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MICROBIOLOGY OF AQUATIC SYSTEMS

Susceptibility to Antibiotics of *Vibrio* sp. AO1 Growing in Pure Culture or in Association with its Hydroid Host *Aglaophenia octodonta* (Cnidaria, Hydrozoa)

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Abstract *Vibrio harveyi* is the major causal organism of vibriosis, causing potential devastation to diverse ranges of marine invertebrates over a wide geographical area. These microorganisms, however, are phenotypically diverse, and many of the isolates are also resistant to multiple antibiotics. In a previous study, we described a previously unknown association between *Vibrio* sp. AO1, a luminous bacterium related to the species *V. harveyi,* and the benthic hydrozoan *Aglaophenia octodonta*. In this study, we analyzed the susceptibility to antibiotics (ampicillin, streptomycin, tetracycline, or co-trimoxazole = mix of sulfamethoxazole and trimetoprim) of *Vibrio* sp. AO1 growing in pure culture or in association with its hydroid host by using microcosm experiments. The results of minimum inhibitory concentration (MIC) experiments demonstrated that *Vibrio* sp. AO1 was highly resistant to ampicillin and streptomycin in pure culture. Nevertheless, these antibiotics, when used at sub-MIC values, significantly reduced the hydroid fluorescence. Co-trimoxazole showed the highest inhibitory effect on fluorescence of *A. octodonta*. However, in all treatments, the fluorescence was reduced after 48 h, but never disappeared completely around the folds along the hydrocaulus and at the base of the hydrothecae of *A. octodonta* when the antibiotic was used at concentration completely inhibiting growth in vitro. The apparent discrepancy between the MIC data and the fluorescence patterns may be due to either heterogeneity of the bacterial population in terms of antibiotic susceptibility or specific chemical–physical conditions of the hydroid microenvironment that may decrease the antibiotic susceptibility of the whole population. The latter hypothesis is supported by scanning electron microscope evidence for development of bacterial biofilm on the hydroid surface. On the basis of the results obtained, we infer that *A. octodonta* might behave as a reservoir of antibiotic multiresistant bacteria, increasing the risk of their transfer into aquaculture farms.

Abbreviations

MIC	Minimal inhibitory concentration
CFU	Colony-forming units
SCUBA	Self-contained underwater breathing apparatus
CB	Cacodylate buffer
SEM	Scanning electron microscope
TCBS Agar	Thiosulfate–citrate–bile salts–sucrose Agar
BP	Band pass
LP	Long pass
FT	*Farb teiler* (German indication for color splitter)
PBPs	penicillin-binding proteins

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Introduction

Luminous vibrios are natural inhabitants of coastal waters [1]. Some luminous *Vibrio* species are pathogenic and can cause vibriosis, a serious infectious disease in both wild and cultured marine organisms [2]. In recent years, vibriosis has become one of the most important bacterial diseases in marine-cultured organisms [2–4].

In particular, *Vibrio harveyi* is a common inhabitant of tropical and temperate marine environments [5]. Once considered an opportunistic pathogen, *V. harveyi* is now known to cause mass mortalities in penaeid prawn farms across the world. Other species of marine organisms have also been affected including pearl oysters (*Pinctada maxima*) [6], fish (*Solea senegalensis* [4], *Hippocampus* sp. [7]), and lobsters (*Panulirus homarus*) [8]. Additionally, *V. harveyi* has also been reported as a major pathogen of an increasing list of aquatic animals, including finfish, bivalves, and *Artemia franciscana* nauplii [6, 9, 10]. To some extent, these diseases occurred because the interactions of microbes with their hosts, their effects on animals, and the host defense mechanisms, at intensive production scales, have not been well understood or have been treated only from a clinical pathology perspective [11]. Animal health can be improved avoiding the presence of pathogenic bacteria or at least by minimizing the effects of pathogens and by improving water quality. In this framework, antibiotics and other chemotherapeutic agents commonly used in fish farms either as feed additives or immersion baths for either prophylactic or therapeutic purposes may result in an increase of drug-resistant bacteria [12, 13]. A few studies on antibiotic resistance in shrimps showed that luminous strains of *V. harveyi* isolated from shrimp larvae are resistant to erythromycin, kanamycin, penicillin G, and streptomycin [14]. *V. harveyi* strains isolated from diseased shrimp were resistant to ampicillin, chlorotetracycline, ceprofloxacin, erythromycin, furazolidone, gentamycin, nalidixic acid, neomycin, nitrofurazole, novobiocin, ofloxacin, oxytetracycline, penicillin G, polymyxin B, rifampicin, streptomycin, sulfamethoxazole, and sulfafurazole [15].

Stabili et al. [16] 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 results of culture-based and culture-independent approaches successively 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 species are highly specific, and this could be explained by the feeding activity of this microorganism on the hydroid chitinous structures. This interaction might have epidemiological as well as ecological implications because of the opportunistic pathogenicity of luminous *Vibrio* species for marine organisms and the wide distribution of the hydrozoans functioning as carriers [17]. In this study, we have analyzed the susceptibility to antibiotics (ampicillin, streptomycin, tetracycline, or co-trimoxazole = mix of sulfamethoxazole and trimetoprim) of *Vibrio* sp. AO1 growing in pure culture or in association with its hydroid host by using microcosm experiments to evaluate the possibility that *A. octodonta* might behave as a reservoir of antibiotic multiresistant bacteria, increasing the risk of their transfer into aquaculture farms.

Methods

Sampling

Batches of 1,000 colonies of the hydrozoan species *A. octodonta* 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 in May 2007. This species was selected since it does not emit its own light [18]. Colonies were transported in the laboratory under controlled temperature (5°C) and utilized for experimentation.

Epifluorescence Microscope Observation

For *A. octodonta* colonies observations, we utilized a Zeiss standard Axioplan epifluorescence microscope equipped with a halogen lamp (Hg 100) light. Blue light excitation with a band pass 485/20 excitation filter, a *farb teiler* (German indication for color splitter) 510 chromatic beam splitter, and a long pass 520 barrier filter were used to observe the slides prepared from each sample. In particular, we observed the fluorescence on the external side of the perisarc (chitinous exoskeleton) around hydrocladia (folds along the hydrocaulus and at the base of the hydrothecae).

Scanning Electron Microscopy on *A. octodonta*

Thirty colonies of *A. octodonta* were washed several times with sterile seawater (0.2 μm pore-filtered) to assure the

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

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.

Effect of Antibiotics on *Vibrio* sp. AO1 Growing in Pure Culture

Minimal inhibitory concentrations (MIC; i.e., the lowest concentration of an antimicrobial that inhibited the growth of a microorganism after overnight incubation) of ampicillin, streptomycin, tetracycline, and co-trimoxazole on *Vibrio* sp. AO1 were determined in nutrient broth (DIFCO) containing 3% NaCl by using a standard broth macrodilution method. Broth, serial two-fold dilution of antibiotics, and a culture of actively growing bacteria (about 10^6 colony forming units (CFU) per milliliter) were dispensed into polyethylene culture tubes (16 by 125 mm, Corning) to a total volume of 2 ml/tube. The tubes were incubated overnight at 30°C. The turbidity was determined spectrophotometrically at 600 nm.

Effect of Antibiotics on *A. octodonta* Fluorescence in Microcosm Experiments

Seawater samples were collected from oligotrophic coastal waters. In the laboratory, the seawater samples were immediately filtered through 200-μm pore-size filters and then through 2- and 0.2-μm pore-size filters. Replicate microcosm experiments were used to test the response of *A. octodonta* to challenge with different antibiotics. All microcosms consisted in beakers of 2 l capacity filled with filtered seawater and subjected to gentle stirring on a magnetic stirrer. Stock solutions of antibiotics were prepared by dissolving the appropriate antibiotics (ampicillin, streptomycin, tetracycline, or co-trimoxazole; Sigma-Aldrich) in distilled water in 10-ml sterile tubes. All antimicrobial compounds, after serial dilutions of the stock solution with natural seawater (previously filtered onto 0.2-μm pore filters), were placed in microcosms, to obtain a final concentration of 10 μg ml^{-1} for ampicillin, streptomycin, and tetracycline and 10 μg ml^{-1} of sulfamethoxazole and 2 μg ml^{-1} of trimetoprim (co-trimoxazole). The final concentrations utilized in the present study fall within the range of antibiotic concentrations usually employed in studies concerning *V. harveyi* antibiotic resistance [15]. Six experimental sets were prepared; each set included three independent replicate microcosms subjected to the same treatments. In the first experimental set of three microcosms, we added ampicillin with a final concentration of 10 μg ml^{-1}; in the second set of three microcosms, we added streptomycin to a final concentration of 10 μg ml^{-1}; in the third set of three microcosms, we added tetracycline (10 μg ml^{-1}, final concentration). In the fourth set of three microcosms, we added ampicillin, streptomycin, and tetracycline, at the same concentrations reported earlier. The fifth experimental set of three microcosms contained 10 μg ml^{-1} of sulfamethoxazole and 2 μg ml^{-1} of trimetoprim (co-trimoxazole). Finally, the sixth experimental set of three microcosms contained only filtered (0.2 μm) seawater, without any treatment, and served as controls. In each microcosm, 40 colonies of *A. octodonta* were added. All microcosms were incubated for 48 h at the in situ seawater temperature. At each sampling time from each microcosm, four colonies of *A. octodonta* were removed and observed using an epifluorescence microscope. Sampling intervals were defined in order to follow decline of fluorescence of *A. octodonta* in response to antibiotics addition. In particular, samples were taken at T0, 30, 60, 120, 180, and 240 min and 24 and 48 h after starting the experiment.

Effect of Co-Trimoxazole on Bacteria Living on *A. octodonta* Surface

A second microcosm experiment was carried out in order to evaluate the effect of co-trimoxazole on bacteria living on the animal surface, because of the highest inhibitory effect of this antimicrobial compound on fluorescence of *A. octodonta*. In particular, we employed two experimental sets, each one composed of three independent replicate microcosms; all microcosms were performed by using beakers of 2 l capacity filled with filtered (0.2 μm) seawater and subjected to gentle stirring on a magnetic stirrer. Co-trimoxazole was added to three microcosms to obtain a final concentration of 10 μg ml^{-1} of sulfamethoxazole and 2 μg ml^{-1} of trimetoprim (treatment). In the second experimental set of three microcosms, we added only filtered seawater (control). All microcosms were incubated for 4 h (T_4) at the in situ seawater temperature. At T_0 (experimental beginning) and T_4 from each microcosm, hydrocauli bearing hydrocladia of *A. octodonta* were examined to obtain about 3,000–3,500 polyps (ca. 1 g) and removed, 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. 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 both treatment and control sonicated samples and appropriate decimal dilutions were plated, in triplicate, in parallel onto Marine Agar 2216 or thiosulfate–citrate–bile salts–sucrose (TCBS) Agar (Beckton Dickinson and Company), and after incubation for 7 days at 22°C and at 30°C for 48 h, respectively, the cultivable bacteria were counted according to the CFU method.

Results

The fluorescence on the external side of the perisarc and around hydrocladia of *A. octodonta* displayed clear differences depending on antibiotic treatment. When compared with the control, ampicillin addition resulted in an about 50% decrease of fluorescence after 60 min of treatment and 60% after 120 min until the end of the experiment (48 h; Figs. 2 and 3a). The same trend was observed when streptomycin was added to *A. octodonta* (Figs. 2 and 3b). The addition of tetracycline had a significant impact on *A. octodonta* fluorescence with a 60% reduction after 120 min and 70% reduction after 48 h (Figs. 2 and 3c). The effect on *A. octodonta* fluorescence was even stronger when all antibiotics (ampicillin, streptomycin, and tetracycline) were simultaneously added. In particular, the fluorescence, in microcosms treated with all the antibiotics, was reduced by 60% after 120 min and 80% after 48 h (Figs. 2 and 3d). The impact of co-trimoxazole treatment on *A. octodonta* fluorescence was clearly detectable already after 30 min (reduction by 30%), and within the first 90 min, the fluorescence was reduced by about 70% with respect to the control. The reduction of fluorescence remained rather constant till the end of the experiment (48 h; Figs. 2 and 3e). However, in all treatments, the fluorescence was reduced after 48 h, but never disappeared completely around the folds along the hydrocaulus and at the base of the hydrothecae of *A. octodonta.*

The fluorescence pattern of the association *A. octodonta–Vibrio* sp. AO1 was quite different compared to results of MIC experiments performed with pure cultures of *Vibrio* sp. AO1. This microorganism was highly resistant to ampicillin (MIC>300 μg ml^{-1}), moderately resistant to streptomycin (MIC=300 μg ml^{-1}), sensitive to tetracycline (MIC= 1.5 μg ml^{-1}), and to the association sulfamethoxazole + trimetoprim (co-trimoxazole). In this latter case, bacterial growth was completely inhibited when a combination of 10 μg ml^{-1} sulfamethoxazole and 2 μg ml^{-1} trimetoprim was used.

Since co-trimoxazole showed the highest inhibitory effect on fluorescence of *A. octodonta*, we selected this antimicrobial compound to evaluate its effect on bacteria living on the animal surface. The results of cultural analysis using in parallel Marine Agar 2216 and TCBS Agar demonstrated that at T_0 luminous vibrios represented a conspicuous component ($6.2\pm0.32\times10^3$ CFU per gram, 20%) of the total cultivable *A. octodonta* surface bacteria ($3.1\pm0.21\times10^4$ CFU per gram). The impact of co-trimoxazole treatment on *A. octodonta* surface bacteria was clearly detectable already after 4 h. Indeed, the luminous vibrios density dropped by about 50% ($3.4\pm 0.18\times10^3$ CFU per gram) as compared to untreated control sample. In addition, not only luminous bacteria were affected by co-trimoxazole treatment but also total cultivable ones ($1.7\pm0.12\times10^4$ CFU per gram).

As the bacterial abundances decreased but never dropped to zero by co-trimoxazole treatment of the hydroid, the presence of a bacterial biofilm on *A. octodonta* surface was hypothesized and consequently supported by scanning electron microscopy data (Fig. 4).

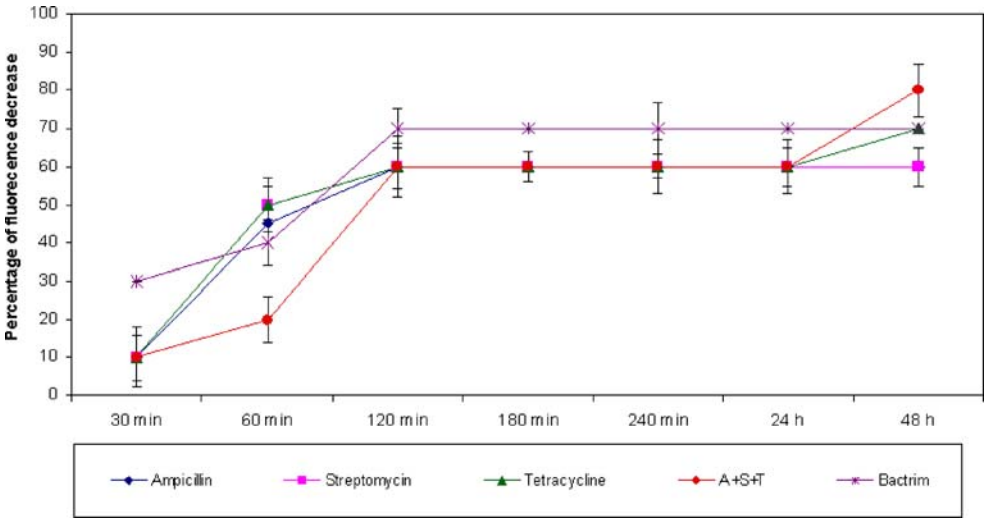

Figure 2 Percentage of fluorescence decrease of *Vibrio* sp. AO1 in association with *A. octodonta* after the antibiotics treatments (from 30 min to 48 h)

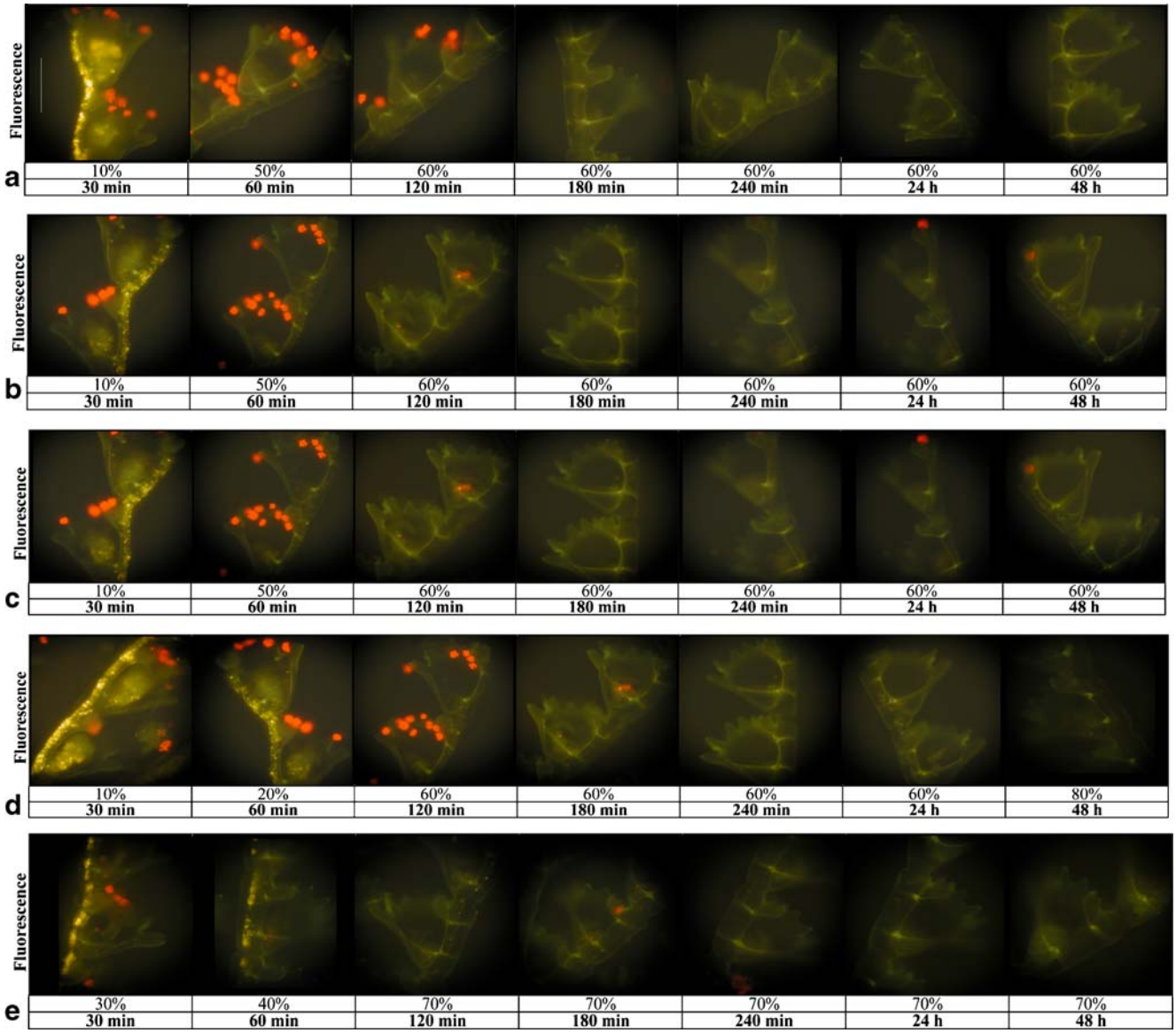

Figure 3 *A. octodonta* photomicrographs at epifluorescence showing the fluorescence decrease from 30 min to 48 h after. **a** Ampicillin (10 μg) treatment. **b** Streptomycin (10 μg) treatment. **c** Tetracycline (10 μg) treatment. **d** All antibiotics (ampicillin 10 μg + streptomycin 10 μg + tetracycline 10 μg) were simultaneously added. **e** Sulfamethoxazole 10 μg + trimetoprim 2 μg mix treatment. Scales: 250 μm

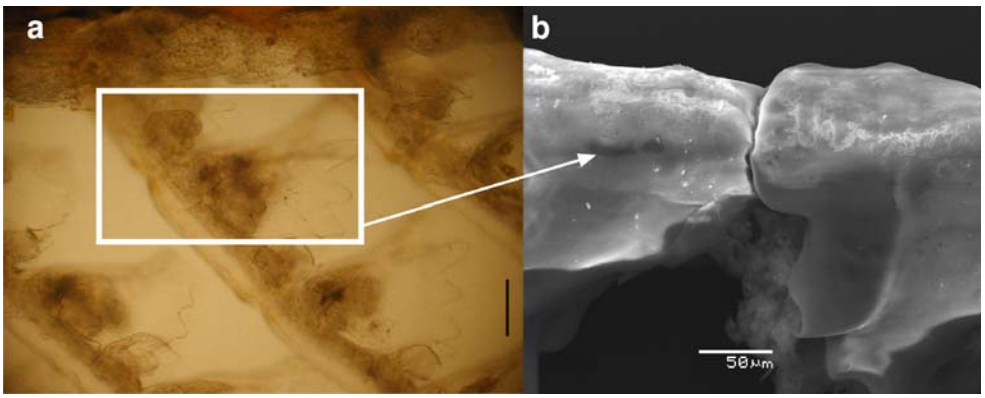

Figure 4 *A. octodonta* micrographs. **a** Hydrocladia at transmitted light. **b** SEM magnification of the microbial biofilm observed on the surface of the investigated hydroid. Scales: **a** 500 μm. **b** 50 μm

Discussion

In a previous study [16], we described the association between *Vibrio* sp. AO1 related to *V. harveyi* and a common member of the shallow water communities of the Mediterranean Sea, the hydrozoan *A. octodonta*. The halo of fluorescence observed around hydrocladia (folds along the hydrocaulus and at the base of the hydrothecae) in the studied invertebrate, examined to the epifluorescence microscope, was due to the presence of luminous vibrios. SEM analysis demonstrated the adhesion of bacterial cells showing a typical morphology of vibrios to the hydroid perisarc. Further magnification showed that a bacteria biofilm was present on the surface of this hydroid [17]. In the present study, we clearly demonstrated by scanning electron microscopy the presence of that bacterial biofilm on the surface of *A. octodonta.* Biofilms can be defined simply and broadly as communities of microorganisms that are attached to a surface. In most natural environments, association with a surface in a structure known as a biofilm is the prevailing microbial lifestyle [19]. Surface association is an efficient means of lingering in a favorable microenvironment rather than being swept away by the current [19]. Therefore, the adhesion of *Vibrio* sp. AO1 on *A. octodonta* may contribute to the survival of this *Vibrio* species in the marine environment providing a suitable growth habitat. The association observed between *Vibrio* sp. AO1 and *A. octodonta* has not only an ecological significance but also an epidemiological one highlighted from the results obtained with the antibiotics treatment.

In particular, in this study, we evaluated the effect of different antibiotics on *Vibrio* sp. AO1 growing in pure culture or in association with its hydroid host. The susceptibility of this luminous bacterium to the antimicrobial compounds was quite different under the two experimental conditions. The results of MIC experiments demonstrated that *Vibrio* sp. AO1 was highly resistant to ampicillin and streptomycin in pure culture. Nevertheless, these antibiotics significantly reduced the hydroid fluorescence when used at sub-MIC values. However, caution is necessary when interpreting these results. In fact, it should be noted that the fluorescence pattern was a measure of the metabolic activity of the luminous vibrios living on *A. octodonta* and not a direct measure of their growth or survival. In addition, MIC experiments allow determining the minimum antibiotic concentration that inhibits growth of every member of a microbial population. However, a bacterial population is heterogeneous in terms of antibiotic susceptibility [20, 21]. Some members may be more resistant than others and determinant for the MIC value. Thus, the reduced hydroid fluorescence after ampicillin and streptomycin treatments at sub-MIC values may be associated to inhibition of metabolic activity of population members more susceptible to the antibiotics.

While we have no information about the molecular basis of streptomycin resistance in *Vibrio* species that are taxonomically related to *Vibrio* sp. AO1, there is evidence that in *V. harveyi*, in addition to chromosomally borne class A β-lactamase genes with low homologies to other β-lactamases of the same class [22], additional mechanisms may contribute to resistance including changing the permeability of outer membrane porin channels (leading to reduced drug influx into bacteria cell) and penicillin-binding proteins (PBPs)-mediated resistance arising when PBPs fail to bind or exhibit reduced affinity to β-lactams [23]. Salt concentration, furthermore, affects the in vitro susceptibility of halophilic vibrios including *V. harveyi* to ampicillin as well as many other antibiotics [23]. It is, therefore, also plausible that the partial sensitivity to ampicillin of *Vibrio* sp. AO1 in association with its hydroid host may be due to the specific chemical–physical conditions of the hydroid microenvironment.

By contrast, *Vibrio* sp. AO1 was highly sensitive to co-trimoxazole in pure culture. However, when this treatment at a concentration completely inhibiting growth in vitro was carried out on the hydroid host, the fluorescence was reduced after 48 h, but never disappeared completely around folds along the hydrocaulus and at the base of the hydrothecae of *A. octodonta.* Similar results were obtained with the tetracycline. These discrepancies could be due to the observed microbial biofilm on *A. octodonta* (Fig. 4). It is clear that microorganisms undergo profound changes during their transition from planktonic (free-swimming) organisms to cells that are part of a complex surface-attached community [24]. These changes are reflected in some phenotypic characteristics developed by biofilm bacteria. Biofilm-associated cells are more resistant to many toxic substances such as chlorine, detergents, and antibiotics [25]. There is evidence that decreased diffusion into the biofilm [26, 27], decreased bacterial growth rate in a biofilm [28], biofilm-specific substances [29], and the quorum-sensing specific effects [30, 31] may be reasons for this resistance [19]. The presence of the bacterial biofilm on *A. octodonta* surface is consistent with the quantitative data of luminous and total cultivable bacteria living on the hydroid in the presence of co-trimoxazole. The bacterial abundances, indeed, decreased but never dropped to zero in presence of this antibiotic. Further studies on *Vibrio* sp. AO1 will be undertaken to evaluate which of the above-mentioned processes is involved in the antibiotic resistance observed. The resistance of *Vibrio* sp. AO1 associated to *A. octodonta* to all the antibiotics employed has epidemiological implications since *V. harveyi* is pathogenic and its carrier, *A. octodonta*, is widely distributed in the Mediterranean Sea [16]. The pathogenic

effects of *V. harveyi* are critical in aquaculture settings, where organisms, e.g., penaeid shrimps and salmonids, are reared at high densities under very artificial and unstable conditions [3, 32, 33]. To maintain the productivity of such an intensive aquaculture, several antibiotics are employed massively. In particular, the antibiotics employed in this study are comprised among those utilized in aquaculture, for therapeutic purposes and as prophylactic agents [34]. On the basis of our results obtained, this practice might be ineffective in presence of *A. octodonta* colonies in the aquaculture plants on account of the *Vibrio* sp. AO1 antibiotic resistance observed when associated to this hydroid. In these conditions, the employment of antibiotics could only have a negative impact on the health of the marine environment. In addition, since *A. octodonta* serves as a reservoir for pathogenic vibrios, we can hypothesize that these microorganisms would endure adverse environmental conditions on the surface of this hydroid in the aquaculture settings. Hydrozoa may, indeed, function as habitat "islands" providing a unique set of environmental conditions for microbial colonization, quite different from those in the surrounding benthic environment [35, 36]. When favorable environmental conditions for vibrios are re-established, these would be able to cause disease in wild animals. Understanding the survival strategies of vibrios in the environment is of utmost importance for control of both water quality and transmission of disease, and this study represents a preliminary contribute on this topic.

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References

1. Ruby EG, Nealson KH (1977) Seasonal changes in the species composition of luminous bacteria in the near shore seawater. Limnol Oceanogr 23:530–533
2. Austin B, Austin DA (eds) (1993) Bacterial fish pathogens, 2nd edn. Ellis Horwood, Chichester
3. Austin B, Austin DA (eds) (1999) Bacterial fish pathogens: disease of farmed and wild fish, 3rd edn. Springer-Verlag KG, Berlin, Germany
4. Zorrilla I, Arijo S, Chabrillon M, Diaz P, Martinez-Manzanares E, Balebona MC, Morinigo MA (2002) *Vibrio* species isolated from diseased farmed sole, *Solea senegalensis*. J Fish Dis 26:103–108
5. O'Brien CH, Sizemore RK (1979) Distribution of the luminous bacterium *Beneckea harveyi* in a semitropical estuarine environment. Appl Environ Microbiol 38:928–933
6. Pass DA, Dybdahl R, Mannion MM (1987) Investigation into the causes of mortality of the pearl oyster, *Pinctada maxima* (Jamson), in Western Australia. Aquaculture 65:149–169
7. Alcaide E, Gil-Sanz C, Esteve D, Sanjuan D, Amaro C, Silveira L (2001) *Vibrio harveyi* disease in seahorse, *Hippocampus* sp. J Fish Dis 24:311–313
8. Jawahar AT, Keleemur RM, Leema JMT (1996) Bacterial disease in cultured spiny lobster, *Panulirus homarus* (Linnaeus). J Aquacult Trop 11:187–192
9. Saeed MO (1995) Association of *Vibrio harveyi* with mortalities in cultured marine fish in Kuwait. Aquaculture 136:21–29
10. Soto-Rodriguez SA, Roque A, Lizarraga-Partida ML, Guerra-Flores AL, Gomez-Gil B (2003) Virulence of luminous vibrios to *Artemia franciscana* nauplii. Dis Aquat Org 53:231–240
11. Moriarty DJW (1998) Control of luminous *Vibrio* species in penaeid aquaculture ponds. Aquaculture 164:351–358
12. Manjusha S, Sarita GB, Elyas KK, Chandrasekaran M (2005) Multiple antibiotic resistences of *Vibrio* isolated from coastal and brackish water areas. Am J Biochem & Biotech 1:201–206
13. Thompson FL, Iida T, Swings J (2004) Biodiversity of vibrios. Microbiol Mol Biol Rev 68:403–431
14. Baticados MCL, Lavilla-Pitogo CR, Cruz-Lacierda ER, Pena LD, Sunaz NA (1990) Studies on the chemical control of luminous bacteria *Vibrio harveyi* and *V. splendidus* isolated from diseased *Penaeus monodon* larvae and rearing water. Dis Aquat Org 9:133–139
15. Abraham TJ, Manley R, Palaniappan R, Dhevendaran K (1997) Pathogenicity and antibiotic sensitivity of luminous *Vibrio harveyi* isolated from diseased penaeid shrimp. J Aquat Trop 12:1–8
16. Stabili L, Gravili C, Piraino S, Boero F, Alifano P (2006) *Vibrio harveyi* associated with *Aglaophenia octodonta* (Hydrozoa, Cnidaria). Microb Ecol 52:603–608
17. Stabili L, Gravili C, Tredici SM, Piraino S, Talà A, Boero F, Alifano P (2008) Epibiotic *Vibrio* luminous bacteria isolated from some Hydrozoa and Bryozoa species. Microb Ecol 56:625–636
18. Bouillon J (1995) Classe des Hydrozoaires (Hydrozoa Owen, 1843). In: Grassé PP, Doumenc D (eds) Traité de Zoologie. Masson, Paris
19. Watnik P, Kolter R (2000) Biofilm city of microbes. Minireview. J Bacteriol 182:2675–2679
20. Amaral L, Trigenis B, Atkinson BA (1982) The radioactive thymidine incorporation method for the determination of antibiotic susceptibility of gram-negative bacilli. Eur J Clin Microbiol 1:149–154
21. Lorian V, Atkinson B (1976) Effects of subinhibitory concentrations of antibiotics on cross walls of cocci. Antimicrob Agents Chemother 9:1043–1055
22. Teo JW, Suwanto A, Poh CL (2000) Novel beta-lactamase genes from two environmental isolates of *Vibrio harveyi*. Antimicrob Agents Chemother 44:1309–1314
23. Ottaviani D, Bacchiocchi I, Masini L, Leoni F, Carraturo A, Giammarioli M, Sbaraglia G (2001) Antimicrobial susceptibility of potentially pathogenic halophilic vibrios isolated from seafood. Int J Antimicrob Agents 18:135–140
24. O'Toole G, Kaplan HB, Kolter R (2000) Biofilm formation as microbial development. Annu Rev Microbiol 54:49–79
25. Costerton JW, Cheng K-J, Geesey GG, Ladd TI, Nickel JC, Dasgupta M, Marrie TJ (1987) Bacterial biofilms in nature and disease. Annu Rev Microbiol 41:435–464
26. De Beer D, Srinivasan R, Stewart PS (1994) Direct measurement chlorine penetration into biofilms during disinfection. Appl Environ Microbial 60:4339–4344
27. Suci PA, Mittelman MW, Yu FP, Geesey GG (1994) Investigation of ciprofloxacin penetration into *Pseudomonas aeruginosa* biofilms. Antimicrob Agents Chemother 38:2125–2133

28. Evans DJ, Brown MRW, Gilbert P (1990) Susceptibility of bacterial biofilms to tobramycin: role of specific growth rate and phase in the division cycle. J Antimicrob Chemother 25:585–591
29. Yildiz FH, Schoolnik GK (1999) *Vibrio cholerae* O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. Proc Natl Acad Sci USA 96:4028–4033
30. Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280:295–298
31. Hassett DJ, Ma JF, Elkins JG, McDermott TR, Ochsner UA, West SE, Huang CT, Fredericks J, Burnett S, Stewart PS, McFeters G, Passador L, Iglewski BH (1999) Quorum sensing in *Pseudomonas aeruginosa* controls expression of catalase and superoxide dismutase genes and mediates biofilm susceptibility to hydrogen peroxide. Mol Microbiol 34:1082–1093
32. Bergh O, Nilsen F, Samuelsen OB (2001) Diseases, prophylaxis and treatment of the Atlantic halibut *Hippoglossus hippoglossus*: a review. Dis Aquat Org 48:57–74
33. Olafsen JA (2001) Interactions between fish larvae and bacteria in marine aquaculture. Aquaculture 200:223–247
34. FAO (2005) Responsible use of antibiotics in aquaculture. In: Serrano PH (ed) Fao fisheries technical paper 469. Food and Agriculture Organization of the United Nations, Rome
35. Carman KR, Dobbs FC (1997) Epibiotic microorganisms on copepods and other aquatic crustaceans. Micros Res Tech 37:116–135
36. Roszak DB, Colwell RR (1987) Survival strategies of bacteria in the natural environment. Microbiol Rev 51:365–379