available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biocon

Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*

Douglas C. Woodhams[a,*], *Vance T. Vredenburg*[b], *Mary-Alice Simon*[c], *Dean Billheimer*[d], *Bashar Shakhtour*[d], *Yu Shyr*[d], *Cheryl J. Briggs*[b], *Louise A. Rollins-Smith*[a], *Reid N. Harris*[c]

[a]*Department of Microbiology and Immunology, A-5301 Medical Center North, Vanderbilt University Medical Center, Nashville, TN 37232, United States*
[b]*Department of Integrative Biology, University of California, Berkeley, CA 94720-3140, United States*
[c]*Department of Biology, James Madison University, Harrisonburg, VA 22807, United States*
[d]*Biostatistics Shared Resources, Vanderbilt University School of Medicine, 571 Preston Building, Nashville, TN 37232, United States*

ARTICLE INFO

Article history:
Received 5 February 2007
Accepted 8 May 2007
Available online 28 June 2007

Keywords:
Antimicrobial peptide
Batrachochytrium dendrobatidis
Herd immunity
Innate immune defense
MALDI mass spectrometry
Rana muscosa
Symbiotic bacteria

ABSTRACT

Symbiotic microorganisms influence health and disease and may contribute to the innate immune defenses of amphibians. The mountain yellow-legged frog, *Rana muscosa*, is currently undergoing unprecedented population declines. One cause of recent declines is the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*). Skin swabs for detection of *Bd*, skin peptide secretions, and symbiotic skin bacteria were collected from 70 adult *R. muscosa* from two populations designated "Sixty Lake" and "Conness" in 2004–2005. The Conness population has persisted with the presence of *Bd* for at least 6 years whereas the Sixty Lake population is newly infected and declining. Of the frogs sampled at Conness, 67.5% were infected; whereas 96.7% of the Sixty Lake frogs were infected. Sixty Lake frogs were also more intensely infected than frogs at Conness. We isolated symbiotic bacteria that may contribute to immune defense. A significantly greater proportion of individuals with at least one anti-*Bd* bacterial species present were found at Conness (85%) than at Sixty Lake (62%). We observed no apparent differences in total skin peptides recovered; however, peptide mixtures from frogs at Sixty Lake showed better growth inhibitory activity against *Bd* than peptides from frogs at Conness. By MALDI-TOF MS analysis, there were no differences between the two populations in the previously described antimicrobial peptides (ranatuerin-2Ma, ranatuerin-2Mb, and temporin-1M). Antimicrobial skin peptides are only one factor in the resistance of *R. muscosa* to *Bd* infection. We suggest that symbiotic bacteria with the ability to persist in the presence of mucosal peptides may inhibit infection and colonization of the skin by *Bd* and increase the effectiveness of innate defense mechanisms in the skin.

* *Corresponding author:* Tel.: +1 615 343 9449; fax: +1 615 343 7392.
E-mail addresses: dwoodhams@gmail.com (D.C. Woodhams), vancev@berkeley.edu (V.T. Vredenburg), mawoodnz@yahoo.com (M.-A. Simon), dean.billheimer@vanderbilt.edu (D. Billheimer), bashar.shakhtour@vanderbilt.edu (B. Shakhtour), yu.shyr@vanderbilt.edu (Y. Shyr), cbriggs@berkeley.edu (C.J. Briggs), louise.rollins-smith@vanderbilt.edu (L.A. Rollins-Smith), harrisrn@jmu.edu (R.N. Harris).
0006-3207/$ - see front matter
doi:10.1016/j.biocon.2007.05.004

1. Introduction

The mountain yellow-legged frog, *Rana muscosa*, is native to cold and remote lakes in the California Sierra Nevada mountains. This once very abundant species is currently undergoing unprecedented population declines (Bradford, 1991; Drost and Fellers, 1996; Sparling et al., 2001; Vredenburg et al., 2005). Although previous declines were attributed to the introduction of non-native trout (Bradford, 1989; Bradford et al., 1993; Knapp and Matthews, 2000; Vredenburg, 2004) or to pesticides (Davidson, 2004; Fellers et al., 2004), recent declines have continued even in apparently unpolluted fishless lakes. One cause of recent declines is the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*) (Briggs et al., 2005; Rachowicz et al., 2006). This fungus is associated with amphibian population declines on several continents (Berger et al., 1998; Waldman et al., 2001; Weldon et al., 2004; Garner et al., 2005; Lips et al., 2006). The factors that lead to extinction or to persistence of amphibian populations with *Bd* are currently under examination (Briggs et al., 2005; Bosch and Martinez-Solano, 2006; Woodhams et al., 2006a,b).

One factor that may contribute to disease resistance is innate immune defense. Innate skin defenses including antimicrobial peptides and symbiotic microbial barriers may be crucial for defending amphibians against the skin-invasive fungus, *Bd* (Harris et al., 2006; Woodhams et al., 2006a,b; Lauer et al., in press). Granular glands in the skin of *R. muscosa* secrete a mixture of peptides including antimicrobial peptides (ranatuerin-2Ma, 2Mb, and temporin-1M). These peptides inhibit the growth of *Bd in vitro* (Rollins-Smith et al., 2006). Symbiotic bacteria of amphibians are not well-known and have not been previously described in *R. muscosa*. However, the salamanders *Plethodon cinereus* and *Hemidactylium scutatum* host beneficial bacteria that inhibit growth of *Bd* in vitro and may be important for resisting *Bd* colonization of skin (Harris et al., 2006; Lauer et al., in press). Here we describe the microbiota of *R. muscosa* that contribute to resistance against *Bd*, and suggest that populations which host beneficial bacteria may be more likely to persist with *Bd*.

2. Methods

2.1. Study species and sites

Adult *R. muscosa* were sampled from two populations in August, 2005. These populations were Lake 11 (elevation 3390 m, depth 3.5 m) in Sixty Lake Basin, Kings Canyon National Park, California (36.82°N, 118.43°W) and Conness (elevation 3175 m, depth 3.9 m) in Yosemite National Park, California (37.97°N, 119.34°W). Populations were in similar high alpine habitats and presumably experienced similar environmental conditions. Using a hand net, we sampled 30 adults from the Sixty Lake site on August 11–12, 2005 and 40 adults from the Conness site on August 15, 2005. Although the Sixty Lake population was thought to be healthy at the time of sampling, a die-off occurred after our sampling between fall 2005 and spring 2006. During subsequent surveys in 2006, no living frogs were found at this site. Fig. 1 illustrates the mass mortality seen in a nearby lake in Sixty Lake Basin in August, 2006. No decline was found at Conness.

2.2. Assessment of Bd infection status

To assess whether frogs were infected with *Bd*, the skin was swabbed as described by Kriger et al. (2006), and real-time Taqman PCR was used to determine the intensity of infection (or the number of zoospore DNA equivalents) (Boyle et al., 2004). Because frogs in each population inhabited the same body of water and were caught in the same net, it is possible that low intensity positive results represent contamination of the swab rather than actual infections. However, precautions were taken to minimize contamination by rinsing the net in lake water between use, and changing gloves before handling a new frog. Frequency of infection (infection prevalence) was compared between populations with Fisher's exact test. Intensity of infection was compared between populations by Mann–Whitney *U*-test.

2.3. Culture and identification of bacteria from frog skin

After capture, frogs were rinsed twice with sterile water to remove transient bacteria (Lauer et al., in press). Symbiotic bacteria from frog skin were sampled using sterile swabs and cultured onto low nutrient Difco R2A media (Becton, Dickinson and Co., Sparks, MD) in the field and quickly transported to James Madison University for isolation. The growth form and color of each isolate was described and each isolate was tested for ability to inhibit the growth of *Bd*. Zoospores were washed from *Bd* culture plates and applied to new 1% tryptone agar plates and allowed to dry in a sterile hood. *Bd*-challenge assays involved adding a streak of an unknown bacterial isolate to one side of the culture plate and a streak of a known bacteria that does not inhibit *Bd* to the other side as a negative control. Bacteria that produced a clear zone with no fungal growth around the streak were considered inhibitory. DNA from these bacteria was then extracted from pure cultures with a MoBio Ultraclean Microbial DNA Isolation Kit (Carlsbad, CA), and then amplified by PCR using universal bacterial 8F and 1492R rRNA primers (Lane, 1991). Products were sent to the Brigham Young University DNA Sequencing Center for sequencing and identified according to similarity with NCBI GenBank entries (http://www.ncbi.nlm.nih.gov) (Harris et al., 2006). The proportion of frogs from each population with at least one species of *Bd*-inhibitory bacteria was compared with a two-tailed Fisher's exact test. All culturable anti-*Bd* symbionts at each site were identified. The microbial species composition was compared between the two populations.

2.4. Collection and quantification of skin peptides

Skin peptides were collected after subcutaneous injection of 10 nmole per gram body weight norepinephrine (bitartrate salt, Sigma, St. Louis, Missouri) by two methods. For a direct survey of all skin peptides, they were absorbed onto 80 μm carbon-imbedded conductive polyethylene film (Goodfellow Cambridge Ltd., Cambridge, England). For collection of larger quantities, the peptides were rinsed from frogs after a 10 min water bath (Chaurand et al., 1999; Rollins-Smith et al., 2002; Woodhams et al., 2006b). For each frog from the Conness population, small pieces (1 × 2 cm) of film were activated by soaking in methanol for 5 s, allowed to air-dry, and