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Association of a luminous *Vibrio* sp., taxonomically related to *Vibrio harveyi,* with *Clytia linearis* (Thornely, 1900) (Hydrozoa, Cnidaria)

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ABSTRACT

A previously unknown association between a luminous *Vibrio* sp., taxonomically related to the species *Vibrio harveyi* and a common member of the shallow/mid water communities of the Mediterranean Sea, the hydrozoan *Clytia linearis* is described. All the specimens of *C. linearis* observed under blue light excitation showed both a natural luminescence appearing as a series of fine dots due to clytin, and a clear fluorescence on the external side of the perisarc around the colonies due to the presence of luminous bacteria. Luminous bacteria were isolated from the surface of *C. linearis*, their phenotypic characterization as isolates was performed by several morphological, biochemical, and cultural tests, completed with 16S rDNA sequence analysis. All the isolates were referred to a *Vibrio* sp. taxonomically related to *V. harveyi*. The association of the *V. harveyi*-related species with *C. linearis*, as already suggested for another hydroid, *Aglaophenia octodonta*, could be explained with the activity of these bacteria of feeding on the chitinous structures present in these hydroids. Moreover, the adhesion of the luminous bacterium (here referred to as *Vibrio* sp. CL1) on *C. linearis* may contribute to the survival of this *Vibrio* species in the marine environment providing a suitable growth habitat.

1. Introduction

In marine organisms, bioluminescence is used as a form of optical communication for attracting mates or prey, or for defence against predation (Herring, 1978; Morin, 1983). In order to make the communication signal effective, light should transmit well through water and be detectable by the appropriate receiver.

Bioluminescent bacteria are common in the ocean, especially in temperate to warmer waters (Dunlap and Kita-Tsukamoto, 2006). Existing information on the ecology of luminous microflora of invertebrates is limited (Herring, 1977; Nair et al., 1979; Venkatiswaran et al., 1981; Nishida et al., 2002). A specific microbial biota is typically associated with marine animals and plants (Bry et al., 1996). The spectrum of direct pairwise interactions of bacteria with marine organisms ranges from mutualisms through commensalisms and competition, to antagonism, determined ultimately by balancing the costs and benefits of the association (Pianka, 1994). Marine invertebrates and their associated microbiota provide a rich source of material for studies that can reveal much about both the diversity in host–bacteria associations and the mechanisms by which symbioses originate and persist (Douglas, 1994; Ostroumov, 2001). Bacteria do not exhibit luminous properties until they have reached sufficiently high concentrations to initiate quorum sensing (Waters and Bassler, 2005; Nealson and Hastings, 2006) and, once induced by a stimulus, they glow continuously. These properties are specific to bacteria, making them uniquely suitable as photogenic symbionts. To profit from the bacterial association, however, the host needs (1) to mantain the bacteria in good health condition (or the light goes out) and to get rid of dead or surplus ones, (2) to restrict the bacterial distribution to the limited site and not let them spread throughout the body, (3) to be able to switch the light on and off at will, and (4) to ensure that the symbionts that are advantageous to the host are transferred to the next generation by vertical transmission. The association is very specific – for each host species there is one kind of bacterium only – and populations seem to be clonal. Pujalte et al. (1999) suggested that the association with host organisms may contribute to the survival and distribution of luminous bacteria in the marine environment. Stabili et al. (2006) described a previously unknown association between *Vibrio* sp. AO1, a luminous bacterium related to the species *V. harveyi*, and the benthic hydrozoan *Aglaophenia octodonta.* Scanning electron microscopy analysis and culture-based and culture-independent approaches led to establish that luminous vibrios represent major constituents of the bacterial community inhabiting the *A. octodonta* surface, suggesting that the interaction between *Vibrio* sp. AO1 and the examined hydrozoan species is highly specific and this could be explained by the feeding activity of this microorganism on the hydroid chitinous structures. The present study describes a previously unknown association between luminous

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bacteria and the benthic hydrozoan *Clytia linearis* (Thornely, 1900). At present, *C. linearis* is one of the most common Mediterranean hydroid species in rocky shallow waters (Bouillon et al., 2004), and possibly it is the first successful Lessepsian migrant, so it can be considered an alien species (Boero et al., 2005). This species has a complex, protective layer, or perisarc, enveloping the colonies mainly composed of chitin and proteins. In Leptomedusae polyps, such as *C. linearis*, the perisarc forms a theca around the hydranths (the hydrotheca), the reproductive organs (the gonotheca), and the stolon (the hydrorhiza). *C. linearis* possesses its own luminescence and, when stimulated, it produces for several seconds a pale bluish light which appears as a series of fine dots (Kato, 1949). Bioluminescence is due to the presence of clytin (syn with phialidin) a photoprotein that was discovered already in the luminous hydroid *Clytia gregaria* (L. Agassiz, 1862). Clytin is located into special light cells present in the ectodermal layer of hydrocaulus, hydrorhiza.

2. Materials and methods

2.1. Sampling

Five groups each one consisting of about 300 *C. linearis* colonies were collected in July 2009 by SCUBA diving along the Otranto Channel (Ionian coast of Apulia, Italy) (40°08′39.8″N, 18°30′23.3″E) (Fig. 1) at 1–3 m of depth; they were transported in the laboratory under controlled temperature and processed for isolation of luminous bacteria within 4 h from collection.

2.2. Microscope observation

Luminous bacteria fluorescence on *C. linearis* colony surfaces was observed by a Zeiss Standard Axioplan epifluorescence microscope equipped with a halogen lamp (Hg 100) light. Slide preparations were observed by blue light excitation with a BP 485/20 excitation filter, a FT 510 chromatic beam splitter and a LP 520 barrier filter.

2.3. Quantitative analysis of bacteria living on *C. linearis* surface and phenotypic characterization of luminous bacterial isolates

In the laboratory, five groups each one consisting of 300 colonies of *C. linearis* with about 3000 polyps for each group (ca. 1 g) were gently washed in sterile seawater (0.2 μm pore filtered) to eliminate the bacteria settled on the surfaces, then 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 each sonicated sample and appropriate decimal dilutions was plated in parallel onto Marine Agar 2216 (Beckton Dickinson and Company) and after incubation for 2 days at 22 °C, the culturable bacteria were counted according to the colony-forming unit (CFU) method. The bacterial colony types having luminous properties were picked out, spread onto Sea Water Complete (SWC) agar to obtain pure cultures. Phenotypic identification followed the schemes of Baumann and Schubert (1984), Alsina and Blanch (1994) and Holt et al. (1994). The performed tests were Gram staining, growth in thiosulphate/citrate/bile salts/sucrose agar (TCBS; Beckton Dickinson and Company), bioluminescence on SWC agar (West and Colwell, 1984), oxidase and catalase assays, sensitivity to the vibriostatic agent O/129, OF test, amino acids decarboxylase reaction, indole production, gelatinase production, lipase hydrolysis, Voges–Proskauer test, utilization of citrate, and acid production from inositol, arabinose and sucrose. Growth at 0, 3 and 8% NaCl, growth at 4 °C, 30 °C and 35 °C were also determined. A further characterization was done by screening the utilization of different carbon sources (L-arabinose, D-glucosamine, xylose, mannose, arabinose, cellobiose, glucose, galactose, trehalose, melibiose, lactose, mannitol, sorbitol, inositol and sucrose) with the addition of NaCl to give a final concentration of 2.5%. The tests of utilization of different carbon sources were performed using the Biolog system.

Fig. 1. Map showing the sampling station in the Northern Ionian Sea off the coast of Otranto, Lecce (Italy).

2.4. 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 STE buffer (500 mM NaCl, 50 mM Tris–Cl pH 8, 5 mM EDTA), and then re-suspended 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: isoamylic alcohol (24:1) extraction according to standard procedures (Sambrook and Russel, 2001), and 15 μg ml^{-1} ribonuclease A was used to remove the RNA. After phenol-chloroform: isoamylic alcohol (24:1) extraction and ethanol-precipitation, high molecular weight DNA was collected by spooling using Shepherd's crooks (Sambrook and Russel, 2001).

Almost the entire 16S rRNA-encoding genes were amplified using the Proteobacteria-specific primers 16SEB20-42-F (5′-TGGCTCAG-GAYGAACGCTGGCGG-3′) and 16SEB1488-R (5′-TACCTTGTTAC-GACTTCACC-3′) (Vigliotta et al., 2007). The two primers were designed to amplify a 1488 bp-long DNA fragment (from nucleotide 20 to nucleotide 1507 in the *Escherichia coli* 16S rRNA gene). All PCR reactions 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 1× TAE 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 Custome Sequencing Service (Germany) or by Primm s.r.l (Italy).

DNA similarity searches were carried out using Basic Local Alignment Search Tool (Altschul et al., 1990) at National Center for