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RESEARCH ARTICLE

Primary co-culture as a complementary approach to explore the diversity of bacterial associations in marine invertebrates: the example of *Nautilus macromphalus* (Cephalopoda: Nautiloidea)

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Abstract The recent application of molecular tools to address associations between bacteria and marine invertebrates has provided access to an immense diversity of unidentified microbes resistant to cultivation. However, the role of bacteria as partners in animal physiology remains unclear and in most cases difficult to investigate in the absence of adequate condition of cell growth and proliferation. In this work, we studied the reservoir of microbes associated with the excretory organs of *Nautilus macromphalus* as a model. Using the bacterial 16S RNA gene as a marker, we compared three complementary approaches for bacterial detection: bacterial DNA extraction from *N. macromphalus* tissues ("molecular approach"), strain isolation to provide a bacterial culture collection ("microbiological approach") and finally, maintenance of *N. macromphalus* excretory organ cells with associated bacteria ("cellular approach"). Our results stress the potential of the "cellular approach" as a promising new tool as it promotes the detection of as yet uncultured β-proteobacteria and spirochaetes associated with *N. macromphalus*, and serves as a foundation for future studies describing potential roles that these bacteria may play in *Nautilus*.

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Introduction

Association with microorganisms is common in marine invertebrates where numerous examples of bacterial associations are known across many phyla (Kushmaro et al. 1996; Dubilier et al. 2001; Webster et al. 2001; Kimura et al. 2003; Rajan 2005; Hentschel et al. 2006). Recent studies of such associations have led to identification of novel bacterial taxa, and also to the characterization of new bioactive substances produced by such bacteria, which opens a promising alternative source of natural products (Holmström and Kjelleberg 1999). However, studies of invertebrate–bacteria associations are usually hampered by the complexity of many bacterial consortia and serious technical challenges such as the resistance of symbionts to culturing attempts (Piel et al. 2005).

Development of molecular tools and more particularly, the comparison of 16S rRNA gene sequences for bacterial taxonomy led to a breakthrough in the identification of noncultivable bacteria, which are estimated to account for over 90% of environmental strains (Amann et al. 1990; Pace 1997). Nevertheless, bacterial identification based either on molecular culture-independent or microbiological culture-dependent techniques is biased and yields different bacterial genotypes depending on the method used (Eilers et al. 2000; Connon et al. 2005). Moreover, characterizing bacterial metabolic potential in the absence of pure cultures presents a serious challenge for microbial ecologists.

Obviously, investigations of the role of bacteria and their interactions with the host biology may benefit from multidisciplinary approaches. This integrative area of research builds on advances in technology and the development of suitable animal–bacterial models (McFall-Ngai 2002; Schirmer et al. 2005).

Among marine invertebrate taxa with bacterial symbiosis potential, cephalopods are a model of particular interest, where bacterial associations have been known for a long time (Pierantoni 1917 and Bloodgood 1977). Among the 700 species of this marine molluscan group with a worldwide distribution, a high diversity of bacteria accumulates in the reproductive organs (accessory nidamental glands) of myopsids, sepiolids, and sepiids (Kaufman et al. 1998; Grigioni et al. 2000; Pichon et al. 2005). The marine luminous bacterium *Vibrio fischeri* is present in the light organs of sepiolids (McFall-Ngai and Ruby 1991) but also *Photobacterium* can colonize sepiolids and loliginids light organs (Nishiguchi et al. 1998; Nishiguchi and Nair 2003).

In the present paper, we focused on bacteria associated in the pericardial appendages of *Nautilus macromphalus* (Cephalopoda: Nautiloidae) (Schipp et al. 1985). *Nautilus* is the last representative of ectocochleate cephalopods. Its distribution is presently restricted to the Indo-Pacific, where it inhabits the outer shelf of the barrier reef, generally at depths between 300 and 500 m (Boucher-Rodoni and Mangold 1994). A high density of bacterial symbionts has previously been localized by transmission electron microscopy (TEM) in the brush border of the pericardial appendages epithelium in two species (*N. macromphalus* and *Nautilus pompilius*) (Schipp et al. 1990). Previous attempts were made to isolate pericardial appendages associated bacteria by classical microbiological culture on artificial media, but this yielded only bacteria characterized as *Pseudomonadales*. Since then, it has been acknowledged that this microbiological approach reveals only a small fraction of total bacteria diversity (Connon et al. 2005). Moreover, it was previously demonstrated that culture on artificial media can induce a bias in favour of γ-proteobacteria comparatively to DNA extraction from host organs (Barbieri et al. 2001; Pichon et al. 2005). Therefore, the use of more than one approach seems necessary for an exhaustive assessment of bacterial diversity.

Although present bacterial identification is achieved using molecular tools, such analysis was lacking for the pericardial appendages bacterial symbionts. The aim of the project was to characterize the diversity of the bacterial classes associated to *N. macromphalus* pericardial appendages, based on 16S rRNA gene comparison, using three complementary approaches: (a) a molecular approach that was culture-independent, based on bacterial DNA extraction from *N. macromphalus* tissues, to obtain a library of 16S rDNAs representative for the associated bacterial community, and two culture-dependent methods based on propagation in artificial media; (b) a microbiological approach which used more classical methods of isolation to provide a bacterial culture collection and (c) a cellular approach which provided a novel in vitro establishment of the bacterial–host symbiotic complex, using primary co-cultures of host pericardial appendages cells with their associated bacteria.

Materials and methods

Specimens' collection

Five *N. macromphalus* were collected (three specimens in 2003 and two in 2005) on the outer shelf of New-Caledonia. Specimens were shipped live to Paris, and subsequently kept in seawater tanks (150 l, 18–20°C, and salinity: 35 ppt). All specimens were dissected aseptically, and for each specimen the four pericardial appendages were processed as follows: the first pericardial appendage was divided in two, one part was stored at −80°C for the molecular approach and the other fixed in 2% paraformaldehyde (PFA) for flow cytometry quantification. The second pericardial appendage was dilacerated in 1 ml sterile seawater to analyse the culturable bacterial fraction by a culture media and laboratory conditions (microbiological approach). The third pericardial appendage was dissociated for the cellular approach, and the fourth pericardial appendage was fixed for in situ histological observations.

DNA isolation

Molecular approach

For three specimens DNA was extracted from pericardial appendages using the DNAeasy tissue Kit (Qiagen, Courtaboeuf, France) protocol and stored in aliquots at −20°C prior to 16S rRNA gene amplification and sequence analysis.

Microbiological approach

For three specimens, 250 mg of pericardial appendages tissue were ground in 1 ml sterile seawater, and serial dilutions (10 and 100) were spread onto marine agar 2216 culture media (DIFCO laboratories, Detroit, MI, USA). Plates were incubated at 18°C for 48–72 h in

order to obtain a collection of cultivable bacteria. Cell titration was estimated by enumerating colony-forming units (CFUs). Twenty isolated strains per specimen were stained to determine Gram reactivity and physiologically characterized. Representative isolates were then selected for 16S rRNA gene sequencing.

The method used to prepare bacterial DNA for PCR was derived from Sritharan and Barker (1991). Colonies grown on marine agar 2216 were suspended in 100 µl of sterile water, boiled for 5 min, centrifuged (15,000×*g*, 5 min) and stored in aliquots at −20°C prior to 16S rRNA gene PCR amplification, purification and direct sequencing.

Cellular approach

Culture media For three specimens, one intact pericardial appendage was rinsed for 90 min at room temperature in 25 ml of modified Locke solution, pH 7.5 (Wimmer et al. 1999) with penicillin (500 U/ml), streptomycin (500 µg/ml) and bacitracin (250 U/ml) to wash off potential surface contaminants. Tissue was then dissociated by two successive digestions in trypsin 0.2% (w:v) in modified Locke's solution at pH 7.5, for 15 min at 25°C. Both successive suspensions were pooled on ice and centrifuged 10 min at 200×*g* to pellet the dissociated cells and associated bacteria. Pellet was washed once in calcium and magnesium-free seawater (CaMgFSW): 14.5 g NaCl/0.38 g KCl/0.5 g Na_2SO_4/0.125 g $NaHCO_3$/20 ml Tris–HCl 0.5 M pH 8.0 per 500 ml of deionized H_2O (Frank et al. 1994). Cephalopod cells were then enriched from the mixed bacterial and cellular suspension by differential density sedimentation over a 2% sucrose cushion in CaMgFSW (10 min centrifugation at 200×*g* at room temperature). The pellet enriched in cephalopod cells was resuspended in the following culture medium without antibiotics: supplemented DMEM (Gibco 41965, Invitrogen, Cergy-Pontoise, France, containing 10.8 g NaCl/0.18 g KCl/0.6 g $CaCl_2$–$2H_2O$/5.1 g $MgCl_2$–$6H_2O$/0.5 g Na_2SO_4/2.38 g Hepes pH 7.8 per 500 ml), half diluted with artificial sea water (14 g NaCl/0.38 g KCl/0.73 g $CaCl_2$–$2H_2O$/5.22 g $MgCl_2$–$6H_2O$/0.5 g Na_2SO_4/0.12 g $NaHCO_3$/2.38 g Hepes pH 7.8 per 500 ml of deionized H_2O). Final pH of the medium was 7.8. Cells and the bacteria still associated after the dissociation and enrichment treatments were plated at about 1×10^6 cells/ml into plastic culture dishes and Lab-tek chambers (Sigma-Aldrich, St Quentin Fallavier, France) pre-coated with collagen 0.01% to promote adherence of cells; cultures were incubated at 18°C with 1 ml medium addition once every week.

Metabolic activity measurements Cephalopod cell density was evaluated by cell counting on an haemocytometer and viability was estimated at 7 and 21 days post plating with trypan blue assay (counting number of cells excluding trypan blue) (Phillips 1973). Global metabolic activity of the co-cultures was assessed at 7 and 21 days post plating by a colorimetric MTT reduction assay adapted from Domart-Coulon et al. (1994). This assay measures the reduction of the yellow tetrazolium salt (MTT) into blue formazan by mitochondrial dehydrogenase enzymes present in active cells. Optical density is proportional to the number of viable cells (Mosmann 1983) which was confirmed by testing linearity between cell and optical density. Global metabolic activity was measured at plating and after 7 and 21 days. Supernatant was discarded from the primary co-cultures and the adhering cells, and bacteria were detached from the substrate by gentle trypsinization (15-min incubation at room temperature with 0.05% trypsin in Locke solution) and scraping. The resulting suspension was centrifuged at 200×*g* for 10 min at room temperature. The supernatant, containing the adherent bacteria of the primary co-culture, was used for bacterial DNA extraction and 16S rRNA gene analysis, and 100 µl was spread on marine agar Difco 2216 to try to isolate bacterial strains. The pellet, containing cephalopod cells and bacteria still associated after gentle trypsinization, was resuspended in cell culture medium. Cephalopod cell density was counted on an haemocytometer and adjusted to about 500,000 cell/ml and 100 µl serial dilutions were placed in a 96-well Nunc microplate with three to four replicates per dilution. Ten microlitres of a 5 mg/ml stock solution of MTT (Sigma-Aldrich, St Quentin Fallavier, France) was added and the plate was incubated for 3 h at 20°C followed by 1 h at 37°C. To dissolve the blue formazan crystals formed, 100 µl isopropanol–HCl 0.04 N was added and the plate was shaken 10 min at room temperature. Absorbance at 570 nm was read on an Elisa plate-reader (Serlabo, Trappes, France) with 630 nm reference. Metabolic activity of 100,000 cells was calculated from the slope of the linear regression analysis of absorbance relative to cell density.

DNA extraction The adherent bacteria of the primary co-cultures, collected at 7 and 21 days after trypsinization of the cell–bacteria layer covering the substrate were taxonomically classified by 16S rRNA gene analysis. Supernatant (1.5 ml) of the low speed 200×*g* centrifugation was sampled and centrifuged at high speed, 15,000×*g* for 5 min. DNA was extracted from the bacterial pellets and stored in aliquots at −20°C for 16S rRNA gene amplification and sequence analysis.

16S rRNA gene analysis

PCR amplification

Bacterial 16S rRNA gene PCR amplification was performed using the universal bacterial primers: 27F-1385R pairs (respectively, *Escherichia coli* position 9: 5′-GAGTTTGATCCTGGCTCA-3′ and position 1385: 5′-CGGTGTGTRCAAGGCCC-3′). The reaction mixture contained 0.3 µmol of each primer, 25 µmol of each deoxynucleoside triphosphate, 1× SuperTaq buffer and 1.25 U of SuperTaq polymerase (ATGC, Marne la Vallée, France) and volume was adjusted with sterile water to 50 µl. PCR reactions were conducted in a thermocycler (T personal PCR system; Biolabo Scientific Instruments, Archamps, France) with an initial denaturing step (94°C for 5 min) followed by 32 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min and a final termination step at 72°C for 7 min. Amplified DNA was checked by 1.5% agarose gel electrophoresis and purified with a QIAquick PCR purification kit (Qiagen, Courtaboeuf, France).

Cloning and sequencing

Purified PCR products from both molecular and cellular approaches were cloned by insertion into plasmid vector PCR 2.1 TOPO TA Cloning (Invitrogen, Cergy-Pontoise, France) following the manufacturer instructions. Positive clones were grown overnight in 1.5 ml Luria-Bertani Lennox culture media (DIFCO Laboratories, Detroit, MI, USA), and plasmids were prepared from pelleted cells with a QIAprep Miniprep kit (Qiagen, Courtaboeuf, France).

Partial (700 bp) sequencing was processed on both clones and isolates by Genome Express (Meylan, France) in a variable region of the 16S rRNA gene (*E. coli* positions 9–710 bp). Putative chimeric sequences were checked with Bellerophon (Huber et al. 2004). Sequences without any PCR artefacts (score < 1) were selected for 16S rRNA gene classification.

16S rRNA gene classification

The obtained sequences were first clustered into operational taxonomic units (OTUs) with similarities > 97% (percentage of similarities defining a genus; Stackebrandt and Goebel 1994) by using the sequence identity matrix function in BioEdit (Hall 1997–2001). Sequences were then assigned to a set of hierarchical taxa using a naïve Bayesian rRNA classifier (RDP Release 9.23 classifier) with a confidence indice estimated by levels of bootstrap based on a 100 resampling data set (Cole et al. 2005).

Nucleotide sequence accession numbers

The EMBL accession numbers for representative sequences of each OTU described in this paper are: AM048782 (*Pseudomonadales*); AM048783 (*Oceanospirillales*); AM048784 (*Alteromonadales*); AM048776 (*Spirochaetes*); AM048778 (*β-Proteobacteria*).

Preparation for in situ observations

Pericardial appendages were fixed in a 3% PFA solution as described by Grigioni et al. (2000) and dehydrated in 100% ethanol overnight before embedding in paraffin. Histological sections (7 µm) were cut and collected on gelatine-coated slides. Paraffin was removed from the sections with xylene (three 10-min treatments) and a rehydration step was performed in decreasing ethanol series (100, 95, 70, 50%). Primary co-cultures were directly fixed in a 3% PFA solution (4°C for 4 h) in Lab-tek chambers.

Diamidino-2-phenylindole (DAPI) staining was performed on both histological sections and primary co-cultures in Lab-tek slides with a 10/1 mix solution of permeability buffer (0.9 M NaCl, 0.02 M Tris–HCl, 0.01% SDS, 30% formamide) and 0.001% DAPI solution (Boehringer, Germany). The slides were visually analysed with a DMLB (Digital Microscope for Laboratory Biology) epifluorescence microscope (Leica Microsystèmes SAS, France).

Flow cytometry measurements

After fixation in 2% PFA solution and storage at −80°C, the organ was ground and centrifuged (3,000×*g*, 15 min). The supernatant was stained with a 5 µM final concentration of SYBRgreen (Qiagen, Courtaboeuf, France) and analysed in a FluxFacsCalibur cytometer (BectonDickinson, San Jose, CA, USA) with an Argon laser (488 nm, 15 mW), calibrated with fluorescent balls (Polysciences Inc., Warrington, PA, USA, 0. 94 µm in diameter). Bacteria density was estimated by measuring fluorescence signal emission at 530 nm and biovolume valued by right-angle light scatter (Marie et al. 1997).

Statistical analysis

In order to analyse the variation of microbial diversity between the two clone libraries (molecular and cellular approaches), coverage indices were calculated, with

the formula $[1 - (n/N)] \times 100$, where n is the number of single clone OTU and N is the total number of sequences for each approach (Good 1953).

Relative frequencies of the various OTUs were first calculated in each *N. macromphalus* specimen, and homogeneity of the data between the three replicate specimens was checked by a chi-square test at the 5% level of significance ($P < 0.05$) (Zar 1999). Throughout the paper, results are presented as mean ± SE.

Results

Diamidino-2-phenylindole staining of *N. macromphalus* pericardial appendages histological sections confirmed a high density of bacteria in close proximity to the surface epithelium (Fig. 1a, b). Flow cytometry based on fluorescence and right-angle light scatter gave estimates of bacterial density between 120 and 160×10^6 bacteria/g [fresh wt]$^-$ of tissue.

The number of colonies formed after 48-h growth in marine agar 2216 indicated that the total heterotrophic bacterial flora amenable to microbiological culture conditions was about 2×10^5 CFU/g [fresh wt]$^-$ of tissue. Thus, only about 0.15% of the total bacterial flora was cultivable in classic marine microbiological medium.

A total of 60 bacterial isolates from three individuals were used for Gram staining and physiological tests (Table 1). Gram staining revealed a diverse community of rod-shaped Gram-negative bacteria. No Gram-positive strains were present among the cultivable heterotrophic flora. Physiological and morphological screening allowed grouping of 60 isolates into three main bacterial profiles. Nine representative strains (three of each physiological profile) were selected for 16S rRNA gene analysis. Sequences identity analysis clustered 16S rRNA gene sequences within three OTUs (similarity > 97%): *Alteromonadales*, *Pseudomonadales* and *Oceanospirillales* (100% in RDP confidence level).

After total DNA isolation and bacterial 16S rRNA gene amplification, random sequencing was performed for 30 clones. Sequence identity analysis on BioEdit designated three OTUs corresponding to 95.8% of coverage (Table 2). Sequence variation within each OTU among the three specimens was very low (0–0.6%) confirming detection of the same phylotype. The RDP classifier assigned OTUs sequences to three bacterial clusters: *β-Proteobacteria*, *Spirochaetes* and

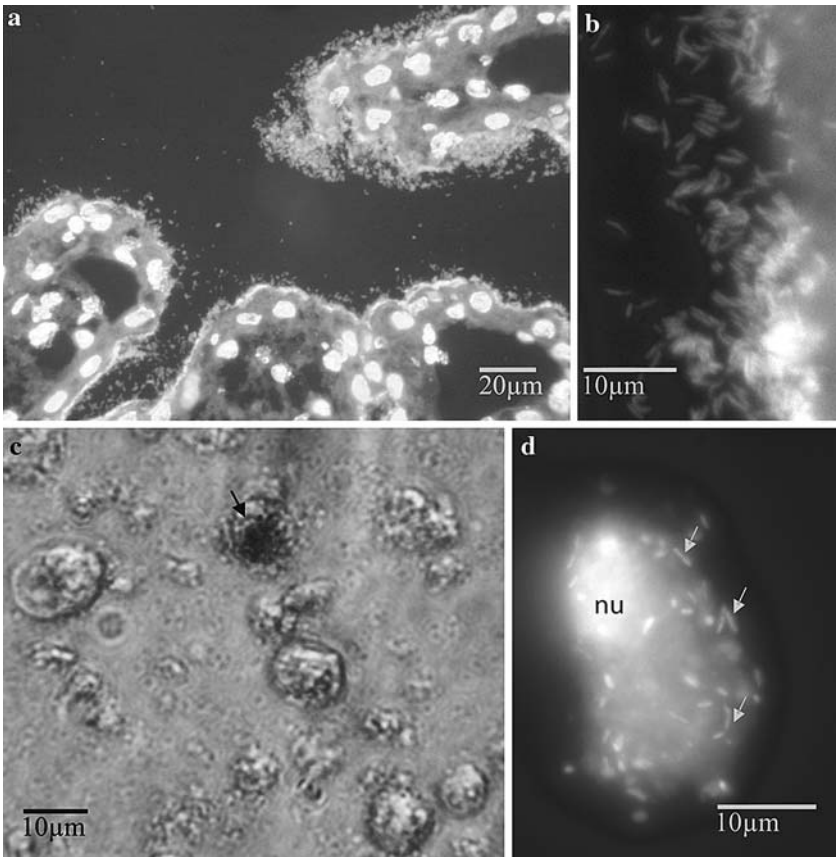

Fig. 1 In situ observations of *N. macromphalus* pericardial appendages associated bacteria. **a** and **b** Histological section of the organ (DAPI staining) showing bacteria in close proximity to the epithelial cell surface; **c** and **d** primary co-culture of pericardial appendages cells and associated bacteria showing **d** close contact between bacteria and an isolated cell (*grey arrows*) after 8 days of primary co-culture (DAPI staining) and **c** reduced formazan crystal (*black arrow*) at the surface of pericardial appendages cell bacteria complex after 21 days primary co-culture. *nu* nucleus

Table 1 Phenotypic traits and RDP classification of isolates from *N. macromphalus* pericardial appendages

Isolates	*Alteromonadales* (RDP 100%)	*Oceanospirillales* (RDP 100%)	*Pseudomonadales* (RDP 100%)
Colony colour on marine agar	Beige	Beige	Creamish
Morphology	Rods	Rods	Straight rods
Gram stain	–	–	–
Catalase	+	+	+
Oxydase	±	–	+
Growth on 2% NaCl media	+	+	+
Growth on 10% NaCl media	+	–	–
N_2 production	–	–	±
H_2S production	–	–	–
Fermentation	–	–	–

Isolates were tested for: presence/absence of catalase; presence/absence of oxydase, growth on 2 and 10% NaCl media, N_2 production on a modified marine agar media with 0.1% of KNO_3, H_2S production on a modified marine agar media with 0.08 g of sodium thiosulfate, fermentation in anaerobic conditions on a modified marine agar media (peptone: 2 g/l; glucose: 10 g/l; 0.0004% of bromothymol blue, pH 7.4)

± positive results were not obtained for all strains tested, *RDP* confidence level in the assignment to a taxonomical group

Table 2 Number of partial sequences and mean relative abundance obtained from bacterial isolates (microbiological approach), cloned PCR products amplified from pericardial appendages tissue (molecular approach) and primary cell cultures with associated bacteria (cellular approach) for three replicate *N. macromphalus* specimens

	N. macromphalus specimen	Number of sequences			
		β-Proteobacteria (RDP: 88%)	*Spirochaetes* (RDP: 88%)	*γ-Proteobacteria* (RDP: 100%)	Chimeras
Microbiological approach ($n = 9$)	N1	–	–	3[a]	–
	N2	–	–	3[a]	–
	N3	–	–	3[a]	–
	Relative abundance	–	–	100%	
Molecular approach ($n = 30$; c =95.8%)	N1	7	1	–	2
	N2	7	1	–	2
	N3	6	1	1	2
	Relative abundance	83.3 ± 2.4%*	12.5%*	4.2 ± 2.4%*	
Cellular approach ($n = 30$; $c = 100\%$)	N2	8	1	–	1
	N3	6	2	–	2
	N4	8	0	–	2
	Relative abundance	88 ± 4.6%*	12 ± 4%*	–	

n total number of 16S rRNA gene sequences per approach, *c* coverage indice of clone libraries, *RDP* confidence level in the assignment to a taxonomical group

*Chi-square test rejected random relative abundance among the three specimens with significant values ($P < 0.05$)

[a] Isolates sequenced were representative of the three physiological profiles

Alteromonadales at, respectively, 88, 90 and 100% RDP confidence level. The null hypothesis of random relative abundance among the three specimens could be rejected by a chi-square test (Table 2; $df = 2$; $P < 0.05$) and mean relative frequencies of each OTU are given in the Table 2.

Primary co-cultures of *N. macromphalus* pericardial appendages cells and associated bacteria displayed small aggregates adhering to the collagen-coated substrate. The number of cephalopod cells decreased during the first week and then remained stable to 70% of the inoculum density over 21 days (total experimental duration). Cephalopod cell viability measured in primary co-cultures by trypan blue exclusion remained above 70%. *N. macromphalus* cell types in primary co-cultures were glandular (10–15 μm in diameter) similar to the morphological cell types observed in tissue sections (Fig. 1a). A high density of bacteria was located close to the cell surface (Fig. 1d). Enzymatic (dehydrogenase) reduction of MTT into formazan indicated that

pericardial appendages cells in primary co-cultures were metabolically active. Optical density was proportional to primary co-culture density and global metabolic activity was maintained to slightly increase over 21 days primary co-culture. Reduced formazan crystals were observed at the surface of pericardial appendages cell–bacteria complex (Fig. 1c), confirming metabolic activity of cells maintained in primary co-cultures. Attempts to separate bacteria from the complex by isolation on marine agar failed to yield any colonies, indicating that bacteria would not grow in the absence of pericardial appendages cells.

Bacterial DNA analysis was performed on the adherent pericardial appendages cell–bacteria complex collected from 7 to 21 days primary co-cultures. Random sequencing of 30 clones, followed by sequences identity analysis on BioEdit, designated two OTUs corresponding to a global coverage of 100% (Table 2). 16S rRNA gene sequences detected in primary co-cultures was assigned to *β-Proteobacteria* and *Spirochaetes* by RDP classifier (88% in confidence level for both groups). The chi-square test rejected the null hypothesis (Table 2; $df = 2$; $P < 0.05$) of random relative abundance among the supernatants sampled at 7 and 21 days for each specimen and among the three *N. macromphalus* specimens. Sequence comparison between clones affiliated to *β-Proteobacteria* and *Spirochaetes* obtained from molecular and cellular approaches exhibited low variation (< 1%) confirming detection of the same OTUs. Mean values of relative frequencies for each OTU are given in Table 2.

Discussion and conclusions

Our results demonstrate that several bacterial groups are associated with *N. macromphalus* excretory organs. 16S rRNA gene analysis of isolated strains and clones detected five bacterial classes, but were only identified by the combination of the three complementary methods used, the microbiological, the molecular and the cellular approach.

The microbiological approach isolated the *Pseudomonadales*, *Oceanospirillales* and *Alteromonadales* belonging to the γ-proteobacteria, a sub-class which is known to be ubiquitous in marine environments and easy to culture on artificial media. These OTUs may be environmental microorganisms which have colonized the excretory organs of *N. macromphalus, Pseudomonadales* and *Alteromonadales* have already been isolated from two Cephalopods organs, respectively, *N. macromphalus* pericardial appendages (Schipp et al. 1990) and the accessory nidamental glands of *Loligo pealei* (Barbieri et al. 2001), which allows speculation on bacteria–host interaction. However, two of the methods used here, the molecular and cellular approach (Table 2), showed that γ-proteobacteria are a minor component of the bacterial diversity associated with *N. macromphalus* (only one *Alteromonadales* clone out of 30 was detected by the molecular approach). These clusters appear to be selected by the marine agar culture media. Interestingly, a culture-related bias in favour of γ-proteobacteria was reported in other cephalopods (Barbieri et al. 2001; Pichon et al. 2005). Moreover, comparative quantification of bacterial density by CFU titration and by flow cytometry evaluated culturable heterotrophic flora at less than 0.2% of the total bacterial density in the pericardial appendages. Our results therefore support the hypothesis of a technical-related bias in the selection of detected γ-proteobacteria.

In congruence with exhaustive molecular results in progress (M. Pernice et al., in preparation), the present molecular and cellular approaches suggest that the β-proteobacteria and spirochaetes are the predominant symbionts and yield clone libraries covering a large proportion of the bacterial diversity (respectively, 95.8 and 100% coverage). These two bacterial groups did not grow on artificial media except with the primary co-culture procedure in presence of pericardial appendages cells which may provide a specific substrate necessary for bacterial growth. Enrichment of pericardial appendages cells from the dissociated organ prior to plating (cellular approach) may have favoured the selection and survival of bacteria closely associated with the cells. This hypothesis is supported by the previous record of close bacterial–tissue association as observed with TEM (Schipp et al. 1990). Further experiments are currently underway to study the specific location and the phylogeny of these bacterial groups and their potential interactions at the pericardial appendages cell surface (M. Pernice et al., in preparation). Primary co-culture may represent a new complementary tool to detect as yet uncultured strains associated with marine invertebrates, and to investigate their interactions with host and their physiological potential.

Our results underline the need for combining culture-dependent and -independent methods to reveal the constant association with β-proteobacteria and spirochaetes and their potential contribution to *N. macrompalus* ecology. The nature of the association be it parasitic, mutualistic or commensalistic is not yet clear. A close bacteria–host interaction is, however, suspected for several reasons: (a) β-proteobacteria and spirochaetes do not grow on classical marine agar but they were

always present among strains associated with active viable cells from *N. macromphalus* excretory organ; (b) β-proteobacteria and spirochaetes 16S rRNA gene sequences diverge from those of bacteria from the marine environment; (c) attempts to propagate β-proteobacteria and spirochaetes from *N. macromphalus* tissue/cells have failed so far. These bacteria may require growth factors released by host cells (Davidson et al. 2004; Rieder et al. 2005) and/or components of the co-culture media. Since the pericardial appendage is a glandular organ that resides in a liquid pouch, it is hypothesized that β-proteobacteria and spirochaetes, maintained in vitro only in primary co-cultures, are closely associated to the pericardial appendage brush border epithelium as in other associations (Lamarcq and McFall-Ngai 1998). Their specificity is presently being analysed by complementary approaches. The γ-proteobacteria, grown on artificial culture media, seem to be negatively selected by the protocol of cephalopod cell enrichment and in the primary co-culture procedure. Clone libraries from whole organs indicate that they are a minor component of the pericardial appendages associations. They are probably present in the urinary liquid and may come from the environment.

Results obtained in this study stress the potential of bacterial–host cells primary co-cultures as a complementary tool for physiological and genetic studies of marine symbiosis, as it allows survival of as yet uncultured strains. Such methods can help to explore mechanisms of cross talk between bacteria and host cells, indicating ways to optimize production of bioactive compounds in various symbiotic systems and can thus contribute to a broader understanding of the ecology of microbial symbiosis.

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