New and potentially toxic isolates from *Noctiluca scintillans* (Dinoflagellata)

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ABSTRACT

Although generally regarded as harmless in European waters, the bloom-forming dinoflagellate *Noctiluca scintillans* has been occasionally associated with fish mortality and reduced shrimp yields in Asia. The reason for this phenomenon is unclear, but it is possible that bacteria may be involved in harmful algal blooms; the bacteria may produce their own toxins or influence the toxic levels of the algae. It is thus noteworthy that up to 1 % of *Noctiluca* occurring in the southern North Sea contain large numbers of intracellular bacteria; these *Noctiluca* cells appear visibly turbid.

Analysis of the diversity and dynamics of bacterial populations associated with *Noctiluca scintillans* by denaturing gradient gel electrophoresis (DGGE) indicates the occurrence of one dominant group of bacteria within *Noctiluca* and different other groups in smaller amounts. In contrast, free-living bacterial populations in the water column consist of several different dominant groups. Up to now 18 bacterial isolates from *Noctiluca* cells have been cultured. The bacteria have been characterized by classic physiological (including antibiotic sensitivity) and molecular biological methods. Phylogenetic analysis of the 16S rDNA of the bacteria revealed a great diversity among the bacterial isolates belonging to different groups of bacteria, i.e. bacteria of the γ-subdivision of the Proteobacteria, and of the Gram positive high G+C% group. Two of the isolates - one belonging to the α-subdivision of the Proteobacteria and *Alteromonas macleodii* - show sodium channel blocking activity. The role and significance of the intracellular bacteria with regard to *Noctiluca* blooms is discussed.

INTRODUCTION

The relationships between bacteria and harmful algal blooms may be very complex. While many algae are able to produce toxins [see reviews by 1, 2] bacteria attached to or associated with algae may also produce toxic substances, or influence the toxicity of the algae [3-7].

Of particular interest are the bacteria living intracellularly in bloom-forming algae, where the conditions for their survival and growth are very different from those in the water column or on the outer cell surfaces. Among the dinoflagellates, intracellular bacteria/cyanobacteria have been found in *Glenodinium foliaceum* Stein and *Gonyaulax diacantha* (Meunier) Schiller [8, 9]; *Amphidinium herdmanii* and *Katodinium glandulum* [10], *Gymnodinium lebourae* Herdman [11], *Gymnodinium splendens* Lebour [9]; *Noctiluca scintillans* Macartney 1810 syn. *miliaris* Suriray 1836 [12, 13]; *Peridinium balticum* [14] and in the genera *Ornithocercus*, *Histioneis* and *Citharistes* [15, 16].

Not all of the above mentioned dinoflagellates form conspicuous or harmful blooms. Although considered as non-toxic in European and American waters, *Noctiluca* has occasionally been implicated in fish mortality and reduced shrimp yields in Asia [17,18]. In many cases, however, the evidence may be circumstantial. Ammonia accumulation and oxygen depletion have also been named as factors in *Noctiluca* toxic phenomena.

In this paper we describe 18 bacterial isolates from *Noctiluca scintillans* cells, all of which have been cultured. These are characterized by classical physiological and molecular biological methods. The role and significance of the intracellular bacteria with regard to *Noctiluca* blooms are discussed.

MATERIALS AND METHODS

The determination of *Noctiluca* abundance in plankton hauls at the Helgoland Roads (German Bight, North Sea), cultivation of *Noctiluca* in the laboratory and experimental conditions are described in [13]. In brief, plankton hauls were examined 5 days a week; clear and turbid cells were counted separately under a stereo microscope. All turbid and clear *Noctiluca* were maintained in the laboratory at 19±1°C in glass vessels containing 50-100 ml of 0.45µm filtered seawater and fed with the unicellular green alga *Dunaliella tertiolecta* Butcher. Cultures were not axenic.

Endocytic bacterial isolates designated as NE1 and NE2 (*Alteromonas macleodii*) were isolated from turbid *Noctiluca* cells [13] and maintained on ZoBell agar slants. Bacterial isolates numbered 1 through 16 originated from both clear and turbid (free living and cultured) *Noctiluca* cells without food vacuoles, treated with cetyl-trimethyl-ammonium bromide (CTAB, 1 µg ml^{-1}), washed 4x with sterile seawater and transferred to 5 ml liquid diluted (10% strength) ZoBell medium (pH range from 3.1 to 7.9). Cells were then pierced and incubated on shakers (90 rpm) at 18°C. After 7 d, 38 µl were plated onto ZoBell agar and incubated further at 18°C.

Isolates NE1 and NE2 were tested by the German Collection of Microorganisms (DSMZ, Braunschweig). Morphological and physiological tests were performed on isolates 1-16 according to [19].

Tests for antibiotic sensitivity of all isolates were based on the method of [20]. Susceptibility discs (Oxoid

Ltd., Hampshire, England) included chloramphenicol 10 µg, penicillin G 5 i.u., tetracycline 30 µg, streptomycin 10 µg, penicillin 1 i.u., and nystatin 100 i.u. All isolates were grown on ZoBell agar. Clear zones around the discs indicating growth inhibition were measured after 4 days at 20°C. *Escherichia coli* B (Kiel) served as control. Screening for production of antibiotic-like substances was performed by streak tests on ZoBell agar [21], modified. Species tested for growth inhibition by the endocytic bacteria were *E. coli* B, two North Sea isolates and 7 marine bacteria species from the NCIMB (National Collections of Industrial and Marine Bacteria Ltd., Aberdeen, Scotland). Laboratory experiments were performed to determine if the endocytic bacteria in turbid *Noctiluca* cells would be visibly reduced by the presence of antibiotics in the culture water. Penicillin G, chloramphenicol and streptomycin were used at a final concentration of 10 mg l^{-1} and also a mixture of all three. Both turbid and clear *Noctiluca* cells were placed in glass vessels (10 cells in 40 ml sterile seawater) with *Dunaliella* food; change of water and food took place every 4 days. Vessels were examined daily and evaluated after 9 days.

The mouse neuroblastome (MNB) assay for the detection of sodium channel blocking (SCB) toxins was performed by the method of [22].

Molecular biological methods were used to describe and classify the culturable as well as non-culturable endocytic bacteria from clear and turbid *Noctiluca* cells, from *Noctiluca* and *Dunaliella* culture water, and from the marine environment.

To characterize the endocytic bacterial population in its entirety within *Noctiluca*, both clear and turbid cells were used. Single *Noctiluca* cells were used for PCR amplification after extensive washing procedure to remove bacteria from the outer cell surface [13]. The ensuing DNA extract was used for PCR amplification and DGGE. Samples of water from the marine environment were taken from 25 June to 6 August 1997. Bacterial biomass was concentrated for nucleic acid extraction using a combination of methods [23]. After prefiltration with 60 µm gauze and 3 µm filters, the water samples were pumped through a 0.22 µm Sterivex filter unit (Millipore Corp.). Filters were stored at -20°C until further processing for PCR and DGGE.

PCR experiments were carried out for *Noctiluca* and bacterial DNA according to [24], briefly described in [13]. DGGE was performed according to [24].

RESULTS

The annual percentages of turbid cells occurring in plankton hauls at Helgoland from 1995 to 1999 were 0.41, 0.46, 0.06, 0.07, and 1.35%, respectively. While the values for 1997-98 are relatively low, there was a large increase in 1999, particularly during the last week in July.

On ZoBell agar all isolates form colonies with a shiny surface; two show yellow pigmentation (1 and 3), the rest are beige. Two are Gram positive, most cells are rod shaped and all but two are motile (Table 1). Isolate no. 1 is oxidase negative; only two do not have the enzyme catalase. Most of the isolates are capable of both oxidative and fermentative hydrolysis of sugars; nos. 1 and 3 are unable to utilize any of the sugars and no. 5 only fermentative.

Table 1. Characterization of bacterial isolates from *Noctiluca* cells (t = turbid *Noctiluca* cell, c = clear; oxid., cat. = presence of cytochrome oxidase and catalase)

Isolate No.	Noctiluca source	Gram stain	Cell form	Motility	Oxid.	Cat.	SCB-blocking activity
NE1	t, free living	-	irreg. rods	+	+	+	+
NE2	t, cultured	-	rods	+	+	+	+
1	t, free living	positive	coccoid	+	-	+	-
2	t, free living	-	curved rods	-	+	-	-
3	c, free living	positive	coccoid	-	+	+	-
4	c, free living	-	spiral rods	+	+	-	-
5	t, free living	-	coccoid	+	+	+	-
6	t, free living	-	spiral rods	+	+	+	-
7	t, free living	-	rods	+	+	+	-
8	t, free living	-	rods	+	+	+	-
9	c, free living	-	rods	+	+	+	-
10	c, cultured	-	rods	+	+	+	-
11	c, cultured	-	rods	+	+	+	-
12	c, cultured	-	rods	+	+	+	-
13	t, cultured	-	rods	+	+	+	-
14	t, cultured	-	rods	+	+	+	-
15	t, cultured	-	coccoid	+	+	+	-
16	c, cultured	-	rods	+	+	+	-

Production of antibiotic-like substances: in streak tests none of the isolates inhibited the growth of ten species tested (data not shown).

Antibiotic sensitivity of endocytic bacteria: clear zones around the antibiotic discs indicating growth inhibition were measured. Results show that all isolates are sensitive to chloramphenicol and streptomycin, and only 3 to nystatin. Isolates nos. 2 and 4 were inhibited by all antibiotics tested.

The addition of antibiotics to the culture water either singly or combined did not visibly reduce the turbidity of *Noctiluca* cells as observed over a 9-day period under a dissecting microscope. The growth rates were determined after 4 days before some cells ceased to divide and formed swarmers. The growth rates µ of turbid *Noctiluca* with antibiotics present ranged from 0.08 to 0.13; the number of cells in the control remained unchanged. Clear cells with antibiotics had µ values from 0.83 to 0.99 (0.95 for the control). Higher growth rates for clear cells compared to turbid have been previously reported [13].

Isolates NE1 and NE2 demonstrate sodium channel blocking activity. The remaining isolates do not.

Molecular biological analyses of endocytic bacteria in their entirety within free-living turbid *Noctiluca* cells by DGGE showed a single band of high intensity, and above this, several weaker bands (Fig. 1). The same pattern was found for endocytic bacteria from laboratory cultured turbid *Noctiluca* cells. This indicates the presence of one

dominant bacterial group and several less abundant groups. There was no change in band patterns corresponding to length of *Noctiluca* cultivation, whether the original turbid cells were free-living or longer in laboratory culture, at the time of analysis (data not shown).

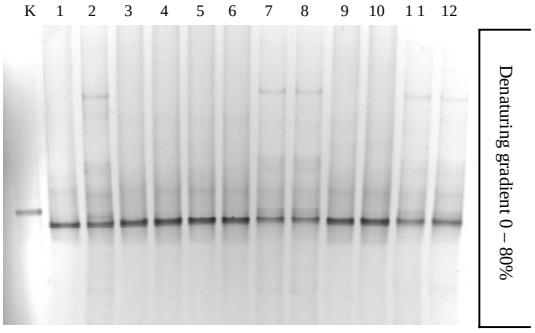

Figure 1. DGGE gel band patterns of endocytic bacteria from free living turbid *Noctiluca* cells. Lanes 1-12 represent samples taken 7 to 11 July 1997.
K : reference strain *E. Coli* J53.

Free-living marine bacteria showed a variable gel pattern with at least 10 bands of different intensities. Thus as many as 10 dominant bacterial groups may occur; no single main band was observed. Bands produced from bacteria from *Dunaliella* and *Noctiluca* culture water did not show any agreement with those from free-living and cultured *Noctiluca* cells (data not shown).

DISCUSSION

Visibly turbid *Noctiluca* cells occur regularly in plankton hauls at Helgoland. Unusually high numbers were recorded at the end of July 1999, at the end of a *Noctiluca* bloom ("red tide"). While endocytic bacteria are transferred to daughter cells during cell division [13] it is not yet known how the bacteria were initially able to infect *Noctiluca* and enter its cytoplasm. Possibly "sloppy feeding" may be involved, whereby bacteria entering the cytostome with other food particles bypass a vacuole membrane and actively invade the host cytoplasm [12]; indeed, most of the endocytic bacterial isolates from *Noctiluca* cells are motile. The bacteria are not enclosed within a membrane as reported for other dinoflagellates with endocytic bacteria [11, 14]. Ingested diatoms with sharp edges could rupture the food vacuole, allowing bacteria to escape. Destruction of the vacuole membrane by bacterial enzymes is also conceivable.

An estimated number of 10^5 - 10^7 bacteria may be found within a single turbid *Noctiluca* cell [13]. The turbidity was not reduced when antibiotics were added to the culture water. Turbid cells in culture have lower growth rates than clear cells, and turbid stock cultures are more prone to collapse than clear cells under the same conditions. Large numbers of intracellular bacteria may therefore be detrimental to the growth of *Noctiluca*.

In the marine environment, free living or attached bacteria may influence the growth of harmful algal species, whereby the effect tends to be more inhibitory than stimulatory [2]. Algicidal activity of bacteria has already been described for various phytoplankton species including dinoflagellates, diatoms and raphidiophytes [25-29]. It is possible that endocytic bacteria in *Noctiluca*, without being directly algicidal, may bring about a decline during a bloom. This would not be without precedent: algicidal marine bacteria (free living, not endocytic) may be involved in the termination of an algal bloom in Hiroshima Bay, Japan [30].

Endocytic bacteria have been found in many species of algae [10-15]. In most of these cases, the algae cells were cultured. The bacteria were described, if at all, as rods or cocci, but were not further identified. With molecular biological methods, it could be shown that endocytic bacteria from turbid and clear *Noctiluca* differ from one another in quality as well as quantity. Turbid *Noctiluca* cells, whether free-living or cultured over different periods of time, harbor similar groups of bacteria consisting of one dominant and several less abundant groups. A cultivation effect may thus be excluded. Bacterial DNA from clear *Noctiluca* cells shows a different pattern from that of turbid cells [13, 31]. Endocytic bacteria produce DGGE gel patterns which are different from those of free-living marine bacteria and again from those present in *Noctiluca* and *Dunaliella* culture water. This indicates that the endocytic bacteria in *Noctiluca* consist of populations which are especially adapted to the environment in the host cytoplasm.

Characterization of 18 isolates from both turbid and clear *Noctiluca* cells shows that the majority belong to the γ-subgroup of Proteobacteria, and one to the α-subgroup. The phylogenetic diversity of these isolates compared with bacteria from other biotopes is discussed in detail elsewhere [31]. Two isolates show sodium channel blocking activity: NE1 and NE2. Isolates 10-13 and 16 show a similarity to isolate PCOB-2 which also shows sodium channel blocking activity, and was isolated from the toxic alga *Protogonyaulax* (= *Alexandrium*) *cohorticula* [32]. Four (nos. 6-9) belong to the *Pseudoalteromonas* group, species of which are generally found in association with marine eukaryotes and show e.g. antibacterial and algicidal activity; several in the group produce toxic substances [29, 33]. Members of the genera *Aeromonas, Alteromonas/Pseudomonas* and *Vibrio* were frequently isolated from dinoflagellate bloom as well as non-bloom waters [3, 7].

Noctiluca is regarded as a non-toxic species in European waters, despite occasional reports of toxic phenomena in connection with *Noctiluca* blooms in other areas of the world [34]. However, *Noctiluca* cells may contain large numbers of endocytic bacteria including strains or species which may be involved in the production of harmful substances. These turbid *Noctiluca* cells demonstrate a reduced growth rate compared to clear cells. Large numbers of endocytic bacteria may

conceivably be involved in the decline of a *Noctiluca* bloom in the North Sea, as e.g. in July 1999 when the proportion of turbid cells was relatively high. The bacteria which had become concentrated within the cells may be released into the water column after a bloom breakdown.

It is known that attached bacteria may alter the toxicity of an algal species [7] or themselves be involved in the production of toxic substances [4, 32, 6,]. With the exception of a *Moraxella* sp. isolated from an apparently axenic culture of *Protogonyaulax* (= *Alexandrium*) *tamarensis*, which possibly was inside the algal cells [4], other toxin-producing bacteria reported appear to be externally associated with the algae and present in the environmental water. There are no reports of bacterial enrichment within the algal host cells.

In conclusion, it is recommended to examine *Noctiluca* cells obtained from other parts of the world, especially where toxic phenomena occur, for turbid cells with endocytic bacteria.

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