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Development, Life Cycle, Ultrastructure and Phylogenetic Position of Pasteuria ramosa Metchnikoff 1888: Rediscovery of an Obligate Endoparasite of Daphnia magna Straus

Dieter Ebert, Paul Rainey, T. Martin Embley and Dimitri Scholz

Phil. Trans. R. Soc. Lond. B 1996 **351**, 1689-1701
doi: 10.1098/rstb.1996.0151

Development, life cycle, ultrastructure and phylogenetic position of *Pasteuria ramosa* Metchnikoff 1888: rediscovery of an obligate endoparasite of *Daphnia magna* Straus

DIETER EBERT[1], PAUL RAINEY[2], T. MARTIN EMBLEY[3] AND DIMITRI SCHOLZ[4]

[1] *NERC Centre for Population Biology, Imperial College at Silwood Park, Ascot, Berks SL5 7PY, U.K. and Institut für Zoologie, Universität Basel, Rheinsprung 9, CH-4051 Basel, Switzerland*
[2] *Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, U.K.*
[3] *Department of Zoology, Natural History Museum, Cromwell Road, London SW7 5BD, U.K.*
[4] *Max-Planck-Institut für Physiologische und Klinische Forschung, Experimentelle Kardiologie, Beneke-Str. 2, 61231 Bad Nauheim, Germany*

SUMMARY

The development, life cycle, ultrastructure and phylogenetic position of an obligate, spore-forming endoparasite of *Daphnia magna* Straus is described. The microparasite was found in the body cavity of three *Daphnia* species (*D. magna*, *D. pulex* and *D. longispina*) collected in England and Russia during 1992–1994 and maintained in artificial culture by co-cultivation with *D. magna*. Transmission of the endoparasite occurred horizontally through waterborne spores released from the remains of dead infected hosts. Progeny of infected hosts were never infected, indicating that vertical transmission does not occur. Egg production by infected mothers ceased soon after infection and death ensued after 46 days (± 7 standard error) at 20 °C. Phase contrast light microscopy and transmission electron microscopy of the infection process showed the endoparasite to have a polymorphic life cycle beginning with the appearance of branched 'cauliflower-like' rosettes and ended with the development of single, oval endospores, nippled at one end and with complex internal structure. Endospore formation resembled that found in endospore-forming bacteria. Morphologically the parasite has strong resemblance to the *Pasteuria ramosa* that Metchnikoff isolated from *D. magna* and *D. pulex* in Ukraine and described in 1888. Identification of this parasite has been an enduring puzzle since Metchnikoff. The previously confused phylogenetic position of *P. ramosa* (it has been classified as bacterium, yeast and protozoa) was resolved by sequencing the 16S rDNA molecule. Fluorescent *in situ* hybridizations confirmed that the 16S rDNA sequence obtained from the spores within the *D. magna* body cavity originated from the endoparasite. Maximum likelihood and maximum parsimony analysis showed that *P. ramosa* belongs to the low G+C Gram positive branch of the eubacteria and resides within a clade containing *Bacillus tusciae*, *Alicyclobacillus cycloheptanicus* and *A. acidocaldarius* as its nearest neighbours. These results confirm suggestions that this parasite is a bacterium and refute its previous tentative placement based on its morphological complexity among the Actinomycetales.

1. INTRODUCTION

Parasites and pathogens often pose a challenge for the taxonomist, as morphology and life cycle are frequently very different from their closest free-living relatives. Reductions of parasite organs and organelles whose functions are provided by the host combined with the evolution of special structures supporting the parasitic lifestyle may lead to extreme specialization. A remarkable example is the microparasites of the genus *Pasteuria* Metchnikoff 1888. All known members of this genus are parasites of invertebrates, in particular, freshwater zooplankton and soil nematodes. Metchnikoff (1888) described *Pasteuria ramosa* from Ukrainian samples of *Daphnia magna* and *D. pulex*, as a spore-forming bacterium with an unusual form of longitudinal division. Since this time, the biology and identity of this parasite has been an enduring problem. Metchnikoff's observations and the classification of *P. ramosa* as a bacterium were repeatedly questioned (e.g. Migula 1900; Hirsch 1972). Rühberg (1933) suggested that it was a *Torula* yeast, while Jirovec placed it first into the microsporidia (Jirovec 1936) and later into the haplosporidian genus *Democystidium* (Jirovec 1939).

Correspondence should be addressed to D. Ebert, Institut für Zoologie, Universität Basel, Rheinsprung 9, CH-4051 Basel, Switzerland.

Weiser (1943) placed it under a new genus *Lymphocystidium*. In his key to parasites of the Cladocera, Green (1974) listed *P. ramosa* twice, once among the bacteria and once under the name *Dermocystidium daphniae* among the Haplosporidia. More recently Sayre and co-workers (1979) stressed the bacterial characters of *P. ramosa* and, based upon its morphological complexity, suggested it to be a member of the bacterial order Actinomycetales.

Further confusion arose because Hirsch (1972) and Staley (1973) applied the name *Pasteuria ramosa* Metchnikoff 1888 to a superficially similar budding and rosette-forming bacteria represented by an isolate labelled strain ATCC 27377. Sayre *et al.* (1983) requested to conserve the name *Pasteuria ramosa* Metchnikoff 1888 and to reject ATCC 27377 as the type strain of the species *Pasteuria ramosa* Metchnikoff 1888. Further work by others supported this request (Schlesner & Hirsch 1984) and the Judicial Commission for the code of nomenclature of bacteria approved this (Judical Commission 1986). Hence the name *Pasteuria ramosa* Metchnikoff 1888 is perfectly valid and should be applied to endoparasitic bacterial parasites of cladocerans (Sayre 1993).

Parasites similar to *Pasteuria* have also been found in other organisms. Sayre & Wergin (1977) had pointed out that a microparasite of root-knot nematodes is similar to *P. ramosa* and later identified this parasite as *Pasteuria penetrans* (ex Thorne) (Sayre & Starr 1985). Interestingly, this parasite, like *P. ramosa*, has also had a confused taxonomic history. For about 70 years it was considered a protozoa belonging to the microsporidia (Cobb 1906; Thorne 1940) and later, it was suggested that it might be a fungus (Canning 1973). Before, placement within the genus *Pasteuria*, *P. penetrans* was classified as a *Bacillus* (Mankau 1975), which for the first time emphasized its bacterial nature and its ability to form endospores. *P. penetrans* is a potential control agent for root-knot nematodes and the search for new control agents has lead to the discovery of several new species. *Pasteuria* parasites in nematodes have been described from some 50 countries and about 200 host species (Sayre 1993), but despite the biological and economic interest the phylogenetic position of the genus *Pasteuria* remains unclear.

The type material of *P. ramosa* appears to be lost, but in an effort to rediscover *P. ramosa*, Sayre and co-workers studied a parasite of the cladoceran *Moina rectirostris* that closely resembles Metchnikoff's description (Sayre *et al.* 1977; Sayre *et al.* 1979; Sayre *et al.* 1983; Sayre & Starr 1989) (NB, Sayre, *et al.* 1977 misspelt the Genus name *Moina* as Monia in the title). Although, this parasite was very similar to Metchnikoff's *P. ramosa* it showed some differences that deterred them (Sayre *et al.* 1983; Sayre & Starr 1989) from declaring it as the neotype for *P. ramosa*.

During field and laboratory work with *Daphnia magna* from ponds in England and Russia we discovered a *P. ramosa*-like parasite in several locations (Stirnadel 1994; Stirnadel & Ebert 1997). Here we first describe the life cycle, development and ultrastructure of this unusual microparasite and show that it is very similar to Metchnikoff's originally described species. In addition we used the polymerase chain reaction (PCR) technique to sequenced the 16S ribosomal DNA (rDNA) gene in an effort to clarify the phylogenetic position of the genus *Pasteuria*. In combination with fluorescent probes it was possible to attribute the different morphological stages of this unculturable microparasites to one species.

2. MATERIAL AND METHODS

(a) *Material*

Daphnia magna Straus infected with a *Pasteuria ramosa*-like endoparasite were found in Summer 1992, and throughout 1993 and 1994 in several ponds in Southern Oxfordshire, Berkshire and in the Children's Boating Pond in Regents Park, London, U.K. (Stirnadel 1994; Stirnadel & Ebert 1997; D. Brunner & D. E., in preparation). In July 1993 infected female *D. magna* were collected from one pond in England (Lat. 51°37′15, Long. 1°21′50; pond 1 in Ebert 1994) and kept in the laboratory in artificial *Daphnia* medium (Klüttgen *et al.* 1994) (this recipe was modified by using only 1/20th of the recommended SeO_2). Newborn females from an uninfected laboratory clone were placed together with infected hosts and the disease was observed to spread to previously uninfected hosts after the infected host had died. Spores released from the cadaver of the previously infected host are waterborne.

Host and parasite material were bred in the laboratory in 0.5–2 litre cultures derived from the original material. These cultures were maintained by adding uninfected newborn at 3 to 4 week intervals. The culture medium was changed every 2 to 4 weeks. We used *Chlamydomonas reinhardii* as *Daphnia* food throughout this study. Unless mentioned otherwise, the cultures were kept at 18–22 °C. Under these conditions, we were able to breed the parasite for more than 20 host generations.

Infected hosts were studied under a dissecting microscope at 25 or 50 × magnification. No fixatives were used. *P. ramosa* in fresh preparations can be seen using light microscopy, but visibility is improved using phase contrast microscopy.

(b) *Development and ultrastructure of spores*

To study the development of the parasite inside their hosts, two hundred newborn *D. magna* were placed in 1 litre of medium containing a spore suspension obtained from the homogenized remains of 20 infected hosts. Every four days 10 *Daphnia* were dissected and the presence of different parasite stages were recorded. The experiment was conducted at 20 °C with a 16:8 hours light:dark cycle. Hosts were fed with 10^4 cells of *C. reinhardii* ml^{-1} daily.

For electron microscopy we fixed pieces of host tissue in 4% freshly prepared gluteraldehyde in PBS (phosphate buffered saline), followed by fixation in 1% OsO_4. After fixation the samples were washed in PBS, dehydrated in ethanol and embedded in EPON. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed using a Philips CM 10 electron microscope.

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(*c*) *Transmission and pathology*

Sayre and co-workers (1979) reported that their *Moina* parasite is transmitted through spore uptake from sediments, but infections were only observed above 25 °C. To compare these observations with our parasite, vessels with a bottom layer of mud (about 2 ml) were filled with 400 ml of water and stocked with 10 uninfected newborn *D. magna*. The mud had been collected from ponds containing infected *D. magna* populations located around Oxford, U.K., and from the Cormorant pond in Moscow Zoo, Russia (two replicates of each of five populations). Animals were fed every second day, and after 20 days animals were dissected and examined for the presence of the disease. Transmission was also examined over two host generations by placing single infected females together with five uninfected newborn in 100 ml beakers in incubators at 15, 20 and 25 °C.

To test for vertical transmission we collected offspring produced by infected *D. magna* and raised the newborn, individually, in 100 ml beakers incubated at 20 °C.

We also examined the ability of Russian and English strains to infect *D. magna* obtained from other sampling locations. For this, 100 ml beakers containing single uninfected two-day-old English and Russian hosts were treated with Russian and English parasite spore suspensions. The animals were checked daily for the presence of offspring and were dissected after death to check for parasites. Uninfected animals were kept as controls. Since the objective of this paper was to compare our parasite with published descriptions of other isolates, we address the pathology only as far as it is necessary to meet this criterion. More detailed results of the cross infection experiments will be described elsewhere (D.E., in preparation).

(*d*) *Sequencing*

(i) *Harvesting spores and extraction of DNA*

Heavily infected single hosts were taken from our cultures and frozen. Freezing caused the carapace to break and it was possible to obtain spores from the thawed hosts simply by flicking the container several times. The body of the host was then removed and the spores were collected by brief centrifugation. To remove contaminating microorganisms the spores were treated as follows. About 10^6 spores were incubated for 30 min at 37 °C in 200 µl of 20 mg ml^{-1} lysozyme solution in Tris-EDTA (TE), pH 8.0, after which sodium dodecylsulphate (SDS) solution was added to a final concentration of 2 % v/v, followed by incubation at 37 °C for a further 30 min. Lysozyme treatment did not destroy the spores. The spore suspension was then washed twice with 500 µl of 10 mM tris HCl buffer, pH 7.0, and DNAase and RNAase were added, each to a concentration of 10 µg ml^{-1}. After incubation at 37 °C for 30 min the spores were harvested by centrifugation and washed twice with Tris HCl buffer, pH 7.0. Proteinase K was added to 50 µg ml^{-1} and the spore suspension was incubated at 55 °C for 2 h. The spores were pelleted by centrifugation and washed twice in Tris HCl buffer, pH 7.0, before resuspending in 500 µl of TE buffer, pH 8.0, for lysis. The absence of vegetative cells was checked using phase contrast microscopy.

The spore suspension was added to a microfuge tube containing 500 µl of glass beads (0.17–0.18 mm diameter) and 500 µl of Tris buffer (pH 8.0) equilibrated phenol. Lysis was achieved by bead-beating in a Braun Mikro-Dismembrator at 2000 r.p.m. for 60 s. The preparation was then centrifuged at 13000 r.p.m. in a microfuge and the aqueous layer was transferred to a fresh tube. Subsequent DNA purification from this supernatant followed the method described by Pitcher *et al.* (1989) but guanidium thiocyanate was omitted. The final concentration stage was achieved using spin dialysis in a Microcon 100 against TE buffer (pH 8.0). The retentate was made to 20 µl using TE buffer, and 1 µl of this was used for PCR.

PCR amplifications (50 µl) were carried out using PCR primers designed to amplify almost the full length of bacterial 16S rDNA (Embley 1991). The single PCR product was precipitated and sequenced directly using published primers (Embley 1991; Lane 1991).

(ii) *In situ probing*

To confirm that the amplified sequences originated from the parasite an oligonucleotide probe 5′-CATTTCTTCTTCCCGATG was designed to bind to a region of variable sequence corresponding to helix 18 (region V3) of *E. coli* secondary structure (Neefs *et al.* 1993). The oligonucleotide was labelled with tetramethylrhodamine during synthesis (Genosys Biotechnologies Inc., Cambridge, U.K.) and used to probe microorganisms present in squashed paraformaldehyde fixed (Stahl & Amann 1991) infected, or uninfected *Daphnia*. A positive control probe (EUB 338) which binds to all bacterial 16S rRNA (Stahl & Amann 1991) was labelled with carboxyfluorescein. Hybridization experiments were carried out on ethanol dehydrated tissue smears at 36 °C for 2 to 4 h in hybridization buffer (Stahl & Amann 1991) containing 20 % formamide. After hybridization, the smears were washed in 0.9 M NaCl/0.1 % SDS at 50 °C for 15 min (twice), rinsed quickly in cold distilled water and air dried. The smears were mounted using Citifluor AF1 antifadant (Citfluor Ltd, London) and observed by epifluorescence microscopy.

(iii) *Analysis of SSU rDNA sequences*

The single sequence recovered from PCR amplifications of DNA from lysed spores has been deposited in GenBank as U34688. Attempts to infer its phylogenetic position proceeded as follows. Preliminary analyses used the SIMILARITY_RANK and SUGGEST_TREE tools provided on the WWW page of the RDP (rdp.life.uiuc.edu/RDP/data/ssu.html). These suggest the most similar sequences to a query sequence and also suggest an approximate placement in the RDP maximum likelihood tree. Additional analyses using neighbour joining (Saitou & Nei 1987)

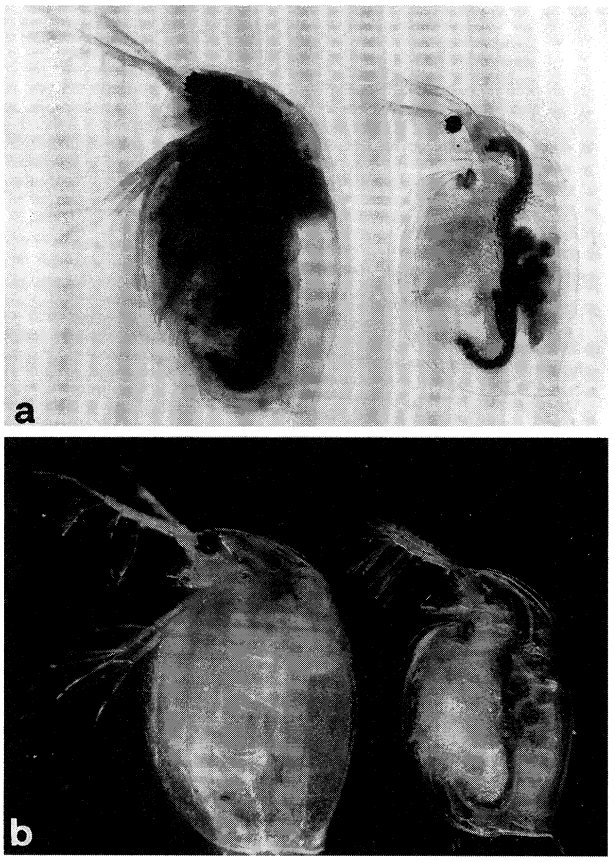

Figure 1. Infected (left) and uninfected (right) female *Daphnia magna* in transmitted light (*a*) and reflected light (*b*). The infections are whitish in reflected light (slightly brownish in live specimens with advanced infections) and dark in transmitted light. The total body length excluding the spina of the left female was 3.1 mm. The uninfected female is smaller because it was younger.

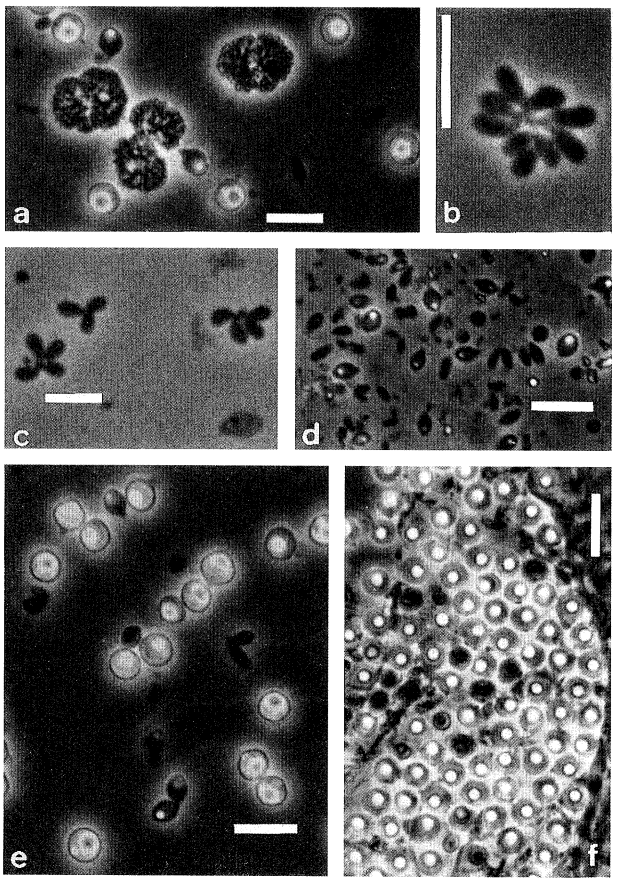

Figure 2. Phase contrast photographs of the *Daphnia* parasite. All pictures were from fresh preparations of infected *Daphnia magna*. Parts (*a*) through (*f*) show the sequence of different life stages of the parasite. (*a*) Several early 'cauliflower' stages. (*b*) A rosette, which formed from a branch of a cauliflower. (*c*), (*d*) Spore clusters become disconnected and increase in size. The pointed end of each spore marks the place of former attachment to the branches. In some spores small vacuole-type structures appear. This stage resembles the 'grape seed' stage by Metchnikoff. (*e*) Spores with vacuole, visible as bright white, phase-light structure opposite the pointed end of the spores. (*f*) Dense packing of final spore stages in a heavily infected female host. The white bars represent 10 μm. Compare with stages as given in table 1.

were carried out using an alignment containing the suggested sequences plus a selection of reference sequences representing all of the main bacterial phyla. All sequence manipulations and analyses were carried out using the Genetic Data Environment (GDE) software version 2.2 (Maidak *et al.* 1994) Distance matrix calculations and neighbour-joining analyses used the programs DNADIST and NEIGHBOR in PHYLIP 3.5 (Felsenstein 1993).

The alignment of our parasite comprised 1256 sites (of which 394 were informative under parsimony) which could be unambiguously aligned for 22 taxa. Three types of analysis were undertaken to find its detailed phylogenetic position. Maximum likelihood analyses using the program fastDNAml version 1.0.6 (Felsenstein 1981; Olsen *et al.* 1994). This program does not assume constancy of evolutionary rate between lineages, and is particularly useful when, as here, the rates are unknown. A transition/transversion ratio of one was applied since this most closely matched the pattern in the aligned data measured using the program mega 1.01 (Kumar *et al.* 1993). Empirical base frequencies were used to estimate substitution model parameters for maximum likelihood calculations. The GLOBAL rearrangement option was used, with analyses repeated using different orders of sequence addition (JUMBLE in fastDNAml). Maximum parsimony (MP) analyses were based on informative positions only and used the heuristic search option in PAUP 3.1.1 (Swofford 1993), with 10 random addition sequences and TBR branch swapping. Bootstrapping (100 replicates), was used to investigate the support for groups in MP analyses, with one random addition sequence per replicate. Distance matrix analysis used the recently developed LogDet transformation that allows for more general models of sequence evolution with asymmetric rates of nucleotide change (Lockhart *et al.* 1994). The correction was implemented on all sites and on parsimony sites only, using the program SplitsTree 1.0 (Huson & Wetzel 1994.). The corrected distances were analysed using neighbour joining (Saitou & Nei 1987) in PHYLIP 3.5.

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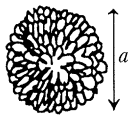
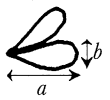
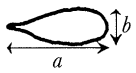
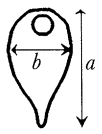
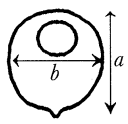
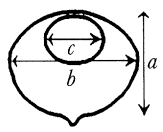
Table 1. *Size of colonies and sporangia of Pasteuria ramosa* [μm]

stages as in figure 2	type of colony or sporangium	this study (ranges of 10 given)		Metchnikoff (1888)[b]		Sayre *et al.* (1977*a*)	
		a	*b*	*a*	*b*	*a*	*b*
a		4.0–6.7				4.4–6.1[c]	
b		2.3–4.4	1.0–1.7	4.8	1.0	3.0–3.8	1.2–2.0
c		3.3–4.4	1.3–2.3			3.0–4.0	1.5–2.2
d		4.0–5.7	3.0–3.4				
e		4.0–6.0	3.9–5.4	5.2	3.9	4.8–5.7	3.3–4.1
f		4.2–5.4[a]	4.9–6.0				
		c = 1.9–2.3					

[a] Length does not include the nipple.
[b] Measurements taken from photographs of Metchnikoff.
[c] From scanning electron microscope.

3. RESULTS

(*a*) *Light microscopic investigation of parasite development*

Parasite spores in living, transparent hosts could be seen floating in the haemolymph, with the majority located in the central body cavity. Spores were visible as dark clusters in transmitted light (figure 1*a*) or as whitish (in heavy infections often light brownish) clusters in reflected light (figure 1*b*).

Dissection of hosts from a cohort of simultaneously infected newborn at four day intervals allowed the developmental stages of the parasite to be determined (figure 2, table 1). Twelve days after the uninfected newborn were placed together with infected adults, the first 'cauliflower' stages (sensu Metchnikoff) (figure 2*a*) were found in three of 10 dissected hosts. A central stem could be identified that branched into secondary and tertiary branches with rounded ends (figure 2*b*). The cauliflower stage of the parasite appeared to be attached to the inner wall of the carapace and to other tissue. Detached from the host tissue they can be seen as spherical objects floating through the haemolymph. Four days later, alongside the cauliflower-type microcolonies, we observed separated fractions of these rosettes (figure 2*b* and *c*), with some cell associations consisting of only two, three or four cells attached to each other at the pointed end (figure 2*c* and *d*). These were branches of the microcolonies, which had broken away. Apparently, during growth of the branches, the rosette fragments into smaller, further growing units. Each branch eventually formed a single spore (figure 2*d–f*). The earliest single cells (Metchnikoff's grape seed stage) were characterized by one pointed and one rounded end and were found 20 days after initial infection (eight days after the first cauliflower stages were found). A small refractile body (Metchnikoff's vacuole-type structure) appeared four days later and was visible as a bright white (phase bright), rapidly growing inclusion, located closer to the rounded end of the spore (figure 2*d* and *e*). This forespore appeared greenish when observed with normal light microscopy. Once the forespore appeared, the cells became more rounded and the pointed end became nipple-like, decreased in size and became less prominent. The forespore increased in size up to 1.9–2.3 µm diameter. Fully developed endospores were found mainly in dying or dead hosts.

The general shape of spore-forming cells changed completely during development. They started as

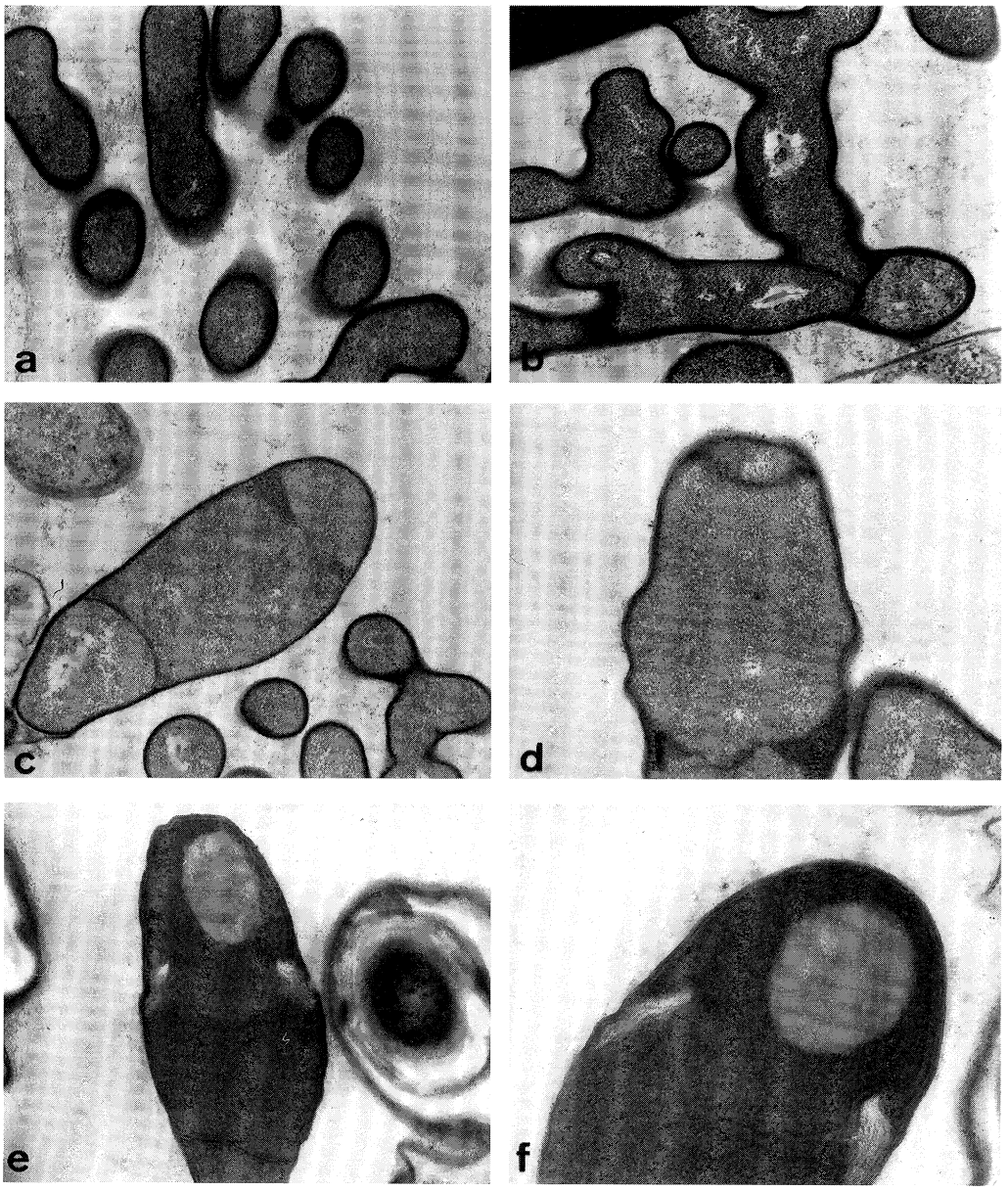


Figure 3. Transmission electron micrographs of vegetative cells of the *Daphnia* parasite. Parts (*a*) through (*f*) represent successive developmental stages of the parasite. (*a*), (*b*) Sections through microcolonies. (*b*) A branch of a microcolony that has begun to sporulate. (*c*), (*d*) Single cells with three compartments. (*e*), (*f*) Sporangia with the forespore.

elongated (tear drop) shaped cells (figure 2*c* and *d*) progressed to spherical, and finally to oval shapes (figure 2*e* and *f*) over the course of about 12 days. The slightly shorter axis of the final stage was originally the longer axis in the earlier stages (compare table 1). In this final stage the nipple was hardly visible and easily overlooked. The forespore's position remained acentric at the same point throughout development, but appeared to be central when observed with the nipple facing away from the observer (figure 2*f*). Hosts dying from this disease were completely filled with the late, oval stages (figure 2*f*), but often earlier stages were also present. In laboratory cultures or field-collected hosts, all parasite stages could be found at the same time, unless infection occurred very recently. If final spore stages were placed on an microscopic slide, covered with a coverslip on which pressure was applied (e.g. with the finger nail) the outer cell wall easily scattered, leaving the circular endospore exposed.

We did not observe longitudinal division as had been reported by Metchnikoff. We also failed to find phagocytosed spores. However, the resistance of some of our laboratory *D. magna* clones to infections with *P. ramosa* (see below) were highly variable, indicating that the ability to phagocytose *Pasteuria* spores might be found only in some clones.

(*b*) *Ultrastructure*

The endogenous spore formation observed in *P. ramosa* closely resembled spore formation of its congeners, as well as that observed in other endospore-forming bacteria. Spore formation started with the enlargement of the terminal filamentous cells of the

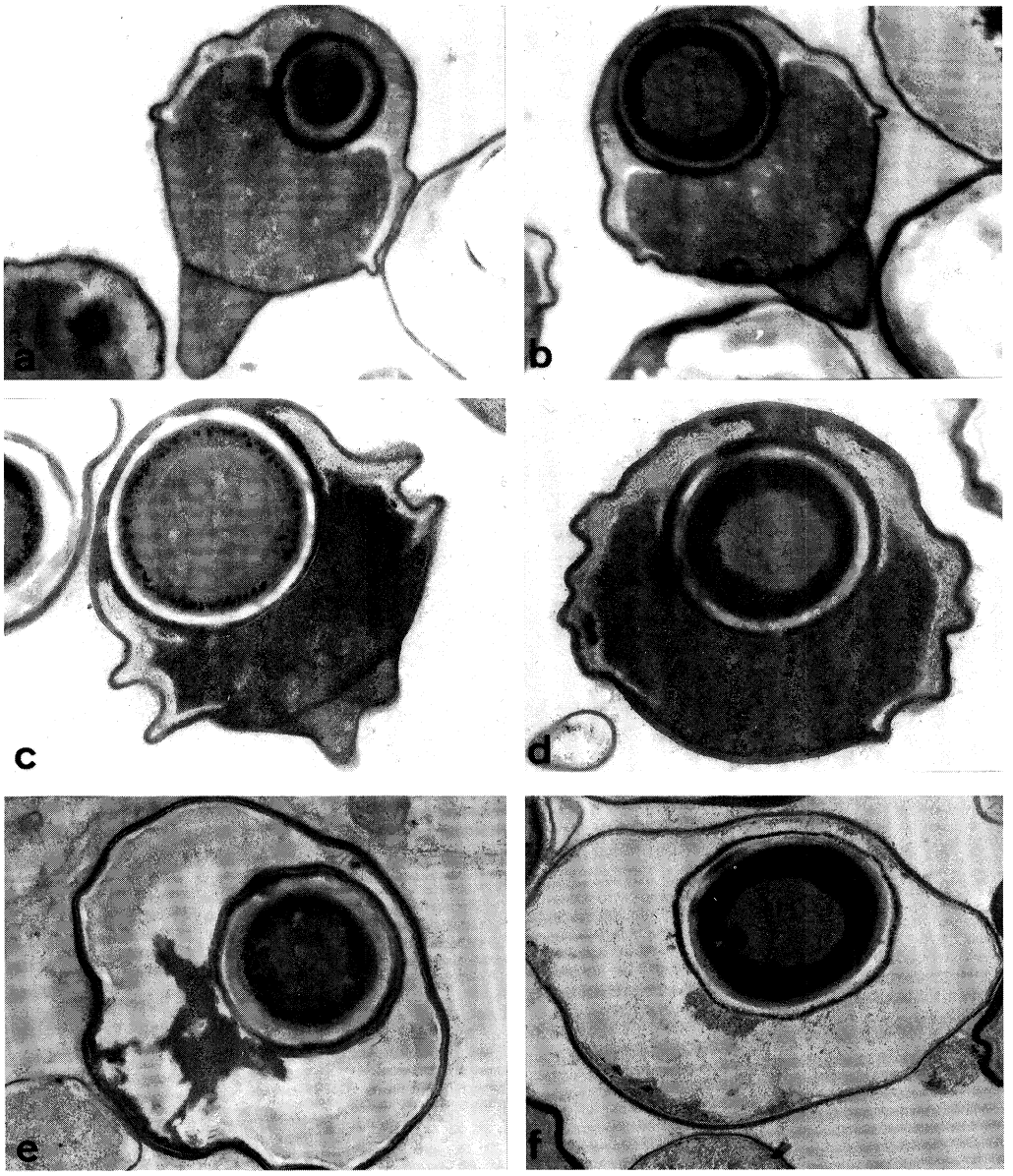


Figure 4. Transmission electron micrographs of vegetative cells of the *Daphnia* parasite. Parts (*a*) through (*f*) represent successive developmental stages of the parasite. (*a*), (*b*) The central compartment disintegrates and the nipple forms. Two equatorial folds can be seen. (*c*) The forespore becomes large and fills out a large portion of the cell. Two equatorial folds are visible. (*d*) The cell assumes a more roundish shape. The folds start to disappear. (*e*) The granular matrix disappears until only a few pieces remain in the posterior part of the cell. (*f*) Final spore stage.

microcolonies (figure 3*a* and *b*). The cytoplasm had a distinct granular appearance, most likely due to the presence of ribosomes (figure 3*a–d*). During the growth of the cell, transverse septa formed and one of these eventually results in the separation of the spore from the microcolony, which at this stage had already fallen into several subunits (figure 3*b*).

Within each of these cells two other transverse septa were usually formed. One separated about one fifth, to one half, of the upper part of the cell (the usually more rounded anterior pole). The other septum separated the lower end of the cell (figure 3*c* and *d*). The lower end eventually formed the nipple-like structure at the pointed, posterior end of the spore. Both septa had double walls. The double membrane in the anterior part then diverged and compartments formed, each surrounded by its own membrane (figure 3*c*). Light (electron translucent) spaces between the compartments becomes visible in the area closest to the outer cell wall (figure 3*e* and *f*). The anterior compartment condensed into a forespore, which was characterized by a circular light area surrounded by a dark, thick, irregular wall. The forespore began as a body of irregular shape, but assumed an increasingly circular shape during later development. The cell expanded more laterally at this stage and the compartment at the posterior pole formed the nipple (figure 4*a*). The dark middle compartment shrank continuously and became detached from the outer cell walls (figure 4*a* and *b*). By the time the forespore assumed an almost circular shape, the middle compartment was only connected to the wall of the most posterior compartment and to the most posterior tip of the forespore (figure 4*b–d*). At about the same stage, the wall of the middle com-

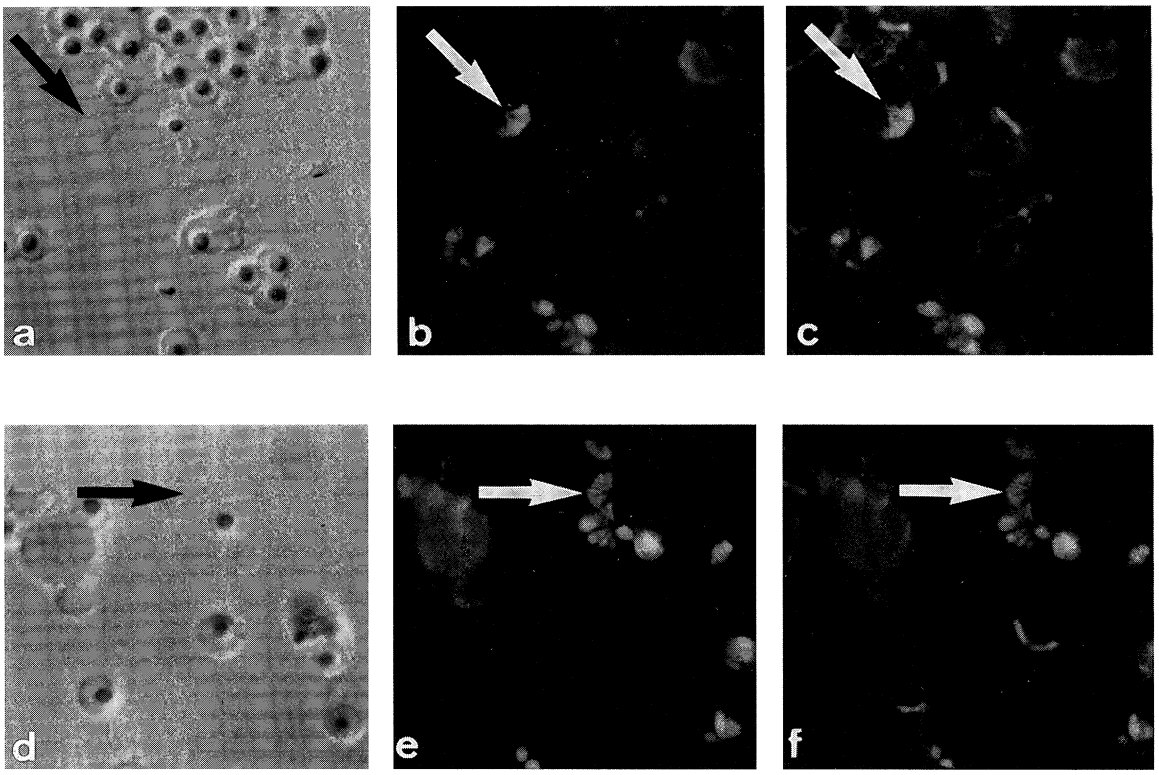


Figure 5. *In situ* probing using fluorescent oligonucleotides to confirm the origin of the *Pasteuria* sequence. The photographs shows two examples (upper and lower row). (*a*), (*d*) Light microscopic pictures with several final stage spores of *P. ramosa*. The earlier stages do not show up well under these conditions. (*b*), (*e*) Fluorescence picture of the same example with the *Pasteuria* specific probe. (*c*), (*f*) Fluorescence picture of the unspecific control probe. The unspecific probe marks the same specimens as the specific probe (figure 5*b* and *e*) and additionally all other bacteria. Some spore clusters can be seen in all three pictures (marked by arrows).

partment disintegrated and finally disappeared. A granular matrix of the same appearance as seen in the disappearing middle compartment was seen between the anterior tip of the forespore and the anterior cell wall, seemingly connecting the forespore with the outer cell wall. As with the middle compartment, this anterior granular matrix disappeared slowly, leaving the forespore apparently detached from the anterior end of the cell (figure 4*d–f*).

During the disintegration of the middle compartment, the forespore differentiated into a multilayered circular structure. During differentiation from the earliest stage with a light central area and a thick granular wall, the central part of the spore became dark, was surrounded concentrically by a very dark ring, followed by a light ring, which was then surrounded by a double membrane. The double membrane separated the forespore from the remaining cell. At this stage all the granular matrix around the forespore disappeared leaving the main body of the cell white, with the dark forespore and some dark areas at the posterior end of the spore. We did not observe lysis of the sporangial wall, as has been described for *P. penetrans* (Sayre 1993).

The outer shape of the cell showed a remarkable change during its development. After the cell had separated from the microcolony it became elongated with one rounded and one pointed pole. The spore increased in diameter and started to form folds around the cell equator. Usually two folds were visible (see figure 4*a–c*), but occasionally more than two folds were visible (figure 4*d*). In later developmental stages these folds disappeared, but even in the final stage, cross-sections maintain an angular appearance, suggesting that traces of the folds remained.

(*c*) *Transmission and pathology*

Transmission of the *Daphnia* parasite was strictly horizontal (waterborne) through spores released from the remains of dead, formerly infected hosts. Offspring of infected females were never infected ($n = 100$). Mud samples from English and Russian ponds with infected populations were infectious as well, indicating the role of pond sediments as a parasite spore bank (Ebert 1995; Ebert *et al.* 1997). Transmission over two host generations was possible at 15, 20 and 25 °C. Transmission from Russian strains to English *D. magna* clones and vice versa was possible, although quantitative variation in transmissibility and pathology across some clones was observed (D. E., in preparation). A comparison of the morphology of the Russian and the English strains did not reveal any differences, suggesting that all strains examined here represent a single species. In most cases parasite infection completely disrupted host reproduction. Females produced a few eggs only within the first 12 days after infection. On average, infected females suffered from a 96%

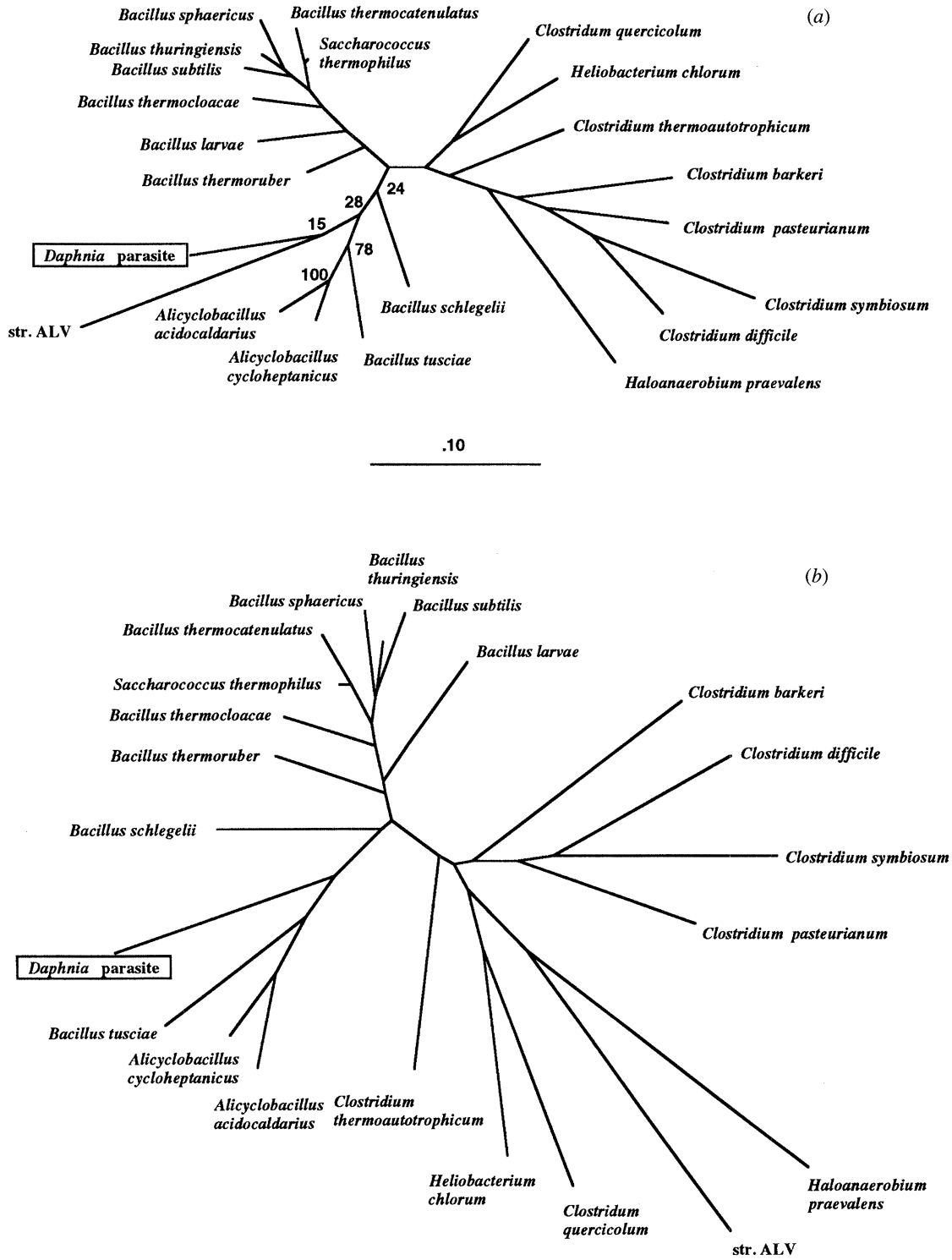


Figure 6. (*a*) Maximum likelihood tree based upon analysis of all 1256 aligned positions. The figures at the nodes are bootstrap proportions (100 replicates) in maximum parsimony analysis of parsimony positions only. the scale corresponds to estimated rate of change per sequence position. (*b*) Tree topology recovered using neighbour joining of LogDet transformed distances calculated for parsimony positions using the program SplitsTree (for details see text). The LogDet is primarily a tool for identifying the correct tree under conditions of asymmetric base compositions, and the branch lengths need not correspond to the amount or rate of change (Lockhart *et al.* 1994; Huson & Wetzel 1994); hence no scale is given.

fecundity reduction in comparison to the controls. Sixty-four per cent of the infected females never produced a single clutch. All infections were lethal. Death occurred after 45.97 days (±6.98 standard error) at 20 °C. By the time the last infected host died, only 2.5% of the controls had died.

(*d*) *Phylogenetic position of the* Daphnia *parasite*

We used the Moscow Zoo strain for the sequence analysis. PCR amplifications yielded a single product, which when sequenced gave a clear unambiguous sequence. Experiments using fluorescent probes to this sequence revealed that it occurred mainly in cells

Table 2. *Base composition table. Percentage of guanine and cytosine for variable positions of compared 16S ribosomal RNA sequence*

	% guanine + cytosine for variable positions	% guanine + cytosine for parsimony positions
Alicyclobacillus acidocaldarius	64.7	64.4
Alicyclobacillus cycloheptanicus	63.3	62.3
Bacillus larvae	54.4	50.7
Bacillus schlegelii	70.1	71.4
Bacillus sphaericus	52.3	47.1
Bacillus subtilis	54.7	50.8
Bacillus thermocatenulatus	64.4	64.2
Bacillus thermocloacae	59.3	57.2
Bacillus thermoruber	58.9	57.1
Bacillus thuringiensis	52.1	47.0
Bacillus tusciae	69.7	71.8
Clostridium barkeri	51.0	46.5
Clostridium difficile	49.8	45.3
Clostridium pasteurianum	48.7	44.0
Clostridium quercicolum	53.2	50.3
Clostridium symbiosum	50.6	44.8
Clostridium thermoautotrophicum	62.3	62.0
Daphnia parasite	59.9	58.9
Haloanaerobium praevalens	47.6	45.5
Heliobacterium chlorum	61.3	60.2
Saccharococcus thermophilus	61.4	59.8
Strain ALV	72.1	73.3

resembling stages c–e (table 1, figure 5). Fully mature spores (stage f in table 1), from which the sequence was originally obtained, usually failed to hybridize with either the symbiont specific probe or the positive control bacterial specific probe. The early cauliflower stages gave faint signals with both probes (figure 5). The failure to find a strong signal was possibly caused by low permeability of spores and colonies or was caused by a low ribosome content of these stages.

Maximum likelihood and maximum parsimony analyses placed the parasite sequence in a clade containing *Alicyclobacillus acidocaldarius*, *Alicyclobacillus cycloheptanicus*, *Bacillus tusciae*, *Bacillus schlegelii* and a thermophilic iron oxidizer called strain ALV (figure 6*a*). Two equally parsimonious trees of 1854 steps were obtained in maximum parsimony analyses, one of which contained the symbiont and strain ALV as sister taxa – as in the maximum likelihood tree. In the other they were recovered separately. Support for most relationships including the position of the *Daphnia* parasite sequence, was low as judged by bootstrap partitions in maximum parsimony analysis: for convenience these particular values are shown at the appropriate nodes on the maximum likelihood tree (figure 6*a*).

Uncertainty in phylogenetic inferences may result in part from the large base composition inequalities observed between the analysed sequences (table 2). To investigate this further we calculated distances between sequences using the LogDet transformation, which allows for asymmetric rates of nucleotide change. Neighbour-joining trees computed using the transformed distances recovered the *Daphnia* parasite sequence as part of a clade comprising *Bacillus tusciae*, *Alicyclobacillus cycloheptanicus* and *Alicyclobacillus acidocaldarius*. *Bacillus schlegelii* was recovered at the base of this clade in the tree based upon parsimony sites (figure 6*b*), but not in the tree based upon all sites (not shown). Strain ALV was not recovered as part of this clade in either analysis.

4. DISCUSSION

(*a*) *Comparison between isolates*

Our morphological description of the *Daphnia* parasite agrees in almost all details with Metchnikoff's (Metchnikoff 1888) original description of *Pasteuria ramosa*. Firstly, the life cycle of the *Daphnia* parasite and its development is virtually identical with Metchnikoff's description. Secondly, our samples and Metchnikoff's samples were obtained from the same host species. Thirdly, Metchnikoff's description of the pathology of the parasite in its hosts agreed well with our observations. The only notable difference was the absence of longitudinal division in the *Daphnia* parasite. Given that Metchnikoff had no electron microscope and no phase contrast equipment, it seems possible that he may have misinterpreted his observations of the mycelial microcolonies.

Development, morphology and pathogenicity of our parasite is also similar to the *P. ramosa*-like parasite described from the cladoceran *Moina rectirostris* (Sayre & Wergin 1977; Sayre *et al.* 1979; Sayre *et al.* 1983). The only discernible difference between *P. ramosa* found in *M. rectirostris* and Metchnikoff's original description is the host species. Sayre and co-workers were not able to infect *Daphnia magna* or *D. pulex* in the laboratory and Sayre *et al.* (1977) also failed to find longitudinal division.

There were, however, some differences between our observation and those of Sayre and co-workers. The

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fold-like structures observed in the *Daphnia* parasite have also been observed in *P. ramosa* from *Moina rectirostris*, but in the *Daphnia* parasite there were usually two equatorial folds, while there was only one in the *Moina* parasite. Further, Sayre (1993, Sayre *et al.* 1979) described the last stage of *P. ramosa* in *M. rectirostris* as a teardrop-shaped sporangium (compare pictures and drawings in Sayre *et al.* (1979) and Sayre (1993)). The last stage in our parasite is a nearly spherical object with a small nipple-like structure opposite the refractile body (table 1, stage f). Metchnikoff's (Metchnikoff 1888) description of the final stage and his drawings of final stage spores inside of leukocytes (Metchnikoff's figures 28–30) correspond well with our parasite, but it appears to be different from the final stage found in *Moina rectirostris* (Sayre 1993). Drawings by Rühberg (1933) and Weiser (1943) of an endoparasite in *D. magna*, which is also believed to be *P. ramosa* (Sayre *et al.* 1979; Sayre 1993), also show an oval or spherical final stage with a small nipple-like structure opposite the refractile body. Finally, the temperature range at which transmission is possible in the laboratory is much wider for our parasite than for the *Moina* isolate. In a field study in three English ponds, our parasite was found all year round, and even at water temperatures below 5 °C (Stirnadel 1994; Stirnadel & Ebert 1997). The *Moina* isolate is only transmitted above 25 °C in the laboratory and only found during summer in natural populations (Sayre *et al.* 1979).

In summary, our observations are in close agreement with the observations made by Metchnikoff (1888). Some differences between our *Daphnia* parasite and the *Moina* parasite were found, namely, the shape of the last spore stage varies, our parasite is transmitted over a wider temperature range, the number of equatorial folds differ and the host species differ. Given the high degree of similarity between our *Daphnia* parasite and Metchnikoff's *Pasteuria ramosa*, we therefore propose our *Daphnia* parasite as the neotype of *Pasteuria ramosa*. Whether the *Moina* isolate is different from *P. ramosa* and represents a new species has to be evaluated in direct comparison between the isolates.

(*b*) *Phylogenetic position*

Given the strong interest in the genus *Pasteuria* over the last 20 years (48 articles since 1990 were found in a literature search for 'Pasteuria' in one data base) it is surprising that its phylogenetic position is still unresolved. Using PCR primers designed to amplify a wide range of prokaryote 16S rRNA, we were careful not to sequence a different microbe. The unambiguous sequence suggests a single organism and not a mixture was sequenced. The fluorescent images (figure 5) confirmed that we sequenced DNA from the parasite, and not host DNA or DNA of another microbe. Our probe was searched through the RDP database of 16S rRNA sequences and proved unique. The *in situ* hybridization confirms that the sequence recovered originates from the parasite, and further demonstrate that the observed morphological variation, which occurs during its life cycle, is indeed from the same organism. This technique has been used successfully in other endosymbionts with complex morphologies (Amann *et al.* 1991; Embley *et al.* 1992).

Maximum likelihood and maximum parsimony analyses agree in placing the symbiont sequence in a clade with sequences from *Alicyclobacillus acidocaldarius*, *Alicyclobacillus cycloheptanicus* and *Bacillus tusciae*. The sequences from *Bacillus schlegelii* and Strain ALV are also members of this clade in these particular analyses, and in some trees ALV formed a sister group with the symbiont sequence. However, there was little positive support from bootstrapping of uncorrected parsimony sites for these relationships (figure 6*a*). Moreover, there are strong base composition inequalities between the compared sequences. Under these conditions it is possible that sequences will group according to their nucleotide compositions rather than their shared evolutionary history. The sequences of *Alicyclobacillus acidocaldarius*, *Alicyclobacillus cycloheptanicus*, *Bacillus tusciae*, *Bacillus schlegelii* and strain ALV are all highly GC rich (table 2). Interestingly, the symbiont sequence has a lower content of guanine and cytosine compared to these sequences, perhaps rendering it less likely that convergence due to G+C content is the only reason for its inferred position. To investigate this further we analysed the sequences using the LogDet transformation which was developed to deal with base inequalities between sequences (Lockhart *et al.* 1994). These analyses also suggest that the *Daphnia* parasite sequence forms a clade (figure 6*b*) with sequences from *Alicyclobacillus* spp. and *Bacillus tusciae*, but they do not support a relationship between the *Daphnia* parasite and strain ALV. The sequence from ALV shifted its position depending on whether all sites or parsimony sites were analysed using LogDet, but in no case did it cluster with the symbiont clade. These results suggest that the inferred phylogenetic position of the symbiont sequence is not due to base composition effects, but that the high G+C content of strain ALV 16S rRNA is compromising its phylogenetic analysis.

The genus *Alicyclobacillus* (formerly *Bacillus* (Wisotzkey *et al.* 1992)) contains mainly thermophilic bacteria (see also Rainey *et al.* (1994)). They are separated from *Bacillus* by omega- alicyclic fatty acids as the major natural membranous lipid component. We do not know if this is the case for our *Daphnia* parasite as well.

The sequence analysis showed that *Pasteuria* does not belong to the *Actinomycetales*, as was suggested previously (Sayre & Wergin 1977; Sayre *et al.* 1977). Our phylogenetic analysis places the *Daphnia* parasite in a clade with endospore-forming bacteria, which are however, morphological less complex. Morphologically the *Thermoacetinomyces* (Williams *et al.* 1989) show some similarity to our parasites; unfortunately there is no sequence available yet. Both produce spores and both are resistant to lysozyme treatment, but more work is required before concluding a closer phylogenetic relationship.

The presented work shows once again that morphological features can be highly misleading in the search for the true phylogeny. The closest neighbours in our phylogeny are all endospore-forming bacteria,

but the complex morphology of the *Pasteuria* endospores is unique in this clade. It remains to be studied if *Pasteuria* endospore formation is an adaptation to the endoparasitic lifestyle or if they are an ancestral trait.

(*c*) *Geographic distribution of* P. ramosa

The geographic locations of our Russian samples and Metchnikoff's samples from the Kiev province in Ukraine are about 750 km apart. Although, this is a large distance, it is closer to the original sampling location than any other sample isolated since Metchnikoff. Our observations of *P. ramosa* extend its host and geographic range. *P. ramosa* has been reported from *D. magna* and *D. pulex* in Ukraine (Metchnikoff 1888), in *D. magna* in North Germany (Rühberg 1933) and in Czechoslovakia (Jirovec 1939). We found it in *D. magna*, *D. pulex* and *D. longispina* in England (Stirnadel & Ebert 1997), where it was the second most common microparasite, and recovered it from mud samples that contained ephippia of *D. magna* and *D. pulex* collected in and close to Moscow, Russia. Given the North American report in *Moina rectirostris* (Sayre *et al.* 1977), the parasite appears to have a very wide distribution. Other endoparasitic members of the genus *Pasteuria* have been reported from about 50 countries and more then 200 host species, most of which were soil nematodes (Sayre 1993).

Thanks are extended to Roland Hübner for help in translating Metchnikoff's original publication from French into German. We thank Lev Yampolsky for providing the Russian mud samples. Pat Dyal provided valuable help with the sequencing and Mark Frikker helped with microscopy. Tom Little and Judy Wearing-Wilde are thanked for valuable comments on the manuscript. This paper is dedicated in great admiration to Elie Metchnikoff.

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Received 19 March 1996; *accepted 25 June 1996*

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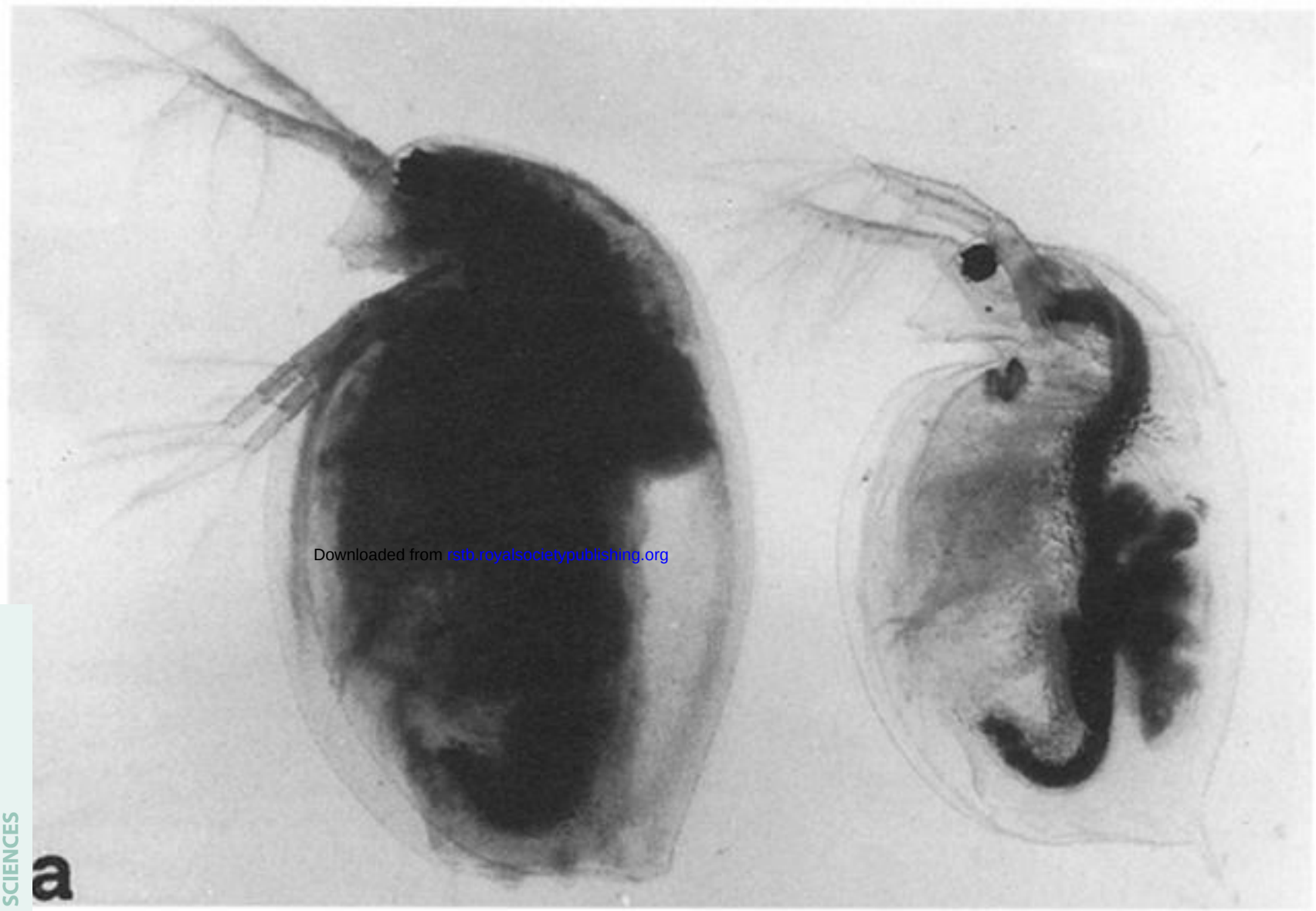

Figure 1. Infected (left) and uninfected (right) female *Daphnia magna* in transmitted light (*a*) and reflected light (*b*). The infections are whitish in reflected light (slightly brownish in live specimens with advanced infections) and dark in transmitted light. The total body length excluding the spina of the left female was 3.1 mm. The uninfected female is smaller because it was younger.

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gure 2. Phase contrast photographs of the *Daphnia* parasite. l pictures were from fresh preparations of infected *Daphnia* *gna*. Parts (*a*) through (*f*) show the sequence of different e stages of the parasite. (*a*) Several early 'cauliflower' ages. (*b*) A rosette, which formed from a branch of a uliflower. (*c*), (*d*) Spore clusters become disconnected and crease in size. The pointed end of each spore marks the ace of former attachment to the branches. In some spores all vacuole-type structures appear. This stage resembles e 'grape seed' stage by Metchnikoff. (*e*) Spores with cuole, visible as bright white, phase-light structure opposite e pointed end of the spores. (*f*) Dense packing of final ore stages in a heavily infected female host. The white bars present 10 μm. Compare with stages as given in table 1.

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Figure 3. Transmission electron micrographs of vegetative cells of the *Daphnia* parasite. Parts (*a*) through (*f*) represent successive developmental stages of the parasite. (*a*), (*b*) Sections through microcolonies. (*b*) A branch of a microcolony that has begun to sporulate. (*c*), (*d*) Single cells with three compartments. (*e*), (*f*) Sporangia with the endospore.

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igure 4. Transmission electron micrographs of vegetative cells of the *Daphnia* parasite. Parts (*a*) through (*f*) present successive developmental stages of the parasite. (*a*), (*b*) The central compartment disintegrates and the pple forms. Two equatorial folds can be seen. (*c*) The forespore becomes large and fills out a large portion of the ll. Two equatorial folds are visible. (*d*) The cell assumes a more roundish shape. The folds start to disappear. (*e*) he granular matrix disappears until only a few pieces remain in the posterior part of the cell. (*f*) Final spore stage.

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gure 5. *In situ* probing using fluorescent oligonucleotides to confirm the origin of the *Pasteuria* sequence. The notographs shows two examples (upper and lower row). (*a*), (*d*) Light microscopic pictures with several final stage ores of *P. ramosa*. The earlier stages do not show up well under these conditions. (*b*), (*e*) Fluorescence picture of the me example with the *Pasteuria* specific probe. (*c*), (*f*) Fluorescence picture of the unspecific control probe. The nspecific probe marks the same specimens as the specific probe (figure 5*b* and *e*) and additionally all other bacteria. me spore clusters can be seen in all three pictures (marked by arrows).