Isolation of antagonistic bacteria associated with the stony coral *Montipora digitata* in Okinawa, Japan

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**Abstract** Coral “holobiont” is defined as a complex assemblage which is comprised of the coral animal and its associated microorganisms. These organisms (including bacteria, archaea, cyanobacteria, fungi and also virus) inhabit coral tissues, coral skeleton, and the coral surface forming the so-called surface mucus layer (SML). The SML bears a diverse assemblage of antagonistic bacteria which suppress overgrowth of other microorganisms and maintain the healthy status of coral holobiont. On the basis of this background, we accomplished the isolation of antagonistic bacteria associated with *Montipora digitata* colonies sampled at Sesoko Island, Okinawa, Japan. As a result, 13 strains of antagonistic bacteria were isolated and identified by the analysis of partial 16S rDNA sequences. The bacteria were identified to belong to either the genus *Pseudoalteromonas*, *Vibrio*, or *Staphylococcus*. We attempted isolation of antifungal compounds from newly isolated *Pseudoalteromonas* sp. S10. By analysis of NMR and ESI-MS spectral data, the antifungal compounds were identified as known macrolactams alteramides A and B.

**Keywords** *Montipora digitata*, antagonistic bacteria, *Pseudoalteromonas* sp., alteramides A and B

**Introduction**

The coral holobiont is defined as a complex assemblage, which is comprised of the coral animal and its associated microorganisms consisting of bacteria, archaea, fungi, viruses, and protists including the dinoflagellate algae *Symbiodinium* that lives in symbiosis with corals providing nutrition to it by translocating their photosynthates (Rohwer et al. 2002; Thompson et al. 2014). Recent studies have revealed the presence of a dynamic status of microorganisms living on the surface, inside the tissues and skeleton of stony and soft corals (Ritchie and Smith 1997; Ben-Haim et al. 1999; Rohwer et al. 2001, 2002; Kooperman et al. 2007). In terms of maintaining coral “health”, the role and contribution of these microorganisms within the holobiont has been discussed by many researchers (Reshef et al. 2006; Bourne et al. 2009; Rosenberg et al. 2009; Barott and Rohwer 2012), however the mechanism of action of these microorganisms is still not well understood. Reshef et al. (2006) advocated the ‘Coral Probiotic Hypothesis’, which explains homeostatic function of symbiotic microbial biota to keep coral in a healthy shape. As a matter of fact, symbiotic bacteria in the surface mucus layer (SML) of corals were indicated to...
function as a first defense line against pathogenic bacteria based on the result that most of bacteria isolated from coral mucus had shown antimicrobial activity (Shnit-Orland and Kushmaro 2009). They also revealed that *Pseudoalteromonas* was the major antagonistic bacteria being abundant in mucus of several soft and stony corals. Furthermore, the characteristic of antagonistic compounds of the strains of *Pseudoalteromonas* were revealed to be heat stable and extracellular-excreted (Shnit-Orland et al. 2012).

Recently we isolated antibacterial compounds montiporic acids from tissues of the stony coral *Montipora digitata* Dana 1846, based on screening of anti-microbial activity (Kodani et al. 2013a; Kodani et al. 2013b). There is the possibility that some of tissue associated bacteria may have antagonistic activity as a part of coral probiotics. To test this possibility, we attempted isolation and identification of antagonistic bacteria from *Montipora digitata*, which is commonly distributed in Okinawan reefs. Regarding *Montipora digitata*, this paper is the first attempt of isolation and identification of antagonistic bacteria. As a result, we found antagonistic bacterium *Pseudoalteromonas* sp. strain S10, which had potent antifungal activity. The antifungal principles of the strain S10 were identified by chemical analysis using a mass spectrometry (MS) and a nuclear magnetic resonance (NMR) spectroscopy.

**Materials and method**

**Collection of specimens**

In May 2013, specimens of *Montipora digitata* were collected from shallow water reefs (< 1 m) around the Tropical Biosphere Research Center Sesoko Station of University of the Ryukyus in the Okinawa Islands, Japan. The collection of coral was performed under the permission of Okinawa Prefecture (Permission Number 24-49). For each specimen, branch tips measuring 5 to 8 cm in length were collected from different colonies.

**Isolation of bacteria**

The surface tissue of *Montipora digitata* was scraped off using sterile steel knife in the clean bench. The scraped tissues were pasted onto TCG agar medium (Tryptone 3.0 g, Casitone 5.0 g, Glucose 4.0 g, agar 15 g, in 1 L of sea water). The plates were incubated at 25°C for 6 days. After observation of emergence of bacterial colonies, each colony was purified by isolation technique and stored in 20% glycerol stock solution at −80°C. A total of 78 strains were isolated and stored as culture collection.

**Microorganisms and culture condition for Antagonistic effect screening**

The bacterium *Bacillus subtilis* Ehrenberg 1835 (NBRC 13719), the yeast *Saccharomyces cerevisiae* Meyen ex E.C. Hansen 1883 (NBRC 2376) and the fungi *Aspergillus oryzae* Cohn 1884 (NBRC 4290), *Aspergillus niger* van Tieghem 1867 (NBRC 33023), *Mucor hiemalis* Wehmer 1903 (NBRC 9405), *Colletotrichum acutatum* Simmonds 1965 (NBRC 31434), *Rhizopus delemar* Wehmer & Hanszawa, 1901 (NBRC 5441) and *Dichotomomyces cepii* Scott 1970 (NBRC 8396) were obtained from the NBRC culture collection (NITE Biological Resource Center, Japan). In addition, two bacteria including *Vibrio coralliilyticus* Ben-Haim 2003 (accession number, AB490821) and *Paracoccus carotinifaciens* Tsubokura 1999 (accession number, AB490820), that were previously reported to induce coral bleaching (Higuchi et al. 2013) were cultured. *V. coralliilyticus* was cultured using TCG medium incubated at 30°C. The all microorganisms except for *V. coralliilyticus* were cultured using ISP2 agar medium (Shirling and Gottlieb 1966) incubated at 30°C.

**Antagonistic effect screening using testing bacterial strains**

Screening for antibacterial activity was performed against testing bacteria, including *Bacillus subtilis*, and the coral pathogens, *Vibrio coralliilyticus* and *Paracoccus carotinifaciens*. The strains from the culture collection were pre-cultured by ISP2 agar medium at 30°C for 7 days. Small amount of bacterial cells of each strain were obtained by sterile toothpick and inoculated onto the agar medium on which the testing bacterium was spread. Antagonistic activity was defined by measuring the radius of the inhibition zone of the growth of testing bacteria around the colony.

**Polymerase chain reaction (PCR) amplification, sequencing, and phylogenetic analysis of 16S rRNA genes**

The extraction of all DNA from the cells of bacterial strain was performed following the procedure described
by Kodani et al. (2002) The 16S rRNA-encoding sequence was amplified from the total DNA by the PCR method with one set of universal primer pairs: 9F (5'-GAGTTTGATCCCTGGCTCAG-3') and 682R (5'-TCTACGCATTTCACCCTACAC-3'). For PCR, the reaction mixture was prepared by adding 1 μL of the total extracted DNA of (approximately 100 ng), 25 μL of Emerald Amp PCR Master Mix (Takara, Japan), 1 μL each of primers (10 μM), and 22 μL of distilled water into the PCR reaction tube. PCR amplification was carried out using a thermal cycler using the following protocol: initial denaturation for 10 min at 94°C, followed by 34 cycles consisting of denaturation for 40 s at 94°C, annealing for 60 s at 55°C, and DNA synthesis for 1 min at 72°C. A final extension of 5 min at 72°C was included at the end of 34 cycles. The PCR product was purified with a QIAGEN PCR PURE kit following the manufacturer’s instructions. The reactions for sequencing were performed using a Beckman DTCS-Quick Start Kit following the manufacturer’s instructions. The primer 9F was used for the reaction. The sequencing was performed with the capillary DNA sequencer CEQ 8000XL (Beckman Coulter).

**Isolation of antibacterial compounds from *Pseudoalteromonas* sp. S10**

TCG agar medium (1 L) was used for culturing *Pseudoalteromonas* sp. S10. After the incubation for 2 days at 30°C, the bacterial cells were harvested by steel spatula. Then an equal volume of methanol (MeOH) was added to the cells for extraction. After centrifugation at 3000 rpm for 5 min, the supernatant was obtained as MeOH extract. The MeOH extract was subjected to reverse-phase HPLC using an ODS column (Nacalai Tesque, Cosmosil C18-MSIII 4.6×250 mm) with an isocratic elution of 60% acetonitrile (MeCN) containing 0.05% trifluoroacetic acid (TFA) at an absorbance of 220 nm to yield alteramides A (5.0 mg) and B (4.3 mg).

**General Methods**

The chromatography system consisted of 2 HPLC pumps (Jasco, PU-980), a UV/VIS detector (Jasco, Model UV-970), and digital integration software (MacIntegrator II). ESI-TOF MS spectra were recorded using a JEOL JMS-T100LP mass spectrometer. 1H NMR spectra were obtained with a JEOL ECA-600 in MeOH-d₄ at 27.0°C. The resonance of residual MeOH at δH 3.30 ppm was used as an internal reference.

**Results and Discussion**

We collected tissues of *Montipora digitata* from coral reefs in Okinawa Island, Japan. Using sea-water based medium, a total of 78 bacterial colonies were isolated from scraped tissues. The 78 bacterial colonies were subjected to antagonistic effect screening against 4 testing microorganisms including a gram-positive bacterium *Bacillus subtilis*, a fungi *Mucor hiemalis*, and two gram-negative bacteria which were indicated to be the possible pathogens; *Vibrio coralliilyticus* and *Paracoccus carotinifaciens*. Briefly, small amount of cells of each isolated bacterium were directly inoculated onto the medium on which the testing bacterial cells were spread. Around a bacterium which had an antagonistic effect, the inhibition zone was observed after incubation at 30°C for 24 h (Fig. 1). As a result, a total of 13 strains showed antagonistic activities against at least one testing microorganism as shown Table 1. To identify the antagonistic bacteria, sequencing of partial 16S rRNA coding gene (approximately, 500 bp) was performed. As a result, the 13 strains were
identified as following: 7 strains (strain number S3, S4, S5, S8, S12, S18, and S19) as *Vibrio*; 4 strains (strain number S10, S11, S17, and S20) as *Pseudoalteromonas*; 2 strains (strain number S6 and S23) as *Staphylococcus*.

Previously Shnit-Orland et al. (2012) isolated and identified antagonistic bacteria from six stony corals. As a result, the bacteria belonging to *Pseudoalteromonas* genus were reported to be a major group of antagonistic bacteria. Furthermore, Shnit-Orland et al. (2012) reported that *Pseudoalteromonas* sp. excreted a heat-tolerant substance which showed inhibitory activity against gram-positive bacteria. However, the inhibitory substance has not been identified yet. On the basis of this background, we tried to isolate and identify antagonistic compounds from newly isolated *Pseudoalteromonas* sp. Among the strains of *Pseudoalteromonas* obtained in this experiment, *Pseudoalteromonas* sp. strain S10 stably grew on TCG medium and showed potent antagonistic activity. Then we subjected the MeOH extract of the strain S10 to anti-microorganism test using four testing microorganisms including *B. subtilis, M. hiemalis, V. coralliilyticus* and *P. carotinifaciens*. As a result, the MeOH extract of the strain S10 only showed antifungal activity against *M. hiemalis*. So we attempted to isolate antifungal compounds from MeOH extract of the strain S10.

*Pseudoalteromonas* sp. strain S10 was cultured for two days using TCG agar medium. The bacterial cells of the strain S10 were harvested and extracted with MeOH. The MeOH extract of the strain S10 was repeatedly subjected to HPLC separation. As a result, two antifungal compounds were isolated (Fig. 2). Both compounds were isolated as powder after lyophilization. The molecular formula of compounds 1 and 2 were respectively determined to be $C_{29}H_{38}N_2O_6$ and $C_{29}H_{38}N_2O_5$, based on by the data of HR-ESI-MS ($m/z$ 511.2812 [M + H], $\Delta$ +0.4 mmu for compound 1, $m/z$ 495.2850 [M + H], $\Delta$ -0.9 mmu for com-

### Table 1  Antagonistic activity of bacterial strains isolated from coral tissue

<table>
<thead>
<tr>
<th>Isolated bacterial strains from coral tissue</th>
<th>Testing microorganisms for antagonistic activity</th>
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<tr>
<td>Genus</td>
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<tr>
<td>Strain number</td>
<td><em>subtilis</em></td>
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<td><em>Vibrio</em></td>
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<td>S18</td>
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<td>S19</td>
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<tr>
<td><em>Pseudoalteromonas</em></td>
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<td>S10</td>
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<td>S11</td>
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<td>S20</td>
<td>+</td>
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<tr>
<td><em>Staphylococcus</em></td>
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<td>S6</td>
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<td>S23</td>
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+: showed inhibitory zone, -: no inhibitory zone, the experiments were duplicated to confirm the antagonistic activity.
To identify the chemical structures, $^1$H NMR spectra and COSY spectra of each compound were measured. $^1$H NMR spectra of both compounds were very similar as shown in Fig. 3. By the comparison of chemical shifts using previous reports, the compound 1 was identified to be a known macrolactam, alteramide A which was originally obtained from marine sponge associated bacterium Alteromonas sp. (Shigemori et al. 1992). In the same manner with compound 1, compound 2 was identified to be a 25-hydroxylated analogue, alteramide B (Moree et al. 2014).

The assigned $^1$H-NMR chemical shifts were following; compound 1 in CD$_3$OD: 0.89 (3H, m, H-30), 0.93 (1H, m, H-9a), 1.05 (3H, d, J = 6.2 Hz, H-31) 1.10 (1H, m, H-29b), 1.22 (2H, m, H-7a, H-26b), 1.33 (1H, m, H-11), 1.40 (1H, m, H-26a), 1.59 (1H, m, H-10), 1.60 (1H, m, H-13), 1.61 (1H, m, H-29a), 1.90 (1H, m, H-7b), 1.93 (1H, m, H-9b), 2.09 (1H, m, H-12), 2.44 (1H, m, H-8), 2.54 (1H, m, H-6), 2.80 (1H, m, H-27b), 3.66 (1H, m, H-27a), 3.90 (1H, t, J = 8.9 Hz, H-14), 3.95 (1H, m, H-23), 4.00 (1H, m, H-25a), 5.67 (1H, dd, J = 14.4, 8.9 Hz, H-5), 5.74 (1H, d, J = 15.1 Hz, H-2), 5.96 (1H, dd, J = 15.1, 8.9 Hz, H-15), 6.07 (1H, dd, J = 14.4, 11.0 Hz, H-4), 6.19 (1H, dd, J = 15.1, 11.0 Hz, H-16), 6.91 (1H, d, J = 15.1 Hz, H-18), 7.35 (1H, dd, J = 15.1, 11.0 Hz, H-17).

The assigned $^1$H-NMR chemical shifts were following; compound 2 in CD$_3$OD: 0.89 (3H, m, H-30), 0.95 (1H, m, H-9a), 1.04 (3H, m, H-31), 1.10 (1H, m, H-29b), 1.17 (1H, m, H-26a), 1.24 (1H, m, H-7a), 1.35 (1H, m, H-11), 1.50 (1H, m, H-26b), 1.58 (1H, m, H-13), 1.62 (1H, m,
H-10), 1.63 (1H, m, H-29a), 1.84 (1H, m, H-25b), 1.92 (1H, m, H-7b), 1.97 (1H, m, H-9b), 2.09 (1H, m, H-25a), 2.12 (1H, m, H-12), 2.44 (1H, m, H-8), 2.53 (1H, m, H-27b), 2.54 (1H, m, H-6), 3.62 (1H, m, H-23), 3.85 (1H, m, H-27a), 3.92 (1H, m, H-14), 5.67 (1H, dd, J = 15.1, 9.6 Hz, H-5), 5.79 (1H, d, J = 15.1 Hz, H-2), 5.95 (1H, dd, J = 15.1, 9.6 Hz, H-15), 6.09 (1H, dd, J = 15.1, 11.0 Hz, H-4), 6.23 (1H, dd, J = 15.1, 10.3 Hz, H-16), 6.92 (1H, dd, J = 15.1, 11.0 Hz, H-3), 7.00 (1H, d, J = 15.1 Hz, H-18), 7.34 (1H, dd, J = 15.1, 10.3 Hz, H-17).

Further, we tested alteramides A and B against testing microorganisms shown in Table 2 using paper a disk method. Both compounds showed similar antifungal activity against *Mucor hiemalis* and *Dichotomomyces cepii* at 10 μg/disk, however only alteramide A showed antifungal activity against *Colletotrichum acutatum* (Table 2). Both compounds did not show activity against other microorganisms including bacteria and yeast as shown in Table 2. Maree et al. (2014) reported that *Pseudoalteromonas* sp. OT59 which was isolated from Pacific octocoral *Leptogorgia alba* displayed light-dependent antifungal properties. They identified the antifungal principles as alteramides A and B which may work as chemical defense against pathogenic fungi. In our experiment, we also found that alteramides A and B released from *Pseudoalteromonas* sp. S10 which was associated with stony coral *Montipora digitata* displayed antifungal activity.

Considering that alteramide A was originally isolated from marine sponge associated bacterium (Shigemori et al. 1992), there may be similar chemical defense system among marine invertebrates such as coral and sponge.

Shnit-Orland and Kushmaro (2009) reported that 25% of screened isolates from stony corals and 13% of isolates from soft corals displayed high antagonistic activity against indicator bacteria. In the present study, we obtained similar results, which shows that 16% of isolated colonies (13 out of 78 colonies) from tissues of *Montipora digitata* displayed antagonistic activity.

In this experiment, we isolated alteramides as antifungal substances from *Pseudoalteromonas* sp. S10. While *Pseudoalteromonas* sp. S10 cells showed antagonistic activity against bacteria including *Bacillus subtilis* and *Vibrio coralliilyticus*, the MeOH extract of the strain S10 did not show activity against these bacteria. There was a possibility that other excreted compounds including high molecular weight compounds like protease may get involved in the antagonistic activity to the bacteria.

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References


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