Genus-specific nested amplifications for *Legionella* were accomplished using the 16S ribosomal RNA (rRNA) gene primer sets Leg225/Leg858 (Miyamoto et al. 1997) and p1.2/cp3.2 (Jonas et al. 1995). Nested amplifications targeting the gene encoding the macrophage infectivity potentiator (*mip*) surface protein of *L. pneumophila* were conducted using mipf1/m1548r (Koide et al. 1993) and mipf1/mipr1 (Templeton et al. 2004).

*Legionella* 16S and *mip* gene PCR nested amplifications were carried out in a 25 µl reaction mixture composed of 5 µl 5 x buffer, 2.5 mM MgCl2, 0.2 mM deoxynucleoside triphosphate, 100 ng of each primer, and 1.25 units of GoTaq Flexi DNA polymerase (Promega). The first reaction was performed in a thermal cycler (GeneAmp 2700 PCR system; Applied Biosystems) for 20 cycles (30 sec at 94°C, 45 sec at 57°C, and 1 min at 72°C) for the 16S rRNA gene and 25 cycles (30 sec at 94°C, 30 sec at 53°C, and 40 sec at 72°C) for the *mip* gene. The second amplification used 1 µl of the previous reaction as the template in a 25 µl reaction. The reactions were run for 40 cycles for both the 16S rRNA gene (30 sec at 94°C, 30 sec 57°C, and 1 min at 72°C) and the *mip* gene (30 sec at 94°C, 30 sec at 53°C, and 40 sec at 72°C). Prior to the cycles, the samples were held at 94°C for 2 minutes, and at the end of the cycles, the samples were held at 72°C for 2 min before going to 4°C. No template added negative control reactions were run for every PCR experiment, as the nested PCR amplifications have a high risk of contamination. If the negative control showed a product, the reagents were discarded and the amplifications rerun. Five microliters of PCR product was examined by gel electrophoresis through a 1.2% agarose gel and visualized by staining with ethidium bromide.

18S rRNA gene amplifications of amoeba culture extracts were carried out in 50-µl reactions with 1 µl template DNA, 10 µl 5 x buffer, 2.5 mM MgCl2, 0.2 mM deoxynucleoside triphosphate, 100 ng of each primer, and 1.25 units of GoTaq Flexi DNA polymerase (Promega). The PCR amplification had an initial denaturation of 95°C for 5 min, followed by 50 cycles of 95°C for 45 sec, 65°C for 45 sec, 72°C for 3 min. A final extension at 72°C for 7 min was run to complete extension products.

To sequence PCR products directly, each reaction was precipitated over night at -20°C using 0.3M sodium acetate and 0.6 volumes of 100% isopropanol. The nucleic acids were pelletized by centrifugation at 20,000 x g for 10 min and the air dried pellets resuspended in 6 µl of sterile distilled water. The quantity of product was checked using a NanoDrop (Thermo Scientific), and approximately 100 ng was sequenced (see below).

To clone and sequence PCR products, each PCR reaction was precipitated and recovered as described above. Two µl were ligated into pGEM vector following the manufacturer’s instructions (pGEM™-T Easy Vector System, Promega). Positive clones were picked and grown for each reaction, and plasmid DNA was recovered using a Genemachines® RevPrep ™ Orbit II automated workstation (Genomic Solutions). M13 primers were used to prime the sequencing reactions.

The 18S rDNA products were also cloned as described above, except that 3 microliters of precipitated product were used in ligations with the pGEM™-T Easy Vector System kit (Promega). Inserts of 1-3 clones from each amoeba isolate culture were sequenced in both directions using internal 18S rDNA primers (Weekers et al. 1994). Sequencing was accomplished following the MBL COHH Genomics Core Facility protocols for 1/8th reactions of the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems; http://jbpc.mbl.edu/facilities-keck.html) using a capillary sequencer (3730XL, Applied Biosystems). Chromatograms were analyzed using the Sequencher™ editing program (Gene Codes Corporation). Only reactions that yielded clear chromatograms without significant overlapping peaks were considered successful.

**REFERENCES**


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