'*Candidatus* Midichloria mitochondrii', an endosymbiont of the tick *Ixodes ricinus* with a unique intramitochondrial lifestyle

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An intracellular bacterium with the unique ability to enter mitochondria exists in the European vector of Lyme disease, the hard tick *Ixodes ricinus*. Previous phylogenetic analyses based on 16S rRNA gene sequences suggested that the bacterium formed a divergent lineage within the *Rickettsiales* (*Alphaproteobacteria*). Here, we present additional phylogenetic evidence, based on the *gyrB* gene sequence, that confirms the phylogenetic position of the bacterium. Based on these data, as well as electron microscopy (EM), *in situ* hybridization and other observations, we propose the name '*Candidatus* Midichloria mitochondrii' for this bacterium. The symbiont appears to be ubiquitous in females of *I. ricinus* across the tick's distribution, while lower prevalence is observed in males (44 %). Based on EM and *in situ* hybridization studies, the presence of '*Candidatus* M. mitochondrii' in females appears to be restricted to ovarian cells. The bacterium was found to be localized both in the cytoplasm and in the intermembrane space of the mitochondria of ovarian cells. '*Candidatus* M. mitochondrii' is the first bacterium to be identified that resides within animal mitochondria.

INTRODUCTION

The genera and the families of the order *Rickettsiales* have recently been reorganized and redescribed by Dumler *et al.* (2001). Based on this taxonomic revision, this order now encompasses two coherent monophyletic groups at the family rank: the *Rickettsiaceae* and the *Anaplasmataceae*. The six genera of the order also form well-defined monophyletic clusters within the two families: *Rickettsia* and *Orientia* within the *Rickettsiaceae* and *Anaplasma*, *Wolbachia*, *Ehrlichia* and *Neorickettsia* within the *Anaplasmataceae*. The family *Anaplasmataceae* might include a fifth genus ('*Candidatus* Xenohaliotis') and a further main branch has been recognized which might represent a third family in the order *Rickettsiales*, the *Holosporaceae* (Garritty *et al.*, 2004; but see Lee *et al.*, 2005). All of these bacteria share a common

characteristic: they have never been cultured outside eukaryotic cells and are thus regarded as obligately intracellular. Different host types have been described for these bacteria, ranging from vertebrates to nematodes (Bandi *et al.*, 1998; Parola, 2004), but several species in both families are associated with arthropods, particularly ticks.

Bacterial 16S rRNA gene sequences obtained recently during analysis of ticks and amoebae showed high similarity to 16S rRNA gene sequences from members of the *Rickettsiales* (Fritsche *et al.*, 1999; Goddard *et al.*, 2003; Parola *et al.*, 2003; Beninati *et al.*, 2004; Mediannikov *et al.*, 2004). In particular, Fritsche *et al.* (1999) obtained almost complete 16S rRNA gene sequences from the symbionts of two strains of *Acanthamoeba* spp. and showed that these sequences formed a new lineage within the *Rickettsiales*. According to Fritsche *et al.* (1999), this lineage could represent a new genus and possibly a new family of this order. Subsequently, bacterial 16S rRNA gene sequences showing similarity (90–92 %) to

Abbreviation: EM, electron microscopy.

The GenBank/EMBL/DDBJ accession number for the *gyrB* gene sequence of '*Candidatus* Midichloria mitochondrii' is AM159536.

those from the endosymbionts of *Acanthamoeba* spp. were obtained from various tick species: *Haemaphysalis wellingtoni* (Parola *et al.*, 2003), *Ixodes ricinus* (Sanogo *et al.*, 2003; Beninati *et al.*, 2004) and *Ixodes persulcatus* (Mediannikov *et al.*, 2004). Phylogenetic analyses of these sequences revealed the existence of a novel clade within the order *Rickettsiales* (Beninati *et al.*, 2004). Moreover, short 16S rRNA gene sequences (376–947 bp) that cluster within this novel clade have also been obtained from ticks (Sanogo *et al.*, 2003; Schabereiter-Gurtner *et al.*, 2003; Goddard *et al.*, 2003), from humans (Mediannikov *et al.*, 2004) and from microbial mats (O. Sanchez, unpublished GenBank submissions AJ871029 and AJ871030).

Most of the above tick-associated 16S rRNA gene sequences were obtained in the context of PCR surveys without any specific microscopical observations. One exception was the study by Beninati *et al.* (2004) that reported electron microscopy (EM) observations of an intracellular bacterium as well as *in situ* hybridization and extensive sequencing of 16S rRNA gene sequence clones (see also Sacchi *et al.*, 2004). This symbiont of *I. ricinus* has provisionally been designated IricES1 (Beninati *et al.*, 2004). IricES1 has been observed in the ovaries of females of *I. ricinus* with 100% prevalence, while lower prevalence is reported in male ticks (Lo *et al.*, 2006). In females, the bacterium inhabits the cytoplasm of ovarian cells and possesses the peculiar ability to invade and destroy the host's mitochondria. IricES1 is thus presently the only member of the novel clade of the *Rickettsiales* for which detailed ultrastructural observations are available in addition to information on its distribution within the host species.

In summary, molecular evidence is available which indicates the existence of a novel major clade within the order *Rickettsiales*. We believe this clade deserves more attention as well as formal taxonomic description. The information available for the IricES1 symbiont of *I. ricinus* now provides the opportunity for the proposal of a new genus and species name, while future studies will address the issue of whether a new family should be created in the order *Rickettsiales*.

This paper reports (i) partial sequencing and phylogenetic analysis of *gyrB*, the gene encoding DNA gyrase subunit B, from IricES1, (ii) phylogenetic analyses of 16S rRNA and *gyrB* gene sequences and (iii) a summary of results of previous studies on IricES1, including EM, *in situ* hybridization and prevalence studies. In view of the fact that cultivation of IricES1 has not yet been achieved, we propose the classification of the bacterium as '*Candidatus* Midichloria mitochondrii'.

METHODS

Cloning and sequencing of the *gyrB* gene. An adult female tick collected in Berlin, Germany, was fed on a hamster and allowed to deposit eggs. DNA was extracted from five pooled eggs using a DNeasy Kit (Qiagen). To control for the presence of bacteria other than the intramitochondrial symbiont, PCR and cloning of 16S

rRNA genes was performed as described in Beninati *et al.* (2004). This experiment indicated that the eggs were infected only by the intramitochondrial symbiont. The *gyrB* gene was then amplified with the universal bacterial primers *gyrBAUP*₂ and *gyrBBNDN*₁ (Santos & Ochman, 2004). Amplifications were performed in 20 µl buffer [10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin] with 0.2 mM each deoxynucleoside triphosphate, 20 pmol each primer, 1 U *Taq* polymerase (Perkin Elmer) and 1 µl DNA sample. The conditions for the PCR on a PTC-100 thermal cycler (Peltier) were as follows: 2 min denaturation at 94 °C; then five touchdown cycles (every cycle with 1 °C lower annealing temperature) 1 min at 94 °C, 1 min at 55 °C (–1 °C per cycle), 1 min at 72 °C; then 25 cycles with 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C and final steps of 6 min at 72 °C, 5 min at 15 °C.

The PCR product was visualized on an agarose gel, purified using a Nucleospin Extract purification kit (Macherey Nagel) and cloned using a pGEM-T Easy Vector System cloning kit (Promega). The inserts from four colonies were sequenced using ABI technology, initially with the primers T7 and SP6. A consensus from the sequences of the four clones was obtained. Eight additional primers were successively designed by a walking approach (Iric-GyrB-F1 GTTGATGGTGTGCGGTTAAG; Iric-GyrB-R1 GATTTGCCATATCGAGATCAC; Iric-GyrB-F2 CTC-TGTGGTTAATGCATTGTCTG; Iric-GyrB-R2 CTCTACAAG-GAAGAGCTCG; Iric-GyrB-F3 GAGTGACAAAGATATTTCCGGC; Iric-GyrB-R3 TTAAGTCCGTTCTCTCTTAC; Iric-GyrB-F4 GACA-ATTTATCAAGAGTGGCTTG; Iric-GyrB-R4 CGCTCAACATAACT-ATTGAAGC). These primers were used on the product of the *gyrBAUP*₂ and *gyrBBNDN*₁ primers in order to generate double-stranded sequence directly from the PCR product obtained from four different tick samples.

Phylogenetic analyses of 16S rRNA and *gyrB* gene sequences. The 16S rRNA gene sequence of '*Candidatus* M. mitochondrii' was subjected to BLAST analysis and closely related, nearly full-length sequences were aligned, taking into account secondary structure, at the Ribosomal Database Project website (Cole *et al.*, 2003). Phylogenetic analysis was performed under Bayesian Inference criteria using the MrBayes program (Huelsenbeck & Ronquist, 2001). Parameters for the selected model of substitution were estimated from the data using MrModeltest (Nylander, 2002). A total of 15 000 trees were obtained (ngen = 1 500 000, sample-freq = 100) and the first 5000 of these were considered as the 'burn in' and discarded. A 50% majority-rule consensus tree of the remaining 10 000 trees, including branch lengths (savebrlens = yes) was produced. Three different replications of these analyses were performed. A second analysis was performed comparing shorter 16S rRNA gene sequences (376–947 bp) that had been determined to be closely related to '*Candidatus* M. mitochondrii' by BLAST analysis.

For the *gyrB* gene, relevant sequences were aligned with the sequence obtained for '*Candidatus* M. mitochondrii' following BLAST searches. Phylogenetic analyses of the *gyrB* gene were performed at the amino acid level due to the high level of nucleotide divergence (61–65% similarity) between '*Candidatus* M. mitochondrii' and other taxa. *GyrB* amino acid sequences were aligned with sequences from various members of the *Alphaproteobacteria* using CLUSTAL_X (Thompson *et al.*, 1997). Minor editing was done manually. A 535-amino acid alignment was used for phylogenetic analyses under the maximum-likelihood Quartet Puzzling criteria using the TreePuzzle 5.2 program (Strimmer & Haeseler, 1996).

Electron microscopy. Adult female ticks collected in České Budějovice (Czech Republic) were fed on guinea pigs until engorgement (3–5 days). Ovaries were dissected out in saline solution and fixed in 0.1 M cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde for 3 h at 4 °C. The samples were washed in the same buffer and then post-fixed in 1% OsO₄ in the same buffer for 1.5 h at

4 °C. All samples were dehydrated in an ethanol series and embedded in Epon 812. Semithin sections (1 µm) for light microscopy were stained with 0.5 % toluidine blue. Thin sections (80 nm) were stained with uranyl acetate and lead citrate and examined under a transmission electron microscope (EM900; Zeiss).

RESULTS AND DISCUSSION

BLAST searches involving the '*Candidatus M. mitochondrii*' 16S rRNA gene sequence revealed the presence of a number of closely related sequences of uncharacterized bacteria obtained from various tick species, amoebae, humans and microbial mats. When compared with the sequence of '*Candidatus M. mitochondrii*', similarities ranged from 90 % (GenBank accession number AF493952 derived from *Ixodes persulcatus*) to 99 % (a different sequence from *I. ricinus*, GenBank accession number AF525482, possibly the same bacterium). The overall level of 16S rRNA gene sequence divergence among these sequences is comparable to that observed among species of the same genus or even genera of the same family within the *Rickettsiales*. For example, within the genus *Ehrlichia*, 16S rRNA gene sequence similarities range from 96 to 98 %. Within the family *Anaplasmataceae*, the level of similarity between different genera varies from ~83 to ~90 %.

Phylogenetic comparisons between the '*Candidatus M. mitochondrii*' 16S rRNA gene sequence and near full-length sequences (>1200 bp) in the databases revealed the existence of a strongly supported monophyletic group containing various unclassified bacteria from ticks and from *Acanthamoeba* spp. (Fig. 1). These results are in agreement with previous studies (Fritsche *et al.*, 1999; Parola *et al.*, 2003; Beninati *et al.*, 2004; Mediannikov *et al.*, 2004) in which the bacteria of this group were found to be the sister group of the family *Anaplasmataceae* (comprising the

genera *Anaplasma*, *Ehrlichia*, *Neorickettsia* and *Wolbachia*). Separate analyses, including partial 16S rRNA gene sequences (<1000 bp), showed that this phylogenetic branch includes other sequences obtained from ticks, humans and a microbial consortium (Fig. 2). '*Candidatus M. mitochondrii*' is closely related to an uncharacterized bacterium from the tick *H. wellingtoni* (Figs 1 and 2). Since these ticks are distantly related (Black & Piesman, 1994), this suggests that '*Candidatus M. mitochondrii*' and its relatives may undergo horizontal transfer.

The *gyrB* gene sequence obtained is 1432 bp long with a DNA G+C content of 39.4 mol%. BLAST analysis showed that the sequence is most closely related to sequences from the genera *Anaplasma*, *Ehrlichia*, *Wolbachia*, *Neorickettsia* and *Rickettsia*. Sequences of the *gyrB* gene from each of these genera were downloaded from the databases and aligned with the sequence from '*Candidatus M. mitochondrii*'. Phylogenetic inference from *gyrB* gene sequence alignments clearly showed that '*Candidatus M. mitochondrii*' is placed within the *Alphaproteobacteria* as a divergent lineage most closely related to members of the family *Anaplasmataceae* (Fig. 3). This placement was slightly different to its position within the 16S rRNA gene sequence tree (Fig. 1), where it was found outside the family *Anaplasmataceae*. This may be due to the fact that fewer *gyrB* gene sequences are available compared with 16S rRNA gene sequences.

Electron microscopy of *I. ricinus* collected in the Czech Republic showed that '*Candidatus M. mitochondrii*' is found within the mitochondria of the ovaries of these ticks (Fig. 4). These results are in agreement with previous EM studies of *I. ricinus* collected in England (Lewis, 1979), Switzerland (Zhu *et al.*, 1992) and Italy (Beninati *et al.*, 2004; Sacchi *et al.*, 2004).

Fig. 1. Phylogenetic tree based on near full-length 16S rRNA gene sequences (>1200 bp) showing the position of '*Candidatus M. mitochondrii*' and selected members of the *Rickettsiales*. The tree was generated using MrBayes. The GenBank accession number for each sequence is indicated. Numbers adjacent to each node represent the posterior probability values. *Acetobacter aceti* (*Rhodospirillales*) was included as an outgroup. Additional analyses in which other *Alphaproteobacteria* were included as outgroups generated trees showing the same overall topology. Host species are shown in parentheses, where relevant. Bar, 0.1 inferred substitutions per site.

Fig. 2. Phylogenetic tree showing the position of 'Candidatus M. mitochondrii' in relation to closely related and short uncharacterized sequences. The tree is based on a 524 bp 16S rRNA gene sequence alignment and was generated using MrBayes. The GenBank accession number for each sequence is indicated. Numbers above each node represent the posterior probability values. *Wolbachia* sp. from *Brugia malayi* was included as an outgroup. Additional analyses in which other *Alphaproteobacteria* were included as outgroups generated trees showing the same overall topology. Host species are shown in parentheses. Bar, 0.05 inferred substitutions per site.

In summary, the information already available in the literature and in databases, as well our new results, clearly show that a further phylogenetic branch exists within the order *Rickettsiales*. This branch may deserve to be elevated at the family rank when further data is obtained, as already suggested (Fritsche *et al.*, 1999). We believe the information currently available for one member of this clade, IricES1, meets the criteria required for the description of new genera and species (for uncultured bacteria, see Murray & Stackebrandt, 1995; see also Zeigler, 2003). In brief, the information available is (i) sequences of two genes (Beninati *et al.*, 2004; this study), (ii) localization in the host tissues by *in situ* hybridization and electron microscopy (Beninati *et al.*, 2004; this study), (iii) detailed ultrastructural observations (Sacchi *et al.*, 2004), (iv) prevalence studies throughout

European and North African populations of the host species (Lo *et al.*, 2006) and (v) PCR evidence for efficient vertical transmission (Lo *et al.*, 2006).

Description of 'Candidatus Midichloria mitochondrii'

'Candidatus M. mitochondrii' appears in EM observations (Lewis, 1979; Zhu *et al.*, 1992; Sacchi *et al.*, 2004) as a Gram-negative bacterium with a bacillus shape of $\approx 0.45 \mu\text{m}$ in diameter and $\approx 1.2 \mu\text{m}$ in length. This bacterium is observed within various cell types (luminal cells, funicular cells and oocytes) of the ovary of the hard tick *I. ricinus* (Ixodidae). In all of the above cell types, the bacteria are observed free in the cytoplasm or included in a host-derived

Fig. 3. Phylogenetic comparison of the *gyrB* gene sequence of 'Candidatus M. mitochondrii' with those from other members of the *Alphaproteobacteria*. The tree was constructed on a 535-amino acid alignment using TreePuzzle 5.2 with the WAG model of substitution, assuming among-site variation (gamma distribution, 4 rates). A tree estimated with the JTT model of substitution showed the same topology at the level of the main branches. For other parameters, defaults were used. GenBank accession numbers are indicated for each sequence. The host species is indicated in parentheses where relevant. Numbers above each node are Quartet Puzzling support values. *Escherichia coli* is included as an outgroup. Additional analyses in which various other *Proteobacteria* were used as outgroups generated trees with the same overall topology. Bar, 0.1 inferred substitutions per site.

Fig. 4. Electron micrographs of ovarian cells of *I. ricinus* containing ‘*Candidatus M. mitochondrii*’ (B) within intact or degraded mitochondrial matrix (Ma). Bars, 2.4 μ m (a), 1.2 μ m (b) and 0.45 μ m (c).

membrane. In addition, in luminal cells and oocytes, the bacterium is also observed within the mitochondria, in the periplasmic space between the two membranes of these organelles. As the development of the oocyte proceeds, the bacteria appear to consume the inner part of the mitochondria and multiply therein. The mitochondrial

matrix is reduced as a result and some mitochondria appear as sacs full of bacteria (Sacchi *et al.*, 2004). Different numbers of bacteria have been observed within the mitochondria, from a single bacterium to over 20. Despite the high number of mitochondria consumed by the bacterium, the eggs of the tick develop normally. *In situ* hybridization with probes designed to target specific 16S rRNA gene regions resulted in the staining of only ovarian cells in female ticks (Beninati *et al.*, 2004). In male ticks, there is only PCR evidence for the presence of the bacterium (Lo *et al.*, 2006). The symbiont appears to be ubiquitous in the females of *I. ricinus* across its distribution (prevalence, 100 %), while a significantly lower prevalence is observed in males (44 %). In males that test positive by PCR, the bacterial load also appears lower compared with females (Lo *et al.*, 2006). Evidence for efficient vertical transmission of the bacterium has been reported based on PCR screening of eggs. Sequencing of the 16S rRNA and *gyrB* genes from ticks from 11 different countries from across the distribution of *I. ricinus* showed a low level of variability in both genes. One substitution in 380 bases was found in the 16S rRNA gene sequence and two substitutions in 519 bases were found in the *gyrB* gene sequence (Lo *et al.*, 2006).

In accordance with the guidelines of the International Committee of Systematic Bacteriology, unculturable bacteria should be classified as *Candidatus* (Murray & Stackebrandt, 1995). Thus we propose the name ‘*Candidatus Midichloria mitochondrii*’ for the novel bacterium. The genus name *Midichloria* (mi.di.chlo’ria. N.L. fem. n.) is derived from the midichlorians, organisms within the fictional Star Wars universe. Midichlorians are microscopic symbionts that reside within the cells of living things and “communicate with the Force”. Star Wars creator George Lucas stated that the idea of the midichlorians is based on endosymbiotic theory. The word ‘midichlorian’ appears to be a blend of the words mitochondrion and chloroplast. The specific epithet, *mitochondrii* (mi.to’chon.drii. N.L. n. *mitochondrium* -i a mitochondrion; N.L. gen. n. *mitochondrii* of a mitochondrion), refers to the unique intramitochondrial lifestyle of this bacterium. ‘*Candidatus M. mitochondrii*’ belongs to the phylum *Proteobacteria*, to the class *Alphaproteobacteria* and to the order *Rickettsiales*. ‘*Candidatus M. mitochondrii*’ is assigned on the basis of the 16S rRNA (AJ566640) and *gyrB* gene sequences (AM159536).

‘*Candidatus M. mitochondrii*’ has so far only been observed within the ovary of *I. ricinus* with 100 % prevalence in female ticks. *I. ricinus* is the main vector of various important human and animal pathogens. This ubiquitous presence suggests that the symbiont plays a role in the biology of female ticks. In addition, ‘*Candidatus M. mitochondrii*’ is certainly an important component of the tick microbial community, possibly interacting with the pathogens transmitted by this arthropod. The most striking feature, however, is its unique intramitochondrial localization which could make this symbiont an important model for various

studies on the biology of this organelle. The presence of various unclassified 16S rRNA gene sequences with high similarity to that of '*Candidatus M. mitochondrii*' suggests the existence of a family of related bacteria which has yet to be explored.

ACKNOWLEDGEMENTS

Support from the Italian Ministry of Universities and Research (MIUR) is acknowledged. N.L. was supported by the Australian Research Council. We thank three anonymous referees for comments on an earlier version of the manuscript. George Lucas kindly granted us permission to base the genus name on the midichlorians.

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