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Community structures of actively growing bacteria stimulated by coral mucus

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ABSTRACT

Coral mucus influences the composition and abundance of bacteria in the surrounding seawater. In this study, the phylogenetic affiliations of actively growing bacteria (AGB) in seawater supplemented with the mucus of *Acropora* sp. during a 24-h incubation period were determined. For this purpose, bromodeoxyuridine magnetic-beads immunocapture and PCR-DGGE (BUMP-DGGE) analysis was used. The coral mucus contained higher concentrations of particulate organic carbon and nitrogen and exhibited higher bacterial abundance than seawater did, and the organic matter and bacteria varied dramatically during the incubation. BUMP-DGGE analysis showed that the AGB stimulated by the coral mucus also varied during the incubation. The bulk of the active growers originated from seawater and not the mucus. However, not all of the bacterial phylotypes in seawater could use the coral mucus as a suitable growth substrate. Cluster analysis revealed that the AGB community structures identified across the incubation period were considerably different in seawater supplemented with coral mucus as compared with those in coral mucus. In total, 12 phylotypes of AGB were identified, and these belonged to Alphaproteobacteria (Rhodobacterales 5 phylotypes), Gammaproteobacteria (Oceanospirillales 3 phylotypes, Vibrionales 1 phylotype, Alteromonadales 1 phylotype), and Bacteroidetes (Flavobacteriales 2 phylotypes). In the coral mucus, phylotypes belonging to Oceanospirillales appeared only at the beginning of the incubation, whereas those belonging to Rhodobacterales appeared only in the late phase of the incubation. Thus, coral mucus can alter the AGB community structure and create an organic matter flux that is specific to a reef environment.

1. Introduction

Corals regularly produce and release mucus into the surrounding seawater. The released coral mucus contains higher concentrations of nutrients such as ammonium and phosphate (Nakajima et al., 2014) and organic carbon and nitrogen (Tanaka et al., 2009, 2011a) than does the surrounding seawater. The coral mucus containing these nutrients and organic matter is used by the organisms present in the surrounding environments (Tanaka et al., 2011b; Wild et al., 2004b) and also by coral holobionts (Rohwer et al., 2002; Thurber et al., 2009). Moreover, in coral mucus, the abundance of prokaryotes is greater than that in the surrounding seawater (Garren and Azam, 2010; Wegley et al., 2004). Coral mucus serves as a carrier of energy and nutrients (Wild et al., 2004a, 2005) and is responsible for supporting biogeochemical processes in reef systems (Bythell and Wild, 2011).

Previous studies have investigated the variations in organic carbon concentration and/or bacterial abundance that occur when corals release mucus into the surrounding seawater. Ferrier-Pages et al. (2000) observed that mucus derived from several corals caused an increase in the abundances of bacteria and autotrophic and heterotrophic flagellates. Furthermore, Nakajima et al. (2009) showed that bacterial abundance increased substantially and organic matter content decreased after the coral mucus of *Acropora nobilis* and *Acropora formosa* was added into natural seawater. Tanaka et al. (2011b) characterized the organic carbons of the mucus of *A. formosa* and *Montipora digitata* and their degradability by bacteria; these researchers showed that the mucus consisted predominantly of easily degradable labile organic carbon, and that 80% of the organic carbon was degraded within 30 days by bacteria. Thus, coral mucus is considered to serve as a favorable substrate for microbial growth. Although previous studies have investigated such variations in bacterial abundance and organic carbon concentration during the incubation of seawater supplemented with coral mucus, the bacterial community structure remains poorly studied.

The coral-associated bacteria present in coral mucus and tissue and in the seawater of reef environments have been investigated (Rohwer

Abbreviations: AGB, actively growing bacteria; PCR, polymerase chain reaction; DGGE, denaturing gradient gel electrophoresis; BrdU, bromodeoxyuridine; BUMP-DGGE, BrdU magnetic-beads immunocapture and PCR-DGGE; SW, seawater; MuS, seawater supplemented with coral mucus; MuFS, 0.22-μm-filtered seawater supplemented with coral mucus.

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et al., 2002; Tout et al., 2014; Wegley et al., 2004). Corals were reported to harbor Rhodobacterales, which belong to Alphaproteobacteria, and Vibrionales, Alteromonadales, and Oceanospirillales, which belong to Gammaproteobacteria (Mouchka et al., 2010). Interestingly, Nelson et al. (2011) reported that dissolved organic carbon was depleted in the coral reef and this changed the overlying bacterial communities. This finding indicates that the mucus derived from the reef is responsible for mediating the organic matter flux in reef environments. Although the coral-associated bacteria present in coral mucus and tissue have been investigated in detail, only a few studies have focused on the change in bacterial community structures that occurs after the release of coral mucus (Allers et al., 2008; Nelson et al., 2013). Allers et al. (2008) used fluorescence *in situ* hybridization (FISH) in order to characterize the variation in the bacterial community structure following the release of coral mucus into the surrounding seawater; the results showed that during incubation for 50 h, the mucus of *Fungia* sp. enhanced gammaproteobacterial growth, especially that of Alteromonadaceae (up to 50% of the total cells) and Vibrionaceae (up to 21% of the total cells), at a mucus-to-seawater concentration ratio of 1:10. Nelson et al. (2013) obtained a distinct result: Alphaproteobacteria, especially in Rhodobacteraceae, were stimulated by coral mucus in various coral species. These studies were focused on the variations in the bacterial community structures that were detected before and after incubation. However, because the total community structure develops as a result of both the growth and the death of bacteria, such analyses cannot readily identify the genuine active growers that are stimulated by coral mucus.

In this study, the objective was to determine the phylogenetic affiliations of actively growing bacteria (AGB) that are stimulated by coral mucus. To monitor the AGB, bromodeoxyuridine (BrdU) was used; BrdU is a halogenated nucleoside that can serve as a thymidine analog and is a useful reagent for analyzing DNA-synthesizing bacteria. Here, bacteria that incorporated BrdU into their DNA were considered to be AGB. BrdU-incorporated DNA can be detected using antibodies (Hamasaki et al., 2004; Mou et al., 2008; Steward and Azam, 1999; Tada and Grossart, 2013), and an immunocapture technique featuring antibody-conjugated magnetic beads has been used for determining the phylogenetic affiliations and functions of bacterial groups present in soil and marine environments (Artursson and Jansson, 2003; Borneman, 1999; Urbach et al., 1999). Previously, the phylogenetic affiliations of AGB and their spatiotemporal changes were investigated in coastal and oceanic environments by employing BrdU magnetic-beads immunocapture and PCR-DGGE (BUMP-DGGE) analysis (Hamasaki et al., 2007; Taniguchi and Hamasaki, 2008; Taniguchi et al., 2011). This BrdU-based technique is a powerful method available for investigating the active bacterial diversity (Zinger et al., 2012) that is responsible for the organic matter flux in reef environments.

2. Materials and methods

2.1. Sample collection

The coral used in this study, *Acropora* sp., was collected in the southern part of Wakayama Prefecture, Japan (N 33°29′11.8″, E 135°41′ 29.2″), in September 2010. The water depth was about 3 m. Water temperature, salinity, and chlorophyll *a* concentration were measured using a modernized, compact and lightweight multi-parameter water-quality meter, AAQ1183-H (JFE Advantec Co., Ltd., Hyogo, Japan). The temperature, salinity, and chlorophyll *a* concentration in ambient seawater were 27.9 °C, 33.21 PSU, and 0.47 μg L^{-1}, respectively. Branches of several colonies of healthy *Acropora* sp. in natural seawater were collected using a chisel, with care being exercised to ensure that the coral was not exposed to air. Duplicate samples of corals from distinct colonies were collected. Immediately after the collection, the branches stored in buckets filled with natural seawater were transferred, at the *in situ* temperature, to the laboratory. Seawater that was obtained from sites far from the coral-collection site was pre-filtered through a 200-μm nylon mesh in order to remove mesozooplankton. In the laboratory, coral mucus was collected using the procedure of Wild et al. (2004a). The corals were washed with 0.22-μm-filtered natural seawater in order to remove the bacteria on the coral surface and then the mucus released during the initial 30 s was discarded, after which the mucus released into a sterilized bottle for 2 min was collected.

2.2. BrdU incubation

Samples were prepared by exposing them to three experimental treatments performed using replicates (Replicates 1 and 2): seawater (SW), seawater supplemented with coral mucus (MuS), and 0.22-μm-filtered seawater supplemented with coral mucus (MuFS). The ratio of the coral mucus to seawater and to filtered seawater was 1:600, a ratio that is sufficiently high for enhancing bacterial growth by using the mucus of *Acropora* (Taniguchi et al., 2014). The samples were mixed gently and after a 30-min pre-incubation period, the samples were incubated for 24 h at the *in situ* temperature. Subsamples were collected before incubation and after incubation for 4, 8, 12, and 24 h, and in order to identify the AGB, each sample was incubated for 3 h with BrdU (final concentration, 1 μmol L^{-1}; Sigma-Aldrich, St. Louis, MO, USA).

2.3. Particulate carbon and nitrogen

During the incubation, 200 mL of each subsample (in duplicate) was filtered onto GF/F filters (Whatman International Ltd., Maidstone, UK) that were pre-combusted at 550 °C for 3 h. The filtered samples were stored at −20 °C until analysis. Particulate organic carbon (POC) and nitrogen (PON) were measured using a CHN micro-coder (JM10, J-SCIENCE LAB Co., Ltd., Kyoto, Japan).

2.4. Bacterial abundance

Each subsample was fixed using 0.22-μm-filtered paraformaldehyde (final concentration, 2%). The fixed samples were filtered onto 0.2-μm-pore Nuclepore membrane filters (Whatman International Ltd.) under <100 mm Hg vacuum and then stained with a 2 μg mL^{-1} solution of 4′,6-diamidio-2-phenylindole (DAPI) (Porter and Feig, 1980). More than 300 cells per sample or at least 10 microscopic fields were counted under an epifluorescence microscope (Olympus BX51; Olympus Corporation, Tokyo, Japan) equipped with a U-MWU2 filter.

2.5. BUMP-DGGE analysis

At the end of each incubation period, the collected subsamples were filtered through 0.22-μm-pore Sterivex filters (Millipore, Billerica, MA, USA) by using a peristaltic pump in order to collect bacterial cells. Immediately after filtration, the Sterivex filters were stored at −20 °C until further analysis. BUMP-DGGE analysis was performed according to procedures described previously (Hamasaki et al., 2007), with slight modifications. Briefly, the Sterivex filters were subjected to xanthogenate-SDS DNA extraction, and 200 ng of the extracted DNA was used for BrdU immunocapture. Total DNA and the immunocaptured, BrdU-labeled DNA were used as templates for PCR amplification of the 16S rRNA gene by using the eubacterial-specific primer 341F-GC that contains a 40-bp GC clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3′; the GC-clamp sequence is underlined) and the universal primer 907R (5′-CCG TCA ATT CMT TTG AGT TT-3′) (Schäfer and Muyzer, 2001). To perform DGGE, the PCR products (approximately 100 ng) were loaded onto 6% polyacrylamide gels in 0.5× TAE buffer, and the denaturing gradient used was 25%–70% from top to bottom. Electrophoresis was performed at 85 V for 16 h at 60 °C in an INGENY PhorU system (Ingeny, Goes, Netherlands). The gels were stained with SYBR Gold (Molecular Probes, Eugene, OR, USA) in 0.5× TAE buffer for 30 min. The Jaccard coefficient was calculated from