

Recovery of an environmental chlamydia strain from activated sludge by co-cultivation with *Acanthamoeba* sp.

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Chlamydiae are a unique group of obligate intracellular bacteria comprising important pathogens of vertebrates as well as symbionts of free-living amoebae. Although there is ample molecular evidence for a huge diversity and wide distribution of chlamydiae in nature, environmental chlamydiae are currently represented by only few isolates. This paper reports the recovery of a novel environmental chlamydia strain from activated sludge by co-cultivation with *Acanthamoeba* sp. The recovered environmental chlamydia strain UV-7 showed the characteristic morphology of chlamydial developmental stages as revealed by electron microscopy and was identified as a new member of the family *Parachlamydiaceae* (98.7% 16S rRNA sequence similarity to *Parachlamydia acanthamoebae*). Infection studies suggested that *Parachlamydia* sp. UV-7 is not confined to amoeba hosts but is also able to invade mammalian cells. These findings outline a new straightforward approach to retrieving environmental chlamydiae from nature without prior, tedious isolation and cultivation of their natural host cells, and lend further support to suggested implications of environmental chlamydiae for public health.

INTRODUCTION

Chlamydiae are important animal and human pathogens causing severe diseases such as trachoma, genital tract infections and pneumonia. They are obligate intracellular, coccoid bacteria characterized by a unique biphasic developmental cycle, which consists of an infectious form, the elementary body (EB), and a vegetative non-infectious form, the reticulate body (RB). Chlamydiae have long been considered a group of evolutionarily deep-branching bacteria comprising only few medically important species (Mahoney *et al.*, 2003). Since the late 1990s, the identification of a number of obligate intracellular chlamydia-related bacteria (i) as endosymbionts of amoebae or arthropods (Amann *et al.*, 1997; Birtles *et al.*, 1997; Fritsche *et al.*, 2000; Horn *et al.*, 2000; Kostanjsek *et al.*, 2004; Michel *et al.*, 1994; Thao *et al.*, 2003), (ii) as contaminants of a laboratory cell culture (Kahane & Friedman, 1995) and (iii) in aborted

bovine fetuses (Henning *et al.*, 2002; Rurangirwa *et al.*, 1999) has led to a new awareness of the diversity and distribution of chlamydiae in nature. This is also reflected in the recent reorganization of chlamydial taxonomy that introduced three new families, *Parachlamydiaceae*, *Simkaniaceae* and *Waddliaceae*, separating these novel chlamydiae from the previously known *Chlamydiaceae* (Everett *et al.*, 1999; Rurangirwa *et al.*, 1999). The term 'environmental chlamydiae' has been suggested to encompass the former three families (Horn & Wagner, 2001).

Several studies suggest an association of some of the environmental chlamydiae with respiratory disease of humans. Based on molecular and serological evidence, *Simkania negevensis* might be implicated in bronchiolitis in infants and community-acquired pneumonia in adults (Friedman *et al.*, 2003). In addition, *Parachlamydia* spp. have been associated with several pneumonia cases, among others in polytraumatized intensive-care patients (Birtles *et al.*, 1997; Corsaro *et al.*, 2001, 2002a; Greub *et al.*, 2003a; Marrie *et al.*, 2001). *Simkania negevensis* and *Parachlamydia* spp. are thus considered potential emerging pathogens (Greub & Raoult, 2002b).

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; EB, elementary body; FISH, fluorescence *in situ* hybridization; p.i., post-infection; RB, reticulate body.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Parachlamydia* sp. UV-7 is AJ715410.

From a public health point of view it therefore deserves attention that a large number of rRNA sequences detected in various clinical and environmental samples (including bronchoalveolar lavage, nose, throat and ocular swabs from humans and animals, fresh water, soil, and activated-sludge samples) represent as yet unknown chlamydiae, indicating that chlamydial diversity is still dramatically underestimated (Bodetti *et al.*, 2003; Corsaro *et al.*, 2001, 2002a, b; Horn & Wagner, 2001; Ossewaarde & Meijer, 1999; recently reviewed by Corsaro *et al.*, 2003; see M. E. Ward's www.chlamydiae.com for up-to-date information). These molecular data also suggest that chlamydiae have an extremely broad host range (comprising protozoa, arthropods, and marsupial and placental mammals) and a ubiquitous, worldwide distribution in nature. However, the currently available environmental chlamydia isolates ($n=12$) do not adequately represent the actual diversity of this group and therefore additional isolates are urgently required to more fully understand the biology and medical significance of those organisms. Isolation of free-living amoebae, naturally infected with environmental chlamydiae, is tedious and extremely time consuming. As members of all four recognized chlamydial families have been shown to be able to thrive within free-living amoebae (Essig *et al.*, 1997; Kahane *et al.*, 2001; Michel *et al.*, 2004), in this study we used co-incubation of an environmental sample (activated sludge from a wastewater treatment plant) with uninfected *Acanthamoeba* sp. to directly retrieve chlamydiae from complex microbial communities. We successfully applied this co-cultivation approach for the recovery of a new *Parachlamydia* sp. In addition, we show that the environmental chlamydia strain (UV-7) obtained in this study is able to infect mammalian cells.

METHODS

Amoebae and mammalian cell lines. Co-cultivation was performed using *Acanthamoeba* sp. UWC1 isolated from corneal tissue (Fritsche *et al.*, 1993, 1998) and maintained axenically in trypticase-soy-yeast extract (TSY) broth at room temperature (Visvesvara, 1999). *Acanthamoeba* sp. UWC1 does not naturally contain endosymbionts but has previously been shown to be able to act as host for phylogenetically diverse bacterial endosymbionts (Fritsche *et al.*, 1998; Gautom & Fritsche, 1995). Mammalian cell lines used in this study included simian Vero (CCL-81), as well as human HeLa 229 (CCL-2.1) and NCI-H292 (CRL-1848) cells obtained from the American Type Culture Collection (Manassas, VA, USA). Cell cultures were maintained under standard laboratory conditions in the recommended growth media.

Co-cultivation of amoebae with activated-sludge samples. Activated-sludge samples were taken from a wastewater treatment plant in Plattling (Germany) processing sewage from a rendering plant, and homogenized for 30 s using an ultraturrax homogenizer (IKA Werke). Amoeba cultures were harvested by centrifugation and resuspended in Page's amoebic saline (Page, 1988). Two millilitre volumes of the amoeba suspension were seeded in six-well tissue culture plates. Tissue culture inserts (pore size 3.0 μm) were placed within the six-well plates. One millilitre of the homogenized activated sludge was applied into the tissue culture inserts submerged in

the Page's amoebic saline and permeable for bacteria but excluding protozoa or amoebal cysts (thus preventing contamination of the amoebal strain UWC1 with autochthonous amoebae). After co-cultivation for 1 day at room temperature, tissue culture inserts containing the activated sludge were removed, amoebae were detached from the surface of the tissue culture plates, and 500 μl amoeba suspension was transferred into a new well containing 3 ml TSY broth and antibiotics (penicillin, 100 $\mu\text{g ml}^{-1}$; streptomycin, 10 $\mu\text{g ml}^{-1}$; amphotericin B, 0.25 $\mu\text{g ml}^{-1}$) to suppress growth of extracellular bacteria. Medium and antibiotics were exchanged daily for the following 2 days. Addition of antibiotics was omitted as soon as no extracellular bacteria were detectable in the medium by light microscopy, and amoebae were further propagated to allow for the spread of a potential infection with intracellular bacteria. Amoebae were further incubated for 4 weeks until intracellular bacteria could be readily detected by staining with 4',6'-diamidino-2-phenylindole (DAPI; 1 $\mu\text{g ml}^{-1}$). *Acanthamoeba* harbouring intracellular bacteria were subcultured and maintained at room temperature by replacing TSY broth weekly. For long-term preservation, amoeba cultures were frozen in TSY broth supplemented with 10% (v/v) dimethylsulfoxide and stored in liquid nitrogen as described elsewhere (Neal *et al.*, 1974).

Electron microscopy. Amoebae and mammalian cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate/cacodylate buffer for 1–2 h. After fixation in 2% osmium tetroxide for 1 h and dehydration in an ascending series of acetate or ethanol, respectively, the samples were embedded in Epon 812 resin (Fluka). Sections (70 nm) were stained with 1% lead citrate and uranyl acetate before examination in a transmission electron microscope (Zeiss EM 10).

DNA isolation, amplification of 16S rRNA genes, cloning and sequencing. Simultaneous isolation of DNA from *Acanthamoeba* and intracellular bacteria was performed using the FastDNA kit (Bio 101) and a bead beater (Bio 101-FP120) following the instructions of the manufacturer. Oligonucleotide primers targeting 16S rRNA gene signature regions which are conserved within the *Chlamydiales* were used for PCR in a thermal cycler (iCycler; Bio-Rad) to obtain near-full-length bacterial 16S rRNA gene fragments. The nucleotide sequences for the forward and reverse primers used for amplification were 5'-CGG ATC CTG AGA ATT TGA TC-3' and 5'-TGT CGA CAA AGG AGG TGA TCC A-3' (Pudjijatmoko *et al.*, 1997). Amplified PCR products were directly ligated into the cloning vector pCRII-TOPO and transformed into competent *E. coli* (TOP10 cells) following the instructions of the manufacturer (Invitrogen). Nucleotide sequences of the cloned DNA fragments were determined using the ThermoSequenase cycle sequencing kit (Amersham Life Science) and an automated DNA sequencer (Li-Cor) under conditions recommended by the manufacturers. Vector-specific primers M13/pUC-V (5'-GTA AAA CGA CGG C-3') and M13/pUC-R (5'-GAA ACA GCT ATG ACC ATG-3') were used for sequencing.

Phylogenetic analysis. The ARB program package (Ludwig *et al.*, 2004) was used for phylogenetic analysis of the retrieved 16S rRNA sequence. The 16S rRNA sequence was added to an alignment of about 16 000 small-subunit rRNA sequences using the alignment tool implemented in ARB and subsequently refined manually. A consensus tree was drawn based on trees calculated using neighbour-joining (Jukes–Cantor correction), PHYLIP distance matrix (Fitch), PHYLIP maximum-parsimony methods (Felsenstein, 1989), and the fastDNaml and TREE-PUZZLE (Strimmer & von Haeseler, 1996) maximum-likelihood approaches. A filter considering only positions that are conserved in at least 50% of all *Chlamydiales* 16S rRNA sequences was used for calculation of all trees.

Fluorescence *in situ* hybridization (FISH) and confocal laser scanning microscopy. Amoebae were harvested from liquid culture by centrifugation and washed with Page's saline. After resuspension in Page's saline, 20 µl aliquots of amoebic suspension were incubated on glass slides for 20 min, and fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature. Mammalian cell cultures were grown on glass coverslips in 24-well plates, fixed with 4% PFA for 20 min at room temperature and dehydrated in ethanol. Hybridizations and staining with DAPI were performed using the protocol, hybridization buffer (containing 25% formamide) and washing buffer described elsewhere (Daims *et al.*, 2005). Slides and coverslips were examined using a confocal laser scanning microscope (LSM 510, Carl Zeiss) equipped with two helium-neon lasers (633 nm, 543 nm), an argon-krypton laser (488 nm) and a UV laser (351–364 nm). Image analysis processing was performed with the standard software package delivered with the instrument (version 3.2).

A new oligonucleotide probe, UV7-763 (5'-TGC TCC CCC TTG CTT TCG-3'; S-St-UV7-763-a-A-18 according to the nomenclature proposed by Alm *et al.*, 1996), exclusively targeting the newly recovered *Parachlamydia* sp. strain UV-7 was designed using the probe functionality of the ARB software (Ludwig *et al.*, 2004) and deposited at probeBase (Loy *et al.*, 2003). In addition, the following oligonucleotide probes were used: (i) Bn₉-658 (5'-TCC GTT TTC TCC GCC TAC-3') (Amann *et al.*, 1997) targeting the 16S rRNA of a subgroup of the *Parachlamydiaceae*, including *Parachlamydia* sp. UV-7; (ii) a mixture of EUB338-I (5'-GCT GCC TCC CGT AGG AGT-3'), EUB338-II (5'-GCA GCC ACC CGT AGG TGT-3') and EUB-III (5'-GCT GCC ACC CGT AGG TGT-3') targeting almost all *Bacteria* (Amann *et al.*, 1990; Daims *et al.*, 1999); and (iii) Euk516 (5'-ACC AGA CTT GCC CTC C-3') (Amann *et al.*, 1990) targeting the 18S rRNA of eukaryotes. Oligonucleotide probes were synthesized and directly 5' labelled with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS), or the hydrophilic sulfoindocyanine fluorescent dyes Cy3 or Cy5 (Thermo Electron).

Infection of mammalian cells. EBs of *Parachlamydia* sp. UV-7 were purified from amoebic host cells by step-gradient centrifugation in 30% Gastrografin (Schering) and 50% sucrose, and quantified by counting DAPI-stained EBs filtered on a nitrocellulose membrane (pore size 0.25 µm; Millipore). Purified EBs were added to confluent cell monolayers grown in 24-well tissue plates at a ratio of one EB per mammalian cell, according to a standard infection protocol (Dowell *et al.*, 2001), but without the addition of antibiotics. Infected cells were incubated at 35 °C with 5% CO₂ and monitored for 5 days.

RESULTS AND DISCUSSION

Recovery of *Parachlamydia* sp. UV-7 from activated-sludge samples by co-cultivation with amoebae

Free-living amoebae are known as natural hosts of environmental chlamydiae (Amann *et al.*, 1997; Fritsche *et al.*, 2000; Horn *et al.*, 2000) and of a variety of phylogenetically diverse obligate intracellular bacterial symbionts (Birtles *et al.*, 2000; Fritsche *et al.*, 1999; Horn *et al.*, 2001, 2002). In addition, they serve as vehicles for dissemination of different bacterial pathogens such as *Legionella pneumophila*, *Francisella tularensis* and *Escherichia coli* O157 (Abd *et al.*, 2003; Barker *et al.*, 1999;

Goebel & Gross, 2001; Harb *et al.*, 2000). Therefore, co-cultivation with free-living amoebae has been used previously to isolate legionellae or other facultative intracellular fastidious bacteria, and to resuscitate *L. pneumophila* from the viable but nonculturable state (Greub *et al.*, 2004; Rowbotham, 1983; Steinert *et al.*, 1997). However, to our knowledge this technique has to date not been applied to the recovery of obligate intracellular bacteria from complex environmental samples. In this study we used the *Acanthamoeba* sp. strain UWC1, which does not naturally contain symbionts, for co-incubation with an activated-sludge sample from an industrial wastewater treatment plant. Activated sludge contains a high diversity of protozoa, including amoebae, which are potential hosts for environmental chlamydiae. Consistently, activated sludge has previously been demonstrated to contain a phylogenetically diverse assemblage of environmental chlamydiae (Horn & Wagner, 2001). After co-cultivation of *Acanthamoeba* sp. UWC1 with the activated-sludge sample, DAPI staining revealed that (after 4 weeks) all amoeba trophozoites were filled with coccoid intracellular bacteria located in few vacuoles, which resembled chlamydial inclusions.

In order to identify the intracellular bacteria observed in *Acanthamoeba* sp. UWC1, a nearly full-length fragment (1516 bp) of the 16S rRNA gene was amplified. The amplicate was subsequently cloned and six clones were sequenced. Comparative sequence analysis revealed that the six 16S rRNA gene sequences determined were identical and showed highest similarity to 16S rRNA gene sequences of members of the genus *Parachlamydia* (98.2–98.7%), and particularly to *Parachlamydia acanthamoebae* strain Bn₉ (98.7%). According to Stackebrandt & Goebel (1994) the 16S rRNA sequence similarity threshold for the unambiguous differentiation of two species is 97.5%. Based on 16S rRNA data it is thus not possible to decide whether the intracellular bacteria recovered in this study belong to the species *P. acanthamoebae* or represent a novel *Parachlamydia* species. The recovered environmental chlamydia was therefore designated *Parachlamydia* sp. strain UV-7 (University of Vienna, isolate number 7). Phylogenetic 16S rRNA gene analysis revealed that *Parachlamydia* sp. UV-7 formed a monophyletic group with all other members of the genus *Parachlamydia* in all treeing methods applied (Fig. 1).

Consistent with these findings, FISH with oligonucleotide probe Bn₉-658 (Amann *et al.*, 1997) specific for a subgroup of the family *Parachlamydiaceae* (including *P. acanthamoebae* and UV-7) was successfully used to demonstrate the recovered bacteria within their *Acanthamoeba* host cells (Fig. 2a). Furthermore, the simultaneous application of the newly developed oligonucleotide probe UV7-763 (specific for the recovered *Parachlamydia* sp. strain UV-7) together with probe Bn₉-658 or the bacterial probe mix EUB338I-III (Amann *et al.*, 1990; Daims *et al.*, 1999) revealed that

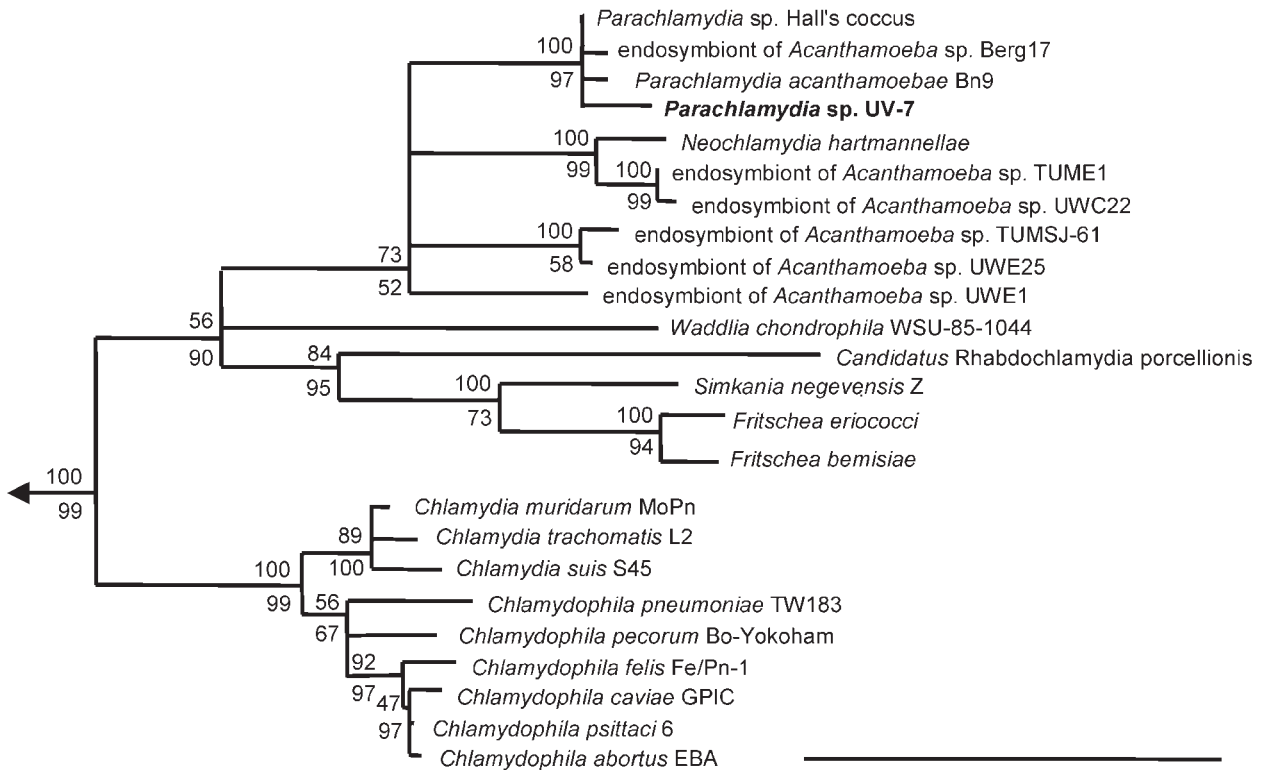


Fig. 1. 16S rRNA-based phylogenetic consensus tree (based on a TREE-PUZZLE tree) reflecting the affiliation of the recovered *Parachlamydia* sp. UV-7. Polytomic branching points indicate those parts of the tree for which different treeing methods produced different topologies. Maximum-parsimony bootstrap values (upper numbers) and TREE-PUZZLE support values (lower numbers) are indicated at each node of the tree. The bar represents 10% evolutionary distance.

Parachlamydia sp. UV-7 was the only chlamydial strain present within the amoeba trophozoites (Fig. 2a), and demonstrated the absence of other phylogenetically different bacteria (data not shown). The recovery of only one environmental chlamydia strain in this study contrasts with previous reports, which suggested that the diversity of intracellular bacteria in activated sludge is much higher (Beier *et al.*, 2002; Horn & Wagner, 2001; Kahane *et al.*, 2004). The most likely explanation for this contradiction is that under the conditions of this approach the recovered environmental chlamydia strain UV-7 was most successful and has outcompeted other bacteria able to thrive in amoebae.

The developmental cycle of *Parachlamydia* sp. UV-7

Electron microscopic analysis of *Acanthamoeba* sp. UWC1 trophozoites harbouring *Parachlamydia* sp. UV-7 demonstrated that the bacteria reside in large vacuoles resembling the host-derived inclusion vacuoles characteristic for chlamydiae (Fig. 2b, c). *Parachlamydia* sp. UV-7 was observed in two morphological forms, representing the two stages of the unique chlamydial developmental cycle (Fig. 2b, c). The electron-dense EB of *Parachlamydia* sp. UV-7 was about

0.3–0.5 μm in diameter and corresponded to the infectious stage in the chlamydial developmental cycle. The RB was slightly larger, with a diameter of 0.5–0.7 μm , and was presumably a replicative, metabolically active form. Electron microscopy also suggested that *Parachlamydia* sp. UV-7 replicated by binary fission (Fig. 2b, c). Thus, the developmental cycle of *Parachlamydia* sp. UV-7 was highly similar to the developmental cycle of *P. acanthamoebae*, which has previously been studied in some detail (Greub & Raoult, 2002a). A crescent-shaped developmental stage, described by Greub & Raoult (2002a) was, however, rarely observed, even at 7 days post-infection (p.i.), questioning the existence of this developmental stage as a general feature of parachlamydiae. The occurrence of crescent-shaped chlamydiae might alternatively be explained by the documented effect of standard fixation techniques used for electron microscopy causing the formation of crescent-shaped cells from coccoid bacteria (Lindsay *et al.*, 1995).

Infection of mammalian cells with *Parachlamydia* sp. UV-7

Environmental chlamydiae are being discussed as potential emerging pathogens, since *Parachlamydia*-like rRNA gene sequences and elevated antibody titres against

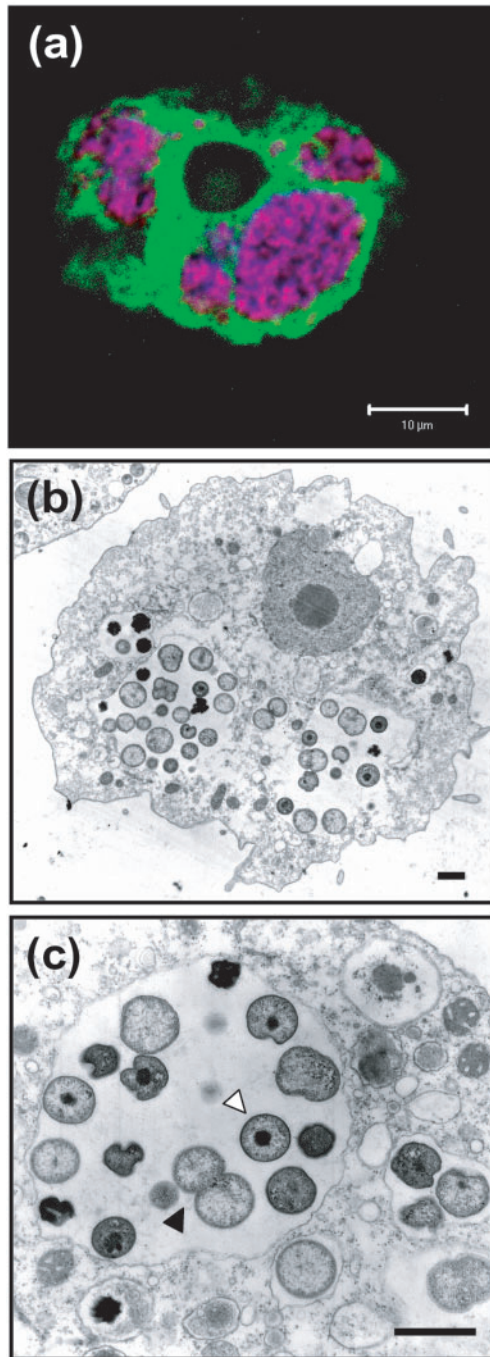


Fig. 2. Intracellular location of *Parachlamydia* sp. UV-7 in *Acanthamoeba* sp. UWC1 as observed after co-cultivation. (a) Visualization of *Parachlamydia* sp. UV-7 within its amoeba host cell by FISH using the UV-7-specific probe UV7-763 labelled with Cy3 (red), probe Bn₉-658 specific for a subgroup of the *Parachlamydiaceae* including UV-7, labelled with Cy5 (blue), and probe Euk516 targeting all eukaryotes, labelled with FLUOS (green). Bar, 10 µm. (b, c) Transmission electron micrographs showing *Parachlamydia* sp. UV-7 in inclusion vacuoles within its amoeba host. The two stages of the chlamydial developmental cycle, EBs (white arrowhead) and RBs (black arrowhead) can be seen. Bars, 1 µm.

parachlamydiae have been detected in patients with respiratory disease of undetermined cause (Greub & Raoult, 2002b). The recent finding that *P. acanthamoebae* is able to infect and multiply in primary human macrophages lends additional weight to this hypothesis (Greub *et al.*, 2003b). In order to investigate whether *Parachlamydia* sp. UV-7 (recovered by co-cultivation in this study) also is able to infect and propagate within eukaryotic host cells other than amoebae, including potential human target cells, we performed qualitative infection assays using one simian and two human cell lines. The course of UV-7 infection was similar in all investigated cell lines (Figs 3 and 4). Single Vero cells 1 day p.i. contained small inclusion vacuoles (Fig. 3a). Electron microscopy showed mostly aberrant chlamydial RBs at days 1 and 2 p.i. (Fig. 3f, Fig. 4). At 2 and 3 days p.i. the fraction of Vero cells containing UV-7 inclusions had increased and a cytopathic effect was evident (Fig. 3b, c, f, g). At 4–5 days p.i. most Vero cells were destroyed and few RBs could be detected by FISH. However, at that time point, a multitude of EBs could be recognized outside of the Vero cells or surrounding cytoplasmic remnants of destroyed cells by DAPI-staining and electron microscopy (Fig. 3d, h). The cytopathic effect of *Parachlamydia* sp. UV-7 on Vero, NCI and HeLa cells is consistent with a previous report showing that *P. acanthamoebae* exhibited similar effects on human macrophages, which have been attributed to the induction of apoptosis (Greub *et al.*, 2003b). Pro- but also anti-apoptotic activities have also been observed for members of the *Chlamydiaceae* after infection of human cells and therefore these effects are still controversial (Greene *et al.*, 2004; Schoier *et al.*, 2001).

The observed invasion of human cells by *Parachlamydia* sp. UV-7, its metabolic activity within these cells as indicated by FISH, the completion of a developmental cycle and the cytopathic effect might indicate a pathogenic potential of the newly recovered *Parachlamydia* strain. In this context it should be noted that recent whole-genome sequence analysis of the *Parachlamydia*-related strain UWE25 (Horn *et al.*, 2004) indeed revealed the presence of several genes that have been associated with virulence of *Chlamydiaceae*, like the type III secretion system, or the chlamydial protease-like activity factor (CPAF) (Zhong *et al.*, 2001). Compared to *Chlamydiaceae*, *Parachlamydia* sp. UV-7 forms only small inclusions in mammalian cell lines and the progression of infection is clearly retarded in Vero, NCI and HeLa cells. This has also been observed for *P. acanthamoebae* during infection of macrophages (Greub *et al.*, 2003b) and for *Simkania negevensis* during infection of Vero cells (Kahane *et al.*, 2002). The decreased efficiency of intracellular multiplication of *Parachlamydiaceae* in mammalian cells might indicate that these bacteria possess a lower pathogenic potential than the *Chlamydiaceae*. Genomic analysis of the *Parachlamydia*-related strain UWE25 indicated that the outer membrane of UWE25 lacks a number of proteins that are characteristic for the *Chlamydiaceae*, among them the polymorphic

outer-membrane proteins, which might be involved in adhesion to mammalian cells (Rockey *et al.*, 2000). These differences in composition could contribute to low infectivity of mammalian cells.

Conclusion

This study has shown that co-cultivation with free-living amoebae is a suitable and straightforward approach for the

direct recovery of environmental chlamydiae from samples containing complex microbial communities. The environmental chlamydia strain UV-7 recovered by this approach represents the first member of the genus *Parachlamydia* isolated from activated sludge of a wastewater treatment plant. These systems, which harbour an enormous diversity of protozoa, might thus represent an important anthropogenic reservoir for environmental chlamydiae (see also Kahane *et al.*, 2004), which contributes to the dissemination

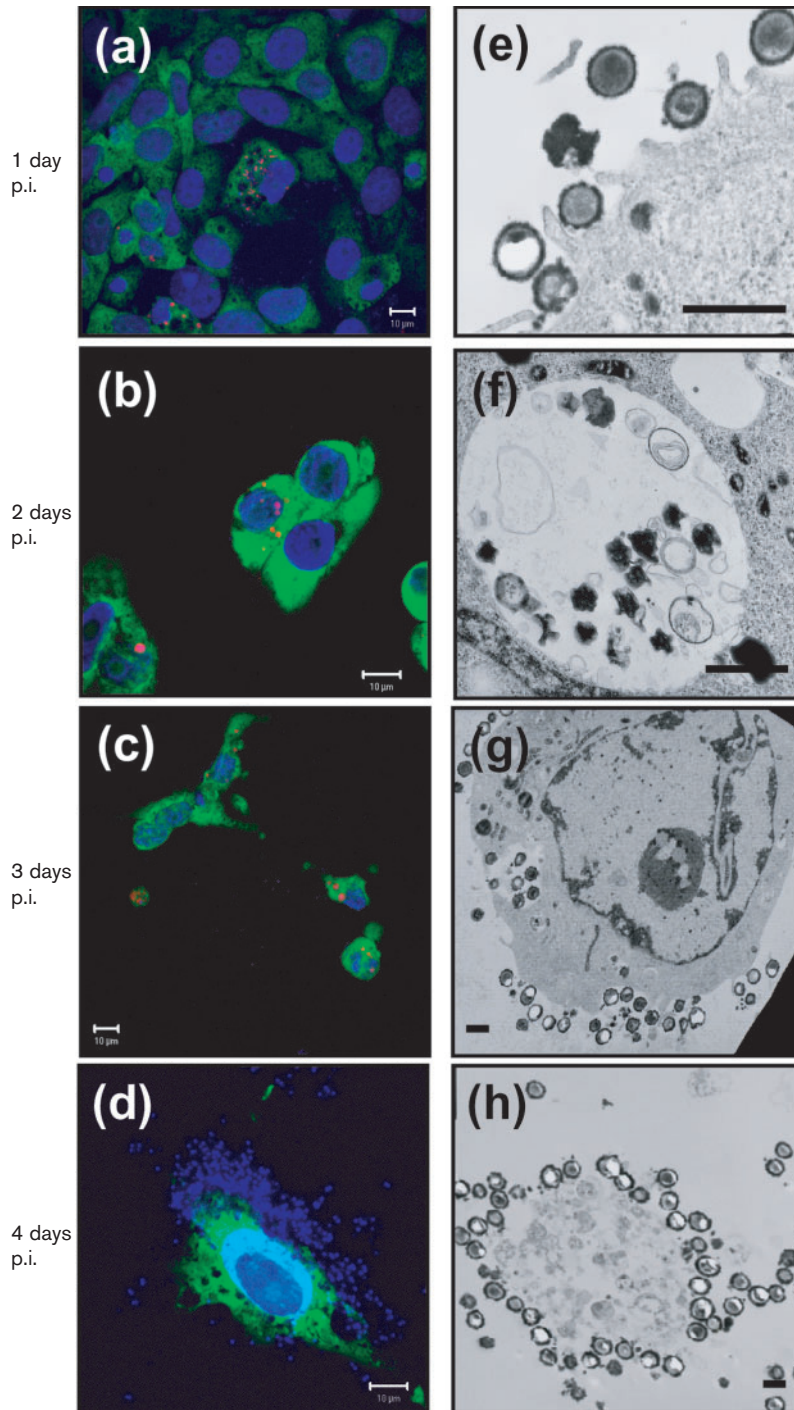


Fig. 3. Infection of Vero cells with *Parachlamydia* sp. UV-7. (a–d) Detection of *Parachlamydia* sp. UV-7 in Vero cells by FISH using the *Parachlamydia*-specific probe Bn₉-658 labelled with Cy3 (red), probe Euk516 targeting eukaryotes and labelled with FLUOS (green), and DAPI staining (blue). Bars, 10 µm. (e–h) Transmission electron micrographs of infected Vero cells. Bars, 1 µm.

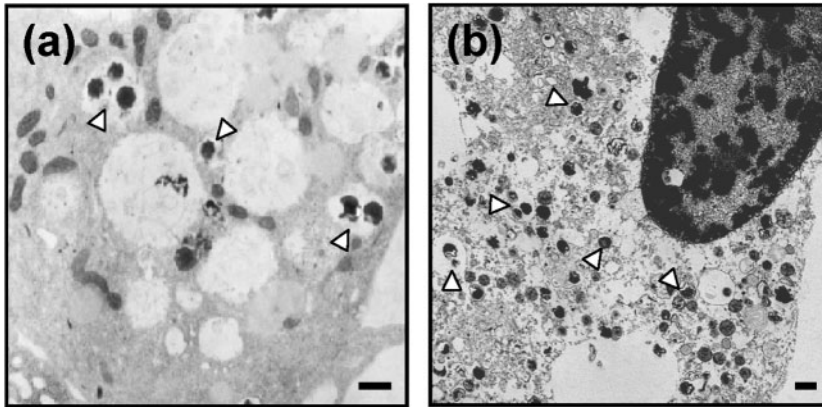


Fig. 4. Infection of NCI and HeLa cells with *Parachlamydia* sp. UV-7. Transmission electron micrographs of (a) NCI cells 1 day p.i., and (b) HeLa cells 2 days p.i. White arrowheads indicate inclusions containing EBs and RBs of *Parachlamydia* sp. UV-7. Bars, 1 µm.

of these bacteria into the receiving water bodies. This might be of particular importance since we could show that strain UV-7 is able to infect mammalian cells, providing further evidence for a possible pathogenic potential of *Parachlamydiaceae* for humans. A more complete understanding of environmental chlamydiae will depend on the availability of a larger number of isolates from different sources, which in the best case will represent the entire diversity and distribution of this recently discovered bacterial group in nature. Comparative examination of such a strain collection will not only increase our knowledge of the biology of environmental chlamydiae, but will eventually also help to answer the question whether *Parachlamydiaceae* indeed represent emerging pathogens.

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