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Neochlamydia hartmannellae gen. nov., sp. nov. (*Parachlamydiaceae*), an endoparasite of the amoeba *Hartmannella vermiformis*

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Free-living amoebae are increasingly being recognized to serve as vehicles of dispersal for various bacterial human pathogens and as hosts for a variety of obligate bacterial endocytobionts. Several *Chlamydia*-like *Acanthamoeba* endocytobionts constituting the recently proposed family *Parachlamydiaceae* are of special interest as potential human pathogens. In this study coccoid bacterial endocytobionts of a *Hartmannella vermiformis* isolate were analysed. Infection of *H. vermiformis* with these bacteria resulted in prevention of cyst formation and subsequent host-cell lysis. Transfection experiments demonstrated that the parasites were not capable of propagating within other closely related free-living amoebae but were able to infect the distantly related species *Dictyostelium discoideum*. Electron microscopy of the parasites revealed typical morphological characteristics of the *Chlamydiales*, including the existence of a *Chlamydia*-like life-cycle, but indicated that these endocytobionts, in contrast to *Chlamydia* species, do not reside within a vacuole. Comparative 16S rRNA sequence analysis showed that the endocytobiont of *H. vermiformis*, classified as *Neochlamydia hartmannellae* gen. nov., sp. nov., is affiliated to the family *Parachlamydiaceae*. Confocal laser scanning microscopy in combination with fluorescence *in situ* hybridization using rRNA-targeted oligonucleotide probes confirmed the intracellular localization of the parasites and demonstrated the absence of other bacterial species within the *Hartmannella* host. These findings extend our knowledge of the phylogenetic diversity of the *Parachlamydiaceae* and demonstrate for the first time that these endocytobionts can naturally develop within amoebae of the genus *Hartmannella*.

Keywords: *Hartmannella*, endoparasite, *Parachlamydiaceae*, *Neochlamydia hartmannellae*, *Chlamydia*

INTRODUCTION

Free-living amoebae (FLA), such as *Hartmannella* spp., *Acanthamoeba* spp., *Naegleria* spp. and *Vahlkampfia* spp., are an important component of soil and water ecosystems, acting as important predators controlling bacterial populations (Rodriguez-Zaragoza, 1994). They are cosmopolitan in distribution, and can be found in fresh water, in marine waters, in soil, on plants and animals, and inside vertebrates, feeding on bacteria, fungi, yeasts, algae and other protozoa. In addition to their environmental significance, some FLA have been identified as human pathogens, causing the diseases amoebic keratitis and meningoencephalitis, and systemic infections (Visvesvara, 1995).

A significant fraction of environmental and clinical FLA isolates harbour, like many other protozoa (Heckmann & Görtz, 1992; Preer & Preer, 1984), bacterial endocytobionts (Fritsche *et al*., 1993; Michel *et al*., 1995). Recent studies have begun to elucidate the phylogenetic diversity of FLA-associated endocytobionts by applying

Abbreviations: EB, elementary body; FLA, free-living amoebae; RB, reticulate body.

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the rRNA approach. The majority of the endocytobionts identified thus far are related to bacterial genera currently recognized as important human pathogens. For example, *Legionella*-related, *Rickettsia*-related and *Chlamydia*-related organisms are known to occur in FLA (Amann *et al*., 1997; Birtles *et al*., 1996; Fritsche *et al*., 1999). In addition, several endocytobionts which group phylogenetically with the *Paramecium caudatum* symbiont *Caedibacter caryophilus* (Springer *et al*., 1993) are known to proliferate within *Acanthamoeba* spp. (Horn *et al*., 1999). Whereas the relationship between hosts and endocytobionts remains largely unexplored, there is increasing evidence that some FLA endocytobionts are of medical importance. Endocytobiont-mediated increase of *Acanthamoeba* cytopathogenicity in tissue culture suggests that these intracellular bacteria enhance FLA virulence (Fritsche *et al*., 1998). Furthermore, some of the endocytobionts have been implicated as causative agents for disease, as indicated by the presence of specific antibodies against *Chlamydia*-related endocytobionts of *Acanthamoeba* in blood from respiratory-disease patients, and by the detection of *Parachlamydia*-like 16S rDNA sequences in specimens from bronchitis patients (Birtles *et al*., 1997; Ossewaarde & Meijer, 1999).

This report describes the investigation of coccoid bacterial endocytobionts of *Hartmannella vermiformis* strain A_1Hsp isolated from the water conduit system of a dental unit, by (i) transfection experiments, (ii) electron microscopy, and (iii) the rRNA approach including comparative 16S rRNA sequence analysis and fluorescence *in situ* hybridization using 16S rRNA-targeted oligonucleotide probes and confocal laser scanning microscopy. Portions of this work were presented as an abstract at the 99th General Meeting of the American Society for Microbiology in Chicago, IL, 1999.

METHODS

Isolation and maintenance of *Hartmannella vermiformis*. The original amoebal host strain *H. vermiformis* strain A_1Hsp was isolated from the water conduit system of a dental unit in Lahnstein, near Koblenz, Germany, by filtering a 100 ml water sample obtained from the source water as described elsewhere (Michel *et al*., 1995). Coccoid endocytobionts recovered from this isolate were transferred to *H. vermiformis* strain OS101 on NN-agar plates (non-nutrient agar; Page, 1988) seeded with *Enterobacter cloacae*. The infected *H. vermiformis* strain OS101 could subsequently be axenized in fluid SCGYE medium (De Jonckheere, 1977) by temporary addition of penicillin and streptomycin (0·2 mg ml^{-1} each). Cultures were incubated at 30 °C and fresh medium was applied every 5–10 d. To investigate the capability of the original amoebal host strain *H. vermiformis* A_1Hsp to form cysts, amoebae were cured of their endocytobionts by treatment with rifampicin as described by Michel *et al*. (1994).

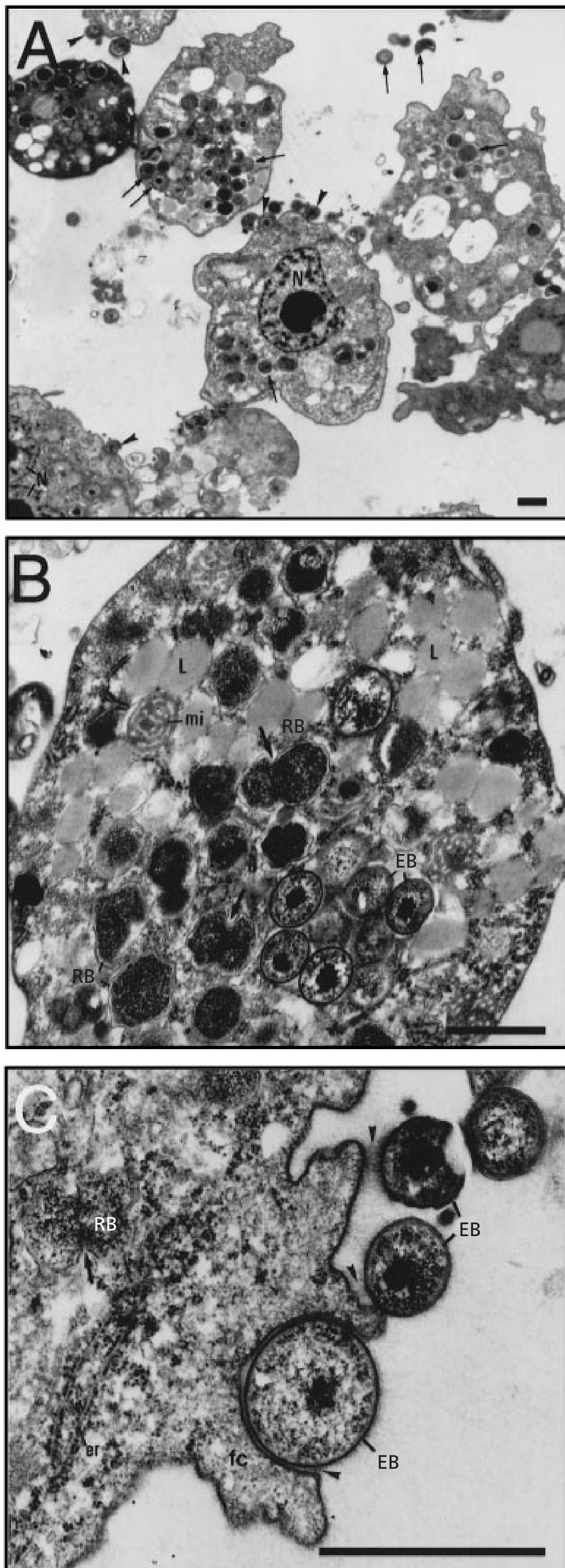
Extracellular growth of *H. vermiformis* endoparasites. Attempts to culture the *Hartmannella* endocytobionts extracellularly included cultivation on blood agar (Becton Dickinson), Casiton-agar (Biotest-Heipha) and SCGYE (De Jonckheere, 1977) at incubation temperatures of 20 and 30 °C. Both whole amoeba cells and filter-purified endocytobionts from lysed amoeba cells were transferred to the respective media. If no growth was observed after 14 d incubation, cultures were considered negative.

Transfection experiments. Following lysis of endocytobiont-infected *H. vermiformis* cells from 4–5-d-old cultures by freeze–thawing, the coccoid bacterial endocytobionts were filter-purified (1·2 µm membrane filter). An aliquot of 80 µl of the resulting suspension was added to strains of different species of FLA, growing either in SCGYE medium or on NN-agar plates covered with a lawn of *Enterobacter cloacae*. The host range of the endocytobiont was investigated by transfection experiments with 14 different strains of FLA (Table 1), and one strain of *Dictyostelium discoideum* isolated from human nasal mucosa. Infection of each host species was monitored by phase-contrast microscopy. After 21 d incubation at 30 or 20 °C the host was considered resistant to infection if no infected cells nor any marked reduction in amoebal numbers (which may have resulted from parasitic activity of the endocytobiont) were observed.

Electron microscopy. For electron microscopical studies, the heavily infected trophozoites from 4–5-d-old cultures were harvested and prefixed in 2·5 % glutaraldehyde in cacodylate buffer (pH 7·2) for 2 h. After prefixation the specimens were fixed in 1 % osmium tetroxide followed by 2 % uranyl acetate in aqueous solution. Subsequently specimens were dehydrated in alcohol and embedded in epoxy resin. Thin sections were stained with 1 % lead citrate and examined with a Zeiss EM 910 electron microscope.

DNA isolation, amplification of 16S rDNA, cloning and sequencing. Simultaneous isolation of DNA from the amoebae and their endocytobionts was performed using a modified UNSET procedure (Hugo *et al*., 1992). Amoebae and their endocytobionts were harvested from axenic cultures by centrifugation (2000 ***g***, 3 min), washed twice with double-distilled water, resuspended in 500 µl UNSET lysis buffer (8 M urea, 0·15 M NaCl, 2 % SDS, 0·001 M EDTA and 0·1 M Tris/HCl at pH 7·5) and incubated at 60 °C for 5 min. Lysates were extracted twice with phenol/chloroform (Roth) and DNA was precipitated for 3 h at −20 °C with 2 vols absolute ethanol. After centrifugation (10000 ***g***, 10 min) at 4 °C the ethanol was removed and the pellet was washed twice with 80 % ice-cold ethanol to remove residual salts. The pellet was air-dried and resuspended in 30 µl double-distilled water.

Oligonucleotide primers targeting 16S rDNA signature regions which are conserved within the *Chlamydiales* were used for PCR to obtain near-full-length bacterial 16S rRNA gene fragments. The nucleotide sequences of the forward and reverse primers used for amplification were 5′-CGGATCC-TGAGAATTTGATC-3′ and 5′-TGTCGACAAAGGAGG-TGATCCA-3′ (Pudjiatmoko *et al*., 1997). 16S rDNA amplification reactions were performed in a thermal capillary cycler (Idaho Technology) using reaction mixtures including 15 pM of each primer, 0·25 µg BSA ml^{-1}, 2 mM $MgCl_2$ reaction buffer and 2·5 IU *Taq* DNA polymerase (Promega). Thermal cycling was carried out as follows: an initial denaturation step at 94 °C for 30 s followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at 52 °C for 20 s, and elongation at 72 °C for 30 s. Cycling was completed by a final elongation step at 72 °C for 5 min. A negative control was performed using a reaction mixture without added DNA. Amplified products were directly ligated into the cloning vector pCRII-TOPO and transformed into competent *Escherichia coli* (TOP10 cells) according to the instructions of the manufacturer (Invitrogen). Nucleotide sequences of the cloned DNA fragments were



determined by the dideoxynucleotide method (Sanger *et al.*, 1977) by cycle sequencing of purified plasmid preparations (Qiagen) with a Thermo Sequenase Cycle Sequencing Kit (Amersham Life Science) and an automated DNA sequencer (Li-Cor) under conditions recommended by the manufacturers. Dye-labelled vector-specific primers M13/pUC V (5′-GTAAAACGACGGCCAGT-3′) and M13/pUC R (5′-GAAACAGCTATGACCATG-3′) were applied.

Phylogenetic analysis. The 16S rDNA sequences obtained were added to the rDNA sequence database of the Technische Universität München (encompassing more than 16 000 published and unpublished homologous small-subunit rDNA primary structures) by use of the program package ARB (O. Strunk and others, unpublished; program available through the homepage of the Technische Universität München: http://www.mikro.biologie.tu-muenchen.de). Alignment of the new rDNA sequences was performed by using the ARB automated alignment tool (version 2.0). The alignments were refined by visual inspection and by secondary-structure analysis. Phylogenetic analyses were performed by applying the ARB parsimony, distance matrix and maximum-likelihood methods to different data sets. To determine the robustness of the phylogenetic trees, analyses were performed with and without the application of various filtersets to exclude highly variable positions.

Fluorescence *in situ* hybridization and confocal laser scanning microscopy. The following oligonucleotide probes were used: (i) EUB338 (5′-GCTGCCTCCCGTAGGAGT-3′), targeting most, but not all, members of the domain *Bacteria* (Amann *et al.*, 1990; Daims *et al.*, 1999), and (ii) S-S-ParaC-0658-a-A-18 (5′-TCCATTTTCTCCGTCTAC-3′), previously designed as complementary to a signature region of the 16S rRNA of the *Parachlamydia*-related endosymbionts of *Acanthamoeba* spp. strains UWC22 and TUME1 (T. R. Fritsche and others, unpublished; probe designation according to the standard proposed by Alm *et al.*, 1996). Oligonucleotides were synthesized and directly 5′-labelled with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS), or the hydrophilic sulphoindocyanine fluorescent dye Cy3 (Interactiva).

For *in situ* hybridization, *Hartmannella* cells were harvested from 4 ml liquid broth culture by centrifugation (2000 ***g***, 3 min), washed twice with double-distilled water, and

Fig. 1. (A) *H. vermiformis* trophozoites harbouring the *Chlamydia*-related endocytobiont (arrows). Free EBs can also be seen outside the eukaryotic cells, some of which are attached to the amoebal cell membrane (arrowheads). (B) Different stages of the endocytobiont of *H. vermiformis* A_1Hsp within the cytoplasm of the amoebal host cell: RBs and EBs can be observed simultaneously and do not reside within vacuoles. Arrows indicate constrictions of RBs undergoing binary fission. In contrast to *Rickettsia*- and *Caedibacter*-related *Acanthamoeba* endosymbionts (Horn *et al.*, 1999; Fritsche *et al.*, 1999) no electron-translucent layers surrounding the intracellularly located bacteria could be observed. (C) Adhesion and phagocytosis of EBs of the endocytobiont by a trophozoite of *H. vermiformis*: adhesion is mediated by fine fibrous material (arrowheads) discernible at the amoebal cellular membrane (glycocalyx) and also at the surface of the EBs. One EB of exceptional size has already been partially engulfed by the amoeba, forming a characteristic foodcup (fc). Within the cytoplasm a constricted stage (arrow) of an RB can be seen. N, nucleus of the host cell; mi, mitochondrion; L, lipid granules; er, endoplasmic reticulum. Bars, 1 μm.

resuspended in 0·05% agarose. Twenty microlitres of this suspension was spotted on a glass slide, air-dried and subsequently dehydrated in 80% ethanol for 10 s. Hybridization was performed using the hybridization buffer (including 30% formamide) and the buffer washing (containing 112 mM NaCl, without SDS) described by Manz *et al.* (1992). Slides were examined using a confocal laser scanning microscope (LSM 510, Carl Zeiss) in combination with a helium-neon laser (543 nm) and an argon-krypton laser (488 nm). Image analysis processing was performed with the standard software package delivered with the instrument (version 2.01).

RESULTS AND DISCUSSION

Coccoid endocytobionts block cyst formation in *H. vermiformis*

The original amoebal host strain A_1Hsp, containing coccoid prokaryotic endocytobionts, was isolated from the water conduit system of a dental unit. Phase-contrast microscopic observation of the amoebae revealed morphological characteristics typical for the genera *Hartmannella* and *Cashia* (Page, 1988). Since no cysts could be observed, the amoebae were provisionally identified as members of the genus *Cashia*. Attempts to grow the amoebal isolate axenically failed. Consequently, bacterial endocytobionts were transferred from the original host strain A_1Hsp into *H. vermiformis* strain OS101, which was subsequently axenized in order to facilitate the following investigations, including evaluation of host spectrum, electron microscopy, and 16S rDNA sequencing. Interestingly, infection with the coccoid endocytobionts prevented cyst formation of *H. vermiformis* strain OS101, a phenomenon which has been previously reported for *Acanthamoeba* sp. Bn_9 and $Berg_{17}$ and *Acanthamoeba castellanii* strain C3 after infection with *Parachlamydia acanthamoebae* (Amann *et al.*, 1997; Michel *et al.*, 1994). Since the ability to form cysts discriminates the genera *Cashia* and *Hartmannella*, this observation forced us to re-evaluate the capability of the original amoebal host strain A_1Hsp to form cysts in the absence of endocytobionts and thus its identification as member of the genus *Cashia*. For this purpose, strain A_1Hsp was cured of its endocytobionts by rifampicin treatment. Despite its toxicity for most amoebae a few trophozoites survived rifampicin treatment and multiplied after transfer to a fresh NN-agar plate containing no rifampicin. Since microscopic observation demonstrated that these endocytobiont-free amoebae were now able to form cysts, strain A_1Hsp was assigned to the genus *Hartmannella* as *H. vermiformis*.

At present, we can only speculate on the ecological significance of the suppression of FLA cyst formation by some endocytobionts. A possible clue might be provided by the observation that *Escherichia coli* (Steinert *et al.*, 1998) is eradicated from artificially infected *Acanthamoeba* cells during cyst formation. Thus, prevention of cyst formation might be a protection mechanism for parasitic endocytobionts, which would be negatively affected by the differentiation of FLA into resting forms. This strategy would contrast with the one used by *Legionella pneumophila* (Steinert *et al.*, 1998; Kilvington & Price, 1990) and several other obligate *Acanthamoeba* endocytobionts (Fritsche *et al.*, 1999; Horn *et al.*, 1999), which survive host-cell cyst formation and thus directly benefit from cyst-mediated host resistance against unfavourable environmental conditions.

Chlamydia-like life cycle and parasitic behaviour of the *H. vermiformis* endocytobiont

Electron micrographs revealed a *Chlamydia*-like morphology and developmental cycle of the endocytobionts (Fig. 1). Stages showing binary fission resembling those of reticulate bodies (RBs, 0·4–0·6 µm in diameter) of *Chlamydia* could be observed. Additionally, the highly condensed coccoid stages (0·5–0·6 µm in diameter) are similar to elementary bodies (EBs) of *Chlamydia*. While RBs of the *H. vermiformis* endocytobiont clearly possess a Gram-negative type cell wall, results of electron microscopic analysis of its EBs are ambiguous. However, since the EBs of the *Hartmannella* endocytobiont showed an outer membrane, we consider them as Gram-negative. This is in noticeable contrast to the Gram-positive type of cell wall which has been observed for the *Chlamydia*-related endoparasite of *Acanthamoeba* sp. strain BN_9 (*P. acanthamoebae*; Amann *et al.*, 1997). In contrast to *Chlamydia* species and *Parachlamydia*-related endocytobionts of *Acanthamoeba* (Amann *et al.*, 1997; T. R. Fritsche and others, unpublished), RBs and EBs of the *Hartmannella* endocytobiont were not surrounded by vacuoles and were thus located directly within the cytoplasm of the host cell, indicating that the endocytobionts possess an escape mechanism from the phagosomes.

Electron microscopic inspection of amoebal cells at different time points showed that massive amounts of mature EBs occurred 3–5 d after infection, and subsequently led to rupture or lysis of heavily infected trophozoites. Shedding of single mature EBs into the environment, not accompanied by host-cell destruction, was, however, already observed at earlier stages of infection (Fig. 1). Since ultimately all infected *Hartmannella* trophozoites are killed by the coccoid endocytobionts, they are considered by us to be intracellular parasites.

Natural stability of this host–parasite association would require an amoebal generation time shorter than the period between parasite infection and host cell lysis. The aggressive parasitic behaviour of the endocytobiont within its *Hartmannella* host suggests a limited adaptation of host and parasite caused by a relatively short evolutionary relationship. *Hartmannella* species may have only recently been infected by these parasites, suggesting their origin from another protist species. Limited adaptation of the endoparasite to the *H. vermiformis* host is also suggested by the suppression of cyst formation, which might protect the parasites from eradication but which may decrease the fitness of the association against environmental stress.

Table 1. Host spectrum of the endoparasite of *H. vermiformis* strain A_1Hsp (*Neochlamydia hartmannellae*)

Amoeba species	Strain	Intracellular multiplication
Incubation at 30 °C		
Acanthamoeba castellanii	C3	−
Acanthamoeba sp.	HLA	−
Acanthamoeba sp.	Renk	−
Acanthamoeba quina-lugdunensis	312-1	−
Acanthamoeba lenticulata	45	−
Acanthamoeba lenticulata	89	−
Naegleria lovaniensis	Aq/9/1/40	−
Naegleria gruberi	NI_1	−
Hartmannella vermiformis	A_1Hsp	+
Hartmannella vermiformis	OS101	+
Willaertia magna	JIII Cl	−
Willaertia magna	NI_4Cl_{1*}	−
Incubation at 20 °C		
Acanthamoeba comandoni	Am 23	−
Acanthamoeba comandoni	Pb 30/40	−
Comandonia operculata	WBT	−
Hartmannella vermiformis	C 3/8	+
Dictyostelium discoideum	$Berg_{25}$	+

Host range of the obligate endoparasite of *H. vermiformis*

Standard cultivation techniques failed to support extracellular growth of the *Hartmannella* endoparasites, suggesting that *Hartmannella* cells are necessary for its growth. This finding is in accordance with the obligate intracellular growth of other endocytobionts of FLA (Amann *et al*., 1997; Fritsche *et al*., 1993).

The host range of the *Hartmannella* endoparasite was determined by transfection experiments encompassing a recently isolated *D. discoideum* strain and 14 different strains of FLA belonging to the genera *Hartmannella*, *Acanthamoeba*, *Naegleria* and *Willaertia* (Table 1). Except for the original host *H. vermiformis* A_1Hsp and two more *H. vermiformis* strains, only *D. discoideum* strain $Berg_{25}$ could be successfully infected. Whereas the extent of parasitic behaviour of the investigated endocytobionts varied slightly between the different *H. vermiformis* host strains, aggregation and stalk and fruiting body formation of *D. discoideum* were not disturbed by the endocytobionts and endoparasite-free spores were formed.

There are remarkable differences in host range between the investigated *Hartmannella* endoparasite and the *Acanthamoeba* endoparasite *P. acanthamoebae*. The *Hartmannella* endoparasite is not able to infect the tested *Acanthamoeba* strains, including *A. castellanii* C3, which is a suitable host for *P. acanthamoebae* (Amann *et al*., 1997). Conversely, *P. acanthamoebae* is unable to infect *H. vermiformis* strains, which serve as host for the investigated *Hartmannella* endoparasite (R. Michel, personal communication). Future studies are required to elucidate the molecular mechanism of a specific recognition system that may mediate specificity of infection.

Phylogeny and *in situ* identification of the *H. vermiformis* endoparasite

Near-full-length 16S rDNA amplicons (1529 bp) retrieved from mixed genomic DNA of amoebal hosts and bacterial endoparasites were successfully cloned and sequenced. Comparative sequence analysis revealed that the retrieved 16S rRNA sequence displayed highest similarity values with 16S rRNA sequences of members of the *Chlamydiales* (Table 2). In particular, the investigated *Hartmannella* endoparasites are moderately related to the *Acanthamoeba* parasite *P. acanthamoebae* strain Bn_9 (92 %), the only validly described member of the new family *Parachlamydiaceae* (Everett *et al*., 1999). It should be noted that even higher sequence similarities of between 96·5 % and 97·1 % to the *Hartmannella* parasite were calculated for 16S rRNA sequences of two recently investigated *Parachlamydia*-

Table 2. Overall sequence similarities for the retrieved 16S rRNA sequence of the endoparasite of *H. vermiformis* strain A_1Hsp (*Neochlamydia hartmannellae*) and representative members of the *Chlamydiales*

		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
A	*Chlamydophila abortus* B577																
B	*Chlamydophila psittaci* 6BC	99·9															
C	*Chlamydophila felis* FP Baker	98·0	98·4														
D	*Chlamydophila caviae* GPIC	98·9	98·9	97·9													
E	*Chlamydophila pecorum* E58	96·2	96·5	95·9	96·1												
F	*Chlamydophila trachomatis* HAR-13	95·0	95·2	94·8	95·3	95·1											
G	*Chlamydia suis* S45	94·3	94·7	94·4	94·5	94·5	97·3										
H	*Chlamydia muridarum* MoPn	95·6	95·7	95·5	95·7	95·5	98·4	97·7									
I	*Chlamydophila pneumoniae* TW-183	95·8	96·2	95·1	95·3	95·8	93·9	93·5	94·6								
J	*Parachlamydia acanthamoebae* Bn_9	86·2	86·7	86·6	86·9	86·4	86·2	87·2	87·0	87·0							
K	*Simkania negevensis* Z	83·5	83·8	83·9	83·9	83·6	84·2	84·4	84·2	83·7	88·2						
L	Endosymbiont of *Acanthamoeba* sp. UWE1	85·8	85·5	84·8	85·4	84·4	84·3	85·4	85·0	85·6	93·1	85·7					
M	Endosymbiont of *Acanthamoeba* sp. UWE25	86·2	86·0	85·6	86·0	85·6	85·4	85·9	86·1	86·3	92·5	85·4	93·0				
N	Endosymbiont of *Acanthamoeba* sp. UWC22	86·8	86·7	86·2	86·7	86·2	85·7	86·4	86·4	86·1	91·2	85·5	92·9	91·9			
O	***Neochlamydia hartmannellae***	87·0	87·5	86·9	87·5	86·5	87·0	87·5	87·4	86·6	92·0	86·5	92·2	91·6	96·5		
P	Endosymbiont of *Acanthamoeba* sp. TUME1	86·8	87·2	86·8	87·2	86·8	86·3	87·0	87·0	86·6	91·2	85·9	92·3	91·4	99·4	97·1	
Q	*Waddlia chondrophila* WSU-85-1044	84·4	84·6	84·4	84·6	84·2	84·7	84·8	84·9	84·4	87·2	84·4	87·1	87·9	87·0	87·0	87·1

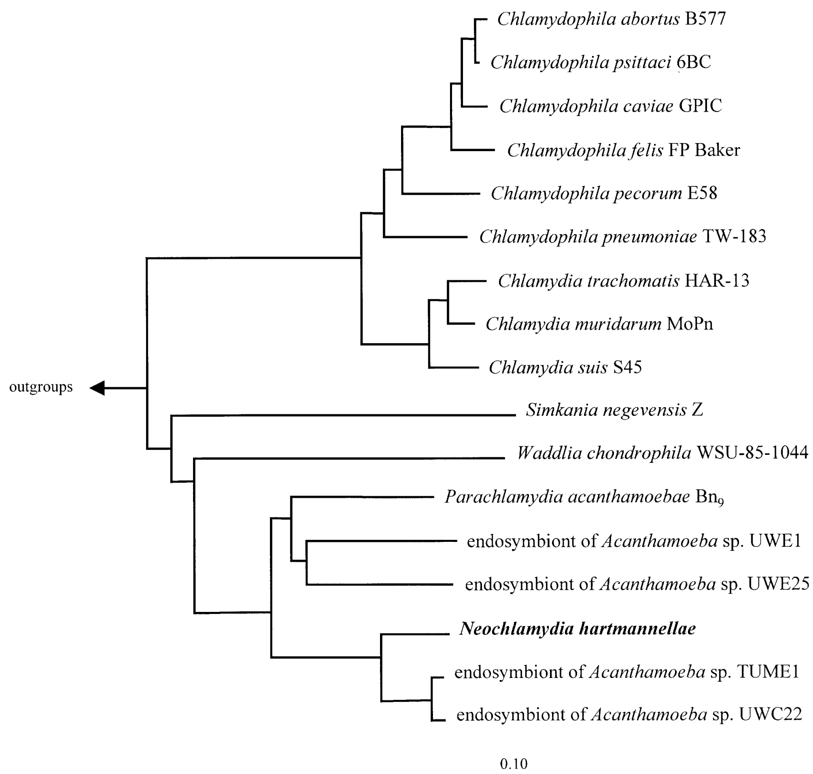


Fig. 2. 16S rRNA-based phylogenetic tree reflecting the affiliation of the endoparasite of *H. vermiformis* strain A_1Hsp (*Neochlamydia hartmannellae*). The tree was obtained using the neighbour-joining method. To exclude highly variable and thus phylogenetically non-informative sequence positions, only those sequence positions which are conserved in at least 50% of the deposited 16S rRNA sequences of the *Chlamydiales* were used for treeing. Nomenclature according to revised taxonomy of the *Chlamydiales* by Everett *et al.* (1999). Bar indicates 10% estimated evolutionary distance.

related endosymbionts of *Acanthamoeba* (Table 2; T. R. Fritsche *et al.*, unpublished).

Phylogenetic analysis using distance matrix, parsimony and maximum-likelihood treeing methods provided consistent evidence for an affiliation of the endoparasites of *H. vermiformis* A_1Hsp with the *Parachlamydiaceae*. Within this family the retrieved sequence forms a monophyletic grouping with the two above-mentioned *Acanthamoeba* endosymbionts (strains UWC22 and TUME1; Fig. 2).

Sequence analysis of 16S rDNA of the investigated endoparasites of *H. vermiformis* revealed the presence of the target site for probe S-S-ParaC-0658-a-A-18, specifically designed previously for the related *Parachlamydia*-like endosymbionts of *Acanthamoeba* sp. TUME1 and UWC22 (T. R. Fritsche *et al.*, unpublished). Simultaneous fluorescence *in situ* hybridization of fixed *Hartmannella* cells with probe S-S-ParaC-0658-a-A-18 and the bacterial probe EUB338 demonstrated that all bacteria detectable by *in situ* hybridization also hybridized with the endocytobiont-specific probe, suggesting the absence of additional, phylogenetically different bacteria within the amoebal host. Confocal laser scanning microscopic analysis confirmed the intracellular localization of the endoparasites of *H. vermiformis* A_1Hsp (Fig. 3).

The genetic data described herein, and the morphological similarity, are consistent with a close relationship between the endoparasite of *H. vermiformis* A_1Hsp and *P. acanthamoebae*. With a 16S rRNA sequence similarity of 92% with *P. acanthamoebae*, a *Chlamydia*-like development cycle, and the ability to multiply and survive within free living amoeba, the endoparasite of *H. vermiformis* A_1Hsp meets the main requirements for inclusion within the family *Parachlamydiaceae* (Everett *et al.*, 1999). However, keeping in mind that 16S rRNA sequence similarities between two bacteria of less than 95% are indicative of their affiliation with two different genera (Ludwig *et al.*, 1998), the *Hartmannella* endoparasite most likely represents a new species of a new genus, since its 16S rRNA similarity to the closest validly described relative, *P. acanthamoebae*, is 92%. In this regard, it should be noted that 16S rRNA sequence similarities of the *Hartmannella* endoparasite with two recently discovered *Parachlamydia*-related endosymbionts of *Acanthamoeba* (T. R. Fritsche and others, unpublished) are higher than 95% but below 97·1%. Nevertheless, we believe that the *Hartmannella* and the *Acanthamoeba* endocytobionts should be assigned to different genera due to their profound differences in host spectra (see above). Consequently, we propose that the endoparasite of *H. vermiformis* A_1Hsp be classified as *Neochlamydia hartmannellae* gen. nov., sp. nov.

Description of *Neochlamydia* gen. nov.

Neochlamydia (Ne.o.chla.my'di.a L. fem. n.; *Neochlamydia* referring to the modest phylogenetic relationship to the *Chlamydiaceae*).

Phylogenetic position: order *Chlamydiales*, family *Parachlamydiaceae*. Members of the genus *Neochlamydia*

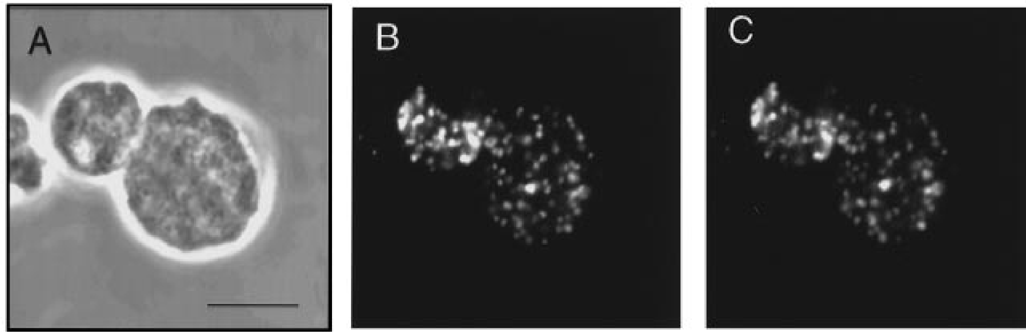


Fig. 3. *In situ* detection of *N. hartmannellae* (endoparasite of *H. vermiformis* strain A_1Hsp) using bacterial probe EUB338 labelled with FLUOS (B), and endocytobiont-specific probe S-S-ParaC-0658-a-A-18 labelled with Cy3 (C). A phase-contrast image is shown in (A). Bar, 10 μm.

should have a 16S rDNA that is >95% identical to the 16S rDNA of the type species, *Neochlamydia hartmannellae* strain A_1Hsp.

Description of *Neochlamydia hartmannellae* sp. nov.

Neochlamydia hartmannellae (hart'mann.el.lae. L. gen. sing. n. of *Hartmannella*, taxonomic name of a genus of *Hartmannellidae*; pertaining to the name of the host amoeba, *Hartmannella vermiformis* strain A_1Hsp, in which the organism was first discovered).

Gram-negative reticulate bodies and Gram-negative elementary bodies; coccoid morphology; 0·4–0·6 μm in diameter. Basis of assignment: 16S rDNA sequence accession number AF177275, nucleotide probe S-S-ParaC-0658-a-A-18 (5′-TCCATTTTCTCCGTCTAC-3′). Not cultivated on cell-free media; obligate intracytoplasmatic parasite of *H. vermiformis* strain A_1Hsp and other *H. vermiformis* strains, therein preventing cyst formation. Host range: able to multiply in *D. discoidum*, but not in *Acanthamoeba* spp.; mesophilic (20–30 °C). Isolated from the water conduit system of a dental unit (Lahnstein, Germany). Type strain, A_1Hsp (=ATCC 50802).

Diversity within the *Chlamydiales* and clinical aspects of *N. hartmannellae*

In a more general perspective, our results and the recent identification of four *Parachlamydia*-related acanthamoebal endocytobionts (T. R. Fritsche and others, unpublished), a *Chlamydia*-like bovine intracellular organism (*Waddlia chondrophila*; Rurangirwa *et al*., 1999) and a *Chlamydia*-related organism observed within tissue culture (*Simkania negevensis*; Kahane *et al*., 1999) demonstrate a previously unrecognized diversity within the *Chlamydiales*. Interestingly, the order *Chlamydiales* still exclusively comprises obligate intracellular bacteria, some of which have developed mechanisms to survive and exploit uptake by protozoa. The adaptation to intracellular growth in the ubiquitously distributed FLA could have functioned as a preadaptation of *Chlamydia*-like ancestors to survival within other host cells of higher eukaryotes, including humans; this raises the question of the clinical significance of members of the family *Parachlamydiaceae*. Few studies have addressed this important issue. Among these, Birtles *et al*. (1997) screened for the presence of specific antibodies against *Parachlamydia*-like endocytobionts of *Acanthamoeba* sp. ('Hall's coccus', displaying more than 99% 16S rDNA similarity to *P. acanthamoebae*) in blood sera from patients with pneumonia of undetermined cause, and found positively reacting sera that did not react with *Chlamydia psittaci*, *C. trachomatis* or *C. pneumoniae*. These researchers therefore suggested that 'Hall's coccus' be considered potentially pathogenic for humans. Another remarkable finding was the recovery of novel *Chlamydia*-like 16S rDNA sequence fragments from specimens of respiratory-disease patients, recently reported by Ossewaarde & Meijer (1999). The sequence similarities of these sequences and *N. hartmannellae* range between 72% and 84%. Reliable phylogenetic analysis of the *Chlamydia*-like sequences and the 16S rRNA sequence of *N. hartmannellae* could not be performed, due to the short length of the *Chlamydia*-like sequence fragments (approx. 220 bp). A stable tree topology could not be obtained by applying different treeing methods and data sets. Further research is needed to clarify whether the FLA endocytobionts of the family *Parachlamydiaceae* are indeed able to infect humans.

Concluding remarks

In conclusion, we have identified an obligate endoparasite of *H. vermiformis*, provisionally classified as *Neochlamydia hartmannellae*, as a new member of the family *Parachlamydiaceae*. These findings broaden our knowledge of the phylogenetic diversity within the *Chlamydiales*. Although it is too early to draw conclusions on the clinical significance of these bacteria, the detection of these organisms in FLA suggests that FLA may act as a general reservoir for *Chlamydia*-like

organisms. More detailed knowledge is needed on the natural habitats, diversity, physiology and virulence of members of the family *Parachlamydiaceae*.

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