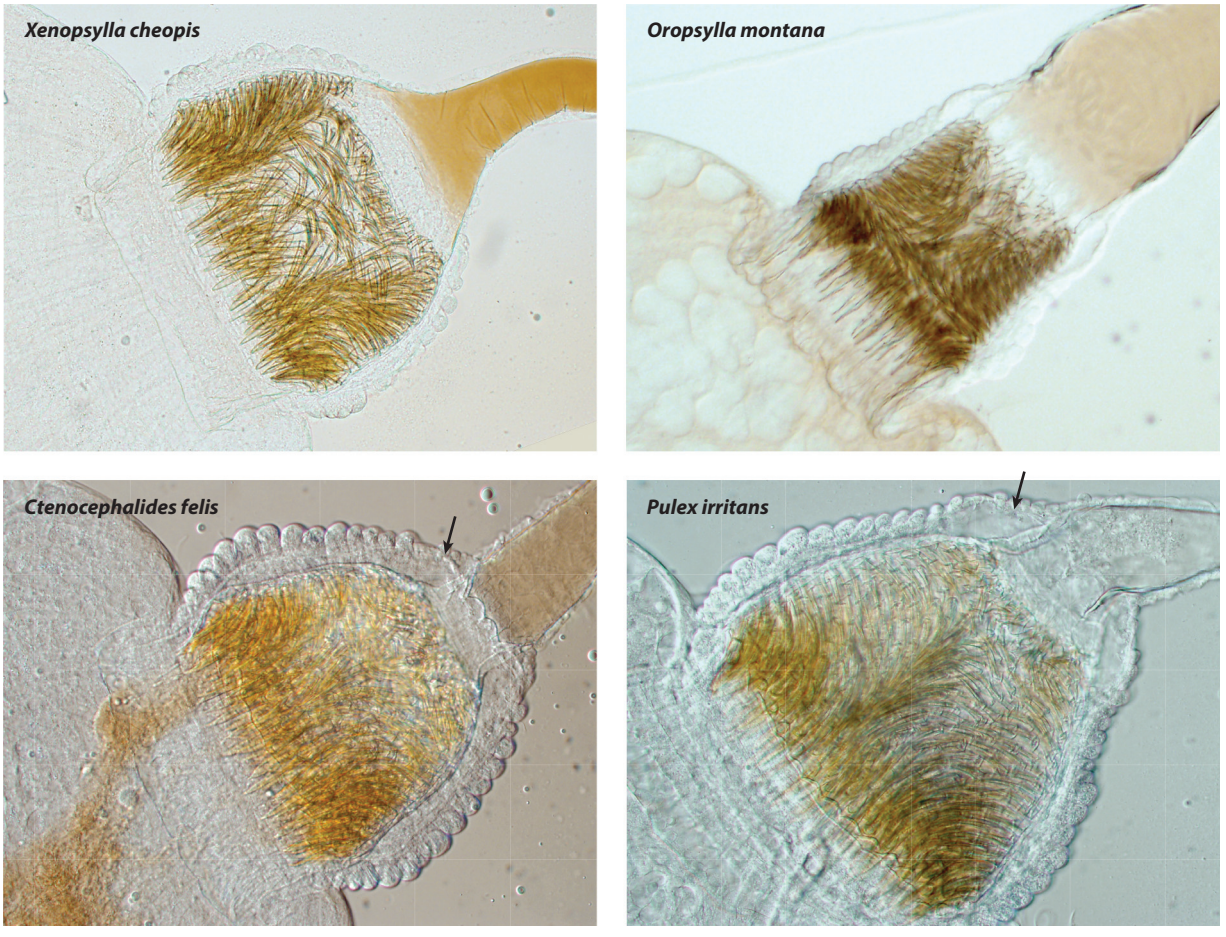

Figure 4

Foregut anatomical differences that correlate with transmission efficiency. The proventriculus of two efficient vectors (*Xenopsylla cheopis* and *Oropsylla montana*) and two inefficient vectors (*Ctenocephalides felis* and *Pulex irritans*) are shown. Note the wide, unrestricted proventricular-esophageal junction of *O. montana* and the thick layers of proventricular muscle (*arrows*) that extend and constrict the base of the esophagus of *C. felis* and *P. irritans*.

~10^9 *Y. pestis*/mL or higher (11). With the notable exception of mice, terminal bacteremias may not routinely reach 10^9/mL in most mammals, or may occur only shortly before death. Thus, if it occurs at all, there is a very brief interval between the early-phase threshold bacteremia level and death. In contrast, a bacteremia level of only 10^7 *Y. pestis*/mL results in infection of 30–50% of fleas, which have the potential to develop partial or complete proventricular blockage (27, 67).

A second delimiting aspect of early-phase transmission is that the average number of *Y. pestis* cells transmitted per flea by this mechanism is very low (41). Published early-phase transmission efficiency estimates include instances that led to seroconversion as well as to overt disease in highly susceptible laboratory mice challenged by groups of about ten fleas. The percentage of transmissions leading to seroconversion is as high as 30% to 50% in mice that received 3 to 12 infected flea bites (B.J. Hinnebusch, unpublished data; 52). These results indicate that the cumulative number

of *Y. pestis* transmitted by fleas in early-phase challenges is not much greater than the LD_{50} of laboratory mice (<10 CFU) (72). The LD_{50} of *Y. pestis* for most wild rodents, including ground squirrels, is typically tenfold higher or more (50, 68, 104, 107). Thus, high flea density is also a likely ecological precondition for early-phase transmission, as intermittent challenges from just a few fleas at a time would frequently in effect vaccinate animals and remove them from the susceptible population, rather than cause the septicemic plague necessary to complete the transmission cycle and drive epizootic spread. The average number of *Y. pestis* transmitted by *X. cheopis* and *O. montana* via the biofilm-dependent mechanism is much higher than during the early phase (41). A single blocked *X. cheopis* flea transmits a few to a few thousand *Y. pestis* per feeding attempt, but because a blocked flea will make repeated attempts to feed before it starves, the cumulative number transmitted can be much greater (14, 57, 67).

These considerations suggest that the late-stage biofilm mode of transmission provides the foundation for ecologically stable plague transmission cycles. The fact that all *Y. pestis* isolates retain the ability to produce mature biofilm in the flea, even though the *hmsHFRS* genes are not required for pathogenesis in the mammal, also indicates that biofilm-dependent transmission is essential (45, 63). Early-phase transmission is predicted to play a significant role in plague cycles involving flea species that purportedly are relatively resistant to chronic proventricular biofilm infection. Further refinement and use of new methods to quantify and compare the efficiency with which important flea vectors transmit at different times following a single, standardized infectious blood meal should lead to a better understanding of the relative importance of the two transmission modes in different ecological contexts (22, 40, 41).

FUTURE ISSUES

1. What are the genetic and molecular mechanisms and effectors of *Y. pestis* that transduce c-di-GMP signal and lead to biofilm development?
2. What is the mechanism for the initial autoaggregation step of biofilm formation in the flea? Are specific bacterial surface adhesins involved?
3. What is the composition of the extracellular polymeric substance associated with the *Y. pestis* biofilm life stage in the flea gut, and how does it affect the mammalian immune response after transmission?
4. What is the physiologic state of *Y. pestis* during long-term infection of the flea gut? Does a dormant or persister cell state develop?
5. What is the flea response to oral infection, and how does *Y. pestis* adapt to it?
6. How does the extent of proventricular colonization correlate with the transmission efficiency of unblocked or partially blocked fleas?
7. What are the physical and biological determinants (both bacterial and insect) of variances in vector competence among different flea species? How do different mammalian host blood types affect flea infection and transmission rates?

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