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Diversity of endocytic bacteria in the dinoflagellate *Noctiluca scintillans*

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ABSTRACT: *Noctiluca scintillans* is one of the largest species of marine dinoflagellates. A small fraction of these bloom forming algae was reported to be turbid due to endocytic bacteria. The diversity of these endocytic bacteria living in turbid *Noctiluca* cells was investigated by denaturing gradient gel electrophoresis (DGGE). The results indicate the occurrence of 1 dominant group of endocytic bacteria and some other groups of less dominance. DGGE profiles were compared between the endocytic bacterial populations of cultivated and non-cultivated turbid *Noctiluca* cells; the latter were directly collected from the North Sea. DGGE profiles displayed no differences between them. In contrast, the comparison of band patterns of endocytic bacteria and free-living marine bacteria were different, indicating the development of a specific bacterial population within *N. scintillans*. The DGGE bands identified by DNA sequencing were assigned to the species *Marinobacter* PCOB-2, to the *Pseudoalteromonas* group, and the *Vibrio* group, all members of the γ subdivisions of Proteobacteria. Another DGGE band was identical to the 18S ribosomal gene of *N. scintillans* itself. Furthermore, 16 bacterial isolates derived from single *Noctiluca* cells were characterized by 16S rRNA phylogenetic analysis. Data revealed that these bacteria belong to several different phylogenetic groups. Most of the isolates (14 strains) belong to several groups of the γ subdivision of Proteobacteria; 2 isolates are related to the *Vibrio* group and 1 isolate to *Moraxella.* The other isolates were assigned to the following groups of the γ subdivision: *Colwellia* group, *Stenotrophomonas* and *Pseudoalteromonas* group. Two of them were closely related to sequences obtained from DGGE bands (*Pseudoalteromonas* group, and *Marinobacter* PCOB-2). Two isolates were assigned to the phylum of Gram-positive bacteria.

KEY WORDS: Endocytic bacteria · *Noctiluca scintillans* · DGGE · Diversity

INTRODUCTION

Noctiluca scintillans (Kofoid 1920), one of the largest species of dinoflagellates (Pratje 1921) is ubiquitously distributed (Hanslik 1987, Uhlig & Sahling 1995). At Helgoland roads (German Bight, North Sea) *Noctiluca* occurs in annual blooms (Uhlig & Sahling 1995) forming orange colored red tides in July and August.

The majority of the *Noctiluca* cells in plankton hauls from Helgoland are clear; only 1% are turbid, which is caused by endocytic bacteria (Kirchner et al. 1999).

Up until now, several endocytic bacteria have been detected in dinoflagellates (Gold & Pollingher 1971, Silva 1978, Lucas 1982, Gordon et al. 1994) and other protists such as ciliates (Fenchel et al. 1993) and cryptomonades (Schnepf & Feith 1992). The endocytic bacteria synthesize nutrients for the host, for example by photosynthesis, nitrogen fixation or by an increase in enzyme activity, and hosts supply optimal habitates (Saffo 1990, Fenchel et al. 1993, Gordon et al. 1994). The bacterial population of *Noctiluca scintillans* has not yet been characterized and mechanisms of symbiosis or parasitism have not been determined.

The elucidation of microbial communities has several obstacles which are due to the low numbers of cul-

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turable bacteria (0.2 to 10%, Hoppe 1978). Molecular tools allow the detection of non-culturable bacteria, and several techniques have been developed to determine the genetic diversity of microbial communities (i.e. DNA reassociation experiments, Torsvik et al. 1990; DNA/DNA hybridization, Lee & Fuhrman 1991; different cloning strategies, Giovannoni et al. 1990, Fuhrman et al. 1993; and DGGE, Muyzer et al. 1995, Nübel et al. 1996, Ferris & Ward 1997).

Originally, DGGE was used to detect single-base changes (Fischer & Lerman 1983, Myers et al. 1985, 1987, Sheffield et al. 1989). However, it has been proved to be a fast, sensitive and direct approach to describe bacterial communities. The technique has been recently applied to study microbial communities of microalgae, bacterial mats and biofilms (Muyzer et al. 1993, Ferris & Ward 1997, Kirchner et al. 1999), hot springs in the marine environment (Muyzer et al. 1995), rhizospheres (Heuer et al. 1997) and soil (Felske et al.1998, Jensen et al. 1998).

Kirchner et al. (1999) provided the first clues using DGGE that turbid and clear *Noctiluca scintillans* cells harbor different intracellular bacteria. However, a systematic investigation is still needed.

The present study shows a first systematic attempt to investigate the diversity of endocytic bacteria in the marine Dinoflagellate *Noctiluca scintillans* with DGGE (V3 region, 16S rRNA). The most prominent DGGE bands were phylogenetically analyzed after cloning and sequencing. Furthermore, endocytic bacteria were isolated and phylogenetically analyzed by 16S rRNA gene sequencing.

MATERIALS AND METHODS

Sampling of *Noctiluca scintillans.* *Noctiluca* cells were obtained from plankton hauls taken at Helgoland Roads (German Bight, North Sea) between June and August 1997. The turbid cells were collected from the hauls (Kirchner et al. 1999), washed in sterilized seawater (0.2 µm filtered) and transferred into autoclaved glass vessels containing sterilized seawater. These cells were used to isolate the endocytic bacteria as well as for cultivation.

Cultivation of *Noctiluca scintillans.* Each culture set up was started with only 1 *Noctiluca* cell, which was incubated (20°C) in glass vessels containing 50 to 100 ml sterilized seawater (salinity 28 S^{-1}, light:dark cycle 16:8 h). The feeding of *N. scintillans* and water replacement was performed as described by Kirchner et al. (1999). The feeding organism *Dunaliella tertiolecta* was cultivated in F/2-Medium (Guillard 1975).

Isolation and cultivation of endocytic bacteria from *Noctiluca scintillans.* Since cytoplasma has a pH of 3.5 (Nawata & Sibaoka 1976), endocytic bacteria were isolated at different acidic pH values. The pH was adjusted with 1 M HCl before autoclaving; after sterilization it remained stable. Media with the following pH values were prepared: 3.1, 3.6, 4.2, 5.4, 6.5, and 7.9. Cultivation was performed in 1/10 Zobell 2216E liquid medium (Oppenheimer & ZoBell 1952).

For isolation of endocytic bacteria, *Noctiluca* cells were starved as described by Kirchner et al. (1999) to exclude the investigation of bacteria from the digestion vacuoles. *N. scintillans* was shown to be free from attached bacteria in earlier studies (Lucas 1982, Kirchner et al. 1999, 2001). In this study, cells were checked for surface-attached bacteria by light microscopy. By adjusting the focus to different levels, it could be seen that the cell wall of *N. scintillans* was in all cases free from bacterial growth. Nevertheless, to ensure the removal of bacteria from the cell surface, dinoflagellate samples were treated with CTAB (cetyl-trimethyl-ammonium-bromide; final concentration 10 µg ml^{-1}, Kirchner et al. 1999). Cells were washed 4 times in sterilized seawater and transferred to 100 µl liquid ZoBell medium. The cell membrane of *Noctiluca* was pierced and the sample material was added as inoculum to a sterilized tube containing 5 ml liquid medium at the different pH values (see above). The tubes were incubated on a shaker (90 rpm) at 18°C for 7 d.

Liquid cultures (38 µl) were plated onto an agar dish containing a similar pH by using a spiral plater (Fa. Spiral System). Samples with low pH values were firstly used as inoculum in liquid medium with a higher pH and adapted in single pH steps to pH 5.4. To obtain pure cultures, colonies were restreaked 3 times on agar dishes.

Sampling of bacterial biomass from seawater samples. Bacterial biomass was collected weekly from June to August 1997. At the Helgoland Roads (German Bight, North Sea), 5 l seawater was taken and prefiltered through a 60 µm gaze and 3 µm filter to exclude plankton and other large particles. Bacterial biomass was collected by 0.22 µm Sterivex filter capsules. Bacterial DNA was extracted from the filter by a combined method from Somerville et al. (1989) and Anderson & McKay (1983) modified according to Gerdts (1997) and stored at –20°C until use for PCR amplification.

PCR of endocytic bacteria in *Noctiluca scintillans.* Amplification of 16S rRNA genes of endocytic bacteria was performed by using single *Noctiluca* cells. To exclude the investigation of bacteria from the digestion vacuoles and the cell surface, *Noctiluca* cells were treated as described for isolation of endocytic bacteria. The starved and CTAB washed cells were rinsed 4 times with 1 ml sterile seawater, and washed in 200 µl sterilized seawater. For each PCR reaction (Saiki et al. 1988),

a single *Noctiluca* cell was transferred into a sterile PCR tube with 10 µl 1 × PCR buffer (Perkin Elmer) and frozen at –20°C for 10 min to destroy the cell wall of the dinoflagellate. After thawing, this mixture was treated with 1 µl Proteinase K (10 µg ml^{-1}, Boehringer). PCR amplification of a 233 bp fragment (V3 region of the 16S rRNA) was performed with specific eubacterial primers P2 and P3 as described by Kirchner et al. (1999). For DNA sequencing of the bacterial isolates, DNA was extracted as described by Wichels et al. (1998). Purified DNA was used as target DNA in the PCR to amplify nearly the whole 16S ribosomal RNA coding region. The sequences of primers used for amplification of the 16S rRNA gene (GM3F and GM4R, from *Escherichia coli* positions 8 to 1507 bp) have been published by Muyzer et al. (1995). PCR amplification (100 µl including 25 pmol of each primer, 125 µM of each nucleotide, 1 to 5 ng DNA, 1U TFL DNA polymerase, Biozym) was performed in a DNA Thermal Cycler 480 (Perkin Elmer Cetus) using the following conditions: 34 cycles of 1 min at 94°C, 1 min at 42°C and 3 min at 72°C. Aliquots (1 µl) of the amplification products were analyzed by electrophoresis in 0.8% (w/v) agarose gels with 0.5 × TBE buffer and stained with ethidium bromide (0.5 µg ml^{-1}). PCR products were purified by using QIAquick Spin columns (Qiagen) and stored at –20°C until use for DNA sequencing.

DGGE. DGGE was performed using a Protean II electrophoresis system (BioRad). Preparation of polyacrylamide gels, electrophoresis parameters, staining of gels by ethidium bromide and gel documentation were performed as described by Kirchner et al. (1999). PCR samples (20 to 45 µl) were applied onto the polyacrylamide gel. A PCR-positive control was applied as a standard on every gel. For sequencing of DGGE bands, 3 samples of turbid *Noctiluca* cells were applied on another DGGE gel (15 to 55% denaturing gradient). After electrophoresis (3 h at 140 V), DGGE bands were excised from the gel and eluted (Sambrook et al. 1989). The reamplified PCR fragments were purified (QIAquick spin column, QIAgen) and cloned into the pGEM-T-Vectorsystem (Promega) according to the manufacturers' instruction.

Table 1. Sequencing primers used for automated sequencing with the Liqor DNA-4200 sequenator

Primer	Sequence
8F-IRD	5'-AGA GTT TGA TCM TGG CTC AG-'3
782F-IRD	5'-AAC AGG ATT AGA TAC CCT GG-'3
800R-IRD	5'-CCA GGG TAT CTA ATC CTG TT-'3
1408R-IRD	5'-TAC CTT GTT ACG ACT T-'3
SP6-IRD	5'-TTT AGG TGA CAC TAT AGA ATA C-'3
T7-IRD	5'-TAA TAC GAC TCA CTA TAG GG-'3

DNA sequencing of PCR products and comparative sequence analysis. DNA sequencing of bacterial isolates was performed according to the manufacturer's instructions on a Liqor DNA 4200 sequencer using the SequiTherm EXEL II long read sequencing Kit-LC (Biozym). SequiTherm EXEL II DNA Polymerase was added according to the instructions of the manufacturer (Biozym). For sequencing the following, infrared labeled primers were used: 8F-IRD, 800R-IRD, 782F-IRD, and 1408R-IRD (Table 1). All sequences were aligned to those obtained from the RDP (Maidak et al. 1996) or GenBank (Benson et al. 1997). Sequence alignment was performed with the sequence editor of CLUSTALW Version 1.7 (Thompson et al. 1994). A phylogenetic tree was created using the neighbor joining algorithm as the model for evolution (Treecon version 1.3b; Van de Peer & De Wachter 1994). Bootstrap analysis (1000 replicates) was used to validate the reproducibility of the branching pattern of the tree.

The purified plasmids (Qiaquick plasmid prep Kit, QIAgen) containing the cloned P2/P3-16S rRNA fragments (DGGE fragments) were sequenced with the SP6-IRD and T7-IRD sequencing primers (Table 1) as described above.

Nucleotide sequence accession numbers. GenBank accession numbers of the sequenced bacterial strains are AF262735 to AF262752. The small subunit rRNA sequences used for phylogenetic analysis have the following GenBank accession numbers: *Microbacterium testaceum* [X77445.1], *Microbacterium maritypicum* [AB004728], *Micrococcus luteus* [AF057289.1], *Stenotrophomonas maltophilum* [AJ1331117], *Moraxella catarrhalis* [A27627], *Psychrobacter immobilis* [U85880.1], *Marinobacter hydrocarbonoclasticus* [X67022], *Flectobacillus* sp. S36 [U14584.1], *Marinobacter* PCOB-2 [AJ000647], *Alteromonas macleodii* [Y18234.1], *Vibrio tapetis* [Y08430.1], *Vibrio orientalis* [X74719.1], *Pseudoalteromonas rubra* [X82147], *Pseudoalteromonas haloplanktis* spp. *tetraodonis* [X82139], *Pseudoalteromonas carrageenovora* [X82134.1], *Escherichia coli* [AE000129.1].

RESULTS AND DISCUSSION

DGGE has been used to determine the genetic diversity of total bacterial communities and of specific populations in many different environments (Muyzer 1999). In the present study, the population diversity of endocytic bacteria in the marine dinoflagellate *Noctiluca scintillans* was investigated with DGGE. Band patterns were compared between the endocytic bacterial populations of cultivated and non-cultivated turbid *Noctiluca* cells. DGGE bands of these bacterial populations as well as endocytic bacteria isolated from single *Noctiluca* cells were characterized by 16S rRNA phylogenetic analysis.

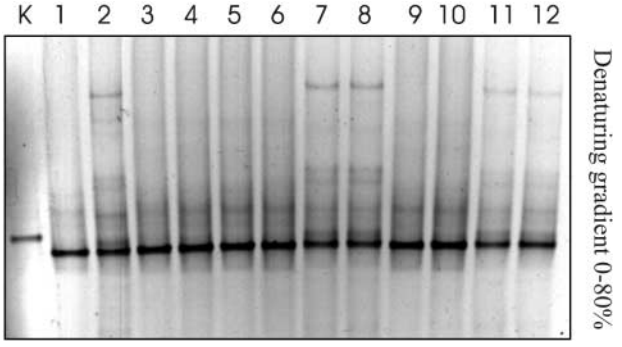

Fig. 1. DGGE profiles of PCR amplified 16S rRNA gene fragments (233 bp) from 12 turbid free-living (non-cultivated) *Noctiluca* cells. Samples were taken from waters of Helgoland Road (North Sea, German Bight) between 7 and 11 July 1997. Control (Lane K) is *Escherichia coli* J53

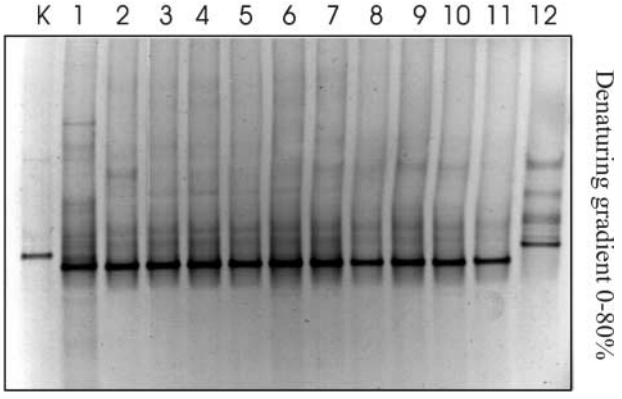

Fig. 2. DGGE profiles of PCR amplified 16S rRNA gene fragments (233 bp) from endocytic bacteria populations in 12 parallel clone cultures of turbid *Noctiluca* cells

Noctiluca scintillans is one of the largest dinoflagellates with a diameter of ca 200 to 500 µm. The first report of intracellular bacteria was provided by Lucas (1982), who investigated *Noctiluca* cells by scanning electron microscopy. His study and the observations of Kirchner et al. (1999) showed that the cell surface was free from bacterial growth. Based on these observations we presume that the cell surface of *Noctiluca* is free from attached bacteria. Indeed there is no proof that all bacteria have been removed from the outer cell surface of the dinoflagellate cell. However, in considering the high number of intracellular bacteria (estimates 10^5 to 10^6 bacterial cells/*Noctiluca* cell, Kirchner et al. 1999), the probability is extremely low that the bacterial isolates or the PCR products derive from the cell surface of the dinoflagellate. It can rather be assumed that bacterial isolates and PCR products described in this paper are derived from the intracellular bacteria of *Noctiluca*.

In most of the samples, the DGGE band patterns of the endocytic bacterial populations in turbid *Noctiluca* cells showed similar profiles, consisting of 1 strong band and diverse weak bands (Figs 1 & 2). The dominant bands appeared almost at the same position. Only in some samples does the dominant band move to a slightly different position. This was shown for endocytic bacterial populations from cultured (see Fig. 2, Lane 12) as well as from non-cultured *Noctiluca* cells (results are not shown). From these results, we conclude that the majority of the *Noctiluca* cells investigated in this study harbor 1 dominant bacterial group. In addition to the single dominant band several weak bands occurred, representing bacterial groups of less dominance. Band patterns of these weak bands were similar within cultured and non-cultured *Noctiluca* cells.

In contrast, DGGE analyses of bacteria in the culture medium of *Noctiluca* and in the feeding organism *Dunaliella tertiolecta* both showed completely different band patterns compared to the endocytic bacteria populations (results are not shown). This presumably indicates that changes of the endocytic bacterial population in *N. scintillans* are not due to cultivation effects. The pattern obtained from DGGE profiles of free-living bacterioplankton showed several predominant bands (Fig. 3). The large number of bands suggests the presence of a relatively large number of different bacteria. This implies high diversity. Furthermore, the intensity of these bands changed over the sampling time. Øvreås et al. (1997) assumed that these changes are due to seasonal changes in temperature and population dynamics of bacterioplankton and co-occurring phyto- and zooplankton.

The high variability of the planktonic bacteria, as shown by DGGE, in contrast to the low variability of the endocytic bacterial community in *Noctiluca scintillans* indicates the development of a specific bacterial

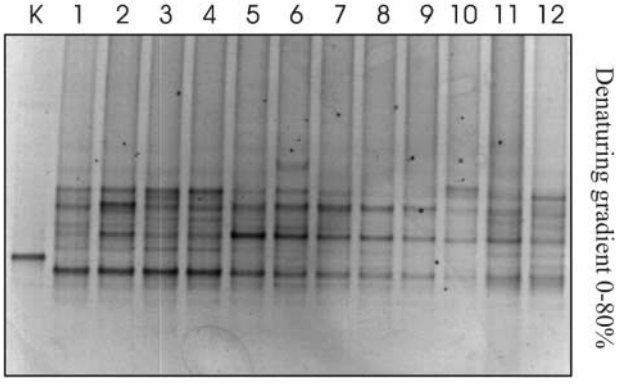

Fig. 3. DGGE profiles of PCR amplified 16S rRNA gene fragments (233 bp) from seawater filtration samples (0.22 to 3 µm fraction). Samples were collected weekly from 11 June 1997 (Lane 1) to 27 August 1997 (Lane 12)

population, adjusted to the physiological conditions in *Noctiluca* cells.

The results are consistent with observations of high bacterial diversity in a number of different aquatic habitats described by DGGE. The microbial populations of hydrothermal vents showed 4 dominant bacterial groups (Muyzer et al. 1995) and in microbial mats of a hot spring up to 10 dominant groups were described in different seasons, different populations, and temperature zones (Ferris & Ward 1997). Øvreås et al. (1997) detected 3 to 6 dominant groups in the bacterioplankton of the meromictic lake Sælenvannet. Variations in the population were detected at different depths of the lake and seasonal changes were observed as well.

From the literature, it can be assumed that the number of bands found in the DGGE gel is proportional to the number of occurring types of bacteria (Borresen et al. 1988, Muyzer et al. 1993). In individual cases, the occurrence of compensating effects in different melting domains (Myers et al. 1985, Muyzer et al. 1993, Nübel et al. 1996) as well as the occurrence of multiple 16S rRNA operons (Mylvalganam & Dennis 1992, Nübel et al. 1996) may falsify the band patterns in the DGGE gel.

Sequence analysis of DNA sequences obtained from cloned DGGE bands (Table 2) revealed that 4 of them belong to the γ subdivision of Proteobacteria. The sequence of another DGGE band (DGGE3) was identical to the *Noctiluca scintillans* 18S ribosomal gene (100%). The amplification of non-bacterial DNA (chloroplasts and plastids) using bacteria-specific DGGE primers (P2/P3) has been reported previously (Riemann et al. 1999). Rapp et al. (1998) amplified DNA of plastidal origin using PCR primers also originally designed for amplification of bacterial SSU rDNA. The DGGE primer P2 is specific for the domains of *Eubacteria*, *Archaea* and *Eukarya*, and primer P3 is specific for most *Bacteria* (Muyzer et al. 1993). This could be one reason for the amplification of 18S rDNA of *N. scintillans*.

Phylogenetic analysis of the 16 bacterial isolates investigated revealed that they belong to several different groups of the domain *Eubacteria* (Fig. 4), Gram-positive bacteria of the high G + C subdivision (NocA1, NocA3) and Proteobacteria of the γ subdivision. Most bacteria (5 isolates) and the sequence of 1 DGGE band (DGGE2) are related to species *Marinobacter* PCOB-2, formerly isolated from dinoflagellate blooms (Ogata et al. 1989). The closest relative of NocA15 is *Alteromonas macleodii* known to be related to toxic dinoflagellate blooms (Gerdts et al. 2000) and belongs, like PCOB-2, to the Colwellia group of the γ subdivision. Four isolates (NocA6, NocA7, NocA8, NocA9) and the sequence of another DGGE band (DGGE5) are closely related to the fast-growing *Pseudoalteromonas* group, which has previously been isolated in waters around Helgoland (Wichels et al. 1998). Two other isolates (NocA2, NocA4) and the sequence of 2 DGGE bands (DGGE1, DGGE4) were related to the *Vibrio* group, a broad group which contains several pathogenic marine bacteria. Both isolates are related to *Vibrio pectenicida* (91 to 97% similarity) isolated from scallops. *V. pectenicida* was shown to cause disease in a scallop hatchery (Lambert et al. 1998). The sequence of the DGGE bands showed high similarity to *Salinivibrio vallismortis* (96 and 97%), a halotolerant, facultative anaerobic *Vibrio*. Two other isolates are related to the *Moraxella* group and *Stenotrophomonas* group of the γ subdivision of Proteobacteria.

DGGE band sequencing revealed that 2 of the DGGE bands (DGGE2 and DGGE5) are related to the isolated bacteria NocA11 (*Marinobacter* group), and NocA9 (*Pseudoalteromonas* group). In contrast to our findings, Suzuki et al. (1997) stated for marine bacterioplankton that there is no agreement between the most frequently occurring bacteria as determined by sequencing after cloning and the culturable bacteria. However, this seems to be not due to the endocytic bacterial population of *Noctiluca scintillans*. The development of specific DNA probes and *in situ* hybridization of *Noctiluca* cells will give us the opportunity to locate the bacteria exactly. Additionally, this technique will provide more information concerning the occurrence and abundance of the specific endocytic bacteria in *Noctiluca scintillans.*

Table 2. DNA sequences of DGGE bands (*E. coli* position: 341f to 519r bp). DGGE bands (part of the bacterial 16S rDNA amplified from 3 turbid *Noctiluca* cells) were excised from a DGGE gel (15 to 50% denaturing gradient) and sequenced after cloning

Isolate	Best match	Accession no.	Similarity (%)	No. of nucleotides compared	Division
DGGE1	*Salinivibrio vallismortis*	AF057016	96	195	γ Proteobacteria, *Vibrio* subgroup
DGGE2	*Marinobacter* PCOB-2	AJO00647	98	195	γ Proteobacteria, *Marinobacter hydrocarbonoclasticus* group
DGGE3	*Noctilluca scintillans*	AF022200	100	167	Eukaryota, Dinophyceae
DGGE4	*Salinivibrio vallismortis*	AF057016	97	194	γ Proteobacteria, *Vibrio* subgroup
DGGE5	*Pseudoalteromonas* sp. 2b	AF132858	99	195	γ Proteobacteria, *Pseudoalteroma*s group

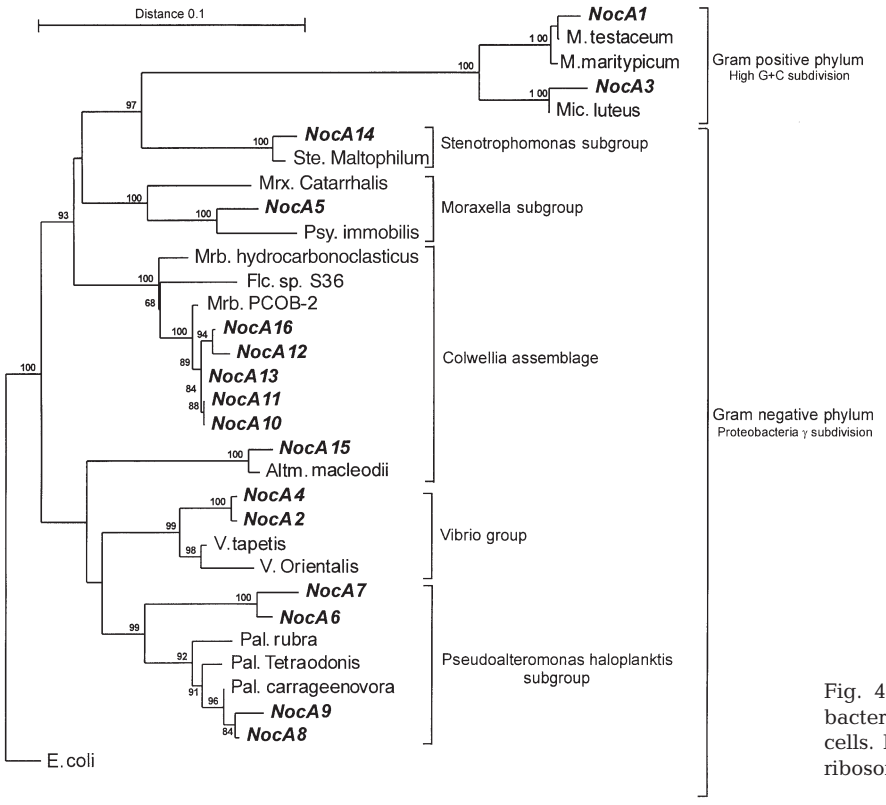

Fig. 4. Phylogenetic affiliation of endocytic bacteria isolated from *Noctiluca scintillans* cells. Distance tree based on 1350 bp of 16S ribosomal RNA. The bootstrap values equal to or greater than 50% are shown

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