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IDENTIFICATION OF BACTERIA ASSOCIATED WITH DINOFLAGELLATES (DINOPHYCEAE) *ALEXANDRIUM* SPP. USING TYRAMIDE SIGNAL AMPLIFICATION–FLUORESCENT *IN SITU* HYBRIDIZATION AND CONFOCAL MICROSCOPY[1]

Isabelle C. Biegala

Station Biologique de Roscoff, CNRS UMR 7127, Université Pierre et Marie Curie, BP 74, F-29682 Roscoff cedex, France

Gabrielle Kennaway

University of Westminster, School of Biosciences, 115 New Cavendish Street, London GB-W1M 8JS, United Kingdom

Elsa Alverca

National Institute of Health Dr. Ricardo Jorge, LME, Av. Padre Cruz, PT-1649-016 Lisboa cedex, Portugal

Jean-François Lennon, Daniel Vaulot,[2] *and Nathalie Simon*

Station Biologique de Roscoff, CNRS UMR 7127, Université Pierre et Marie Curie, BP 74, F-29682 Roscoff cedex, France

In the marine environment, phytoplankton and bacterioplankton can be physically associated. Such association has recently been hypothesized to be involved in the toxicity of the dinoflagellate genus *Alexandrium*. However, the methods, which have been used so far to identify, localize, and quantify bacteria associated with phytoplankton, are either destructive, time consuming, or lack precision. In the present study we combined tyramide signal amplification–fluorescent *in situ* hybridization (TSA-FISH) with confocal microscopy to determine the physical association of dinoflagellate cells with bacteria. Dinoflagellate attached microflora was successfully identified with TSA-FISH, whereas FISH using monolabeled probes failed to detect bacteria, because of the dinoflagellate autofluorescence. Bacteria attached to entire dinoflagellates were further localized and distinguished from those attached to empty theca, by using calcofluor and DAPI, two fluorochromes that stain dinoflagellate theca and DNA, respectively. The contribution of specific bacterial taxa of attached microflora was assessed by double hybridization. Endocytoplasmic and endonuclear bacteria were successfully identified in the nonthecate dinoflagellate *Gyrodinium instriatum*. In contrast, intracellular bacteria were not observed in either toxic or nontoxic strains of *Alexandrium* spp. Finally, the method was successfully tested on natural phytoplankton assemblages, suggesting that this combination of techniques could prove a useful tool for the simultaneous identification, localization, and quantification of bacteria physically associated with dinoflagellates and more generally with phytoplankton.

***Key index words:* *Alexandrium*; bacteria; confocal microscopy; dinoflagellates; fluorescent *in situ* hybridization (FISH); *Gyrodinium instriatum*; phytoplankton; tyramide signal amplification (TSA)**

***Abbreviations:* CLSM, confocal laser scanning microscopy; CY3, cyanine 3; DAPI, 4,6-diamidino-2-phenylindole; FISH, fluorescent *in situ* hybridization; HRP, horseradish peroxidase; TSA, tyramide signal amplification**

Phytoplankton and bacterioplankton are fundamental components of marine pelagic ecosystems, and the specificity of their association has been demonstrated in coastal areas (Azam et al. 1983, De Long et al. 1993). Although alpha and gamma proteobacteria, as well as Cytophaga-Flavobacter and Planctomycetes, have been recognized as important members of coastal bacterioplankton (Hagström et al. 2000, Glöckner et al. 1999), epsilon proteobacteria have been more rarely detected and have been found to be specifically associated with a phytoplankton bloom off New Jersey (USA (Kerkhof et al. 1999). Other studies have demonstrated that within a phytoplankton bloom, alpha proteobacteria dominate the free-living bacterioplankton, whereas bacteria attached to phytoplankton are mainly identified as belonging to Cytophaga-Flavobacter, gamma proteobacteria, and Planctomycetes groups (De Long et al. 1993, González and Moran 1997). In the context of harmful algal blooms, the specificity of these associations has also been investigated (Doucette et al. 1998). For some dinoflagellates, such as *Alexandrium tamarense*, it has been hypothesized that bacteria, either intracellular or attached to the dinoflagellate, could be involved in the production of toxins such as paralytic shellfish toxins (Silva 1982, Kodama et al. 1990). In fact, paralytic shellfish toxin has been detected in bacteria isolated from *Alexandrium* sp. cultures (Kodama et al. 1990, Gallacher et al. 1997). These bacteria were identified as members of the *Roseobacter* and *Alteromonas* clades, which belong to alpha and gamma proteobacteria, respectively (Doucette and Tricks 1995, Gallacher et al. 1997).

Identification, localization, and quantification of specific bacteria taxa, closely associated with phytoplank-

[1]Received 5 March 2001. Accepted 31 December 2001.
[2]Author for correspondence: e-mail vaulot@sb-roscoff.fr.

ton, are thus of prime importance for a better understanding of the occurrence of toxic blooms and more generally to assess bacteria–phytoplankton association in marine pelagic ecosystems. So far the physical association between bacteria and phytoplankton has been studied by different technical approaches: scanning, electron, or epifluorescence microscopy for localization and quantification (e.g. Franca et al. 1995, Paerl 1976, Rausch de Traubenberg et al. 1995, Vaqué et al. 1990), whereas identification of bacteria involved destructive techniques such as DNA extraction followed by sequencing and dot blot hybridization (Lafay et al. 1995, Riemann et al. 2000). However, these techniques cannot provide a simultaneous acquisition of both the identity and spatial localization of bacteria physically associated with phytoplankton. Both types of information could be obtained by *in situ* hybridization using oligonucleotide probes targeting 16S rRNA, associated with a precise method of detection such as confocal microscopy (Doucette et al. 1998).

Within the last 10 years, fluorescent *in situ* hybridization (FISH) has been widely used in aquatic environments to identify free-living and intracellular bacteria (Amann 1995). However, in many cases probes labeled with a single fluorescent molecule (i.e. monolabeled probes) do not yield sufficient fluorescence to be easily detected (Lebaron et al. 1997). This can be due either to a slow rate of cell growth, which translates into a low concentration of rRNA, or to the interference of the autofluorescence of the organism or of the background (Lee et al. 1993, Christensen et al. 1999). Among the different amplification techniques that have been proposed, the tyramide signal amplification (TSA) system uses probes labeled with horseradish peroxidase (HRP) that allow the fixation of radicalized fluorochrome-tyramide substrate in proximity to HRP (Amann et al. 1992, Bobrow et al. 1989). The TSA-FISH technique has been tested successfully for the gram-negative proteobacteria *Escherichia coli* (Schönhuber et al. 1997) and free-living marine cyanobacteria, which have thicker cell walls (Schönhuber et al. 1999).

Observation of *in situ* hybridized cells with a standard epifluorescence microscope does not allow the unambiguous localization of bacteria associated with three-dimensional surfaces, because emission light from different planes (z axis) contributes to the image. In contrast, a confocal laser scanning microscope (CLSM) takes thin optical sections (<1 μm) that remove contribution from planes that are not in focus. This technique is increasingly used to assess natural bacterial diversity associated with three-dimensional substrates such as biofilms, soil, or sludge (Manz et al. 1999, Crocetti et al. 2000).

In this study we investigate the combination of TSA-FISH with confocal microscopy to simultaneously identify, localize, and quantify the amount of bacteria associated (either attached or intracellular) with dinoflagellates (*Alexandrium* spp. and *Gyrodinium instriatum*). Group- and genus-specific eubacteria probes, targeting 16S rRNA, were tested on bacteria associated with dinoflagellates in cultures and on natural phytoplankton assemblages.

MATERIALS AND METHODS

Strains, cultivation, and sampling. Information on dinoflagellate and bacterial strains is summarized in Table 1. *Alexandrium* spp. strains and *G. instriatum* were grown on f/2 Guillard medium (Sigma-Aldrich, Saint Quentin Fallavier, France) in a 200-mL tissue culture flask (Sarstedt, Newton, NC, USA) at 15° C and 20° C, respectively, under a light:dark cycle of 14:10 h, with an intensity of 54 μmol photons·m^{-2}·s^{-1}. The different cultures were sampled in the early stationary phase (21 days old) to obtain the highest cell concentration for FISH analysis. To induce encystment, cells of *A. fundyense* were grown in nitrogen-deficient f/2 medium (Sigma-Aldrich). Encystment was observed after 2 to 3 weeks, and single cysts were selected for analyses. Bacteria strains were grown in marine broth (DIFCO, Elancourt, France) and were either directly sampled for FISH analysis or added to the axenic culture of *A. tamarense* CCMP 1771 and sampled after 34 days to observe bacterial attachment. Bacteria were introduced at 2×10^3 cells·mL^{-1} to the axenic dinoflagellate culture and within 5 days reached 10^5 to 10^6 cells·mL^{-1} and stayed stable until sampling. This cell concentration mimics bacterial abundance usually found in the natural environment (e.g. Azam et al. 1995). Natural samples were collected in May in surface coastal waters off Roscoff (Brittany, France) and filtered through 200-μm mesh size to exclude mesozooplankton.

Cell fixation and dehydration. Samples were fixed with 1% paraformaldehyde at 4° C for 1 h or 24 h, immobilized onto either 0.2-μm pore size Anodisc (Whatman Int. Ltd., Maidstone, Kent, UK) or 12-μm pore size Isopore (Millipore S. A., Saint Quentin, France), depending on the need to collect either both free and attached bacteria or only the bacteria attached to phytoplankton. The cells immobilized on filters were then dehydrated in an ethanol series (50%, 80%, 100%, 3 min each; Amann 1995), dried, and kept at room temperature in the dark until FISH analysis.

Probes. Oligonucleotide probes (Table 2) were purchased with a 5′-aminolink (C6) from Interactiva (Saint Malo, France). Probes were labeled with HRP (Roche Diagnostic Boehringer, Meylan, France) according to Urdea et al. (1988) and Amann et al. (1992).

FISH. Whole cell *in situ* hybridization with TSA amplification was adapted from Amann et al. (1992) and Schönhuber et al. (1997, 1999). Before hybridization, prokaryotic cells were partially lysed by placing the filters for 5 min at room temperature in 5 mL of 100 μg·mL^{-1} lysozyme (47,000 U·mg^1, Sigma-Aldrich) in 0.1 M Tris-HCl and 0.05 M EDTA (pH 7.7). The enzyme reaction was stopped by rinsing the filter three times in 5 mL sterile H_2O for 1 min. Filters were then dehydrated in a second ethanol series (50%, 80%, 100%, 3 min each), dried, and cut into pieces with razor blade. Filter fragments with cells immobilized on top were placed on a well of a Teflon glass slide (Poly Labo, Strasbourg, France) containing 1 μL of oligonucleotide probe labeled with HRP (50 ng·mL^{-1}) and 10 μL hybridization buffer (40% or 50% deionized formamide, Fluka, Sigma-Aldrich [Table 2]; 900 mM NaCl; 20 mM Tris-HCl pH 7.7; 0.01% SDS, Bio-Rad, Ivry sur Seine, France; and 20% of a stock blocking buffer solution, 10% [w/v] of blocking reagent, Roche Diagnostic Boehringer, in maleic acid buffer: 100 mM maleic acid, Sigma-Aldrich; 150 mM NaCl, pH 7.5; sterile filtered). Hybridization was performed at 35° C for 2 h in a prewarmed polypropylene humid hybridization chamber (Poly Labo), in which a piece of Whatman paper, soaked in 800 μL of hybridization buffer, was placed. After hybridization, filters were washed twice for 30 min at 37° C in prewarmed 5 mL hybridization buffer, in which formamide was replaced by an NaCl solution of equivalent stringency (56 mM NaCl for 40% formamide and 28 mM for 50% formamide). Filters were quickly rinsed in 5 mL H_2O and equilibrated for 15 min in 5 mL of TNT buffer (approximately 7% Tween 20, Sigma-Aldrich; in 150 mM NaCl, 100 mM Tris-HCl, pH 7.7) and placed on wells of a Teflon glass slide, with immobilized cells on top.

TABLE 1. Strains used in this study.

Group	Organism	Strains[a]	Characteristics	Origin
Dinoflagellate	*Alexandrium tamarense* (Lebour) Balech	CCMP 1771	Axenic, nontoxic	Plymouth, UK
Dinoflagellate	*Alexandrium tamarense*	PLY 173a similar to CCMP115 and NEPCC 183	Nonaxenic, nontoxic	Plymouth, UK
Dinoflagellate	*Alexandrium tamarense*	NEPCC407	Nonaxenic, toxic	Jerico Beach, Vancouver, BC
Dinoflagellate	*Alexandrium fundyense* Balech	CCMP1719	Nonaxenic, toxic	Gulf of Maine, NH, USA
Dinoflagellate	*Gyrodinium instriatum* Freudenthal and Lee	LEM184	Nontoxic, nonaxenic	Sto. André Lagoon, Portugal,(Silva and Franca 1985)
Bacteria	*Roseobacter* clade (alpha proteobacteria)	AJ294351 (253-11 similar as 253-13)	Toxic	Isolated from *A. lusitanicum* (NEPCC 253), Gallacher et al. (1997)
Bacteria	*Alteromonas* genus (gamma proteobacteria)	AJ294360 (407-2)	Toxic	Isolated from *A. tamarense* (NEPCC 407), Gallacher et al. (1997)
Bacteria	*Zobellia galactonovorans* (Cytophaga-Flavobacter)	DSM12802	Nontoxic	Isolated from a red alga *Delesseria sanguinea* (Huds) Lamour, English Channel, France (Barbeyron et al. 2001).

[a] CCMP, Provasoli-Guillard National Center for Culture of Marine Phytoplankton; PLY, Plymouth Marine Laboratory; NEPCC, North East Pacific Culture Collection; LEM, Laboratory of Experimental Microbiology, Instituto National de Saúde Dr Ricardo Jorge, Lisbon, Portugal; AJ, Fisheries Research Services Marine Laboratory, Aberdeen, UK; DSM, Deutsche Sammlung von Mikroorganismen and Zell Kulturen (German Collection of Microorganisms and Cell Cultures).

For TSA reaction, 10 μL of the fluorochrome-tyramide substrate mixture was added to each filter and left to incubate 30 min at room temperature in the dark. The mixture consisted of dextran sulfate stock solution, 40% (w/v) in sterile H_2O aliquoted and stored at −20° C and mixed (1:1) with the amplification diluent of the TSA-Direct kit (NEN Life Science Product Inc., Boston, MA, USA) (Van Gijlswilk et al. 1996) that was added (50:1) to fluorescein-tyramide (Figs. 1, a, b, d, and e; 2, b–g; and 3, b, d, f, and h), tetramethylrhodamine-tyramide, or cyanine 3-tyramide (TSA-Direct kit). In this study, tetramethylrhodamine was used first in combination with fluorescein for double hybridization but presented a signal-to-noise ratio four times lower than fluorescein (Fig. 2, a–c and g). Tetramethylrhodamine has been subsequently changed to cyanine 3 (CY3) (Fig. 2, d and f). To remove unreacted fluorochrome-tyramide and stop the enzyme reaction, filters were washed twice (20 min at 55° C) in prewarmed 5 mL TNT buffer. Filters were then rinsed in 5 mL sterile H_2O for 1 min. At this stage, filters could be either kept in the dark at room temperature for a day to run a second hybridization or immediately processed for additional staining. DNA and dinoflagellate theca staining were performed by adding 10 μL of a mix containing 5 $\mu g \cdot mL^{-1}$ 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) and 100 $\mu g \cdot mL^{-1}$ calcofluor (Sigma-Aldrich) and incubating 7 min in the dark at room temperature. Calcofluor stains cellulose specifically, the main component of the dinoflagellate theca (Taylor 1987). Filters were then rinsed twice in 5 mL sterile H_2O for 1 min and 15 min, dried, and mounted in antifade AF1 or AF3 (Citifluor Ltd., Canterbury University, UK). Coverslips were sealed with nail varnish. These whole cell *in situ* preparations were kept at 4° C in the dark ready for confocal observation over the next 2 weeks without significant loss of fluorescence.

Microscopy. Epifluorescence microscopy was done with a BH-2 microscope (Olympus Optical Co. Ltd., Tokyo, Japan) equipped with a mercury lamp HBO 100 W/2, OSRAM (Eurosep Instruments, Cergy Pontoise, France), to excite fluorescein through a 400- to 500-nm bandpass filter. Green emission fluorescence was collected at a wavelength greater than 500 nm. The epifluorescence image was acquired with an oil immersion objective 40× (UVFL, numerical aperture 1.30, Olympus), and the micrograph was taken with P1600 Kodak (Rochester, NY, USA) film. Optical sections (0.7 μm) were acquired with CLSM (Fluoview, Olympus) equipped with two lasers: an argon-krypton laser (643R-OLYM-A03 Omnichrome, Melles Griot, Carls-

TABLE 2. Oligonucleotide probes used in this study.

Probe	Specificity	Sequence (5′-3′) of probe	Target[a] site (rRNA positions)	% Formamide in ISH buffer	Reference
EUK1209R	Eukarya	GGGCATCACAGACCTG	18S (1209–1223)	40	Giovannoni et al. (1988)
EUB338R	Bacteria	GCTGCCTCCCGTAGGAGT	16S (338–355)	50	Amann et al. (1990)
AMAC137R	*Alteromonas* clade (gamma proteobacteria subclass)	TGTTATCCCCCTCGCAAA	16S (137–154)	50	Brinkmeyer et al. (2001)
CF319a	Cytophaga-Flavobacterium	TGGTCCGTGTCTCAGTAC	16S (319–336)	50	Manz et al. (1996)

[a] *Escherichia coli* numbering of the ribosomal RNA operon.
ISH, *In situ* hybridization.

bad, CA, USA) to excite at 488 and 568 nm fluorescein and tetramethylrhodamine or CY3, respectively, and a pulsed laser (Mira 900, Coherent, Santa Clara, CA, USA) that produces a 760 nm wavelength, which gave an equivalent two-photon excitation around 380 nm for DAPI and calcofluor stains (Denk et al. 1990). The blue, green, and red emission fluorescence, produced by the different fluorochromes, were collected at wavelengths below 480 nm, between 510 and 550 nm, and above 585 nm, respectively. Confocal images were acquired with different objectives: oil immersion 60× (UplanFi, numerical aperture 1.25, Olympus) and 20× (UplanApo, numerical aperture 0.7, Olympus). Scanning speed was 7.6 s per optical section, and the distance between each slice was 1 μm. Artificial colors were chosen to match the emission wavelengths.

RESULTS AND DISCUSSION

Our major goal was to detect bacteria that are physically associated with the surface of dinoflagellate cells. Because dinoflagellates produced strong autofluorescence, *in situ* identification of bacteria attached to cells was not possible using nonamplified fluorescent signal generated by the hybridization of monolabeled probes (data not shown). In contrast, the TSA system associated with FISH allowed a clear identification of bacteria attached and associated with dinoflagellates when using the general eubacterial probe (EUB338R, Fig. 1a). However, despite the use of the TSA, standard epifluorescence microscopy analysis did not allow discrimination of all labeled bacteria because of the autofluorescent background generated by the dinoflagellates, whereas confocal microscopy overcame this limitation (Fig. 1b). In addition to dinoflagellate autofluorescence, lysozyme and TSA-FISH reagents were also significant sources of background fluorescence (data not shown). Lysozyme was necessary to permeabilize the peptidoglycan layer of the eubacterial cell wall (Madigan et al. 1997), an important step considering the large size of the HRP-labeled probes. However, when used in excess, lysozyme is thought to bind HRP-labeled probes for unknown reasons (it has been hypothesized that lysozyme may display affinity to negatively charged molecules, such as oligonucleotides; Jollès and Jollès 1984). Likewise, dextran sulfate, a polymer added to TSA reagents to increase signal sensitivity and localization (Schönhuber et al. 1999), can produce spotty fluorescent background (Van Gijlswilk et al. 1996). Most of the background fluorescence was successfully removed by increasing the incubation duration, number, and volume of washes after lysozyme, hybridization, and TSA steps from published protocols (Schönhuber et al. 1997, 1999).

It is critical to localize and quantify precisely bacteria attached within the dinoflagellate sulcus or cingulum, avoiding the misidentification of extracellular bacteria as intracellular, as well as to distinguish attached bacteria associated with morphologically intact dinoflagellate cells from those associated with empty theca. For this purpose, we used two additional fluorochromes, calcofluor and DAPI, which stain dinoflagellate theca and DNA, respectively (Fig. 1c). Once identified and located, it was possible to enumerate bacteria attached to dinoflagellates observed on each CLSM optical sections and to visualize their density by adding optical sections (Fig. 1e). Even though no statistical data were provided in this study, it can be clearly seen, when comparing Figure 1a and each optical slice merged together to form Figure 1e, that confocal microscopy allows a more accurate quantification of attached bacteria compared with epifluorescence microscopy.

A routine count of bacteria attached to dinoflagellate was successfully realized in two recent studies using TSA-FISH and confocal microscopy (N. Simon and D. Vaulot, personal communication). However, before these latter works, few studies have tried to quantify bacteria attached to phytoplankton (Kogure et al. 1982, Vaqué et al. 1989, 1990). This was due to the difficulty in distinguishing bacteria labeled with fluorescent dyes from the background generated by phytoplankton pigments. Some authors circumvented the problem by dislodging attached bacteria by sonication and subsequently acquired a precise count with the help of epifluorescence microscopy (Albright et al. 1986). However, information concerning the localization of these bacteria was lost. The association of techniques presented in this study provides a way to discriminate the substrates to which bacteria are attached and to ensure a clear quantification and localization of all attached microflora. However, in any of the method proposed so far, including ours, it is not certain that the counts obtained reflect exactly the number of bacteria attached to phytoplankton at the time of sampling. Collection of sample by filtration may induce some additional attachment of free bacteria on phytoplankton; on the other hand, the different steps of hybridization may dislodge some cells from their substrate.

To determine the relative contribution of specific bacterial taxa to the attached microflora, we initially tried to use DAPI to counterstain all prokaryotes previously labeled with group- or genus-specific probes (e.g. DeLong et al. 1999). However, the blue fluorescence produced by the theca and the DNA of the dinoflagellate masked the bacterial signal. We therefore tested the use of double hybridization with a general 16S eubacteria probe combined with a group- or genus-specific 16S probe targeting the Cytophaga-Flavobacter or *Alteromonas* spp., respectively (Fig. 2). To visualize these probes we used tetramethylrhodamine or CY3 for the general probe (EUB338R) and fluorescein for the specific probes. Cytophaga-Flavobacter were successfully identified either on an artificial mixture of bacterial strains (Fig. 2, a–c) or on bacteria attached to natural phytoplankton or macroalgae fragment (Fig. 2, d and f). Likewise, bacteria belonging to the *Alteromonas* genus could be identified attached to the axenic *A. tamarense* CCMP 1771 strain (Fig. 2g). The EUB338R probe hybridized, in addition of bacteria, the plastids of some phytoplankton cells (Fig. 2d) and in particular that of diatoms (Fig. 2e). However, the probe hybridized neither mitochondria nor the plastids of an unidentified macroalgae fragment (Fig. 2f) and of the dinoflagellates used in this study (Fig. 2g). The latter result is confirmed by the fact that the EUB338R probe sequence does not match with the five dinoflagellate

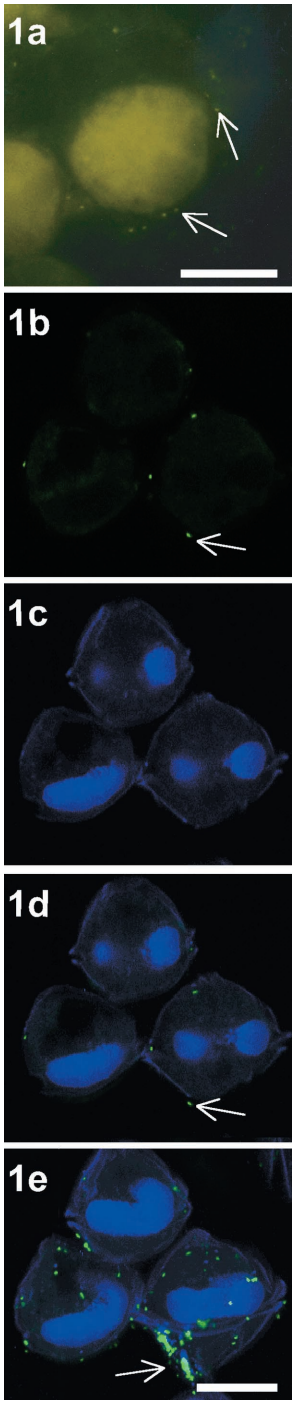

FIG. 1. Vegetative stage of *Alexandrium tamarense* (PLY 173a, nonaxenic, nontoxic) with its associated bacteria. (a and b) Bacteria hybridized with EUB338R oligonucleotide probe, labeled with fluorescein (green) with TSA-FISH technique. (a) Picture taken with an epifluorescence microscope with an excitation bandpass of 400–500 nm. (b) CLSM optical section (0.7 μm thick) with 488 nm excitation. (c) Same optical slice as b, excited at 380 nm, showing the DAPI-labeled nucleus (blue) surrounded by the calcofluor-labeled cellulose (blue) of dinoflagellate theca. (d) Image produced by merging b and c. (e) Superposition of 20 optical slices, combining 380-nm and 488-nm excitations, through the dinoflagellate and its attached bacteria. Bars, 20 μm. Arrows point to bacteria hybridized with the general EUB338R probe.

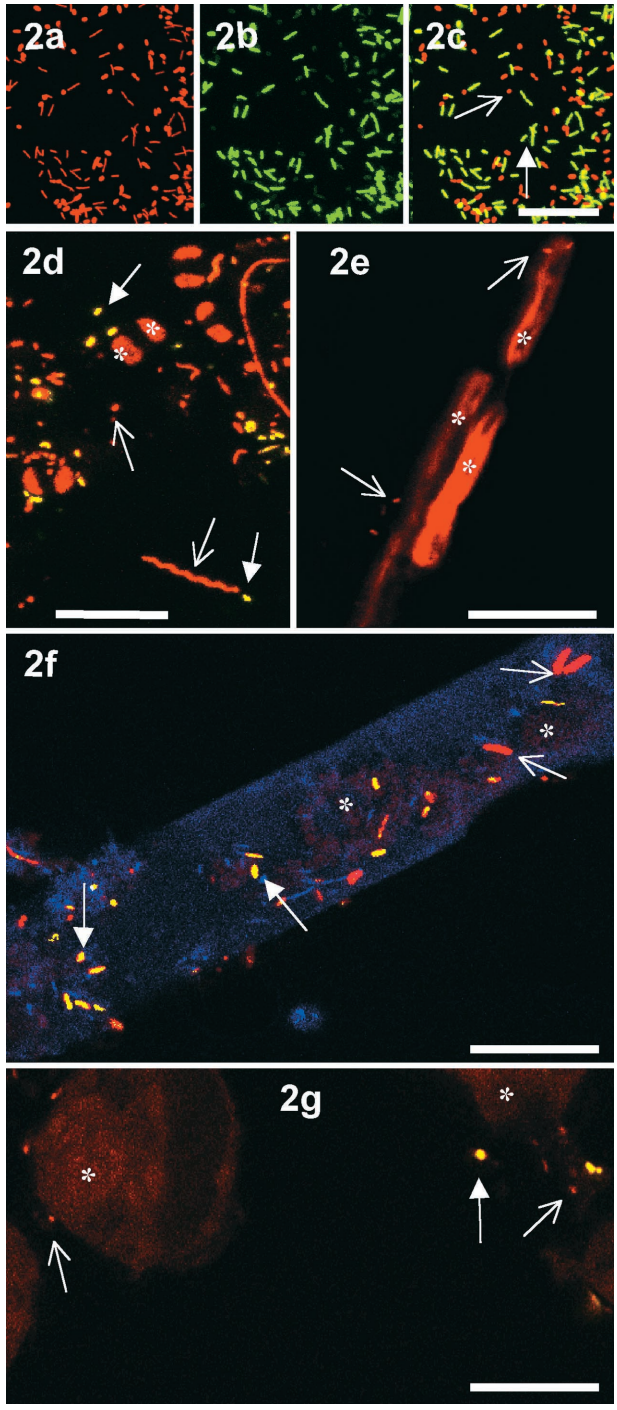

FIG. 2. CLSM optical sections (0.7 μm thick) of double hybridized 16S rRNA TSA-FISH on artificial or natural mixture of bacteria and phytoplankton. (a–c) Optical sections through an artificial mix of two bacteria types: round shaped *Roseobacter sp.* 253-11 and rod-shaped *Zobellia galactanovorans*. (a) Both bacteria types hybridized with the general EUB338R probe labeled with tetramethylrhodamine (red). (b) *Zobellia galactanovorans* hybridized with the CF319a probe (specific for Cytophaga-Flavobacter), labeled with fluorescein (green). (c) Merged image of a and b, the yellow color observed in the middle of each *Z. galactanovorans* corresponds to the superposition of the red and the green colors due to double hybridization. (d) Superposition of 10 optical sections of merged images (488-nm and 568-nm excitations) through

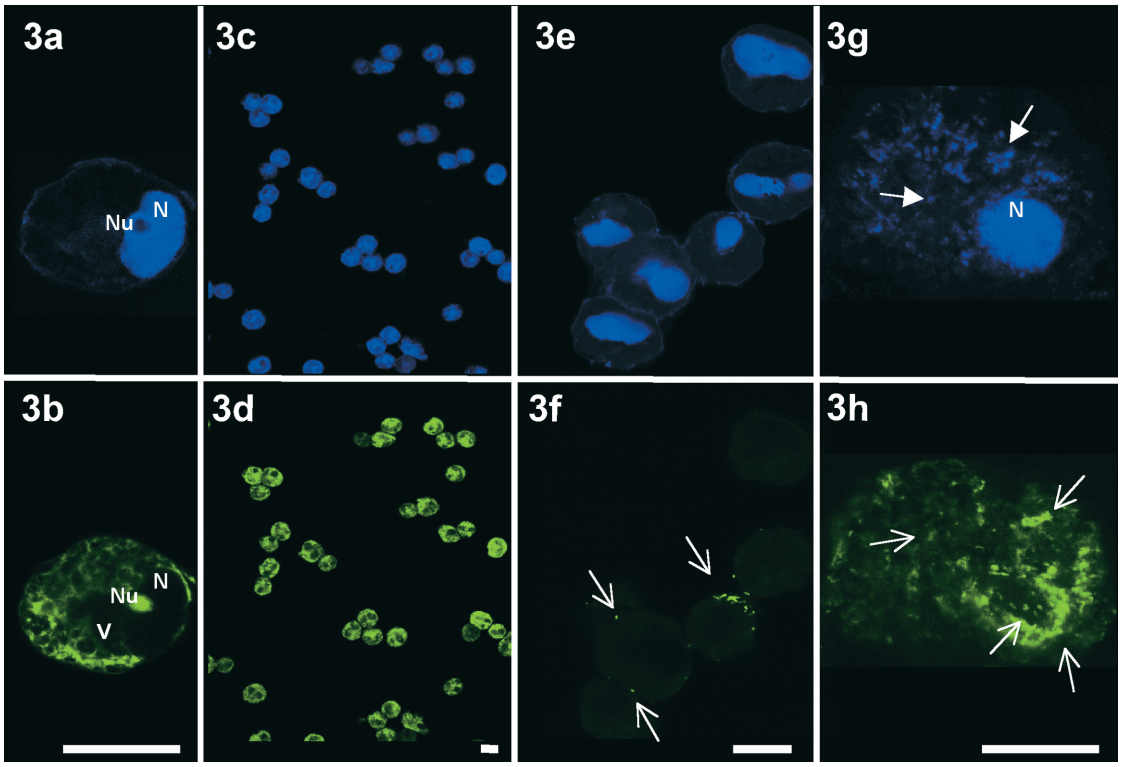

FIG. 3. CLSM optical section (0.7 μm thick) through different dinoflagellates species and developmental stages. (a, c, e, and g) DNA and cellulose stained with DAPI and calcofluor, respectively (blue, 380-nm excitation). (b, d, f, and h) HRP-labeled probes stained with fluorescein (green, 488-nm excitation). (a) *Alexandrium fundyense* cyst showing a highly condensed nucleus, stained with DAPI, surrounding a unstained nucleolus. (b) Same optical slice as a showing eukaryotic rRNA hybridized with EUK1209R probe. The unstained regions correspond to the condensed DNA and to an unidentified vesicle, respectively. (c, d, e, and f) Vegetative stages of thecate *A. fundyense*. (d) Eukaryotic rRNA hybridized with EUK1209R probe. (f) Prokaryotic rRNA hybridized with EUB338R probe. (g and h) Nonthecate *Gyrodinium instriatum* vegetative stage. (g) DAPI-stained eukaryotic nuclear DNA and prokaryotic DNA present in the dinoflagellate cytoplasm. (h) Endocytoplasmic and endonuclear 16S rRNA bacteria hybridized with EUB338R probe. Bars, 20 μm. Open arrows point to bacteria hybridized with the general EUB338R probe. Closed arrows point to prokaryotic DNA stained with DAPI. N, dinoflagellate nucleus; Nu, nucleolus; V, vesicle.

plastid sequences available from public database (*Gymnodinium breve*, AF172718; *G. aureolum*, AF172717; *Gyrodinium galatheanum*, AF172716; *G. mikimotoi*, AB027236; and *Heterocapsa triquetra*, AF130038, http://www.ncbi.nlm.nih.gov/GenBank/). When dealing with the phytoplankton groups, from which plastids are recognized by the general eubacteria probe, it is thus critical to use group-specific probes to distinguish the labeled bacteria from the labeled plastids (Fig. 2, d and e).

Our next step was to see whether these associations of techniques could also detect intracellular bacteria. Therefore, we tested the penetration of HRP-labeled probes inside cells using a general eukaryotic probe (EUK1209R), targeting 18S rRNA in encysted *A. fundyense* cells, because this stage possesses condensed DNA surrounding a non-DAPI-stained nucleolus (Fig. 3a). Using the standard treatment, the HRP-labeled eukaryotic probe penetrated and hybridized successfully

a natural assemblage of phytoplankton and bacterioplankton, where all bacteria and plastids are hybridized with the general EUB338R probe labeled with CY3 (red). In addition, Cytophaga-Flavobacter are hybridized with CF319a probe labeled with fluorescein and appeared yellow. (e) Optical sections of merged images (488-nm and 568-nm excitation) through a natural assemblage of phytoplankton (pennate diatoms) with attached bacteria, where all bacteria and plastids were hybridized with the general EUB338R probe labeled with CY3, no bacteria were hybridized with the CF319 probe, labeled with fluorescein. (f) Superposition of 10 optical sections of merged images (380-, 488-, and 568-nm excitations) through a fragment of macroalgae with associated bacteria. The cellulose of the algae cell wall was labeled with calcofluor (blue). Bacteria were hybridized with EUB338R probe, labeled with CY3. In addition, Cytophaga-Flavobacter were hybridized with CF319a probe, labeled with fluorescein, and appear yellow. Plastids of the macroalga were, however, not hybridized by the general EUB338R probe. (g) Optical section of merged images (488-nm and 568-nm excitations) through the axenic strains of *Alexandrium tamamrense* CCMP 1771, mixed with two strains of bacteria, *Roseobacter* sp. and *Alteromonas* sp., respectively. Both bacteria types were hybridized with EUB338R probe stained with tetramethylrhodamine. In addition, *Alteromonas* sp. were hybridized with AMAC137R probe (specific for *Alteromonas* clade) and labeled with fluorescein, which appear yellow. Bars, 20 μm. Open arrows point to bacteria hybridized only with the general EUB338R probe. Closed arrows point to bacteria double hybridized with the general EUB338R probe and the specific probes either CF319R or AMAC137R. Asterisks show plastids position.

18S rRNA of both cytoplasm and nucleolus (Fig. 3b). When applied to vegetative stages, eukaryotic probes labeled 100% of *A. fundyense* cells (Fig. 3, c and d) and of *A. tamarense* cells (strains PLY173a and NEPCC407, data not shown). However, despite the possibility that probes penetrated dinoflagellate cells, the general eubacteria probe did not reveal the presence of intracellular bacteria in any of the three *Alexandrium* strains, whereas extracellular bacteria were clearly labeled (data shown only for *A. fundyense*, Fig. 3f), suggesting that these cells did not contain any intracellular bacteria at the time of sampling. On the other hand, these *Alexandrium* strains revealed, with the help of TEM, the presence of bacteria-like structures inside the cells, albeit in low abundance (Kennaway, unpublished data, Lewis et al. 2001). If these structures correspond indeed to bacteria, one reason for the inability to stain them could be that they are probably not very active and had a very small number of ribosomes (Lee et al. 1993). However, TSA-FISH was successfully used to localize a single locus on a human chromosome (Schmidt et al. 1997), suggesting that low copy numbers of target sites should not be a problem. Alternatively, probes may not penetrate intracellular bacteria if their cell wall is insensitive to a moderate lysozyme treatment, as is the case for gram-positive bacteria (Madigan et al. 1997). However, TEM observations and RFLP analysis of small ribosomal subunit rDNA suggest that intracellular bacteria of *Alexandrium* spp. strains are gram negative (Franca et al. 1995, Doucette and Tricks 1995).

To test further the accessibility of HRP-labeled probes to intracellular bacteria, we used *Gyrodinium instriatum*, a nonthecate dinoflagellate, which is known for its numerous endocytoplasmic and endonuclear gram-negative bacteria (Silva and Franca 1985). Successful labeling of intracellular bacteria was achieved in this species with the EUB338R probe (Fig. 3h) and further characterized as members of proteobacteria (unpublished data). This demonstrates that the HRP-labeled probe can access intracellular bacteria when present. Consequently, it is highly likely the vegetative cells of the observed *Alexandrium* strains did not harbor intracellular bacteria.

The technique detailed here combines the high taxonomic specificity and sensitivity of the HRP probes targeting 16S rRNA and the very precise localization made possible by CLSM. This approach should, therefore, be very useful to assess the relationships between specific groups of bacteria and specific phytoplankton populations both in the natural environment and in controlled conditions, which would be of prime importance for the study of toxic dinoflagellate blooms and more generally for the understanding of marine pelagic ecosystems.

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