

Biofilm formation is not required for early-phase transmission of *Yersinia pestis*

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Early-phase transmission (EPT) is a recently described model of plague transmission that explains the rapid spread of disease from flea to mammal host during an epizootic. Unlike the traditional blockage-dependent model of plague transmission, EPT can occur when a flea takes its first blood meal after initially becoming infected by feeding on a bacteraemic host. Blockage of the flea gut results from biofilm formation in the proventriculus, mediated by the gene products found in the haemin storage (*hms*) locus of the *Yersinia pestis* chromosome. Although biofilms are required for blockage-dependent transmission, the role of biofilms in EPT has yet to be determined. An artificial feeding system was used to feed *Xenopsylla cheopis* and *Oropsylla montana* rat blood spiked with the parental *Y. pestis* strain KIM5(pCD1)+, two different biofilm-deficient mutants ($\Delta hmsT$, $\Delta hmsR$), or a biofilm-overproducer mutant ($\Delta hmsP$). Infected fleas were then allowed to feed on naïve Swiss Webster mice for 1–4 days after infection, and the mice were monitored for signs of infection. We also determined the bacterial loads of each flea that fed upon naïve mice. Biofilm-defective mutants transmitted from *X. cheopis* and *O. montana* as efficiently as the parent strain, whereas the EPT efficiency of fleas fed the biofilm-overproducing strain was significantly less than that of fleas fed either the parent or a biofilm-deficient strain. Fleas infected with a biofilm-deficient strain harboured lower bacterial loads 4 days post-infection than fleas infected with the parent strain. Thus, defects in biofilm formation did not prevent flea-borne transmission of *Y. pestis* in our EPT model, although biofilm overproduction inhibited efficient EPT. Our results also indicate, however, that biofilms may play a role in infection persistence in the flea.

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INTRODUCTION

Yersinia pestis infection is characterized as a rapidly spreading disease that cycles between rodents and fleas (Gage & Kosoy, 2005; Pollitzer, 1954). Humans are also seriously affected, as indicated by the occurrence of three historical pandemics that killed millions (Perry & Fetherston, 1997). The transmission of *Y. pestis* from flea to host occurs primarily via flea bite; however, the mechanisms of transmission remain under investigation. Fleas could also play a significant role as reservoirs of infection, providing that they are capable of sustaining *Y. pestis* infections over extended periods of time. The most studied form of transmission of *Y. pestis* by fleas is the Bacot and Martin model, in which transmission depends on development of a biofilm-dependent proventricular

blockage that begins to form when a flea ingests a blood meal from an infected mammalian host (Bacot & Martin, 1914; Bacot, 1915; Eskey & Haas, 1940). In this process, the bacteria multiply and produce a biofilm inside the flea that eventually forms a blockage in the proventriculus of certain fleas such as *Xenopsylla cheopis*, which is a species that is the major vector of plague in many foci around the world (Gage & Kosoy, 2005; Hinnebusch *et al.*, 1996; Jarrett *et al.*, 2004). The blockage prevents the flea from taking a full blood meal, resulting in eventual starvation and repeated attempts by starving fleas to take blood meals from multiple hosts. During these feeding attempts, it is presumed that pieces of the blockage matrix containing the bacteria are regurgitated into the host, thereby infecting them (Burroughs, 1947; Eskey & Haas, 1940; Pollitzer, 1954). The possible role of biofilms in the maintenance of *Y. pestis* in fleas has received little attention, although it has been suggested that they may be important for colonization

Abbreviations: c-di-GMP, cyclic-dimeric GMP; CR, Congo Red; EPT, early-phase transmission; p.i., post-infection.

of fleas by the bacteria, and of soil organisms such as nematodes and protists, which have been suggested by some to be potential reservoirs of *Y. pestis* in soils (Darby *et al.*, 2002; Udovikov *et al.*, 2009).

Although this blockage-dependent mechanism is well described and undoubtedly important, it cannot support the rapid rate of spread that characterizes many plague epidemics or epizootics, particularly those that involve transmission by fleas that require a long extrinsic incubation period of perhaps two or more weeks to become blocked, and then experience a short window of infectivity before flea death (Drancourt *et al.*, 2006; Eisen *et al.*, 2006; Wood *et al.*, 2003). In addition, blockage formation is rare in many species of fleas, including those that, based on epidemiological data, are thought to be important vectors for plague (Eisen *et al.*, 2009). For example, despite its poor blocking capability, *Oropsylla montana* is considered a primary vector of *Y. pestis* to humans in the USA, and is the major flea found on rock squirrels and ground squirrels that routinely succumb to plague epizootics. Even in blockage-forming fleas, such as *X. cheopis*, the rate of blockage formation is only 25–45% (Burroughs, 1947; Eskey & Haas, 1940; Gage & Kosoy, 2005; Hinnebusch *et al.*, 1996). Recently, Eisen *et al.* (2006) described an alternative form of transmission termed ‘early-phase transmission’ (EPT). In this model, fleas are fed a blood meal, and were then allowed to feed on naïve mice for 3–96 h after infection. Transmission occurs each day for at least 4 days after fleas become infected, and is efficient enough to provide an explanation of the temporal dynamics in fast-moving plague epizootics (Eisen *et al.*, 2006, 2007b). One day, or even 4 days, provides insufficient time to form a block in *X. cheopis*, and this suggests that an alternative mechanism of transmission is required to explain these results.

The *hms* genes that create and regulate biofilms are located in three identified operons: *hmsHFRS*, *hmsT* and *hmsP* (Hinnebusch *et al.*, 1996; Jones *et al.*, 1999; Kirillina *et al.*, 2004; Perry *et al.*, 1990). The *hmsHFRS* operon is a region within the *pgm* locus, whose gene products are responsible for the adsorption of vast quantities of exogenous haemin and structurally similar Congo Red (CR) dye (Jackson & Burrows, 1956; Surgalla & Beesley, 1969). Together, these gene products are predicted to synthesize the β -1,6-*N*-acetylglucosamine-like polymers that form biofilms (Bobrov *et al.*, 2008; Hinnebusch *et al.*, 1996; Itoh *et al.*, 2005; Kirillina *et al.*, 2004; Perry *et al.*, 1990, 2004). Synthesis of biofilm exopolysaccharide is initiated by the putative formation of a cyclic-dimeric GMP (*c*-di-GMP)-dependent HmsR–HmsS complex (Bobrov *et al.*, 2008). HmsR is a putative glycosyltransferase with a conserved glycosyltransferase D,D,D35QXXRW motif, and is critical for biofilm formation and CR binding in *Y. pestis* (Forman *et al.*, 2006). The amount of local *c*-di-GMP is proposed to be tightly regulated by the gene products of *hmsT* and *hmsP* (Bobrov *et al.*, 2008; Kirillina *et al.*, 2004). HmsT is a diguanylate cyclase with a signature GGDEF domain, and

promotes the formation of higher levels of *c*-di-GMP, thus increasing biofilm production, while HmsP contains an EAL phosphodiesterase motif, and is required for degrading *c*-di-GMP (Bobrov *et al.*, 2005; Jones *et al.*, 1999; Kirillina *et al.*, 2004; Perry *et al.*, 2004; Simm *et al.*, 2005). Deletion of *hmsT* leads to a large decrease in *c*-di-GMP-dependent production of the poly- β -1,6-*N*-acetylglucosamine-like polysaccharide, and deletion of *hmsP* results in overproduction of biofilm due to the loss of the encoded *c*-di-GMP phosphodiesterase (Bobrov *et al.*, 2008; Kirillina *et al.*, 2004).

Our study sought to determine whether biofilm formation was necessary for EPT. We used two different species of fleas (*X. cheopis* and *O. montana*), to investigate whether biofilm formation differed in a vector (*X. cheopis*) which routinely becomes blocked compared with another epidemiologically important vector (*O. montana*) which does not become blocked. Our results showed that biofilm was not necessary for EPT, and that the overproduction of biofilm was actually inhibitory to EPT. Moreover, studies that quantified bacterial loads in fleas indicated a potential role for biofilms in the maintenance of the bacteria in the flea.

METHODS

Bacterial growth conditions and construction of mutants.

Bacterial strains used in this study are listed in Table 1. The biofilm-defective mutants [Δ *hmsR* (KIM6-2118) and Δ *hmsT* (KIM6-2051.6+)] form white colonies on CR plates incubated at 26 °C, in contrast to the red ‘pigmented’ colonies formed by their parental Hms⁺ strain (KIM6+). The biofilm-overproducing strain KIM6-2090.7+ (Δ *hmsP*) is characterized by the formation of pigmented colonies on CR plates at both 26 and 37 °C (Bobrov *et al.*, 2008; Jones *et al.*, 1999; Kirillina *et al.*, 2004; Perry *et al.*, 2004). Although the *hms* locus is part of the *pgm* locus, none of the *hms* mutations interferes with the yersiniabactin-mediated iron-acquisition system of the *pgm* locus or affects virulence in the mammalian host (Abu Khweek *et al.*, 2010; Bearden *et al.*, 1997; Burrows & Jackson, 1956a, b; Fetherston *et al.*, 1992; Lillard *et al.*, 1999; Perry *et al.*, 1990).

The *hmsT* deletion mutant (KIM6-2051.6+) was constructed using lambda red recombinase (Datsenko & Wanner, 2000). The kanamycin-resistance (Km^r) cassette was amplified from pKD4 using primer pair deltaT-5′ (5′-ATGCAGAGTAAATTGAATATGAATAGCCACT-CCTACGATCGTGTAGGCTGGAGCTGCTTC-3′) and deltaT-3′ (5′-AGGGGAAGACTGTACATTTGATAATTCATCTTTAGCAAATCAT-ATGAATATCCTCCTTAG-3′). The PCR product was electroporated into KIM6(pKD46)+, and the culture was plated on CR plates containing kanamycin. Cells that formed white colonies were checked using primers 10-2inv (5′-GTGAAATTAACGTGCAG-3′) and 11BinV (5′-TATTTGTCGTGATGTCGG-3′) for the deletion of *hmsT* and the insertion of the Km^r cassette, which was eliminated by introducing pCP20 [encoding the F₁ recombinase, and ampicillin (Ap^r) and chloramphenicol resistance (Cm^r)] (Cherepanov & Wackernagel, 1995; Datsenko & Wanner, 2000). To remove pCP20, isolated colonies were streaked onto tryptose blood agar (TBA) base plates and grown at 40 °C. Individual colonies from these plates were grown in a 96-well dish at 37 °C, and then replica-plated onto TBA plates containing ampicillin and TBA containing chloramphenicol. Sensitivities to kanamycin and the presence of the *pgm* locus were checked as described elsewhere (Perry & Bearden, 2008). One colony,

Table 1. Bacterial strains and plasmids

Y. pestis strains marked with '+' carry the wild-type 102 kb *pgm* locus, while those without '+' have one or more mutations, or a deletion, in the *pgm* locus. Hms⁻ indicates the presence of an intact *pgm* locus, but with deletions in the *hmsHFRS*, *hmsT* or *hmsP* operons; these render the strain incapable of producing wild-type levels of biofilm. Hms^C indicates an intact *pgm* locus coupled with the loss of *hmsP*; this allows for temperature-independent expression of biofilm.

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
KIM6+	Pgm ⁺ Lcr ⁻ Pla ⁺ ; pPCP1, pMT1	Fetherston <i>et al.</i> (1992)
KIM5(pCD1Ap)+	Ap ^r Pgm ⁺ , pPCP1+, pMT+, pCD1Ap (' <i>yadA::bla</i> '); derived from KIM6+	Gong <i>et al.</i> (2001)
KIM6-2051.6+	Hms ⁻ (Δ <i>hmsT</i>); Lcr ⁻ Pla ⁺ ; pPCP1, pMT1	This study
KIM5-2051.6(pCD1Ap)+	Ap ^r Hms ⁻ (Δ <i>hmsT2090.2</i>), pPCP1, pMT1, pCD1Ap (' <i>yadA::bla</i> '); derived from KIM6-2051.6	This study
KIM6-2090.1+	Cm ^r Hms ^C (Δ <i>hmsP::cam2090.1</i>) Lcr ⁻ Pla ⁺ ; pPCP1, pMT1	Kirillina <i>et al.</i> (2004)
KIM6-2090.2+	Hms ^C (Δ <i>hmsP::cam2090.1</i>) Lcr ⁻ Pla ⁺ ; pPCP1, pMT1; derived from KIM6-2090.1+	This study
KIM6-2090.7+	Hms ^C , Δ <i>hmsP</i> , pPST+, pMT+, pCD ⁻ , Km ^r ; derived from KIM6-2090.2+	This study
KIM5-2090.7(pCD1Ap)+	Km ^r Ap ^r Hms ^C (Δ <i>hmsP2090.2</i>) Δ y2360:: <i>kan2093.1</i> , pPCP1, pMT1, pCD1Ap (' <i>yadA::bla</i> '); derived from KIM6-2090.7	This study
KIM6-2118	Hms ⁻ (in-frame Δ <i>hmsR2118</i>) Lcr ⁻ Pla ⁺ ; pPCP1, pMT1	Forman <i>et al.</i> (2006)
KIM5-2118(pCD1Ap)	Ap ^r Hms ⁻ (in-frame Δ <i>hmsR2118</i>) Lcr ⁺ Pla ⁺ ; pPCP1, pMT1, pCD1Ap (' <i>yadA::bla</i> '); derived from KIM6-2118	This study
Plasmids		
pKD4	3.3 kb, Km ^r , template plasmid	Datsenko & Wanner (2000)
pKD46	6.3 kb, Ap ^r , Red recombinase expression plasmid	Datsenko & Wanner (2000)
pCP20	Suicide plasmid with temperature-sensitive replication and thermally induced expression of FLP recombinase; Ap ^r	Datsenko & Wanner (2000)
pCD1Ap	70.5 kb pCD1 with <i>bla</i> cassette inserted into ' <i>yadA</i> '; 71.7 kb Lcr ⁺ Ap ^r	Gong <i>et al.</i> (2001)

which was kanamycin-sensitive and retained the *pgm* locus, was selected for further analysis.

The *hmsP* mutant used in this study was derived from KIM6-2090.1+ (Δ *hmsP::cam2090.1*). The Cm^r cassette was first excised by introducing the pCP20 plasmid, as described above, creating KIM6-2090.2+ (Δ *hmsP2090.1*). To mark the *pgm* region with a Km^r cassette, red-mediated recombination (Datsenko & Wanner, 2000) was again employed to insert the Km^r cassette into y2360, which is located in the *pgm* locus, and encodes a putative endopeptidase. This created KIM6-2090.7+ [Δ *hmsP2090.2* (Δ y2360::*kan2093.1*)]. The Δ y2360::*kan2093.1* mutation was confirmed by PCR using primers previously described for confirming a similar mutation, Δ y2360::*cam2093* (Bobrov *et al.*, 2008; Jones *et al.*, 1999; Kirillina *et al.*, 2004; Perry *et al.*, 2004).

The KIM6-2051.6+ (Δ *hmsT*), KIM6-2090.7+ (Δ *hmsP*) and KIM6-2118 (in-frame Δ *hmsR2118*) strains were electroporated with pCD1Ap to restore the type III secretion and effector protein virulence factors, creating strains KIM5-2051.6(pCD1Ap)+, KIM5-2090.7(pCD1Ap)+ and KIM5-2118(pCD1Ap), respectively (Table 1). Transformants were then tested by using PCR and plasmid profile analysis to confirm the presence of the plasmid (Chu, 2000). To confirm virulence, 1×10^4 c.f.u. of each strain was inoculated subcutaneously into two 6-week-old naïve Swiss Webster mice. Upon appearance of signs of plague illness (described below), each mouse was euthanized. Each strain was then reisolated from the liver of each mouse, and glycerol stocks [0.5 ml aliquots containing $\sim 1 \times 10^6$ c.f.u. in heart infusion broth (HIB) supplemented with 10% (v/v) glycerol] were made to use with each subsequent artificial feeding trial. This procedure was also used to confirm the virulence of each strain for each artificial feed trial performed. When required,

carbenicillin was used at a concentration of 50 μ g ml⁻¹ and kanamycin at 25 μ g ml⁻¹.

To determine the Pgm⁺ phenotype retention rate in the fleas, 10 fleas from each artificial feeding that were infected with either the parent KIM5(pCD1Ap)+ strain or the biofilm-overproducing strain (Δ *hmsP*) were selected at random. A 0.1 ml aliquot of a 10^{-3} dilution of the flea homogenate was plated on CR plates in duplicate, and the ratio of red to white colonies was counted. In all, about 1000 colonies were counted for each group.

Infection of fleas. Fleas were fed using an artificial feeding system described previously (Eisen *et al.*, 2006). Briefly, on day 0, batches (150 fleas per feeder) of colony-reared *O. montana* or *X. cheopis* were allowed to feed on artificial feeders containing blood infected with approximately 1×10^9 c.f.u. ml⁻¹ of KIM5(pCD1Ap)+, KIM5-2090.7(pCD1Ap)+, KIM5-2118(pCD1Ap) or KIM5-2051.6(pCD1Ap)+. To prepare infected blood, a 0.5 ml aliquot of each bacterial stock (prepared as described above) was inoculated into 60 ml HIB. The culture was grown for 16 h at 28 °C and 175 r.p.m. to $\sim 1 \times 10^9$ c.f.u. ml⁻¹. Bacteria were collected by centrifugation, and resuspended in 60 ml defibrinated Sprague Dawley strain rat blood (Bioreclamation). The fleas were allowed to feed for 1 h, and were then examined by light microscopy. Only fed fleas with red blood in the proventriculus or mid-gut were kept, while unfed fleas were discarded. Potentially infected fleas were held at 23 °C and 85% relative humidity for 1–4 days post-infection (p.i.). An artificial feeding of either species of flea normally results in 50–95% of fleas taking an infectious blood meal, and of those fed fleas, 90% are infected (Eisen *et al.*, 2006).

Transmission of *Y. pestis* from fleas to mice. On each of the 4 days p.i., pools of 10 potentially infectious fleas were added to a

feeding capsule affixed to seven 6-week-old naïve Swiss Webster mice, and the fleas were allowed to feed for 1 h (Eisen *et al.*, 2008b). The fleas were then removed by a mechanical aspirator, and examined by light microscopy to determine whether they had consumed a blood meal. Normally, 5–10 of 10 fleas take a blood meal upon the mouse (Eisen *et al.*, 2006, 2007a, 2008a). The fed fleas were stored in individual centrifuge tubes at -80°C until flea bacterial loads could be determined by serial dilution (Eisen *et al.*, 2006). The mice were housed in individual cages, and monitored daily for signs of *Y. pestis* infection (e.g. shivering, lethargy, ruffled fur). Mice showing signs of infection were euthanized and necropsied, and slide preparations of their livers and spleens were examined for the presence of bacteria through the use of an immunofluorescent antibody to the *Y. pestis* F1 antigen (Chu, 2000). Mice that did not show signs of illness were euthanized 21 days after exposure to the infected fleas and tested serologically for exposure to *Y. pestis*. All animal procedures were approved by the Division of Vector-Borne Infectious Diseases (Centers for Disease Control and Prevention) Institutional Animal Care and Use Committee.

Statistical analysis. The estimation of EPT efficiency was based on the number of infected fleas that fed on an individual mouse, and whether or not transmission was observed for that recipient mouse, by using the Microsoft Excel Add-In PooledInfRate version 3.0 (Biggerstaff, 2006; Eisen *et al.*, 2006, 2007b). The resulting number is a prediction of the expected number of transmission events in a sample pool of 100 mice. The bacterial load of each flea was categorized as high ($>1 \times 10^4$ c.f.u. per flea), low (1×10^1 – 1×10^4 c.f.u. per flea) or uninfected (0 c.f.u. per flea). The percentage of fleas in the high category was compared with those in the other two categories combined by using two-tailed Fisher's exact tests. Results

were included from at least three artificial feeding experiments for each bacterial strain and each flea species tested. All statistical comparisons were performed using JMP statistical software (SAS Institute), and were considered statistically significant at $P < 0.05$.

RESULTS

Biofilm is not required for EPT

When assessed in *X. cheopis* fleas, which routinely become blocked, EPT of *Y. pestis* was observed at three of the four time points in both the $\Delta hmsT$ mutant and the $\Delta hmsR$ mutant (Table 2). Transmission was demonstrated for the diguanylate cyclase mutant KIM6-2051.6(pCD1Ap) + ($\Delta hmsT$) on days 1–3 p.i. in 86 % (six of seven) to 57 % (four of seven) of mice exposed to infected fleas. When fleas were infected with the $\Delta hmsR$ mutant KIM6-2118(pCD1Ap), transmission events were observed in 43 % (three of seven) to 57 % (four of seven) of mice on days 1–3 p.i. of the fleas. We did not observe EPT of either the $\Delta hmsT$ or the $\Delta hmsR$ strain on day 4 p.i. Transmission efficiencies of these two mutants were comparable with those of the parent strain KIM5(pCD1Ap) + (Table 2). Fleas infected with KIM5(pCD1Ap) + were able to transmit infection on days 1, 2 and 4, with transmission events occurring in 14 % (one of seven) to 42 % (three of seven) of mice exposed.

Table 2. Transmission efficiencies of *X. cheopis* for KIM6+ and *hms* mutant strains

Strain	Day p.i.	No. of transmission events*	Estimated transmission efficiency per time point†	Estimated transmission efficiency per strain for all time points†
Parent KIM5(pCD1Ap) +	1	3 (7)	7.60 (2.46–18.86)	3.48 (1.48–7.02)
	2	2 (7)	4.38 (0.84–13.97)	
	3	0 (7)	0.00 (0.00–6.24)	
	4	1 (7)	2.10 (0.12–10.19)	
Biofilm-negative $\Delta hmsR$ KIM6-2118(pCD1Ap)	1	3 (7)	5.75 (1.6–15.74)	5.14 (2.66–9.15)
	2	4 (7)	9.37 (3.13–24.03)	
	3	3 (7)	6.09 (1.67–16.09)	
	4	0 (7)	0.00 (0.00–5.21)	
Biofilm-reduced $\Delta hmsT$ KIM6-2051.6 (pCD1Ap) +	1	6 (7)	20.28 (10.22–40.28)	9.83 (5.66–16.11)
	2	3 (7)	8.78 (2.53–23.02)	
	3	4 (7)	13.04 (4.31–33.04)	
	4	0 (7)	0.00 (0.00–6.95)	
Biofilm-overproducer $\Delta hmsP$ KIM6-2090.7(pCD1Ap) +	1	0 (7)	0.00 (0.00–5.55)	0.00 (0.00–1.99)
	2	0 (7)	0.00 (0.00–6.85)	
	3	0 (7)	0.00 (0.00–6.90)	
	4	0 (7)	0.00 (0.00–7.10)	

*Total number of mice tested in parentheses.

†95 % confidence interval in parentheses.

When tested with *O. montana*, which is a flea that rarely becomes blocked, biofilms were again not required for EPT (Table 3). Both $\Delta hmsT$ and $\Delta hmsR$ mutants were transmitted from infected fleas to naïve mice at all time points, ranging in efficiency from 14 % (one of seven mice) to 71 % (five of seven mice), and 28 % (two of seven mice) to 86 % (six of seven mice), for all instances in which infected fleas were fed on plague-susceptible mice, respectively. The parent KIM5(pCD1Ap) + strain was transmitted on days 1, 2 and 4, with between 14 and 42 % of exposed mice becoming infected. As seen in *X. cheopis*, *O. montana* could transmit the biofilm-deficient strains at rates comparable with those of the parent strain. Although one flea-mediated EPT event, as indicated by seroconversion, was demonstrated on day 2 p.i. for the biofilm-overproducing strain, the level of transmission was significantly lower than that observed for the parent strain (Table 3).

Biofilm effects in the flea

Bacterial loads were quantified for all potentially infected fleas that fed on naïve mice. Preliminary observations of the data suggested that bacterial loads in the fleas fell into two reasonably distinct groups: one group harbouring $>10^4$ c.f.u. per flea, and the other with $<10^4$ c.f.u. per flea. Accordingly, the bacterial load for each flea was then categorized as high bacterial load ($>10^4$ c.f.u. per flea), low bacterial load

($1-10^4$ c.f.u. per flea) or uninfected (Figs 1 and 2). *X. cheopis* infected with the parent strain KIM5(pCD1Ap) + retained a high bacterial load over 4 days p.i. (>85 %) (Fig. 1a). In contrast, *X. cheopis* infected with either the biofilm-reduced strain [$\Delta hmsT$, KIM5-2051.6(pCD1Ap) +] or the biofilm-negative strain [$\Delta hmsR$, KIM5-2118(pCD1Ap)] experienced a decrease in bacterial load over 4 days p.i. The percentage of fleas infected with high bacterial loads of the biofilm-reduced strain was significantly decreased ($P=0.003$) compared with non-infected fleas and fleas harbouring low bacterial loads, on days 1–4 p.i. (93.2–68.1 %, respectively) (Fig. 1c), or found to be uninfected. The percentage of fleas infected with high bacterial loads of the biofilm-negative strain on days 3 and 4 p.i. was significantly lower than for fleas tested on day 1 p.i. ($P=0.003$). Bacterial loads in fleas tested on day 2 p.i. did not differ significantly from those observed in fleas tested on day 1 p.i.

Results with the poorly blocking species *O. montana* were similar to those obtained with *X. cheopis* (Fig. 2). *O. montana* infected with the parent strain experienced a gradual decrease from day 1 to day 4 p.i. in the proportion of fleas infected with high bacterial loads, with significantly fewer ($P=0.001$) fleas with high bacterial loads (51.0 %) on day 4 p.i. compared with those with a similar load on day 1 p.i. (82.9 %) (Fig. 2a). Fleas infected with either the biofilm-reduced or the biofilm-negative strain showed a much more dramatic decrease in the proportion of

Table 3. Transmission efficiencies of *O. montana* for KIM6+ and *hms* mutant strains

Strain	Day p.i.	No. of transmission events*	Estimated transmission efficiency per time point†	Estimated transmission efficiency per strain for all time points†
Parent KIM5(pCD1Ap) +	1	1 (7)	1.72 (0.10–8.42)	3.21 (1.33–6.65)
	2	3 (7)	6.46 (1.76–18.48)	
	3	0 (7)	0.00 (0.00–6.14)	
	4	2 (7)	4.76 (0.88–15.70)	
Biofilm-negative $\Delta hmsR$ KIM6-2118(pCD1Ap)	1	2 (7)	3.36 (0.63–11.06)	7.88 (4.34–13.41)
	2	6 (7)	18.75 (8.53–38.76)	
	3	4 (7)	9.69 (3.46–23.18)	
	4	5 (7)	14.75 (5.91–34.01)	
Biofilm-reduced $\Delta hmsT$ KIM6-2051.61(pCD1Ap) +	1	5 (7)	10.93 (4.33–26.27)	10.85 (6.77–16.82)
	2	5 (7)	17.33 (6.63–37.33)	
	3	1 (7)	2.15 (0.13–10.54)	
	4	1 (7)	2.55 (0.15–12.18)	
Biofilm-overproducer $\Delta hmsP$ KIM6-2097.7(pCD1Ap) +	1	0 (7)	0.00 (0.00–5.36)	0.46 (0.03–2.23)
	2	1 (7)	1.76 (0.11–8.49)	
	3	0 (7)	0.00 (0.00–5.43)	
	4	0 (7)	0.00 (0.00–5.81)	

*Total number of mice tested in parentheses.

†95 % confidence interval in parentheses.

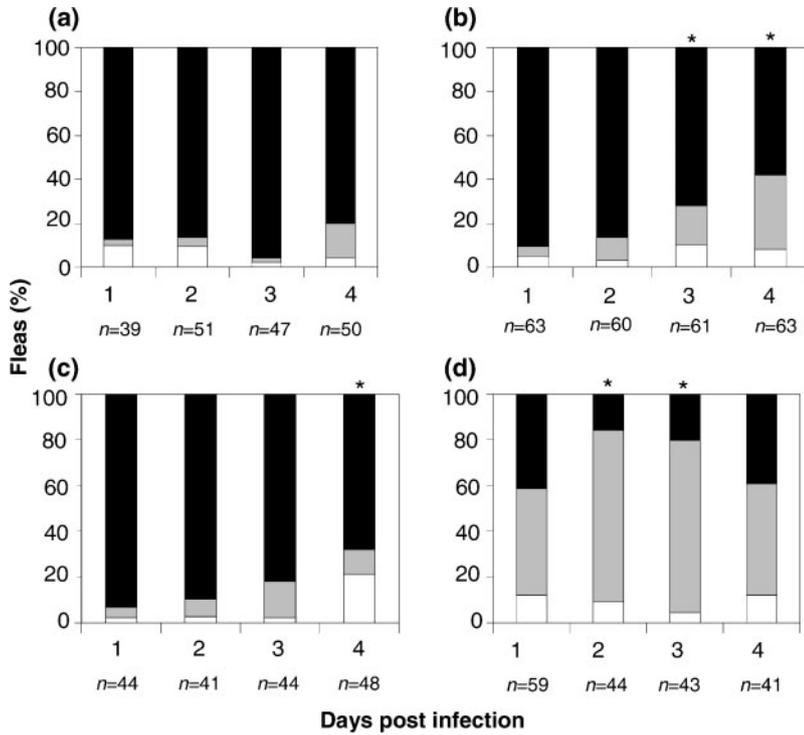


Fig. 1. Percentages of *X. cheopis* that were uninfected, infected with high bacterial load (>10⁴) or infected with low bacterial load (<10⁴) at 1–4 days p.i. Fleas received an infectious blood meal with *Y. pestis*: (a) parent strain, (b) biofilm-negative strain, (c) biofilm-reduced strain and (d) biofilm-overproducing strain. The infected fleas were then allowed to feed on naïve mice for 1–4 days p.i. Fleas taking blood meals on naïve mice were harvested after the blood feed, and it was determined whether their bacterial load was high (>1×10⁴ c.f.u. per flea; black bar), low (>0, but <1×10⁴ c.f.u. per flea; grey bar) or zero (0 c.f.u. per flea; white bar). An asterisk indicates that the percentage of fleas harbouring >1×10⁴ c.f.u. *Y. pestis* was significantly different (P<0.05) from that of fleas carrying <1×10⁴ c.f.u. of the same strain on day 1 p.i., as determined by two-tailed Fisher’s exact test.

fleas with high bacterial loads, with each group experiencing a significant drop (P<0.001 and P=0.001, respectively) in fleas with a high bacterial load by day 2 p.i. (Fig. 2b, c).

Results for the biofilm-overproducing strain KIM5-2090.7(pCD1Ap)+ ($\Delta hmsP$) were different from those for the parent and biofilm-deficient strains. In *X. cheopis*, bacterial loads were lower in fleas infected with this strain

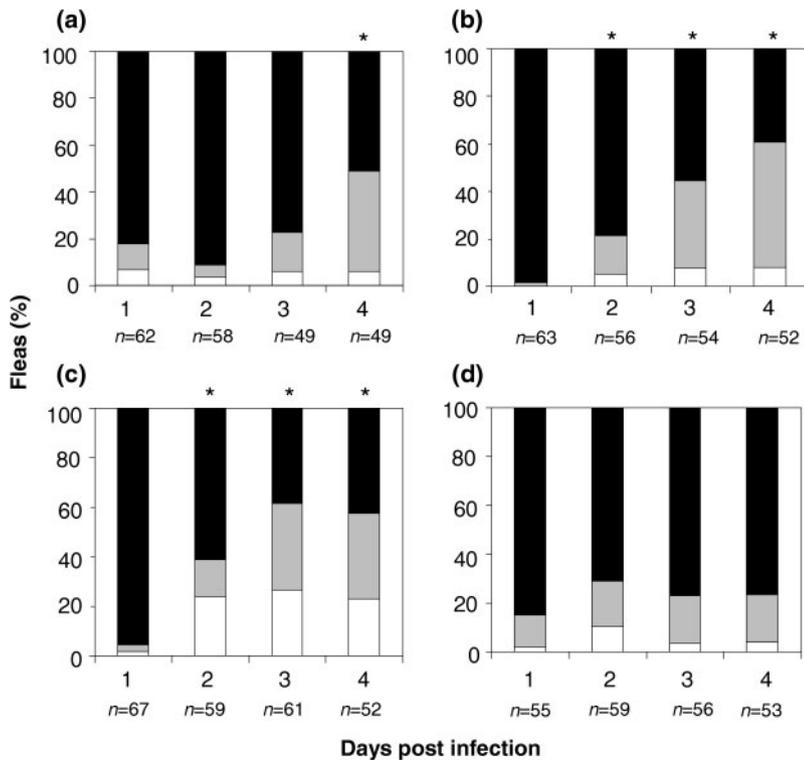


Fig. 2. Percentages of *O. montana* that were uninfected, infected with high bacterial loads (>10⁴) or infected with low bacterial loads (<10⁴) at 1–4 days p.i. Fleas received an infectious blood meal with *Y. pestis*: (a) parent strain, (b) biofilm-negative strain, (c) biofilm-reduced strain and (d) biofilm-overproducing strain. The infected fleas were then allowed to feed on naïve mice for 1–4 days p.i. Fleas taking blood meals on naïve mice were harvested after the blood feed, and it was determined whether their bacterial load was high (>1×10⁴ c.f.u. per flea; black bar), low (>0, but <1×10⁴ c.f.u. per flea; grey bar) or zero (0 c.f.u. per flea; white bar). An asterisk indicates that the percentage of fleas harbouring >1×10⁴ c.f.u. *Y. pestis* was significantly different (P<0.05) from that of fleas carrying <1×10⁴ c.f.u. of the same strain on day 1 p.i., as determined by two-tailed Fisher’s exact test.

compared with those infected with the parent strain (Fig. 1d). To show that this effect on c.f.u. was indeed due to fewer bacteria in the flea, and not to clumping of the bacilli because of increased biofilm production, which would result in artificially low c.f.u. values, we performed quantitative PCR on flea lysates (Gabitzsch *et al.*, 2008). Quantitative PCR assay results were highly correlated with the bacterial counts (data not shown), confirming that the bacterial loads observed in *X. cheopis* infected with the biofilm-overproducing strain were indeed lower than the bacterial loads in *X. cheopis* infected with the other strains. Moreover, this strain did not show a growth defect in *O. montana*, as indicated by the c.f.u. counts of bacteria in infected fleas (Fig. 2). However, unlike the other strains, the percentage of fleas with a high or low bacterial load in both *X. cheopis* and *O. montana* infected with the biofilm-overproducing strain did not significantly change over 1–4 days p.i. The proportions of fleas with high bacterial loads on days 1–4 p.i. were similar for both *X. cheopis* and *O. montana* (Figs 1d and 2d).

The ability of each strain to retain its Pgm⁺ status (a virulence characteristic conferred by the yersiniabactin biosynthetic and transport loci encoded in this region) was then tested. As expected, fleas harbouring the parent strain KIM5(pCD1Ap)⁺ contained nearly 100% Hms⁺ colonies (Table 4). However, in both flea species, the biofilm-overproducing strain KIM5-2090.7(pCD1Ap)⁺, isolated from *X. cheopis*, lost the Hms⁺ phenotype at a higher frequency than the parent strain. Interestingly, this phenomenon was not observed in the $\Delta hmsP$ mutant cells

isolated from *O. montana* (Table 4). Only 5–13% of KIM6-2090.7(pCD1Ap)⁺ colonies isolated from *X. cheopis* were CR-negative at days 3 and 4 p.i., presumably indicating either a partial or an incomplete excision of the *pgm* locus, and suggesting that most colonies retained the *hms* genes required for the pigmentation phenotype; this is unlikely to explain the lack of EPT of the biofilm-overproducing strain in this flea species (Table 4). While maintaining a high proportion of Pgm⁺ cells (>98%), the EPT rate for this biofilm overproducer was also low in *O. montana* (Table 3).

DISCUSSION

EPT is the period of time during which fleas can transmit *Y. pestis* bacilli before a blockage can form. EPT of plague bacteria by *X. cheopis* and *O. montana* occurs for at least 4 days following the ingestion of an infectious blood meal, and it has been proposed as a mechanism to explain the temporal dynamics of rapidly moving plague epizootics in rodent populations (Eisen *et al.*, 2006, 2007a). Our study used two biofilm-defective mutants to determine whether biofilms are necessary for EPT. Both $\Delta hmsT$ and $\Delta hmsR$ mutants are biofilm-negative *in vitro*. However, while the putative glycosyltransferase HmsR is essential for biofilm formation, HmsT is only one of several potential diguanylate cyclases in the *Y. pestis* KIM genome. Thus, it is possible that one of these other diguanylate cyclases produces c-di-GMP in the flea, allowing *in vivo* biofilm development. Consequently, we tested both mutants.

Table 4. Percentage of Pgm⁺ colonies on days 1–4 p.i. of the parent or biofilm-overproducing strain from either *X. cheopis* or *O. montana*

Flea species	Bacterial strain	Day p.i.	Mean percentage of Pgm ⁺ colonies*
<i>X. cheopis</i>	Parent KIM5(pCD1Ap) ⁺	1	99.9 (99.1–100.0)
		2	99.8 (99.5–100.0)
		3	99.9 (99.3–100.0)
		4	99.9 (99.8–100.0)
	Biofilm overproducer $\Delta hmsP$ KIM6-2097.7(pCD1Ap) ⁺	1	99.4 (98.1–100.0)
		2	96.3 (95.0–100.0)
		3	87.4 (68.3–100.0)
		4	95.8 (78.4–100.0)
<i>O. montana</i>	Parent KIM5(pCD1Ap) ⁺	1	98.6 (87.5–100.0)
		2	100.0 (100.0–100.0)
		3	100.0 (100.0–100.0)
		4	100.0 (100.0–100.0)
	Biofilm overproducer $\Delta hmsP$ KIM6-2097.7(pCD1Ap) ⁺	1	100.0 (100.0–100.0)
		2	99.4 (95.3–100.0)
		3	99.5 (97.2–100.0)
		4	99.8 (78.4–100.0)

*The range is given in parentheses.

Unlike the biofilm-dependent classic model of transmission, our results show that *hms*-mediated biofilm formation is not required for EPT, which likely involves an alternative mechanism. One potential alternative mechanism involves mechanical transmission, in which bacteria survive without multiplication on the external surface of the mouthparts or in the gut of the flea prior to being transmitted to a susceptible host (Burroughs, 1944; Eskey & Haas, 1940). However, this mechanism is unlikely to explain EPT because *Y. pestis* can only survive on mouthparts for up to 3 h, which is much shorter than the infectious time period presented here (Bibikova, 1965). Furthermore, Rose *et al.* (2003) have also demonstrated the lack of survivability of *Y. pestis* on certain environmental surfaces, indicating that one or more additional mechanisms besides mechanical transmission is likely to be involved in EPT (Rose *et al.*, 2003). An alternative mechanism may involve aggregates of *Y. pestis* bacilli that develop in the anterior portion of the digestive tract of the flea (Eisen & Gage, 2009). Presumably, the formation of such aggregates might depend on biofilm formation, although our results indicate that strains that overproduce biofilm are less likely to be transmitted than parent strains. These results were somewhat surprising, and it is possible that a biofilm overproducer would form bacterial aggregates too large to pass through the mouthparts and into the bite site of a naïve mammalian host. Regardless, aggregate formation, even at the lower levels exhibited by the parent strain in our experiments, clearly is not required for EPT, as demonstrated by our experiments with biofilm-negative *Y. pestis* strains.

Results shown in our study provide strong evidence to suggest that biofilms are critical for maintenance of *Y. pestis* infection in fleas. Fleas fed biofilm-defective strains were more likely to recover from infections, and show reduced bacterial loads, compared with fleas infected with the parent strain. In this study, we found that both $\Delta hmsR$ and $\Delta hmsT$ mutants, which are biofilm-negative *in vitro*, did not maintain a high-bacterial-load infection in fleas to the same extent as the parent or the biofilm-overproducing strains. Kuttyrev and co-workers found that an *hms*-negative strain is eliminated within 4 days from *Nosopsyllus laeviceps* fleas, the established vector for transmission of *Y. pestis* to voles in the natural plague foci of the former USSR (Kuttyrev *et al.*, 1992). Hinnebusch *et al.* (1996) noted that an *hms*-negative mutant was more likely to be cleared from *X. cheopis* than its parent strain. Moreover, our study found that the biofilm overproducer maintained its infection in the flea better than the parent strain. Over the 4 days p.i., fleas infected with the biofilm-overproducing strain maintained a constant, albeit somewhat lower, bacterial load in the flea, while fleas infected with the parent strain showed a significant drop in the percentage of fleas with a high bacterial load over 4 days p.i. (Figs 1 and 2). Taken together, these results suggest that biofilms are indeed important for *Y. pestis* persistence in the flea gut. Further studies will be conducted to determine whether fleas infected

with a biofilm-deficient strain of *Y. pestis* will, over a longer period of time, experience a complete clearing of infection instead of simply a decrease in bacterial load.

Generally, compared with *X. cheopis*, *O. montana* experienced a more dramatic reduction over 4 days p.i. in the proportion of fleas assigned to the high-bacterial-load class. Since biofilm-dependent blockage is rare in *O. montana* compared with *X. cheopis*, this suggests a role for biofilms in non-blocking flea species. Ours is not the first study to document different bacterial behaviours in different flea species. Engelthaler *et al.* (2000) showed that *Y. pestis* infection primarily develops in the mid-gut of *O. montana*, while in *X. cheopis*, infection develops simultaneously in the proventriculus and the mid-gut (Engelthaler *et al.*, 2000). Although *Y. pestis* bacteria seem to colonize the two flea species differently, the effect on EPT efficiency appears to be negligible, since both flea species transmit at similar rates. This is not the case with all flea species examined so far. *Aetheca wagneri*, *Oropsylla tuberculata cynomuris*, *Oropsylla hirsuta* and *Ctenocephalides felis* are not as efficient as *X. cheopis* and *O. montana* in EPT (Eisen *et al.*, 2006, 2007b, 2008b; Wilder *et al.*, 2008a, b). Previous studies have suggested that differences in proventriculus structure explain why some flea species are better at blocking, and therefore transmission (Konnov *et al.*, 1979; Korzun & Nikitin, 1997). Since EPT occurs before blockage of the proventriculus, more studies are needed to determine the role of the proventriculus in EPT, and whether other anatomical differences between flea species contribute to varying transmission efficiencies.

In *X. cheopis*, overproduction of biofilms also limited the growth of bacteria. However, we do not believe the lack of EPT of the overproducing strain was a result of reduced numbers of bacteria in the flea. Other studies have shown that a decrease in bacterial load in individual fleas does not lead to decreases in transmission rates, and transmission of a wild-type strain has been seen with a median bacterial load of 2.65×10^2 , which is a quantity lower than the lowest median value seen in fleas in this study (Eisen *et al.*, 2007b, 2008a, b; Wilder *et al.*, 2008a, b). Another possible explanation for seeing lower transmission rates could be an accumulation of non-pigmented (Δpgm) mutants that spontaneously appear in the flea gut over the time that it remains infected. Wild-type strains of *Y. pestis* spontaneously delete the *pgm* locus at a rate of 10^{-5} (Brubaker, 1969). In the laboratory, it has been noted that the biofilm-overproducing $\Delta hmsP$ strain has a higher accumulation of *pgm* excision mutants. Since the *pgm* locus contains the yersiniabactin genes required for siderophore-mediated iron acquisition in *Y. pestis*, deletion of this locus renders the bacteria attenuated with respect to plague virulence (Bearden *et al.*, 1997; Burrows & Bacon, 1958; Fetherston *et al.*, 1995; Une & Brubaker, 1984). While it is possible that deregulation of intracellular c-di-GMP in the $\Delta hmsP$ mutant (Bobrov *et al.*, 2008) could contribute to a higher rate of spontaneous deletion of the *pgm* locus than that observed in wild-type strains, these spontaneous mutants

may simply be able to outgrow biofilm-overproducing cells, and this effect may be amplified in the flea-gut environment. However, 88 % of *X. cheopis* infected with the biofilm-overproducing strain retained an intact *pgm* locus on day 4 p.i. (Fig. 1b). Thus, even if loss of CR binding was due to a deletion of a portion of or the entire *pgm* locus, this loss was still probably not sufficient to affect the transmission efficiency.

The synthesis of biofilms is tightly regulated by HmsP and HmsT, and levels of c-di-GMP. High levels of c-di-GMP close to an HmsR–HmsS–HmsT–HmsP complex activate biofilm formation. HmsT, characterized as a diguanylate cyclase, increases the local levels of c-di-GMP near this complex, while HmsP, a phosphodiesterase, degrades c-di-GMP (Bobrov *et al.*, 2008). The coordinating levels of HmsT and HmsP control the amount of exopolysaccharide synthesized. Regulation of HmsT (as well as HmsR) is post-translational and dependent on temperature, resulting in lower protein levels above 30 °C, suggesting that biofilms must be important in the environment of the flea gut, which is normally at a lower temperature (Kirillina *et al.*, 2004). Moreover, it has been demonstrated that biofilms are not critical for the pathogenesis of bubonic or pneumonic plague in mice, thus supporting the hypothesis that a function of *Y. pestis* biofilm production in fleas could be long-term survival of *Y. pestis* in its flea vectors (Abu Khweek *et al.*, 2010; Lillard *et al.*, 1999). The results shown here suggest that too much biofilm reduces EPT, while too little biofilm may not allow bacteria to be maintained over extended periods, a factor that could help sustain enzootic plague cycles. It is possible that this regulation is crucial for the balance between transmission and maintenance. Although not required for EPT, biofilms undoubtedly remain critical in the classic model of transmission, which occurs later in the period when fleas are infectious, and is likely to play an important role in long-term maintenance of *Y. pestis* infection in flea populations.

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