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Enigmatic dual symbiosis in the excretory organ of *Nautilus macromphalus* (Cephalopoda: Nautiloidea)

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Symbiosis is an important driving force in metazoan evolution and the study of ancient lineages can provide an insight into the influence of symbiotic associations on morphological and physiological adaptations. In the 'living fossil' *Nautilus*, bacterial associations are found in the highly specialized pericardial appendage. This organ is responsible for most of the excretory processes (ultrafiltration, reabsorption and secretion) and secretes an acidic ammonia-rich excretory fluid. In this study, we show that *Nautilus macromphalus* pericardial appendages harbour a high density of a β-proteobacterium and a coccoid spirochaete using transmission electron microscopy, comparative *16S* rRNA sequence analysis and fluorescence *in situ* hybridization (FISH). These two bacterial phylotypes are phylogenetically distant from any known bacteria, with ammonia-oxidizing bacteria as the closest relatives of the β-proteobacterium (above or equal to 87.5% sequence similarity) and marine *Spirochaeta* species as the closest relatives of the spirochaete (above or equal to 89.8% sequence similarity), and appear to be specific to *Nautilus*. FISH analyses showed that the symbionts occur in the baso-medial region of the pericardial villi where ultrafiltration and reabsorption processes take place, suggesting a symbiotic contribution to the excretory metabolism.

Keywords: symbiosis; cephalopods; *Nautilus*; excretion; bacteria; *16S* rRNA

1. INTRODUCTION

The acquisition and maintenance of one or more organisms by another often leads to novel structures and metabolic pathways. Hence, the study of symbiotic systems has resulted in exciting discoveries, such as the role of symbiotic luminescent bacteria in the morphogenesis of the light organ in squid (*Euprymna scolopes*/*Vibrio fischeri* association; McFall-Ngai & Ruby 2000) or in the assimilation of inorganic molecules from hydrothermal vents by giant tube worms (*Riftia pachyptila* with its sulphide-oxidizing symbionts; Cavanaugh *et al.* 1981; Felbeck *et al.* 1981).

The evolutionary history of both the partners is also a major component of the symbiotic relationship, symbiosis being an important driving force of metazoan evolution (McFall-Ngai 2002). Therefore, the study of symbiotic associations in ancient lineages might provide an insight into the origin of major physiological adaptations. Thus, the symbiotic associations in the excretory organs of *Nautilus* (Schipp *et al.* 1985) are of particular interest, as these invertebrates are considered as 'living fossils' from the Cambrian (542–500 Myr ago). They are also the only extant cephalopods with an external chambered shell (filled with over 90% nitrogen gas) resembling that of extinct ammonoids (figure 1*a*; Norman 2000). A better comprehension of their life history, behaviour, ecology, physiology and metabolism is a means of understanding the success and eventual demise of the ammonoids (Boutilier *et al.* 1996; Staples *et al.* 2000).

In contrast to other extant cephalopods in which several organs are involved in excretion, in *Nautilus*, only the pericardial appendages are responsible for most of the three excretory processes (ultrafiltration, reabsorption and secretion; Martin 1983). The main end product of excretion is ammonia (Boucher-Rodoni 1989) and the contractile villi of the four pericardial appendages collect the blood from capacious sinuses (figure 1*b*) and secrete the acidic ammonia-rich fluid (up to 200 p.p.m., Schipp & Martin 1987) into the pericardial coelom from which it is finally excreted into the mantle cavity (figure 1*a*; Mangold *et al.* 1989).

Each pericardial appendage consists of numerous finger-like villi that are divided into an apical region covered with microvilli and a basal-medial region with crypt-like invaginations. The basal-medial villus segment is filled with a branched lacunar system (Schipp *et al.* 1985). An ultrastructural study of *Nautilus* pericardial appendages revealed symbiotic bacteria forming a dense fringe on the outer epithelium of the villi and its baso-medial invaginations (Schipp *et al.* 1985). Only a single bacterial morphotype was observed with a thin rod shape (0.25–0.35×1.5–2.2 μm) and a typical gram-negative cell wall (Schipp *et al.* 1990). The presence of bacteria in pericardial appendages, an ecological niche rich in

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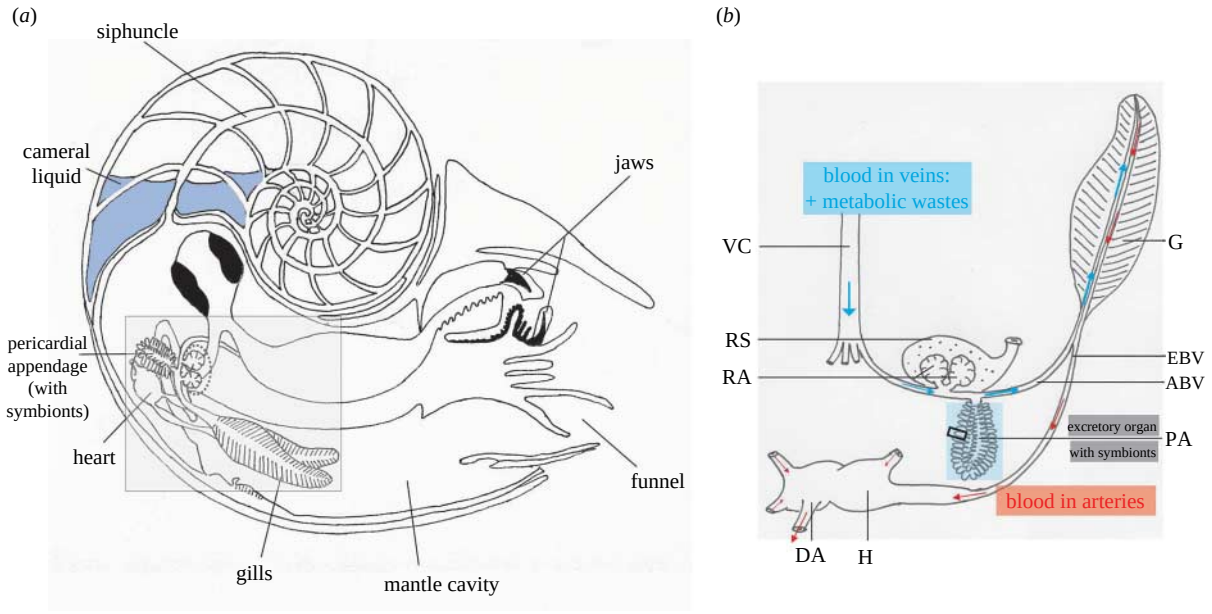

Figure 1. Morphology of *Nautilus macromphalus*. (*a*) Schematic of a longitudinal section through *Nautilus*. (*b*) Details of the inset in (*a*): simplified diagram of the excretory organ (with symbionts) and associated circulatory structures of *Nautilus*. ABV, afferent branchial vein; DA, dorsal aorta; EBV, efferent branchial vein; G, gill; H, heart; PA, pericardial appendage; RA, renal appendage; RS, renal sac; VC, vena cava. Inset, figure 4.

ammonia, has led to several hypotheses about their potential role in the nitrogen metabolism of *Nautilus*, such as (i) detoxification by the symbionts via ammonia oxidation (Schipp *et al.* 1990) or (ii) the use of nitrogenous waste by the bacteria to produce the nitrogen gas filling the *Nautilus* shell and responsible for its neutral buoyancy (Boucher-Rodoni & Mangold 1994).

Nautilus pericardial appendage bacteria were physiologically characterized as Pseudomonodales after their isolation in artificial culture media (Schipp *et al.* 1990). However, it is now widely acknowledged that culture methods reveal only a small fraction of the total bacterial diversity (Amann *et al.* 1990; Pace 1997). The aim of the present study was therefore to characterize the symbiotic bacteria in *Nautilus macromphalus* (Cephalopoda: Nautiloidea) excretory organs using culture-independent molecular approaches. The phylogenetic position of bacteria associated with the pericardial appendages was investigated by comparative sequence analysis of the *16S* rRNA gene. Their localization within the organ was determined by catalysed reporter deposition (CARD) fluorescence *in situ* hybridization (FISH) and transmission electron microscopy (TEM). We also examined the potential function of the bacteria by investigating genes characteristic for nitrogen metabolism, namely *amoA*, *nirS*, *nirK* and *nosZ*, as respective indicators for aerobic ammonia oxidation, nitrite reduction and nitrous oxide reduction (Braker *et al.* 1998; Scala & Kerkhof 1998; Kowalchuck & Stephen 2001). The specificity of this bacterial–invertebrate association and its possible implications in *Nautilus* metabolism are discussed.

2. MATERIAL AND METHODS

(a) *Specimen collection*

Four *N. macromphalus* individuals were collected on the outer shelf of New Caledonia in September 2005. All specimens were dissected aseptically and for each specimen, the four pericardial appendages were processed as follows: two organs were divided into two; one part was immediately frozen in liquid nitrogen and stored at −80°C for DNA extraction and the other fixed in 2% paraformaldehyde for CARD–FISH (see §2*h*). The last two pericardial appendages were fixed for transmission electron microscopy (see §2*b*).

(b) *Transmission electron microscopy*

Pericardial appendages from the four individuals were fixed at 4°C for 12 h in a fixative solution containing 3.2% glutaraldehyde in sterile seawater (pH 7.4) and washed in 0.05% of NaN_3 in sterile seawater (pH 7.4). After a brief rinse in 0.1 M pH 7.2 cacodylate buffer, adjusted to 900 mOsm with NaCl and $CaCl_2$ to improve membrane preservation, samples were fixed for 45 min in 1% osmium tetroxide at room temperature, then rinsed in distilled water, and post-fixed with 2% aqueous uranyl acetate for 1 h before embedding in epon–araldite. Semi-thin sections (0.5 µm thick) and ultra-thin sections (60 nm thick) were obtained using an Ultracut E microtome (LEICA). Transmission electron microscopy analyses were performed on a Philips 201 microscope operated at 75 kV accelerating voltage.

(c) *DNA extraction*

DNA was extracted from the pericardial appendages of four *N. macromphalus* specimens by the method described by Zhou *et al.* (1996) using proteinase K for cell digestion and a standard chloroform–isoamyl alcohol extraction procedure. DNA was precipitated in isopropanol, washed with 70% ethanol, resuspended in 1× TE buffer and stored in aliquots at −20°C.

(d) 16S *rRNA gene PCR amplification*

Bacterial *16S* rRNA gene PCR amplification was performed using GM3 (5′-AGAGTTTGATCMTGGC-3′) and GM4 (5′-TACCTTGTTACGACTT-3′) bacterial primers (*Escherichia coli* positions 8–23 and 1492–1477, respectively, Muyzer *et al.* 1995). The reaction mixture contained 50 pmol of each primer, 6 µg of bovine serum

albumin, 2.5 μmol of each of deoxynucleoside triphosphate, 1× SuperTaq buffer and 0.2 U of Eppendorf Master Taq (Eppendorf, Hamburg, Germany) and the volume was adjusted with sterile water to 20 μl. PCR reactions were conducted in a thermocycler (Eppendorf, Hamburg, Germany), with an initial denaturing step (96°C for 5 min) followed by 25 cycles at 94°C for 1 min, 45°C for 1 min and 72°C for 3 min and a final elongation step at 72°C for 5 min. PCR bias was minimized by using only 25 amplification cycles and pooling four replicate PCR reactions. Amplified DNA was checked by 1.5% agarose gel electrophoresis and purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany).

(e) *Cloning and sequencing*

A *16S* rRNA clone library was constructed for each of the four *Nautilus* specimens. Purified PCR products were cloned by an insertion into a plasmid vector PCR4 TOPO TA Cloning (Invitrogen, Carlsbad, Ca), following the manufacturer's instructions. Positive clones were grown overnight in Luria–Bertani medium and *E. coli* plasmid inserts amplified in a 35 cycle PCR using M13F and M13R primers. The reaction mixture contained 50 pmol of each primer, 6 μg of bovine serum albumin, 2.5 μmol of each deoxynucleoside triphosphate, 1× SuperTaq buffer, 1× Enhancer and 0.2 U of Eppendorf Master Taq (Eppendorf, Hamburg, Germany) and the volume was adjusted with sterile water to 20 μl. The size of PCR products was checked by 1.5% agarose gel electrophoresis.

For each of the four individuals, 87–105 clones were sequenced partially in a variable region of the *16S* rRNA (*E. coli* positions from 518 to approx. 1000), and a total of 81 representative clones were fully sequenced in both directions. After sequence assembly and alignment with SEQUENCHER v. 4.5 (Gene Codes Corporation, Michigan), putative chimeric sequences were eliminated after analysis with BELLEROPHON (Huber *et al.* 2004). Sequencing reactions were performed using the BigDye Terminator v. 3.1 Cycle Sequencing Kit from Applied Biosystems on the ABI Prism 3130xl Genetic Analyser (Applied Biosystems, Foster City, Ca).

(f) amoA, nirK, nirS *and* nosZ *amplification and sequencing*

Functional gene amplification was performed by PCR using specific primers and cycling conditions described by Holmes *et al.* (1995), Rotthauwe *et al.* (1997) and Purkhold *et al.* (2000) for the *amoA* gene (with A189–A682, AmoA1F–AmoA2R and AmoA3F–AmoA4R as specific primers and *Nitrosomonas europea* as a positive control), Braker *et al.* (1998) for the *nirK* gene (with NirK1F–NirK5R as specific primers and *Ochrobactrum anthropi* as a positive control) and the *nirS* gene (with NirS1F–NirS6R as specific primers and *Paracoccus denitrificans* as a positive control) and Scala & Kerkhof (1998) for the *nosZ* gene (with Nos661F, Nos2230R and Nos1773R as specific primers and *Paracoccus denitrificans* as a positive control). Thirty-five PCR cycles were used and PCR products were cloned and sequenced as described previously for the *16S* rRNA gene.

(g) *Phylogenetic reconstruction*

Sequences were compared with those in the database by using BLAST (Altschul *et al.* 1994), FASTA (Pearson & Lipman 1988) and RDP (Cole *et al.* 2005), and highly similar sequences were included in the analysis. The *16S* rRNA sequence data were analysed using the ARB software package (Ludwig *et al.* 2004; www.arb-home.de) and assigned to a database of approximately 40 000 *16S* rRNA sequences. Only sequences above 1300-nt were used for alignment and tree construction. Sequence alignment was performed according to the *16S* rRNA secondary structure with the Integrated Editor and then corrected manually. Preliminary analysis of 35 full-length sequences of *Nautilus* symbionts was performed using the quick-add parsimony methods of ARB to select an appropriate dataset. Phylogenetic trees were then reconstructed using distance and parsimony methods with different filter sets (none, termini, eubacteria and β-proteobacteria) on ARB (Ludwig *et al.* 2004; www.arb-home.de). The 28 sequences were finally analysed by maximum-parsimony (dnapars) and maximum-likelihood (dnaml) methods in the PHYLIP software package (Felsenstein 2002) with bootstrap analyses (1000 replicates) to test the robustness of each topology.

(h) *CARD–FISH*

Pericardial appendage tissues from the four *Nautilus* specimens were fixed for CARD–FISH in 2% paraformaldehyde in sterile seawater at 4°C for 4 h. After two washes in 1× phosphate buffered saline (PBS: 10 mM sodium phosphate and 130 mM NaCl), samples were stored at −20°C in a 1 : 1 mixture of 0.5× phosphate buffered saline and 50% ethanol. Fixed tissues were dehydrated in an increasing series of ethanol and xylene and embedded in paraffin. Histological sections (4 μm) were collected on gelatin-coated slides. Paraffin was removed from the sections with xylene (three 10 min treatments), and the sections were rehydrated in a decreasing ethanol series. Hybridization was performed in preheated chambers containing tissue wetted with hybridization buffer (0.9 M NaCl, 0.02 M Tris–HCl, 0.01% SDS, 1% blocking reagent, 10% dextran sulphate and 35% formamide). The sections were covered with a mixture containing 50 ng of horseradish peroxidase-labelled probe in 150 μl of hybridization buffer and then hybridized as described by Pernthaler *et al.* (2002). The slides were washed with 1× PBS for 10 min, MilliQ water for 1 min, 96% ethanol for 1 min, air dried and covered with an antibleaching agent (e.g. Citifluor AF1, Citifluor Ltd, London) amended with DAPI (final concentration 1 $\mu g\,ml^{-1}$) and a cover-slip. A total of about 120 pericardial appendage sections obtained from the four *Nautilus* specimens were visually examined for symbiont distribution and relative abundance using a DMLB epifluorescence microscope (Leica Microsystèmes SAS, France).

A specific probe was designed for each of the two *16S* rRNA phylotypes found in *N. macromphalus* by using the PROBE_DESIGN function of ARB and the *16S* rRNA accessibility information provided by Behrens *et al.* (2003). The specificity was checked by using BLAST (Altschul *et al.* 1994). The probe specific for the β-proteobacterial symbiont, NauBet66 (5′-AAGCTTCTCTCGTTCCG-3′), was labelled with $Alexa_{488}$ (Invitrogen, Carlsbad, Ca) and the probe specific for the spirochaete symbionts, NauSpiro255 (5′-TGGTACGCTC TTACCCTA-3′), was labelled with $Alexa_{546}$ (Invitrogen, Carlsbad, Ca). Stringency was evaluated at different formamide concentrations (10–50%) and 35% formamide chosen for all hybridizations, as this concentration led to optimal images (high specific signal and low background fluorescence). The general bacteria probe EUB338 (Amann *et al.* 1990) was used as a positive control and the NON338 probe (Wallner *et al.* 1993) as a negative control.

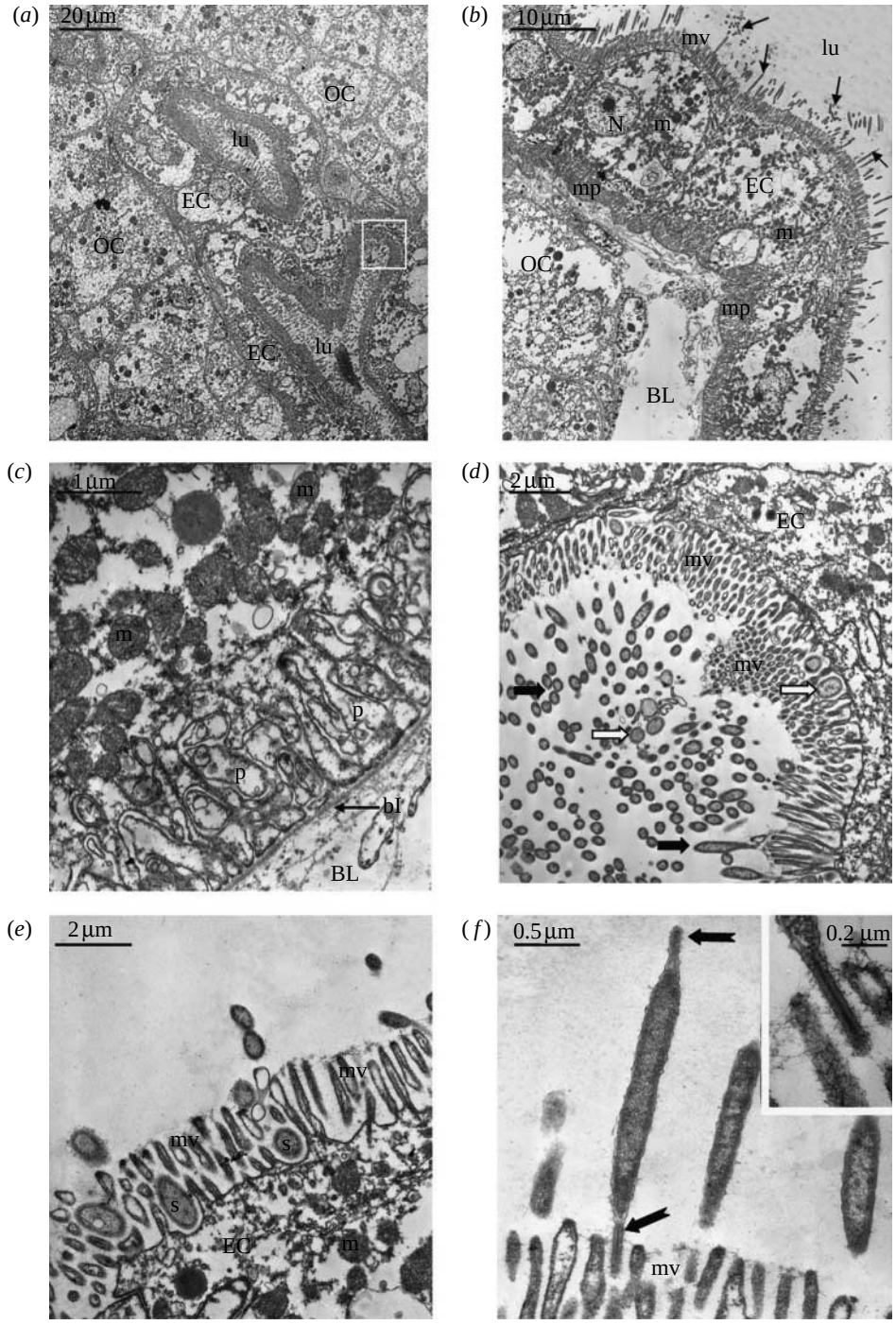

Figure 2. Transmission electron micrographs of *Nautilus macromphalus* pericardial appendages with symbiotic bacteria. (*a*) Transverse section of epithelial invaginations of a pericardial villus. The basal pole of each epithelial cell (EC) constituting the outer excretory epithelium is in contact with a blood lacuna, which is also in contact with ovoid cells (OC) characterized by numerous spherical electron-dense bodies. The apical pole of ECs is covered by bacteria, which are located in the pericardial lumen (lu). (*b*) Higher magnification of the excretory epithelium. Epithelial cells are characterized by numerous mitochondria (m) and the presence of basal infoldings (mp) in contact with the blood lacuna (BL). Bacteria (arrows) are observed either in contact with the microvilli (mv) of the apical pole or in the pericardial lumen (lu). N, nucleus; OC, ovoid cells. (*c*) Details of active transporters next to the blood lacunae. The basal pole of the epithelial cells possesses numerous podocyte-like structures (p) in contact with a strong basal lamina surrounded by mitochondria (m). These structures could represent an active ultrafiltration barrier consuming a large amount of energy provided by the mitochondria. BL, blood lacuna; bl, basal lamina. (*d*) Details of the inset in photo (*a*): distribution of symbiotic bacteria in the pericardial lumen. Two morphotypes of bacteria can be observed in TEM sections (identified by CARD–FISH as β-proteobacteria and spirochaetes; figure 4). The larger bacterial morphotype (white arrows) is located in the lumen or between the microvilli differentiated by the epithelial cells (EC), whereas the smaller bacteria are observed as small transverse sections in the lumen or as elongated bacteria with a parallel orientation to the microvilli (mv, black arrows). (*e*) Details of the spirochaete symbionts. The spirochaetes (s) possess the typical double membrane of gram-negative bacteria and are located between the microvilli (mv) of the epithelial cells (EC). m, mitochondria. (*f*) The β-proteobacteria symbionts are attached to the apical part of the microvilli (mv). These bacteria have a parallel orientation to the microvilli of the epithelial cells. They are characterized by bipolar appendages (black arrows). Inset: details of the polar appendage with a dense central filament. This appendage seems to be in contact with the microvilli through glycocalix fibres.

Table 1. Number of partial (from nucleotide 518 to approx. 1000 based on *E. coli* numbering) and nearly full *16S* rRNA gene sequences (approx. 1450 nucleotides) obtained from cloned PCR products amplified from pericardial appendage DNA of four *Nautilus macromphalus* specimens.

	number of partial sequences			number of full sequences		
specimen source	β-proteobacteria	*Spirochaeta*	total	β-proteobacteria	*Spirochaeta*	total
N1	49	54	103	14	8	22
N2	52	35	87	13	7	20
N3	76	29	105	12	6	18
N4	68	21	89	13	8	21
total	245	139	384	52	29	81

(i) ***Nucleotide sequence accession numbers***

The EMBL accession numbers for the two sequences described in this paper are AM399076 (β-proteobacteria) and AM399077 (spirochaetes).

3. RESULTS

(a) *Transmission electron microscopy*

Ultrastructural observations of pericardial villi in the baso-medial region showed an epithelial layer of excretory cells and a branched lacunar system (figure 2*a*,*b*). This epithelium was characterized by a polar organization consisting in (i) 'labyrinth structures' characteristic of active transporters next to the blood lacunae in the basal part (figure 2*c*), (ii) a high density of mitochondria in the medial part, and (iii) an apical border of high microvilli (figure 2*d*). Higher magnification confirmed a high density of extracellular bacteria associated with the microvilli, with two bacterial morphotypes regularly observed (figure 2*d*). In almost all the tissue sections examined, the coccoid-shaped bacterium ($1.53 \pm 0.20 \times 0.8 \pm 0.07$ μm) was located basally within the microvilli (figure 2*e*), while the rod-shaped bacterium ($0.23 \pm 0.06 \times 2.2 \pm 0.39$ μm) appeared more apically attached by fine filamentous material to the microvilli (figure 2*f*).

(b) *Bacterial diversity and phylogeny*

Comparative bacterial 16S rRNA gene sequence analysis of a total of 385 partially sequenced and 81 fully sequenced clones from four *N. macromphalus* pericardial appendages revealed two distinct bacterial phylotypes (table 1) with low sequence variation within each phylotype (below 0.4%). Phylogenetic analyses placed the two phylotypes within two phylogenetically distinct bacterial groups, the β-proteobacteria and the *Spirochaeta* (figure 3). Neither phylotype is closely related to symbionts from other hosts species or to free-living bacteria (below 90% sequence similarity) and thus they appear to be specific to their host.

The *N. macromphalus* β-proteobacterial symbiont is phylogenetically associated with a clade of free-living, ammonia-oxidizing bacteria from the family Nitrosomonadaceae with *Nitrosospira multiformis* and *Nitrosospira briensis* (Teske *et al.* 1994) as its closest relatives (87.5 and 87.4% sequence similarity, respectively). This phylogenetic affiliation is not strongly supported by bootstrap values (below 60%), but remained stable using different tree-building algorithms (parsimony and maximum likelihood) and filter sets (Hillis & Bull 1993).

The *N. macromphalus* spirochaete symbiont belongs to a monophyletic group that includes free-living spirochaetes and symbionts of gutless marine oligochaetes. Within this group, the *N. macromphalus* spirochaete symbiont forms a highly supported clade (bootstrap value above 99% in all treeing methods) with two free-living spirochaetes: *Spirochaeta bajacaliforniensis* (Fracek & Stolz 1985) and *Spirochaeta smaragdinae* (Magot *et al.* 1997; 89.8 and 89.3% sequence similarity, respectively).

Numerous attempts to amplify genes characteristic for aerobic ammonia oxidation (*amoA*), nitrite reduction (*nirSK*) and nitrous oxide reduction (*nosZ*) were unsuccessful, despite the use of a wide array of different conditions and successful amplification of these genes in positive controls.

(c) In situ *identification*

Examination of pericardial appendage longitudinal sections after hybridization with the universal Eub388 probe confirmed the presence of bacteria in the baso-medial region of *N. macromphalus* pericardial villi, whereas the apical part of these villi was devoid of any bacteria (figure 4*a*). Hybridization with probes specific to the β-proteobacterial and spirochaetal sequences found in *N. macromphalus* showed the coexistence of these two symbiotic bacterial phylotypes in all the examined specimens (figure 4*b*). The observed distribution of the spirochaetal and β-proteobacterial symbionts was similar in FISH and TEM sections. The β-proteobacterium-specific probe (NauBet66) hybridized to thin, rod-shaped bacteria was distributed principally in the invaginated epithelial areas (figure 4*c*,*d*). The spirochaete-specific probe (NauSpiro255) hybridized with coccoid bacteria was generally found in the peripheral areas and closely associated with the pericardial epithelium (figure 4*c*,*d*). A non-quantitative, visual comparison of pericardial appendages from the four *N. macromphalus* specimens showed a similar coexistence and distribution of the two symbiont phylotypes in most of the 120 sections examined. The hybridization patterns of the probes specific to the β-proteobacterial and spirochaetal symbionts of *N. macromphalus* corresponded to those from the general eubacterial probe Eub388, suggesting that the two specific probes revealed all the bacteria present in the pericardial appendages.

4. DISCUSSION AND CONCLUSION

The present exhaustive molecular analysis (more than 100 clones per specimen) of *N. macromphalus* pericardial appendages in combination with FISH clearly shows that these organs harbour only two symbionts, a β-proteobacterium and a spirochaete. This result supports

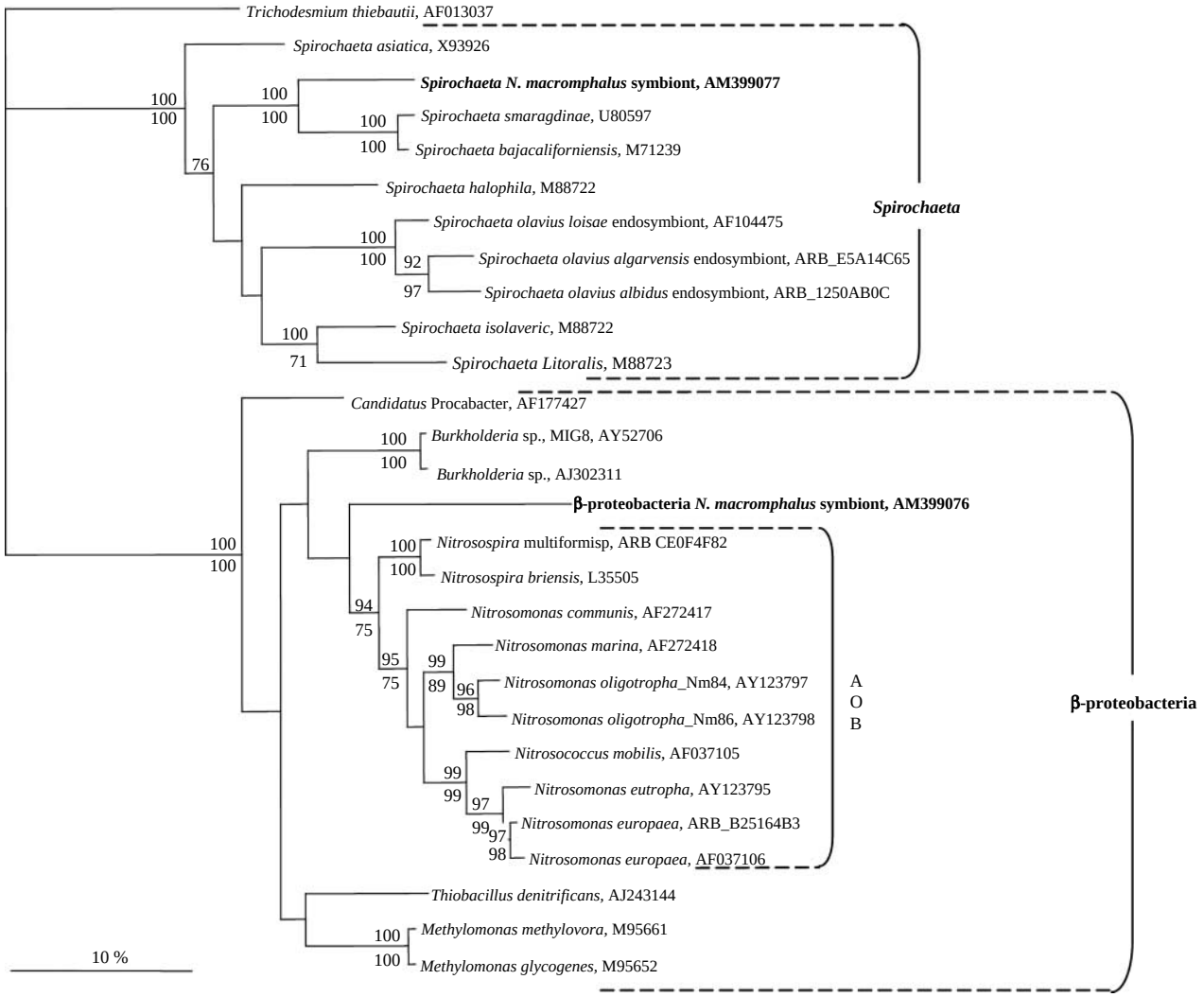

Figure 3. The phylogenetic relationships of the spirochaetal and β-proteobacterial symbionts of *N. macromphalus* (bold) inferred from *16S* rRNA gene analysis using maximum likelihood (ML; 1411 sites analysed). Numbers at each branch point are the bootstrap values for percentages of 1000 replicate trees calculated by MP (upper) and ML (lower) methods. Only values above 60% are shown. Two cyanobacterial species (*Trichodesmium thiebautii*: AF013027 and *Merismopedia glauca*: AJ781044) were used as outgroups. AOB, ammonia-oxidizing bacteria.

the hypothesis of a culture-related bias in the detection of γ-proteobacteria isolated in previous studies from cephalopod symbiotic organs (Schipp *et al.* 1990; Barbieri *et al.* 2001; Pichon *et al.* 2005; Pernice *et al.* 2007).

Symbioses for recycling nitrogenous waste products have evolved in a number of terrestrial invertebrates' lineages (Cochran 1985; Sasaki *et al.* 1996; van Borm *et al.* 2002; Schramm *et al.* 2003) and in gutless marine oligochaetes (Woyke *et al.* 2006). The *N. macromphalus* bacterial symbiotic association occurs in a highly specialized organ, unique among cephalopods, and responsible for most of the excretory processes. The bacteria associated with the pericardial appendages are not closely related to any known bacteria. In addition, the β-proteobacteria symbionts are phylogenetically affiliated to Nitrosomonadaceae, an ammonia-oxidizing lineage that, to our knowledge, has not previously been found in symbiotic associations with animals.

The exact mechanisms by which the symbionts are involved in *N. macromphalus* excretion remain to be clarified. However, these bacteria are assumed to be involved in such a symbiotic function for several reasons: (i) the attachment and concentration of symbionts at the outer epithelium in the baso-medial region of the pericardial villi suggest that they could be active in ultrafiltration and/or reabsorption processes (figure 4*a*), (ii) the ultrastructure of this outer epithelium and its polar organization are characteristic of an energy-requiring, transport active tissue (Martin 1983; figure 2*a–c*), and (iii) the bacteria live in an ammonia-rich environment (Schipp & Martin 1987).

Cephalopods are ammonotelic, i.e. more than 70% of their nitrogenous waste is excreted as ammonia (Boucher-Rodoni 1989). Excretion rates of ammonia are surprisingly low in *Nautilus*, and it has been suggested that this waste compound is used for buoyancy through bacterial transformation of ammonia to dinitrogen gas (Denton 1974; Boucher-Rodoni & Mangold 1994). Direct transformation of ammonia to nitrogen is only known to occur under anaerobic conditions in the so-called anammox reaction driven by the recently discovered anaerobic planctomycete bacteria (Strous *et al.* 1999). In contrast, there are no known aerobic bacteria that can directly oxidize ammonia to nitrogen. Under oxic conditions in a process called nitrification, ammonia is first oxidized to nitrite by (among others) β-proteobacteria of the genera *Nitrosomonas* and

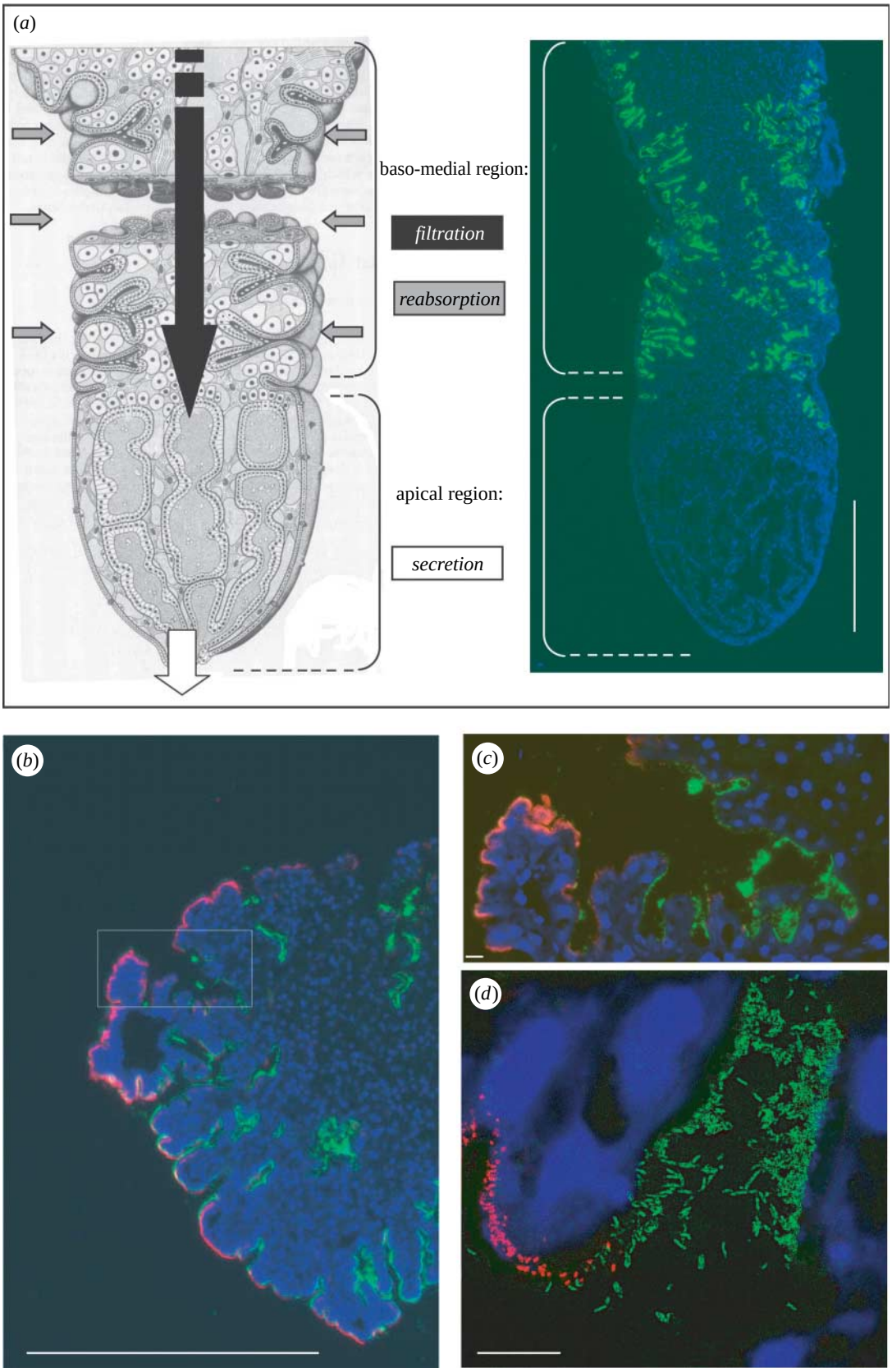

Figure 4. Schematic diagram (modified from Schipp *et al.* 1985, with kind permission of the author and Springer Science and Business Media) and distribution of *N. macromphalus* symbionts in a pericardial appendage villus. (*a*) Details of the inset in figure 1*b*: schematic diagram of a longitudinal section through a villus and general distribution of the symbiotic bacteria (CARD–FISH with universal eubacterial probe EUB388 in green). Direction of blood flow: secretion (white arrow), filtration (black arrow) and reabsorption processes (grey arrows). Scale bar, 500 μm. (*b–d*) Multicolour CARD–FISH images of *N. macromphalus* symbionts in transversal sections of a pericardial villus. The spirochaete symbionts (NauSpiro255 probe in red) are closely associated with the pericardial epithelium in peripheral areas. The β-proteobacterial symbionts (NauBet66 probe in green) are found in peripheral and invaginated areas and are less closely associated with the epithelium. In blue, *Nautilus* tissue is stained with DAPI. (*b*) Scale bar, 500 μm; (*c*,*d*) scale bar, 10 μm.

Nitrosococcus and nitrite is further oxidized to nitrate mainly by α-proteobacteria of the genus *Nitrobacter*. Transformation of nitrate and nitrite to dinitrogen gas, i.e. denitrification, occurs under anoxic conditions and is driven by a wide range of very different bacteria.

No bacteria belonging to the Planctomycetales that are to date the only bacteria known to oxidize ammonia anaerobically to nitrogen were found in *N. macromphalus*. What we did find is β-proteobacteria most closely related to the nitrifiying bacteria that oxidize ammonia to nitrite. However, none of the genes involved in these processes such as *amoA*, *nirKS* and *nosZ* were found in DNA extracted from *N. macromphalus* pericardial appendages so that other metabolic pathways of the symbionts must be considered.

An alternative hypothesis is that the *N. macromphalus* β-proteobacterial symbionts are protein degraders, as suggested for the *Acidovorax* symbionts of earthworms (Schramm *et al.* 2003; Davidson & Stahl 2006) that are also β-proteobacteria, although only very distantly related to the *N. macromphalus* β-proteobacterial symbionts. The coelomic fluid proteins could thus be degraded to peptides and amino acids by the symbionts. This would facilitate their reabsorption and enable the host to conserve valuable nitrogen compounds otherwise lost through excretion.

Free-living spirochaetes are not only widespread in marine and limnic environments, but are also commonly found as commensals or parasites in animals and humans (Dubilier *et al.* 1999; Cabello *et al.* 2001; Prescott *et al.* 2003; Dröge *et al.* 2006). One of the closest relatives to the *N. macromphalus* spirochaete symbiont is *S. smaragdinae*, a free-living, obligate anaerobe that ferments carbohydrates (Magot *et al.* 1997), suggesting fermentation as one possible metabolic pathway of the *N. macromphalus* spirochaetes. However, these symbionts could also have a completely different metabolism, just as spirochaete symbionts in termites, which are involved in the nitrogen nutrition of their host (Tayasu *et al.* 1994; Lilburn *et al.* 2001).

Unexpectedly, the morphology of the *N. macromphalus* spirochaete symbionts observed with TEM (figure 2*e*) and FISH (figure 4*d*) was not the typical helical-shaped spirochaete but rather a coccoid morphotype. However, the existence of coccoid spirochaetes was recently shown in anoxic freshwater sediments (Ritalahti & Loffler 2004) and in the digestive tract of termites (Dröge *et al.* 2006). The presence of such coccoid spirochaetes in *N. macromphalus* pericardial appendages supports the hypothesis that 'non-spiral spirochaetes' are widespread in nature and not restricted to specialized ecosystems or to the termite hindgut (Dröge *et al.* 2006).

The high density of bacteria in *N. macromphalus* pericardial appendages, as estimated previously by flow cytometry (approx. 150×10^6 bacteria per gram of fresh tissue; Pernice *et al.* 2007), indicates that these symbionts may play an important role in the physiology of their host. Further investigations are needed to understand how exactly the β-proteobacteria and spirochaete symbionts are involved in *N. macromphalus* excretion. This could include the use of ^{15}N tracer experiments coupled with multiple isotope imaging mass spectrometry to measure the assimilation of nitrogen compounds by symbionts in *N. macromphalus* pericardial appendages tissue at the cellular level (Luyten *et al.* 2006).

A further point of interest in the *N. macromphalus* symbiosis is the understanding of the factors that contribute to the distribution patterns of β-proteobacteria and spirochaete symbionts in the pericardial appendages. Indeed, the predominance of the β-proteobacteria symbionts in the cavities formed by baso-medial invaginations was clearly observed by CARD–FISH (figure 4*b–d*), while the spirochaete symbionts were evidenced mainly in the peripheral areas. Such spatial distribution may reflect an ecological equilibrium between the two symbiotic phylotypes. This allows the comparison of the *N. macromphalus* pericardial appendages with a micro-ecosystem harbouring two bacterial groups that have established their own ecological niche. Additional examination of *N. macromphalus* pericardial appendages by combining CARD–FISH with microsensor technology will help to relate the distribution of β-proteobacteria and spirochaete symbionts to microscale gradients of key factors, such as oxygen, ammonia, nitrite and nitrate (De Beer *et al.* 1997).

Finally, preliminary analyses of 16S rRNA sequences from *N. pompilius* specimens showed that *N. macromphalus* and *N. pompilius* share highly similar symbiont phylotypes (above 99.5% identity), suggesting that this symbiosis could be widespread among *Nautilus* species. With symbiosis being an important driving force of metazoan evolution (McFall-Ngai 2002), it would then be of interest to explore the *N. macromphalus* dual symbiosis from an evolutionary point of view to better understand where and when this symbiosis evolved.

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