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Phylogenetic Characterization and Prevalence of "*Spirobacillus cienkowskii*," a Red-Pigmented, Spiral-Shaped Bacterial Pathogen of Freshwater *Daphnia* Species▽

Jorge L. M. Rodrigues,[1]† Meghan A. Duffy,[2]‡ Alan J. Tessier,[2]§ Dieter Ebert,[3] Laurence Mouton,[3] and Thomas M. Schmidt[1,2]*

Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, Michigan 48824[1]; W. K. Kellogg Biological Station, Michigan State University, Hickory Corners, Michigan 49060[2]; and Zoologisches Institut der Universität Basel, Evolutionsbiologie, Vesalgasse 1, 4051 Basel, Switzerland[3]

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Microscopic examination of the hemolymph from diseased daphniids in 17 lakes in southwestern Michigan and five rock pools in southern Finland revealed the presence of tightly coiled bacteria that bore striking similarities to the drawings of a morphologically unique pathogen, "*Spirobacillus cienkowskii*," first described by Elya Metchnikoff more than 100 years ago. The uncultivated microbe was identified as a deeply branching member of the *Deltaproteobacteria* through phylogenetic analyses of two conserved genes: the 16S rRNA-encoding gene (*rrs*) and the β-subunit of topoisomerase (*gyrB*). Fluorescence in situ hybridization confirmed that the rRNA gene sequence originated from bacteria with the tightly coiled morphology. Microscopy and PCR amplification with pathogen-specific primers confirmed infections by this bacterium in four species of *Daphnia*: *Daphnia dentifera*, *D. magna*, *D. pulicaria*, and *D. retrocurva*. Extensive field surveys reveal that this bacterium is widespread geographically and able to infect many different cladoceran species. In a survey of populations of *D. dentifera* in lakes in Michigan, we found the bacterium in 17 of 18 populations studied. In these populations, 0 to 12% of the individuals were infected, with an average of 3% during mid-summer and early autumn. Infections were less common in rock pool populations of *D. magna* in southern Finland, where the pathogen was found in 5 of 137 populations. The broad geographic distribution, wide host range, and high virulence of *S. cienkowskii* suggest it plays an important role in the ecology and evolution of daphniids.

Daphniids are key links in aquatic food webs: they are a primary food source for numerous fishes and important grazers of phytoplankton (39). As a result, they influence ecosystem-level properties such as productivity, water clarity, and nutrient cycling (7, 8, 25, 40). Due to their pivotal ecological roles and relative ease of cultivation, daphniids have become model organisms for ecological, evolutionary, genetic, and ecotoxicology research (9).

Daphnia spp. are host to many parasites (18, 22). These parasites affect *Daphnia* population dynamics (see, for example, references 3, 12, and 14), the evolution of *Daphnia* populations (see references 16, 17, and 18), and the interactions of *Daphnia* with other species (see references 15, 27, and 44). Several of these *Daphnia* pathogens were first described by Elya Metchnikoff (31, 32, 33), and these seminal studies contributed to a body of work for which he was ultimately awarded a Nobel prize (2). Two of the pathogens described by Metchnikoff, the bacterium *Pasteuria ramosa* and the yeast *Metschnikowia bicuspidata*, have been the subject of numerous ecological and evolutionary studies (see, for example, references 4, 16–19, and 28).

A third parasite described by Metchnikoff, "*Spirobacillus cienkowskii*," has received less attention from bacteriologists. Metchnikoff described this species from *Daphnia magna* in a pond in Odessa, Ukraine. Sporadic studies by zooplankton ecologists have noted infections in a wide range of other *Daphnia* species (18, 22) and in members of other cladoceran genera, including *Ceriodaphnia*, *Chydorus*, *Diaphanosoma*, *Sida*, and *Simocephalus* (18, 22; M. A. Duffy, unpublished data).

Populations of *S. cienkowskii* multiply in the hemolymph of its host, turning infected *Daphnia* a dramatic pink-red color due to the production of carotenoids by the bacterium (21). Carotenoids are well known as accessory pigments for harvesting light in photosynthetic organisms, and it is an intriguing possibility that *S. cienkowskii* may be able to derive energy from light, but carotenoids are also well known in strictly heterotrophic organisms, where they can serve as antioxidants and photoprotective agents. Infected individuals of *D. dentifera* rarely have eggs and inevitably die within 1 to 4 days, even at moderate (15°C) temperatures (13; D. Ebert, unpublished data). In addition, infected *Daphnia* are preferentially fed upon by planktivorous fish, leading to an even higher mortality in lake populations (15). Not surprisingly, given this high virulence, epidemics of *S. cienkowskii* are associated with large reductions in *Daphnia* population densities (13).

Despite these strong impacts of *S. cienkowskii* on the ecology

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, Biomedical and Physical Sciences Building, Michigan State University, East Lansing, MI 48824. Phone: (517) 355-6463, ext. 1606. Fax: (517) 353-8957. E-mail: tschmidt@msu.edu.

† Present address: Department of Biology, University of Texas at Arlington, Arlington, TX 76019.

‡ Present address: School of Biology, Georgia Institute of Technology, 310 Ferst Drive, Atlanta, GA 30332.

§ Present address: Division of Environmental Biology, National Science Foundation, Arlington, VA 22230.

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TABLE 1. Primer and probe sequences, their lengths, and target sites used in this study

Primer or probe	Oligonucleotide sequence (5′–3′)	Length (bases)	Position[a]	Source or reference
Primers				
8F	AGAGTTTGATCCTGGCTCAG	20	8–27*	42a
1492R	GGTTACCTTGTTACGACTT	19	1474–1492*	42a
gyrBBAUP2	GCGGAAGCGGCCNGSNATGTA	21	57–78†	35
gyrBBNDN1	CCGTCCACGTCGGCRTCNGYCAT	23	1485–1508†	35
recABDUP1	CCCCAGTCCTCCGGNAARACNAC	23	ND	35
recABGDN2	CGTTGCCGCCGGKNGTNRYYTC	22	ND	35
fusAF	CATCGGCATCATGGCNCAYATHGA	24	ND	35
fusAR	CAGCATCGGCTGCAYNCCYTTRTT	24	ND	35
S-*-Smet-0058-a-S-18	AAGTCGGACGGAGGTAGC	18	58–76*	This study
S-*-Smet-0828-a-S-18	GTCAACTCCTCCAACACC	18	828–846*	This study
S-*-Smet-1459-a-A-18	CACAAGCCTTGGCAAGCT	18	1459–76*	This study
Probes				
S-D-Bact-0338-a-A-18	GCTGCCTCCCGTAGGAGT	18	338–356*	1a
S-D-Bact-0338-a-S-18	ACTCCTACGGGAGGCAGC	18	338–356*	1a

[a] *, Positions corresponding to the *E. coli* 16S rRNA gene; †, positions corresponding to the *E. coli gyrB* gene; ND, not determined.

of daphniids, little is known about the organism. Here, we establish the phylogenetic position of *S. cienkowskii* through analysis of genes encoding the 16S rRNA and β-subunit of topoisomerase and confirm the presence of the pathogen in multiple species of *Daphnia*. We also report on the prevalence of this pathogen in 155 *Daphnia* populations in Europe, where the bacterium was originally described, and in North America, where preliminary studies suggested it was more prevalent. Finally, we report on the development and testing of specific primers that provide a tool for future studies of the ecology of this important pathogen.

MATERIALS AND METHODS

Study system and field sampling: North America. During previous studies on lakes in southwest Michigan, zooplankton infected with a bacterium that turned the animal's hemolymph a distinct pinkish-red color were observed repeatedly, initially in populations of *D. dentifera* (formerly *D. galeata mendotae* and *D. rosea*). Eighteen lakes in southwestern Michigan were selected for the present study: Baker, Bassett, Bristol, Cloverdale, Deep, Fine, Hall, Lawrence, Little Mill, Long, Pine, Pleasant, Shaw, and Warner Lakes in Barry County; and Sherman, Whitford, Wintergreen Lakes, and Three Lakes Two in Kalamazoo County. *Daphnia* spp. are key links in the food webs in these lakes, strongly influencing algal communities and serving as a key food resource for planktivorous fish (39). These lakes are dominated by two species of *Daphnia*: *D. pulicaria* and *D. dentifera*. Members of a third species, *D. retrocurva*, are also commonly observed, but less abundant (39).

These 18 lakes were chosen because they all contain *D. dentifera* and yet also encompass a wide range of productivities, ranging from oligotrophic to hypereutrophic (spring total phosphorus between 5 and 200 μg/liter). All are relatively small lakes (surface areas of 1.8 to 131.5 ha), with maximum depths between 5 and 17 m (4, 5). The lakes are of glacial (kettle) origin, well buffered with an alkaline water chemistry, and are typically thermally stratified in the late summer months (4).

Lakes were sampled biweekly from July through November in 2003 and 2004 (with the exception of Wintergreen Lake, which was only sampled in 2004). Pleasant Lake contained too few *D. dentifera* in 2004 to estimate infection prevalence, and we therefore only report data from this lake for 2003. *Daphnia* samples were collected with plankton tows at three locations in the deep portion of each lake using a 153-μm-mesh Wisconsin bucket net; these three samples were then pooled, yielding one sample per lake. *D. dentifera* from this sample (generally 400 to 500 animals/sample) were then examined within 12 h at ×25 to ×50 magnification for infections by using a stereomicroscope. Animals with late-stage infections were easily detected, since they are opaque and a bright, pink-red color; their opacity distinguished them from animals containing hemoglobin. Early-stage infections are not readily recognized visually. Infection prevalence was calculated as the number of visibly infected *Daphnia* divided by the total number of *Daphnia* in the sample. The mean infection prevalence for each year was calculated as the arithmetic average of the infection prevalence for all samples for a given lake in a given year. The peak infection prevalence is the maximum infection prevalence in a given lake in a given year. More information on the seasonal dynamics of parasitism in a subset of these lakes is presented elsewhere (13, 15). During these visual inspections of the zooplankton samples, infections of this parasite in other zooplankton species were also recorded. All data on the proportion infected were arcsine square root transformed prior to statistical analysis.

In 2003, fecundity was also analyzed on a subsample of adult females (mean subsample size of 72 adult females; minimum subsample size, 26; maximum subsample size, 247). For each of these individuals, we recorded the number of eggs carried, as well as the infection status. Animals infected with parasites other than *Spirobacillus* were excluded from the analysis presented below.

Study system and field sampling: Europe. A metapopulation of *Daphnia* on the coast of the Baltic Sea in Southern Finland, at Tvärminne on the Hanko Peninsula (59°50′35″N, 23°15′E), was also studied. Approximately 40% of the rock pools in this area are inhabited by at least one *Daphnia* species. For the study of the parasites of *D. magna*, *Daphnia* samples were collected from 138 rock pools. A total of 34% of all pools also contained populations of either *D. longispina* or *D. pulex*. None of the pools harbored all three *Daphnia* species.

To investigate daphniids for the presence of pathogens, samples were collected from each rock pool that harbored *D. magna*, cooled on ice, and brought to the laboratory, where they were kept at 12°C and inspected within 3 days. At least 150 adult hosts from each sample were screened visually, using a stereomicroscope for *S. cienkowskii*. Screening for pathogens was done between 14 June and 30 July 1998. In a similar rock pool habitat on the Baltic coast of Sweden (60°25′93″N, 18°31′34″E), 30 pools with *D. magna* on four islands were inspected for the presence of *S. cienkowskii* on 23 August 2007.

Cloning and sequencing of phylogenetic markers. Individual daphniids were collected with a Pasteur pipette and washed with sterile phosphate saline buffer (pH 7.0). Carapaces were punctured with a sterile needle, and 2 μl of the hemocoel fluid was added to 4 μl of 0.05 N NaOH. Samples were heated at 95°C for 5 min, and then 2 μl of the digested material was used for PCR. The 16S rRNA gene was amplified by PCR using primers that encompass a majority of bacteria (Table 1). Amplification reactions were performed in a total volume of 25 μl containing 0.2 μM concentrations of each primer, 200 μM concentrations of each deoxynucleoside triphosphate, 1× *Taq* buffer, 1.5 mM $MgCl_2$, 2.0 U of *Taq* DNA polymerase (Invitrogen/Life Technologies, Carlsbad, CA), and 2 μl of the DNA template. Amplification was initiated with a 3 min denaturation step at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 30 s, and extension at 72°C for 2 min, with a final extension incubation for 7 min at 72°C. For the *gyrB* gene, previously published degenerate PCR primers (Table 1) and thermal cycling conditions were used (35). Aliquots (5 μl) of the PCRs were visualized on ethidium bromide-stained 0.8% agarose gels. PCR amplification of *recA* and *fusA* was conducted as previously published (35) with an initial denaturation at 94°C for 2 min, followed by 10 cycles of 94°C for

1 min, 60°C for 1 min (−1°C/cycle), and 72°C for 1 min, followed in turn by 21 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, with a final extension of 72°C for 5 min.

Cloning and transformation were carried out according to the instructions provided with the TOPO TA cloning kit (Invitrogen/Life Technologies). *E. coli* transformants were grown in LB liquid medium containing ampicillin (50 μg/ml). Plasmids were isolated with a Promega Wizard Kit (Promega Corp., Madison, WI). Both strands of the cloned PCR products were sequenced on an ABI Prism 3100 automatic sequencer using BigDye chemistry (Applied Biosystems, Foster City, CA) at the Michigan State University sequencing facility (East Lansing, MI). Sequences were assembled and edited with the software BioEdit v.7.0.4.1. (26). Chimera detection software was used to screen the assembled sequences for a chimeric origin (http://rdp8.cme.msu.edu/cgis/chimera.cgi).

Microscopy. The pathogen's morphology was visualized with phase-contrast microscopy after the carapace of *D. dentifera* was punctured and the hemolymph for wet mounts was collected. Images were captured by using a Zeiss Axioskop microscope (Carl Zeiss, Inc., Thornwood, NY) with a charged-coupled device camera and processed with SPOT software (Diagnostic Instruments, Inc., McHenry, IL). The length and width of cells was calculated by using CMEIAS v.1.27 (29) operating in the UTHSCSA ImageTool v.1.27 (43) (http://macorb.uthscsa.edu/dig/itdesc.html). Detailed images were obtained by scanning electron microscopy of freshly collected *D. dentifera* organisms that were fixed with 4% glutaraldehyde in 100 mM sodium phosphate buffer (pH 7.4) for 30 min. Samples were dehydrated in an ethanol series (25, 50, 75, and 95% ethanol and a total of three times in 100% ethanol) and subjected to critical point drying with CO_2 in a Balzers critical point dryer. Samples were mounted on aluminum stubs and coated with gold in an EMScope SC500 Sputter Coater. Images were collected with the JEOL 6300F field emission scanning electron microscope (Japan Electron Optics, Ltd., Peabody, MA).

Phylogenetic analyses. Consensus sequences of 16S rRNA genes were aligned against the most similar sequences in the ARB small subunit rRNA database using the alignment algorithm in the ARB software package (30). The alignment was refined manually based on elements of primary sequence and secondary structure. For construction of a phylogenetic tree of the 16S rRNA gene sequences, 1,298 shared nucleotide positions were included for both maximum-likelihood analyses in ARB and maximum-parsimony analyses with PAUP*, including 1,000 bootstrap replicates (38).

For GyrB phylogenetic analyses, the partial gene sequence of *gyrB* was translated to an amino acid sequence and aligned with other GyrB protein sequences in ARB, using the alignment provided by the ICB database (www.mbio.co.jp/icb) (42). A total of 233 shared amino acid positions were used for phylogenetic analyses with the same algorithms as described above.

Primer and probe design. Species-specific, 16S rRNA gene-targeted primers were designed by using the ARB software package and their specificities further tested against 128,376 16S rRNA aligned sequences by using the CHECK PROBE function of the Ribosomal Database Project II (RDP; http://rdp.cme.msu.edu/index.jsp) (10) and the basic local alignment search tool (BLAST) program from the National Center for Biotechnology Information (1).

Fluorescence in situ hybridization (FISH). Infected daphniids were ground and washed twice with phosphate-buffered saline (PBS; 10 mM phosphate buffer [pH 8.0] and 130 mM NaCl) and then resuspended in 100 μl of the same buffer, followed by addition of freshly prepared paraformaldehyde solution (4% final concentration). Cells were fixed for 12 h at 4°C, washed twice with PBS, resuspended in PBS-ethanol (50:50), and stored at −20°C until use (34).

Samples (3 μl) were spotted in duplicate on eight-well Teflon-coated slides (Cel-Line Associates) containing 1% gelatin and dried at room temperature for 2 h. Slides were dehydrated for 3 min each in 50, 80, and 95% ethanol and then air dried. A total of 10 μl of hybridization solution containing 100 mM Tris-HCl (pH 8.0), 0.9 M NaCl, 20% formamide, 0.1% sodium dodecyl sulfate, and 50 ng of biotin-labeled oligonucleotide probe (Integrated DNA Technologies, Inc., Coralville, IA) was added to each well. Hybridization was conducted in moisturized chambers for 12 h in the dark at 37°C. Slides were then washed with 100 mM Tris-HCl (pH 7.2), 10 mM NaCl, 0.1% SDS, and 5 mM EDTA for 20 min at 39°C and dipped briefly in cold water.

Tyramide signal amplification. After a rinsing step, slides were immediately incubated with blocking buffer (100 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% blocking reagent [Perkin-Elmer, Boston, MA]) for 30 min at room temperature, followed by incubation with horseradish peroxidase diluted 1:100 in blocking buffer for 30 min at room temperature. Slides were washed three times (5 min each) with 100 mM Tris-HCl (pH 7.5)–150 mM NaCl–0.05% Tween 20 and incubated with cyanide tyramide for 10 min at room temperature. The slides were rinsed three times for 5 min each time with 100 mM Tris-HCl (pH 7.5)–150 mM NaCl–0.05% Tween 20, air dried at room temperature, and mounted with Citifluor (Vector Laboratories, Inc., Burlingame, CA). Fluorescence was visualized with a Zeiss Axioskop epifluorescence microscope equipped with appropriate filter sets. Images were captured with a charge-coupled device camera and processed with SPOT software (Diagnostic Instruments).

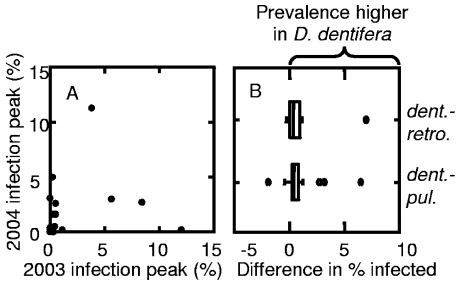

FIG. 1. Peak prevalence of *S. cienkowskii* infections in lake *Daphnia* populations. (A) Peak prevalence in *D. dentifera* populations in 2003 (*x* axis) versus peak prevalence in *D. dentifera* in 2004 (*y* axis). Each point represents a different lake population. (B) Difference in infection prevalence between *D. dentifera* and *D. retrocurva* populations (top) and between *D. dentifera* and *D. pulicaria* populations (bottom). The prevalence in *D. retrocurva* or *D. pulicaria* in a given lake on a given date was subtracted from the prevalence in *D. dentifera* for the same lake date; therefore, values greater than 0 indicate a higher prevalence in *D. dentifera*.

Whole-cell probe specificity. Conditions for optimal probe stringency were determined by incremental 5% increases of formamide in the hybridization buffer at three hybridization temperatures (37, 42, and 46°C). The specificity of the fluorescently labeled probe was tested with target and nontarget microorganisms.

Nucleotide sequence accession numbers. 16S rRNA and *gyrB* gene sequences were submitted to GenBank under accession numbers EU220836 and EU220837, respectively. Selected gene sequences in phylogenetic analyses are from fully sequenced genomes of *Agrobacterium tumefaciens* strain C58 (AE008265, AAK85837), *Borrelia burgdorferi* strain B31 (AE000783, AAC66802), *Burkholderia mallei* strain ATCC 23344 (AF110188, AAU48919), *Campylobacter jejuni* strain NCTC 11168 (CJ11168X1, CAB72496), *Caulobacter crescentus* strain ATCC 19089 (AJ227757, AAK22147), *Escherichia coli* strain K12-MG1655 (U00096, AAC76722), *Geobacter sulfurreducens* strain ATCC 51573^T (U13928, AAR33338), *Helicobacter hepaticus* strain ATCC 51449 (U07573, AAP77724), *Helicobacter pylori* strain 26695 (AE000511, AAD07566), *Nitrosomonas europaea* strain ATCC 19718 (BX572607, CAD83914), *Pseudomonas aeruginosa* strain PAO1 (AE004949, AAG03394), *Rhodopseudomonas palustris* strain CGA009 (BX572607, CAE25448), *Treponema denticola* strain ATCC 35405 (AF139203, AAS10500), *Treponema pallidum* strain Nichols (M88726, AAC65954), and *Vibrio cholerae* strain El Tor N 16961 (AE004106, AAF93193). *Myxococcus xanthus* only has accession numbers for the 16S rRNA (MXAV2823) and *gyrB* (MXAN0264) through the J. Craig Venter Institute database (http://www.tigr.org).

RESULTS

Infection quantification: North America. Infections by this pathogen were observed in *D. dentifera* in all but one (Fine Lake) of the 18 lakes in Michigan during the course of the 2-year study period (Fig. 1A). In general, infections with this bacterium were rare during summer months and increased in autumn. However, in most lakes, infection prevalence was always low (<3%). In five lakes (Bassett, Hall, Pine, and Shaw lakes and Three Lakes Two) the infection rate exceeded 3%; the maximum infection prevalence observed in those lakes was 12% (Fig. 1A). Given that only infections in late stages are visible, it should be noted that these prevalences underestimate the true prevalence. There was no between-year correlation in peak infection prevalence in the 16 lakes which were sampled in both 2003 and 2004 (Fig. 1A; $r = 0.31$, $P = 0.24$);

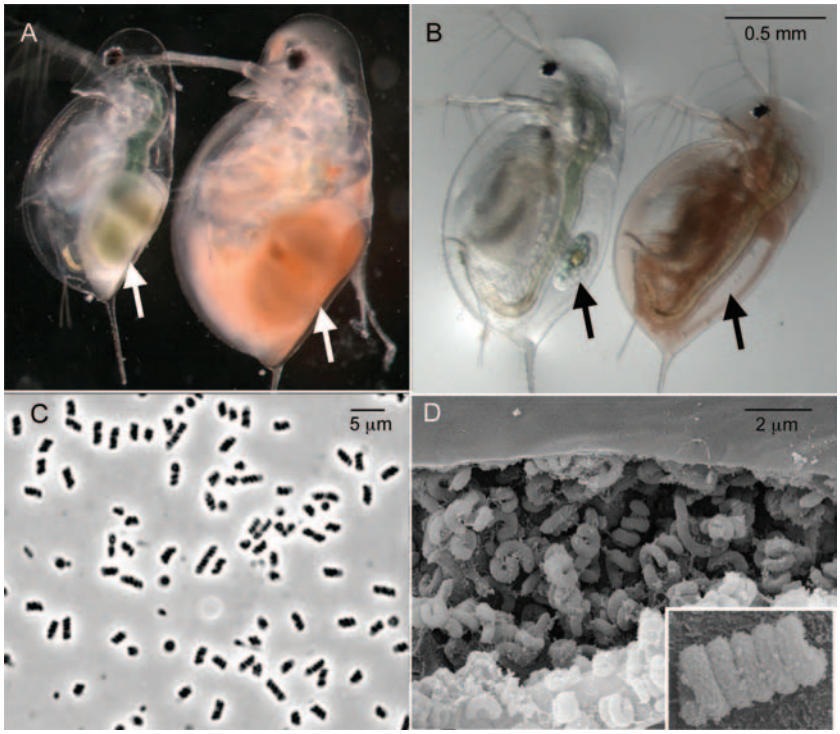

FIG. 2. (A and B) Micrographs of healthy (transparent) and diseased (pink-red) *Daphnia dentifera.* Panel A shows *D. dentifera* carrying diapausing eggs (indicated by arrows). Note that the diapausing egg case appears to contain the bacterium. Panel B shows uninfected *D. dentifera* carrying an asexually produced egg, whereas the infected *D. dentifera* has an empty brood chamber (indicated by arrows). (C) Phase-contrast micrograph of bacterial content extruded from *D. dentifera*. (D) Scanning electron micrograph of the bacterial content taken inside the carapace of the *D. dentifera*. The inset shows a close-up of the spiral morphotype.

the same is true for mean infection prevalence ($r = 0.22$, $P = 0.42$).

Infections were also observed in *D. pulicaria* and *D. retrocurva*. In general, infection prevalences were higher in *D. dentifera*, though the difference in infection prevalence was usually fairly small (Fig. 1B). The difference in infection prevalence between *D. dentifera* and *D. retrocurva* was not significant (paired *t* test: $t_6 = 1.96$, $P = 0.10$). The difference in prevalence between *D. dentifera* and *D. pulicaria* was significant (paired *t* test: $t_{17} = 3.26$, $P = 0.005$), with infection prevalence higher in *D. dentifera*. *Ceriodaphnia reticulata* and *Diaphanosoma birgei* were also observed with infections; however, the incidence of infection was generally lower in these *Cladocera* than in the *Daphnia* spp.

Infection quantification: Europe. A much smaller proportion of Finnish *D. magna* populations contained infected *Daphnia*. Infected *D. magna* organisms were found in 5 of 137 rock pools in early summer. The prevalence of infected individuals was always low, with the highest incidence of infection being 10%. Infections were also observed in *D. pulex* but not in *D. longispina*. Prevalence in *D. pulex* hosts was also low; the highest infection prevalence observed in these hosts was 10%. In the Swedish rock pools, *S. cienkowskii* was only found in one *D. magna* population, at a prevalence below 1%.

Transmission and effects on fecundity. Infected *D. dentifera* have greatly reduced fecundity. Only 2.3% of visibly infected adult females carried any eggs; in contrast, 62% of adult uninfected females carried eggs. During the present study, subitaneous (that is, asexually produced, immediately hatching) eggs, which are the normal mode of reproduction during the summer, were never observed to be infected. Therefore, transmission of this parasite appears to be primarily horizontal.

However, we observed infected *D. dentifera* females with sexually produced diapausing egg cases that appear to contain the pathogen (Fig. 2A) and at least one case of an infection that developed in an animal that hatched from a diapausing egg. Together, these findings suggest that the parasite can reside in or on diapausing egg cases.

Morphological description. The hemolymph of infected individuals (Fig. 2A and B) supported a dense culture of bacteria (Fig. 2C and D), with 99.2% of bacterial cells distinguished by a tightly coiled morphology. The average length of the coiled bacterial cells was 3.50 μm (±1.02), with an average width of 1.07 μm (±0.15). Few cells were observed in a loosely coiled condition, and in this state the average cell length stretched to approximately 8.57 μm with a width of 0.42 μm. Scanning electron microscopy not only confirmed the tightly coiled cell shape (Fig. 2D, inset) but also revealed a dense bacterial infection in infected daphniids (Fig. 2D).

Phylogenetic analyses. The phylogenetic affiliations of the pathogen from three different *Daphnia* species (*D. dentifera*, *D. pulicaria*, and *D. retrocurva*) was determined after amplification and sequencing of a 1,473-bp region of the 16S rRNA-encoding gene. All three sequences were identical to each other; there was no evidence of a chimeric origin for any of these sequences. Searches of public databases indicated that the

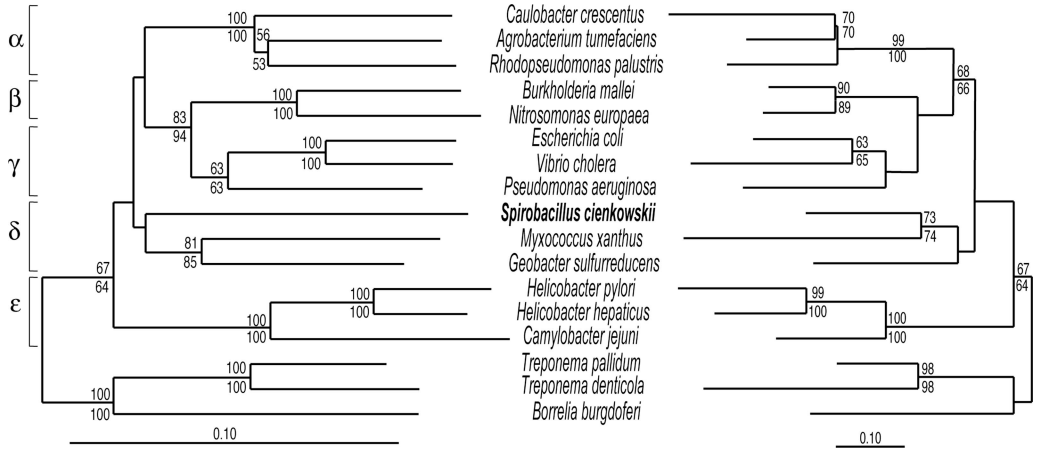

FIG. 3. Phylogenetic trees based on the 16S rRNA gene (left) and shared amino acids of the translated *gyrB* gene (right) of *S. cienkowskii* and other bacterial species. The scale is the expected number of substitutions per position. Numbers above and below the nodes are shown only for percentage bootstrap values above 50% of 1,000 resamplings using parsimony and maximum-likelihood methods, respectively.

sequences were 99% similar to an uncultivated *Deltaproteobacteria* strain (GenBank accession no. AY707570). PCR amplifications with primers designed for genes *recA* and *fusA* yielded no visible products.

Detailed phylogenetic analyses using data representing different subdivisions of the *Proteobacteria* revealed that the microorganism was most closely related to the *Deltaproteobacteria* (Fig. 3). The deep branching point of these sequences was consistent both maximum-parsimony and maximum-likelihood analyses. Grouping of the pathogen with the *Deltaproteobacteria* subdivision was confirmed with phylogenetic analyses of the translated *gyrB* gene sequences (Fig. 3).

One stem-loop structure that was not included in the phylogenetic analysis due to considerable variation in length among the Proteobacteria, is the region consisting of positions 179 to 197 (*E. coli* numbering [23]). The secondary structure of this region in *S. cienkowskii* assumes an 8-bp stem that is characteristic of many *Deltaproteobacteria* (Fig. 4).

Primer design and evaluation. Primers specific for amplification of the pathogen's 16S rRNA gene were designed to target regions 58 to 76 (S-*-Smet-0058-a-S-18), 828 to 846 (S-*-Smet-0828-a-A-18), and 1459 to 1476 (S-*-Smet-1459-a-A-18). These primer target regions were unique to *S. cienkowskii* even when allowing for a single mismatch with any of the 210,059 rRNA sequences available through the RDP II.

The specificity of the 16S rRNA gene-targeted primers combined in pairs was tested experimentally with infected and healthy individuals from four different species of *Daphnia*. In all cases, general bacterial primers yielded a positive amplification product, demonstrating that the DNA was suitable for amplification (Fig. 5). When the PCR primer combination S-*-Smet-0828-a-A-18 and S-*-Smet-0058-a-S-18 was used, a product of 648 bp was amplified only for infected *Daphnia* samples: no amplification products were visible from DNA obtained from uninfected, lab-reared *D. dentifera*. The same results were observed with the primer set S-*-Smet-0058-a-S-18 and S-*-Smet-1459-a-A-18, yielding the expected PCR amplification products of 1,418 bp (Fig. 5). These primers were also used to determine the phylogenetic affiliation of the pathogen in two infected *D. magna* collected in Europe. The sequences from the European samples were 99.7% identical to the sequences from infected *Daphnia* collected in North America.

FISH. Staining with DAPI (4′,6′-diamidino-2-phenylindole) (Fig. 6C), revealed an extensive colonization of the hemolymph of a diseased daphniid. To confirm that the tightly coiled bacterium in these specimens contained the 16S rRNA encoding gene characterized above, the contents of infected *Daphnia* were subjected to whole-cell in situ hybridization using fluorescently labeled general and specific oligonucleotide probes (Fig. 6A). A majority of bacterial cells were visualized with the general probe, confirming that these cells contained sufficient number of ribosomes to be readily visualized. The specificity of the hybridization conditions was confirmed by the absence of hybridization with the fluorescently labeled probe that is identical rather than complementary to bacterial 16S rRNAs (S-D-

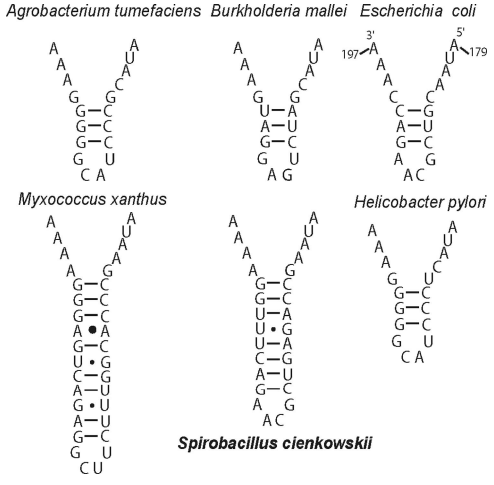

FIG. 4. 16S rRNA stem-loop structures of representative *Proteobacteria*. *E. coli* nucleotide numbering is provided according to Guttell (23). The symbols used in the secondary structure are as follows: —, canonical base pairs; ·, for G-U base pairs; and ●, for noncanonical base pairs.

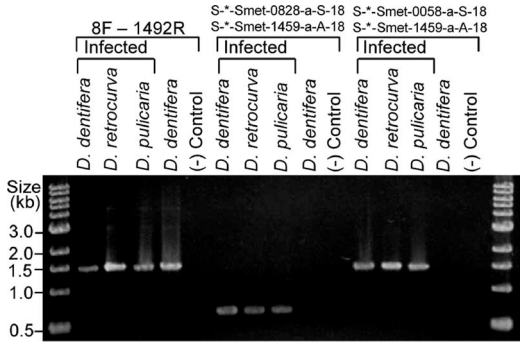

FIG. 5. Ethidium bromide-stained agarose gel of PCR products after amplification of the 16S rRNA gene with bacterial or *S. cienkowskii*-specific primers. The expected sizes for the specific primer sets S-*-Smet-0828-a-S-18/S-*-Smet-1459-a-A-18 and S-*-Smet-0058-a-S-18/S-*-Smet-1459-a-A-18 are 770 and 1,401 bp, respectively. Sterile H_2O was used as a negative control.

Bact-0338-a-S-18). Furthermore, the probe specific for *S. cienkowskii* hybridized with cells sharing the unique coiled morphology (Fig. 6B), confirming the identity of this abundant morphotype.

DISCUSSION

Although the Russian biologist Elya Metchnikoff (1845 to 1916) described *S. cienkowskii* infections in *D. magna* over 100 years ago, this system has received little subsequent attention. Here, we confirm that the hemolymph of infected daphniids contains bacteria with the unusual, tightly coiled morphology described by Metchnikoff. Moreover, we show that this bacterium is a deeply branching relative of the *Deltaproteobacteria* and that it infects a wide range of *Cladocera*. We also show that this bacterium is geographically widespread but that there appear to be geographic and specific differences in infection prevalence.

Analysis of two gene sequences that are commonly used for phylogenetic placement of bacteria, that of the 16S rRNA-encoding gene and the translated sequence of the β-subunit of DNA gyrase (*gyrB*), identified the pathogen as a deeply branching member of the *Deltaproteobacteria*. FISH confirmed that the PCR-amplified 16S rRNA gene was associated with the tightly coiled morphotype seen in phase-contrast microscopy and scanning electron microscopy. Efforts to amplify two other genes frequently used in phylogenetic analyses (*recA* and *fusA*) did not yield amplification products; this is typical for members of the *Deltaproteobacteria* (35). Also consistent with the placement of the pathogen into the *Deltaproteobacteria* is the inferred secondary structure of the stem-loop structure of the 16S rRNA between position 179 to 197: while the *Alpha-*, *Beta-*, and *Gammaproteobacteria* subdivisions generally have a stem-loop structure consisting of 3 bp, the stem-loop structure in members of the *Deltaproteobacteria* varies between 3 and 11 bp. There are currently few distinguishing metabolic features shared among the *Deltaproteobacteria* but, as additional genomes and representative isolates in this group are characterized, features common to this eclectic group of bacteria should be revealed.

The prevalence of this pathogen was documented in three

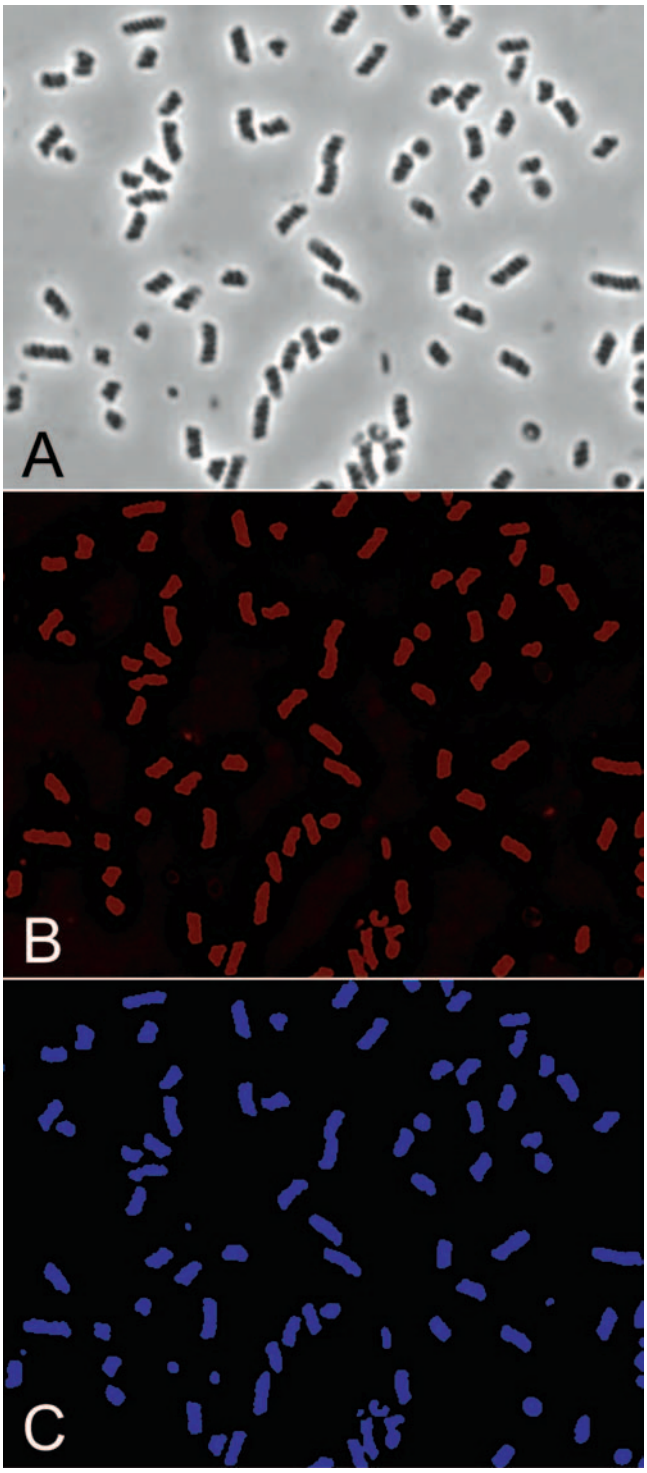

FIG. 6. Micrographs showing the same microscopic field of extruded material from *D. dentifera.* (A) Phase-contrast micrograph; (B) epifluorescence micrograph of bacterial cells hybridized with biotin-labeled probe specific to *S. cienkowskii*; (C) DAPI-stained preparation.

Daphnia species in North American lakes. For *D. dentifera*, the North American species studied most intensively, the parasite was found in 17 of the 18 populations examined. These lakes spanned a gradient of depth and productivity, suggesting that

the parasite is likely to be widespread, at least in stratified lakes in the Midwestern United States. The association between *D. dentifera* and *S. cienkowskii* in these lakes also appears to be enduring; Green (22) reports finding infected animals in one of our study lakes, Wintergreen Lake, in 1970. The parasite also appears to be endemic regionally in the European rock pools, although at levels consistently lower than in Michigan lakes. Endemism of *S. cienkowskii* in these habitats is supported by an earlier survey (20), which found *S. cienkowskii* infections in rock pool populations of *D. magna*, *D. pulex*, and several other *Cladocera* in southern Finland. We show that North American and European *Daphnia* were infected by closely related pathogens whose 16S rRNA gene sequences were nearly identical (99.7%). Molecular evidence also suggests that closely related bacteria are present in other aquatic environments, including the rhizoplane of a floating angiosperm (37).

Although *S. cienkowskii* is never particularly common in *D. dentifera* populations (maximum prevalence, 12%), it is likely to be important to their ecology and evolution. Infected *D. dentifera* has a greatly reduced fecundity and life span, and there is evidence that there are significant effects of epidemics on *D. dentifera* densities and dynamics (13). In addition, *S. cienkowskii* infections alter interspecific interactions: the increased opacity of infected hosts renders them much more susceptible to predation by bluegill sunfish (*Lepomis macrochirus* Rafinesque), the dominant planktivores in these populations (15). Given these large effects on *D. dentifera* fecundity, life span, and predation risk, parasite-mediated selection on the host populations seems likely. This assumes that there is genetic variation in susceptibility to infection by *S. cienkowskii*; this assumption seems justified, given that genetic differences in susceptibility have been found in other *Daphnia*-parasite systems (6, 16, 18). Whether *S. cienkowskii* is also under selection and whether there are host-pathogen genotype interactions (as have been found in the *Daphnia-Pasteuria* system [6, 19]) remains to be determined.

The possibility that *S. ciencowskii* resides in diapausing egg cases produced by *Daphnia* spp. is intriguing. First, it represents an excellent strategy for a pathogen to deal with host dormancy and may explain why epidemics of this pathogen, but not others, are sometimes observed in spring shortly after *D. dentifera* populations are refounded by hatching from diapausing eggs (C. E. Cáceres, unpublished data). For a vertically transmitted microsporidian parasite of *Daphnia*, it has been shown that dormancy in the diapausing egg ensures pathogen survival (41). In addition, it might provide an opportunity for studies of host-pathogen interactions over long time scales, given that these diapausing eggs form an "egg bank" in the sediments of lakes and ponds (11, 24). The probes and primers developed and tested in the present study provide a tool for addressing the distribution of the pathogen in the environment and should help resolve questions about its transmissibility.

Based on the characterization provided above and the recommendation of the Ad Hoc Committee on Bacterial Species (36), we propose formal acceptance of the genus and species epithets for *S. cienkowskii* with the following description:

> *Spirobacillus cienkowskii*, described initially by Elya Metchnikoff (1889), *cienkowskii* in memory of L. S. Cienkowsky. Deeply branching *Deltaproteobacteria*. Morphologically distinct as a tightly coiled bacterium (3.50 μm in length by 1.07 μm in width), production of a pink carotenoid. Infects the hemolymph of multiple species of *Daphnia*. Basis of assignment: 16S rRNA gene sequence GenBank accession number EU220836, oligonucleotide sequence S-*-Smet-1459-a-A-18 complementary to unique region of the 16S rRNA gene. Yet to be cultivated.

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