Evaluation of the Role of the *Yersinia pestis* Plasminogen Activator and Other Plasmid-Encoded Factors in Temperature-Dependent Blockage of the Flea

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*Yersinia pestis*, the plague bacillus, has a plasminogen activator (*pla*) gene on the 9.5-kb plasmid *pPla* that is hypothesized to play a role in producing the foregut blockage in the flea vector that precedes transmission. In this study, however, *Y. pestis* that lacked *pPla*, the 70-kb virulence plasmid, or both plasmids, proved able to block *Xenopsylla cheopis* fleas normally. Blockage rates decreased with increasing environmental temperature for fleas infected with either wild type or *pPla*+ *Y. pestis*. Thus, procoagulant ability of the *Y. pestis pla* gene product does not mediate blockage, nor does its ability to induce fibrinolysis at >28°C account for failure to block at elevated temperatures. A *Y. pestis* strain that lacked all or part of the third plasmid of 110 kb, however, failed to colonize the flea midgut normally, indicating that one or more genes on the large plasmid may be required for vectorborne transmission.

Bubonic plague, which historically has visited human populations in the form of devastating pandemics, is a zoonosis that primarily affects rodents. The plague bacillus, *Yersinia pestis*, is well-established within wild rodent populations in many parts of the world and is transmitted primarily by fleas [1, 2]. Many of these wild rodent species are relatively resistant to overt disease and constitute the natural reservoir of *Y. pestis*. The ecology of plague is complex: *Y. pestis* has been isolated from ~200 species of rodents worldwide, and at least 80 species of fleas have been implicated in maintaining cycles of plague [2]. The disease exhibits a pattern of periodic eruptive epizootics among certain species of highly susceptible rodents, resulting in rapid amplification and geographic spread followed by regression to focal areas [1]. Risks to humans increase during these epizootics, particularly if the disease crosses over into urban rat populations. Most human cases of plague in the United States result from flea bite during exposure to rural plague foci [3]. Disease can also result from direct contact with infected animal tissues or by inhaling aerosolized respiratory secretions from an animal with pneumonic plague [2, 3]. Plague remains an international public health concern, and recent outbreaks in India and parts of Africa, where the disease had been dormant for decades, suggest that it is undergoing a resurgence [4].

The manner in which fleas transmit plague was first described in 1914 by Bacot and Martin [5]. Blood containing *Y. pestis* enters the midgut of a flea that is feeding on a septicemic host. The bacteria multiply within the midgut, eventually forming a large cohesive mass that can lodge in the flea’s proventriculus, a valvelike chamber that connects the midgut to the esophagus. The lumen of the proventriculus, which is lined with cuticulated spines, eventually becomes completely filled with bacteria. Fleas in this condition are said to be “blocked” because they are unable to pump blood into the midgut. Transmission occurs as bacteria are flushed from the proventriculus to the bite wound during persistent but futile attempts by blocked fleas to feed. Several studies have demonstrated that only blocked fleas are important for biologic transmission [2, 6–8].

In 1971, Cavanaugh [9] proposed that proventricular blockage of fleas depended on a coagulase activity of *Y. pestis*, which was known to produce a fibrin clot from rabbit plasma at ≤28°C. In concert with a trypsin-like enzyme secreted by the flea midgut, the bacterial coagulase was thought to precipitate flea blockage by embedding the plague bacilli in a fibrin matrix that anchors them to the proventricular spines. This hypothesis is often cited in infectious disease textbooks. Conversely, at temperatures >28°C, *Y. pestis* demonstrates potent plasminogen activator capability that results in fibrinolysis. A single bacterial surface protease, the product of the plasminogen activator (*pla*) gene that is present on a 9.5-kb plasmid of *Y. pestis*, has been found to be responsible for both activities. Expression of the *pla* gene is not affected by temperature, but its protein product displays opposite coagulase and plasminogen activator functions depending on temperature [10, 11]. Cavanaugh invoked the fibrinolytic activity to explain the fact that fleaborne bubonic plague epidemics abruptly terminate with the onset of hot weather. According to this hypothesis, as ambient temperatures rise above 28°C, the fibrin matrix of the bacterial mass...
in infected fleas is degraded via the plasminogen activator function of the *Y. pestis* Pla protease, precluding blockage and transmission.

As part of a study to identify *Y. pestis* plasmid genes that are required in the insect host, we have tested the Cavanaugh hypothesis. Wild type *Y. pestis* isolates typically harbor three plasmids [12–14]. The smallest (pPla, 9.5 kb) contains the *pla* gene as well as genes for the bacteriocin pesticin and its antidote [11, 15]. The *Yersinia* virulence plasmid (pYVp, 70 kb) contains an extensive array of genes that encode a multicomponent system that functions to deliver cytotoxic proteins directly into eukaryotic cells on contact, presumably to prevent phagocytosis and to curtail the immune response in the mammal. This plasmid is required for pathogenesis in the mammal, is highly conserved among all three pathogenic yersiniae (*Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*), and has been the primary focus of *Yersinia* research for the last decade (reviewed in [16, 17]). The third and largest plasmid (pFra, 110 kb) is the least characterized. It contains the biosynthetic genes for the *Y. pestis* fraction 1 capsular antigen and a toxin, but the genetic content of the rest of this large plasmid is unknown [14, 18]. For this study, we compared the ability of wild type *Y. pestis* and isogenic plasmid-cured derivative strains to infect and block fleas maintained at different temperatures.

**Materials and Methods**

*Y. pestis* strains. *Y. pestis* 195/P-wt was originally isolated from a human plague patient in India and is fully virulent [19]. The isogenic plasmid-cured strains *Y. pestis* 195/P-1 (pPla'), *Y. pestis* 195/P-2 (pPla' pYVp'), and *Y. pestis* 195/P-3 (pPla' ΔcafΔ yum') were generated as described below. *Y. pestis* KIM6' (pYVp') and *Y. pestis* KIM10' (pPla' pYVp'), plasmid-cured derivatives of the KIM strain originally isolated from a human in Iran, were provided by Robert Perry (University of Kentucky, Lexington) [20]. *Y. pestis* 195/P-1 (pPla') and KIM10' (pPla' pYVp'), which express the gene encoding green fluorescent protein, were obtained by electroporation (GenePulser; Bio-Rad Laboratories, Hercules, CA) with pGFP (Clontech Laboratories, Palo Alto, CA).

Cloning and characterization of plasmid-cured strains. Isogenic plasmid-cured strains of *Y. pestis* were cloned from the wild type 195/P parent strain after 5–10 serial passages in brain-heart infusion (BHI) broth media at 8°C. Clones lacking the 9.5-kb Pla plasmid were detected by means of a fibrinolysis assay [21]. Bovine thrombin (Miles Laboratories, Kankakee, IL; 25 NIH units in 0.5 mL of sodium borate buffer) was mixed with 10 mL of 0.5% bovine fibrinogen (ICN Pharmaceuticals, Costa Mesa, CA) in a petri plate to form a fibrin layer. Individual *Y. pestis* clones were spotted onto the surface of the fibrin and incubated at 37°C for 4 h. Clones that contained the plasminogen activator (*pla*) gene produced a zone of clearing due to fibrinolysis. A fibrinolysis-negative (*Pla−*) clone, designated *Y. pestis* 195/P-1, was selected for further analysis. A clone lacking both the pPla and pYVp plasmids, designated *Y. pestis* 195/P-2, was selected by culturing *Y. pestis* 195/P-1 on magnesium oxalate medium at 37°C. Growth of pYVp' but not pYVp cells is inhibited on this calcium-chelated medium at 37°C [22, 23]. A clone that appeared to lack the 110-kb Fraser plasmid was obtained after 10 additional serial 8°C passages of *Y. pestis* 195/P-1, followed by plating at 37°C on tryptone-glucose–yeast extract medium to differentiate smooth and rough colony phenotypes [24]. Because a smooth phenotype is typical of F1 capsule expression, encoded by genes on pFra, the plasmid profiles of 80 individual rough colonies were examined. Three of these 80 rough clones appeared to have lost pFra and 1, designated *Y. pestis* 195/P-3, was chosen for further analysis.

Plasmid content of the strains was examined by using a modified alkaline lysis plasmid purification protocol (Qiagen, Chatsworth, CA) followed by electrophoresis in 0.7% agarose in Tris-acetate/EDTA buffer [25]. Gel-purified 9.5-kb and 70-kb plasmid DNA was individually radiolabeled by nick translation (Boehringer Mannheim, Indianapolis) and used to probe *Y. pestis* DNA that had been subjected to electrophoresis and transferred to a nylon membrane (GeneScreen; NEN, Boston) by vacuum blotting (VacuGene; Pharmacia, Piscataway, NJ). A 9.4-kb segment of pFra that includes the F1 capsule operon [26] and polymerase chain reaction (PCR)-generated segments of the *pla* gene and the chromosomal *fur* gene were radiolabeled by using a random primer labeling kit (Boehringer Mannheim) and used to probe Southern blots of EcoRI- or EcoRI- and HindIII-digested *Y. pestis* genomic DNA preparations [27]. After overnight hybridization at 37°C, the membranes were washed twice at 70°C for 20 min in 0.1× standard saline citrate before autoradiography [25]. PCR was done on whole-cell *Y. pestis* DNA as described [28], using primers for the *Pla* gene (5'-ATCTTACTTTTCCGAGAAG-3' and 5'-CTTGGATTGTGACCTCTTA-3'), the pYVp gene *lcrV* (5'-GATTGCCCATGACAGTGTCCCG-3' and 5'-TCTAGCAGACGTTGCATC-3') [29], the *fra* gene *ytm* (5'-GATACAGATGGGATACAAAAATATGC-3' and 5'-CCCAAGAGTGGTTGGAAC-3') [18, 30], and the chromosomal gene *fur* (5'-GAAGGTGTTGCAAATCCTCCTGCG-3' and 5'-AGTGGACCTTAAATACAGC-3') [30, 31].

**Flea infection.** Oriental rat fleas (*Xenopsylla cheopis*) were from colonies of the Rocky Mountain Laboratories [32]. Adult fleas, starved for 6 days, were infected by allowing them to feed on blood containing ∼5 × 10^7* Y. pestis* mL by use of an artificial feeding system [33, 34]. To prepare the blood meal, bacteria were cultured at 37°C for 16 h in BHI broth, quantitated in a Petroff-Hausser counting chamber by phase microscopy, centrifuged, resuspended in 1 mL of PBS, and added to 5 mL of fresh heparinized mouse blood containing 5 mM ATP. After a 1-h feeding period, cohorts of equal numbers of male and female blooded fleas (sample size, 85–110, with two exceptions noted in the footnote to table 1) were maintained at 20°C, 25°C, or 30°C and 75% relative humidity.

Flea blockage, mortality, and infection rates. Fleas were monitored for incidence of blockage and mortality during a 4-week period after infection as described [33]. During this month, fleas were fed twice weekly on normal uninfected mice and examined microscopically immediately afterwards to identify blocked fleas, which had bright red blood in the esophagus but none in the midgut [33]. Blocked fleas were then segregated to a separate container. After 4 weeks, 20 surviving unblocked female fleas were stored at −60°C. The percentage of infected fleas in these samples (infection or colonization rate) was determined by plating dilutions of individual triturated fleas onto BHI agar plates containing 1 μg/mL.
Irgasan (Ciba-Geigy, Greensboro, NC). Y. pestis colony-forming units were quantitated after incubation at 28 °C for 3 days.

**Electron microscopy.** Dissected midguts of fleas blocked with Y. pestis 195/P-wt were fixed in 4% paraformaldehyde–2.5% glutaraldehyde in 100 mM cacodylate buffer, pH 7.4, at 4 °C for 16 h. Samples were postfixed in 0.5% OsO₄–0.8% K₃Fe(CN)₆ in 100 mM cacodylate buffer and stained for 1 h with 1% tannic acid at room temperature and overnight with 0.5% uranyl acetate at 4 °C. Flea midguts were then dehydrated in a graded ethanol series and subjected to critical point drying (cpd 030; Bal-Tec, Balzers, Liechtenstein). The midguts were mounted on aluminum studs, teased open, coated with 110 Å of chromium in an IBS/TM200S ion beam sputterer (VCR Group, South San Francisco), and viewed with using an S-4500 cold field emission scanning electron microscope (Hitachi, Tokyo).

Proventriculi dissected from uninfected and blocked fleas were prepared for transmission electron microscopy by fixing, postfixing, and staining as described above. After ethanol dehydration, the proventriculi were embedded in Spurr’s resin. Thin sections were cut with an MT-7000 ultramicrotome (Research and Manufacturing Company, Tucson), stained with 1% uranyl acetate and Reynolds’s lead citrate, and viewed at 80 kV on a CM-10 transmission electron microscope (Philips, Eindhoven, The Netherlands).

**Results**

**Isolation and characterization of isogenic plasmid-cured Y. pestis strains.** Wild type Y. pestis typically contains three native plasmids, designated here as pPla (9.5 kb), pYVp (70 kb), and pFra (110 kb). Clones lacking one or more of these plasmids were obtained after serial culture at 8 °C, which enhances spontaneous loss of plasmids [35]. Two Y. pestis strain backgrounds were used. The isogenic series derived from wild type Y. pestis 195/P included Y. pestis 195/P-1 (pPla⁻), 195/P-2 (pPla⁻ pYVp⁺), and Y. pestis 195/P-3 (pPla⁻ ΔcafΔymt). Plasmid DNA profiles of these strains are shown in figure 1A. Another isogenic set, Y. pestis KIM6⁻ (pYVp⁻) and KIM10⁻ (pPla⁺ pYVp⁻), was independently generated from a different isolate [20].

In addition to the expected plasmid profiles (figure 1A), loss of pPla and pYVp was confirmed by Southern hybridizations of Y. pestis total DNA, using isolated pPla and pYVp plasmid DNA as probes; lack of in vitro fibrinolytic activity (plasminogen activator function) for pPla⁻ strains; and failure to PCR-amplify pPla-specific pla and pYVp-specific lcrV gene sequences (data not shown). The absence of the pla gene from the genome of pPla⁻ strains was also verified by Southern blot (figure 1B). The case of Y. pestis 195/P-3, which appeared to have lost pFra (figure 1A), was more problematic. Southern blot and PCR results verified that this strain lacked the two pFra loci that have been described to date (the caf operon encoding the F1 capsule and the murine toxin gene, ymt). Because the bulk of pFra is uncharted, however, and contains multicopy insertion sequence elements that reportedly can mediate the integration

![Figure 1](https://academic.oup.com/jid/article-abstract/178/5/1406/810623/1408-Hinnebusch-et-al.-JID-1998-178-(November)

**Table 1.** Blockage and mortality of X. cheopis fleas during 4-week period following infection with wild type or plasmid-cured Y. pestis.

<table>
<thead>
<tr>
<th>Fleas infected with Y. pestis 195/P-wt</th>
<th>Plasmid content</th>
<th>% of fleas blocked</th>
<th>Flea mortality (%) at 20°C</th>
<th>% of fleas blocked</th>
<th>Flea mortality (%) at 25°C</th>
<th>% of fleas blocked</th>
<th>Flea mortality (%) at 30°C</th>
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<tr>
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NOTE: Results of replicate experiments are shown. Total no. of fleas was 37, 46, and 102 for 3 experiments using virulent Y. pestis 195/P-wt at 20°C; n = 85–110 for all other experiments. All samples comprised equal numbers of male and female fleas, which were monitored for 4 weeks after single infectious blood meal. Data for Y. pestis 195/P-wt and KIM6⁻ at 20°C are from [33]. ND = not done.

a Y. pestis 195/P-3 lacks at least caf and ymt operons of pFra (ΔcafΔymt).
of portions of this plasmid into the chromosome [36], we could not be confident that pFra DNA had been completely eliminated from the 195/P-3 strain. Hence, the Y. pestis 195/ P-3 strain is designated ΔcaflΔsymt, although it may actually lack more or all of pFra. No difference in the in vitro growth rates of the strains was observed at 20°C or 30°C (unpublished data).

The 9.5-kb pPla and 70-kb pYVp Y. pestis plasmids are not required in the flea. To investigate the role of the Y. pestis pla gene and other plasmidborne genes in transmission, we tested the ability of plasmid-cured strains to infect and block fleas. The results of flea infections with the two series of Y. pestis strains are shown in Table 1. Y. pestis 195/P-wt blocked 32%–45% of fleas kept at 20°C in three independent experiments. Similar blockage rates were observed in fleas infected with Y. pestis 195/P-1, which lacked the 9.5-kb pla-containing plasmid pPla (χ² = 0.72; P > 2), and Y. pestis 195/P-2, which lacked both pPla and the 70-kb yersinia virulence plasmid pYVp (χ² = 0.18; P > .5). Although blockage rates observed for the KIM strains appeared to be slightly lower than those of the 195/P strains, again no significant difference was seen between Y. pestis KIM6, which lacks pYVp only, and KIM10 *, which lacks both pYVp and pPla (χ² = 2.01; P > .1). Thus, for both Y. pestis strains, the ability to block fleas did not depend on the presence of pPla. Results for the isogenic Y. pestis 195/P series indicate that the virulence plasmid, pYVp, is not required either.

Because blocked fleas died within a few days due to dehydration and starvation, normal levels of blockage at 20°C correlated with flea mortality rates (Table 1) that were significantly higher than that of uninfected control fleas (χ² = 57–101; P < .001). In agreement with previous studies with X. cheopis [6, 8, 33, 37–39], the time from the infectious blood meal to the appearance of blockage (the extrinsic incubation period) ranged from 6 to 27 days and peaked between the second and third week after infection (median, 15–18 days) for Y. pestis wild type as well as the pPla-, pYVp- and pPla pYVp- strains. Male fleas tended to become blocked sooner than did the larger females, as has been noted before [37].

Normal development of proventricular blockage was also directly visualized in fleas infected with pPla- and pYVp- bacteria expressing the gene for green fluorescent protein. At 3, 8, 17, and 22 days after infection, digestive tracts of 10 fleas infected with Y. pestis 195/P-1 (pPla- pGFP) and 10 fleas infected with KIM10* (pPla- pYVp- pGFP) were dissected and examined as previously described [33]. In a normal infection at 20°C, bacteria ingested in a blood meal multiply and form large masses during the first week in the flea midgut. The dense masses of bacteria appear to be contained within an irregular, brown-pigmented layer of degraded cellular debris, possibly derived from blood meal erythrocytes [5, 8, 33]. A typical mass or microcolony of Y. pestis from the digestive tract of a blocked flea is shown in Figure 2. Colonization of the proventriculus is usually evident during the second week, followed by blockage resulting from growth and consolidation of the bacterial mass among the spines in the lumen of the proventriculus [33]. Fleas infected with either pPla- strain followed this typical course of infection. Transmission electron micrographs of thin proventricular sections prepared from fleas blocked with Y. pestis 195/P-1 (pPla-) were also indistinguishable from those of fleas blocked with Y. pestis 195/P-wt: The entire lumen of the proventriculus was packed with bacteria, which appeared to be embedded in an electron-dense matrix (Figure 3).

Temperature dependence of flea blockage is not due to the Y. pestis plasminogen activator. The Y. pestis pla gene product induces very little fibrinolysis at 20°C but is a potent plasminogen activator at higher temperatures [9, 10]. To test the effect of the plasminogen activator on the ability to block, we maintained additional cohorts of infected fleas at 25°C and 30°C. Blockage rates decreased with temperature, and none of the fleas kept at 30°C developed blockage (Table 1). There was no difference in this regard between fleas infected with pla- or pla- strains, however. Thus, decreased flea blockage at the elevated temperatures is not due to the action of the Y. pestis plasminogen activator on the putative fibrin matrix that consolidates a blocking bacterial mass. Although blockage rates declined, the mortality of fleas kept at 25°C was as high as that observed for fleas kept at 20°C. At 30°C, uninfected controls as well as infected fleas experienced high mortality (Table 1).

Infection rates of fleas kept at the three temperatures were determined by quantitating Y. pestis from surviving, unblocked female fleas 4 weeks after the infectious blood meal. As has been noted before, Y. pestis infection of the flea is an inefficient process—a significant percentage of fleas normally clear themselves of bacteria even after feeding on highly septicemic blood, probably by simply excreting the bacteria before they have a chance to produce large masses in the midgut [2, 6, 8, 33, 40]. Fleas that are successfully colonized, however, remain infected with large numbers of bacteria [8, 33]. The experiments depicted in Figure 4 indicate that a significant percentage of fleas were colonized at all three temperatures, even though proventricular blockage rates decreased with increasing temperature and fleas held at 30°C did not block at all. Furthermore, >10⁷ Y. pestis were present in all infected fleas kept at the three temperatures, in keeping with the masses or microcolonies seen in infected midguts (Figure 2). Thus, the decline in blockage rates at the higher temperatures cannot be explained by an inability of the bacteria to establish infection in these fleas. Some qualitative differences related to temperature were seen in fleas infected with the green fluorescent protein—expressing Y. pestis strains described above. The microcolonies formed by Y. pestis in the midgut of fleas kept at 25°C and 30°C appeared to be less dense than the masses in fleas kept at 20°C. Y. pestis morphology in the flea midgut also changed with temperature: The bacteria were rod-shaped at 20°C, coccobacillary at 25°C, and coccoid at 30°C.
Figure 2. A. Digestive tract dissected from *X. cheopis* flea blocked with *Y. pestis* 195/P-wt. Proventriculus-blocking, brown-colored bacterial mass extends into anterior portion of midgut; independent large mass is also present in midgut (arrows). E = esophagus, PV = proventriculus, MG = midgut, HG = hindgut, MT = Malpighian tubules. Original magnification, \( \times 10 \). B. \( \times 1000 \) magnification view of edge of typical bacterial mass from midgut of infected flea. Individual bacteria cannot be seen because mass is covered with amorphous layer of what appears to be partially digested cellular material. This layer is removed during fixation prior to scanning electron microscopy (C), which reveals mass to be microcolony of *Y. pestis*. Bar = 6 \( \mu \)m.
Figure 3. Section of the proventriculus from uninfected flea (A) and from blocked fleas infected with *Y. pestis* 195/P-wt (B) or 195/P-1 (pPla<sup>−</sup>) (C). Interior wall of proventriculus is arrayed with cuticized proventricular spines (PS) that extend posteriorly into lumen and are hexagonal in cross-section. Bar = 2 μm.
Figure 4. Percentages of fleas still infected 4 weeks after single infectious blood meal containing wild type or plasmid-cured *Y. pestis*. Cohorts of fleas were kept at indicated temperatures. Bars indicate average, and vertical lines indicate range of 3 separate experiments, except for fleas infected with *Y. pestis* 195/P-wt and -pPla* kept at 20°C (2 experiments each) and *Y. pestis* 195/P-wt-infected fleas kept at 25°C and 30°C (1 experiment each). For each experiment, number of *Y. pestis* cfu in 20 individual, unblocked female fleas still alive 4 weeks after infection was determined. For all experiments, average number of cfu/positive flea was >10⁴. * Not done.

A possible role in fleaborne transmission for the 110-kb pFra plasmid. The preceding results indicate that the pPla and pYVp plasmids are not required for *Y. pestis* to infect and produce normal proventricular blockage in its flea vector. In contrast, the strain missing at least part of the pFra plasmid, *Y. pestis* 195/P-3 (pPla-ΔcaΔymt), was markedly deficient in the ability to block fleas. Only 1 (0.3%) of 323 fleas examined in three separate experiments developed blockage at 20°C following infection with this strain, compared to the 28%–45% blockage rate of the other 195/P strains tested (table 1). In this case, the defect could be explained by a simple inability of these bacteria to initiate normal colonization of the flea midgut. Normally, 40%–80% of fleas kept at 20°C are successfully infected and contain 10⁴–10⁵ *Y. pestis* 1 week after an infectious blood meal, and they remain infected throughout the 4-week observation period or until blockage and death occur (figure 4) [33]. In three experiments, only 1 (0.2%) of 60 fleas was still infected with *Y. pestis* 195/P-3 1 week after the infectious blood meal, and this flea contained <500 *Y. pestis*. Only 4 (6.7%) of 60 fleas examined 4 weeks after infection were positive, although these contained a normal bacterial load (average of 9.1 × 10⁴ *Y. pestis* per flea).

Discussion

Early this century, the Lister Institute’s Indian Plague Commission reported that fleaborne bubonic plague epidemics abruptly terminated with the onset of hot, dry weather [41, 42], an observation further confirmed by Cavanaugh in Vietnam during the 1960s [2, 9, 43]. Fleas of the genus *Xenopsylla*, chiefly *X. cheopis*, are the main vectors in those tropical locations [2, 43]. Laboratory studies later demonstrated that proventricular blockage does not develop normally in fleas kept at elevated temperatures [2, 9, 38, 44, 45], which, because blockage is prerequisite for biologic transmission of plague, could account for seasonal attenuation of plague epidemics. These epidemiologic and experimental data fit nicely with known in vitro abilities of *Y. pestis* to coagulate plasma weakly at ≤28°C but to rapidly
lyse fibrin at 28°–37°C [9]. Cavanaugh tied these observations together into a model for plague transmission. He interpreted the brown-colored, cohesive masses of plague bacilli that form in the flea midgut (figure 2), which previous workers had described as “colloidal” [46] or “gelatinous” [5, 8], as being fibrinous. This putative fibrin matrix was proposed to help anchor the bacteria in the proventriculus, where blockage eventually develops. Cavanaugh suggested that the Y. pestis coagulase activity, along with an endogenous flea midgut trypsin-like enzyme, serve to form the fibrin matrix of the blocking bacterial mass in the flea, but as the ambient temperature rises above 28°C, the Y. pestis fibrinolytic activity rapidly dissolves this matrix, thus precluding blockage and transmission [9].

The opposite, temperature-determined coagulase and fibrinolytic activities were later attributed to a single outer surface protease, the Y. pestis plasminogen activator, encoded by the pla gene present on the 9.5-kb Y. pestis plasmid [10, 11]. According to this model, then, the Y. pestis pla gene product has a critical role in fleaborne transmission.

In this study, we tested both parts of the Cavanaugh hypothesis, as well as the role of other Y. pestis plasmidborne genes in the flea vector. By means of an artificial feeding device, fleas were infected with a uniform dose of 1 of 2 different Y. pestis isolates or with isogenic derivatives that were cured of pPla. Flea infection and blockage rates were similar for both isolates or with isogenic derivatives that were cured of Y. pestis genes in the flea vector. By means of an artificial feeding device, hypothesis, as well as the role of other genes in the matrix, thus precluding blockage and transmission [9]. The opposite, temperature-determined coagulase and fibrinolytic activities were later attributed to a single outer surface protease, the Y. pestis plasminogen activator, encoded by the pla gene present on the 9.5-kb Y. pestis plasmid [10, 11]. According to this model, then, the Y. pestis pla gene product has a critical role in fleaborne transmission.

An alternative role for the Y. pestis plasminogen activator in transmission by flea bite has recently been described, one that occurs in the mammal and not the flea. Sodeinde et al. [48] found that the Y. pestis pla gene is required for the bacteria to disseminate from a subcutaneous inoculation site in mice. This would predict that susceptible mice fed upon by fleas blocked with pPla Y. pestis would not develop plague beyond an initial localized infection at the subcutaneous bite site, resulting in a sort of natural vaccination. We have not yet done such transmission experiments to directly test this. Not every Y. pestis strain requires the pla gene for spread from a subcutaneous site, however, and a role for the Pla protease in oral transmission of Y. pestis has also been proposed [49, 50]. Sodeinde et al. [48] also determined that the clot-forming ability of the Y. pestis pla gene product is not due to a true (thrombin-activating) coagulase mechanism but to a procoagulant, fibrinogen-free coagulase mechanism that does not yield the covalent cross-links between fibrin monomers that characterize a normal fibrin clot. Since we detected no role for the Y. pestis pla gene product in the insect host, it seems likely that the primary biologic function of the Pla protease occurs in the mammal and is based on its potent plasminogen activator capability at 37°C. The fact that the weak, low temperature-dependent procoagulant activity of the Y. pestis pla gene product has been detected only in rabbit plasma and not in mouse, rat, guinea pig, squirrel, or human plasma also suggests that this activity is not biologically relevant [21, 48, 51].

The course of Y. pestis infection that we observed in fleas kept at 30°C matches that of Y. pestis hms mutants, which also are able to establish long-term infection of the midgut but which never block fleas, even at 20°C [33]. The Y. pestis chromosomal hms (hemin storage) locus encodes two outer surface proteins that sequester large amounts of exogenous hemin (or structural analogs such as Congo red dye) to the Y. pestis outer membrane, giving rise to pigmented colonies on appropriate culture media; these proteins also confer a hydrophobic, autoaggregative cell phenotype [52–54]. An Hms+ phenotype is required for proventricular blockage of fleas, but expression of
the hms genes is down-regulated at ≥28°C [14, 52, 54]. Thus, decreased expression of the hms genes, rather than fibrinolysis due to the pla gene product, may explain the decreased ability of Y. pestis to block fleas at elevated temperatures.

Unlike blockage rates, mortality of infected fleas was high at all three experimental temperatures (table 1). Rapid starvation of blocked fleas accounted for excess mortality (above that seen for uninfected control fleas) at 20°C. Blocked fleas constituted fewer than half of excess deaths at 25°C; the remainder may have resulted from dehydration stress at the higher temperature due to incomplete blockage (bacterial colonization of the proventriculus causing impairment of its normal valvular function and decreased ingestion of blood) [55]. We previously found no flea morbidity associated with chronic infection with hms’ Y. pestis at 20°C, which can colonize the flea midgut but not the proventriculus [33]. At 30°C, even uninfected control fleas experienced high mortality. Although the fleas were maintained at constant 75% relative humidity, the saturation deficiency (the absolute difference between the actual humidity and the humidity of a saturated atmosphere at the same temperature) increases from ~4.5 to 7.9 mm Hg with a change from 20° to 30°C [42, 56]. Saturation deficiency more accurately reflects the drying capacity of an atmosphere, so twice-weekly feedings may have been insufficient to completely prevent dehydration of the 30°C fleas. A shortened flea lifespan, along with the decreased tendency to block, have been used to explain why X. cheopis–borne bubonic plague epidemics rapidly subside with the onset of hot, dry weather [43, 57].

We reconfirmed in this study that the 70-kb Yersinia virulence plasmid, which is required by all pathogenic yersiniae to produce disease in the mammal, is not required for Y. pestis to infect and block the flea [33, 58, 59]. The Y. pestis 195/P-3 strain, however, which lacks all or part of the 110-kb pFra plasmid, colonized <7% and blocked <1% of fleas at 20°C, much lower than normal rates. Adult fleas pulled randomly from laboratory colonies were used in these experiments, and the few individuals that were susceptible to infection by this strain may have been aged or otherwise compromised. The results indicate that one or more genes on pFra are required for normal flea infection or that a portion of pFra integrated into and disrupted a required chromosomal gene in the 195/P-3 strain. We are currently investigating the flea-specific genetic defect of this strain. One pFra gene candidate is ymt, which, like the hms genes, is up-regulated at the low temperatures that typify the flea gut environment and is down-regulated at 37°C [60]. The only other pFra genetic locus that has been characterized to date is the caf operon, which encodes the Y. pestis F1 capsule and which is known to be expressed at 37°C in the mammal but not in the flea [14, 61, 62]. Thus, the 110-kb plasmid may contain genes important for both the mammalian and insect host, whereas the two smaller plasmids are required only in the mammal. These results further support the concept that arthropod-borne bacteria require distinct sets of genes for life in their two very different hosts. Genes that underlie infection and pathogenesis in the mammalian host are referred to as virulence factors. The analogous genes that are specifically required to establish a transmission-competent infection in the arthropod vector we have termed transmission factors [33].

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