Marine Biology (2005) 147: 1323–1332
DOI 10.1007/s00227-005-0014-5

RESEARCH ARTICLE

**Delphine Pichon · Valeria Gaia · Mark D. Norman**
**Renata Boucher-Rodoni**

# Phylogenetic diversity of epibiotic bacteria in the accessory nidamental glands of squids (Cephalopoda: Loliginidae and Idiosepiidae)

Received: 25 August 2004 / Accepted: 14 April 2005 / Published online: 30 July 2005


**Abstract** Bacterial communities were identified from the accessory nidamental glands (ANGs) of European and Western Pacific squids of the families Loliginidae and Idiosepiidae, as also in the egg capsules, embryo and yolk of two loliginid squid species, and in the entire egg of one idiosepiid squid species. The results of phylogenetic analyses of 16S RNA gene (rDNA) confirmed that several phylotypes of α-proteobacteria, γ-proteobacteria and *Cytophaga-Flavobacteria-Bacteroides* phylum were present as potential symbiotic associations within the ANGs. Several identified clones were related to reference strains, while others had no known close relatives. Gram positive strains were rare in loliginid squids. Several bacterial groups may play important roles in the function of the ANGs, such as production of the toxic compounds involved in egg protection and carotenoid pigments. Within the eggs, no bacteria were associated with embryo or yolk of *Loligo vulgaris* and *Sepioteuthis lessoniana*, but α- and γ-proteobacteria were present in the egg capsules. Most bacterial strains detected in the egg capsules were the same as those found in the ANGs. The cephalopods of temperate regions (European cuttlefishes and the squid *L. vulgaris*) appear to be associated with one *Agrobacterium* strain (Agro2) while tropical-subtropical strains (Asian and Australian loliginids) are associated with *Silicibacter*-related strains, suggesting a biogeographic clustering for the *Agrobacterium*-like strains.

Communicated by S.A. Poulet, Roscoff

D. Pichon (✉) · R. Boucher-Rodoni
Département Milieux et Peuplements aquatiques (MNHN), Biologie des Organismes marins et Ecosystème (UMR5178, CNRS), 55 rue Buffon, 75005 Paris, France
E-mail: cephalo@mnhn.fr
Tel.: +33-1-40793097
Fax: +33-1-40795734

V. Gaia
Cantonal Institute of Microbiology, University of Geneva, 6500 Bellinzona, Switzerland

M. D. Norman
Museum Victoria, GPO Box 666E, Melbourne, 3001 VIC, Australia

## Introduction

The female reproductive systems of certain cephalopod taxa possess a paired organ associated with egg laying that contains dense bacterial communities (Bloodgood 1977). Known as the "accessory nidamental glands" (ANGs), these organs are present in pencil and reef squids (family Loliginidae), cuttlefishes (family Sepiidae), bobtail squids (family Sepiolidae), bottletail squids (family Sepiadariidae) and pygmy squids (family Idiosepiidae). The function of these glands remains unclear but is considered to play a role in the elaboration of the outer capsule layer of the eggs. The tubules of this gland are filled with a dense bacterial community, which accumulates carotenoids during sexual maturation of the host (Van den Branden et al. 1978; Lum-Kong and Hastings 1992). As females of these cephalopod species mature, the glands change colour from cream white to bright red-orange. The bacterial communities present in the ANGs of some cephalopod species have been identified in recent studies by bacterial 16S RNA gene (rDNA) sequencing (Barbieri et al. 1997, 2001; Grigioni et al. 2000; Bonnaud et al. 2005; Pichon et al. 2005). These studies have also shown that among the ANGs strains, some are closely related to free-living strains while others lack any known relatives and might be specifically related to the taxonomy of the cephalopod host.

The present paper describes the bacterial diversity in the ANGs of a number of European and Western Pacific squid species, compares them to previously reported cephalopod symbiotic strains (Barbieri et al. 1997, 2001; Kaufman et al. 1998; Grigioni et al. 2000), and analyses the phylogenetic results in taxonomic and biogeographic contexts. The identification of the bacterial strains in many species is important in recognizing true symbionts

and in building hypotheses on their physiological role(s). Loliginid squids are common in most tropical and temperate continental margins around the world and represent approximately 10% of the total cephalopod world catch (Roper et al. 1984). Some are widely distributed (*Sepioteuthis lessoniana*, *Photololigo edulis*, *P. duvaucelii*) while others have more restricted areas of distribution (*Idiosepius biserialis*, *I. pygmaeus*, *P. chinensis*, *Loligo forbesi*, *L. vulgaris*, *Loliolus beka* and *L. uyii*).

## Materials and methods

### Specimen collection

Accessory nidamental glands of four genera of the family Loliginidae (*Loligo*, *Photololigo*, *Sepioteuthis* and *Loliolus*), and two species of the pygmy squid genus *Idiosepius* were examined in this study. Capture locations, DDBJ/EMBL/GenBank accession numbers for the bacteria 16S rDNA sequences and references are given in Table 1.

Specimens were caught live and anesthetized, or were freshly dead. All tissue samples (ANGs, embryo, yolk, and egg capsules) were obtained by aseptic dissection and preserved in 100% ethanol prior to DNA extraction and fluorescent in situ hybridization (FISH) analyses. For two ANGs samples of *L. vulgaris*, paraformaldehyde fixation was performed (PFA 4%). After dehydration, organ fragments were paraffin embedded and prepared as 7 $\mu$m histological sections.

*Sepioteuthis* egg capsules, embryos and ANGs $\gamma$-proteobacteria were cultured in DSMZ 308 *Vibrio* Medium (10 g Tryptone, 10 g NaCl, 4 g $MgCl_2*6H_2O$, 1 g KCl, 1l distilled water, pH 7.5). The plates were incubated at 25°C for 48–72 h in order to obtain a collection of cultivable bacteria. Isolates were sampled for DNA extraction and sequencing.

### Bacterial analyses

#### *Gram staining*

A standard Gram staining (Gerhard et al. 1994) was carried out on histological sections for all individuals.

#### *Fluorescent in situ hybridization (FISH)*

Whole-cell in situ hybridization (Amann et al. 1990; Hahn et al. 1992; Zarda et al. 1997; Grigioni et al. 2000) of eubacterial cells was performed with specific fluores-

**Table 1** List of cephalopods studied and their origin

| Host species | Origin | Number of samples | DDBJ/EMBL/GenBank accession numbers | Reference |
|---|---|---|---|---|
| *Sepia officinalis* | English Channel | | APR244780, APR244786, APR244791, APR244796, APR244799, APR244802, APR244807, APR244810 | Grigioni et al. 2000 |
| *Photololigo duvaucelii* ANGs | Taiwan | *3* | AJ633960, AJ633961, AJ633962, AJ633963, AJ633964, AJ633965, AJ633966, AJ633967, AJ633968, AJ633969, AJ633970 | Present paper |
| *P. opalescens* ANGs + Ecs | Pacific coast USA | | AF026460, AF026461, AF026462, AF026463 | Kaufman et al. 1998 |
| *P. chinensis* ANGs | Taiwan | *4* | AJ633950, AJ633951, AJ633952, AJ633953, AJ633954, AJ633955 | Present paper |
| *Loligo edulis* ANGs | Taiwan | *1* | AJ633956, AJ633957, AJ633958, AJ633959 | Present paper |
| *L. pealei* ANGs + Ecs | Atlantic coast USA | | AF022392, AF022393, AF022395, AF022402, AF022403, AF022404, AF022407, AF022408, AF022409, AF034937, AF034938 | Barbieri et al. 2001 |
| *L. forbesi* ANGs | English Channel | *2* | AJ633945, AJ633946, AJ633947, AJ633948, AJ633949 | Present paper |
| *L. vulgaris* ANGs<br>Ecs | English Channel | *3*<br>*3* | AJ633937, AJ633938, AJ633939, AJ633940, AJ633941, AJ633942, AJ633943, AJ633944 | Present paper |
| *Loliolus uyii* ANGs | Taiwan | *1* | AJ633988, AJ633989; AJ633990 | Present paper |
| *Loliolus beka* ANGs | Taiwan | *1* | AJ633981, AJ633982, AJ633983, AJ633984, AJ633985, AJ633986, AJ633987 | Present paper |
| *Sepioteuthis lessoniana* ANGs | Taiwan | *2* | AJ633975, AJ633977 | Present paper |
| *S. lessoniana* ANGs<br>Ecs | Australia | *1*<br>*1* | AJ633971, AJ633972, AJ633973, AJ633974, AJ633976, AJ633991, AJ633992, AJ633993, AJ633994, AJ633995 | Present paper |
| *Idiosepius biserialis* Es | Thailand (Andaman Sea) | *5* | AJ633996, AJ633997 | Present paper |
| *I. pygmaeus* ANGs | Australia | *1* | AJ633978, AJ633979, AJ633980 | Bonnaud et al. 2005 |

DDBJ/EMBL/GenBank accession numbers for the 16S rDNA sequences and reference
*ANGs* accessory nidamental glands, *ECs* egg capsules, *Es* eggs

cent (Cy3 or Fluorescein) labelled probes (Table 2) (OPERON Biotechnologies).

Prior to the FISH technique, the organ sections were deparaffinized in xylene and dehydrated in 100% ethanol. The hybridisation was carried out in 9 $\mu$l hybridisation buffer (0.9 M NaCl, 20 mM Tris/HCl, 30% N–N-dimethylformamide, 0.01% SDS) and 1 $\mu$l of probe (100 $\mu$M) during 90 min at 48°C. Washing lasted 20 min at 48°C (1.02 M NaCl, 20 mM Tris/HCl pH 7.2, 10 mM EDTA pH 8, 0.01%SDS). The stringency conditions were evaluated with a gradient of formamide (10–50%). Thirty percent formamide gave the optimal images (high specific fluorescent reaction, and low background fluorescence) and was chosen for all hybridizations.

Control sections were hybridized without a probe to check background autofluorescence. The eubacterial universal probe (EUB338) was used as a positive control for $\alpha$- and $\gamma$-proteobacteria. The probes ARC and BET42A were considered as negative controls.

Ten microliter of a 0.0001% solution of DAPI (DNA intercalating dye 4′,6-diamidino-2′-phenylindole solution, Sigma) was applied and incubated for 10 min in darkness at room temperature, then rinsed with distilled water and air dried.

Samples were mounted with Citifluor immersion oil solution (Chemical laboratory, The University Canterbury, England) and immediately observed with a Leica epifluorescence microscope, equipped with a high-pressure mercury bulb using filter sets I2 (Leica) for fluorescein, (360/40, 400DCLP, 460/50), F41-007 for Cy3 (535/50, 565LP, 610/75) and A513824 (340-380/425nm) for DAPI.

### *Amplification and cloning of bacterial 16S rDNA*

Total DNA extraction from ANGs was performed with DNeasy Tissue Kit (Qiagen), following the specific protocol for bacteria. PCR was conducted with dNTP (0.2 mM) (Eurogentec), primers (10 $\mu$M each), *Taq* polymerase (2.5 U) and buffer (10 mM Tris–HCl, 15 mM $MgCl_2$, 500 mM KCl) (A.T.G.C. Biotechnologie) in a GeneAmp PCR System (Perkin Elmer) with a denaturing step of 94°C for 5 min, 32 cycles of 94°C (30 s), 55°C (30 s), and 72°C (1 min) and a final elongation step of 72°C for 7 min. Universal prokaryote primers were used: 27F-1385R pairs (respectively *Escherichia coli* position 9: 5′-GAGTTTGATCCTGGCTCA-3′ and position 1385: 5′-CGGTGTGTRCAAGGCCC-3′), which produced almost the entire 16S rRNA gene fragment (ca. 1,400 bp). Each PCR product was checked by electrophoresis in 1.5% agarose gel. Purified PCR products (QIAquick PCR Purification Kit, Qiagen Inc.) were cloned by insertion into plasmid vector PCR 2.1 TOPO TA Cloning (Invitrogen) following the instructions of the manufacturers.

### *16S rDNA sequencing and analysis*

Ten to twenty clones per amplification were sequenced. All sequenced clones were analysed for the presence of chimeras using the Chimera Check program (version 2.7) (Maidak et al. 1997) from the Ribosomal Database Project (RDP-II) and the Bellerophon program (Huber et al. 2004). Sequences suspected of being chimeric were not included in further analyses. Final sequences were aligned using the ClustalW software (Thompson et al. 1994) with a subset of bacterial 16S rDNA sequences obtained by comparison with the EMBL GenBank database using FASTA3.0 program (Pearson and Lipman 1988).

Phylogenetic trees were calculated using neighbour-joining algorithms with Kimura "2 parameters" model (Kimura 1980), and with maximum-parsimony and maximum-likelihood methods (Olsen et al. 1994), contained in PHYLIP 3.6 package. Bootstrap analyses (1,000 replicates) were performed for distance analyses to test each topology for robustness in PHYLIP version 3.6 (Felsenstein 1993).

The different sequences were arbitrarily clustered into operational taxonomic units (OTU) with similarities $>97\%$. The percentage of coverage of the diversity by the clone libraries was calculated with the formula [1-(n/N)]*100, where n is the number of single clone OTU and N is the total number of sequences (Good 1953).

## Results

### Egg analyses

For *I. biserialis* the entire eggs were fixed and analysed, due to their small size (2 mm) and because they were freshly spawned. The eggs of *L. vulgaris* and *S. lessoniana* were larger and well developed. As a result, they were dissected and the different parts (embryo, yolk, egg

**Table 2** List of probes used for FISH

| Probe name | Sequence (5′-3′) | *Escherichia coli* position | Specificity | Reference |
|---|---|---|---|---|
| EUB338*Cy3 | GCTGCCTCCCGTAGGAGT | 16S rDNA (388–355) | Eubacteria | Amann et al. 1990 |
| ARC*Fluo | GTGCTCCCCCGCCAATTCCT | 16S rDNA (915–934) | Archaebacteria | Stahl and Amann 1991 |
| ALFA1B*Fluo | CGTTCGYTCTGAGCCAG | 16S rDNA (19–35) | $\alpha$-Proteobacteria | Manz et al. 1992 |
| GAM42A*Cy3 | GCCTTCCCACATCGTTT | 23S rDNA (1027–1043) | $\gamma$-Proteobacteria | Manz et al. 1992 |
| BET42A*Fluo | GCCTTCCCACTTCGTTT | 23S rDNA (1027–1043) | $\beta$-Proteobacteria | Manz et al. 1992 |

capsules) analysed separately. Gram staining did not detect Gram positive strains in any part of the eggs.

The results of FISH (DAPI staining, EUB338, ALFA1B, GAM42A probes) and PCR show that bacteria are present in the eggs of *I. biserialis*, but only in the egg capsules of *L. vulgaris* and *S. lessoniana*.

16S rDNA sequencing of egg case bacteria identified γ-proteobacteria (*Vibrio*, *Shewanella*) among cultured strains (*S. lessoniana*) and α-proteobacteria (*Agrobacterium*, *Roseobacter, Rhodobium*) among clones (*S. lessoniana* and *L. vulgaris*).

## Accessory nidamental glands analyses

In all squid species, Gram stained histological sections of ANGs detected four morphotypes: rod-shaped, small coccoid-shaped and large coccoid-shaped Gram negative and some rare large coccoid-shaped Gram positive.

Fluorescent in situ hybridization results after ethanol fixation were the same as those obtained after PFA fixation in *L. vulgaris*. These supported the analyses of specimens for which only ethanol-preserved organs were available. The results show the presence of targeted bacteria strains, but do not allow precise quantification. Different morphotypes corresponding to α−proteobacteria and γ−proteobacteria were detected in the lumina of the ANGs of all the investigated species.

Results of ANGs clone sequencing are summarized in Table 3. In our study we obtained 128 sequences with 15 unique OTU that represented a calculate coverage of 88.8%, meaning that the probability of an additional clone sequence falling into a not yet observed OTU is 11.2%.

All phylogenetic criteria for tree calculations (maximum-parsimony, maximum-likelihood and neighbor-joining) produced the same phylogenetic groups (Figs. 1, 2), i.e. one group related to *Cytophaga-Flavobacteria-Bacteroides* Phylum, four groups of γ-proteobacteria, and three groups of α-proteobacteria.

*Cytophaga*–it Flexibacter–it Bacteroidetes related strains were identified in five cephalopod taxa (Fig. 1). Among γ−proteobacteria, one cluster gathered cephalopod ANGs strains and various sulphur-oxidizing endosymbionts from hydrothermal vent taxa (87–90%

**Table 3** Phylogenetic affiliation of the clones from loliginid ANGs. C: Percentage of coverage (Good 1953); P: Percentage of clone libraries for each host species

| Phylogenetic affiliation | Host species | Clones | P (%) | Reference strains (Accession n°) | % of similarity |
|---|---|---|---|---|---|
| α-proteobacteria (C = 92%) | | | | | |
| *Agrobacterium–Silicibacter* | *L. vulgaris* | 3 | 18 | *A. atlanticum* D88526 | 97–98 |
| | *P. chinensis* | 7 | 30 | *A. meteori* D88527 | 97–99 |
| | *P. duvaucelii* | 7 | 39 | *Silicibacter lacuscaerulensis* SLU77644 | 98–99 |
| | *Loliolus beka* | 1 | 12 | *Silicibacter lacuscaerulensis* SLU77645 | 90–92 |
| | *S. lessoniana* | 8 | 29 | *Silicibacter lacuscaerulensis* SLU77646 | 96–97 |
| | *I. pygmaeus* | 1 | 8 | *A. meteori* D88527 | 96–97 |
| *Roseobacter* | *L. vulgaris* | 2 | 12 | *R. gallaeciensis* RG16SRR | 97–98 |
| | *L. forbesi* | 2 | 29 | *R. gallaeciensis* RG16SRR | 91–92 |
| | *P. chinensis* | 3 | 12 | *Crassostrea virginica* symbiont AF114484 | 95–96 |
| | *P. duvaucelii* | 5 | 28 | *R. algicola* RA16SRNA1 | 97–98 |
| | *P. edulis* | 1 | 17 | *R. gallaeciensis* RG16SRR | 93–94 |
| | *Loliolus beka* | 2 | 25 | *R. algicola* RA16SRNA1 | 92–98 |
| | *Loliolus uyii* | 2 | 25 | *R. gallaeciensis* RG16SRR | 90–92 |
| | *S. lessoniana* | 2 | 7 | *R. gallaeciensis* RG16SRR | 92–93 |
| *Rhodobacter* | *L. vulgaris* | 11 | 65 | *Rhodobium orientis* RO16SRNAC | 93–95 |
| | *L. forbesi* | 4 | 57 | *Rhodobium orientis RO16SRNAC* | 92–94 |
| | *P. chinensis* | 7 | 30 | *Rhodobium orientis* RO16SRNAC | 90–94 |
| | *P. duvaucelii* | 3 | 17 | *Rhodobium orientis* RO16SRNAC | 92–94 |
| | *P. edulis* | 2 | 33 | *Rhodobium orientis* RO16SRNAC | 90–92 |
| | *Loliolus beka* | 1 | 12 | *Rhodobium orientis* RO16SRNAC | 90–92 |
| | *Loliolus uyii* | 5 | 63 | *Rhodobium orientis* RO16SRNAC | 92–94 |
| | *S. lessoniana* | 18 | 64 | *Rhodobium orientis* RO16SRNAC | 90–92 |
| | *I. pygmaeus* | 11 | 84 | *Rhodobium orientis* RO16SRNAC | 90–92 |
| γ-proteobacteria (C = 58%) | *L. forbesi* | 1 | 14 | *Pseudoalteromonas* sp. AF227238 | 85–86 |
| | *P. chinensis* | 6 | 26 | *Riftia pachyptila* endosymbiont RPU77478 | 90–95 |
| | *P. duvaucelii* | 1 | 5 | *Pseudoalteromonas* sp. AF227238 | 85–86 |
| | *P. edulis* | 1 | 17 | *Riftia pachyptila* endosymbiont RPU77478 | 91–92 |
| | *Loliolus beka* | 1 | 12 | *Riftia pachyptila* endosymbiont RPU77478 | 89–90 |
| | *Loliolus uyii* | 1 | 12 | *Colwellia psychroerythrus* CSU85841 | 86–87 |
| *Cytophaga-Flexibacter-Bacteroidetes* | *P. duvaucelii* | 2 | 11 | *Empedobacter brevis* FBRR16K | 76–78 |
| | *P. edulis* | 2 | 33 | *Cytophaga* sp. AF235114 | 83–85 |
| | *Loliolus beka* | 3 | 39 | *Empedobacter brevis* FBRR16K | 81–82 |
| Gram positive bacteria | *L. vulgaris* | 1 | 5 | *Clostridium cellulovorans* CC16SRNAD | 80–81 |
| | *I. pygmaeus* | 1 | 8 | *Clostridium cellulovorans* CC16SRNAD | 80–81 |


**Fig. 1** Consensus tree obtained after neighbour-joining analysis of ca. 1,000 bp $\gamma$-proteobacteria 16S rDNA sequences. All sites are taken into account. Only bootstrap values higher than 60% are indicated. *Doubled lines* stress the relations supported by maximum-parsimony and maximum-likelihood analysis

of similarity, i.e. 1250 to 1350 identical nucleotides). These strains have no known related environmental strains and might be highly adapted to symbiotic life. Strains related to *Vibrio, Shewanella* (isolates) and *Pseudoalteromonas* (clones) were also present in the ANGs of some Western Pacific loliginid squids. A large number of cephalopod bacterial clones were gathered within two $\alpha$-proteobacteria groups: a solid *Rhodobium-Stappia* related group, and a *Roseobacter–Agrobacterium–Silicibacter* related group (Fig. 2). The third group isolated one deep branching cluster gathering clones of *L. forbesi, L. pealei*, *Loliolus uyii* and *L. beka*; their closest relative from the DDBJ/EMBL/GenBank is *Roseobacter* (90% similarity).

The cluster related to *Rhodobium–Stappia* included clones present in all the host species.

Within the *Roseobacter–Agrobacterium–Silicibacter* group, two subgroups were distinguished, (1) the *Roseobacter* subgroup, and (2) the *Agrobacterium–Silicibacter* subgroup.

*Roseobacter*-related strains were identified in many loliginid squids from all geographical regions. They cluster with the non-phototroph *Roseobacter* reference strains.

Several clones present only in European and Western Pacific species (*L. vulgaris*, *S. lessoniana, P. duvauceli*, *P. chinensis*, *Loliolus beka*, and *I. pygmaeus*) showed a high similarity with the *Agrobacterium–Silicibacter* subgroup which includes both symbiotic and free-living taxa (only marine free-living *Agrobacterium* are used as reference). In our study, sequence results showed three *Agrobacterium* subgroups: Agro1, Agro2, and *Silicibacter*. Agro1

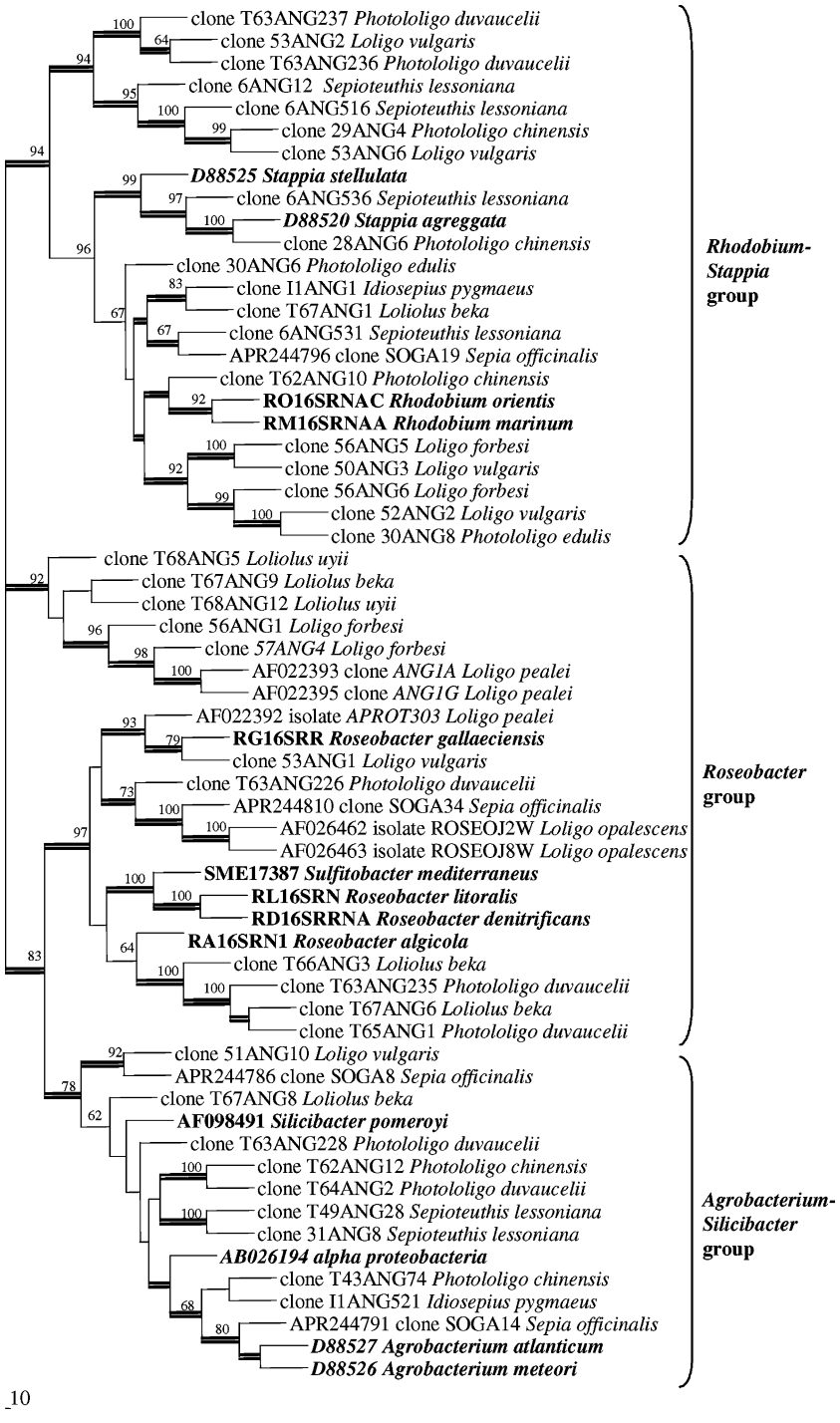


**Fig. 2** Consensus tree obtained after neighbour-joining analysis of ca. 1,300 bp α-proteobacteria 16S rDNA sequences. All sites are taken into account. Only bootstrap values higher than 60% are indicated. *Doubled lines* stress the relations supported by maximum-parsimony and maximum-likelihood analysis

was present in *P. chinensis, Idiosepius* and *Sepioteuthis* (Fig. 3). Agro2 was found only in one European loliginid squid (*L. vulgaris)*. Several bacterial clones from tropical loliginids appeared related to *Silicibacter* (Fig. 3).

There is no relation between the associated strains and the range of distribution of the host.

In order to include the data from a previous study of *L. pealei* (Barbieri et al. 2001) and from egg case sequences from the present work, an analysis taking into account only 500 bp was performed (data not shown). The same clusters of α-proteobacteria groups were supported, although bootstrap values were reduced. Pairwise comparison of sequences from egg capsules and ANGs confirm the identity of the strains.

## Discussion and conclusions

### Potential roles of symbiotic ANGs bacteria

In contrast to octopuses, cuttlefishes and the vast majority of squids deposit their eggs and leave them to

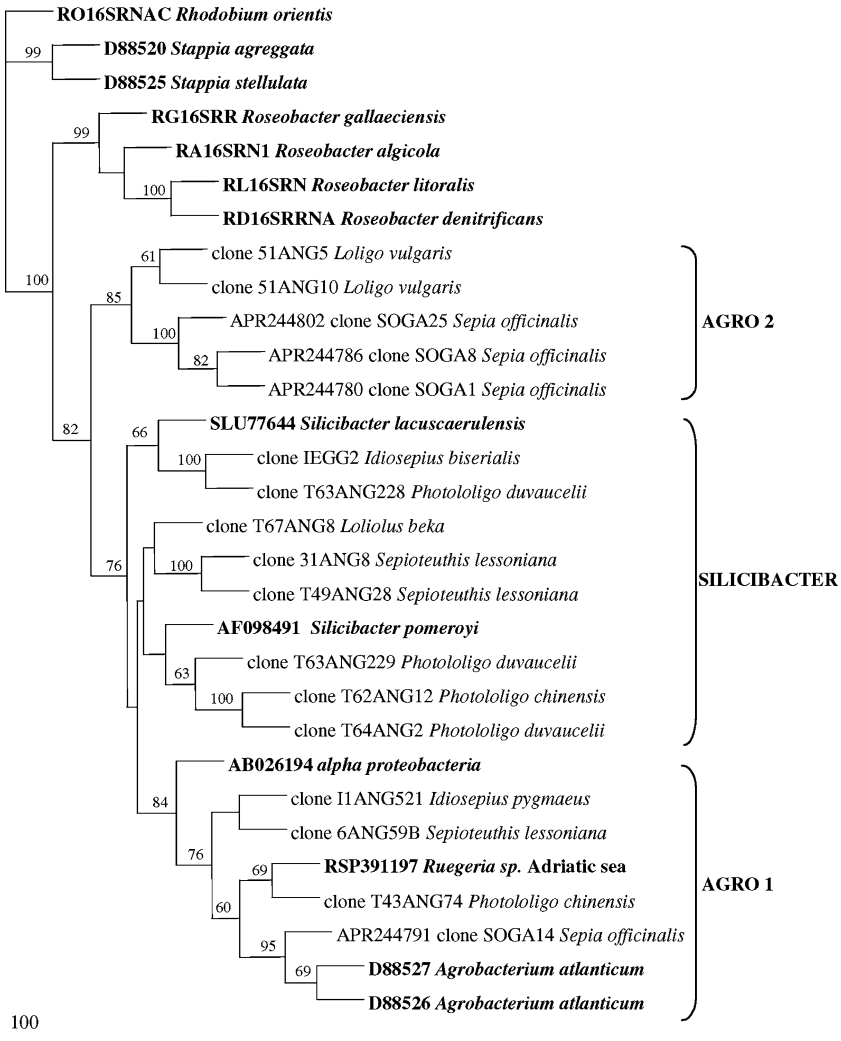


**Fig. 3** Consensus tree obtained after neighbour-joining analysis of ca 950 bp 16S rDNA sequences of *Agrobacterium sensu lato* strains. All sites are taken into account. Only bootstrap values higher than 60% are indicated

fend for themselves. It is unknown why these eggs are not immediately consumed by predators, as is the case for untended octopus eggs. Similarly, untended octopus eggs rapidly fall victim to algal, fungal or bacterial infections, whereas the eggs of cuttlefishes and many squids appear to resist such infections (Norman, personal observation).

Cephalopods that possess ANGs produce eggs with a gelatinous outer coating. Certain bacterial strains within the accessory nidamental glands may be associated with chemical protection of the unguarded eggs (protection from direct predation and infection), through inclusion of toxic bacteria and/or their chemical compounds in the outer layers of the egg capsule. Accordingly, the production of antibiotics and toxic compounds by cultured strains from ANGs and egg capsules was suggested for *L. pealei* (Barbieri et al. 2001) and is presently being investigated in other species.

Combinations of bacterial strains may enable chemical protection. The presence of *Stappia* is known to inhibit the proliferation of a *Roseobacter* pathogen of Juvenile Oyster Disease (Boettcher et al. 2000). Two groups of bacterial strains detected in cephalopod ANGs (*Roseobacter*-like and *Stappia*-like) may act in concert to inhibit the proliferation (and hence toxic impact) of the *Roseobacter* related strains until the latter bacteria are released into the gelatinous outer egg casings during egg laying. Isolation of the embryo from the bacteria, as evident from the presence of bacterial strains in the egg case and absence in the embryo, would effectively protect the developing cephalopod from toxic bacterial by-products.

The bacteria found in cephalopod ANGs accumulate carotenoids during sexual maturation of the host, evident as bright red glands visible through the ventral mantle wall of mature females in many species. *Rhodobium*-like strains and *Roseobacter* strains are known to produce carotenoids. The role of this pigment production remains unknown.

In the bobtail squids (family Sepiolidae), acquisition of luminous bacteria (*Vibrio fisheri*) from the environment instigates remodelling of the complex bacterial light organ from a sterile structure at birth that remains undifferentiated in the absence of the appropriate bacteria (McFall-Ngai and Ruby 1991, 1998). The accessory nidamental gland is undeveloped at birth. The role of

symbiotic bacteria (such as *Agrobacterium* or *Vibrio*) in organogenesis in these cephalopods is currently being investigated.

## Sources of transmission

Kaufman et al. (1998) suggested "horizontal transmission" for *L. opalescens*, where bacteria are acquired within a generation, i.e. not passed down from the parental generation. The absence of bacteria in the embryos of *Sepioteuthis* and *L. vulgaris* would support this hypothesis. However, if loliginid embryos lack bacteria (i.e. are "aposymbiotic"), the egg capsules harbor dense populations of bacteria, most of which are also present in the ANGs (Kaufman et al. 1998; Barbieri et al. 2001; present study). Some have no reference related strains from the environment. Either ANGs colonization is strictly horizontal and these bacteria belong to the vast amount of as yet unidentified species, or infestation takes place at hatching from the egg capsules, in the same way as termites feed the newly hatched juveniles feces of the adults (Nalepa et al. 2001). This hypothesis is being investigated by experimental approach, in aquarium-reared animals (water tank enrichment at hatching, bacteria sampling from egg capsules and maternal ANGs at spawning). However, some γ-proteobacteria present only in symbiotic associations in cephalopod ANGs and in other taxa from hydrothermal vents, have not yet been identified in egg capsules: their origin remains unresolved.

## Bacteria diversity across cephalopod hosts, biogeography

The DNA analyses of bacteria from the ANGs of loliginid squids show the presence of α-proteobacteria (*Roseobacter*, *Agrobacterium, Silicibacter, Stappia, Rhodobium*-like), of γ-proteobacteria (*Vibrio, Shewanella, Pseudoalteromonas*) and of *Cytophaga-Flavobacteria-Bacteroides*-like strains.

In the *L. pealei* study (Barbieri et al. 2001), all the bacterial taxa were present in the same species. In our study, the various strains were present in different species. Results concerning γ-proteobacteria may be biased as *L. pealei* cloning of bacterial DNA from tissue extracts mainly detected α-proteobacteria, whereas in cultured isolates γ-proteobacteria were mainly present. This is confirmed by a calculated coverage of 92% for α-proteobacteria and only 58% for γ-proteobacteria among cloned sequences (Table 3). Only two groups of γ-proteobacteria were detected by clone sequencing in our study (γ−symbionts in three cephalopod species, *Pseudoalteromonas* in two cephalopod species). The identification of the other two γ-proteobacteria groups (*Vibrio, Shewanella*) occurred only in cultured isolates of the ANGs and egg capsules of *Sepioteuthis*, as was the case for *L. pealei* and *L. opalescens*. Thus, the presence or absence of γ-proteobacteria in some cephalopod groups may be due to problems with bacterial DNA extraction rather than actual bacterial distributions amongst taxa. As our procedure concentrates on cloning bacterial DNA from tissue extracts, only α-proteobacteria were included in interpretations of host/bacteria relationships.

When compared to the reported bacterial consortium present in the ANGs of *Sepia officinalis* (Grigioni et al. 2000), it appears that most α-proteobacteria were present in both cuttlefishes and loliginid squids, whereas differences between the two cephalopod groups were detected in relation to Gram species and γ-proteobacteria.

Gram positive and negative strains have previously been reported in juvenile *L. opalescens* (Kaufman et al. 1998). Gram positive strains from adult loliginids have not previously been detected (Kaufman et al. 1998; Barbieri et al. 2001), and are reported here for the first time. There appears to be a specific difference in Gram strains between loliginid squids and cuttlefishes: Gram positive strains are abundant in adult cuttlefishes (Grigioni et al. 2000); they are present but rare in adult loliginids and life cycle related in *L. opalescens* (Kaufman et al. 1998). The parallel analysis of cephalopod phylogeny and ANGs Gram positive symbiotic strains suggests high specificity of Gram positive strains to cephalopod taxonomy at higher taxonomic levels (Bonnaud et al. 2005). They may represent interesting candidates for co-evolutionary studies.

A specific difference exists also between cuttlefishes and loliginid squids in relation to γ-proteobacteria: they are more abundant in loliginids.

It has previously been shown (Pichon et al. 2005) that the α-proteobacterium *Roseobacter* is present in many cephalopod species and includes three main strains, two of which are specific to cephalopod taxa, the third being included in a cluster with known reference strains. No geographical grouping was evident. Our results support these findings.

The marine *Agrobacterium* species were recently reclassified, *A. atlanticum* being transferred to the genus *Ruegeria* together with *R. algicola* (Uchino et al. 1998) and *Silicibacter* (Gonzalez et al. 2003). In our study, as in the cuttlefishes (Grigioni et al. 2000), *Silicibacter* was included in the *Agrobacterium* cluster, clearly distinct from *Roseobacter*. An additional *Ruegeria (Agrobacterium) atlantica* strain is present in the GenBank (Jansen et al., unpubl., AF124521) which appears effectively close to *Roseobacter*. But it was not the latter that was considered in the reclassification (Gonzalez et al. 2003), but the *Ruegeria* (*Agrobacterium*) atlantica sequence (D88527) of Uchino et al. (1998). In our results the sequence D88527 appeared related to *Silicibacter*, but not to *Roseobacter*. Obviously, the nomenclatural status of these taxa deserves further clarification. To avoid confusion, we still use the name *A. atlanticum* and restrict it to the sequence D88527.

*Agrobacterium*-related strains are abundant in the ANGs of all the European and West Pacific species analyzed. They were not reported from *L. opalescens* and *L. pealei* (Kaufman et al. 1998; Barbieri et al. 2001). Several *Agrobacterium* strains present in loliginid ANGs are related to those present in *Sepia* and *Idiosepius* and to the recently described *Silicibacter pomeroyi* (Gonzalez et al. 2003). Subgroups of *Agrobacterium* were clearly delineated in *S. officinalis* (Grigioni et al. 2000), one subgroup being closely related to the free-living strain (Agro1), the other (Agro2) having no known close relative. Agro2, the most represented *Agrobacterium* strain in *S. officinalis*, was found only in one European loliginid squid (*L. vulgaris*).

*Agrobacterium–Silicibacter* strains appear to reflect a geographical grouping: Agro1 and Agro2 are present in temperate hosts and are related to temperate reference free-living strains; *Silicibacter* strains are present in tropical loliginids and are related to tropical *Silicibacter* reference strains. Thus from the present results it appears that only bacterial strains within the *Agrobacterium sensu lato* group are related to the host origin. Nishiguchi et al. (1998) interpreted the specificity of bacterial strains present in Mediterranean sepiolids versus those present in Pacific sepiolids as evidence of co-evolution. This could also be the case for the *A. sensu lato* strains, confirming that in these strains adaptive evolution might take place under the pressure of symbiosis, as already suggested for the same bacterial group in cuttlefishes (Grigioni et al. 2000). Moreover, the presence of closely related strains in phylogenetically distinct cephalopod taxa from similar geographic regions suggests a regional evolution of symbiotic bacterial associations. Data on additional cephalopod taxa from diverse regions are currently being analysed and should shed further light on this fascinating and complex system.

**Acknowledgements** The authors wish to thank: Dr. C.C. Lu for his help during collection of specimens in Taiwan and for providing *Loliolus* samples; Dr. J. Nabhitabhata for *Idiosepius biserialis* egg samples from Thailand; J. Finn for assistance in field collection in Australia; Dr. H. Rybarczyk for assistance in statistical analysis. This work was supported by a CNRS grant (PICS 743) which provided 3 months salary and funds for field collection in Australia for the second author. The experiments complied with the current laws of the country in which the experiments were performed.

## References

Amann RI, Krumholz L, Stahl DA (1990) Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J Bacteriol 172:762–770

Barbieri E, Barry K., Child A, Wainwright N (1997) Antimicrobial activity in the microbial community of the accessory nidamental gland and egg capsules of *Loligo pealei* (Cephalopoda: Loliginidae)). Biol Bull 193:275–276

Barbieri E, Paster BJ, Hughes D, Zurek L, Moser DP, Teske A, Sogin ML (2001) Phylogenetic characterization of epibiotic bacteria in the accessory nidamental gland and egg of the squid *Loligo pealei* (Cephalopoda: Loliginidae). Environ Microbiol 3:151–167

Bloodgood R (1977) The squid accessory nidamental gland: ultrastructure and association with bacteria. Tissue Cell 9(2): 197–208

Boettcher KJ, Barber BJ, Singer JT (2000) Additional evidence that juvenile oyster disease is caused by a member of the *Roseobacter* group and colonization of non affected animals by *Stappia stellulata*-like strains. Appl Environ Microbiol 66(9):3924–3930

Bonnaud L, Pichon D, Boucher-Rodoni R (2005) Molecular approach of Decabrachia phylogeny: is *Idiosepius* definitely not a sepiolid? Phuket mar biol Cent Res Bull (in press)

Felsenstein J (1993) Phylip Version 3.5c. University of Washington, Seattle. [Online] http://sdmc.krdl.ord.sg:8080/~lxzhang/phylip)

Gerhard P, Murray RE, Wood WA, Krieg R (eds) (1994) Methods for general and molecular bacteriology. American Society for Microbiology, Washington DC

Gonzalez JM, Covert JS, Whitman WB, Henriksen JR, Mayer F, Scharf B, Schmitt R, Buchan A, Fuhrman JA, Kiene RP, Moran MA (2003) *Silicibacter pomeroy* sp nov and *Roseovarius nubinhibens* sp nov, dimethylsulfoniopropionate-demethylating bacteria from marine environments. Int J Syst Evol Microbiol 53:1261–1269

Good IJ (1953) The population frequencies of species and the estimation of population parameters. Biometrica 40:237–264

Grigioni S, Boucher-Rodoni R, Demarta A, Tonolla M, Peduzzi R (2000) Phylogenetic characterisation of bacterial symbionts in the accessory nidamental glands of the sepioid *S officinalis* (Cephalopoda: Decapoda). Mar Biol 136:217–222

Hahn D, Amann RI, Ludwig W, Akkermans ADL, Schleifer K-H (1992) Detection of micro-organisms in soil after in situ hybridisation with rRNA-targeted fluorescently labelled nucleotides. J Gen Microbiol 138:879–887

Huber T, Faulkner G, Hugenholtz P (2004) Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics 20:2317–2319

Kaufman MR, Ikeda Y, Patton C, Van Dykhuizen G, Epel D (1998) Bacterial symbionts colonize the accessory nidamental gland of the squid *Loligo opalescens* via horizontal transmission. Biol Bull 194:36–43

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Lum-Kong A, Hastings T (1992) The accessory nidamental glands of *Loligo forbesi* (Cephalopoda, Loliginidae). Characterization of symbiotic bacteria and preliminary experiments to investigate factors controlling maturation. J Zool Lond 228:395–403

Maidak BL, Olsen GJ, Larsen N, Overbeek R, McCaughey MJ, Woese CR (1997) The RDP (ribosomal database project). Nucleic Acids Res 25:109–111

Manz W, Amann R, Ludwig W, Wagner M, Schleifer K-H (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. Syst Appl Microbiol 15:593–600

McFall-Ngai MJ, Ruby EG (1991) Symbiotic recognition and subsequent morphogenesis as early events in an animal-bacterial mutualism. Science 254:1491–1494

McFall-Ngai MJ, Ruby EG (1998) Sepiolids and Vibrios: when first they meet. Bioscience 48(4):257–265

Nalepa CA, Bignell DE, Bandi C (2001) Detritivory, coprophagy, and the evolution of digestive mutualisms in Dictyoptera. Insect Soc 48: 194–201

Nishiguchi MK, Ruby EG, McFall-Ngai MJ (1998) Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in sepiolid squid—*Vibrio* symbioses. Appl Environ Microbiol 64(9):3209–3213

Olsen GJ, Matsuda H, Hagstrom R, Overbeek R (1994) FastDNAmL: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. Comput Appl Biosci 10:41–48

Pearson WR, Lipman DJ (1988) Improved tools for biological sequence comparison. Proc Natl Acad Sci USA 85(8):2444–2448

Pichon D, Grigioni S, Favet J, Boucher-Rodoni R (2005) Symbiotic associations between Cephalopods and the *Roseobacter* bacteria strain. Phuket Mar Biol Cent Res Bull (in press)

Roper CFE, Sweeney MJ, Nauen CE (1984) Cephalopods of the world. An annotated and illustrated catalogue of species of interest to fisheries. FAO fisheries synopsis, vol 3, no. 125. FAO, Rome

Stahl DA, Amann R (1991) Development and application of nuceic acid probes in bacterial systematics. In: Stackenbrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, Chichester, pp 205–248

Thompson JD, Higgins DG, Gibson TJ (1994) ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and Weight matrix choice. Nucleic Acids Res 22:4673–4680

Uchino Y, Hirata A, Yokota A, Sugiyama J (1998) Reclassification of marine *Agrobacterium* species: proposals of *Stappia stellulata* gen nov, comb nov, *Stappia aggregate* sp nov, nom rev, *Ruegeria atlantica* gen nov, comb nov, *Ruegeria gelatinovora* comb nov, *Ruegeria algicola* comb nov, and *Ahrensia kieliensis* gen nov sp nov, nom rev. J Gen Appl Microbiol 44:201–210

Van den Branden C, Richard A, Lemaire J, Decleir W (1978) La glande nidamentaire accessoire de *Sepia officinalis* L : analyses biochimiques des pigments des bactéries symbiotiques. Ann Soc R Zool Belg 108:123–139

Zarda B, Hahn D, Chatzinotas A, Schönhuber W, Neef A, Amann R, Zeyer J (1997) Analysis of bacterial community structure in bulk soil by in situ hybridization. Arch Microbiol 168:185–192