Thermophilic Amoebae and *Legionella* in Hot Springs in Yellowstone and Grand Teton National Parks

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ABSTRACT

Microscopic examination of samples from an algal mat biofilm community in Nymph Creek, Yellowstone National Park, WY, showed the presence of free-living, vahlkampfiid amoebae. Sequence analysis of portions of 18S rRNA genes from a community DNA clone library revealed, among other sequences, several that matched *Vahlkampfia*. Because some vahlkampfiids, including the pathogens *Acanthamoeba* and *Naegleria fowleri*, can cause severe and often fatal infections in humans, we developed a rapid method for sampling and analyzing the mat community using primers targeting *Naegleria* species. We identified sequences that closely match *Acanthamoeba* sp. and *N. fowleri*, as well as *Naegleria* sequence types not previously described, in Nymph Creek and a variety of other aquatic geothermal sites in Yellowstone and Grand Teton National Parks. Since a number of bacteria, including the intracellular human pathogen *Legionella pneumophila*, are known to survive in microbial biofilms and as endosymbionts in free-living amoebae, we used traditional culture-based methods in addition to PCR-amplification with primer sets that target 16S rRNA genes, to identify five *Legionella* species in Nymph Creek. In addition, one potentially new *Legionella* species was identified in a *Euglena* enrichment culture obtained from Nymph Creek.

Key Words

Acanthamoeba
biofilms
endosymbionts
Euglena
Naegleria
phylogenetics
sequence analysis

1.0 INTRODUCTION

Thermophilic free-living amoebae (Vahlkampfiidae) grow at elevated temperatures in hot water systems, natural hot springs, farm ponds, thermal effluents from power plants, and spas worldwide. The amoebae live as phagotrophs, feeding on biofilms and detritus (De Jonckheere 2002; Ramaley et al. 2001). However, given suitable conditions some opportunistic species can infect humans and other mammals causing serious illnesses (Kollars and Wilhelm 1996; Szénáis et al. 1998).

One valhkampfiid, *Naegleria fowleri*, causes primary amoebic meningoencephalitis in humans (Hannish and Hallagan 1997; John 1993, 1998), a rare but fatal disease that usually occurs in otherwise healthy persons with a history of swimming and diving in heated, contaminated water. The amoebae invade the nasal passages, move along the olfactory nerves to the brain, and cause severe damage and death approximately 10-14 days after exposure (Martinez and Visvesvara 1997).

Another species, *Acanthamoeba*, also can infect the central nervous system (CNS) in humans causing an illness called granulomatous amoebic encephalitis. In addition, *Acanthamoeba* can infect other tissues, including skin, eyes (amoebic keratitis), and lungs. Unlike infection by *N. fowleri*, an *Acanthamoeba* infection is not usually contracted while swimming. Instead, the amoebae reach the CNS via the bloodstream, generally in immunocompromised elderly or diabetic persons after exposure to contaminated water (John 1993).

Vahlkampfiid amoebae such as *Naegleria* and *Acanthamoeba*, and at least two protozoans, can be host cells for bacterial endosymbionts including human pathogens *Mycobacterium* spp., *Escherichia coli* 157, and *Legionella pneumophila* (Fields 1996; Steinert et al. 2002; Molmeret et al. 2005). Bacteria that are engulfed by grazing amoebae have evolved mechanisms to avoid phagocytosis. These mechanisms may also allow the bacteria to proliferate in human lung macrophages (Molofsky and Swanson 2004; Swanson and Isberg 1995). In addition, there is evidence that bacteria released from protozoan hosts are more virulent in mammalian cells *in vitro* (Cirillo et al. 1999). Amoebae act as natural reservoirs for the bacteria and have been described by some as the "Trojan horses" of the microbial world because they enable the pathogens to persist in the environment (Molmeret et al. 2005; Barker and Brown 1994).

In some cases following intracellular replication in protozoan hosts, bacteria have become more resistant to harsh extracellular conditions such as high temperature, acidity, and osmolarity, further increasing their survival under stressful conditions (Abu Kwaik et al. 1998). Often the endosymbionts are impossible to culture outside their hosts, making identification difficult (Amann et al. 1991).

L. pneumophila was first isolated in 1977 and shown to cause Legionnaire's disease—a severe pneumonia contacted by breathing contaminated, aerosolized water droplets from manmade hot water sources such as whirlpools, fountains, and air conditioning cooling towers (Harb et al. 2000; Molmeret et al. 2005). Since the initial isolation of *L. pneumophila*, more than 46 *Legionella* species have been characterized and at least half of the species have been reported to be pathogenic in humans, causing infections generally referred to as legionellosis. To date, no person-to-person infections have been documented (Miyamoto et al. 1997; Steinert et al. 2002; Molmeret et al. 2005). Prevention of illness requires avoiding contaminated water.

Since swimming and soaking is allowed in some areas of Yellowstone and Grand Teton National Parks that are suitable habitats for thermophilic amoebae and *Legionella* species, it is important to more accurately describe the natural distribution and diversity of these microorganisms and to address any public health issues.

PCR-based approaches for direct recovery and analysis of rRNA gene sequences from the environment provide a relatively rapid and effective means of detection; enhance identification beyond that obtainable by cultivation and/or microscopy; and often reveal previously unknown diversity (Amaral Zettler et al. 2002; Dawson and Pace 2002; DeLong and Pace 2001). We surveyed 23 different hot springs for pathogenic amoebae using PCR amplification, cloning, and sequencing methods, as well as culture methods. We further analyzed Nymph Creek (using culture- and sequence-

based methods) and three additional sites, the Boiling River, Terrace Springs, and Bathtub (using sequence-based methods) for the presence of legionellae.

2.0 METHODS

2.1 Sample collection and microscopy

Nymph Creek (**Figure 1**) has been studied and described previously by our laboratory (Sheehan et al. 2003a, 2003b, 2005; Ferris et al. 2003, submitted). Additional sites where people swim and bathe in Yellowstone and Grand Teton National Parks were identified and sampled between August 2001 and September 2002 with the assistance of the Yellowstone Center for Resources, park rangers, and volunteers. Samples (~5 mL) of sediment, algal mat, or biofilms were obtained from at least five locations within individual thermal pools or streams across temperatures that ranged from 3-51°C, covering the growth range (25-50°C) for thermophilic amoebae. The samples were immediately frozen in the field in dry ice and kept frozen until further processing in the laboratory. At remote sampling sites where transport of dry ice was not feasible, samples were mixed 1:1 in absolute ethanol: sterile STE (10 mM Tris-HCl, pH 7.5; 10 mM NaCl; 1 mM EDTA, pH 8). Samples stored at ambient temperature were examined microscopically or cultured for amoebae and legionellae within 24 hours of collection (see methods, Sheehan et al. 2005). Briefly, GPAV culture medium (BYCEα supplemented with glycine) specific for *Legionella* sp., and PAV(–) negative control medium were prepared following protocols by Gorman et al. (1994). Mat samples from Nymph Creek were plated directly on selective media at a range of 3–7 pH and incubated at 37°C in a candle jar, or plated on SAG medium (Sammlung von AlgenKulturen Gottingen Culture Collection of Algae, *Cyanidium* Medium 17, pH 2.9) at 25°C for culturing of amoebae.

↑ **Figure 1. Nymph Creek, a thermal, acidic hot spring-fed stream, flows for about 150 m to Nymph Lake.** A striking, vivid green algal mat covers the streambed. (Image courtesy of K.B. Sheehan).

2.2 PCR amplification, cloning, sequencing, and phylogenetic analysis

PCR amplification, cloning, and sequencing approaches were used to detect taxonomically informative 16S, 18S, and ITS rRNA gene sequences directly from extracted DNA, as described previously (Sheehan et al. 2003a, 2003b, 2005; Ferris et al. 2003, submitted). Primer sets shown in **Table 1** targeted *Naegleria* and *Legionella* spp. and are described in detail in Sheehan et al. (2003a, 2003b, 2005). Genomic DNAs extracted from *L. pneumophila* subsp. *pneumophila* (ATC 33152D; American Type Culture Collection) and from an *N. fowleri* isolate (ATC 30863; American Type Culture Collection) were used as positive controls in all reactions. All rDNA sequences were deposited in GenBank (accession numbers AY274812-14, AY267537, and AY682851-AY682873).

3.0 RESULTS AND DISCUSSION

Twenty-three different sampling sites in warm water pools, hot springs, or locations where heated water flowed into freshwater streams were sampled. The sites varied considerably with regard to temperature and pH, which included

Table 1. Locations, sample dates, environmental conditions, and primer sets used in the study. Primers ITS f/r and FW 1/2 target *Naegleria*. LEG 225/858 is specific for *Legionella*. (+ = sequences were identified in at least one of the samples; – = no sequences were identified in any samples despite repeated attempts; NT=not tested)

Sampling Site	Sample Date	Temperature	pH	*Naegleria* Primers ITS f/r	*Naegleria* Primers FW1/2	*Legionella* Primers LEG225/858
Boiling River	8/23/01	35°C	7.1–7.4	+	+	+
Nymph Creek	8/23/01 9/18/01	28°C	3.1–3.4	+	–	+
Dead Savage Spring	5/30/02	35°C	6.0–7.0	–	–	NT
Ranger Pool	5/30/02	35°C	6.2–6.8	–	–	NT
Hillside Springs	5/30/02	35°C	5.9–7.8	+	–	NT
Seismic Geyser	5/30/02	32–40°C	7.3–7.7	+	–	NT
Mallard Lake Trail	5/30/02	30–38°C	3.3	+	–	NT
Madison Campground	10/2/01	35–38°C	6.7	+	–	NT
Firehole Swim Area	10/2/01	12–16°C	6.18–7.47	–	–	NT
Terrace Springs	5/30/02	40°C	7.1	+	–	–
Bathtub	5/30/02	36°C	6.1	+	–	–
Spirea Creek	7/8/02	40–42°C	8.2–8.4	–	–	NT
Huckleberry Hot Springs	6/20/02	36°C	6.3–7.3	+	–	NT
Upper Polecat Creek	6/20/02	40°C	6.2	+	–	NT
Lower Polecat Creek	6/20/02	36–39°C	6.2–7.0	–	–	NT
Kelly Warm Springs	10/12/01	20°C	7.5–8.4	–	–	NT
Obsidian Creek	9/6/02	37°C	2.1–2.3	–	–	NT
Water Tower Road	9/1/02	38–40°C	6.2-–7.3	–	–	NT
Dunanda Falls	9/23/02	40–45°C	NT	–	–	NT
3 Rivers	9/20/02	25–51°C	NT	–	–	NT
Scout Pool	9/27/02	21°C	NT	–	–	NT
Morning Falls	9/28/02	25°C	NT	–	–	NT
Sheepeater Cliffs	10/18/02	3°C	7.2	–	–	NT

mildly alkaline, circumneutral, and strongly acidic environments (**Table 1**).

Clone sequence analysis demonstrated the presence of *Naegleria* phylotypes not previously described, in these aquatic geothermal sites (Sheehan, et al. 2003a, 2003b, 2005; **Figure 3, p. 323**), as well as sequences in cultured isolates of potential microbial hosts—*Acanthamoeba* (95% similarity to AY026245.1) and *Euglena* (95% similarity to EMU532403). In addition, three of the four *Naegleria* sequence types detected represented populations distinct from those represented by cultivated species described in GenBank (**Figure 3**).

Other than *N. fowleri*, there are few reported studies of the occurrence of *Naegleria* species from continents other than Europe and Australia (De Jonckheere 2002). A previous survey of *Naegleria*-like amoebae in Yellowstone and Grand Teton National Parks utilized cultivation and microscopy approaches for detection, identification, and determination of virulence (Ramaley et al. 2001), and reported that *Naegleria*-like species isolates accounted for almost half of the amoebae observed. Although no pathogenic isolates were found in that study, isolates of *Naegleria australiensis* that were virulent in mice were identified and verified by isoenzyme analysis. Even though *Naegleria*-like species were cultured in the Ramaley study, genus and species designa-

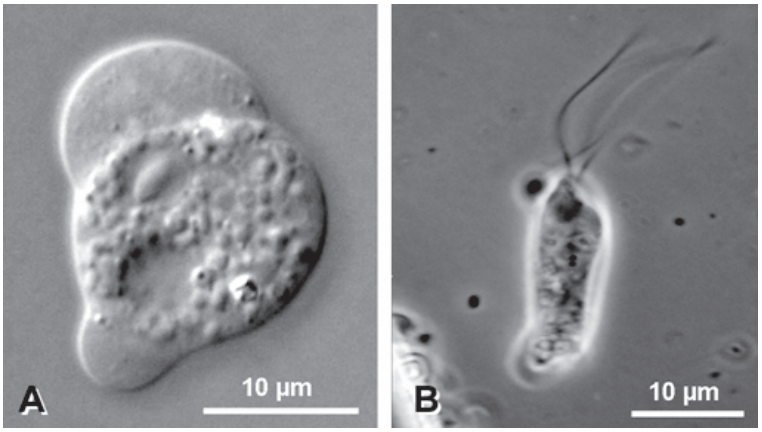

Figure 2. Microscopic examination of microbial mat samples from Nymph Creek, revealed vahlkampfiid-like amoebae: A. amoeboid trophozoite, and **B.** flagellated forms typical of vahlkampfiids. (Images courtesy of D.J. Patterson and K.B. Sheehan).

Table 2. *Legionella* sequences (16S rDNA) obtained from Nymph Creek mat samples or enrichment cultures.

Sequences	GenBank Accession #	Closest GenBank Match	% Similarity
30°C site	AY682852	*L. sainthelensii*	99%
35°C site	AY682856	*Legionella* sp.	96%
38°C site	AY682860	LLAP (*Legionella*-like amoebal pathogen	98%
Pure culture isolate	AY682872	*L. micdadei*	99%
Detected in an advanced *Euglena* enrichment	AY682859	*L. cherrii*	97%
Detected in an advanced *Acanthamoeba* enrichment	AY682859	*Legionella* sp.	99%

tions in the family Vahlkampfiidae are impossible to classify using morphology alone (De Jonckheere 2002). Our microscopic examination of samples from Nymph Creek found vahlklampfiid-like amoebae (**Figure 2**). Our PCR-based strategy of identification provided a rapid means of detection and enhanced identification of the amoebae beyond that obtainable by cultivation and/or microscopy. Furthermore, we detected a sequence type that may represent a novel, potentially pathogenic *Naegleria* species (**Figure 3**, AY274812) in Nymph Creek (Sheehan et al. 2003a, 2003b).

Further analysis of Nymph Creek, Boiling River, Terrace Springs, and Bathtub samples (**Table 1**)—with PCR amplification using *Legionella*-specific primers targeting 16S rRNA genes—detected in mat samples from Nymph Creek and the Boiling River, and cultivated *Legionella* isolates from Nymph Creek, four known *Legionella* species (**Table 2**), as well as one potentially new *Legionella* species (**Table 2**, AY682860) not represented in sequence databases. We were unable to amplify *Legionella* sequences from any of the Terrace Springs and Bathtub samples, despite repeated attempts. In addition, potentially novel *Legionella* species were identified in advanced *Euglena* and *Acanthamoeba* enrichment cultures obtained from Nymph Creek (**Table 2**, AY682859; Sheehan et al. 2005), providing preliminary evidence that *Euglena* and *Acanthamoeba* act as host organisms for *Legionella*.

Ours is the first sequence-based analysis survey of free-living amoebae and *Legionella* from environmental samples obtained from numerous Yellowstone and Grand Teton hot springs. We found sequences that closely match known *Naegleira* and *Legionella* species in GenBank, as well as phylotypes not previously described. In addition, we uncovered species diversity across a range of environmental conditions, including the extremely acidic Nymph Creek mat. We found *Naegleria* species in many of the samples collected at temperatures favorable for the growth of thermophilic amoebae (25-50°C). Our data provide evidence that free-living amoebae and *Legionella* species flourish in a variety of physical and chemical environments in the parks. The potentially pathogenic amoebae may serve as host organisms for legionellae, as well as potential human pathogens. Further study utilizing molecular methods of detection will be valuable in determining the ecological significance of these organisms in natural consortia, as well as their persistence in the environment, and

will aid park officials in making informed decisions regarding public health issues in the parks.

ACKNOWLEDGMENTS

This research was funded by grants from the Department of the Interior, National Park Service; the Thermal Biology Institute at Montana State University; and the National Science Foundation (Microbial Observatory grant 9977922). Jennifer Fagg was supported by the Thermal Biology Institute Undergraduate Internship Program. Thanks to David J. Patterson for his expert microscopic analysis and micrographs. We are grateful for laboratory and field assistance from Dean Snow, Tai Takenaka, Emily Kuhn, Mary Bateson, Bob Seibert, Wes Miles, Kathleen O'Leary, Lane Baker, David Daniels, Mike Keller, Mark Sheehan, Mike Sheehan, and the Bechler Ranger Station staff. The project was conducted under the direction of the Yellowstone Center for Resources following guidelines for scientific research in Yellowstone National Park. We especially thank John Varley, Director, and staff members Tom Oliff, Anne Deutch, Christie Hendrix, and Liz Cleveland for their enthusiastic support and assistance.

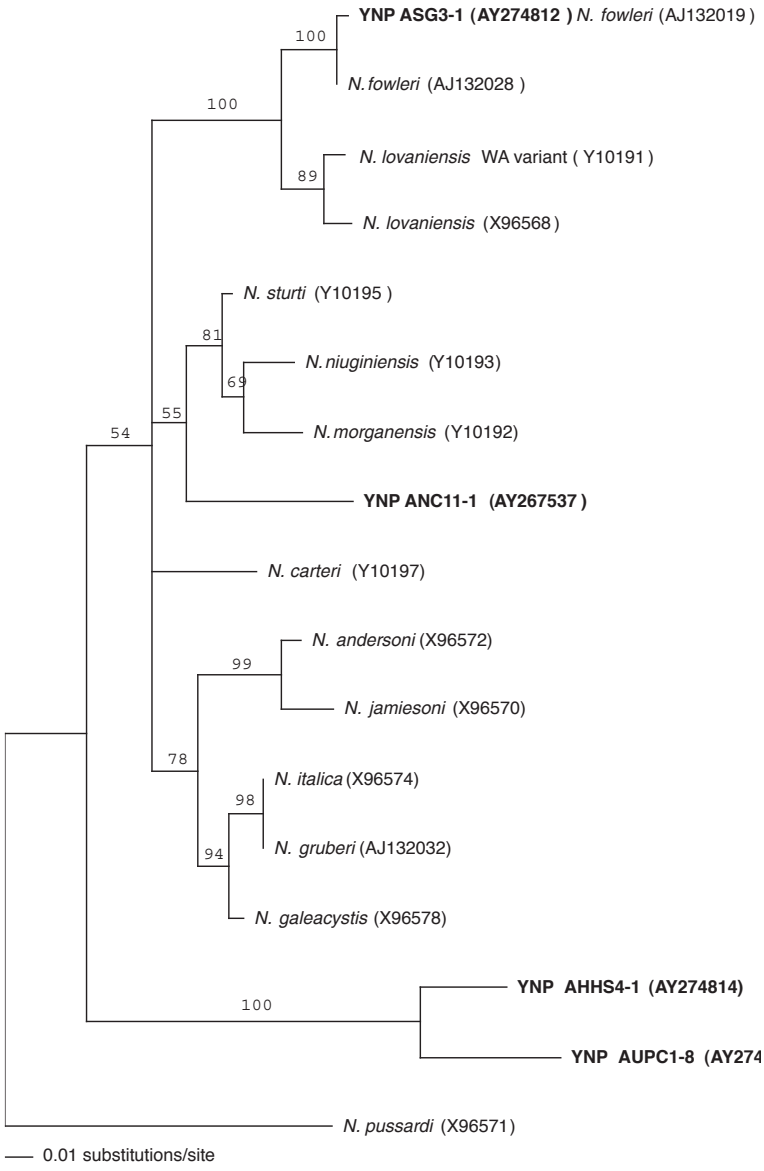

Figure 3. Neighbor-joining analysis using the 5.8S rRNA gene sequences, and portions of the adjacent ITS-1 and ITS-2 regions of *Naegleria* species from GenBank and those PCR-amplified from bulk DNA extracted from hot springs in Yellowstone and Grand Teton National Parks. The tree was generated using PAUP*, distances were calculated with the Kimura two-parameter model, and bootstrap values of >50% are indicated. YNP, Yellowstone National Park (Sheehan et al. 2003a).

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