Identification of the bacterial endosymbionts of the marine ciliate *Euplotes magnicirratus* (Ciliophora, Hypotrichia) and proposal of 'Candidatus Devosia euplotis'

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This paper reports the identification of bacterial endosymbionts that inhabit the cytoplasm of the marine ciliated protozoan *Euplotes magnicirratus*. Ultrastructural and full-cycle rRNA approaches were used to reveal the identity of these bacteria. Based on analysis of 16S rRNA gene sequences, evolutionary trees were constructed; these placed the endosymbiont in the genus *Devosia* in the α -Proteobacteria. The validity of this finding was also shown by fluorescence *in situ* hybridization with a *Devosia*-specific oligonucleotide probe. Differences at the 16S rRNA gene level (which allowed the construction of a species-specific oligonucleotide probe) and the peculiar habitat indicate that the endosymbiont represents a novel species. As its cultivation has not been successful to date, the provisional name 'Candidatus Devosia euplotis' is proposed. The species- and group-specific probes designed in this study could represent convenient tools for the detection of 'Candidatus Devosia euplotis' and *Devosia*-like bacteria in the environment.

Endosymbiotic bacteria in ciliates have mainly been studied by protozoologists, using morphological and functional approaches. They were originally described as cytoplasmic or nuclear particles, designated by a single Greek letter (Preer *et al.*, 1974; Nobili *et al.*, 1976; Rosati *et al.*, 1976; Heckmann *et al.*, 1983; for a review, see Görtz, 2002), and most of them have not yet been redescribed or given a binomial name. The only exceptions are represented by the following genera with validly published names, which inhabit different species of *Paramecium*: *Caedibacter* (Preer & Preer, 1982) (five species), *Lyticum* (Preer & Preer, 1982) (two species), *Pseudocaedibacter* (Quackenbush, 1982) (three species) and *Holospora* (*ex* Hafkine, 1890; Gromov & Ossipov, 1981) (four species), and by a single *Euplotes* endosymbiont that was redescribed as *Polynucleobacter necessarius* (Heckmann & Schmidt, 1987). Molecular

phylogenetic affiliation is available for an even smaller number of ciliate bacterial symbionts (Amann *et al.*, 1991; Springer *et al.*, 1993, 1996; Beier *et al.*, 2002).

Beier *et al.* (2002) found that the two *Caedibacter* species so far characterized do not belong to the same subclass of proteobacteria, despite their morphological and functional similarity. This indicates that both morphological and molecular approaches are needed for satisfactory identification of endosymbionts.

In the present study, the identity and phylogenetic affiliation of an as-yet-undescribed symbiont of the marine species *Euplotes magnicirratus* was investigated by electron microscopy and the full-cycle rRNA approach. This cycle includes comparative 16S rRNA gene sequence analysis and detection of the endosymbionts within their host cells by fluorescence *in situ* hybridization (FISH), using group- and species-specific, 16S rRNA-targeted oligonucleotide probes (Amann *et al.*, 1991, 1995; Petroni *et al.*, 2000).

These analyses showed that the endosymbiont is a novel species and was the same in all *E. magnicirratus* strains examined, despite their different geographical provenance. Comparative phylogenetic analysis indicated that this species could be included in the genus *Devosia* in the α -Proteobacteria. Cultivation of these bacteria has not been successful to date and we therefore propose the provisional status *Candidatus* for this bacterial endosymbiont: 'Candidatus Devosia euplotis'.

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Abbreviation: FISH, fluorescence *in situ* hybridization.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of 'Candidatus Devosia euplotis' from *Euplotes magnicirratus* strains LIV5, CO and CAMP4.4 are AJ548825, AJ548823 and AJ548824, respectively. The accession number for the 16S rRNA gene sequence of *Devosia riboflavina* DSM 7230^T is AJ549086. The accession numbers for the 18S rRNA gene sequences of *Euplotes magnicirratus* strains LIV5 and CAMP4.4 are AJ549209 and AJ549210, respectively.

A complete similarity matrix and a colour version of Fig. 3 are available as supplementary material in IJSEM Online.

Three ciliate strains of different geographical origin were studied: LIV5 from the Ligurian Sea (Italy), CO from the Caribbean Sea (Colombia) and CAMP4.4 from the Sardinian Sea (Italy). By morphological observations and characterization of their 18S rRNA gene, they were all identified as *E. magnicirratu*s. Clonal cultures were obtained from cells isolated from the marine samples and maintained in artificial sea water (salinity, 33 ‰) at 20 °C. Ciliates were fed regularly with pure cultures of the nanoflagellate *Dunaliella tertiolecta* [for details, see Dini & Nyberg (1994)].

Ciliate morphology was studied by using scanning and transmission electron microscopy, according to protocols used for other marine ciliates (Modeo *et al.*, 2003).

Host and symbiont DNA extraction was performed by following the method of Wisotzkey *et al.* (1990) with slight modifications. Amplification of the 18S rDNA of *E. magnicirratu*s strains was carried out by using the forward primer 18S F9 (Medlin *et al.*, 1988) and the reverse primer 18S R1513 Hypo (Petroni *et al.*, 2002). 16S rRNA genes of bacterial endosymbionts and of *Devosia riboflavina* DSM 7230^T (Nakagawa *et al.*, 1996) were amplified by using primers that were designed specifically for the α -*Proteobacteria* subclass: forward, 16S alfa F19a (5'-CCT-GGCTCAGAACGAACG-3') and reverse, 16S alfa R1517 (5'-TGATCCAGCCGCAGGTTTC-3'). In order to obtain higher specificity, a 'touchdown' PCR (Don *et al.*, 1991) was performed. Amplified and purified fragments were sequenced directly in both directions with the same primers that were used for amplification and with proper internal primers for the ciliate 18S rRNA gene sequence (Rosati *et al.*, 2004) or the bacterial 16S rRNA gene sequence (16S F7a: 5'-AGAGTTTGTATCCTGGCTCA-3'; 16S F515 ND: 5'-GTGCCAGCAGCCGCGGT-3'; 16S F1099 ND: 5'-GCAACGAGCGCAACCC-3'; 16S F343 ND: 5'-TACGG-GAGGCAGCAG-3'; 16S F785 ND: 5'-GGATTAGATAC-CCTGGTA-3'; 16S R785 ND: 5'-TACCAGGGTATCTA-ATCC-3'; 16S R515 ND: 5'-ACCGCGGCTGCTGGCAC-3'; 16S R1522a: 5'-GGAGGTGATCCAGCCGCA-3').

Sequences were aligned by using the sequence editor and aligner from the ARB program package (Ludwig & Strunk, 1997). In a preliminary analysis, sequences were added by using interactive parsimony to a general tree that comprised over 10 000 bacterial species. Further phylogenetic analyses were performed with different subsets of closely related species. Some sequences of unidentified organisms that shared >95 % similarity with those of the endosymbionts were also included; only almost-complete sequences were included in analyses. Two filters were used to selectively remove or retain the more variable positions, before performing the phylogenetic analysis: filter bact_rr5_nov98 and filter alp_rr5_nov98 (Ludwig & Strunk, 1997). Phylogenetic analyses were performed as described previously (Petroni *et al.*, 2002). The two filters were used for each reconstruction method. Topologies of obtained trees were compared to recognize stable nodes (Ludwig *et al.*, 1998b).

*E. magnicirratu*s starved cells that belonged to the three strains and bacterial cells from growth media were fixed as described elsewhere (Petroni *et al.*, 2003). *In situ* hybridization was performed according to Manz *et al.* (1992) without formamide in the hybridization buffer. In order to identify the endosymbiont group, the following oligonucleotide probes were used: ARCH915 (Stahl & Amann, 1991), EUB338 (Amann *et al.*, 1990), ALF1b, BET42a, GAM42a, (Manz *et al.*, 1992) and one probe that is specific for most members of the δ -*Proteobacteria* (Amann *et al.*, 1995). *Devosia* group probe Dev_819 (5'-CCAACGGCTA-GCTCTCAT-3') and 'Candidatus *Devosia euplotis*' probe DevEup_993 (5'-AAGTCGTCCTGGTATGTC-3') were designed to hybridize to variable regions of the obtained sequence of endosymbiont 16S rRNA by using the special option of the ARB package, then refined and synthesized according to Petroni *et al.* (2003). Their specificity was checked with the probe-match tool from the ARB package (Ludwig & Strunk, 1997). Both probes were checked on *E. magnicirratu*s bearing symbionts and on *D. riboflavina* DSM 7230^T (Nakagawa *et al.*, 1996). Double hybridizations of species-specific probe DevEup_993 together with eubacterial universal probe EUB338 (Amann *et al.*, 1990) or α -subclass-specific probe ALF1b (Manz *et al.*, 1992) were carried out, in order to exclude the presence of other eubacterial endosymbionts.

Starved *E. magnicirratu*s cells were filtered and concentrated. They were then rapidly surface-sterilized with streptomycin (400 mg l⁻¹) and washed with sterilized sea water. A suspension with sterilized, distilled water was sufficient to obtain osmotic lysis of the cells. The homogenate was then transferred to culture media and incubated at 28 and 20 °C. Growth attempts of endosymbionts were performed on the following media: nutrient agar and broth (Difco) with KH₂PO₄ (0.45 g l⁻¹) and Na₂HPO₄.12H₂O (pH 6.8), tryptone soya agar and broth (Oxoid) and marine agar and broth (Difco). The presence of *E. magnicirratu*s symbionts was checked regularly every week for 1 month by *in situ* hybridization with the species-specific probe DevEup_993.

The three *Euplotes* strains used in this study have been assigned to *E. magnicirratu*s, based on analysis of the morphological characters that are generally used to distinguish *Euplotes* species: size, shape, number of dorsal kineties, cirral arrangement and argyrome pattern on the dorsal surface (dargyrome). The assignment was congruent with the results obtained by comparative analysis of 18S rRNA gene sequences. Both the newly determined sequences of CAMP4.4 and LIV5 are identical to the already published *E. magnicirratu*s CO strain 18S rRNA gene sequence (Petroni *et al.*, 2002; GenBank accession no. AJ305250).

Morphologically identical bacterial endosymbionts were found in all specimens examined at the ultrastructural level, independent of the strain they belonged to (CO, LIV5 or CAMP4.4). In all cases, bacteria were distributed equally throughout the cytoplasm and were contained

Fig. 1. Electron micrograph of 'Candidatus Devosia euplotis' in *Euplotes magnicirratu*s. Bacteria are contained individually in small cytoplasmic vacuoles. Bar, 1 µm.

individually in vacuoles (symbiosomes), which probably originated from the host rough endoplasmic reticulum. This can be inferred by the presence of numerous ribosomes on their external surface (Fig. 1). The symbionts are rod-shaped bacteria, 0.5 µm wide and up to 2.5 µm long, that possess two membranes. The innermost membrane is adherent to the protoplasm; the outermost appears wavy and, at some points, contacts the membrane of the vacuole. Flagella are not present. The protoplasm is rich in ribosomes; dense nucleoids were not observed. The bacteria divide by transverse binary fission, accompanied by division of the vacuole to result in separate enclosure of each new bacterium.

The almost-complete 16S rRNA gene sequence (1422 bp) of *D. riboflavina* DSM 7230^T was determined; six nucleotide differences were observed from the originally described sequence (Nakagawa *et al.*, 1996). Almost full-length

sequences (1423 bp) of the bacterial endosymbiont 16S rRNA gene were obtained from the three strains of *E. magnicirratu*s. Validity of the sequences was confirmed by the positive results of *in situ* hybridization, performed with the DevEup_993 probe (see below). The sequences from strains CO and LIV5 are identical and differ by a single nucleotide substitution (position 592, relative to *Escherichia coli* 16S rRNA) from that obtained from strain CAMP4.4. This means that, despite their different geographical origin, endosymbionts of the three strains can reasonably be assigned to the same bacterial species. This species can now be added to the short list of molecularly characterized ciliate endosymbionts.

Endosymbionts of *E. magnicirratu*s belong to the α -subclass of the *Proteobacteria*. The sequences share rather low similarity with those of *Holospora obtusa* (82.7%) and *Caedibacter caryophilus* (85.7%), the only two described symbionts of ciliates that belong to the same subclass of proteobacteria. Similarity towards two bacteria of the genus *Devosia*, family *Hyphomicrobiaceae*, order 'Rhizobiales', is surprisingly high (96.2 and 96.9%). The complete similarity matrix is available as supplementary data in IJSEM Online. The genus *Devosia*, proposed by Nakagawa *et al.* (1996), currently includes two species: *D. riboflavina* (Nakagawa *et al.*, 1996), formerly *Pseudomonas riboflavina*, isolated from soil, and *Devosia neptuniae*, a nitrogen-fixing legume endosymbiont (Rivas *et al.*, 2002, 2003). A value of 95% similarity between 16S rRNA gene sequences is widely accepted to circumscribe a genus of bacterial organisms (Ludwig *et al.*, 1998b; Rosselló-Mora & Amann, 2001), whilst a similarity value of 97% has been proposed as a critical boundary for species level (Stackebrandt, 2000; Ludwig & Klenk, 2001; Rosselló-Mora & Amann, 2001). On this basis, the endosymbiont of *E. magnicirratu*s can be assigned to the genus *Devosia*, but the differences shown at the 16S rRNA gene level and its peculiar habitat suggest that it could represent a novel species.

Fig. 2. Maximum-parsimony tree, inferred from 16S rRNA gene sequences by using filter alp_rr5_nov98. Numbers at bifurcations represent bootstrap values on 1000 pseudo-replicates (values <70% are not shown). Branch-length was calculated with the parsimony interactive tool from the ARB package. Bar, 10 nucleotide substitutions in 100 nt.

Phylogenetic analysis strongly confirmed these results (Fig. 2). Obviously, *E. magnicirratu*s symbionts form a steady cluster and their association within the *Devosia*-like clade is highly supported, regardless of the reconstruction method and filter set used. Sequences of uncultured proteobacteria from different habitats also belong to the same group, suggesting that the genus *Devosia* could be broader and more diversified than considered previously. As already stressed by Nakagawa *et al.* (1996), the *Devosia*-like cluster occupies an independent position in the α -*Proteobacteria* and it is constantly associated with the included species of the rhizobia group in all trees calculated, although these associations are not supported by high bootstrap values.

The preliminary use of oligonucleotide probes for *Archaea* and the major subclasses of *Proteobacteria* revealed that the *E. magnicirratu*s endosymbionts belong to the α -*Proteobacteria*.

Comparison with all 16S rRNA gene sequences in the ARB database confirmed the specificity of the two designed probes. The Dev_819 probe detects all *Devosia* species, *E. magnicirratu*s endosymbionts and all uncultured organisms that are related closely to the described *Devosia* species. No extra-target-group organisms were recognized by this probe; therefore, Dev_819 can be considered as an efficient and satisfactory tool for the detection of *Devosia*-like bacteria. DevEup_993 matched only the three endosymbiont sequences and this indicated its high specificity.

In situ hybridization confirmed these results. Dev_819 hybridized both with endosymbionts from the three strains of ciliate and with *D. riboflavina* DSM 7230^T. DevEup_993 gave a positive result only with ciliate endosymbionts (Fig. 3), whereas there was no detectable signal with *D. riboflavina*. This shows the different specificity of the designed probes, which allows discrimination at the species level within the genus as it is at present. As already stated by Ludwig *et al.* (1998a), the use of an appropriate set of oligonucleotide probes can represent a good and useful tool for rapid identification of bacteria at different taxonomic levels (Amann & Schleifer, 2001). Finally, double hybridization performed with probes DevEup_993 and EUB338 or ALF1b excluded the presence of other α -proteobacterial endosymbionts and, reasonably, of other eubacteria.

Any attempt to grow the symbionts in liquid or solid nutrient broth with added phosphates, a classical culture medium that is also suitable for the related species *D. riboflavina*, failed both at 20 °C and at higher temperature (28 °C). The same negative results were obtained with tryptone soya agar and broth, as well as with marine agar and broth. These results suggest that endosymbionts of *E. magnicirratu*s may have particular metabolic needs, therefore it is likely that they are hardly cultivable with standard cultivation techniques.

Morphological and molecular identities indicate that the

Fig. 3. FISH, demonstrating the identity of 'Candidatus *Devosia euplotis*' by using species-specific bacterial probe DevEup_993. (a) Phase-contrast; (b) corresponding fluorescent picture. Bars, 20 µm. Arrows indicate the host cell in (c), which shows the bacterium at higher magnification. Bar, 10 µm. A colour version of this figure is available as supplementary material in IJSEM Online.

endosymbionts of the three *E. magnicirratu*s strains analysed here belong to the same species. *E. magnicirratu*s is a cosmopolitan, sand-welling ciliate and the strains used were collected in geographical regions as far apart as the Mediterranean and Caribbean Seas. The symbionts were also maintained for years in laboratory cultures. As far as we know, similar symbionts have not been found in

Euplotes-related species that share the same habitat as *E. magnicirratu*s. Therefore, this symbiotic relationship appears to be permanent and species-specific. Phylogenetic analysis assigned the *E. magnicirratu*s endosymbionts to the genus *Devosia*. Until we succeed in cultivation or additional phenotypic data become available, we assign them the provisional status *Candidatus*, as proposed by Murray & Schleifer (1994) and implemented by Murray & Stackebrandt (1995). Based on our characterization, we designate the symbiont as '*Candidatus Devosia euplotis*'.

Moreover, we provide two new probes for the rapid and unambiguous recognition of this novel *Candidatus* species (DevEup_993) and the whole *Devosia*-like group of cultured and uncultured organisms (Dev_819).

Description of '*Candidatus Devosia euplotis*'

'*Candidatus Devosia euplotis*'. The generic name is that of the genus to which, according to phylogenetic analysis, the species was assigned; the specific name is taken from the genus of the ciliate host: *Euplotes* Ehrenberg, 1830.

The 16S rRNA gene sequence obtained from symbionts of *E. magnicirratu*s strain LIV5 from the Ligurian Sea (Italy) was chosen as the reference sequence.

'*Candidatus Devosia euplotis*' [(α -*Proteobacteria*) NC; NA; R (approx. 0.5 μ m wide and up to 2.5 μ m long); NAS (GenBank/EMBL no. AJ548825), oligonucleotide sequence complementary to unique region of 16S rRNA 5'-AAG-TCGTCCTGGTATGTC-3'; S (*Euplotes magnicirratu*s, cytoplasm)]. Vannini *et al.*, this study.

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