'*Candidatus* Protochlamydia amoebophila', an endosymbiont of *Acanthamoeba* spp.

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The obligately intracellular coccoid bacterium UWE25, a symbiont of *Acanthamoeba* spp., was previously identified as being related to chlamydiae based upon the presence of a chlamydia-like developmental cycle and its 16S rRNA gene sequence. Analysis of its complete genome sequence demonstrated that UWE25 shows many characteristic features of chlamydiae, including dependency on host-derived metabolites, composition of the cell envelope and the ability to thrive as an energy parasite within the cells of its eukaryotic host. Phylogenetic analysis of 44 ribosomal proteins further confirmed the affiliation of UWE25 to the '*Chlamydiae*'. Within this phylum, UWE25 could be assigned to the family *Parachlamydiaceae* based on comparative analyses of the 16S rRNA, 23S rRNA and endoribonuclease P RNA genes. The distinct dissimilarities from its closest relative, *Parachlamydia acanthamoebae* Bn₉^T (7.1, 9.7 and 28.8 %, respectively), observed in this analysis justify its classification in a new genus. Therefore, the name '*Candidatus* Protochlamydia amoebophila' is proposed for the designation of the *Acanthamoeba* sp. symbiont UWE25 (= ATCC PRA-7).

The symbiont UWE25 was originally found intracellularly within an *Acanthamoeba* sp. isolated from a soil sample in western Washington State, USA (Fritsche *et al.*, 1993, 2000). All attempts to cultivate UWE25 in host-cell-free media have failed, suggesting that these bacteria are obligately intracellular symbionts. UWE25 is able to multiply in various *Acanthamoeba* hosts and even in the distantly related amoeba *Dictyostelium discoideum* (Fritsche *et al.*, 1998; Skriwan *et al.*, 2002). UWE25, however, had a cytopathic effect on its original host amoebae, and was therefore transferred to another host, *Acanthamoeba* sp. UWC1 (Fritsche *et al.*, 1998; Gautom & Fritsche, 1995), which was axenically cultivated in trypticase/soy/yeast extract broth (Visvesvara, 1999) at 20–24 °C.

In a previous study, UWE25 was identified as a Gram-negative chlamydia-like organism belonging to the family *Parachlamydiaceae* (Fritsche *et al.*, 2000). This family was introduced by Everett *et al.* (1999) and comprises obligately intracellular symbionts of free-living amoebae that are closely related to the medically important *Chlamydiaceae* (Everett *et al.*, 1999; Mahoney *et al.*, 2003). Within the *Parachlamydiaceae*, two genera have been described, represented by *Parachlamydia acanthamoebae* Bn₉^T (Amann *et al.*,

1997) and *Neochlamydia hartmannellae* A₁Hsp^T (Horn *et al.*, 2000).

Fluorescence *in situ* hybridization and electron microscopy demonstrated that UWE25 resides inside its host cells within small inclusions (containing only one or few bacteria) dispersed in the cytoplasm (Fig. 1), differentiating UWE25 from other members of the *Parachlamydiaceae*, which form large inclusions inside their host cells or seem to reside directly in the cytoplasm (Amann *et al.*, 1997; Fritsche *et al.*, 2000; Horn *et al.*, 2000). Occasionally, however, small clusters or morulae of UWE25 cells could be found inside the host cells (Fig. 1a). In contrast to *N. hartmannellae*, UWE25 does not prevent encystation of its amoeba host and can be found in *Acanthamoeba* trophozoites and cysts (Fritsche *et al.*, 2000; Horn *et al.*, 2000). UWE25 showed a chlamydia-like developmental cycle including morphological stages resembling chlamydial elementary bodies (0.5–0.8 µm in diameter; Fig. 1b) and reticulate bodies (0.7–1.0 µm in diameter; Fig. 1c) (Fritsche *et al.*, 2000).

Complete genome sequencing of UWE25 (Horn *et al.*, 2004) revealed that its genome is about twice as large as those of all members of the family *Chlamydiaceae* sequenced to date (2 414 465 bp versus 1–1.2 Mb; Kalman *et al.*, 1999; Read *et al.*, 2000, 2003; Shirai *et al.*, 2000; Stephens *et al.*, 1998), and has an overall G + C content that is much lower than those of genomes of *Chlamydiaceae* (35.8 versus 39.2–41.3 mol%). Like members of the family *Chlamydiaceae*,

Published online ahead of print on 22 April 2005 as DOI 10.1099/ijs.0.63572-0.

The GenBank/EMBL/DDBJ accession number for the RNase P RNA gene (*rnpB*) of '*Candidatus* Protochlamydia amoebophila' is AJ748539.

Fig. 1. Ultrastructure of '*Candidatus Protochlamydia amoebophila*'. Transmission electron micrograph showing '*Candidatus Protochlamydia amoebophila*' inside its *Acanthamoeba* host cell (a). The two developmental stages, an elementary body (b) and dividing reticulate bodies (c), are shown separately. Bars, 1 µm (a, c) and 0.5 µm (b).

UWE25 has only limited biosynthetic capabilities and relies on the import of several host-derived metabolites. In particular, UWE25 is not able to synthesize nucleotides or the electron carrier NAD^+ de novo but uses specific transporters to import these compounds from its host (Haferkamp *et al.*, 2004; Schmitz-Esser *et al.*, 2004), and produces only a limited number of amino acids (glycine, alanine, serine, aspartic acid, glutamine, glutamic acid and proline) and cofactors (riboflavin, haem, folate and menaquinone), illustrating its obligately intracellular lifestyle. The outer membrane of UWE25 contains cysteine-rich proteins characteristic of members of the *Chlamydiaceae* although no homologue for the major outer-membrane protein (MOMP), a primary antigen of known chlamydiae, has been identified so far. This is consistent with the failure of polyclonal antibodies directed against the MOMP of members of the *Chlamydiaceae* to bind to elementary bodies of UWE25 in micro-immunofluorescence tests (data not shown). In addition, monoclonal antibodies (CF2) directed against the *Chlamydiaceae*-specific lipopolysaccharide epitope did not react with UWE25 (data not shown), further illustrating differences in cell wall composition between UWE25 and the *Chlamydiaceae*.

The taxonomic affiliation of UWE25 was examined by phylogenetic analyses of the 16S rRNA, 23S rRNA and endoribonuclease P (RNase P) RNA genes, and a concatenated dataset of 44 ribosomal proteins, by using the ARB software package (Ludwig *et al.*, 2004). Phylogenetic analysis of the ribosomal proteins confirmed the ultrastructure- and genome-based assignment of UWE25 to the '*Chlamydiae*' (Fig. 2a). The ribosomal protein trees were in agreement with 16S and 23S rRNA gene trees published previously and thus support the current rRNA gene-based classification of '*Chlamydiae*' (Everett *et al.*, 1999). The 16S rRNA and 23S rRNA genes of UWE25 showed sequence identities above 82.1 and 83.4 %, respectively, with all other members of the *Chlamydiales*. UWE25 hence meets the requirements of Everett *et al.* (1999) for inclusion in this order. Consistent with this finding and independent of the phylogenetic marker and treeing method applied, UWE25 formed a monophyletic group with all chlamydiae to the exclusion of all other bacteria (Fig. 2).

The rRNA genes of UWE25 were more than 90 % identical to those of members of the *Parachlamydiaceae*, indicating the affiliation of UWE25 with this family. Within the *Parachlamydiaceae*, the highest sequence identities were observed with *P. acanthamoebae* Bn₉^T (92.9 % for the 16S rRNA gene and 90.3 % for the 23S rRNA gene). According to Everett *et al.* (1999), 16S rRNA and/or 23S rRNA gene sequence identities to the closest relative of less than 95 % are indicative of a new genus. UWE25 can thus not be assigned to any previously described genus of the family *Parachlamydiaceae*. Consistently, all calculated phylogenetic trees clearly showed the monophyletic grouping of UWE25 with all other members of the family *Parachlamydiaceae*, but within this group UWE25 formed a deeply branching evolutionary lineage (Fig. 2b, c).

To confirm the rRNA gene-based phylogenetic inference, the RNase P RNA gene (*rnpB*) was analysed. *rnpB* has previously been shown to be an evolutionarily ancient and ubiquitous gene that is well-suited as a phylogenetic marker, and has been used as a tool for the discrimination of chlamydiae down to the species level (Hartmann & Hartmann, 2003; Herrmann *et al.*, 2000). The *rnpB* sequence similarity of UWE25 to its closest relative *P. acanthamoebae* Bn₉^T (71.2 %) was lower than that between members of the genera *Chlamydia* and *Chlamydophila* (73.8–79.7 %), providing further support for the classification of UWE25 in a new genus. The distinct relationship of UWE25 to *P. acanthamoebae* Bn₉^T was also reflected in the *rnpB*-based phylogenetic tree (Fig. 2d). Therefore, based on Murray & Schleifer (1994), we propose the name '*Candidatus Protochlamydia amoebophila*' for the designation of the *Acanthamoeba* sp. endosymbiont UWE25.

'*Candidatus Protochlamydia amoebophila*'

'*Candidatus Protochlamydia amoebophila*' (Pro.to.chla.my'di.a. Gr. adj. *protos* first, foremost; N.L. fem. n.

(a) Ribosomal proteins

(b) 16S rRNA

(c) 23S rRNA

(d) rnpB

Fig. 2. Relationship of 'Candidatus Protochlamydia amoebophila' to other chlamydiae. TREE-PUZZLE-based consensus trees. Polytomies indicate branching points that were not consistently supported by TREE-PUZZLE (Strimmer & von Haeseler, 1996), neighbour-joining (Jukes-Cantor correction) and PHYLIP maximum-parsimony (Felsenstein, 1989) treeing methods. Numbers at nodes indicate TREE-PUZZLE support values (left numbers) and PHYLIP maximum-parsimony bootstrap values (right numbers). Only positions conserved in at least 20% (16S rRNA gene, *rnpB* – considering 1547 and 328 alignment positions, respectively) or 30% (ribosomal proteins – considering 4897 alignment positions) of all full-length sequences were used for treeing calculations. Bars, 10% estimated evolutionary distance.

Chlamydia taxonomic name of a bacterial genus; N.L. fem. n. *Protochlamydia* referring to the similarity of these bacteria to the chlamydial ancestor; a.moe'bo.phi.la. N.L. n. *amoeba* an amoeba; Gr. adj. *philos* loving; N.L. fem. adj. *amoebophila* loving amoebae, referring to the intracellular lifestyle within amoebae).

Phylogenetic position: phylum '*Chlamydiae*', order *Chlamydiales*, family *Parachlamydiaceae*. '*Candidatus Protochlamydia amoebophila*' represents a novel genus within the family *Parachlamydiaceae*. Other members of this tentative genus should have 16S rRNA genes with > 95% identity to the 16S rRNA gene of '*Candidatus Protochlamydia amoebophila*'.

Coccoid Gram-negative reticulate bodies and elementary bodies, 0.5–1.0 µm in diameter. Not cultivable on cell-free media. Obligately intracellular symbiont of *Acanthamoeba* spp. surrounded by vacuolar membranes and dispersed in the host cell cytoplasm, occasionally in small clusters or morulae. Basis of assignment: 16S rRNA, 23S rRNA and RNase P RNA (GenBank accession no. AJ748539) genes, and complete genome sequence (genome size 2 414 465 bp; overall G+C content 35.8 mol%; GenBank accession number BX908798) (Horn *et al.*, 2004). The original host *Acanthamoeba* sp. was isolated from a soil sample in western Washington State, USA; the current host is *Acanthamoeba* sp. UWC1 (Fritzsche *et al.*, 1993, 1998). Represented by isolate UWE25 (= ATCC PRA-7).

Acknowledgements

We gratefully acknowledge Waltraud Klepal and the team of the Ultrastructure Laboratory (University of Vienna) for advice and assistance with electron microscopy. This work was supported by the German Ministry for Education and Science (bmb+f) grant PTJ-BIO/03U213B and the Austrian Science Fund (FWF) grant P16566-B14.

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