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Molecular Identification of a Phage-infected *Protochlamydia* Strain Naturally Harboured by Non-Encysting *Naegleria*

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Abstract. A thermophilic strain of *Naegleria clarki*, isolated from a pond, has previously been investigated for its peculiarity to host a cytoplasmic symbiont, which causes a loss of the ability to form cysts. This endosymbiont, called Pcb, was itself infected by a phage, and exhibited chlamydia-like features resembling to another symbiont of *Naegleria* previously described as *Protochlamydia naegleriophila*. We report in this study, the results of amoeba host range and 16S rDNA molecular phylogeny of this strain, showing that Pcb is a new strain of the *Naegleria* endosymbiont chlamydial species *Protochlamydia naegleriophila* (Chlamydiae: *Parachlamydiaceae*).

Key words: *Naegleria*, *Protochlamydia*, Chlamydiae, phage.

INTRODUCTION

Naegleria spp. (Excavata, Heterolobosea, Vahlkampfiidae) are free-living amoebae widely present in soil and in natural as well as artificial water systems, feeding mostly on bacteria. Their life cycle comprises a vegetative amoeboid stage, a dispersal flagellate stage and a dormant cyst (Marciano-Cabral 1988). *Naegleria* spp. may cause opportunistic infections in vertebrates, falling in the heterogeneous group of amphizoic amoebae, which comprises various unrelated pathogenic amoeboids (Visvesvara 2010). More than 40 species are presently recognized, mainly on the basis of the internal transcribed spacer (ITS) molecular typing (De Jonckheere 2004). In humans *Naegleria fowleri* causes a fulminant primary amoebic meningoencephalitis (PAME), and in laboratory mice a milder disease is caused by *Naegleria australiensis* and *Naegleria italica* (Visvesvara 2010, De Jonckheere 2011), while various species infect gills and internal organs of fishes (Dyková *et al.* 2006) and may also be recovered in the herpetofauna (Hassl and Benyr 2003).

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Amphizoic amoebae like *Acanthamoeba* and *Naegleria* were shown to play a role as environmental niches for *Legionella pneumophila* in the Legionnaires Disease (Rowbotham 1980). While *Acanthamoeba* may harbour a wide panel of endosymbionts, *Naegleria* was rarely reported infected by intracellular organisms, e.g. possible pathogenic Gamma-Proteobacteria like *Legionella* spp. or *Stenotrophomonas maltophilia* (Corsaro *et al.* 2010b, 2013a). However, these amoeboflagellates are the natural hosts of *Protochlamydia naegleriophila* (Chlamydiae, *Parachlamydiaceae*) (Michel *et al.* 2000). Members of the family *Parachlamydiaceae* mainly infect Amoebozoa like *Acanthamoeba*, *Vermamoeba* and *Saccamoeba* (Amann *et al.* 1997; Fritsche *et al.* 2000; Horn *et al.* 2000; Corsaro and Venditti 2006; Corsaro *et al.* 2010a, 2013b). Studies on amoeba host range of chlamydiae showed however that some *Naegleria* strains were susceptible to infection allowing intracellular growth of bacteria (Michel *et al.* 2001a, 2004, 2005).

A *Naegleria* strain, N-DMLG, isolated from a garden pond containing ornamental fishes, was found to harbour intracytoplasmic chlamydia-like organisms as well as endonuclear symbionts (Michel *et al.* 1999). The amoeba was unable to encyst but it readly transformed to the flagellate stage when submitted to the transformation test (Michel *et al.* 1999). By applying a combination of distinct temperature incubations, rounds of antibiotics or filtration steps, both monoxenic (i.e., containing only one type of symbiont) and aposymbiotic (symbiont-free) *Naegleria* subcultures were obtained. The amoeba was identified as a thermophilic (growth at 37°C) strain of *Naegleria clarki* able to induce cytopathic effects (CPE) in cell cultures (Walochnik *et al.* 2005). Thermophily (growth at ≥ 37°C) and/or production of CPE in cultured mammalian cells are phenotypic traits suggesting for a pathogenic status, even if for *Naegleria* no direct correlation exists with the pathogenicity tests in animal models (Marciano-Cabral 1988). *Naegleria* regained its ability to encyst only once the chlamydia-like symbiont was eliminated (Michel *et al.* 1999, Walochnik *et al.* 2005). This latter, called Pcb, infected the cytoplasm of both the amoeba and flagellate stages, and was further infected by phages (Michel *et al.* 2001b).

In this study, we have identified the cytoplasmic symbiont Pcb by means of 16S rDNA-based molecular phylogenetic analysis as a new strain of *Protochlamydia naegleriophila*. We have tested its amoeba host range, compared with that of the type strain, and we provided a review of the literature.

MATERIAL AND METHODS

Organisms and culture. *Naegleria clarki* strain N-DMLG (GenBank acc. no. KC527832), harbouring intracytoplasmic (Pcb) and endonuclear (Pnb) symbionts, was isolated from a garden pond, in Germany, and kept in both bacterised non-nutritive agar (NNA) and axenic SCGYE medium (Michel *et al.* 1999). Monoxenic cultures of amoebae infected with only the chlamydia-like Pcb symbiont were obtained after 5-µm filtration and low temperature incubations. *Protochlamydia naegleriophila* strain KNic infected a non-thermophilic *Naegleria* sp. recovered from an ornamental aquarium and successively kept in *Naegleria lovanensis* (Michel *et al.* 2000). The host range for the chlamydia-like Pcb was further tested by using various members of other Amoebozoa and Heterolobosea amoebae (Table 1), as well as mammalian Vero cells. Amoebae were grown under different culture conditions in agar plates, liquid media (SCGYE or PYG), and when possible, also prepared as monolayers in 6-well microplates in Page's Amoeba Saline (PAS). Simian Vero cells were cultured in Cellstar® cell culture flasks (Greiner bio-one No. 690160) as monolayers in RPMI 1640 medium (GIBCO No. 52400-025, supplemented with 5% newborn calf serum) at 37°C. Amoebae and Vero cells were inspected daily at light microscope to record the intracellular growth of the chlamydia-like symbiont, and eventually further screened by staining and/or PCR. Full protocols were described previously (Michel *et al.* 2004, 2005, 2006; Corsaro *et al.* 2009, 2010a). Electron microscopy was performed according to previous studies (Walochnik *et al.* 2005, Michel *et al.* 2006).

Molecular analysis. Infected amoebae were harvested and washed in PAS (three times at 200 × *g*), and further centrifuged after freezing-thawing before DNA extraction, as described (Corsaro *et al.* 2010a). Pcb 16S rDNA was amplified and sequenced with a pan-chlamydia primer set (Corsaro and Venditti 2009, Corsaro and Work 2012). Multiple alignments were performed with Muscle and edited with BioEdit, and molecular phylogeny was performed by using Maximum Likelihood (ML, GTR, G + I) with Treefinder (Jobb *et al.* 2004), and Neighbour-Joining (NJ, p-distance) and Maximum Parsimony (MP) with MEGA5 (Tamura *et al.* 2011), with bootstrap test of 1000. Pair-wise similarity values were calculated with BioEdit, using all the sites and indels and by removing common and terminal gaps.

RESULTS AND DISCUSSION

Amoeba host range. Pcb successfully infected other *Naegleria* spp., inhibiting encystation but not transformation into the flagellate stage, as well as *Willaertia* and *Tetramitus*, which are closest relatives of *Naegleria* and *Vahlkampfia*, respectively. Almost all the amoebozoans tested, including several *Acanthamoeba* and *Vermamoeba* strains, also proved to sustain the growth of Pcb. Some other *P. naegleriophila* strains, isolated through *Acanthamoeba* coculture but for which the natural host is unknown (Corsaro and Venditti

Table 1. Free-living amoebae host range for *Protochlamydia naegleriophila.*

Amoebae	Strain	Source	IC growth[1]	
			Pcb	Knic
Excavata, Heterolobosea				
Naegleria clarki[2,3]	N-DMLG0	garden pond, Germany	+++	+++
Naegleria sp.[2]	Nic	aquarium, Germany	n.t.	+++
Naegleria gruberi	Nbeck	aquarium	+++	n.t.
Naegleria sp.[3]	Ng-FW21	puddle, France	+++	+++
Naegleria lovaniensis[3]	Aq/9/1/45D	aquarium, Belgium	+++	+++
Naegleria pagei	CCAP 1518/1e	unknown	+++	n.t.
Naegleria philippinensis	RJTM CCAP 1518/20	CSF patient, Philippines	n.t.	+++
Willaertia magna	A1PW1CL2	River Nile, Egypt	++	–
Willaertia magna	NI4C11	freshwater pond, India	n.t.	++
Willaertia magna	PAOBP40	brook, Spain	n.t.	++
Tetramitus pararusselli	Rhodos CCAP 1581/4	puddle, Greece	+++	+++
Vahlkampfia avara	Va-env1	surface water, Germany	–	n.t.
Paravahlkampfia ustiana	Vsp	freshwater pond, Germany	–	–
Amoebozoa, Lobosa				
Acanthamoeba castellanii T4[3]	C3 ATCC 50739	water reservoir	+++	+++
Acanthamoeba sp. T4[3]	ATCC 30010	soil	+++	+++
Acanthamoeba sp. T4[3]	Ac4-15	freshwater pond	+++	+++
Acanthamoeba lugdunensis T4	312-1	human nasal mucosa	n.t.	+++
Acanthamoeba lenticulata T5	45 ATCC 50703	human nasal mucosa	+++	–
Acanthamoeba lenticulata T5	118 ATCC 50706	human nasal mucosa	(+)	n.t.
Acanthamoeba lenticulata T5	89a	human nasal mucosa	n.t.	+++
Acanthamoeba sp. T6	WBT	raw water reservoir	–	–
Acanthamoeba astronyxis T7	Am23	physiotherapeutic unit	n.t.	–
Acanthamoeba comandoni T9	Pb30/40	greenhouse	+++	–
Balamuthia mandrillaris	CDC:VO39	*Papio sphinx*, brain	+++	+++
Vermamoeba vermiformis[3]	C3/8	water reservoir	+++	+++
Vermamoeba vermiformis[3]	Hv-22	puddle, France	+++	+++
Vermamoeba vermiformis	Os101	hospital tap water	n.t.	+++
Saccamoeba lacustris	CCAP 1572/4	freshwater pond, Germany	(+)	(+)
Ripella platypodia	Vp-env1	surface water, Germany	–	n.t.
Vannella placida	Pp-aq1	aquarium, Germany	++	n.t.
Flamella aegyptia	A1	River Nile, Egypt	n.t.	–
Amoebozoa, Mycetozoa				
Dictyostelium discoideum	Berg25	human nasal mucosa	+++	+++
Dictyostelium discoideum	Sör2wild	fountain	–	n.t.
Hyperamoeba-like	AH1P2PE	maple bark	–	n.t.
Hyperamoeba-like	G1	physiotherapy bath	–	n.t.
Hyperamoeba-like	Wi2i	physiotherapy bath	+++	n.t.

[1] IC growth: intracellular growth, as determined by microscopical inspection. – – failure of infection; ++, +++ – successfull infections; (+) – successfull infection, but loss of endobacteria during subcultures; n.t. – no tested.
[2] N-DMLG0 and Nic are natural hosts of Pcb and Knic, respectively.
[3] Cocultures performed both at room temperature and 37°C.

2009), also have successfully infected *Naegleria* spp., behaving like KNic (not show).

The amoeba host range for the *Protochlamydia naegleriophila* type strain KNic was largely studied previously (Michel *et al.* 2000), and a few new amoebal strains were tested in this study. We recorded the same ability for KNic to infect several *Naegleria* strains leaving them the ability to transform into flagellates but by losing the ability to form cysts (Michel *et al.* 2000; this study). When comparing Pcb and KNic host ranges, a remarkable overlapping at level of amoeba strain may be observed (Table 1).

Strain Pcb appeared to multiply slower compared to KNic, about 4–5 vs. 2–3 days, respectively (this study). This delay is presumably caused by phage infection in Pcb, while no phage was detected in KNic (Michel *et al.* 2000, 2001b).

Some amoebae strains subjected to both room temperature and 37°C incubations, showed no difference in the behaviour of both chlamydial strains (this study). Previous reports using a model with a unique *Acanthamoeba* strain, have associated temperatures ≥ 37°C with an increased virulence for amoeba endoparasites (Birtles *et al.* 2000, Greub *et al.* 2003a). However, as already highlighted (Corsaro and Venditti 2004), this phenomenon may be a typical feature of the non-termophilic amoeba strain used, rather than a virulence trait of the endoparasites.

Mammalian cell culture. Pcb was able to enter a few Vero cells, forming multiple vacuoles in intact monolayers (Fig. 1). Other *Parachlamydiaceae* strains tested by us in Vero cells formed by contrast a unique large vacuole. Vero cells infected by Pcb burst about 4 to 5 days post-infection, in an apoptotic manner. Our results are largely in accordance with the available literature, reporting very limited growth of *Parachlamydiaceae* in non-amoebae cells and induction of apoptosis (e.g., Greub *et al.* 2003b, Collingro *et al.* 2005, Ito *et al.* 2012, Sixt *et al.* 2012).

Molecular phylogeny. The almost complete 16S rDNA of Pcb (GenBank acc. no. JX846629) was obtained from infected naegleriae. Pcb showed highest pair-wise similarity values (99.7%) with two strains of *Protochlamydia naegleriophila*, CRIB36 and cvE26, recovered by *Acanthamoeba* coculture from a Spanish water treatment plant (Corsaro *et al.* 2009) and an Italian freshwater (Corsaro and Venditti 2009), respectively, and a value of 99.3% with the type strain KNic. Similarity values were 96.3–97.2% with *Protochlamydia amoebophila*, and 96.9–97.3% with the putative *Protochlamydia* sp. represented by strains CRIB40 and CRIB44 (Corsaro *et al.* 2010b), and < 95% with other species and clades of *Parachlamydiaceae* as defined in previous studies (Corsaro and Venditti 2006, 2009; Corsaro *et al.* 2013b). For full or nearly full 16S rDNA sequences, threshold values of 95% and 97% were generally considered to delimite genus and species taxa, respectively. In phylogenetic trees (Fig. 2), Pcb emerged unambigously within the species *P. naegleriophila* in the holophyletic *Protochlamydia* clade. Molecular phylogeny and genetic similarity > 99% both strongly indicated that Pcb is a new strain of the species *P. naegleriophila* naturally harboured by *Naegleria clarki*.

Electron microscopy. Pcb cells are present in the cytoplasm of *Naegleria*, showing strong spiny appearances (Fig. 3). Elementary bodies (EB) are about 0.8 µm in diameter and appear very prickly, while reticulate bodies (RB) are sligthly larger and less wrinkled. Crescent bodies were not observed (Michel *et al.* 1999; this study). In KNic, crescent bodies had been observed extracellularly in *Naegleria* culture (Michel *et al.* 2000), and intracellularly, but with different shapes,

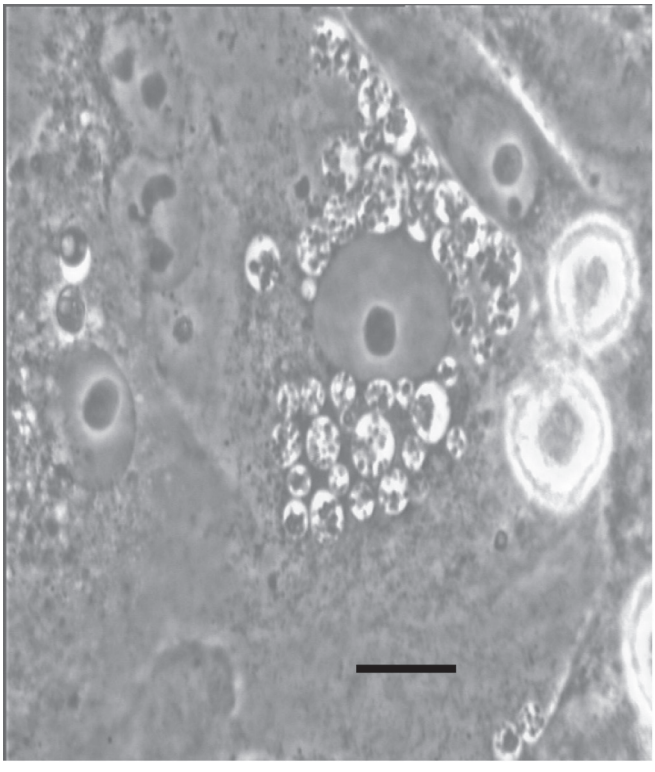

Fig. 1. Light microscopy of Pcb-infected Vero cells monolayer at 3 days p.i., showing multiple vacuoles. Scale bar: 20 µm.

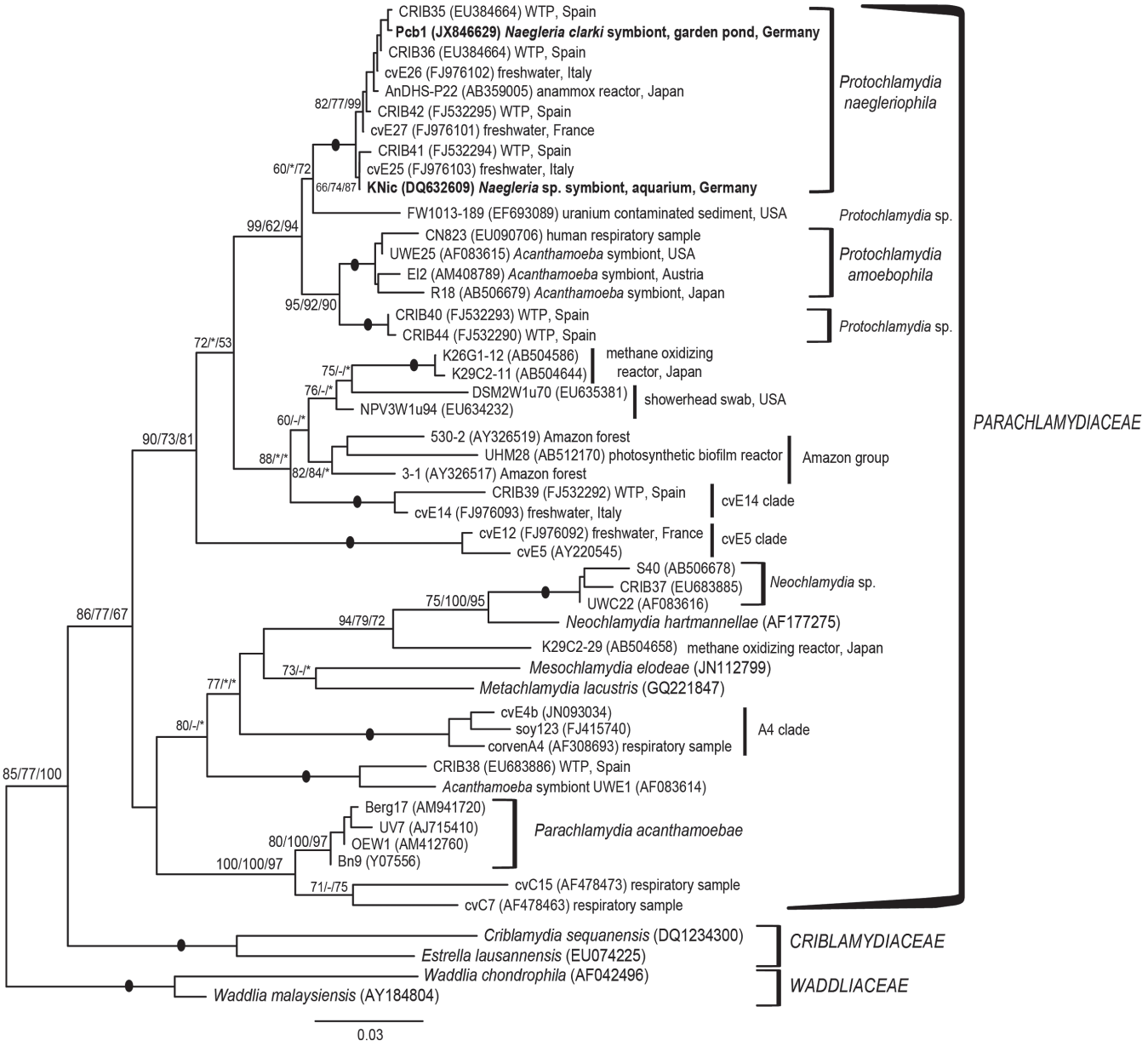

Fig. 2. Maximum likelihood 16S rDNA tree of *Parachlamydiaceae*, showing the major lineages and sublineages, and all the phylotypes assigned to *Protochlamydia naegleriophila*. The Pcb1 strain recovered here and the reference strain KNic (in bold) are both natural endosymbionts of *Naegleria* spp. The tree was rooted on members of *Criblamydiaceae* and *Waddliaceae*. Bootstrap values (BV) after 1,000 replicates for ML/NJ/MP were indicated at nodes. Filled circle – node 100% supported with all three methods; asterisk – node supported but BV < 40%; hyphen – node not supported. Scale bar represents substitution/site.

in *Acanthamoeba* culture (Casson *et al.* 2008), resembling the *Chlamydia trachomatis* crescent-shaped RB induced by penicillin (Skilton *et al.* 2009). All these observations thus suggest that crescent bodies may likely be unusual aberrant shapes, rather than a third developmental stage of *Parachlamydiaceae*.

Some enlarged RB present 70-nm hexagonal phages (Figs 4, 5), that were named Neo-Ph2 (Michel *et al.* 2001b) because they resemble to the Neo-Ph1 phages found within *Neochlamydia hartmannellae* (Schmid *et al.* 2001). Similar 50–70-nm phages have also been found in another parachlamydia, *Mesochlamydia elo-*

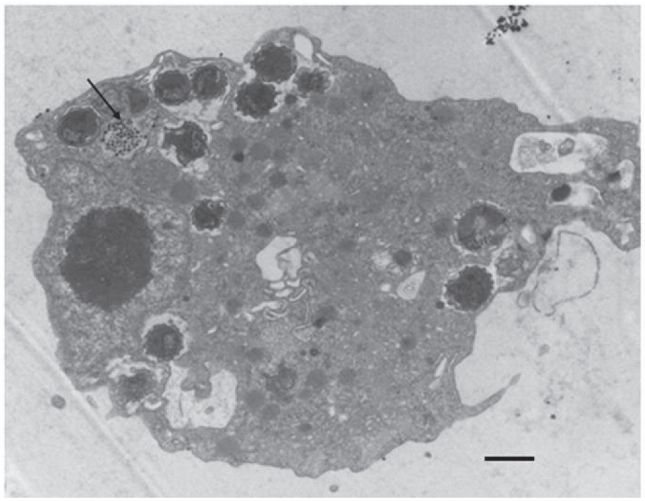

Fig. 3. Electron microscopy of *Naegleria lovanensis* infected by Pcb. Several wrinkled EB and RB are visible, as well as an RB containing phage particles (arrow). Scale bar: 1 µm.

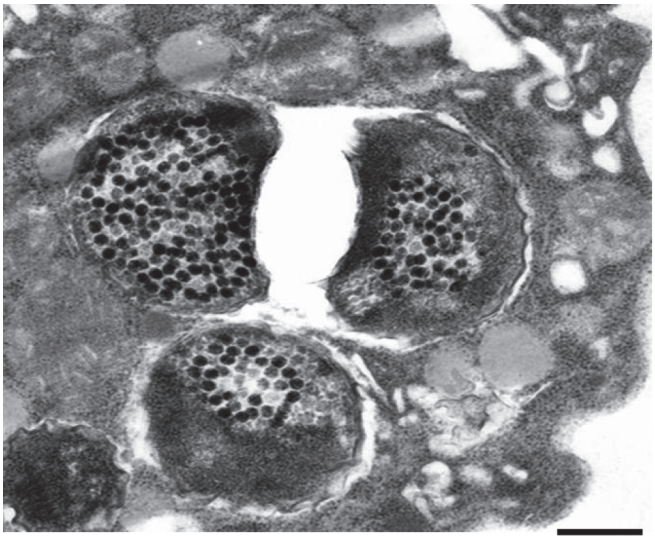

Fig. 5. Detail of electron microscopy of *Naegleria clarki* infected by Pcb, showing three enlarged RBs containing filled and empty phages. A normal-size wrinkled EB is also visible. Scale bar: 0.5 µm.

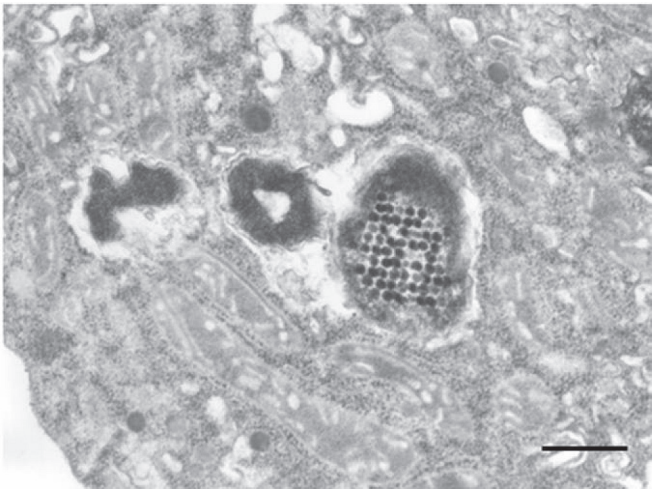

Fig. 4. Detail of electron microscopy of *Naegleria clarki* infected by Pcb, showing enlarged RB with phages. Scale bar: 0.5 µm.

deae (Michel *et al.* 2010, Corsaro *et al.* 2013b), and in an uncharacterized 'chlamydia-like' organisms infecting marine invertebrates (Harshbarger *et al.* 1977, Comps and Tigé 1999), appearing distinct in size from the smaller 25-nm phages infecting only members of *Chlamydophila* (family *Chlamydiaceae*) (Everson *et al.* 2003). These latter phages are all included in the viral genus *Chlamydiamicrovirus* (*Microviridae*: Gokushovirinae), however recent metagenomic studies revealed huge diversity and distribution within the entire family *Microvidae* (Roux *et al.* 2012).

Ecology and medical relevance. *Naegleria* spp. are the main natural hosts for *Protochlamydia naegleriophila*. Strains KNic and Pcb were found in a non-thermophilic *Naegleria* sp. from an ornamental aquarium (Michel *et al.* 2000), and in a thermophilic *Naegleria clarki* from a garden pond (Michel *et al.* 1999), respectively, and both strains were successfully grown in various other *Naegleria* spp. (Table 1). While heavily infected naegleriae were finally lysed, a symbiotic relationship seemed to be established in moderately infected naegleriae. The amoeboid stage continously secreted chlamydiae into the environment, and these latter could also be transported over long distances by residing within the flagellate stage. All *Naegleria* strains tested have lost their ability to encyst after being infected by both strains, as well as by strains isolated through *Acanthamoeba* coculture (this study). However, *Protochlamydia naegleriophila* could easily infect other amoebae, including more resistent cyst-forming ones, like *Acanthamoeba*, thus ensuring their survival under stress conditions, like the closely related *Protochlamydia amoebophila* (Nakamura *et al.* 2010).

Casson *et al.* (2008) have analysed 134 bronchoalveolar lavages from Swiss patients with and without pneumonia, by applying KNic-specific real-time PCR.

They have found a unique sample from an immunocompromised patient positive out of 65 samples from patients with pneumonia, and then they concluded that *Protochlamydia naegleriophila* is an etiologic agent of pneumonia. However, subsequent studies failed to detect this species within respiratory samples, either through real-time PCR (e.g., Lamoth *et al.* 2011, Niemi *et al.* 2011) or pan-chlamydia PCR (Haider *et al.* 2008). While studies using real-time PCR raised doubts about the correct identification of the taxa, Haider *et al.* (2008) have clearly shown, by almost complete 16S rDNA sequencing, that a complex of chlamydial species might be detected in those samples.

By contrast, *Protochlamydia naegleriophila* was easily recovered from both natural and artificial water environments in several European countries (see Fig. 2), both as natural endosymbionts of *Naegleria* spp. (Michel *et al.* 1999, 2000; this study) and/or through *Acanthamoeba* or mixed cocultures (Corsaro and Venditti 2009; Corsaro *et al.* 2009, 2010b). Molecular analyses of microbial communities allowed to identify *Protochlamydia naegleriophila* (clone AnDHS-P22) also in an anammox down-flow hanging sponge (DHS) reactor in Japan, while the clone FW1013-189, recovered from uranium contaminated sediment in the USA (Cardenas *et al.* 2008), is the closest related phylotype and could represent a possible new species (96.5% sequence similarity with KNic) (Fig. 2). All these data indicate a common and widespread occurrence of *Protochlamydia naegleriophila* in the environment, and its very rare recovery in clinical samples may be due to very occasional infections, as reported also for other rare novel chlamydial and parachlamydial strains (Corsaro *et al.* 2001, 2002; Haider *et al.* 2008). It may also be possible that *Parachlamydiaceae*, and closely related lineages, are effectively limited to amoebae or protists in general, and the successful expansion in animals for other chlamydiae has been possible by developing anti-apoptotic mechanisms (Sixt *et al.* 2012, Matsuo *et al.* 2013). The real importance of these bacteria in the medical and veterinary fields is thus far to be established and needs further investigations.

In conclusion, we have identified by 16S rDNA molecular phylogeny, the phage-infected chlamydia-like cytoplasmic symbiont Pcb of *Naegleria clarki* as a new strain of *Protochlamydia naegleriophila*, and we have demonstrated by coculture its ability to grow in a large spectrum of free-living amoebae. Further studies will be aimed to identify the endonuclear symbiont of the same *Naegleria clarki* host strain, as well as to characterise the various phages infecting some strains of *Parachlamydiaceae* recovered during our other studies.

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