Description of ‘Candidatus Berkiella aquae’ and ‘Candidatus Berkiella cookevillensis’, two intranuclear bacteria of freshwater amoebae

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Two novel bacteria of the phylum Proteobacteria were isolated during searches for amoeba-resistant micro-organisms in natural and constructed water systems. Strain HT99 was isolated from amoebae found in the biofilm of an outdoor hot tub in Cookeville, Tennessee, USA, and strain CC99 was isolated from amoebae in the biofilm of a cooling tower in the same city. Both bacteria were Gram-stain-negative cocci to coccobacilli, unculturable on conventional laboratory media, and were found to be intranuclear when maintained in Acanthamoeba polyphaga. The genomes of both isolates were completely sequenced. The genome of CC99 was found to be 3.0 Mbp with a 37.9 mol% DNA G+C content, while the genome of HT99 was 3.6 Mbp with a 39.5 mol% DNA G+C content. The 16S rRNA gene sequences of the two isolates were 94% similar to each other. Phylogenetic comparisons of the 16S rRNA, mip and rpoB genes, the DNA G+C content and the fatty acid composition demonstrated that both bacteria are members of the order Legionellales, and are most closely related to Coxiella burnetii. The phenotypic and genetic evidence supports the proposal of novel taxa to accommodate these strains; however, because strains HT99 and CC99 cannot be cultured outside of the amoeba host, the respective names ‘Candidatus Berkiella aquae’ and ‘Candidatus Berkiella cookevillensis’ are proposed.

Free-living amoebae are natural environmental predators of bacteria and other micro-organisms found in soil and water, including anthropogenic systems such as drinking water systems, air conditioners and cooling towers, spas and swimming pools (Rodríguez-Zaragoza, 1994; Benkel et al., 2000; Greub & Raoult, 2004; Berk et al., 2006). Some bacteria only survive engulfment by amoebae whereas other bacteria not only survive but multiply within the amoebal host (Barker & Brown, 1994; Horn & Wagner, 2004; Molmeret et al., 2005). Recent reviews list over 40 genera of bacterial symbionts of amoebae that are human pathogens, making amoebae important environmental reservoirs of virulent bacteria (Lamoth & Greub, 2010; Moliner et al., 2010). Evidence also suggests that amoebae can aid in the selection of virulence traits and the adaptation of bacteria for intracellular survival in human macrophages and other cell types (Cirillo et al., 1994; Greub & Raoult, 2004; Evstigneeva et al., 2009). This report describes two bacterial isolates, HT99 and CC99, that were recovered from amoebae in biofilm samples from constructed aquatic environments (Berk et al., 2006). Both bacteria are amoeba-resistant, intranuclear bacteria. We have determined, on the basis of both genotypic and phenotypic characteristics, that these two bacteria do not belong to known genera, justifying the proposal of novel taxa. However, due to the...
intracellular lifestyle of the bacteria and the lack of pure cultures, we propose the classification of these two bacteria as representatives of 'Candidatus Berkiella aquae' and 'Candidatus Berkiella cookevillensis' and in this report describe the characteristics of both novel bacteria.

Bacterial strain HT99 was recovered from an amoeba isolated from an outdoor hot tub spa, and strain CC99 was recovered from an amoeba isolated from a hospital cooling tower. The amoebae were found in biofilm material that was swabbed from the sides of a cooling tower and an uncovered hot tub spa that were both filled with chlorinated water from a municipal water system in Cookeville, Tennessee, USA. The biofilm material was made into a slurry and plated onto the centre of non-nutrient agar plates seeded with UV-killed Escherichia coli and processed as previously described (Berk et al., 2006). After 48 h, plates were examined microscopically for the presence of amoebae. Plates positive for amoebae were washed with approximately 2 ml of sterile Tris-buffered saline solution and 0.1 ml aliquots of the wash were added to individual wells of a 96-well flat-bottom microplate. Microplates were incubated at the temperature of the original agar plate and wells were checked daily for the presence of infected amoebae. Both CC99 and HT99 were recovered from samples incubated at 30 °C that contained native amoebae infected with highly motile bacteria. Aliquots from microplate wells positive for infected amoebae were added to Acanthamoeba polyphaga monolayers. Monolayers of A. polyphaga (ATCC 30461) were grown in 25 cm² flat-bottomed cell culture flasks in 5 ml tryptic soy broth (TSB, Becton Dickinson). Once amoebae were confluent, the growth medium was removed, and the monolayer washed and medium replaced with 5 ml of filter-sterilized spring water (SSW; Carolina Biological Supply), which is osmotically balanced but nutrient poor. The plate and wells were checked daily for the presence of amoebae infected with sheep blood agar (all from Sigma-Aldrich) to assess the numbers of contaminants, and to check individual colony types for their infectivity for amoebae. For both of the described isolates, contaminating bacteria were eliminated using these described techniques. However, the infecting bacteria were unable to be cultured on conventional laboratory medium as determined by plating amoeba lysates of the co-cultures onto BCYE differential agar, TSA and TSA II sheep blood agar with incubation at temperatures from 25 to 37 °C in ambient air, 5 % CO₂, anaerobic (90 % H₂, 10 % CO₂; GasPak, Becton Dickinson) and microaerophilic (5 % O₂, 10 % CO₂, 85 % N₂; CampyPak, Becton Dickinson) atmospheres. Cultivation of the micro-organisms was also attempted with the more recently described ACCM-2 medium (kindly provided by Dr Robert Heinzen) for axenic growth of Coxiella burnetii (Omsland et al., 2011). Gram, endospore, capsular and RYU flagellar (Remel) staining were performed on bacteria in amoeba lysates.

Because isolates CC99 and HT99 did not grow on the conventional laboratory media described above, their growth in A. polyphaga was examined by Giemsa staining and transmission electron microscopy (TEM). A. polyphaga monolayers were maintained in TSB and transferred to SSW for infection with the bacteria. Both Giemsa staining and TEM were performed periodically following infection of A. polyphaga. For Giemsa staining, a 0.1 ml aliquot of dislodged cell suspension was centrifuged through a Shandon cytofunnel (Thermo Scientific) onto a microscope slide and Giemsa stained (Sigma-Aldrich) as described by Newsome et al. (1998). For TEM, bacteria and A. polyphaga co-cultures were fixed in 2.5 % (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h, followed by post-fixation with 2 % (w/v) aqueous osmium tetroxide for 2 h at room temperature. Fixed specimens were dehydrated in an ascending ethanol series from 30 to 100 % (v/v) with final dehydration in propylene oxide. Samples were embedded in Epon-Araldite (Electron Microscopy Sciences) and 70 nm sections stained with 5 % (w/v) uranyl acetate and lead citrate. Microscopy was performed with a Hitachi H-7650-II transmission electron microscope.

The 16S rRNA genes from isolates CC99 and HT99 were amplified from amoeba co-cultures by the PCR using universal eubacterial primers 8F (5'-AGTTACTTCTCCAGGTTGATCTG-3') and 1541R (5'-GCTTAAGGATTCCAGTTAAGGAGTGWTC-3'). PCR products were cloned into plasmid vectors and sequenced using internal primers 515F (5'-AGGTACCTTCCGACCTATGATCTGCTCAG-3'), 1541R, 911F (5'-GCTAAGGATTCCAGTTAAGGAGTGWTC-3'), 806R (5'-GGACTACAGGGTACCTAAT-3'), and 1392R (5'-AGCGGCGGTTGWTC-3') as previously described (Berk et al., 2006). The plasmid inserts were sequenced in both directions, combined into a single consensus sequence, and compared with sequences available in the GenBank database using the BLASTN program on the NCBI website (http://www.ncbi.nlm.nih.gov). For whole genome sequencing, genomic DNA was purified from both isolates following their growth in A. polyphaga co-culture. After complete lysis of the amoebae, CC99 and HT99 cells were separated from host cell debris using Renografin density-gradient centrifugation as described by Shannon & Heinzen (2008). Genomic DNA was extracted from the purified isolates using a DNeasy Blood and Tissue kit.
The genomic DNA was sequenced at the University of Maryland School of Medicine, Institute for Genome Sciences (IGS), Genome Resource Center. The genome sequences were generated using genomic paired-end sequencing on an Illumina MiSeq and sequence reads were assembled using Newbler software v2.5.3 (Roche). The contig data were annotated using the annotation pipeline at the IGS Informatics Resource Center (http://www.igs.umaryland.edu/). Protein coding sequences were identified using Glimmer3 algorithm as part of the IGS annotation engine. Non-coding RNA genes were predicted using RNAmer, and tRNA-scanSE was utilized to predict tRNA genes. Searches for all predicted genes were performed using NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast/). Phylogenetic trees were reconstructed from conserved gene sequences, 16S rRNA (1356 bp), mip (587 bp) and rpoB (384 bp). The 16S rRNA, mip and rpoB gene sequences were aligned with similar sequences from the NCBI nucleotide database using CLUSTAL_X 2.0 and MEGA6 software (Tamura et al., 2011). Bayesian analyses were run using MrBayes (v3.2.2) under the general time reversible (GTR) model along with gamma-distributed rates of evolution and a proportion of invariant sites (Ronquist & Huelsenbeck, 2003). Analyses were performed for 2 000 000 generations using four Monte Carlo Markov chains, and sample trees were taken every 1000 generations. Posterior probabilities calculated over 5000 best trees were used as support values for nodes in the tree. Maximum-likelihood analyses were performed using PhyML 3.0 with input tree generated by BIONJ with the GTR model of nucleotide substitution incorporating invariant sites and a discrete gamma distribution. The node reliabilities of the phylogenetic tree generated were estimated using bootstrap values calculated over 1000 replicates.

Cellular fatty acid analysis was performed using bacterial isolates purified from amoebal co-cultures using Renografin density-gradient centrifugation as described above. Fatty acid methyl esters (FAME) were obtained by saponification, methylation and extraction as described in the MIDI standard protocol. FAME analyses were carried out by MIDI using a Hewlett Packard 5890 gas chromatograph. The MIDI software package was used to automatically integrate peaks as well as to name the predominant fatty acids and determine their composition as a percentage of total peak area.

Both strains CC99 and HT99 were transferred to cultures of A. polyphaga and initially resembled Legionella-like amoebal pathogens (Adeleke et al., 1996) due to their failure to be cultivated on traditional culture media and their intracellular growth and high motility within A. polyphaga. No growth of either bacterium occurred on any of the culture media tested under any incubation conditions employed for 30 or more days, including the ACCM-2 growth medium, as determined by both optical density measurements and PCR amplification of 16S rRNA gene. TEM (Fig. 1) and Giemsa staining (Fig. S1, available in the online Supplementary Material) showed that both

**Fig. 1.** Transmission electron micrographs of cells of CC99 (a) and HT99 (b). Bacteria are seen within the double nuclear membrane of A. polyphaga (arrows; bars, 2 μm). Although bacteria often appear in the cytoplasm as they travel to the nucleus or extracellular following cell lysis, the structures present in the cytoplasm seen in these micrographs are mitochondria, which are similar in size to the bacteria. Insets of the bacteria show the condensed chromatin and that their sizes are between 300 and 500 nm (inset bars, 0.5 μm).
isolates were contained within the nuclei of amoebal cells. Bacteria were visible in nuclei as early as 12 h post-infection (p.i.) and filled the nuclei over the following 24–48 h, resulting in cytopathogenicity of the amoeba population within 72 h. Bacteria remained motile for 12–24 h, after which time, the bacterial cells became non-motile and adhered tightly to the bottom of culture flasks. Gram staining of lysed amoebal cultures showed that both CC99 and HT99 were Gram-stain-negative. Bacterium CC99 was coccoid with diameters ranging from 0.30 to 0.60 μm, occurring singly or as diplococci. HT99 was a coccobacillus, 0.45–0.65 μm in length and 0.30–0.55 μm in width, occurring singly or in pairs. Both CC99 and HT99 exhibited electron-dense, condensed chromatin. Neither endospores nor capsules were detected by staining. Flagella staining revealed polar flagella in both isolates, although no flagella were observed for either bacterium by TEM. Because TEM only showed intracellular bacteria, it is likely that, as with Legionella pneumophila, motility is associated with their growth phase such that bacteria multiplying within the host are non-motile, while bacteria from later stages of infection or in cell lysates are flagellated and highly motile (Pruckler et al., 1995; Byrne & Swanson, 1998). CC99 and HT99 were infectious for at least 50–60 days when in suspensions of lysed amoebae in SSW. When tested for intracellular growth at various temperatures, both CC99 and HT99 grew in A. polyphaga and were detected in the nuclei at 28, 30 and 37 °C.

The genomes of both isolates were sequenced for total reads of 11 839 536 and 11 009 736 for CC99 and HT99, respectively. The reads were assembled into 44 contigs containing 2,990,361 bp for CC99 and 56 contigs containing 3,626,027 bp for HT99. The DNA G+C content of the CC99 and HT99 genomes was 37.9 mol% and 39.5 mol%, respectively, and is similar to those reported for other species of the genus Legionell (Pagnier et al., 2014). The 16S rRNA gene sequences were 95–100 % (CC99) and 90–96 % (HT99) similar to sequences from uncultured organisms in the NCBI GenBank database. CC99 had 100 % identity to an uncultured isolate from a Korean oyster shell waste in the NCBI GenBank database. CC99 had 100 % identity to each other, thus demarcating them as representatives of separate species. However, because both CC99 and HT99 exhibit similar morphologies, intranuclear growth and fatty acid compositions, they have been placed within the same genus. The invasion of the amoeba host nucleus by both strains distinguishes them from other members of the family Coxiellaceae, and they contain minimal amounts of branched-chain fatty acids, which is unlike C. burnetii and species of the genus Aquicella of the family Coxiellaceae and species of the genus Legionella of the family Legionellaceae.

Description of Candidatus Berkiella aquae

‘Candidatus Berkiella aquae’ [Berk.i.e’l’la. L. dim. suff. -ella; N.L. fem. n. Berkiella after the US microbiologist, Sharon G. Berk, for her contributions to the study of interactions between protozoa and bacteria; a’qua. L. fem. gen. n. aquae of water, referring to the source of isolation and for the Roman term, aquae, referring to settlements with mineral springs such as Aquae Sulis, now Bath, England, and Aquae Spadanae, near Liége, Belgium (Sarton, 1954; Bunson, 1991)].

Represented by strain HT99, which was isolated from an amoeba in the biofilm of an outdoor hot tub spa in Cookeville, TN, USA. The strain is in axenic culture with the amoeba host A. polyphaga. Cells stain Gram-negative and are short coccobacillus-shaped cells, 0.45–0.65 μm in length and 0.30–0.55 μm in width, occurring singly or in pairs. Non-spore-forming, motile and exhibits intranuclear growth in the amoeba host. Phylogenetic analyses of the 16S rRNA, mip and rpoB genes indicate that this strain is different from all other recognized genera and belongs to the class Legionellales, family Coxiellaceae. The mole G+C ratio of the DNA is 39.5 mol%. Predominant fatty acids are monounsaturated, 18:1ω9c, 18:1ω7c and 16:1ω7c. The bacterium does not grow on conventional laboratory culture medium, but grows in A. polyphaga at temperatures ranging from 25 to 37 °C.

Description of Candidatus Berkiella cookevillensis

‘Candidatus Berkiella cookevillensis’ (cooke’vill.ens. L. adj. cookevillensis referring to Cookeville, TN, USA, where the first strain was isolated).
Represented by bacterial strain CC99, which was isolated from an amoeba in the biofilm of a cooling tower in Cookeville, TN, USA. The strain is in axenic culture with A. polyphaga. Cells stain Gram-negative and are coccoid shaped with diameters ranging from 0.30 to 0.60 μm, occurring singly or as diplococci. Non-spore-forming, motile and exhibits intranuclear growth in the amoeba host. Phylogenetic analyses of the 16S rRNA, mip and rpoB genes indicate that this strain is different from all other recognized genera and from 'Ca. Berkiella aquae', and belongs to the order Legionellales, family Coxiellaceae. The mole G+C ratio of the DNA is 39.5 mol%. Predominant fatty acids are monounsaturated, 18:1 ω9c, 18:1 ω7c and 16:1 ω7c. The bacterium does not grow on conventional laboratory culture medium, but grows in A. polyphaga at temperatures ranging from 25 to 37 °C.

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