Bacterial Communities Associated With Flea Vectors of Plague

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ABSTRACT The microbial flora associated with fleas may affect their ability to transmit specific pathogens, including *Yersinia pestis*, and also could be used to develop paratransgenesis-based approaches to interfere with transmission. To begin addressing this hypothesis, the microbial flora associated with the relatively efficient *Y. pestis* vectors *Xenopsylla cheopis* (Rothschild) (Siphonaptera: Pulicidae) and *Oropsylla montana* (Baker) (Siphonaptera: Ceratophyllidae), and the inefficient vector *Ctenocephalides felis felis* (Bouché) (Siphonaptera: Pulicidae) were investigated using polymerase chain reaction amplification of 16S rDNA genes. DNA sequencing revealed that these species harbor distinct communities of microbial flora and suggest that *Acinetobacter* sp. might be used in developing anti-transmission strategies.

KEY WORDS *Xenopsylla cheopis*, plague transmission, paratransgenesis

Blood-feeding arthropods generally carry bacteria that can markedly influence their physiology and development, including supplementing their restricted diets but also affecting their competence as vectors of infectious disease. Bacterial symbionts have been proposed as a route for the creation of paratransgenic insects that are unable to transmit pathogens. Populations of reduviid kissing bugs that normally transmit *Trypanosoma* parasites that cause Chagas' disease have been rendered incompetent via this approach (Beard et al. 2002). Infection of arthropods with specific types of symbiotic bacteria also can prevent other pathogens from successfully establishing infections. For example, ticks that are normally susceptible to the Rocky Mountain spotted fever agent *Rickettsia rickettsii* are protected from infection when they carry the closely related symbiont *Rickettsia peacockii* (Burgdorfer et al. 1981, Baldridge et al. 2004). Pea aphids can harbor *Regiella insecticola* bacteria that protect them from acquiring the fungal pathogen *Pandora neoaphidis* (Scarborough et al. 2005). In addition, bacterial flora of arthropods represent a vast pool of genetic material that could influence the evolution of novel pathogenic microbes or the acquisition of novel traits by existing pathogens such as antibiotic resistance (Waterfield et al. 2004). For example, the frequency of plasmid transfer among *Escherichia coli* and *Yersinia pestis* is greatly enhanced in the flea midgut, well above the maximal levels observed under laboratory conditions (Hinnebusch et al. 2002), which could contribute to the emergence of antibiotic-resistant strains of *Y. pestis.*

Recently, the bacterial communities associated with the rock squirrel and prairie-dog fleas *Oropsylla montana* (Baker) and *Oropsylla hirsuta* (Baker) (Jones et al. 2008) and the cat flea *Ctenocephalides felis* (Bouché) (Pornwiroon et al. 2007) have been investigated using 16S rDNA clone library approaches. The rat flea *Xenopsylla cheopis* (Rothschild) acts as a vector for plague (*Y. pestis*), murine typhus (*Rickettsia typhi*), and as an intermediate host for cyclophyllidean tapeworms (*Hymenoplepis nana*, *Hymenoplepis diminuta*, and *Dipylidium caninum*) (Gage 2005). In an effort to identify bacteria potentially suitable for paratransgenesis or that could influence the vector competences of these flea species, we have surveyed the bacteria associated with *X. cheopis*, in comparison with *O. montana* and *C. felis.*

Materials and Methods

X. cheopis and *O. montana* colonies were housed within glass jars at 26°C and 80% RH and fed biweekly on neonatal mice. The bedding consisted of 80% sand, 10% dried sheep's blood, and 10% powdered mouse chow. *C. felis* were caught live from two separate cats that were treated by a local veterinarian. Fleas were surface sterilized by washing twice in 70% ethanol followed by two rinses in sterile distilled water, transferred to 20 μl of sterile glass sand in phosphate-buffered saline, and thoroughly homogenized with a pestle. Total DNA was extracted using the DNeasy tissue kit (QIAGEN, Valencia, CA) from pools of 10 fleas. In addition to the whole fleas, DNA was obtained from 15 dissected *X. cheopis* midguts. Bacterial 16S rDNA was amplified using the 27 forward (F) and 1492 reverse (R) primers (5′-GAGTTTGATCMTGGCTCAG-3′ and 5′-TACGGYTACCTTGTTAGGACTT-3′, respectively) under the following conditions: 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 2 min, and 72°C for 7 min. Amplification products were cloned using the TOPO TA

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Table 1. Bacteria identified in *X. cheopis* (XC isolates), *O. montana* (OM isolates), and *C. felis* (CF isolates) based on 16S rDNA gene sequences

Source-isolate	Length (bp)	Closest BLASTn match (accession no.)	No. identical/total (% identity)	Prevalence in clones analyzed	Accession no.
XC-1	1406	*Rickettsiales* bacterium 'Montezuma' (AF493952.1)	1338/1352 (98)	39/110 ([a]MG-7/42)	FJ981659
XC-2	1498	*Pseudomonas collierea* (AM421016.2)	1414/1423 (99)	29/110 (MG-4/42)	FJ981658
XC-3	1164	*Proteus mirabilis* strain HI4320 (AM942759.1)	1141/1161 (98)	17/110 (MG-27/42)	FJ981657
XC-4	776	*Wolbachia* endosymbiont of *Cubitermes* (EF417900)	770/776 (99)	13/110 (MG-ND[b])	FJ981656
XC-5	945	*Roseateles aquatilis* (AM501446.1)	940/958 (98)	11/110 (MG-1/42)	FJ981655
XC-6	1204	*Staphylococcus epidermidis* strain JPLtot1-5 (DQ870748.1)	1190/1233 (96)	8/110 (MG-3/42)	FJ981654
XC-7	1483	*Pseudomonas brenneri* (AM933521.1)	1082/1158 (93)	2/110 (MG-ND)	FJ981653
OM-1	1246	*Wolbachia* (EU137480.1)	1147/1169 (98)	86/132	FJ981665
OM-2	1492	*Staphylococcus epidermidis* strain KL-096 (AY030342.1)	1419/1419 (100)	21/132	FJ981664
OM-3	1385	*Brevibacterium epidermidis* strain ZJB-07021 (EU046495)	1346/1384 (97)	12/132	FJ981663
OM-4	1224	*Bacteroidetes* bacterium K6-27 (EF612322.1)	1141/1170 (97)	10/132	FJ981662
OM-5	1435	*Oceanobacillus* sp. R-34296 (AM910349.1)	1434/1435 (99)	4/132	FJ981661
OM-6	1331	*Cerasibacillus quisquiliarum* (AB107894.1)	1200/1253 (95)	1/132	FJ981660
CF-1	1258	*Acinetobacter* sp. TM6_6 (DQ279315.1)	1255/1258 (99)	36/157	FJ981675
CF-2	1196	*Rickettsiales* bacterium Huangshan-1 (AB297807)	1108/1131 (97)	34/157	FJ981673
CF-3	1133	Uncultured *Wolbachia* bacterium clone Om_02_O2A (EU137455)	863/877 (98)	33/157	FJ981672
CF-4	1001	Uncultured *Roseateles* bacterium clone nbt28c08 (EU535626)	993/1004 (98)	27/157	FJ981671
CF-5	1543	*Bartonella henselae* (AJ223779.1)	1437/1447 (99)	8/157	FJ981670
CF-6	1024	Uncultured Burkholderiaceae bacterium clone COREB72 (EF562258)	1021/1024 (99)	7/157	FJ981669
CF-7	1016	Uncultured *Massilia* sp. clone GI7-8-C13 (FJ193801)	981/1023 (95)	6/157	FJ981668
CF-8	1289	*Exiguobacterium* sp. MY02 (DQ083948)	1274/1288 (98%)	2/157	FJ981667
CF-9	1353	*Delftia* sp. 5.7 (EF426439.1)	1345/1349 (99)	2/157	FJ981666
CF-10	1207	*Stenotrophomonas maltophilia* (AJ516049)	1130/1206 (93)	2/157	FJ981674

[a] MG, prevalence in midgut-derived library.
[b] ND, not detected.

(pCR4-TOPO) cloning kit (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. Individual inserts from separate colonies were amplified by colony polymerase chain reaction (PCR) by using the primers TOPOF (5′-TTATGCTTCCG-GCTCGTATG-3′) and TOPOR (5′-GTG CTG CAA GGC GAT TAA GT-3′) (Lane 1991) and quantified by agarose gel electrophoresis. The PCR products were sequenced using M13 forward and reverse primers and ABI BigDye version 3.1 (Applied Biosystems, Foster City, CA) dye terminator chemistry. Alignment of forward and reverse sequences, trimming of poor-quality ends, and generation of a phylogenetic tree were completed using the Geneious software package (Biomatters Ltd., Auckland, New Zealand). The 16S rDNA sequences were compared with sequence data deposited in GenBank by using the BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/) search algorithm. Database sequences showing the greatest identity were recorded.

Results and Discussion

X. cheopis and *O. montana* are both relatively efficient vectors for *Y. pestis* via blocked fleas as well as through early phase transmission; in contrast, *C. felis* is an inefficient vector (Eisen et al. 2006, 2007, 2008). The bacteria colonizing these fleas might influence their vector competence as well as provide opportunities to develop antitransmission strategies. We amplified bacterial 16S rDNA sequences from total DNA isolated from laboratory colonies of *X. cheopis* and *O. montana*, as well as *C. felis* collected from two cats. We obtained sequences for a total of 110 16S rDNA clones from *X. cheopis*, 132 from *O. montana*, and 157 from *C. felis* fleas. The unique isolates, frequency of detection, as well as their best match in GenBank are listed in Table 1. In total, seven unique clones were detected in *X. cheopis* compared with six for *O. montana* and 10 for *C. felis.* Phylogenetic analysis of the 16S rDNA sequences (Fig. 1) indicated that clones from the individual flea species do not strictly cluster together. However, a greater diversity of γ-Proteobacteria were detected in the *X. cheopis* library compared with the *O. montana* library, which was dominated with Firmicutes or the *C. felis* library, which contained a higher number of β-Proteobacteria.

The most commonly detected clone from the *X. cheopis* libraries was a *Rickettsia*-like species with high sequence identity to the bacterium 'Montezuma', which has unknown pathogencity and has been detected in ticks from the Far East (Shpynov et al. 2006,

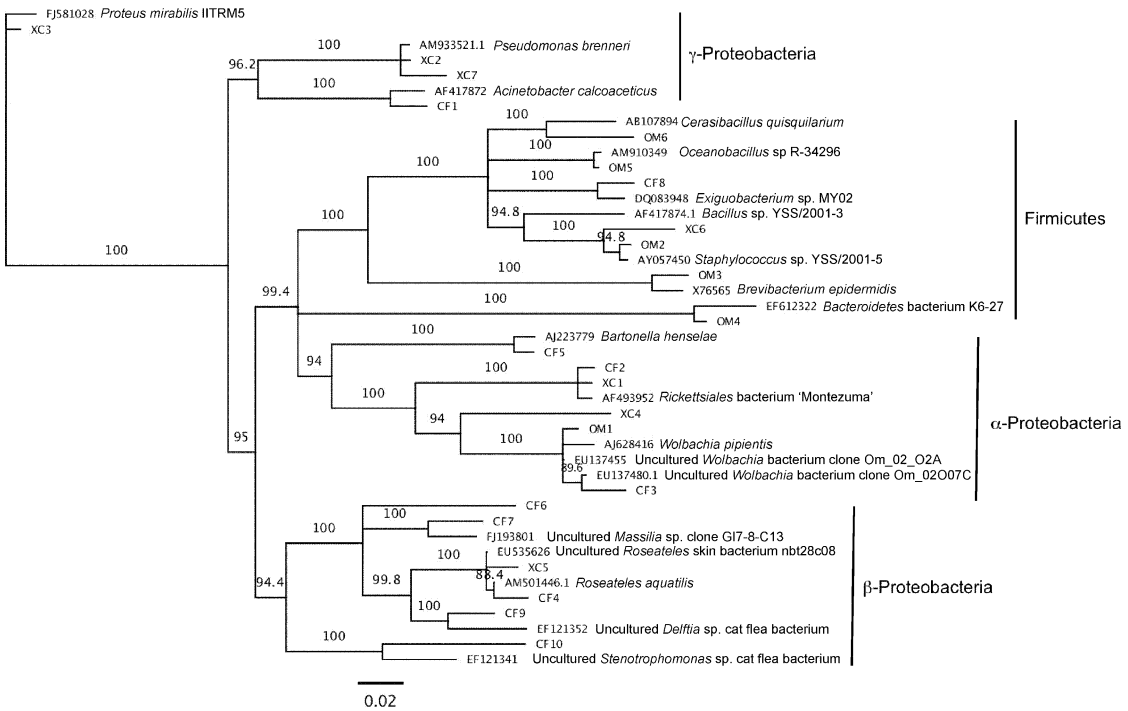

Fig. 1. Neighbor-joining tree of all 16S rDNA clones from this study (XC, OM, CF sequences) and selected database sequences. The tree was generated from a MUSCLE alignment by using Tamura–Nei distance parameters. The bootstrap values with 500 resamplings are indicated.

Eremeeva et al. 2007). This clone was not present in our *O. montana* or *C. felis* libraries although a separate *Rickettsia* species was detected in cat fleas (Table 1). In contrast, an *Acinetobacter* sequence was the most commonly detected clone in our cat flea library. It is interesting that two unique *Acinetobacter* strains were detected in colonized *C. felis* (Pornwiroon et al. 2007) and in wild-caught *O. hirsuta* (Jones et al. 2008) and were the dominant phylum residing in the midguts of *Culex quinquefasciatus* Say mosquitoes (Pidiyar et al. 2004). However, we did not detect any *Acinetobacter* spp. in the *X. cheopis* library. Because some members of the genus *Acinetobacter* exhibit diverse metabolic capabilities as well as natural competence (Young et al. 2005), they might be useful for delivery of transmission-blocking molecules in fleas and other insects.

To be useful in paratransgenesis, candidate bacteria should be localized to the digestive tract for interference with initial pathogen colonization. In previous histology analyses of flea guts in response to infection (Erickson et al. 2007), we noted the presence of bacteria in the midguts of fleas that had not been infected with *Y. pestis*. Some were in close association with the midgut epithelia. The digestive tract of fleas is likely to be a harsh environment for bacteria. They must overcome rapid changes in osmolarity, digestive enzymes such as proteases and lipases, and immune effectors such as reactive oxygen or nitrogen species and antibacterial peptides secreted by epithelial cells. To investigate the midgut-specific bacterial population of *X. cheopis*, we repeated our analysis on dissected midguts from 15 *X. cheopis* fleas (Table 1). The *Proteus mirabilis*-like strain dominated this population. We did not detect the *Wolbachia* or the *Pseudomonas brenneri*-like strains in the midgut but did detect all the other clones from the whole-flea library. All bacteria detected in the midgut population also were detected in the whole-flea library.

Many of the bacteria that inhabit these fleas could be considered environmental microbes commonly isolated from soil or water such as the *Pseudomonas*, *Acinetobacter*, and *Roseateles*-like isolates. These bacteria may colonize larvae (which feed on organic debris as well as adult feces) could survive molting and thus remain in the adult fleas. These bacteria also may reside on the surface of the fleas, particularly under the overlapping sclerites of the adults preventing their removal by washing with ethanol. In addition to these environmental microbes, there also were bacteria that are commonly found on the surface of host skin (*Staphylococcus epidermidis*, *Brevibacterium epidermidis*), and these may colonize the flea specifically during blood feeding. We also detected *Bartonella henselae*, the agent of cat scratch disease, which colonizes cat erythrocytes and vascular endothelial cells. Cat fleas that feed on a bacteremic cat can remain infected for at least 9 d (Higgins et al. 1996) and excrete *Bartonella* in their feces.

The presence of particular bacteria within an insect may correlate with the ability of pathogenic microbes to colonize or be transmitted by that vector. Mechanistically, this could be because resident bacteria pro-

duce antibiotics or bacteriocins that preclude other species from colonizing. We detected bacteria most closely related to *Pseudomonas*, *Burkholderia*, and *Stenotrophomonas* in the *C. felis* and *X. cheopis* libraries and bacteria from these genera are known to produce bacteriocins (Guerrieri et al. 2008). *Stenotrophomonas* and *Burkholderia*-like isolates also were detected by Pornwiroon et al. (2007) in their analysis of *C. felis.*

Rather than direct interaction, normal flora also could modulate particular immune responses effective against colonizing pathogens. For example, the presence of gut flora stimulates the Toll pathway in *Aedes* mosquitoes, thereby enhancing resistance to Dengue virus (Xi et al. 2008), although the particular bacteria that are responsible were not identified. Similarly, infection with certain *Wolbachia* strains enhances *Drosophila* resistance to RNA viruses (Hedges et al. 2008, Teixeira et al. 2008) and pathogenic fungi (Panteleev et al. 2007), but the precise mechanism is not known. We detected three separate *Wolbachia*-like sequences in our libraries. It will be important to determine whether all *Wolbachia* are equal in their ability to induce a generalized immune response in other insects, including fleas and whether this response is effective against pathogenic bacteria. Because fleas do not produce a peritrophic matrix, their midgut epithelia are particularly vulnerable to contact with microbes ingested during a bloodmeal. It is not known whether the presence of the normal flora we have detected in this study affect the response of fleas to oral infection. To answer these questions we are working to rid *X. cheopis* fleas of their normal flora by adding antibiotics to adult blood meals and to the larval bedding.

We can conclude from this pilot study that fleas harbor a diverse microbiota, including members of the Firmucute, α-, β-, and γ-Proteobacteria classes. Specific bacteria may be better suited to colonizing *X. cheopis*, *O. montana*, or *C. felis.* The impact of prior colonization with specific bacteria we have identified on pathogen infection and transmission deserves further study. Finally, *Acinetobacter* spp. are common (and sometimes dominant) inhabitants of vector insects including fleas (Pidiyar et al. 2004, Jones et al. 2008), suggesting their promise for use in paratransgenesis.

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