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Original article

Host blood meal-dependent growth ensures transovarial transmission and transstadial passage of *Rickettsia* sp. phylotype G021 in the western black-legged tick (*Ixodes pacificus*)

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ABSTRACT

In this study, we explored the growth dynamics of *Rickettsia* sp. phylotype G021 during transovarial transmission and transstadial passage by *Ixodes pacificus* using real-time quantitative PCR. Four parental engorged *I. pacificus* females were allowed to complete their developmental stages until the F2-generation eggs yielded unfed larvae. All eggs, larvae, nymphs, and adults tested through 2 generations were found to be infected with phylotype G021. Hence, we conclude that the efficiency of transovarial transmission and transstadial passage of this phylotype in *I. pacificus* was 100%. Acquisition of a blood meal by all 3 parasitic stages (larva, nymph, adult) significantly increased the rickettsial burden as fed larvae, nymphs, and adults had respective 19-, 12-, and 313-fold increases of rickettsiae compared with unfed ticks representing each developmental stage. *I. pacificus* eggs contained high rickettsial burdens at the time of oviposition. While *I. pacificus* egg cells underwent rapid proliferation during early embryonic development, the rickettsiae remained relatively quiescent, which resulted in depressed numbers of phylotype G021 per tick cell. However, the rickettsial burden remained constant over a period of 56 days, as the rate of *I. pacificus* cell division slowed during later embryonic development.

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Introduction

Long-lasting interactions between arthropods and bacteria are commonly found in nature (Baumann, 2005). Although the maintenance of bacterial infections in arthropods is driven by several transmission routes, vertical transmission is crucial for the stable maintenance of some bacterial species from one generation to the next (Bright and Bulgheresi, 2010; Buchner, 1965). Many bacteria transmitted by ticks are transmitted vertically via transovarial transmission and transstadially from stage to stage, such as certain *Rickettsia* species in ticks (Horta et al., 2006; Socolovschi et al., 2009), cat fleas (Azad et al., 1992) or mites (Takahashi et al., 1988), and *Borrelia* species (Lane and Burgdorfer, 1987; Scoles et al., 2001), as well as *Francisella*-like endosymbionts and *Anaplasma* species in ticks (Baldridge et al., 2009). Compared with other transmission routes, transovarial transmission is a highly efficient mechanism for perpetuating certain bacteria (Matsumoto et al., 2005).

The western black-legged tick, *Ixodes pacificus* Cooley & Kohls, which is broadly distributed in the far-western United States, is the primary vector of the bacteria causing Lyme borreliosis and anaplasmosis (Burgdorfer et al., 1985; Clover and Lane, 1995; Foley et al., 2008; Piesman et al., 1999). Its life cycle requires 3 years to complete and includes the egg and 3 parasitic stages, the larva, nymph, and adult. The larva and nymph require a blood meal in order to develop into the next stage, whereas the adult female needs blood to mature a batch of about 900–1000 eggs (Padgett and Lane, 2001). During the blood meal, ticks undergo profound expansion in mass and regulation of internal cellular and molecular pathways. In response, the quantity and location of bacteria in ticks also undergo dramatic changes (Liu et al., 2011; Radolf et al., 2012).

Spotted fever group rickettsiae are Gram-negative, intracellular bacteria commonly found in association with ixodid ticks (Boretti et al., 2009; Dalton et al., 1995). Unlike pathogenic rickettsiae that cause human and animal diseases, some nonpathogenic rickettsiae in the spotted fever group are bacterial endosymbionts (Douglas, 2007). Endosymbiotic rickettsial species are nonpathogenic and intracellular, are transmitted transovarially from female arthropods to their offspring, and have an intimate and persistent relationship with the hosts (Douglas, 2007; Perlman et al., 2006; Sakurai et al., 2005). The rickettsial endosymbionts have been identified in many tick hosts,

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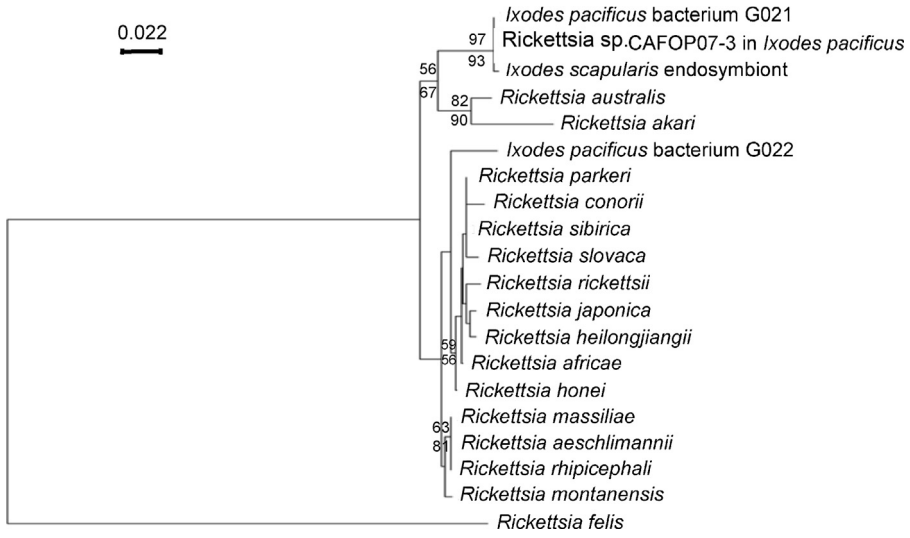

Fig. 1. Phylogenetic tree of the *ompA* genes of *Rickettsia* phylotypes G021 and G022 in *Ixodes pacificus*. Other selected sequences on the phylogenetic tree of the *ompA* gene are *Rickettsia* sp. CAFOP07-3 (EU544297.1), *I. scapularis* endosymbiont (AB002268.1), *R. aeschlimannii* (DQ379981.1), *R. massiliae* (DQ212707.1), *R. rhipicephali* (EU109177.1), *R. australis* (AF149108.1), *R. montanensis* (AF045223.1), *R. sibirica* (AABW01000001.1), *R. rickettsii* (AY319293.1), *R. slovaca* (EU622810.1), *R. honei* (AF018075.1), *R. africae* (EU622980.1), *R. parkeri* (EU715288.1), *R. heilongjiangii* (AF179362.2), *R. conorii* (U43794.1), *R. felis* (AY727036.1), *R. japonica* (U83442.1), and *R. akari* (L01461.1). The tree is inferred from the comparison of *ompA* sequences by the neighbor-joining method using PHYLO_WIN. The numbers at nodes are the bootstrap values obtained from 1000 replicates. Bootstrap values >50 are shown at the nodes. Bootstrap values determined by the neighbor-joining method and maximum-parsimony are shown above the line and below the line, respectively. Bar, nucleotide distance.

The phylogenetic tree of rickettsial *ompA* genes was provided with permission of use by the journal of "Vector-borne and Zoonotic Diseases" (Phan et al., 2011).

such as *I. scapularis*, *I. ricinus*, and *Amblyomma americanum*, to name but a few (Jasinskas et al., 2007; Weller et al., 1998).

Recently, 2 rickettsial phylotypes, G021 and G022, were detected in host-seeking *I. pacificus* collected in Napa, California. Phylogenetic analysis suggested that phylotype G021 is closely related to a rickettsial endosymbiont infecting *I. scapularis*, whereas phylotype G022 is a novel, deeply branched spotted fever group *Rickettsia* (Fig. 1) (Phan et al., 2011). The prevalence of phylotype G021 in *I. pacificus* collected from 7 counties in California was 100%, which suggested that it is an endosymbiotic rickettsia that is transmitted transovarially and passed transstadially (Cheng et al., 2013).

The objective of the current study was to experimentally evaluate the rate of transovarial transmission and transstadial passage of G021 in *I. pacificus* through the first (F1) and part of the second (F2) generation by real-time quantitative PCR. In addition, the temporal pattern of bacterial growth in all developmental stages of *I. pacificus*, as determined by real-time quantitative PCR, was determined to further study the interaction of phylotype G021 with its tick host.

Materials and methods

Ticks

Host-seeking *I. pacificus* adults were collected by dragging a white flannel cloth, 1 m × 1.25 m, over low vegetation in October 2009 at the University of California Hopland Research and Extension Center in Mendocino County, California (Universal Transverse Mercator coordinates: 10N 492815 4316885). Additionally, 18 engorged female ticks were collected from dogs brought into the Mendocino County Animal Care Services in Ukiah, California, in May, June, and July 2010. All *I. pacificus* were maintained in desiccators (Fisher Scientific, Houston, TX) at 25 °C and 90% relative humidity. The day/night cycle was set at 12 h light:12 h dark.

Laboratory rearing and experimental design

All procedures followed protocols approved by the Institutional Animal Care and Use Committee at Humboldt State University. To study the transmission routes of phylotype G021 by *I. pacificus*, 16 ten-week-old male New Zealand white rabbits (*Oryctolagus cuniculus*) (Western Oregon Rabbit Co., Philomath, OR) were used for feeding ticks. Four rabbits were used for each of the parasitic developmental stages, including the parental female, F1-generation larvae, F1-generation nymphs, and F1-generation females. The ticks were placed in tin capsules (1.5 × 1 in.) previously glued onto preshaven skin on the rabbits' dorsal thoracic and abdominal regions. Engorged ticks were removed from the capsules following detachment (Eisen et al., 2003).

Initially, 30 female and 30 male ticks were fed on each rabbit. Next, 4 engorged *I. pacificus* parental females were allowed to complete their developmental cycles. Offspring from the 4 lineages in the F1 generation including 80 eggs, 40 larvae, 20 engorged larvae, 40 nymphs, 20 engorged nymphs, and 56 adults, were preserved in 95% ethanol at 4 °C for further studies. In the F2 generation, 2 engorged adults were used in each lineage to construct 2 sublineages. In total, 8 sublineages including 8 engorged adults, 160 eggs, and 160 larvae, were saved in 95% ethanol at 4 °C for further studies (Fig. 2).

To study the growth dynamics of phylotype G021 in eggs of *I. pacificus*, 50 eggs from a laboratory-reared engorged female were collected 1, 4, 10, 16, 32, 45, and 60 days after tick oviposition and stored in 95% ethanol at 4 °C, prior to DNA extraction. For field-derived engorged *I. pacificus* females, 5 eggs were collected from each of the 18 engorged females. Spent females and their eggs were preserved in 95% ethanol at 4 °C.

DNA extraction

The DNA extraction method was described previously (Zhong et al., 2007). Briefly, ticks representing all stages were extracted individually. Eggs, flat and engorged larvae, flat and engorged nymphs, and flat adults were surface-sterilized in 70% ethanol 3

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