Rickettsia felis Infection in a Common Household Insect Pest, *Liposcelis bostrychophila* (Psocoptera: Liposcelidae)^{∇†}

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Many species of *Rickettsia* are well-known mammalian pathogens transmitted by blood-feeding arthropods. However, molecular surveys are continually uncovering novel *Rickettsia* species, often in unexpected hosts, including many arthropods that do not feed on blood. This study reports a systematic molecular characterization of a *Rickettsia* infecting the psocid *Liposcelis bostrychophila* (Psocoptera: Liposcelidae), a common and cosmopolitan household pest. Surprisingly, the psocid *Rickettsia* is shown to be *Rickettsia felis*, a human pathogen transmitted by fleas that causes serious morbidity and occasional mortality. The plasmid from the psocid *R. felis* was sequenced and was found to be virtually identical to the one in *R. felis* from fleas. As *Liposcelis* insects are often intimately associated with humans and other vertebrates, it is speculated that they acquired *R. felis* from fleas. Whether the *R. felis* in psocids causes disease in vertebrates is not known and warrants further study.

Many species of *Rickettsia* are well-known mammalian pathogens that are transmitted by blood-feeding arthropods via bites or feces and can cause mild to fatal diseases in humans (33). Some species are also considered potential bioterrorism agents (4). Most *Rickettsia* research has focused on pathogens that are found in two closely related species groups, the typhus and spotted fever groups, such as *Rickettsia prowazekii*, *Rickettsia rickettsii*, and *Rickettsia typhi*, the causal agents of epidemic typhus, Rocky Mountain spotted fever, and murine typhus, respectively (3, 4, 33). However, recent surveys suggest that *Rickettsia* bacteria are much more widespread than previously suspected and that they are being detected in novel hosts, the vast majority of which are arthropods, including many that do not feed on blood (29, 45).

The number of new rickettsial species that cause diseases in humans is rapidly increasing (33). One such species that has been generating much interest in recent years is *Rickettsia felis*, the causative agent of a murine typhus-like disease (1, 2, 13, 16, 17, 28, 44). The disease is often unrecognized, and even though it is considered clinically mild, it can cause severe illness and death in older patients and in cases of delayed diagnosis (2). *R. felis* was identified only in 1990 (1) and has since been found worldwide in fleas, where it is maintained transovarially and can reach high infection rates (e.g., 86% to 94% in cat fleas) (2, 3, 44), as well as in ticks and mites (34). While experimental infections have confirmed that *R. felis* is transmitted to vertebrate hosts via blood feeding and that *R. felis* occurs in an infectious extracellular state (39), it is not known whether transmission can also occur through contamination of broken skin by infected vector feces, as in *R. typhi* (3, 34).

A number of features distinguish *R. felis* from species in both

the typhus and spotted fever groups. Lately, it has been proposed that *R. felis* be in its own group, allied with *Rickettsia akari* and *Rickettsia australis*, the causal agents of rickettsial pox and Queensland tick typhus, respectively, and a number of recently discovered strains infecting insects that do not feed on blood (16, 17, 29, 45). Moreover, *R. felis* was the first *Rickettsia* species shown to have a plasmid (28). While plasmids now appear to be quite widespread in the genus, the *R. felis* plasmid stands out with respect to its relatively large size and distinctive gene content (5, 6, 9, 14, 17).

This study reports that a common and cosmopolitan insect, the psocid *Liposcelis bostrychophila* (Psocoptera: Liposcelidae) harbors *R. felis*. Liposcelids are the closest free-living relatives of parasitic lice (19) and are well-known for their close proximity to humans, particularly as pests in houses and grain storage facilities (8, 41). Through 16S rRNA gene sequencing, *L. bostrychophila* was recently shown to harbor a strain of *Rickettsia* (29, 30, 42). A systematic molecular characterization of this *Rickettsia* was conducted, demonstrating that it is authentic *R. felis*. Furthermore, the psocid symbiont plasmid was sequenced and was shown to be virtually identical to the plasmid from *R. felis* that infects cat fleas.

MATERIALS AND METHODS

Insects. *L. bostrychophila* insects were collected by Manoj Nayak (Queensland Department of Primary Industries) in the Darling Downs region of Queensland, Australia, in 1997. The culture was maintained in confined vessels on a culture medium consisting of whole wheat and rice at 27 ± 1°C and 70% ± 2% relative humidity (RH). Sequences from an *L. bostrychophila* culture collected by George Opit (Entomology and Plant Pathology, Oklahoma State University) from a grain elevator in Manhattan, KS, in 2006 and from an *L. bostrychophila* culture collected on organic rice by Alex Wilson from Tucson, AZ, in 2004 were also obtained in order to confirm the presence of *R. felis* in insects from different locales.

DNA extraction. Insects were dipped in a soap solution, quickly surface disinfected in 70% ethanol, and washed in sterile phosphate-buffered saline (PBS) and processed whole. Total DNA was extracted from pooled adult *L. bostrychophila* insects ($n = 20$) using a DNeasy Purification Kit (Qiagen, Hilden, Germany), with modifications. Samples were first incubated with enzymatic lysis buffer for 2 h at 37°C. Samples were then frozen in liquid nitrogen for 5 min and

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heated to 65°C for 10 min, and this freeze-thaw cycle was performed three times. The remaining steps were as described in the manufacturer's protocol for Gram-positive bacteria. Samples were processed independently to remove the possibility of contamination.

Molecular characterization and phylogenetic analyses. 16S rRNA, *gltA*, *ompA*, *ompB*, and cell surface antigen *sca4* (gene D) were amplified and sequenced using previously described primers and methods (see Table S1 in the supplemental material). All PCR amplifications were performed on a Mastercycler Gradient (Eppendorf, Hamburg, Germany) with 1 μ M each primer in 3 mM MgCl₂, 20 μ M each deoxyribonucleoside triphosphate, and 1.25 units of *Taq* polymerase in a total volume of 25 μ l of 1 $\times$ reaction buffer (Invitrogen, Carlsbad, CA). PCR products were separated in a 1% (wt/vol) agarose gel in TBE buffer (45 mM Tris-borate, 1 mM EDTA) and stained with ethidium bromide (0.1 mg/ml). Negative controls (no DNA added) were always performed in parallel. No products were obtained from these controls. All PCR products were sequenced in both directions at the Macrogen, Inc., Sequencing Center (Seoul, South Korea). Sequence chromatograms were visually inspected, verified, aligned, and annotated using Geneious Pro (Biomatters).

In order to assess the phylogenetic relationships of the psocid bacterium, the 16S rRNA, *gltA*, *ompA*, *ompB*, and cell surface antigen *sca4* (gene D) sequences obtained from our analyses were compared with the appropriate sequences from all validated *Rickettsia* spp. (12, 18, 22, 35–37). Phylogenetic analysis was performed using maximum likelihood, implemented in PhyML (11, 20). Modeltest, version 3.7 (32), was used to determine the most appropriate model of nucleotide substitution. Each gene was analyzed separately, and analyses were also performed on a concatenated data set of the slowly evolving genes *gltA* and 16S rRNA and a concatenated data set of *ompA*, *ompB*, and gene D. To assess confidence in nodes, 100 bootstrap replicates were performed. Maximum-parsimony analyses implemented in PAUP*, version 4.0 (40), yielded similar topology.

Cloning and sequencing of the plasmid. To determine the possible presence of a plasmid, the diagnostic primer sets pRFc-pRFd and pRFh-pRFi were initially used (28). To rule out the possibility that the fragments amplified by these primers were chromosomal and to confirm the presence of a plasmid, plasmid sequences from the completed *R. felis* URRWXCal2 genome were used to design primers (described in Table S2 in the supplemental material) that would amplify large overlapping fragments that would close the circle of the plasmid. Large fragments were amplified using an Expand Long Template PCR system (Roche, Mannheim, Germany) according to the manufacturer's protocol. Once the presence of a plasmid via long PCR was confirmed, primers were designed in order to sequence the entire plasmid genome (also described in Table S2). All new primers in Table S2 were designed using Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). The plasmid from *R. felis* infecting fleas has been previously reported to occur in up to two forms that may differ in stability (13, 28). The present study focused on the large form.

PCR products were cloned using a StrataClone PCR cloning kit (Stratagene, San Diego, CA) according to manufacturer's instructions. Clones from selected colonies were purified with a QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Clones were then sequenced in both directions (Macrogen, Seoul, South Korea). The plasmid was annotated using NCBI ORF Finder (open reading frame finder) (<http://www.ncbi.nlm.nih.gov/projects/gorf/>) and the CGView server (http://stothard.afns.ualberta.ca/cgview_server/index.html).

Nucleotide sequence accession numbers. The nucleotide sequences of the 16S rRNA, *gltA*, *ompA*, *ompB*, and gene D genes were deposited in the GenBank database under accession numbers GQ329872 to GQ329880 and GQ385243. The nucleotide sequences of the plasmid diagnostic genes were deposited in the GenBank database under accession numbers GQ385244 to GQ385247. The pRF plasmid sequence was deposited in the GenBank database under accession number GQ329881.

RESULTS

Sequencing and phylogenetic analysis of five genes from *L. bostrychophila* originally collected in Australia revealed that the psocid *Rickettsia* is authentic *R. felis* (Fig. 1). Citrate synthase (*gltA*) (382 bp) and cell surface antigen *sca4* (gene D) (2,013 bp) sequences were identical to those of *R. felis* URRWXCal2. 16S rRNA (1,205 bp), outer membrane protein rOmpA (*ompA*) (1,705 bp), and outer membrane protein rOmpB (*ompB*) (4,893 bp) sequences exhibited three, one, and

five nucleotide differences, respectively, compared to *R. felis* URRWXCal2. 16S rRNA (1,205 bp) and rOmpB (*ompB*) (750 bp) sequences obtained from *L. bostrychophila* from the Kansas culture and 16S rRNA (1,202 bp) and rOmpB (*ompB*) (5,180 bp) sequences obtained from *L. bostrychophila* from the Arizona culture were identical to those obtained from the Australia culture, thus confirming that *R. felis* is present in *L. bostrychophila* from different origins.

Diagnostic PCR, long PCR, and, finally, sequencing of the complete plasmid show that the psocid *Rickettsia* harbors a plasmid that is virtually identical to the *R. felis* URRWXCal2 pRF plasmid. The plasmid sequenced from the psocid *Rickettsia* (Queensland) was 62,882 nucleotides in length, had a GC content of 33.63%, and was 98% identical in the DNA level to the pRF plasmid from *R. felis* URRWXCal2. There were no differences in gene order between the plasmids. The few differences in gene content are summarized in Table 1 and Fig. 2. These can be largely attributed to accumulation of nonsense mutations that have caused shortening of 14 predicted genes. Two *R. felis* URRWXCal2 plasmid genes (pRF09 and pRF39) have been split into two separate genes in the psocid *Rickettsia* plasmid as a result of a new stop codon. Two *R. felis* URRWXCal2 plasmid genes (pRF54 and pRF55) have been merged into one in the psocid *Rickettsia* plasmid, and five genes (pRF22, pRF38, pRF57, pRF58, and pRF60) are absent. Finally, diagnostic PCR and sequencing also identified the plasmid in psocid cultures collected in Kansas and Arizona.

DISCUSSION

This study identifies a new host for *R. felis*: *L. bostrychophila*, a cosmopolitan insect pest. These highly polyphagous insects are infamous for their close associations with human dwellings and food storage facilities (8, 41). Furthermore, there are reports of intimate contact between liposcelids and humans, including feeding on dead skin of human toe nails (23) and infesting scalps (41). In addition, these insects are often found in close associations with vertebrates as they were recovered from rat nests, bird nests, and even on puppy fur (7, 26, 41). They also eat dead insects and insect eggs (41). Thus, it is feasible that during the course of evolution, while sharing the same microhabitat and in close association with humans or other vertebrates, *R. felis* was horizontally transmitted from fleas to *L. bostrychophila*.

Previous studies on *Rickettsia* from *Liposcelis* suggest that its distribution and transmission may be similar to that in fleas. *Rickettsiae* are present in the ovaries and oocytes of the insects (30, 42), suggestive of maternal transmission. They also occur within host fat bodies and digestive tracts (10, 30), suggesting that like flea *R. felis*, the bacteria may be transmitted through host feces. Moreover, within the gut epithelium and gut lumen, the *Rickettsia* from *Liposcelis* was shown to be free in the cytosol and surrounded by a "halo" (10, 30), as was described for *R. felis* in various flea tissues (1, 34) and in tick-derived cell culture (31).

The role of *R. felis* in *L. bostrychophila* remains a great mystery. *L. bostrychophila* reproduces via parthenogenesis (although males were recently discovered in at least one population in Hawaii [27]); no other parthenogenetic *Liposcelis* species are known. No *L. bostrychophila* insects without *Rickettsia* infection have yet been

FIG. 1. Unrooted maximum-likelihood phylogenetic tree of *Rickettsia* species based on a concatenated alignment of *ompA*, *ompB*, and cell surface antigen *sca4* (gene D), using a general time-reversible and gamma-distributed rate heterogeneity (GTR+G) model of nucleotide substitution (a), and a concatenated alignment of 16S rRNA and *gltA* genes, using a transversion and invariable sites (TVM+I) model of nucleotide

TABLE 1. Summary of differences in plasmid ORFs between *R. felis* URRWXCal2 and *R. felis* from *L. bostrychophila*

ORF	Annotation	Characteristic of psocid <i>R. felis</i> ORF vs <i>R. felis</i> URRWXCal2 ORF	
		% Difference	Description
pRF07	Type I restriction-modification system, methyltransferase subunit	1	Shorter ORF
pRF08	Hypothetical protein, similar to a part of CheY-like receiver domain	1	Longer ORF
pRF09	<i>R. felis</i> -specific protein (hypothetical protein)	8	Split into two small ORFs
pRF18	Tetratricopeptide repeat domain	2	
pRF19	Chromosomal replication initiator protein DnaA-like protein	17	Shorter ORF
pRF20	Chromosomal replication initiator protein DnaA-like protein	2	Longer ORF
pRF22	Similar to <i>Pseudomonas syringae</i> plasmid Ppsr1 ORF12	2	ORF is absent
pRF23	ParA, possible cytokinesis regulatory protein	1	Shorter ORF
pRF25	Cell surface antigen <i>sca12</i>	4	
pRF30	Transposase, mutator family (transposase_mut)	1	Longer ORF
pRF34	DNA polymerase III, epsilon subunit-like protein; WGR domain	7	
pRF37	Conjugative transfer protein TraD_Ti	2	Shorter ORF
pRF38	Conjugative transfer protein TraA_Ti (<i>Agrobacterium tumefaciens</i> Ti plasmid)	6	ORF is absent
pRF39	MobA_MobL (plasmid transfer)/RecD (exonuclease V) hybrid (conjugative transfer protein TraA)	3	Split into two small ORFs
pRF43	Putative conjugative transfer protein TraD	1	Longer ORF
pRF44	Putative conjugative transfer protein TraD	1	Shorter ORF
pRF46	Putative conjugative transfer protein TraG	1	Shorter ORF
pRF47	Putative conjugative transfer protein TraG	1	Shorter ORF
pRF50	Hypothetical protein	1	Shorter ORF
pRF52	Small heat-shock protein 1	1	Shorter ORF
pRF53	DNA polymerase III, epsilon subunit-like protein	1	Shorter ORF
pRF54	Transposase, mutator family	3	Merged with pRF55
pRF57	Transposase	10	Absent
pRF58	Transposase	2	Absent
pRF60	Hypothetical protein, similar to element transposase	1	Absent
pRF62	<i>R. felis</i> -specific protein; possible TldD/PmbA protein	12	Shorter ORF
pRF63	<i>R. felis</i> -specific protein; hypothetical protein; similar to <i>Wolbachia</i> RepA	11	Shorter ORF
pRF64	Transposase 14	4	Shorter ORF
pRF67	Hypothetical protein, similar to transposase	1	Shorter ORF

found; cultures from Europe (United Kingdom and Czech Republic) (30, 42) and Asia (China) are infected (30), just like those from Australia and the United States presented in this study; *R. felis* has also not yet been found in any sexual *Liposcelis* species (A. Behar and S. J. Perlman, unpublished data). Furthermore, treating *L. bostrychophila* with antibiotics results in sterility (42). Thus, it is possible that *R. felis* is interfering with reproduction (38). In addition to the well-known insect symbionts *Wolbachia* (36) and *Cardinium* (47), one other *Rickettsia* species has been implicated in inducing parthenogenesis in its hosts (21). However, unlike *L. bostrychophila*, all of the insects thus far implicated in parthenogenesis induction have haplodiploid sex determination, and whether *R. felis* causes asexuality in *L. bostrychophila* remains unresolved. It should also be noted that, as yet, no symbionts

other than *Rickettsia* have been conclusively demonstrated in *L. bostrychophila*. Recent studies reporting *Wolbachia* (24) and *Cardinium* (43) infection in *L. bostrychophila* do not provide sequences, only PCR results, and these results could not be repeated during the course of this study.

Alternatively, the bacterium could be providing its host with an essential function. A recent study proposed that the *Rickettsia* from *Liposcelis* is supplementing host nutrition (30) although this is hard to believe for a number of reasons. *Rickettsia* genomes are highly reduced and lacking most metabolic genes, leaving the bacterium totally dependent on host resources for growth and survival (15, 17). Although it is possible that the strain of *R. felis* infecting *L. bostrychophila* has acquired new genes, this seems unlikely, given that plasmid and

substitution (b). Values above and below branches indicate likelihood and parsimony bootstrap support, respectively. Only bootstrap values greater than 70% are shown. Whenever possible, we used *Rickettsia* spp. for which a genome sequence was available: *R. bellii* strain RML369-C (NC_007940), *R. bellii* strain OSU85 389 (NZ_AARC000000000), *R. canadensis* strain McKiel (NZ_AAFF010000001), *R. prowazekii* strain Madrid E (NC_000963), *R. typhi* strain Wilmington (NC_006142), *R. akari* strain Hartford (NZ_AAFE010000001), *R. felis* strain URRWXCal2 (NC_007109), *R. conorii* strain Malish 7 (NC_003103), *R. rickettsii* strain Sheila Smith (NC_009882), *R. rickettsii* strain Iowa (NC_010263), *R. massiliae* strain MTU5 (NC_009900), and *R. sibirica* strain 246 (NZ_AABW010000001). For *Rickettsia* spp. for which full genome sequence is not available, we used the following accessions from previous studies (21–25): *R. australis* strain Phillips (L36101, U59718, AF149108, AF123709, and AF187982), *R. helvetica* strain C9P9 (L36212, U59723, AF123725, and AF163009), *R. montanensis* strain M56 (L36215, U74756, U43801, AF123716, and AF163002), *R. aeschlimannii* strain MC16T (U74757, U59722, U43800, AF123705, and AF163005), *R. rhipicephali* strain 376 (L36216, U59721, U43803, AF123719, and AF155053), *R. japonica* strain YM (L36213, U59724, U43795, AF123713, and AF155055), *R. slovacica* strain 13B (L36224, U59725, U43808, AF123723, and AF155054), *R. parkeri* strain Maculatum 20 (L36673, U59732, U43802, AF123717, and AF155059), *R. africana* strain ESF-5 (L36098, U59733, U43790, AF123706, and AF151724), *R. honei* strain TT118 (L36220, U59726, U43809, AF123724, and AF163004), and *R. rickettsii* strain R (L36217, U59729, U43804, X16353, and AF163000).

FIG. 2. Circular representation of the *L. bostrychophila*-associated *R. felis* pRF plasmid generated by using the CGView server (http://stothard.afns.ualberta.ca/cgview_server/index.html). The inner circle represents GC content bias $[(G - C)/(G + C)]$, with positive values in green and negative values in purple; the second circle represents G+C content; the third circle represents a nucleotide BLAST comparison with *R. felis* URRWXC2 pRF plasmid, with matches in light blue, differences in dark blue, and gaps in white. The outer 12 circles represent predicted ORFs on the reverse and forward strands. Sp, split ORF; A, absent ORF; M, merged ORF; L, longer ORF; Sh, shorter ORF. The inner scale is marked in kilobases.

outer membrane genes are virtually identical to those of the *R. felis* found in fleas. Moreover, closely related sexual species with the same ecology and nutritional requirements do not harbor *Rickettsia* (30). Finally, it is also possible that *R. felis* is maintained via addition (25), for example, through a toxin-antitoxin system. The fact that *L. bostrychophila* is unable to reproduce when treated with antibiotics is especially intriguing since *R. felis* does not appear to induce such an effect in fleas (34).

The discovery of *R. felis* in the non-blood-feeding *L. bostrychophila* has a number of implications. This may be a much easier system potentially to study the molecular interactions between *Rickettsia* species (and *R. felis* specifically) and their arthropod hosts as the psocid can be cultured easily, grows rapidly, and does not require blood. Furthermore, comparative studies between *R. felis* from fleas and the psocid *R. felis* may provide researchers with valuable information on key

processes regarding *R. felis* biology and epidemiology, including mechanisms of host recognition, maintenance, pathogenicity, and virulence.

Finally, future studies will need to determine the potential pathogenicity of the psocid *R. felis* for vertebrates. Thus far, there is no evidence that *L. bostrychophila* constitutes a threat to public health. Nevertheless, the results of the current study, combined with the intimate associations between *Liposcelis* and humans, suggest that this novel *R. felis* strain warrants further study.

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