Transmission of *Yersinia pestis* from an Infectious Biofilm in the Flea Vector


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Transmission of plague by fleas depends on infection of the proventricular valve in the insect’s foregut by a dense aggregate of *Yersinia pestis*. Proventricular infection requires the *Y. pestis* hemin storage (*hms*) genes; here, we show that the *hms* genes are also required to produce an extracellular matrix and a biofilm in vitro, supporting the hypothesis that a transmissible infection in the flea depends on the development of a biofilm on the hydrophobic, acellular surface of spines that line the interior of the proventriculus. The development of biofilm and proventricular infection did not depend on the 3 *Y. pestis* quorum-sensing systems. The extracellular matrix enveloping the *Y. pestis* biofilm in the flea appeared to incorporate components from the flea’s blood meal, and bacteria released from the biofilm were more resistant to human polymorphonuclear leukocytes than were in vitro–grown *Y. pestis*. Enabling arthropod-borne transmission represents a novel function of a bacterial biofilm.

*Yersinia pestis*, the cause of bubonic and pneumonic plague in humans, persists in populations of wild rodents in many parts of the world and is transmitted primarily by the bites of infected fleas [1]. *Y. pestis* undergoes a characteristic development in the flea that leads to transmission [2, 3]. During the first week after being taken up by a flea in a blood meal, the bacteria multiply in the lumen of the flea gut to form dense aggregates. In some fleas, the infection also involves the proventriculus, a valve that connects the midgut to the esophagus. The interior wall of the proventriculus is lined with rows of spines covered with cuticle, the same hydrophobic, acellular material that composes the insect exoskeleton. A *Y. pestis* aggregate can adhere to these spines, continue to grow and consolidate, and eventually fill the proventriculus, impeding the passage of blood into the midgut. Physical blockage and interference with the action of the proventricular valve are required for efficient transmission [4–6], which occurs when a complete or partially blocked flea attempts to feed.

Two genetic factors of *Y. pestis* have been shown to be specifically required for fleaborne transmission. Initial survival and growth after the infectious blood meal requires a plasmid-encoded phospholipase D that protects the bacteria from lysis in the flea midgut [7]. A transmissible infection, however, further depends on the *Y. pestis* hemin storage (*hms*) gene products, which are required for colonization and blockage of the proventriculus. *Y. pestis* hms mutants colonize the flea midgut but are unable to infect the proventriculus [8]. The *hms* genes constitute 2 unlinked operons on the *Y. pestis* chromosome: *hmsHFRS* and *hmsT* [9–12]. HmsR and F have amino acid sequence similarity to the glycosyl transferase and acetylase enzymes of *Staphylococcus epidermidis*, which participate in the synthesis of an extracellular polysaccharide that is required to produce a biofilm [13–16]. The *hms* genes were so named because they are responsible for a pigmentation pheno-
Table 1. Susceptibility pattern of the extracellular matrix surrounding Hms-positive Yersinia pestis in the flea.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Resulta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent</td>
<td></td>
</tr>
<tr>
<td>0.1% SDS</td>
<td>–</td>
</tr>
<tr>
<td>Acetone</td>
<td>–</td>
</tr>
<tr>
<td>Chloroform plus methanol</td>
<td>+</td>
</tr>
<tr>
<td>Chloroform</td>
<td>–</td>
</tr>
<tr>
<td>Methanol</td>
<td>+ (slight)</td>
</tr>
<tr>
<td>3% H₂O₂</td>
<td>– (decolorizes)</td>
</tr>
<tr>
<td>0.01 N NaOH</td>
<td>– (decolorizes)</td>
</tr>
<tr>
<td>Enzyme</td>
<td></td>
</tr>
<tr>
<td>Proteinase K, E</td>
<td>–</td>
</tr>
<tr>
<td>Plasmin</td>
<td>–</td>
</tr>
<tr>
<td>Lipase</td>
<td>+ (slight)</td>
</tr>
<tr>
<td>Phospholipase C</td>
<td>–</td>
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</tbody>
</table>

*a*, +, Degraded matrix; –, no effect on matrix.

Figure 1. Development of temperature- and Hms-dependent biofilm in Yersinia pestis. A–C: Interior surface of glass flowcell inoculated with Hms-positive Y. pestis KIM6+ after 0 (A) and 48 (B) h of media flow at 21°C and after 48 h of media flow at 37°C (C). D–F: Flowcells after 48 h of media flow at 21°C inoculated with Hms-negative Y. pestis KIM6 (D), Hms-positive Y. pestis KIM6(pHMS1) (E), and Hms-positive, quorum-sensing–negative Y. pestis KIM6+yepI::kan yepI::kan ΔluxS (F). Bar, 20 μm.

Type based on the ability of Y. pestis colonies to bind hemin or the structurally analogous Congo red dye [17, 18].

Previous investigations have indicated that the dense aggregates of Y. pestis that develop in the flea midgut and proventriculus are surrounded by an extracellular matrix, resembling a biofilm [3, 19]. Y. pestis and Yersinia pseudotuberculosis also produce biofilm-like growth on the external mouthparts of Caenorhabditis elegans nematodes, and this phenotype also is dependent on the hms genes [16, 20]. Many bacteria adhere to and accumulate on a surface by forming a biofilm, a physiologically distinct community of cells embedded in an extracellular matrix of bacterial origin [21]. The formation of biofilm has been implicated in many persistent and chronic bacterial infections, including dental plaque and periodontitis, infections of implanted medical devices, and Pseudomonas aeruginosa infection of the lungs of patients with cystic fibrosis [22]. In the present study, we link Hms-dependent proventriculus infection to formation of biofilm and examine the phenotype of the bacteria that are released from the biofilm and transmitted when a blocked flea attempts to feed.

MATERIALS AND METHODS

Bacteria. The Y. pestis strains KIM6+, KIM6 (the Hms-negative KIM6+ derivative that lacks the chromosomal locus containing hmsHFRS), and KIM6 complemented with pHMS1, which contains a wild-type (wt) copy of the hmsHFRS locus, were used in the present study [23, 24]. A Y. pestis KIM6+ quorum-sensing (QS)–defective mutant was constructed by use of sequential allelic exchanges using the suicide vector pCVD442 [25] to replace the wt N-acyl-homoserine lactone (AHL)–type autoinducer synthase genes yepI (YPO0984; identical to ytbI of Y. pseudotuberculosis; GenBank accession number AF079136) and ypeI (YPO2456; identical to ypsI of Y. pseudotuberculosis; GenBank accession number AF071401) [26] with insertion mutant alleles. The suicide vectors used for mutagenesis contained the ypeI or ypeI gene, into which a kanamycin (kan) resistance marker was inserted. Integration of the suicide vector into the wt gene on the chromosome was determined by screening for pCVD442-encoded resistance to ampicillin. After growth of the merodiploids in nonselective conditions, sucrrose-resistant, ampicillin-sensitive clones [25] were tested by use of polymerase chain...
reaction (PCR) and nucleotide sequencing, to verify the desired mutation. Loss of function of the *Y. pestis* AHL autoinducers was also verified in a bioassay of culture supernatant using a *Chromobacterium violaceum* reporter strain [27]. A third allelic exchange using pCVD44 generated a triple-QS mutant (*Y. pestis* KIM6+ yepL:kan ypel:kan ΔluxS), in which the luxS homolog (YPO3300) was replaced with a mutant luxS allele with a 411-bp in-frame deletion that truncated the predicted protein after aa 8. The luxS deletion in this strain was confirmed by use of PCR, sequence analysis, and Northern blot. The KIM6, KIM6+, and triple-QS mutant strains were transformed with pGFP (Clontech) by use of electroporation.

**Infection of fleas.** *Xenopsylla cheopis* fleas were infected by allowing them to feed on fresh heparinized mouse blood containing $5 \times 10^8$ *Y. pestis* organisms/mL, using an artificial feeding system [3, 8]. Fleas (50 males and 50 females) that took an infectious blood meal were kept at 21°C, fed twice weekly thereafter on normal mice, and monitored for 4 weeks for infection and proventricular blockage rates, as described elsewhere [8].

**Enzyme, solvent, and histological stain treatments of *Y. pestis* biofilms.** Bacterial aggregates were dissected from fleas’ digestive tracts 1–3 weeks after infection, washed with PBS, and placed on microscope slides. Slides were submerged in each of the solvents listed in table 1 for 12 h at 21°C. Additional slides

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**Figure 2.** Scanning electron microscopy of Hms-positive (A) and Hms-negative (B) *Yersinia pestis* grown on agar plates at 21°C. Bar, 0.5 μm.

**Figure 3.** Biofilm-like growth of Hms-positive *Yersinia pestis* in the proventriculus of blocked fleas. A. Digestive tract dissected from an uninfected *Xenopsylla cheopis* flea, indicating the location of the proventricular valve (PV), which connects the esophagus (E) to the midgut (MG). Bar, 0.25 mm. Scanning electron microscopy of the spines lining the interior of a proventriculus from an uninfected flea (B), a flea infected with Hms-negative *Y. pestis* (C), and a flea blocked with Hms-positive *Y. pestis*, showing bacteria embedded within an extracellular matrix (D). Asterisks indicate proventricular spines. Bar, 5 μm.
Figure 4. Comparison of flea infections produced by Hms-positive and Hms-negative Yersinia pestis. Digestive tracts dissected from a flea infected and blocked with Hms-positive Y. pestis KIM6+ (A) and a flea infected with Hms-negative Y. pestis KIM6 (B). Bar, 0.25 mm. Higher-magnification views of bacterial masses from the midgut of fleas infected with Hms-positive Y. pestis KIM6+(pGFP) (C and E) or Hms-negative Y. pestis KIM6(pGFP) (D and F) examined by brightfield and fluorescence microscopy. Arrows, border of the viscous extracellular matrix surrounding the Hms-positive Y. pestis aggregate, which was not seen for the Hms-negative aggregate. Bar, 0.05 mm.

were treated with proteinase K (200 µg/mL), proteinase E (250 µg/mL), fibrinolysin (plasmin, 2.5 U/mL), phospholipase C (6.25 U/mL), and lipase (25,000 U/mL), for 12–24 h at 37°C in buffers recommended by the supplier (Sigma). The ability of these agents to dissolve the brown extracellular matrix was evaluated by use of microscopy before and after treatment. Dense suspensions of Y. pestis from 48-h, 21°C cultures on heart-infusion agar (Difco) containing 0.2% galactose or liquid TMH media [28] containing 1% (wt/vol) N-acetylglucosamine were also prepared. Slides were stained with periodic acid–Schiff (PAS), calcofluor white, ruthenium red, Sudan black, and OsO4 reagents, according to established protocols [29, 30].

**Flowcell experiments.** Y. pestis that had been incubated for 48 h at 21°C in N-minimal medium [31] containing 0.1% casamino acid, 38 mmol/L glycerol, and 8 µmol/L MgCl2, were quantitated in a Petroff-Hausser counting chamber and diluted to $1 \times 10^7$ cells/mL with fresh medium; 1.5 mL of the bacterial suspension was injected into a 12-cm-long section of 0.5-mm-thick, 3.5-mm² glass tubing that was connected to a reservoir of sterile medium via a peristaltic pump at the influent end and to a discard reservoir at the effluent end. After a 20-min period to allow bacteria to attach to the glass surface (designated $t = 0$), sterile medium was pumped through the flowcell at 1 mL/min. The interior surface of the flow cell was photographed 48 h later using differential interference contrast microscopy.

**Scanning electron microscopy.** Samples were prepared as described elsewhere [32], with the following modifications: 0.1% poly-l-lysine coated coverslips were applied to bacterial
Table 2. Differential staining properties of Hms-positive (Hms+) and Hms-negative (Hms−) Yersinia pestis.

<table>
<thead>
<tr>
<th>Source</th>
<th>Y. pestis KIM6+ (Hms+)</th>
<th>Y. pestis KIM6 (Hms−)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vivo (flea)</td>
<td>In vitro culture</td>
</tr>
<tr>
<td>Sudan black</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>OsO4</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>PAS</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Ruthenium red</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Calcofluor white</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>IFA (α-Y. pestis AS)</td>
<td>−a</td>
<td>+</td>
</tr>
</tbody>
</table>

**NOTE.** AS, antiserum; IFA, indirect fluorescent antibody; PAS, periodic acid–Schiff; +, positive; −, negative.  
*a* Surface of bacterial aggregates did not stain; external bacteria were positive (see figure 5).

lawns after 48 h of growth at 21°C on heart-infusion agar containing 0.2% galactose. Adherent bacteria were fixed overnight at 4°C with 75 mmol/L lysine acetate, 0.075% (wt/vol) alcan blue, 2% paraformaldehyde, and 2.5% glutaraldehyde buffered with 100 mmol/L sodium cacodylate. Samples were washed with 100 mmol/L sodium cacodylate, postfixed with 1% OsO4 for 1 h, and dehydrated in a graded ethanol series. Samples were critical point–dried under CO2 with a Bal-Tec cpd 030 drier (Balzers), mounted on aluminum studs, and coated with 125 Å of iridium in an IBS/TM200S ion beam sputterer (South Bay Technologies) before viewing at 5 kV on a S-4500 field emission scanning electron microscope (Hitachi). The proventriculus was dissected from fleas, bisected with a 27-gauge needle, and fixed with 2.5% glutaraldehyde and 4% paraformaldehyde in 100 mmol/L sodium cacodylate buffer (pH 7.4). Samples were postfixed, mounted, and viewed as described above.

**Indirect fluorescent antibody (IFA) assays.** Hyperimmune serum was generated by injecting rabbits intravenously twice weekly for 8 weeks with 0.2 mL of PBS containing 1 × 10^9 killed Y. pestis KIM6+ grown at 21°C in N-minimal medium containing 2% glucose and 1% glucosamine. The serum was used in IFA assays of bacterial aggregates dissected from the midguts of infected fleas and of in vitro biofilms produced on the walls of glass culture tubes by Y. pestis grown in TMH, 1% N-acetylglucosamine, and 100 mmol/L hemin at 21°C. Washed, unfixed aggregates were incubated with a 1:500 dilution of the antiserum for 30 min at 37°C, washed, and reincubated with a 1:50 dilution of goat anti–rabbit IgG labeled with rhodamine (Kirkegaard & Perry). Slides were examined by fluorescence microscopy.

**Interaction with human polymorphonuclear leukocytes (PMNLs).** The midguts of 50 fleas were dissected 1–3 weeks after infection and placed in 100 μL of RPMI 1640 medium (Gibco) with 10 mmol/L HEPES. Twenty microliters of glass sand was added, and the midguts were triturated and vortexed thoroughly. After this procedure, both the Y. pestis KIM6+ and KIM6 supernatants predominantly contained individual bacteria, with a few clusters of 2–5 bacteria but no large clumps. In vitro control bacteria were grown in TMH and 1% N-ace-
tylglucosamine for 48 h at 21°C, volumes containing $1 \times 10^8$ bacteria were centrifuged, and the cells were resuspended in 100 μL of RPMI 1640 medium plus HEPES. The dissected midguts of 50 uninfected fleas were added to each in vitro–grown bacterial suspension, triturated, and vortexed. All bacterial supernatants were quantitated by direct count, adjusted to $1 \times 10^7$ Y. pestis organisms/mL in RPMI 1640 medium plus HEPES, and opsonized with 25% normal human serum for 30 min at 37°C. Phagocytosis and killing of Y. pestis by human PMNLs was determined by adding 0.1 mL of each bacterial suspension to triplicate wells of a 96-well tissue culture plate containing fresh human PMNLs in 0.1 mL of RPMI 1640 medium plus HEPES, prepared as described elsewhere [33]. Matched control wells without PMNLs were also inoculated. The plates were then centrifuged at 350 g for 8 min at 4°C. After 10, 30, and 60 min of incubation at 37°C in 5% CO₂, the contents of individual wells were removed and treated with saponin (final concentration, 0.1%) for 15 min at 4°C to lyse PMNLs, and serial dilutions were plated on Luria-Bertani agar. The percentage of killing was calculated by comparing the average number of colony-forming units in the presence and absence of PMNLs for each time point.

**RESULTS**

**Temperature- and Hms-dependent production of Y. pestis biofilm in vitro.** We used a glass flowcell system to determine whether Y. pestis can adhere to a hydrophobic surface and form a biofilm in vitro. At room temperature, the Hms-positive Y. pestis strain produced biofilm-like growth on the glass surface (figure 1). An isogenic Y. pestis Hms-negative mutant did not form a biofilm, but this ability was restored when the mutant was complemented with the hms genes. Y. pestis did not form a biofilm when the flowcell was incubated at 37°C, which correlates with the lack of hms-dependent Congo red–binding (pigmentation phenotype) at temperatures ≥28°C [17, 18, 34]. Growth-rate determinations at 21°C and 37°C showed no differences among the Y. pestis strains in the medium. Thus, along with the temperature-dependent ability of Y. pestis colonies to bind hemin and Congo red, the ability to produce an in vitro biofilm is also Hms dependent.

Given the similarity of Y. pestis hmsR and hmsF to the S. epidermidis genes that are responsible for producing the extracellular polysaccharide required for the formation of biofilm, we examined bacterial lawns on agar plates by scanning electron microscopy. Of note, extracellular material was produced by Hms-positive, but not Hms-negative, Y. pestis grown at room temperature (figure 2).

**Biofilm-like growth of Y. pestis in the flea: effect of hms gene products.** Previous studies have shown that wt and Hms-negative strains of Y. pestis colonize the flea equally after an infectious blood meal and achieve equivalent bacterial loads in the midgut [8]. The most striking difference between the 2
strains is that Hms-negative *Y. pestis* never proceed to infect the proventriculus but remain confined to the midgut. As a result, *Y. pestis hms* mutants are completely unable to block fleas, whereas Hms-positive *Y. pestis* infect both the proventriculus and the midgut, leading to proventricular blockage in 25%–50% of *X. cheopis* fleas [8]. Examination of a blocked proventriculus by scanning electron microscopy confirmed the presence of a bacterial mass associated with extracellular material fixed among the proventricular spines (figure 3).

Both Hms-positive and -negative *Y. pestis* grew in the form of large aggregates in the flea midgut but were dissimilar. The aggregates formed by Hms-positive *Y. pestis* in the flea were darkly pigmented, had complex structures, and were enveloped in a viscous extracellular matrix (figure 4A). In contrast, Hms-negative *Y. pestis* aggregates were light colored, had little 3-dimensional structure, and were not associated with an obvious extracellular matrix, although individual bacilli in the aggregate were difficult to resolve, even at high magnification (figure 4B and 4D). Hms-positive *Y. pestis* aggregates dissected from fleas were also more cohesive and less easily disrupted than were Hms-negative *Y. pestis* aggregates.

In an initial attempt to characterize the amorphous brown matrix that surrounds *Y. pestis* in the flea gut, bacterial aggregates were dissected from infected fleas and exposed to different solvents, degradative enzymes, and histological stains (tables 1 and 2). The flea gut–associated extracellular matrix was not soluble in aqueous solvents but was degraded by polar lipid solvents; it also was resistant to proteases and to plasmin, an enzyme that hydrolyzes fibrin. Bacterial masses dissected from the flea midgut could be decolorized by treatment with the hemin solvent 0.01N NaOH and by oxidation with 3% H2O2, suggesting that hemin derived from the flea’s blood meal was responsible for the brown color of the extracellular matrix. Because the extracellular matrix of bacterial biofilms is typically composed of bacterially-derived polysaccharide, we analyzed *Y. pestis* aggregates dissected from infected fleas with differential stains. The flea gut–associated extracellular material surrounding Hms-positive *Y. pestis* stained with PAS, which reacts with polysaccharides and other complex polymers. It did not stain with ruthenium red or calcofluor white, which react with poly-anionic and β 1,3-linked polysaccharides, respectively. Hms-positive *Y. pestis* aggregates dissected from fleas also stained positive with lipid-soluble dyes. In contrast, the Hms-dependent extracellular matrix produced by *Y. pestis* on agar media did stain with ruthenium red but not with PAS or the other polysaccharide and lipid stains. Hms-negative *Y. pestis* aggre-
Independent experiments are shown. Biological activity of Y. pestis insect forms was evaluated by comparing the resistance of fleas to Y. pestis strains. The Y. pestis strain used for the studies was a wild-type strain (Y. pestis 2104). The Y. pestis strain was used to infect and block fleas just as wt fleas do [3], but the matrix in which the bacteria are embedded in the fleas gut is completely resistant to the fibrinolytic enzyme plasmin and to other proteases (table 1). Although it is not required in the flea, the Y. pestis Pla is believed to be important after transmission into the dermis, where it facilitates dissemination from the site of the flea bite [41].

DISCUSSION

Y. pestis exhibits a distinct life stage in the flea vector. Unlike infection in the mammal, the bacteria are not invasive in the flea. The bacteria do not adhere to or penetrate the flea gut epithelium but remain confined to the lumen of the digestive tract (figure 4). This presents 2 potential problems. First, because fleas feed and excrete frequently, the bacteria are at constant risk of being eliminated in the feces. In fact, nearly half of X. cheopis fleas clear themselves of infection in this manner even after feeding on highly septicemic blood [4, 7, 8]. Second, transmission requires that Y. pestis, which are nonmotile, move against the direction of blood flow when the flea feeds. Y. pestis overcomes both of these problems by adopting a biofilm mode of growth in the flea gut. Successful infection of the flea depends on the formation of large, dense bacterial aggregates in the gut lumen that are too large to pass from the midgut to the hindgut.

The flea-blocking bacterial aggregates have long been assumed to be enveloped in a fibrin clot matrix formed from the blood meal by a procoagulant activity of the Y. pestis plasminogen activator (Pla) [40]. This model is often cited in infectious-disease textbooks. Our results provide further evidence against the Pla-generated fibrin clot model of flea blockage. Not only are Y. pestis strains that lack the pla gene able to infect and block fleas just as wt strains do [3], but the matrix in which the bacteria are embedded in the fleas gut is completely resistant to the fibrinolytic enzyme plasmin and to other proteases (table 1). Although it is not required in the flea, the Y. pestis Pla is believed to be important after transmission into the dermis, where it facilitates dissemination from the site of the flea bite [41].

Y. pestis QS systems and biofilm-like growth in the flea. Bacterial QS systems have been implicated in regulating biofilm development [35, 36]. Three such systems have been identified in Y. pestis [25, 26], 2 that use autoinducer-1 type AHL signals and 1 that uses the autoinducer-2 LuxS. We tested a Y. pestis strain with loss of function mutations in all 3 autoinducer synthase genes for its ability to form a biofilm and block fleas. This Y. pestis triple-QS-negative mutant was able to produce a biofilm on a glass surface (figure 1f) and to infect and block fleas as well as the wt parent strain did (figure 6).

Y. pestis phenotype at the flea-host transmission interface. A transmissible infection in the flea fits the description of a bacterial biofilm—surface colonization by a dense microcolony embedded in an extracellular matrix (figure 3D). In a completely blocked flea, the biofilm fills the lumen of the proventriculus. Bacteria embedded in the viscous matrix, either contiguous with the blocking mass in the proventriculus or detached pieces of it, were exuded into the esophagus after application of a coverslip during preparation of dissected digestive tracts for microscopy, indicating that the matrix was cohesive but somewhat fluid (figure 7). The bacteria transmitted by fleas—either individual cells, small clusters, or both—undoubtedly derive from this viscous biofilm-like mass.

Bacteria embedded in a biofilm have been shown to be more resistant to uptake or killing by phagocytes [37]. For some bacteria, antiphagocytic properties have been attributed to the extracellular matrix itself [38, 39]. To examine the effect of the fleaspecific phenotype on phagocytosis, aggregates of Hms-positive and -negative Y. pestis were dissected from infected fleas and triturated and vortexed to release individual cells into suspension, which were then added to human PMNLs. Bacteria from fleas were significantly more resistant to killing by PMNLs than were the same bacteria grown in liquid media under conditions in which the Hms-dependent extracellular matrix is synthesized (figure 8). The increased resistance was attributable to reduced uptake of flea-derived Y. pestis by the PMNLs, because the kinetics of phagocytosis mirrored those of killing (data not shown).
As an alternative to the fibrin matrix model, our results support the biofilm model proposed by Darby et al. [16], who showed that both Y. pestis and Y. pseudotuberculosis produce an Hms-dependent biofilm that accumulates on the external mouthparts and that blocks the feeding of C. elegans and who suggested that the same phenomenon is responsible for proventricular blockage in the flea. Infection of the proventriculus by Y. pestis (figures 3, 4, 5, and 6) fits the operational definition of a bacterial biofilm, which is formed by many bacteria in many different environments. Developing a matrix-enclosed biofilm is a common means by which bacteria colonize a surface in spite of currents and shear forces [37]. For example, many aquatic bacteria can alternate between a commensal, surface-attached biofilm and a dispersed planktonic life stage. Certain chronic bacterial infections, such as periodontitis, endocarditis, and infections of implanted medical devices, result from biofilm formation in vivo [22, 37]. Y. pestis appears to use a similar means to infect the hydrophobic surface of the proventricular spines, thus overcoming the pulsating valvular action of the proventriculus and the inward flow of blood during feeding that would tend to wash the bacteria back into the midgut. Transmission of Leishmania parasites also depends on a foregut-blocking phenomenon in the sandfly [42], but Y. pestis is unique among arthropod-borne bacteria in using biofilm development to produce a transmissible infection in the vector.

Growth of Y. pestis in the biofilm phenotype, both in vitro and in the flea, requires the temperature-dependent synthesis of hms gene products. HmsH and HmsF are outer membrane proteins [43]. However, the presence of these 2 surface proteins is not sufficient to produce blockage of fleas or binding of Congo red and biofilm-like growth in vitro, because hmsR and hmsT mutations abolish these phenotypes [8, 11, 12] (authors’ unpublished data). Complementation analyses further indicate that all 5 identified hms genes are required for binding of Congo red in vitro [10, 43]. In contrast, none of the 3 known QS systems of Y. pestis is required to form a biofilm in vitro or to cause proventricular blockage in fleas, despite the high cell densities achieved (figure 6).

The biochemical composition of the Hms-dependent extracellular material associated with Y. pestis in vitro and in the flea is not known. Given the similarity of hmsF and hmsR to nucleotide sugar transferase genes involved in polysaccharide biosynthesis and the differential staining results of Hms-positive Y. pestis colonies (table 2), it is likely that the in vitro extracellular material is polysaccharide. However, the extracellular matrix around the bacteria in the flea gut appeared to be heterogeneous and more complex. It was dark brown, probably as a result of adsorbed hemin derived from red blood cells in the flea’s diet. In addition, the hydrophobic, viscous nature and the staining and solubility characteristics of the flea-associated matrix (tables 1 and 2) indicated that it contained lipid, perhaps also derived from blood. Thus, unlike the extracellular matrix produced in vitro, the flea gut–associated matrix may be composed of both bacterially derived polysaccharide and exogenous digestive products of blood present in the flea gut. The matrix was not recognized by hyperimmune serum (figure 5), which also suggests that it does not consist entirely of bacterial products.

Y. pestis from fleas were more resistant to human PMNLs than were Y. pestis from in vitro cultures (figure 8). This phenomenon was not Hms dependent, however, suggesting that other, unknown Y. pestis factors specific to the flea environment are responsible. Lillard et al. [14] also found that the Hms phenotype did not affect the resistance to human PMNLs of Y. pestis grown in vitro in the presence or absence of hemin. Adsorbed material from the flea gut may be responsible for increased resistance to PMNLs, and unknown Y. pestis antiphagocytic gene products whose expression is specifically induced in the flea may also contribute. In any case, as bacteria are released from the biofilm and enter the mammalian host, the flea-specific phenotype may confer some protection against the innate immune response. This may be important immediately after transmission, because known Y. pestis antiphagocytic factors, such as the F1 capsule and the type III secretion system, are not produced in the flea [34, 44]. In addition, if the matrix does contain lipid or other generic material derived from the flea’s blood meal, this may mask bacterial antigens.

Y. pestis is a clonal variant of Y. pseudotuberculosis that emerged within the last 1500–20,000 years [45]. Presumably, the change from the food- and waterborne transmission of the Y. pseudotuberculosis ancestor to the fleaborne transmission of Y. pestis occurred during this short evolutionary period. The hms genes are not required in the mammal; thus, their primary biological function is to enable fleaborne transmission [8, 14]. Of interest, the 2 chromosomal hms loci are present in both Y. pestis and Y. pseudotuberculosis, but the hms phenotype of the 2 species differs. Colonies of most laboratory strains of Y. pseudotuberculosis strains do not bind hemin or Congo red, which is the signature Hms-positive phenotype in Y. pestis [46, 47]. Y. pseudotuberculosis, even Congo red–binding strains, do not block the proventriculus of fleas, although they are able to establish a chronic midgut infection [7] (authors’ unpublished data). Thus, the evolutionary transition of Y. pestis to fleaborne transmission appears to have involved the recruitment of endogenous chromosomal genes for a new function—the production of a proventriculus-blocking biofilm.

**Acknowledgments**

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2. Baco AW, Martin CJ. Observations on the mechanism of the trans-
ganization, 1954.
5. Burroughs AL. Sylvatic plague studies: the vector efficiency of nine
9. Fetherston JD, Schuetze P, Perry RD. Loss of the pigmentation phe-
notype in Yersinia pestis is due to the spontaneous deletion of 102 kb of chromosomal DNA which is flanked by a repetitive element. Mol Microbiol 1992; 6:2693–704.
10. Lillard JW, Fetherston JD, Pedersen L, Pendrak ML, Perry RD. Se-
11. Hare JM, McDonough KA. High-frequency RecA-dependent and
12. Jones HA, Lillard JW, Perry RD. HmsT, a protein essential for ex-
14. Lillard JW, Bearden SW, Fetherston JD, Perry RD. The haemin storage
(Hms+) phenotype of Yersinia pestis is not essential for the pathogenesis of bubonic plague in mammals. Microbiology 1999; 145:197–209.
23. Perry RD, Pendrak ML, Schuetze P. Identification and cloning of a 
24. Fetherston JD, Perry RD. The pigmentation locus of Yersinia pestis
26. Atkinson S, Throup JP, Stewart GSB, Williams P. A hierarchical quo-
30. Mills J, Pulliam L, Dall L, Marzouk J, Wilson W, Costerton JW. Exopoly-
saccharide production by viridans streptococci in experimental endo-
39. Meluleni GJ, Grout M, Evans DJ, Pier GB. Mucoid Pseudomonas aeu-
gínosa growing in a biofilm in vitro are killed by opsonic antibodies to the mucoid exopolysaccharide capsule but not by antibodies produced during chronic lung infection in cystic fibrosis patients. J Immunol 1995; 155:2029–38.
46. Baco AW, Martin CJ. Observations on the mechanism of the trans-