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 ($\approx 10^8$ 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 regurgitated 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

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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*. Hemocoel infection was performed by piercing the integument between posterier 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 nl of a suspension of *Y. pestis* strain KIM6+ ($\approx 1 \times 10^8$ CFU/ml) in phosphate buffered saline (PBS) or the same volume of sterile PBS as a control.

Suppression Subtractive Hybridization. After infection, fleas were kept at 21°C and 80% humidity for 24 or 48 h. Groups of 50 fleas were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) for 60 s using a hand-held tissue homogenizer. Total RNA was then isolated from homogenized flea tissues in TRIzol reagent according to manufacturer's instruction. The isolated RNA was treated with TURBO DNase (Applied Biosystems/Ambion, Austin, TX) and purified with RNA MinElute column kit (Qiagen, Germantown, MD). RNA integrity and concentration was checked by agarose gel electrophoresis and ultra violet spectrophotometry. Double-stranded cDNA was then synthesized using SMART polymerase chain reaction (PCR) cDNA Sythesis Kit (Clontech Laboratories, 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′-TTA TGC TTC CGG CTC GTA TG-3′) and TOPO reverse (5′-GTG CTG CAA GGC GAT TAA 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 1×10^{-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 Biosysthems) 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).

Measurements of Midgut Peroxide Levels. The midguts (groups of 10) from fleas that were unfed for 7 d, fed with sterile blood, or fed with infected blood (1×10^8 *Y. pestis* CFU/ml) from three separate feed-

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

Gene (source)	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product length	Fold change[a] (mean ± SEM)	*P* value (unpaired *t*-test)
Serine protease (OR1)	ACTTATGCAGTGGCAACAACTCCG	TATATCCGCAACCTGGTGGATGCT	186 bp	2.108 ± 0.198	0.011
Serine protease (OR2)	TTTCTGCAAGTTCAACTGGTGCCC	AGATACGTGCTGGTGAATGGGACA	162 bp	1.935 ± 0.210	0.021
Phospholipase A2, group VI (OR2)	ATGGAGGCATCAACACCAGGCTAT	AACATGTAAAGCAGTGCGGCCTTC	192 bp	0.630 ± 0.622	0.594
Thioredoxin reductase (OR2)	GGTATTCCTTGACGGAAGCTGTGCAA	CGGCCGCCAACAGCAATAACAATA	199 bp	1.512 ± 0.221	0.103
Cadherin-N (OR2)	TAGCTCCACATGTGCACTCAGCTA	CATGGTCGGGATTGACGAATGCTT	161 bp	1.421 ± 0.218	0.149
Cytochrome P450 (OR2)	GCGCCCAGGAAGATGAGAATAACT	TCGATTTGCGCTCTGCTATCACCT	195 bp	1.668 ± 0.212	0.051
Proline oxidase (HM1)	CGGCAACAAGTCCATGCCACAATA	TCCTCTCGCAAGTAGCTTCGTTCA	113 bp	1.773 ± 0.237	0.047
Glycogenin (HM1)	GGAGTTGCGAGGACCACCAATTTA	GACGCTTGCGACAAACGACTCATA	102 bp	1.868 ± 0.238	0.036
Dual oxidase maturation factor (OR1)	TTGGAA CGGCGT TAGCAA GTTGG	GGCCTC GTAGGC AACATC AGATAGTA	146 bp	1.701 ± 0.154	0.045
XC-71 ribosomal protein L13 (Andersen et al. 2007)	AAAGCCCGTGTCGTTACTGAGGAT	ACATCATCTGGATTCTCAGCCGCA	134 bp	N/A	N/A

[a] 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.

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 infe for enumeration of *Y. pestis.*

Enumeration of *Y. pestis* in Fleas. *Y. pestis* KIM6+ wild type as well as its isogenic Δ*oxyR* mutant and the complemented Δ*oxyR* (pJEToxyR) strain (Erickson et al. 2011) that have altered sensitivity to reactive oxygen were used to infect fleas. Fleas were starved for 7 d before feeding on infected blood as described above. Groups of 20 were collected immediately after infection as well as 3 d after infection from three separate feeding experiments. Fleas were individually triturated with sterile glass sand. The homogenates were serially diluted and plated on Brain-Heart Infusion or Terrific Broth agar containing 1 μg/ml irgasan, 0.5 μg/ml crystal violet, and 1 mg/ml bile salts to select for *Y. pestis* (Ber et al. 2003). Colonies were counted after incubation at 30°C for 48 h.

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 hemocoel injection (HM1). From these libraries, colonies were randomly picked for sequence analysis (391 colonies from OR1, 343 from OR2, and 312 from HM1). 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 HM1, which may indicate that hemocoel injection induces a stronger immune response than oral infection. This result was expected because bacteria injected into the hemocoel bypass the midgut epithelia and face hemocoel immunity directly (Liehl et al. 2006, Lemaitre 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

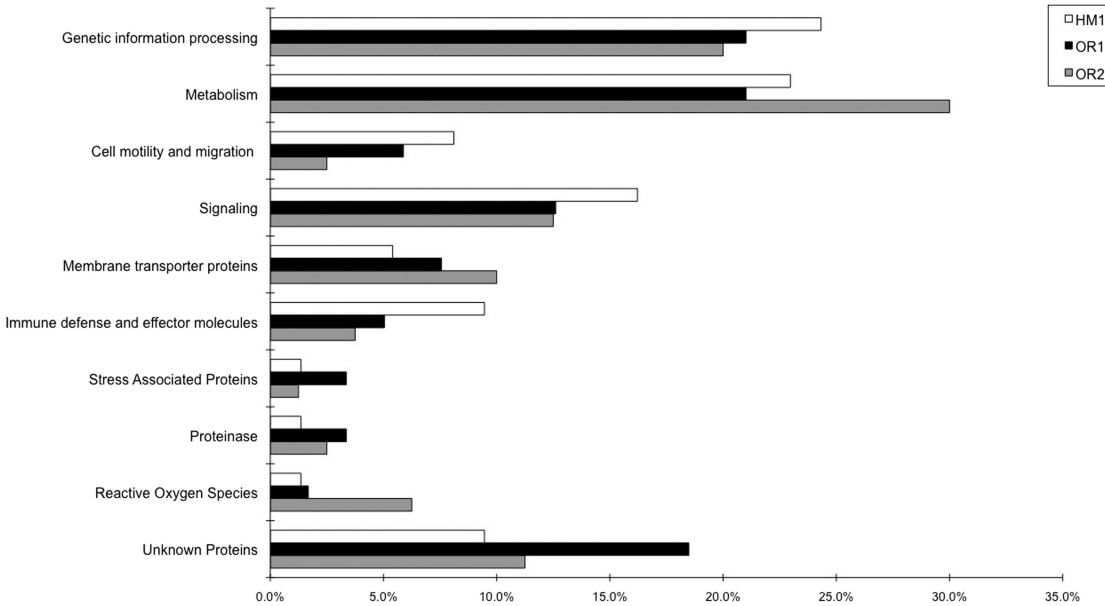

Fig. 1. Distribution of immune-inducible *X. cheopis* transcripts after *Y. pestis* infection. Suppression subtractive hybridization libraries were prepared after oral or hemocoel infection (OR1: 1 d postoral infection, OR2: 2d postoral infection, HM1: 1d posthemocoel infection) with *Y. pestis* KIM6+.

than one *X. cheopis* cDNA library. These included a gene encoding glycogenin (HM1 and OR2), genes encoding matrix metalloproteinase, vitellogenin, chromaffin 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 injection). 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 ß-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 (Mavrouli et al. 2005). Insect cellular immunity is also regulated by Rho, a member of the monomeric G-protein family that also includes Rac and Cdc42 (Marmaras and Lampropoulou 2009). Several genes were identified that encode Rho members and guanine nucleotide exchange factors potentially involved in the Rho

signaling pathway in OR1 (guanine nucleotide exchange factor for Rho/Rac/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 byproducts 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 dismutatses, and catalases is important to prevent damage to host tissues (Ha et al. 2005a, b; Lemaitre 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 fleas that had not been fed for 7 d, uninfected fleas 3 and 24 h after sterile bloodmeals, or infected fleas after bloodmeals containing *Y. pestis* were measured (Fig. 2). In the absence of bacteria in the bloodmeal, ROS levels in fleas decreased after ingestion of a bloodmeal. In the presence of bacteria, ROS levels were significantly higher in midguts from infected fleas compared with the uninfected fleas 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 fleas with the antioxidant *N*-acetylcysteine before oral infection with *Y. pestis.* The treatment significantly lowered the amount of ROS in

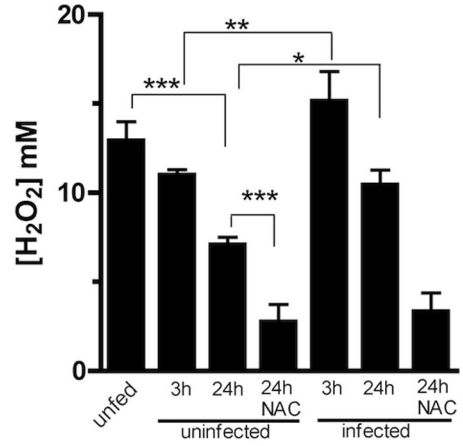

Fig. 2. Peroxide levels in *X. cheopis* midguts increase after *Y. pestis* infection. Midguts were dissected from three groups of 10 female fleas just before a bloodmeal (unfed for 7 d), and at 3 and 24 h after blood feeding with or without *Y. pestis* infection. Fleas that had been treated with 20 mM *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, $*P < 0.05$, $**P < 0.005$, $***P < 0.001$.

midguts from uninfected as well as infected fleas (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 fleas (75 and 72%, respectively). However, after 3 d the *Y. pestis* load per flea 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 fleas were infected with the wild-type *Y. pestis* KIM6+ strain, its isogenic Δ*oxyR* 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 fleas immediately after infection. After 3 d, the percentages of fleas 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 Δ*oxyR* mutant was significantly reduced after 3 d, whereas the wild-type and complemented strains achieved similar loads in fleas. Taken together, these results suggest that ROS defense reduces *Y. pestis* growth during early colonization of fleas.

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-

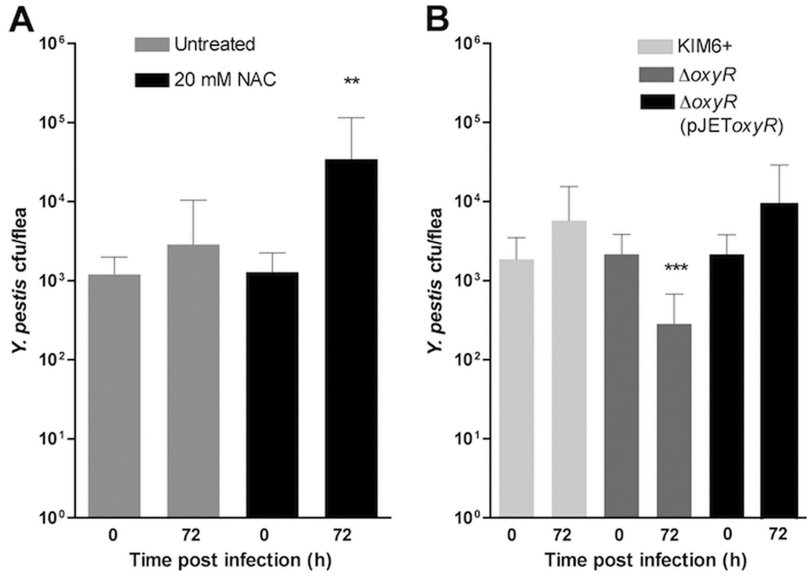

Fig. 3. ROS limits the survival of *Y. pestis* in fleas. (A) Pretreatment of fleas with the antioxidant *N*-acetylcysteine before infection increases the growth of *Y. pestis*. Data are shown as the mean ± SD *Y. pestis* CFU recovered from individual fleas immediately after or 3 d after an infectious 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 Δ*oxyR* 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).

tween 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.

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