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SYMBIOTIC ASSOCIATIONS BETWEEN CEPHALOPODS AND THE *ROSEOBACTER* BACTERIA STRAIN

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ABSTRACT: The accessory nidamental glands (ANG) of sepiids and loliginids harbour a dense population of bacteria of unknown functions. The α-proteobacterium *Roseobacter* is present in most species. The present results show that 3 *Roseobacter* strains are associated with the cephalopod ANG. Two strains are specific to cephalopod taxa, the third is grouped together with reference strains of known physiological activity. The latter suggest a possible on the function of *Roseobacter* strains associated with the cephalopod ANG, in carotenoids production and egg protection by antibiotics and toxins production.

INTRODUCTION

Among cephalopods, loliginids, sepiids and sepiolids are known to possess accessory nidamental glands (ANG) hosting various strains of symbiotic bacteria. The role of this association is still not clearly understood. Characterization of the bacteria associated with the ANG of various cephalopod species reveals that the genus *Roseobacter* is present in most loliginids and sepiids studied so far.

The genus *Roseobacter* was created by Shiba (1991), and originally included 2 species (*R. denitrificans, R. litoralis*). *Roseobacter* is common in marine environments. Molecular investigation of marine habitats has revealed the importance of this group of coastal marine bacteria, which is related to the *Rhodobacter* grouping of the a-proteobacteria. Accordingly, *Roseobacter* are also called the "marine α bacteria" (Gonzales and Moran, 1997). This group includes free-living strains as well as strains that can be epiphytes and symbionts of marine organisms. Four species have been described and given a specific name so far, which include *R. litoralis* and *R. denitrificans* (isolated from the surface of green seaweeds (Shiba, 1991, 1992; Ashen and Goff, 2000)), *R. algicola* (isolated from the phycosphere of the toxic dinoflagellate *Prorocentrum lima* (Lafay *et al.*, 1995; Patten and Glick, 1996)) and *R. gallaeciensis* (a symbiont of the scallop *Pecten maximus* larvae (Ruiz-Ponte *et al.*, 1998)). Strains related to the *Roseobacter* group are also found in the galls of the marine red alga *Prionitis lanceolata* (Ashen and Goff, 2000), in squid and sepia accessory glands and egg cases (Barbieri *et al.*, 1996, 2001; Kaufman *et al.,* 1998; Grigioni *et al.*, 2000) and in juvenile oysters where they cause important mortalities of hatchery-produced larvae (Boettcher *et al.*, 2000).

The *Roseobacter* lineage is physiologically rather versatile. Some strains of the group are nonphototrophs (*R. algicola* and *R. gallaeciensis*), while others are phototrophs (*R. denitrificans* and *R. litoralis*).

In this paper, symbiotic strains of *Roseobacter* isolated from cephalopod ANGs were identified by 16S rDNA sequencing and fluorescent *in situ* hybridisation (FISH) in various cephalopod species. Sequences comparison allows to relate bacteria phylogeny to cephalopod taxonomy and/or to geographical environmental conditions.

MATERIALS AND METHODS

Roseobacter strains were identified in the ANG of various loliginids and sepiids (Table 1) by 16S rDNA sequencing, and FISH analyses.

Table 1. List of cephalopod species used for phylogenetic analyses of symbiotic *Roseobacter* strains.

Taxon	Origin	Reference
Sepia officinalis	Mediterranean and English Channel	Grigioni *et al.* 2000
Sepia elegans	Mediterranean	present paper
Sepia orbignyana	Mediterranean	present paper
Loligo vulgaris	Mediterranean	present paper
Loligo forbesi	English Channel	present paper
Loligo opalescens	Pacific coast USA	Kaufman *et al.*, 1998
Loligo pealei	Atlantic coast USA	Barbieri *et al.* 2001

Amplification and cloning of 16S rRNA genes

Extraction of total DNA from ANGs was performed by the specific protocol for bacteria of the DNeasy Tissue Kit (Qiagen). PCR reaction mixtures contained 0,5 µl of sample DNA, 0,125 µl of each primer (100 µm), 2 µl of premixed dNTPs (Eurogentec, 200 µm), 2,5 U of *Taq* DNA polymerase (ATGC), 5 µl of 10x *Taq* DNA polymerase buffer (15 mM $MgCl_2$), and 41.75 µl of sterile water. PCR was conducted 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. The universal prokaryote primers used [27F-1385R pairs (respectively *Escherichia coli* position 9 : 5'-GAGTTTGATCCTGGCTCA-3' and position 1385 : 5'-CGGTGTGTRCAAGGCCC-3')] produced ca 1350 bp of the 16S rDNA gene (ca 1500 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 gene sequencing and analysis

Minipreps of the clones were sequenced directly. 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 algorithm (Pearson and Lipman, 1988) .

Phylogenetic trees were calculated using Neighbour-joining algorithms with Kimura "2 parameters" model (Kimura, 1980), bootstrap analyses (100 replicates) were performed for distances analyses to test each topology for robustness in Phylip (Felsenstein, 1993).

Fluorescent *in situ* Hybridisation (FISH)

For fluorescent *in situ* hybridisation, ANG were processed as described by Amann *et al.* (1990) with some modifications. ANG were fixed and dehydrated in 100% ethanol, then embedded in paraffin. Histological sections (7 µm) were thoroughly disembedded (xylene) and rehydrated (ethanol) before hybridisation.

The protocol for whole-cell hybridisation was adapted from Hahn *et al.* (1992) and Zarda *et al.* (1997), following Grigioni *et al.* (2000). The hybridisation was carried out in 9µl hybridisation buffer (0.9 M NaCl, 20 mM Tris/HCl, 30% N-N-dimethylformamide, 0.01% SDS) and 1 µl probe (25 $ng.\mu l^{-1}$) 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).

Fluorescent in situ hybridisation was also performed with the specific fluorescein labelled probe ROSE (5'-TGGTAAGTTCTGCGCGT-3'). Ten microlitres of a 0.0001% solution of 4',6-diamino-2'-phenylindole (DAPI) was applied; samples were incubated for 10 min, then rinsed with distilled water and air dried. Samples were mounted with cityfluor immersion oil solution (Chemical Laboratory, The University of Canterbury, England) and immediately observed

with an epifluorescence microscope, equipped with a high-pressure mercury bulb using filter sets (Leica) for fluorescein (480/40, 527/30) and for DAPI (340-380/425 nm).

RESULTS

Results of the phylogenetic analyses of the *Roseobacter* strains present in the ANGs of various cephalopod species are presented in Figure 1. They show the separation of the strains into different groups. The phylogenetic tree clearly separates two groups of *Roseobacter* strains with a high bootstrap value (100) : a group of phototroph strains including only reference strains (*i.e. R. denitrificans* and *R. litoralis*) and a large group of non-phototroph strains including reference strains (*i.e. R. algicola* and *R. gallaeciensis*) and cephalopod symbionts, which are present in 3 clusters, named A, B and C.

Cluster A includes a solid cephalopod group of sepiids and loliginids *Roseobacter* symbionts. No closely related reference strain could be found in the databases. Within cluster A each symbiont appears specific: different strains correspond to different cephalopod species. Sequence similarity between strains that are sister to one another is above 99%. Cluster B associates bacteria specific to the loliginid ANGs. This group is solid and branches at the root of the tree. Cluster C includes mainly reference and 3 cephalopod strains (two loliginids and one sepiid). But this cluster is not solidly supported.

FISH analyses confirm the presence of *Roseobacter* strains in the tubules of the ANG of all the species investigated. Their morphology is mainly rod-shaped

DISCUSSION

The presence of different α-proteobacteria has been previously reported from the ANGs of various cephalopod species, but also from the egg capsules of loliginids. The ANGs are supposed to be involved in the production of the bacteria coating of the egg cases. *Roseobacter* strains were observed in the ANG of *Sepia officinalis* (Grigioni *et al.*, 2000), in the ANG and egg cases of *Loligo pealei* (Barbieri *et al.*, 2001) and in the egg cases of *L. opalescens* (Kaufman *et al.*, 1998).

Results presented here identify three different *Roseobacter* strains associated with the cephalopod ANGs. These groupings are unrelated to geographical distribution, but might have a phylogenetic meaning. Clusters A and B correlate with the host taxonomic status, they appear thus as good candidates in support of a coevolution, which could be resolved with a larger sample of cephalopod species.

In the red algae gall symbiont, a ca. 1% 16S rDNA sequence difference has significant ecological implications, particularly in terms of host-symbiont specificity (Ashen and Goff, 2000). The percentage of difference in 16S rDNA among cephalopod *Roseobacter* symbionts is the highest (over 12%) between cluster B and all other clusters, and lower (below 10%) between all other clusters, the lowest (4%) between clusters A and C. This pattern might mean a more ancient adaptation to symbiotic life for cluster B, and a more recent one for cluster A.

Clusters A and B include cephalopod strains only and are not associated to any known reference taxa. The third cluster (cluster C), relates cephalopod strains to reference strains of known activity. Similar activities are thus plausible for the related cephalopod ANGs *Roseobacter* strains.

Ruiz-Ponte *et al.* (1999) suggest an antimicrobial activity for the symbiotic bacteria *R. gallaeciensis* present in the larvae of *Pecten maximus*. This strain is closely related to a *L. vulgaris* bacteria strain. Such an antimicrobial and anti fungal role of the bacteria coating egg capsules was suggested (Barbieri *et al.*, 2001; Kaufman *et al.*, 1998), but attributed rather to *Pseudalteromonas* and *Shewanella* than to *Roseobacter* in *Loligo pealei*. The cephalopod *Roseobacter* strain (V53-1 *Loligo vulgaris*) is related to toxin producing strains from shellfish and dinoflagellates. Accordingly a repulsive role and shelter from predation in the egg case coating cannot be excluded (Dang and Lovell, 2000). Moreover, *Roseobacter* strains are suggested to be involved in biofilm formation, and exoenzyme production

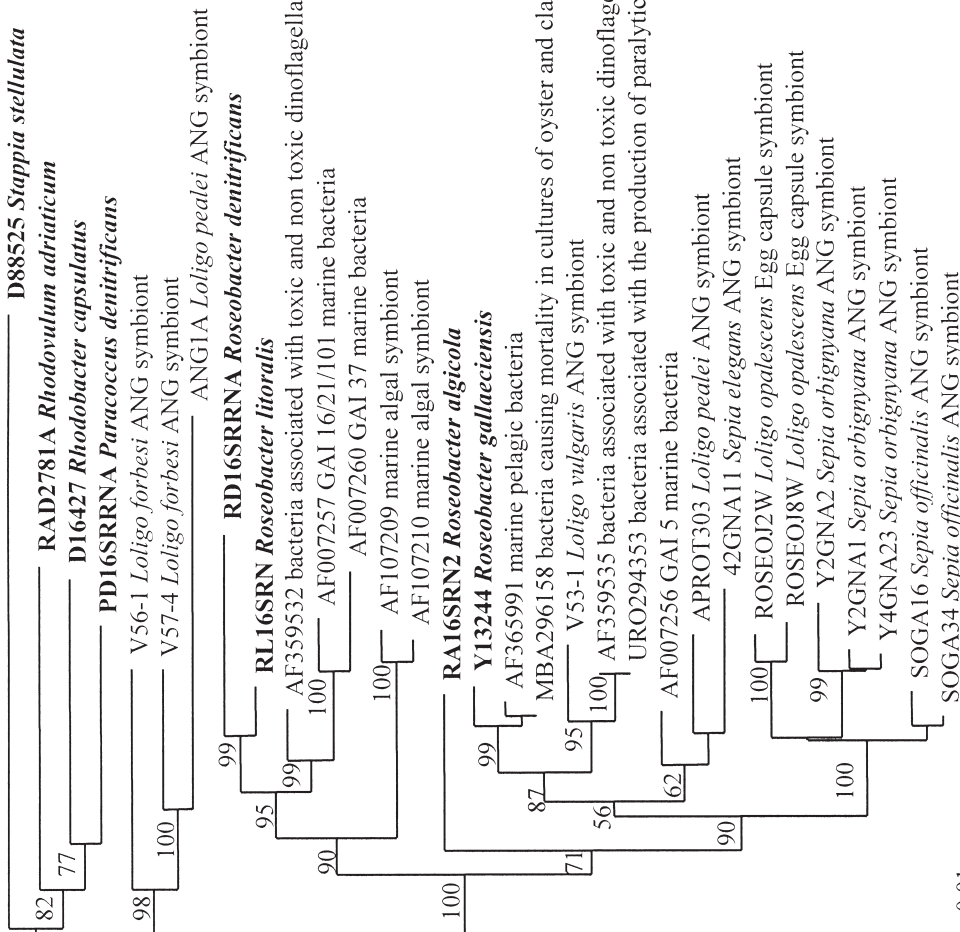

Figure 1. Distance tree obtained after neighbour-joining analysis of 1500bp 16Sr DNA sequences. All sites are taken into account. Only bootstrap values higher than 50% are indicated.

(Gram *et al.*, 2002). Such an ability to colonize surfaces provides important advantages in egg protection via bacteria coating of the egg cases.

In oyster disease, it appears that *Roseobacter* is a pathogenic strain (Boettcher *et al.*, 2000). The presence of another genus (*Stappia*) inhibits the proliferation of the pathogen *Roseobacter*. Such regulation may exist in the ANGs, where other bacterial species are present.

Two cephalopod *Roseobacter* strains are related to an environmental strain known to produce carotenoids (AF007256; Gonzales and Mora n, 1997). Cephalopod ANG bacteria are known to accumulate carotenoids during sexual maturation of the host. Accordingly, this function is plausible for some of the *Roseobacter* strains. The role of this production is still unknown (sexual attraction, UV protection).

The hypotheses of the probable role of the ANGs in egg protection via bacteria coating of the egg cases are presently being tested by physiological studies on cultured strains of *Roseobacter*. The presence of *Roseobacter* has been known from the egg cases of loliginids as well as in the egg cases of *Sepia officinalis* (unpubl.res.). The identity of *Roseobacter* strains in the ANG and in the egg cases may further elucidate the role of the accessory nidamental glands in the reproductive process of Cephalopods.

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