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Epibiotic *Vibrio* Luminous Bacteria Isolated from Some Hydrozoa and Bryozoa Species

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Abstract Luminous bacteria are isolated from both Hydrozoa and Bryozoa with chitinous structures on their surfaces. All the specimens of the examined hydroid species (*Aglaophenia kirchenpaueri*, *Aglaophenia octodonta*, *Aglaophenia tubiformis*, *Halopteris diaphana*, *Plumularia setacea*, *Ventromma halecioides*), observed under blue light excitation, showed a clear fluorescence on the external side of the perisarc (chitinous exoskeleton) around hydrocladia. In the bryozoan *Myriapora truncata*, luminous bacteria are present on the chitinous opercula. All the isolated luminous bacteria were identified on the basis of both phenotypic and genotypic analysis. The isolates from *A. tubiformis* and *H. diaphana* were unambiguously assigned to the species *Vibrio fischeri*. In contrast, the isolates from the other hydroids, phenotypically assigned to the species *Vibrio harveyi*, were then split into two distinct species by phylogenetic analysis of 16S rRNA gene sequences and DNA–DNA hybridization experiments. Scanning electron microscopy analysis and results of culture-based and culture-independent approaches enabled us to establish that luminous vibrios represent major constituents of the bacterial community inhabiting the *A. octodonta* surface suggesting that the interactions between luminous bacteria and the examined hydrozoan and bryozoan species are highly specific. These interactions might have epidemio-logical as well as ecological implications because of the opportunistic pathogenicity of luminous *Vibrio* species for marine organisms and the wide-distribution of the hydrozoan and bryozoan functioning as carriers.

Introduction

In the marine environment, only certain biological surfaces resist bacterial colonization for more or less extended periods, whereas, as a rule, bacteria rapidly colonize both living and nonliving submerged surfaces [20]. Despite their potentially important role in marine ecology, reports of interactions of epibiotic bacteria with marine macroorganisms are scarce and often circumstantial. A wide range of epibacterial densities (0 to 10^8 cells per square centimeter) is reported from the surfaces of algae [27, 44], sponges [49], Cnidaria [18, 50], and bryozoans [60]. Epibionts are able to survive in the natural environment longer than free-living forms and, by means of adhesive strategies, they can adapt to adverse conditions, e.g., organic matter limitation [15, 45]. The symbiosis of micro with macroorganisms is such a widespread phenomenon that it should have a profound impact on the physiology, ecology, and evolution of both hosts and symbiotic partners [38]. Thus, it is not surprising that the evolutionary patterns of closely related hosts and symbionts are often congruent. However, the mechanisms underlying the processes of cospeciation and host–symbiont specificity are poorly understood because most of animal–bacterial interactions cannot be experimentally initiated.

Some *Vibrio* species elaborate an extracellular chitinase and can feed on the chitinous structures of supporting organisms [6]. The colonization of the integument of copepods by *Vibrio* species is a well-described phenome-

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non especially in fecal-polluted vs. nonpolluted coastal zones [14, 28, 34, 51, 56]. Luminous bacteria, the simplest light-emitting organisms, can be either free-living in the water column with densities ranging from 10^2 to 10^4 cells per liter [37] or can be associated with other organisms [5]. The best-characterized species among luminous bacteria are *Photobacterium phosphoreum*, *Vibrio fischeri*, and *Vibrio harveyi*. Pujalte et al. [41] suggested that the association with guest organisms might contribute to survival and distribution of luminous bacteria in the marine environment. In contrast, the nature of the relationships between marine Metazoa and their associated luminous bacteria is not well known.

In a previous study, we described a novel association between *V. harveyi* and a benthic hydrozoan, *Aglaophenia octodonta* (Hydrozoa, Cnidaria) [50], suggesting that the association could be explained by the feeding activity of this microorganism on the hydroid chitinous structures. In the present study, we investigated the presence of luminous bacteria in some species of Hydrozoa and one species of Bryozoa, all characterized by the presence of chitinous structures on their surfaces. The selected species are commonly found at shallow depths on rocky coast of the Mediterranean Sea [12, 43].

Methods

Sampling

Batches of 50 colonies of the hydrozoan species *Aglaophenia kirchenpaueri*, *Aglaophenia octodonta*, *Aglaophenia tubiformis*, *Halopteris diaphana*, *Plumularia setacea*, *Ventromma halecioides* and 20 colonies of the bryozoan *Myriapora truncata* were collected by SCUBA diving along the Ionian coast of Apulia, Italy (Otranto Channel; 40° 08′ 39.8″ N, 18° 30′ 23.3″ E; Fig. 1) at 0–2 m of depth from May until November 2007. These species were selected because they do not emit their own light and avoid any interference with the epifluorescence microscope observations [11]. Colonies were transported in the laboratory under controlled temperature (5°C) and processed for isolation of luminous bacteria within 4 h from collection.

Epifluorescence Microscope Observation

Taking into account that luminous bacteria are able to emit fluorescence (excitation wavelength=450 nm; emission wavelength=540 nm), we utilized an epifluorescence microscope for our observations. Both the hydrozoans and the bryozoan colonies, in particular the hydrothecae and hydrocauli for hydrozoans and the opercula for bryozoan, were observed using a Zeiss Standard Axioplan microscope
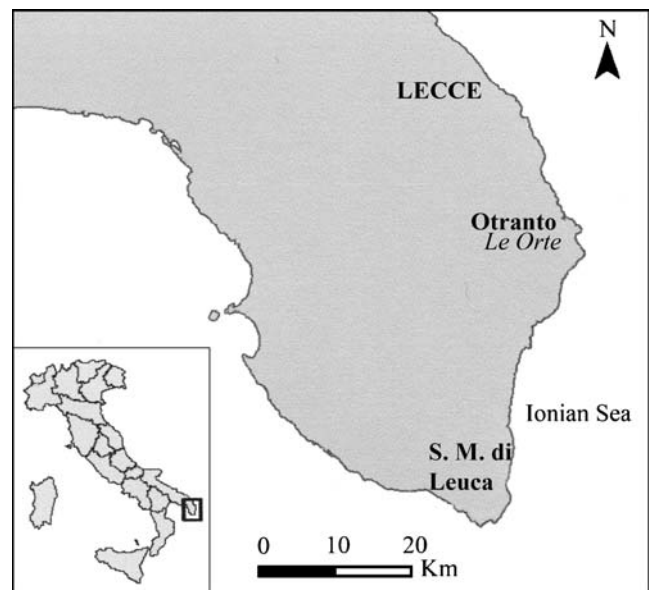
Figure 1 Map showing the sampling station in the Northern Ionian Sea off the coast of Otranto, Lecce (Italy)

equipped with a halogen lamp (Hg 100) light. Blue light excitation with a BP 485/20 excitation filter, a FT 510 chromatic beam splitter and a LP 520 barrier filter were used to observe the slides prepared from each sample for light-emitting bacteria.

Phenotypic Characterization of Bacterial Isolates

In the laboratory, hydroids and bryozoans were washed in sterile seawater (0.2 µm pore-filtered). Hydrocauli-bearing hydrocladia of the hydroid species examined to obtain about 3,000–3,500 polyps (approximately 1 g) and calcified stolons of *M. truncata* (approximately 1 g) were diluted with an equal volume of phosphate-buffered peptone water (Beckton Dickinson and Company) and homogenized for 90 s in a sterile Waring blender at 5°C. Serial dilutions of each homogenate were plated onto Marine Agar 2216 (Beckton Dickinson and Company; seeding with 0.1 ml). The plates were incubated for 24–48 h at 30°C. At the end of the incubating period, bioluminescence was detected in a dark room from the emission of visible light. The luminous bacterial colony types were picked out and streaked onto Marine Agar to obtain pure cultures. Phenotypic identification followed the schemes of Baumann and Schubert [7], Alsina and Blanch [3], and Garrity et al. [22]. Tests performed were: Gram stain, growth in thiosulfate–citrate–bile salts–sucrose agar (TCBS; Beckton Dickinson and Company), bioluminescence on luminescence agar [62], oxidase and catalase assays, sensitivity to the vibriostatic agent O/129, OF test, amino acids decarboxylase reaction. Growth at 0%, 3%, and 8.0% NaCl, growth at 4°C, 30°C, and 35°C, indole production, gelatinase production, lipase

hydrolysis, Voges–Proskauer test, utilization of citrate, and acid production from inositol, arabinose, and sucrose were also determined. The assay of Berger and Reynolds [8] was employed to determine the chitinolytic activity of the isolates. A further characterization was done by screening the utilization of different carbon sources in a concentration of 1% (L-arabinose, D-glucosamine, xylose, mannose, arabinose, cellobiose, glucose, galactose, trehalose, melibiose, lactose, mannitol, sorbitol, inositol, and sucrose) added in Bacto Phenol Red Agar Base (Beckton Dickinson and Company) with the addition of NaCl to give a final concentration of 2.5%.

16S rRNA Gene Sequence Analysis of Bacterial Isolates

High-molecular-weight genomic DNA from the different bacterial isolates was prepared according to standard procedures. Strains were grown in 100-ml nutrient broth (Beckton Dickinson and Company) containing 3% NaCl with shaking at 30°C to an optical density at 550 nm of 0.8. After centrifugation, pellets were washed with 50 ml of sodium chloride–Tris–ethylenediaminetetra-acetic acid (EDTA) buffer (500 mM NaCl, 50 mM Tris–Cl pH 8, 5 mM EDTA) and then resuspended in 4 ml of a solution containing 50 mM Tris–Cl pH 8, 25% sucrose, 1 mM EDTA. Lysozyme (1 mg ml^{-1}) treatment was carried out at 0°C for 10 min, then EDTA was added to a final concentration of 40 mM, and samples were incubated at 0°C for 10 min. Proteinase K (100 μg ml^{-1}) treatment was performed for 2 h at 65°C after addition of sodium dodecyl sulfate (SDS) to a final concentration of 1%. Nucleic acids were extracted by phenol-chloroform and isoamylic alcohol (24:1) extraction according to standard procedures [48], and 15 μg ml^{-1} ribonuclease A were used to remove RNA. After phenol-chloroform and isoamylic alcohol (24:1) extraction and ethanol precipitation, high-molecular-weight DNA was collected by spooling using Shepherd's crooks [48].

The 16S-rRNA-encoding genes were amplified using the bacterial-specific primers 16SEB20-42-F (5′-TGGCTCAG GAYGAACGCTGGCGG-3′) and 16SEB1488-R (5′-TACCTTGTTACGACTTCACC-3′) [58]. The two primers were designed to amplify a 1,488-bp-long DNA fragment (from nucleotide 20 to nucleotide 1507 in the *Escherichia coli* 16S rRNA gene). Polymerase chain reactions (PCR) were performed as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 45 s, annealing for 30 s at 58°C and extension at 72°C for 2 min. They were carried out in a Perkin-Elmer Cetus DNA Thermal Cycler 2400. PCR products were isolated through 1% agarose gels in 1X Tris–acetate-EDTA buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8.0), recovered by using the Quiaex II Gel extraction kit (Quiagen), cloned into

pGEM® -T Easy Vector by using the TA Cloning kit (Promega), and finally sequenced using primers M13F and M13R as a service by MWG Biotech Custom Sequencing Service (Germany) or by Primm s.r.l (Italy).

DNA similarity searches were carried out using Basic Local Alignment Search Tool [4] at National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Sequence alignments were performed with Clustal W [53] at European Bioinformatics Institute (http://www.ebi.ac.uk/). Phylo_win software [21] was used to infer evolutionary trees according to neighbor-joining methods [47] using the Kimura two-parameter model [29]. Tree robustness was assessed by bootstrap resampling (1,000 replicates each) [13].

DNA–DNA Hybridization Analyses

To perform DNA–DNA hybridization experiments, the genomic DNAs (5 μg) from the different bacterial isolates were restricted with *Bam*HI, serially diluted in a buffer containing 2×sodium citrate–sodium chloride (SSC buffer) [48] and 50% (v/v) formamide, heated at 95°C for 5 min, and immobilized onto nylon filters by slow filtration in a slot-blot apparatus (Schleicher and Schull) in duplicate. A prehybridization step was performed by adding a solution containing 5×SSC (1×SSC: 0.015 M NaCl, 15 mM Na–citrate [pH7]), 5×Denhardt's solution [48], 0.1% SDS, 50 mM sodium phosphate buffer (pH 6.5), 50% (v/v) formamide, 500 μg denatured salmon sperm DNA per milliliter for 6 h at 36°C. This temperature represents stringent conditions for *V. harveyi*, for which the optimal renaturation temperature (34.05°C) is calculated as [(0.51× G+C content)+47]−36 [23], where 36°C is the correction for the presence of 50% formamide [36]. A G + C content of 45.2 mol% was assumed for *V. harveyi* according to previous study [54]. Hybridization was carried out for 12 h at 36°C, a hybridization buffer similar to the prehybridization solution but containing ^{32}P-labeled *Bam*HI-restricted genomic DNAs from the different *Vibrio* strains in place of salmon sperm DNA. The genomic DNA from the type strain *V. harveyi* LMG 4044^T was used as a reference. The *Bam*HI-restricted genomic DNAs were labeled with ^{32}P by random priming according to standard procedures [48]. After hybridization, filters were washed three times with a solution containing 2×SSC, 0.2% SDS at 36°C, and then subjected to autoradiography. The semiquantitative analysis of the hybridization signals was performed by densitometry using a Scanmaster 3 (Howtek, Inc., Hudson, NH, USA), a high-performance desktop flatbed color scanner equipped with RFLPrint^{TM} (Pdi, Huntington Station, NY, USA) software package or by directly counting the radioactivity bands by a PhosphoImager SI (Molecular Dynamics, Inc., Sunnyvale, CA, USA).

GenBank Accession

EU031643: *V. fischeri* HD-1, partial 16S rRNA gene; EU031644: *V. fischeri* AT-1, partial 16S rRNA gene; EU031646: *V. harveyi* AK-1, partial 16S rRNA gene; EU031649: *Vibrio sp*. MT-1, partial 16S rRNA gene; EU031648: *Vibrio sp*. PS-1, partial 16S rRNA gene; EU031647: *Vibrio sp*. VH-1, partial 16S rRNA gene.

Scanning Electron Microscopy on *Aglaophenia octodonta*

Twenty colonies of *A. octodonta* were washed several times with sterile seawater (0.2 μm pore-filtered) to assure the elimination of any bacteria settled on the surfaces and then fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (CB), pH 7.5, overnight. The colonies were then washed three times in CB and postfixed for 1 h in 2% osmium tetroxide in CB. After washing, the colonies were dehydrated in a graded acetone series, dried in a critical-point drier, and coated with gold. Colonies were observed with a Philips 515 scanning electron microscope (SEM) operated at 20 kV.

Quantitative Analysis of Bacteria Living on *Aglaophenia octodonta* Surface

In the laboratory, *A. octodonta* colonies (approximately 1 g) were gently washed in sterile seawater (0.2 μm pore-filtered) to assure the elimination of any bacteria settled on the surfaces, suspended in sterile seawater and sonicated three times (Branson Sonifier 2200, 60 W, 47 kHz for 1 min in an ice bath) to optimize surface bacteria detachment. The sonication was interrupted for 30 s every minute, during which time the samples were shaken manually. To enumerate surface bacteria including luminous bacteria, 1 or 5 ml of the sonicated sample and appropriate decimal dilutions were plated in parallel onto Marine Agar 2216 or TCBS Agar (Beckton Dickinson and Company) and after incubation for 2 days at 22°C and at 30°C, respectively, the culturable bacteria were counted according to the colony-forming units (CFU) method.

PCR–Single-Strand Conformational Polymorphism Analysis

Bacteria were recovered from *A. octodonta* colonies (about 1 g) surface and high-molecular-weight genomic DNA was extracted as described [17, 58]. Due to limited amount of the microbial sample (about 3×10^4 CFU/g), the protocol for DNA extraction was adapted to smaller (20-fold) volumes. After the extraction, the DNA was amplified using the bacteria-specific primers Com1-F (5′-CAG CAGCCGCGGTAATAC-3′) and Com2-R (5′-

CCGTCAATTCCTTTGAGTTT-3′) [30]. These primers were designed to amplify 409-bp-long DNA fragments (from nucleotide 519 to nucleotide 927 in the *E. coli* 16S rRNA gene) that could be resolved by single-strand conformational polymorphism (SSCP) as described [17, 58]. To this purpose, PCR products were purified by High Pure PCR product Purification Kit (Boehringer Mannheim, Germany), denatured and resolved on 10% polyacrylamide gel (acrylamide–*N,N*-methylenebisacrylamide 49:1) in 0.8X Tris–borate–EDTA (72 mM Tris–borate, 1.6 mM EDTA) containing 5% glycerol. Bands identified after silver staining were excised with razorblades and single-strand DNAs were eluted from the gel by using the Quiaex II DNA purification kit (Quiagen). The resulting DNAs were reamplified, cloned into pGEM® -T Easy Vector by using the TA Cloning kit (Promega), and finally sequenced using primers M13F and M13R as a service by MWG Biotech.

Results

Microscope Evidence for Luminous Bacteria Biofilm in Hydrozoa and Bryozoa Species

In thecate hydroids, such as *A. kirchenpaueri* (Fig. 2a), *A. octodonta* (Fig. 2c), *A. tubiformis* (Fig. 3a), *H. diaphana* (Fig. 3c), *P. setacea* (Fig. 4a), *V. halecioides* (Fig. 4c), hydrocauli and hydrocladia are ectoendodermal tubular prolongations of hydranths' gastric cavities, enveloped by a protective chitinous layer, or perisarc. Perisarcal structures are complex, being mainly composed of chitin and proteins; in some Hydrozoa, they are associated with calcareous elements (coenosteum). The chitinous perisarc forms a solid theca around the hydranths (the hydrotheca), the reproductive organs (the gonotheca), and the protective polyps, or dactylozooids (the dactylotheca or nematotheca). All the specimens of the examined hydroid species, observed under blue light excitation, showed a clear fluorescence on the external side of the perisarc (chitinous exoskeleton) around hydrocladia. In particular, this fluorescence was concentrated in the folds along the hydrocaulus and at the base of the hydrothecae (Figs. 2b,d, 3b,d, and 4b,d). Localization of fluorescence in the chitinous structures of these hydroids suggested, as previously observed in *A. octodonta* [50], an involvement of luminous bacteria known for their capacity to elaborate an extracellular chitinase and feed on the chitinous structures of supporting organisms [6]. This hypothesis was supported by isolation of luminous bacteria from all hydrozoan homogenates. Moreover, an interesting issue is that all the bacterial isolates were chitinase positive. Taking into account that in the bryozoan *M. truncata*, the operculum is made up of chitin, we checked and found luminous bacteria on the skeletal structure near to the

Figure 2 *A. kirchenpaueri* photomicrographs, living material: **a** hydrothecae at transmitted light, **b** hydrothecae at epifluorescence, the halo of fluorescence is due to bacterial biofilm; *A. octodonta* photomicrographs, living material **c** hydrothecae at transmitted light, **d** hydrothecae at epifluorescence, the halo of fluorescence is due to bacterial biofilm. *Scales*: (**a**–**d**) 500 μm
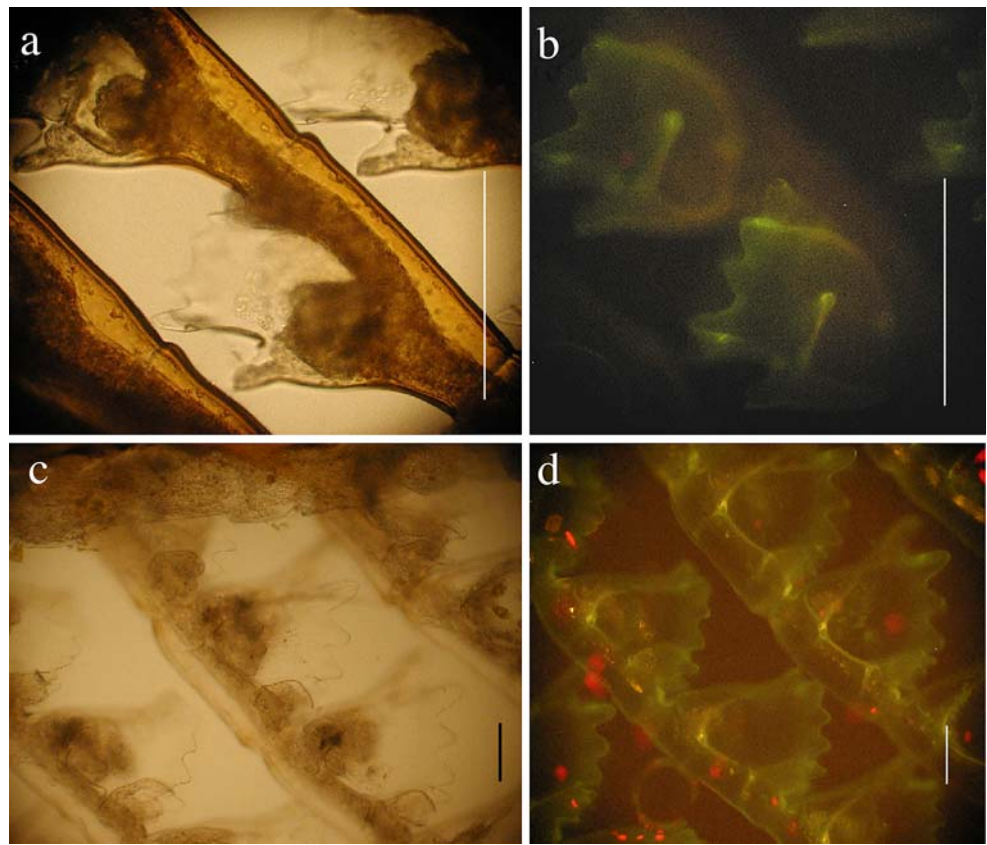
Figure 3 *A. tubiformis* photomicrographs, living material: **a** hydrotheca at transmitted light, **b** hydrotheca at epifluorescence, the halo of fluorescence is due to bacterial biofilm; *H. diaphana* photomicrographs, living material: **c** hydrotheca at transmitted light, **d** hydrotheca at epifluorescence, the halo of fluorescence is due to bacterial biofilm. *Scales*: (**a** and **b**) 200 μm, (**c** and **d**) 250 μm
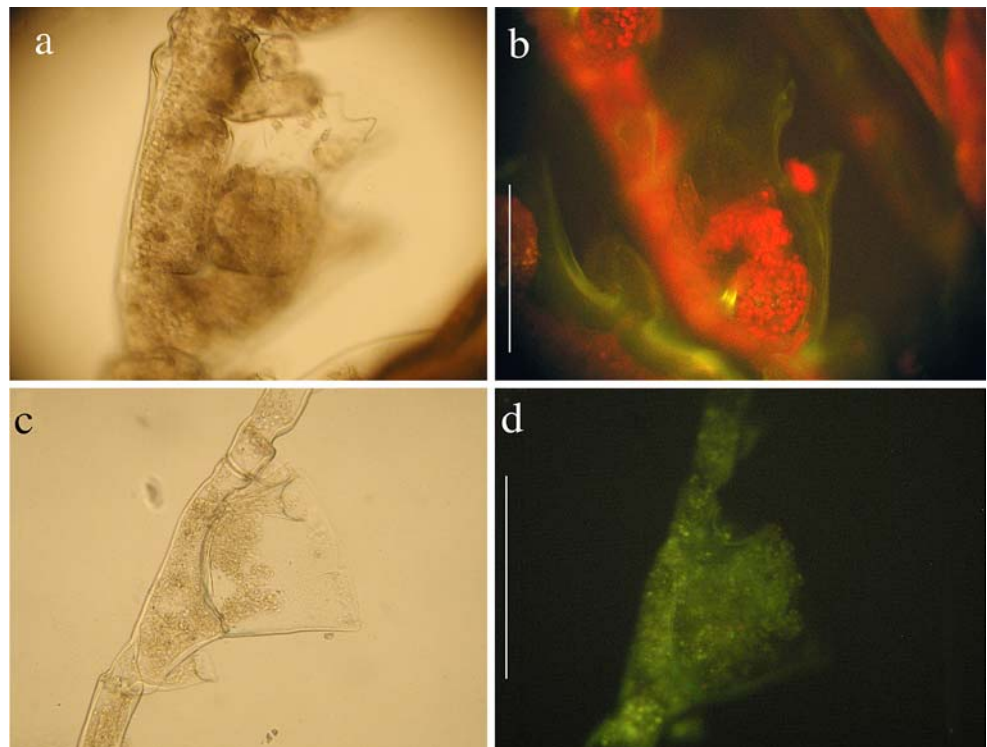
Figure 4 *P. setacea* photomicrographs, living material: a particular of hydrocladia at transmitted light, b particular of hydrocladia at epifluorescence, the halo of fluorescence is due to bacterial biofilm; *V. halecioides* photomicrographs, living material: c particular of hydrocladia at transmitted light, d particular of hydrocladia at epifluorescence, the halo of fluorescence is due to bacterial biofilm. Scales: (**a** and **b**) 100 μm, (**c**) 500 μm, (**d**) 250 μm
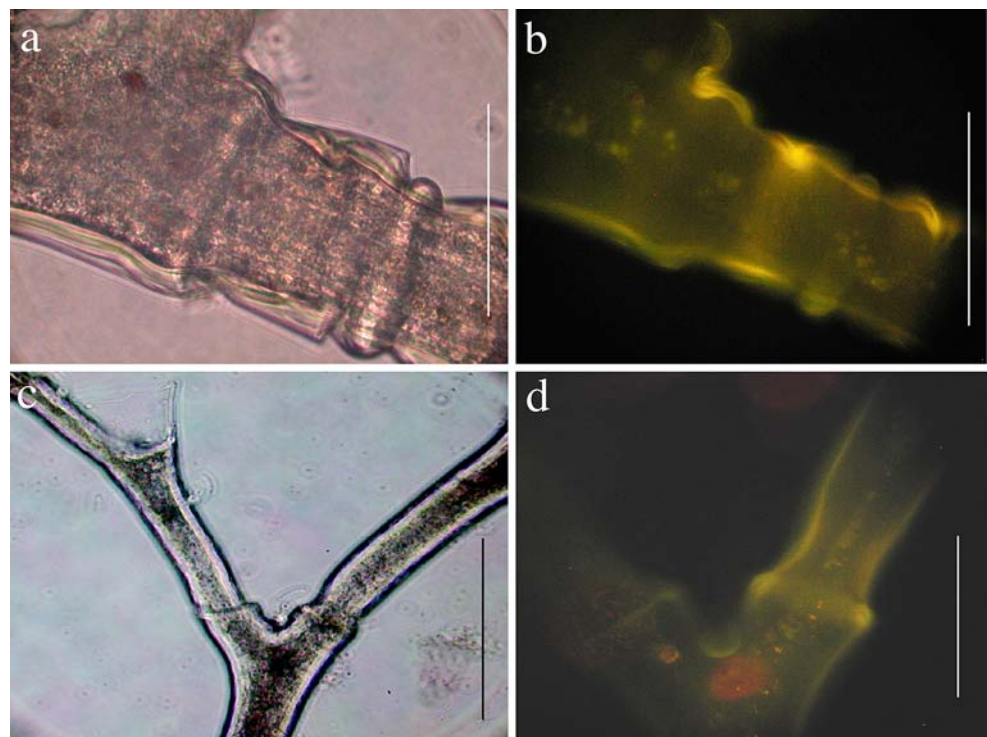

opercula. The halo of fluorescence observed in the invertebrates studied was due to the development of a bacterial biofilm. Indeed, when the examined species were treated with antibiotics, the fluorescence disappeared suggesting the involvement of bacteria (data not shown).

Identification of Luminous Bacteria

All the isolated luminous bacteria were identified on the basis of both phenotypic and genotypic analysis. Five independent isolates were examined from each type of animal. The identity of the isolates was investigated by phylogenetic analysis using almost the entire sequences of the 16S-rRNA-encoding genes (Fig. 5). All the 16S rRNA sequences of luminescent vibrios from *A. tubiformis* and *H. diaphana* were identical and exhibited an identity of 99.8% to the 16S rRNA sequence of the *V. fischeri* type strain ATCC 7744[T]. These isolates were named, respectively, *V. fischeri* AT1 to AT5 and *V. fischeri* HD1 to HD5. The isolates from *A. kirchenpaueri* (AK1 to AK5) grouped with the *V. harveyi* type strains LMG 4404[T] and ATCC 35084[T], while those from *P. setacea* (PS1 to PS5), *V. halecioides* (VH1 to VH5), and *M. truncata* (MT1 to MT5) clustered with V.-harveyi-related strains LMG 20370 and R-14913.

These two strains were previously assigned to the species *V. harveyi* on the basis of phenotypic criteria but later suspected to belong to a new species on the basis of DNA–DNA similarity, amplified fragment length polymor-

Figure 5 Phylogenetic analysis of 16S rRNA gene sequences of luminous *Vibrio* species isolated from the hydroids: taxonomic position of *V. fischeri* HD1 and *V. fischeri* AT1, *V. harveyi* AK1, and *Vibrio* sp. MT1, VH1, and PS1 is indicated with respect to several type strains. AO1 is a *Vibrio* species previously isolated form *A. octodonta* [50]. Bootstrap values ≥60 are reported at the branch points
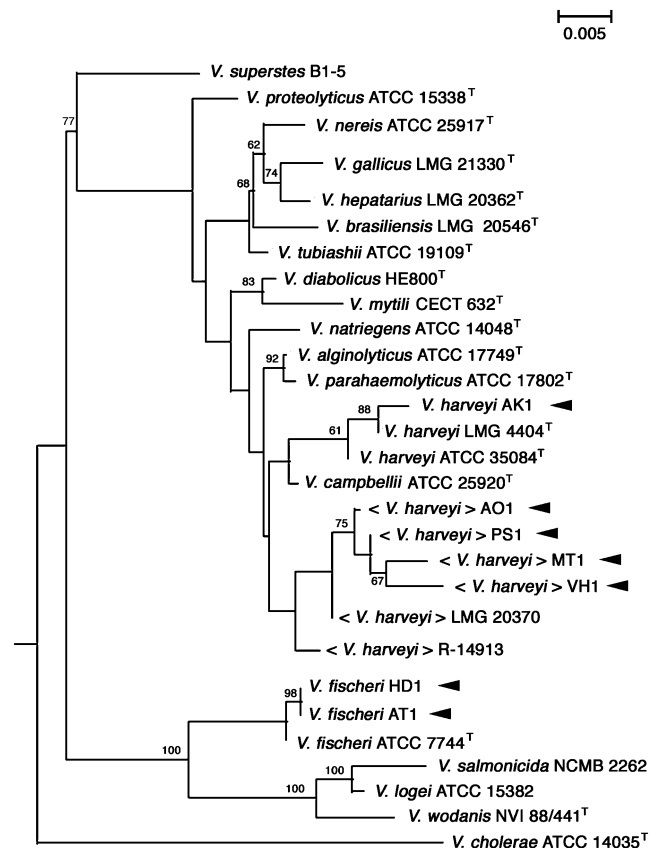
phism, and multilocus sequence analyses [24, 52, 55]. 16S rRNA gene sequences identities between AK1–AK5, PS1–PS5, VH1–VH5, MT1–MT5, and the *V. harveyi* type strain ATCC 35084[T] were, respectively, 99.7%, 98.1%, 97.7%, and 97.9%. On the basis of these results, only the five isolates AK1–AK5 could be unambiguously assigned to the species *V. harveyi* and indicated as *V. harveyi* AK1 to AK5, while PS1–PS5, VH1–VH5 and MT1–MT5 were assigned to a new V.-harveyi-related species together with the luminous *Vibrio* species (*Vibrio* sp. AO1 in Fig. 5) from *A. octodonta* previously described [50].

This conclusion was supported by DNA–DNA hybridization experiments under stringent conditions (see "Methods") using the DNA from *V. harveyi* type strain ATCC 4404[T] as a reference. AK1–AK5 were confirmed to belong to the species *V. harveyi* because the DNA similarity value with *V. harveyi* ATCC 4404[T] was 91.2%, while values below the 70% proposed by Wayne et al. [61] to delimit a species were obtained with the other isolates (Table 1).

The assignment of the bacterial isolates to the above-mentioned species was consistent with the results of morphological, cultural, and biochemical tests (Table 2) according to the schemes proposed by Baumann and Schubert [7], Alsina and Blanch [3], Garrity et al. [22], and Farmer et al. [19]. However, due to lower resolution with respect to phylogenetic and DNA–DNA hybridization analyses, these tests were not able to discriminate between the *V. harveyi* and the V.-harveyi-related isolates.

Scanning Electron Microscopy and Quantitative Estimation of *A. octodonta* Surface Bacteria

We further investigated the specificity of the association between hydroids and epibiotic bacteria by both SEM inspection of *A. octodonta* surface and quantitative assessment of luminous bacteria on the hydroid perisarc by using culture-based and culture-independent approaches. We chose *A. octodonta* as a model because, among the hydroids employed in this study, it is the most common along rocky coasts of the Mediterranean Sea [50]. SEM analysis demonstrated the adhesion of several vibrios to the hydroid perisarc (magnification ×500; Fig. 6a). Further magnification

(×25,000) showed that the bacteria observed had the typical morphology of vibrios (Fig. 6b).

The results of cultural analysis using in parallel Marine Agar 2216 and TCBS Agar demonstrated that luminous vibrios represented a conspicuous component (6×10^3 CFU/g, 20%) of the total culturable *A. octodonta* surface bacteria (3×10^4 CFU/g) that was dominated by *Vibrio* species (>80%). This finding was supported by culture-independent approach (Fig. 7). To this purpose, three distinct batches of *A. octodonta* (sampled in May, August, and November 2007) were analyzed for the presence of epibiotic bacteria by PCR–SSCP using bacteria-specific primers Com1-F and Com2-R. These primers target a 409-bp-long (in *E. coli*) central region of prokaryotic 16S rRNA gene and allow high taxonomic resolution, often at the species level [30]. The SSCP profile of the amplified 16S rRNA gene pool revealed a relatively low degree of complexity of the microbial community with predominance of few common bands in all samples (a–d; Fig. 7, lanes 1–3). These bands were excised from the gel, eluted, cloned, and subjected to nucleotide sequence analysis. To avoid any bias due to the reamplification step, only PCR products showing the same SSCP profile as the original bands were further processed. The sequence corresponding to bands a, when challenged in the databank, poorly matched (81% of identity) with that of *Rubritalea marina* strain Pol012 (accession number DQ302104.1) and other *Verrucomicrobiaceae*, suggesting that it might belong to an organism phylogenetically distant from any known bacterial division. Bands b and d represented to the two complementary DNA strands of the 16S rRNA gene whose sequence exhibited the highest similarity (91.2%) with that of an unknown microorganism from deep-sea octocoral phylogenetically close to as-yet-uncultivated Spirochaeta (accession number DQ395500.1). Band c comigrated with the major signal (band c') resulting from PCR amplification of luminous *Vibrio* sp. AO1 DNA (Fig. 7, lane 4). Indeed, when subjected to nucleotide sequencing, band c and c' gave the same result corresponding to 16S rRNA gene of the *Vibrio* core group including the species *V. harveyi* (and V.-harveyi-related), *Vibrio alginolyticus*, *Vibrio pelagius, Vibrio natriegens*, and *Vibrio mytili*.

Table 1 DNA–DNA hybridization values between *V. harveyi* and V.-harveyi-related isolates

	V. harveyi ATCC 4404[T]	*V. harveyi* AK1	*Vibrio* sp. PS1	*Vibrio* sp. VH1	*Vibrio* sp. MT1
V. harveyi ATCC 4404[T]	100				
V. harveyi AK1	91	100			
Vibrio sp. PS1	52	43	100		
Vibrio sp. VH1	44	38	89	100	
Vibrio sp. MT1	48	42	89	78	100

Values are averages of hybridizations with quintuplicate samples

Table 2 The result of analysis of *V. harveyi* and *V. fischeri* morphological and physiological properties

Source	A. kirchenpaueri, A. octodonta, P. setacea, V. halecioides, M. truncata			A. tubiformis, H. diaphana	
Characteristics–test	Isolated bacteria	Bauman and Schubert [7]	Alsina and Blanch [3]	Isolated bacteria	Bauman and Schubert [7]
Gram reaction	−	−	−	−	−
Cell morphology	r	r	r	r	r
Luminescence	+	d	d	(+)	(+)
0/129 sensitivity					
10 μg	+	d	d	(−)	(−)
150 μg	+	+	−	−	−
Oxidation–fermentation	F	F	F	F	F
Decarboxylase					
Arginine	−	−	−	−	−
Lysine	+	+	+	+	+
Ornithine	+	+	+	−	−
Growth in % NaCl					
0	−	−	−	−	−
3	+	+	+	+	+
8	+	d	d	−	−
Oxidase	+	+	+	+	+
Catalase	+	nd	nd	+	nd
Voges–Proskauer	−	−	−	−	−
Indole	+	nd	+	−	nd
Gelatinase	+	+	+	−	−
Lipase	+	+	nd	+	+
Citrate	−	nd	d	d	nd
Chitinase	+	+	nd	+	+
Growth at °C					
4	−	−	−	(−)	(−)
30	+	+	+	+	+
35	+	+	+	d	d
Acid from					
Inositol	−	nd	−	−	nd
Arabinose	(−)	d	−	−	−
Sucrose	−	d	d	(−)	(−)
Carbon sources					
L-arabinose	−	−	−	−	−
D-glucosamine	−	nd	−	−	nd
Xylose	−	−	nd	−	−
Mannose	+	+	nd	+	+
Arabinose	−	−	−	−	−
Cellobiose	+	+	nd	+	+
Glucose	+	+	+	d	d
Galactose	−	d	nd	+	+
Trehalose	+	+	nd	−	−
Melibiose	−	−	−	−	−
Lactose	−	−	−	−	−
Mannitol	+	+	+	d	d
Sorbitol	−	−	−	−	−
Inositol	−	−	−	−	−
Sucrose	−	d	d	−	−
Identification	*Vibrio harveyi*	*Vibrio harveyi*	*Vibrio harveyi*	*Vibrio fischeri*	*Vibrio fischeri*

F Fermentative, *d* diverse, *nd* no data, + positive reaction, − negative reaction, *(−)* negative for 25–11%, *r* rod-shaped

Figure 6 Scanning electron micrographs: **a** *A. octodonta* surface, note the adhesion of several vibrios, **b** magnification of one of the vibrios observed on the surface of the investigated hydroid. *Scales*: (**a**) 50 μm, (**b**) 1 μm
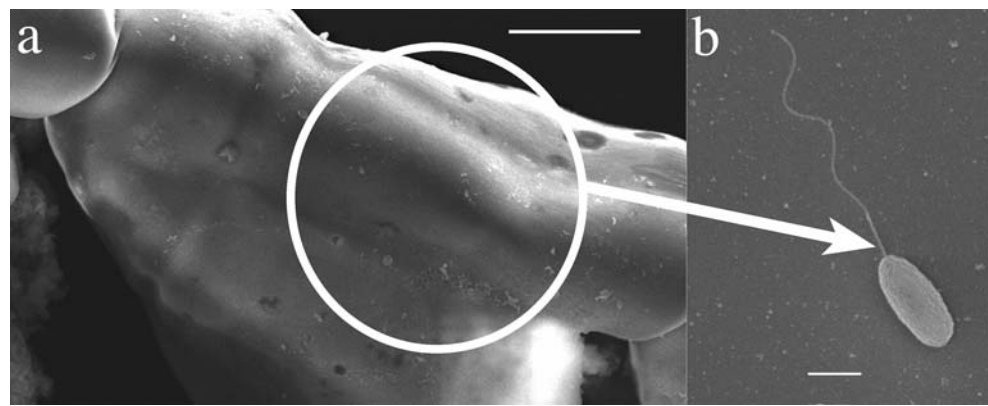
Discussion

Available information on the interaction of luminous bacteria with marine invertebrates is limited [42]. By contrast, a wide literature is available on the symbiotic colonization of *V. fischeri* and the Hawaiian bobtail squid, *Euprymna scolopes* [10, 16, 35, 46, 63]. In this association, newly hatched squids acquire *V. fischeri* from the surrounding seawater in which they are present at a few hundred CFU per milliliter [33] in a total background of about 10^6 other marine bacteria per milliliter. The relationship between *V. fischeri* and its squid host is complex and highly specific. The specificity of the association suggests the specialized colonization mechanisms in the bacterial symbiont have coevolved with cognate recognition mechanisms in the squid host [59].

As regards the interaction between the luminous bacteria (*V. fischeri* and *V. harveyi*) and hydrozoans or bryozoan observed in our study, the exact molecular mechanism involved is still unknown. All the investigated invertebrate species are characterized by the presence of chitinous surface on which the luminous bacteria identified could

find a suitable substrate. Because the luminous bacteria isolated showed a chitinolytic activity, the results of the present study support the original hypothesis of Hood and Meyers [25] that a primary role of some vibrios could be the colonization and initiation of the degradation of chitinous material in aquatic ecosystems.

In the present study, *V. fischeri, V. harveyi*, and V.-harveyi-related species were the luminous bacteria isolated with the selected hydrozoans and bryozoan. *V. harveyi* is almost ecologically identical to *V. fischeri*. Almashanu et al. [2] observed that the β subunit of *V. fischeri* luciferase forms active enzyme with the α subunit of *V. harveyi* luciferase showing the affinity of these two bacterial species. This result supports the specificity of the interaction observed in our study between these luminous bacteria and the investigated invertebrates: in fact, among the strains of luminous bacteria, only *V. fischeri*, *V. harveyi*, and V.-harveyi-related species were found on the surfaces of the examined invertebrates. Moreover, quantitative analyses demonstrated that luminous vibrios represent a conspicuous component of the total culturable bacteria colonizing the surface of *A. octodonta* (about 20% of the total count). This conclusion was consistent with the result of the PCR–SSCP analysis of 16S rRNA gene pool of the local bacterial community. In addition to bacteria belonging to the *Vibrio* core group (including the luminous V.-harveyi-related strain AO1), this analysis revealed the presence of at least two additional uncultivated microorganisms phylogenetically distant from any known species (bands b or d) or bacterial division (band a; Fig. 5). It is noteworthy that PCR–SSCP patterns were very similar irrespective of the sampling time (May to November). Thus, as observed for *V. fischeri* and the Hawaiian bobtail squid *E. scolopes*, also in the case of *A. octodonta*, the relationship between luminous V.-harveyi-related species and this hydroid is highly specific. This issue was also supported by scanning electron microscopy images demonstrating the colonization of this hydroid perisarc by vibrios (Fig. 6a).

The ancestral position of the Hydrozoa with respect to the Metazoa [9] suggests that these invertebrates functioned

Figure 7 PCR–SSCP analysis of the bacterial community colonizing *A. octodonta* surface. Three distinct batches of *A. octodonta* sampled in May, August, and November (*lanes 1–3*, respectively) were analyzed for the presence of epibiotic bacteria by PCR–SSCP using 16S rRNA gene-specific primers. The PCR–SSCP profiles were compared with that obtained by amplifying the DNA from *Vibrio* sp. AO1, the luminous strain isolated from the hydroid, with the same primers. *Arrowheads* mark the positions of specific DNA bands (*a, b, c, d, c′*) that were eluted from the gel, cloned, and subjected to nucleotide sequence analysis
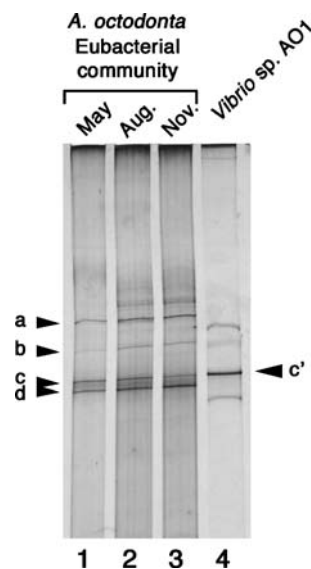
from the past until now as habitat "islands" providing a unique set of environmental conditions for the survival of the luminous bacteria isolated over the time [50]. The possibility of using chitin as a feeding substrate evidently exploited other chitin-coated Metazoa, from the Crustacea (as is well known for copepods) to the Bryozoa (as shown here).

Further studies will be undertaken to investigate the molecular mechanisms involved in such host–bacteria relationships and to better understand whether the interaction observed is only related to chitin utilization by *V. fischeri*, *V. harveyi*, and the V.-harveyi-related species (parasitic association) or if it has also an ecological role for the examined hydrozoan and bryozoan species. None of the invertebrate species studied, in fact, is able to emit its own light. Thus, the presence of luminous bacteria, able to emit light continuously, probably provides a behaviorally useful light source (symbiotic association) as already suggested [50].

Furthermore, both luminous bacterial isolated are considered opportunistic pathogens, as suggested by the name of the disease commonly referred to as luminous vibriosis: the expression of luminescence has long been associated with the virulence in pathogenic strains of these bacteria. In particular, *V. harveyi* is now known to cause mass mortalities in several marine organisms including pearl oysters (*Pinctada maxima*) [39], fish (*Solea senegalensis*) [64], sea horses (*Hippocampus* sp.) [1], and lobsters (*Panulirus homarus*) [26]. Virulence in *V. harveyi* has been attributed the production of an extracellular protein referred to as toxin T1 [40]. *V. harveyi* and *V. fischeri*, together with other luminous vibrios, have been involved in disease outbreaks in shrimp larviculture facilities [31] and, to a lesser extent, in grow-out ponds [32] as well as penaeid prawn farms across the world [57]. In this framework, the interaction between luminous bacteria with *A. kirchenpaueri*, *A. octodonta*, *A. tubiformis*, *H. diaphana*, *P. setacea*, *V. halecioides*, and *M. truncata* might have epidemiological implications because these hydrozoan and bryozoan species are widely distributed in the Mediterranean Sea and might constitute natural reservoirs of the pathogens for marine organisms.

Moreover, the interaction between the above-mentioned invertebrates and luminous bacteria provides an unusually tractable system for studying the evolution of these relationships because the selected species have a wide biogeographic distribution.

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