Gene Expression Analysis of Xenopsylla cheopis (Siphonaptera: Pulicidae) Suggests a Role for Reactive Oxygen Species in Response to Yersinia pestis Infection

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ABSTRACT Fleas are vectors for a number of pathogens including Yersinia pestis, yet factors that govern interactions between fleas and Y. pestis are not well understood. Examining gene expression changes in infected fleas could reveal pathways that affect Y. pestis survival in fleas and subsequent transmission. We used suppression subtractive hybridization to identify genes that are induced in Xenopsylla cheopis (Rothschild) (Siphonaptera: Pulicidae) in response to oral or hemocoel infection with Y. pestis. Overall, the transcriptional changes we detected were very limited. We identified several genes that are likely involved in the production or removal of reactive oxygen species (ROS). Midgut ROS levels were higher in infected fleas and antioxidant treatment before infection reduced ROS levels and resulted in higher bacterial loads. An ROS-sensitive mutant strain of Y. pestis lacking the OxyR transcriptional regulator showed reduced growth early after infection. Our results indicate that ROS may limit Y. pestis early colonization of fleas and that bacterial strategies to overcome ROS may enhance transmission.

KEY WORDS Xenopsylla cheopis, Yersinia pestis transmission, reactive oxygen species, flea gene expression

Plague, caused by Yersinia pestis, is a life-threatening infectious disease usually transmitted between rodents by infected fleas. At least 80 flea species are known to carry plague, although their role in disease transmission varies (Gage and Kosoy 2005). For instance, the rat flea Xenopsylla cheopis (Rothschild) is considered to be the most efficient and is an important vector to humans worldwide. One mechanism for X. cheopis transmission of Y. pestis involves blockage of the digestive tract by biofilm-forming bacteria (Bacot and Martin 1914, Bacot 1915), and recently a separate early-phase transmission by unblocked fleas has been described (Eisen et al. 2007a).

Although several bacterial factors required for establishment of a transmissible infection in fleas have been identified, the role of flea gene expression in this process has not been investigated. The responses that occur in X. cheopis immediately after ingestion of Y. pestis in a bloodmeal are evidently effective in limiting bacterial growth. Approximately 25% of fleas that feed on highly septicemic (∼10⁶ CFU/ml) blood will clear themselves of the infection within 1 wk (Lorange et al. 2005), through mechanisms that are not understood. The bacteria are confined to the digestive tract and coalesce into clumps in the midgut, perhaps as a protection against soluble immune effectors or digestive enzymes. Y. pestis that survive this initial challenge can be transmitted during subsequent feedings during the early phase transmission window which lasts up to 7 d (Eisen et al. 2006, Eisen et al. 2007b).

For efficient transmission to occur beyond 1 wk, the bacteria must form a biofilm on the cuticle surface of the proventricular valve (Hinnebusch and Erickson 2008). The biofilm grows to fill the spaces between these spines, ultimately preventing the flow of blood into the midgut. Part of the blocking mass can be re-gurgitated back into the bite site during feeding attempts by blocked fleas. Under laboratory conditions, biofilm formation and blockage of X. cheopis peak between 2 and 3 wk after the infectious bloodmeal.

In flea species that are not competent vectors, Y. pestis may not survive in the midgut, colonize the proventriculus and thereby block the normal flow of blood, or induce the as-yet-unknown effects associated with early-phase transmission. It is not known how different fleas alter expression of gene products in response to Y. pestis in their midguts, and whether differences in these responses affect the ability of Y. pestis to establish a transmissible infection. Unlike...
many blood-feeding insects, fleas do not produce a chitinous peritrophic membrane around the bloodmeal. However, *Y. pestis* does not penetrate the midgut epithelia to invade other tissues, suggesting that the immune defense mechanisms are effective in limiting the growth of the bacteria and keeping them contained in the digestive tract. Differences in these responses could influence whether a particular flea is able to transmit *Y. pestis* or reveal new opportunities for interrupting transmission.

Understanding the genetic basis for the ability of *X. cheopis* to transmit *Y. pestis* may lead to the development of anti-transmission strategies. However, there are as yet no complete genome sequences available for any flea species. We have identified genes expressed in *X. cheopis* infected with *Y. pestis* at 24 and 48 h postinfection, using suppression subtractive hybridization (SSH). We identified transcripts predicted to be involved in genetic information processing, immune defense effector molecules, stress associated proteins, metabolism, cell motility and migration, and signaling. In particular, several genes involved with reactive oxygen species (ROS) production and removal were detected, suggesting an important role for these molecules in regulating early colonization of *X. cheopis*.

**Materials and Methods**

**Flea Infections.** *X. cheopis* were orally infected with *Y. pestis* through shaved mouse skins by the means of previously described membrane feeder apparatus (Erickson et al. 2007, 2008). Female fleas were used in all experiments, and were fed on 5 ml of heparinized human blood containing $\approx 1 \times 10^8$ colony-forming units (CFU) *Y. pestis* per milliliter. Fleas were infected with *Y. pestis* KIM6+ lacking the pCD1 virulence plasmid for gene expression experiments. Feedings lasted 1 h, and the blood was kept at 37°C during the feeding. Control fleas were fed under the same conditions, on the same blood source but without *Y. pestis*. Hemocoeal infection was performed by piercing the integument between posterior dorsal tergites with a 36-gauge needle attached to a UMP2 Microsyringe Injector and Micro4 Controller (World Precision Instruments, Inc., Sarasota, FL). Fleas received either 65 CLS Injector and Micro4 Controller (World Precision Instruments, Inc., Mountain View, CA) and suppression subtractive hybridization was performed using the PCR-Select cDNA Subtraction Kit (Clontech Laboratories, Inc.) according to the protocol of the manufacturer.

**cDNA Library Cloning and Amplification of Inserts.** For each SSH procedure, amplified cDNA fragments were inserted into the pCR II-TOPO vector (Invitrogen) and transformed into *Escherichia coli* TOP10 competent cells. The cDNA inserts were amplified from each clone via colony PCR using TOPO forward (5'-TAA TGC TTC CGG CTC GTA TG-3') and TOPO reverse (5'-GTA AAA CGA CGG CCA GT-3') primers that bind 250 bases upstream and downstream from the insertion site.

**Sequencing of Inserts and Analysis.** Excess primers and dNTPs from the colony PCR reactions were removed using EXO-SAP (exonuclease-shrimp alkaline phosphatase) treatment. After the clean-up reaction, BigDye terminator v.3.0 ready cycle sequencing reaction (Applied Biosystems/Ambion) was performed using M13 forward (5'-GTA AAA CGA CGG CCA GT-3') or M13 reverse 5'-CAG GAA ACA GCT ATG AC-3' to sequence the cDNA inserts. The BigDye sequencing reactions were cleaned of excess dye using Sephadex G-50 resin (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The forward and reverse sequences were assembled and aligned using the Geneious Pro v. 4.6.2 software package (Biomatters Ltd., Auckland, New Zealand). The adaptor sequences at both ends of the sequenced products were removed. The high quality sequences were searched by TBLASTX at an E-value cut off less or equal to 1 x $10^{-5}$. Sequences of bacterial origin and those with no homologues were removed.

**Quantitative Real-Time RT-PCR.** Total RNA was isolated from infected and uninfected fleas from three separate feeding experiments and cDNA was synthesized using oligo-dT primers and SuperScript VILO cDNA Synthesis Kit (Invitrogen) according to the protocol of the manufacturer. The cDNA was used in qPCR reactions with Power SYBR Green PCR master mix (PE Applied Biosystems) and gene-specific primers (0.5 μM) in a Light cycler 480 system (Roche Applied Science, Mannheim, Germany). The thermal cycling program was set as one cycle of at 94°C for 3 min, followed by 40 cycles of at 95°C for 30 s, 60°C for 30 s, and 72°C for 1.5 min, and one cycle of final extension at 72°C for 7 min. The threshold cycles (Ct) were calculated by the Light cycler 480 software and these values were used to calculate the fold-change in gene expression using the two -ΔΔCt method (Livak and Schmittgen 2001). *X. cheopis* clone XC-71 ribosomal gene L13 (Andersen et al. 2007) was used as a control gene. Gene-specific primers (Table 1) were designed with Primer3 software (Steve Rozen, Helen J. Skaletsky. Code available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html).
ing experiments, or had been treated with antioxidant (see below) were rapidly hand-dissected and triturated in 100 µl PBS containing 2 mg/ml aminotriazol (Sigma-Aldrich Corp., St. Louis, MO) under a dissecting microscope. Peroxide levels were determined with the PeroXOquant Quantitative Peroxide Assay Kit (Pierce Biotechnology Inc., Rockford, IL), which is based on oxidation of ferrous to ferric ion in the presence of xylenol orange (Jiang et al. 1991). The data were expressed as the mean and standard deviation of three different pools of fleas.

Antioxidant Treatment. Groups of ~100 X. cheopis female fleas were fed on two consecutive occasions, 3 d apart on heparinized human blood containing 20 mM N-acetyl-cysteine (Sigma). A separate group was fed with the same untreated blood. Three days after the second treatment the fleas were orally infected with Y. pestis as described above. After each feeding, fleas were visually inspected and those that had not fed were removed. Groups of 20 fleas were collected 3 d after infection from three different infested pools of fleas.

Table 1. Quantitative Real-Time PCR Analysis of Selected X. cheopis Genes From Whole Flea Tissues

<table>
<thead>
<tr>
<th>Gene (source)</th>
<th>Forward primer (5′-3′)</th>
<th>Reverse primer (5′-3′)</th>
<th>Product length</th>
<th>Fold change</th>
<th>P value (unpaired t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine protease (OR1)</td>
<td>ACTTATCGCTGGGCAACACTCCG</td>
<td>TATATCCGCAACCTGGTGGATGCT</td>
<td>186 bp</td>
<td>2.108 ± 0.198</td>
<td>0.011</td>
</tr>
<tr>
<td>Serine protease (OR2)</td>
<td>TTTCGGCAATTGAAGCTCTGCCC</td>
<td>AGATAACGTTGGTGACGAGACA</td>
<td>162 bp</td>
<td>1.935 ± 0.210</td>
<td>0.021</td>
</tr>
<tr>
<td>Phospholipase A2, group VI (OR2)</td>
<td>ATGGAGGCCATACCAACCGCTAT</td>
<td>AACATGGAAGAAGCTGCGGCCTTC</td>
<td>192 bp</td>
<td>0.630 ± 0.622</td>
<td>0.594</td>
</tr>
<tr>
<td>Thioredoxin reductase (OR2)</td>
<td>GGATTCCTGACGGAACCTGTCGAA</td>
<td>CCGGCACCACAGCAGAACACTAAT</td>
<td>199 bp</td>
<td>1.512 ± 0.210</td>
<td>0.103</td>
</tr>
<tr>
<td>Cadtherin-N (OR2)</td>
<td>TAGGTCGCGATCCTAGGCTGA</td>
<td>CATGCGGGCCATGGCAATGCTT</td>
<td>161 bp</td>
<td>1.421 ± 0.218</td>
<td>0.149</td>
</tr>
<tr>
<td>Cytochrome P450 (OR2)</td>
<td>GCCGACAGACGCGGTCTACG</td>
<td>TCATCCGPTGCTGTACGACCT</td>
<td>195 bp</td>
<td>1.668 ± 0.212</td>
<td>0.051</td>
</tr>
<tr>
<td>Proline oxidase (HMI)</td>
<td>GGATCATCCGCTGGGATCCTG</td>
<td>TCACCCTCTCCTAGGTCGG</td>
<td>146 bp</td>
<td>1.706 ± 0.154</td>
<td>0.045</td>
</tr>
<tr>
<td>Glycogenin (HM1)</td>
<td>GCAGTTGCGAGGACCAATTTA</td>
<td>GACGCTTGCGACAAACGACTCATA</td>
<td>102 bp</td>
<td>1.865 ± 0.235</td>
<td>0.036</td>
</tr>
<tr>
<td>Dual oxidase maturation factor (OR1)</td>
<td>GGATTCCTGACGGAACCTGTCGAA</td>
<td>CCGGCACCACAGCAGAACACTAAT</td>
<td>199 bp</td>
<td>1.512 ± 0.210</td>
<td>0.103</td>
</tr>
<tr>
<td>XC-71 ribosomal protein L13 (Andersen et al. 2007)</td>
<td>AAAGCCGCTGGCCCTACTGAGGAT</td>
<td>ACATCTCGAGATTCTCAGCGCA</td>
<td>134 bp</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*The fold change in expression between uninfected and infected fleas was calculated using the ribosomal protein L13 as a control gene. A value >1.0 indicates that expression was higher in infected fleas.

Results and Discussion

General Sequencing Results and Gene Distributions. cDNA libraries were created from orally infected X. cheopis fleas (OR1 and OR2) early after infection. The OR1 and OR2 libraries represented the X. cheopis genes expressed after a bloodmeal containing Y. pestis KIM6+ 24 or 48 h (respectively) after oral infection. In addition to the natural oral infection route, a cDNA library was created representing X. cheopis genes expressed 24 h after hemocoeal injection (HMI). From these libraries, colonies were randomly picked for sequence analysis (391 colonies from OR1, 343 from OR2, and 312 from HMI). After amplification of the inserts, the sequences were compared with the NCBI database and the closest sequences identified. From these sequences, 274 genes were identified and categorized by their different predicted functions: genetic information processing, immune defense effector molecules, stress associated proteins, metabolism, cell motility and migration, signaling proteins, proteases, reactive oxygen species production and defense, and genes of unknown function (Supplementary Tables 1–3, available online only).

Gene distribution in the three X. cheopis libraries showed some similarities (Fig. 1) but also had several important differences. For instance, there were more genes for immune defense and effector molecules and signaling proteins in HMI, which may indicate that hemocoeal injection induces a stronger immune response than oral infection. This result was expected because bacteria injected into the hemocoeal bypass the midgut epithelia and face hemocoeal immunity directly (Liehl et al. 2006, Lemaître and Hoffmann 2007), whereas the midgut response must be carefully regulated to maintain healthy interaction with the normal gut flora. There were a greater abundance of proteases, stress associated proteins, and immune defense and effector molecules in OR1 compared with OR2, suggesting that the X. cheopis immune response to Y. pestis in the gut is higher initially postinfection but is down-regulated to avoid excessive host damage. The initial immune-related stress in the midgut could clear a significant portion of the bacteria, but those that remain may not face the same types of acute responses. Y. pestis may also contribute to down-regulation of the initial immune responses. There were relatively few individual transcripts detected in more
than one X. cheopis cDNA library. These included a gene encoding glycogenin (HM1 and OR2), genes encoding matrix metalloproteinase, vitellogenin, chromatin granule amine transporter, and a conserved hypothetical protein with a chromatin organization modifier domain (HM1 and OR1) and a gene encoding muscle myosin heavy chain (OR1 and OR2).

**Quantitative Real-Time PCR Analysis of Selected Target Genes.** To determine the magnitude of gene expression changes, quantitative real-time PCR (qPCR) was performed on a subset of genes using RNA from infected fleas compared with uninfected controls (Table 1). Transcript levels for each gene were quantified relative to the gene for ribosomal protein L13, whose transcription was not significantly influenced by the treatments. The qPCR results showed that most of these genes were very modestly upregulated in infected fleas (1.5–2.5× increase). Thus, we can infer that the majority, but not all, of the genes in our libraries represent differentially expressed genes. Small transcriptional changes may reflect a relatively benign effect of Y. pestis on fleas; however, they likely do not represent the full extent of cellular responses that occur after infection. Additionally, the greatest changes in gene expression may occur in cells directly exposed to the bacteria (i.e., midgut epithelial cells in oral infection or hemocytes in systemic infection). Significant localized changes could be masked when isolating RNA from whole fleas. However, localized infection can elicit systemic effects, so we chose not to limit our initial investigations to the site of infection. Future studies will focus on tissue-specific responses in X. cheopis after Y. pestis infection.

**Specific Genes Associated With Selected Functional Categories.** Several classes of transcripts that are induced by bacterial infection in other insects were noted, whose presence suggests they may be important in X. cheopis immune interactions. These included pathogen recognition molecules such as β-1,3-glucan recognition protein one (HM1), leukocyte receptor cluster (lrc) member (OR2), and a matrix metalloproteinase with a putative peptidoglycan binding domain (OR1 and HM1). Ferritins (detected in OR2) are important intracellular iron storage molecules (Andrews et al. 1992) whose expression can be induced by iron overload, such as might occur after a bloodmeal to protect against oxidative stress, as well as in response to infection as an iron-withholding strategy (Ong et al. 2005). Other potential immune defense effectors included a putative small lysozyme protein (HM1), a serine protease inhibitor (HM1), two palmitoyl-protein thioesterases that function in lysosomal degradation (OR2), and a deoxyribonuclease I, responsible for DNA fragmentation during apoptosis (Apostolov et al. 2009).

Three predicted serine protease genes (OR1 and OR2) with homology to prophenoloxidase activating (ProPO) factors were observed. ProPO is activated by proteolytic cleavage, through the action of secreted serine proteases whose expression is regulated by MAP kinases and is enhanced after infection (Mavrouri et al. 2005). Other potential immune defense effectors included a putative small lysozyme protein (HM1), a serine protease inhibitor (HM1), two palmitoyl-protein thioesterases that function in lysosomal degradation (OR2), and a deoxyribonuclease I, responsible for DNA fragmentation during apoptosis (Mavrouri et al. 2005).

Fig. 1. Distribution of immune-inducible X. cheopis transcripts after Y. pestis infection. Suppression subtractive hybridization libraries were prepared after oral or hemocoel infection (OR1: 1 d postoral infection, OR2: 2d postoral infection, HM1: 1d posthemocoel infection) with Y. pestis KIM6+.
signaling pathway in OR1 (guanine nucleotide exchange factor for Rho/Bac/Cdc42-like GTPases, RhoGAP, and a FERM, RhoGEF, and pleckstrin domain-containing protein) and HM1 (guanine-nucleotide-exchange-factor, kalirin, RhoGEF kinase).

Finally, a gene with homology to phospholipase A2 in OR2 was identified. Phospholipase A2 is induced in bacterial-challenged tobacco hornworms and releases arachidonic acid from phospholipids (Tunaz et al. 2003). Arachidonic acid and related fatty acids are precursors for eicosanoids, which regulate phagocytosis and nodulation and can also trigger ProPO release (Downer et al. 1997, Figueiredo et al. 2008).

**ROS Production is Enhanced After Infection and Limits *Y. pestis* Growth.** In all three SSH libraries, several genes were identified that are predicted to contribute to either production (e.g., Dual oxidase maturation factor in OR1; cytochrome P450, cytochrome oxidase subunit I, and uricase in OR2) or removal (e.g., peroxidasin in HM1; thioredoxin reductase in OR2) of ROS. This indicates that *Y. pestis* infection of *X. cheopis* very likely induces ROS defenses. Production of reactive oxygen species (ROS) is the first-line defense that protects the epithelial cells from high numbers of bacteria. ROS are natural by-products of aerobic respiration but can also be produced by enzymes such as NADPH oxidase in phagocytes and dual oxidases in epithelial cells (Ha et al. 2005a). Superoxide and hydrogen peroxide production by dual oxidases at midgut epithelial surfaces are especially important for maintaining midgut microbial homeostasis in fruit flies (Ha et al. 2005a, Ha et al. 2009) and mosquitoes (Kumar et al. 2010). Dual oxidase production of hydrogen peroxide is affected by their associated maturation factors, which form complexes during posttranslational modifications during passage through the Golgi and assembly at the cell membrane (Morand et al. 2009). Subsequent removal of excess ROS by peroxidases, superoxide dismutases, and catalases is important to prevent damage to host tissues (Ha et al. 2005a, b; Lemaître and Hoffmann 2007).

The identification of several *X. cheopis* ROS production and detoxification genes in the cDNA prompted further investigation of the production of ROS in response to *Y. pestis* colonization. Peroxide levels in midguts of uninfected flies that had not been fed for 7 d, uninfected flies 3 and 24 h after sterile bloodmeals, or infected flies after bloodmeals containing *Y. pestis* were measured (Fig. 2). In the absence of bacteria in the bloodmeal, ROS levels in flies decreased after ingestion of a bloodmeal. In the presence of bacteria, ROS levels were significantly higher in midguts from infected flies compared with the uninfected flies at both time points.

Incorporation of antioxidants in insect bloodmeals can reduce the oxidative stress in insect midguts and enhance the growth of some pathogens (MacLeod et al. 2007). We treated flies with the antioxidant N-acetylcysteine before oral infection with *Y. pestis*. The treatment significantly lowered the amount of ROS in midguts from uninfected as well as infected flies (Fig. 2). Both groups were infected with similar numbers of bacteria immediately after the feeding. Antioxidant treatment did not affect the infection rate after 3 d compared with untreated flies (75 and 72%, respectively). However, after 3 d the *Y. pestis* load per fly was significantly higher in the treated group compared with the untreated group (Fig. 3A).

Production of ROS in response to infection suggested that *Y. pestis* ROS detoxification mechanisms could promote survival in the flea digestive tract. *Y. pestis* OxyR regulates catalases and peroxidases, and is important for survival in the presence of hydrogen peroxide (Erickson et al., submitted for publication). Groups of flies were infected with the wild-type *Y. pestis* KIM6+ strain, its isogenic ΔoxyR mutant, or the complemented mutant strain containing a functional oxyR gene on a plasmid (Fig. 3B). There was no difference in the bacterial loads in flies immediately after infection. After 3 d, the percentages of flies that remained infected were not significantly different, with a mean ± SEM of 80.00 ± 2.887% versus 71.67 ± 4.410% for the wild-type and mutant, respectively. The numbers of bacteria per infected flea for the ΔoxyR mutant was significantly reduced after 3 d, whereas the wild-type and complemented strains achieved similar loads in flies. Taken together, these results suggest that ROS defense reduces *Y. pestis* growth during early colonization of flies.

We conclude that insects mount effective immune responses tailored to the nature of the invading organism and the infection route. Many of the immune pathways and effector molecules are conserved be-

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**Fig. 2.** Peroxide levels in *X. cheopis* midguts increase after *Y. pestis* infection. Midguts were dissected from three groups of 10 female fleas just before a bloodmeal (unfed for 7 d), and at 3 and 24 h after blood feeding with or without *Y. pestis* infection. Fleas that had been treated with 20 mM N-acetylcysteine before infection were also tested. Peroxide levels in the pooled midguts were determined by xylenol orange assay. Data are shown as mean ± SD concentration. The data were analyzed by analysis of variance (ANOVA) followed by Tukey’s Multiple Comparison Test using GraphPad Prism, *P* < 0.05, **P* < 0.005, ***P* < 0.001.
Between diverse insects that span the orders Diptera, Hymenoptera, Lepidoptera, and Coleoptera, but there are also important differences between individual species and groups. Until recently (Andersen et al. 2007, Dreher-Lesnick et al. 2010), there has been a paucity of genome, transcriptome, and proteome data available for molecular studies of transmission of infectious microbes by Siphonaptera, despite their medical and veterinary importance.

We have shown here a glimpse of *X*. *cheopis* gene expression at early time points after *Y*. *pestis* infection, which will contribute to understanding flea-*Y*. *pestis* interactions during plague transmission. To obtain a more complete understanding of the flea genes that influence transmission, it will be necessary to examine additional time points, varying bacterial loads, and the effect of the pCD1 virulence plasmid of *Y*. *pestis* on gene expression changes. It is likely that signaling proteins, immune effector molecules, proteases, and pathogen recognition proteins that we have identified in our libraries contribute in responding to *Y*. *pestis* infection, and will be the focus of future studies. The presence of ROS-related genes and direct biochemical measurements show that ROS production is a significant component of early flea responses to *Y*. *pestis* infection. Antioxidant treatment increased the *Y*. *pestis* survival in fleas, and bacterial defenses regulated by the OxyR transcriptional regulator that counter ROS likely contribute to the establishment of transmissible infections.

Fig. 3. ROS limits the survival of *Y*. *pestis* in fleas. (A) Pretreatment of fleas with the antioxidant N-acetylcysteine before infection increases the growth of *Y*. *pestis*. Data are shown as the mean ± SD *Y*. *pestis* CFU recovered from individual fleas immediately after or 3 d after an infectious bloodmeal. Fleas that were treated with antioxidant had higher bacterial loads than untreated (*N* = 60 for both groups, **P < 0.005 via unpaired t-test**). (B) Fleas were infected with wild-type *Y*. *pestis* KIM6+, an ΔoxyR mutant, or the mutant carrying a functional oxyR gene on a multi-copy plasmid (pJEToxyR). Data are shown as the mean ± SD *Y*. *pestis* CFU recovered from individual fleas immediately after or 3 d after an infectious bloodmeal. Fleas infected with the mutant strain had lower bacterial loads than those infected with the wild-type (*N* = 60 for all groups, ***P < 0.001 via unpaired t-test***).

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References Cited


