Marine Environmental Research 125 (2017) 90–98

Contents lists available at ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

Spatial distribution of the culturable bacterial community associated with the invasive alga *Caulerpa cylindracea* in the Mediterranean Sea

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ARTICLE INFO

Article history:
Received 26 October 2016
Received in revised form
31 January 2017
Accepted 1 February 2017
Available online 3 February 2017

Keywords:
Bioinvasions
Alien seaweeds
Metabolic profiles
Biolog assay
Functional diversity
Culturable heterotrophic bacteria

ABSTRACT

Understanding the mechanisms underlying the complex seaweed-bacteria associations in nature may provide information on the fitness of an invasive host. This may require the use of different approaches. In this study, we employed, for the first time, the Biolog system-Ecoplates™ to analyze the functional diversity of the culturable fraction of the bacterial assemblages associated with the surface of *Caulerpa cylindracea*, the invasive seaweed of the Mediterranean Sea. Seaweed samples were collected at five sites across the basin. A high similarity in the bacterial activity, expressed as Average Well Color Development (AWCD), among the study sites was observed. Culturable heterotrophic bacteria at 22 °C showed mean values ranging from 1.4×10^5 CFU g^{-1} at Porto Cesareo (Ionian Sea, Italy) to 5.8×10^6 CFU g^{-1} at Othonoi, Diapontine Island (Ionian Sea, Greece). The analysis of the DNA sequences on isolated bacteria demonstrated that the genera *Shewanella*, *Marinobacter*, *Vibrio*, *Granulosicoccus* and the family Rhodobacteraceae are consistently present on *C. cylindracea*, irrespective of its geographical origin. The present study provided new insights into the complex association between bacteria and this algal species, suggesting a specific composition and function of the associated culturable bacteria across the basin.

1. Introduction

In the marine environment, bacteria regulate rates of organic matter mineralization, nutrient cycling, and energy transfer through their metabolism and the utilization of several molecules (Simon et al., 2014). In particular, polysaccharides, proteins and lipids are the product of carbon fixation representing the main component of dissolved organic matter (DOM) and particulate organic matter (POM) pools (Nagata, 2008). Microbial extracellular enzymes (EE) may control organic polymer degradation and utilization (Cunha et al., 2010). Released enzymes with diverse specificities include proteases, chitinases, cellulases, amylases, phosphatases, and others (Zhang and Kim, 2010). The metabolic diversity of bacteria is perhaps as noteworthy as their taxonomic and evolutionary diversity, and the quality of DOM can influence the bacterial community structure (Kujawinski, 2011).

Seaweeds may play a role in driving the development of specific bacterial communities, since it provides a suitable substrate for the settlement of microorganisms (Goecke et al., 2010; Wahl et al., 2010; Sneed and Pohnert, 2011). Epiphytic bacteria communities are fast colonizers consisting of complex bacterial assemblages growing on the surfaces of seaweeds, capable of rapid metabolization of algal exudates (Aires et al., 2013; Singh and Reddy, 2014; Stratil et al., 2014; Vieira et al., 2016). Recently, it has been proven that bacterial communities associated with invasive seaweeds can be species-specific, variety-specific (Aires et al., 2013, 2015), or made of different bacteria with similar metabolic functions (Aires et al., 2016), as theorized by Burke et al. (2011). These associated bacterial communities can produce bioactive compounds promoting algal growth, quorum sensing signals and other substances influencing algal morphology, development and survival (Singh and Reddy, 2014).

Caulerpa cylindracea (Sonder) (Belton et al., 2014), previously known as *C. racemosa* var.*cylindracea* (Sonder) Verlaque, Huisman

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http://dx.doi.org/10.1016/j.marenvres.2017.02.001
0141-1136/

and Boudouresque (Verlaque et al., 2003), is a non-indigenous invasive algal species introduced in the Mediterranean Sea from South-Western Australia (Klein and Verlaque, 2008). Since then, it has colonized all available habitats and substrates. In 2000 the seaweed *C. cylindracea* was also found in the Adriatic (Žuljević et al., 2003) leading to profound structural and functional alterations of indigenous benthic assemblages and fish metabolism (Holmer et al., 2009; Cebrian et al., 2012; Felline et al., 2012; Pusceddu et al., 2016). Recent phylogenetic analyses confirmed the presence of *C. cylindracea* also in northeastern Australia and New Caledonia and identified a potential colonized area in Port Adelaide, South Australia (Sauvage et al., 2013). At present, seaweeds represent up to 40% of all introduced marine species (Schaffelke et al., 2006), and some seaweeds can significantly affect the composition and functioning of resident marine benthic communities (Williams and Smith, 2007). When an alien species invades a new range, different scenarios are possible due to lack of co-evolution between the newly arrived invader and local foes including the not "recognition" by the foes of the potential biotic target (Enemy release hypothesis, Keane and Crawley, 2002), invader's particularly efficient defences (Novel weapon hypothesis, Enge et al., 2012), and rapid defence adaptation mechanisms (Saha et al., 2016). These different mechanisms could facilitate the fitness and thereby success of invasive hosts, as in the case of *Caulerpa*. Taking into account its potential impact and fast spread on resident assemblages, *C. cylindracea* has been inserted in the 100 worst invasive species of the Mediterranean Sea (Streftaris and Zenetos, 2006). In addition the homogenization of the Mediterranean coastal system observed in invaded sites lead to hypothesize deep alterations in the structure and functioning of ecosystems (Pacciardi et al., 2011). Recent analyses revealed that endobacterial communities of *C. cylindracea* [*C. racemosa* var.*cylindracea* in Aires et al. (2013)] can be effective tracers of the origin of invasions and have a potential role in supporting their eukaryotic host's adaptation to new environments (Aires et al., 2013). In this study, we combined both cultural and metabolic methods to describe the active microbial community associated with the surface of *C. cylindracea* collected from five different sites in the central Mediterranean Sea, hundreds of kilometers apart. The Biolog EcoPlate system (Biolog, Hayward, CA, USA), was used to describe their metabolic profiles. Among the available methods used to identify environmental bacteria, the Biolog EcoPlate system offers a standardized rapid method for determining bacterial oxidation of 31 ecologically relevant carbon substrates with a redox-sensitive tetrazolium indicator of microbial respiration (Truu et al., 2009). Recent studies on *C. cylindracea* evidenced that the seaweed can modify the organic matter composition of the surrounding sediments (Matijević et al., 2013). Moreover, as a significant donor of organic carbon, the surface of this seaweed provides a microhabitat that is favorable for bacterial colonization and reproduction. Therefore, the associated culturable bacteria, through their metabolic processes and utilization of substrates, could assist the pattern of colonization of this algal species. In this framework, the aim of this study was to assess the metabolic capabilities of the bacterial community associated with the surface of *C. cylindracea,* hence the functional diversity of a microbial community and provide specific insights into this complex seaweed–bacteria association in nature.

2. Methods

2.1. Sampling

Caulerpa cylindracea was collected in the period of maximum spreading (September–October) by SCUBA divers in the Bay of Kotor (Montenegro) (42°29′06.6″N, 18°41′28.6″E), in Othonoi (Diapontine Islands, Greece) (39° 50.257′N, 19° 24.037′E), in the Marine Protected Area of Torre Guaceto (Brindisi, Italy) (40°42′59.25″N, 17°48′5.12″E), in Otranto (Lecce, Italy) (40° 9′5.94″N, 18°29′27.16″E) and in the Marine Protected Area of Porto Cesareo (Lecce, Italy) (N 40°12.772′, E017°48.218′) at shallow water (5–10 m depth) on rocky substrates (Fig. 1) to assess potential differences in the metabolic capabilities of the bacterial community associated with the surface of *C. cylindracea* among sites. The morphological identification was performed through analysis of different parts of the thalli. In detail, the following morphological features of the thalli were analyzed: height, width and attachment to stolons of the fronds; height, diameter, shape, and arrangement of the ramuli; diameter of the stolons; length, width, spacing, and morphology of the rhizoids. Algal morphological features fit the description given by several authors for the invasive species *C. cylindracea* (Verlaque et al., 2000; Klein and Verlaque, 2008; Guillén et al., 2010; Cebrian et al., 2012; Belton et al., 2014). In each site, about three replicates of 300 g each of *C. cylindracea* were collected and transferred to the laboratory under controlled temperature and processed for culturable heterotrophic bacteria enumeration within 4 h from sampling.

2.2. Bacteria enumeration

In the laboratory, the seaweed was washed three times in sterile seawater (using seawater samples from each sampling area filtered on 0.2 μm pore size filters, salinity between 37.1 ± 0.13 and 38.5 ± 0.20), to eliminate any loosely associated bacteria, then suspended in sterile glass flasks with sterile seawater (ca. 10 g of seaweed in 90 ml of seawater to obtain a 1:10 (w/v)) and sonicated three times (Branson Sonifier 2200, 60 W, 47 kHz for 1 min in an ice bath) to optimize surface bacteria detachment (Stabili et al., 2008; Rizzo et al., 2016a,b). The sonication was interrupted for 30 s every minute, during which time the samples were shaken manually. To enumerate surface bacteria 1 mL of the sonicated sample and appropriate decimal dilutions (10^1-10^8) were plated onto Marine Agar 2216. After incubation for 2 days at 22 °C the culturable bacteria were counted according to the colony forming units (CFU) method (Stabili et al., 2008). All the suspected colony types grown were picked out and streaked on to Marine Agar to obtain pure cultures. Phenotypic identification of the bacterial isolates was performed in order to identify common bacteria present in the examined sites.

2.3. 16S rRNA gene sequencing

For phylogenetic assignments of the bacterial isolates, the 16S rRNA-encoding genes were amplified using the bacteria-specific primers Com1-F (5′-CAGCAGCCGCGGTAATAC-3′) and Com2-R (5′–CCGTCAATTCCTTTGAGTTT-3′). These primers were designed to amplify 409 bp long DNA fragments central region (from nucleotide 519 to nucleotide 927 in the *Escherichia coli* 16S rRNA gene) of prokaryotic small subunit ribosomal RNA gene (16S rRNA gene), and allowed high taxonomic resolution, often at the species level (Lane et al., 1985). PCRs were performed as follows: initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at 55 °C and extension at 72 °C for 1–2 min, and the final elongation step at 72 °C for 5 min, carried out in a Perkin-Elmer Cetus DNAThermal Cycler 2400. PCR products were isolated through 1% (wt/v) agarose gels in 1X TAE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8.0), recovered using the Qiaex II Gel extraction kit (Qiagen). PCR products finally were sequenced as a service by MWG Biotech Custom Sequencing Service (Germany). The amplicons were sequenced by Sanger method. All sequences were compared with those of closely related reference strains using the EzTaxon-e server (Kim et al., 2012).