In Vitro Anti-methicillin-resistant *Staphylococcus aureus* Activity Found in Extracts of Marine Algae Indigenous to the Coastline of Japan

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Resistance to treatment for methicillin-resistant *Staphylococcus aureus* (MRSA) causing nosocomial infection has become a serious medical issue and currently, there is no effective antibiotic against MRSA but vancomycin, teicoplanin and arbekacin. MRSA first emerged in the late 1970s[1], and subsequently, the number of methicillin-resistant strains have increased, causing severe clinical and management problems in hospitals throughout the world.

Although many studies have reported that marine algae extracts contain antibacterial activity[2~9], these reports were published from 1960 to 1980, when MRSA was relatively rare. The current situation concerning MRSA has prompted re-evaluation of these reports and has aroused new interest in using marine algae sources for developing novel antibiotics.

Therefore, in this study, we first screened a wide variety of Japanese marine algae for the presence of antibacterial activity against 10 different isolates of MRSA. Then we evaluated the potency of MeOH-extracts from algae which tested positive for anti-MRSA activity using a dose-response assay.

Materials and Methods

Sample Algae and Extracts

A total of 857 algae samples comprising of 308 species (91 brown algae, 154 red algae, 55 green algae and 8 others) were collected and their extracts were prepared as described in our previous paper[10]. These filtered extracts were concentrated up to 10 fold by freeze-drying what was used in the antibacterial assays.

Bacteria and Culture Medium

Ten isolates of methicillin-resistant *Staphylococcus aureus* (MRSA, strains GIFU 12361, GIFU 12364, 7B29, E31224, E31237, E31243, E31256, E31271, E31280, and E31283) were used and compared with methicillin-sensitive *Staphylococcus aureus* (MSSA, strain IFO 15035). These MRSA strains were clinically isolated from the supplies and off the fingers of nurses or patients in different hospitals around Japan. To determine specific antibacterial activity, we also used *Bacillus subtilis* (strain IFO 14419), *Pseudomonas aeruginosa* (strain IFO 13736), and *Vibrio parahaemolyticus* (strain IFO 12711) as controls.

Antibacterial Assays

First, MRSA or MSSA strains were inoculated in 5 ml of TSB medium (Difco Laboratories Inc.), and incubated by shaking (120 rpm) at 36°C for 1 day. After culturing, 20 ml of TSB medium containing 0.8% agar was gently mixed with each test culture fluid to give approximately 10^6 CFU/ml and overlayed on a TSA medium (Difco Laboratories Inc.). Antibacterial assays for the other strains, *B. subtilis* and *P. aeruginossa* were carried out on a TSA, and *V. parahaemolyticus* on a Marine agar media (Difco Laboratories Inc.). Sterilized paper disks (i.d. 8 mm, Advantec), which were permeated with 50 μl of PBS- or MeOH-extracts and dried completely, were put on the double layer agar media. These plates were incubated at 36°C for 1 day for MRSA, MSSA and the control *P. aeruginosa* strains. *B. subtilis* and *V. parahaemolyticus* were incubated at 25°C for 1 day.

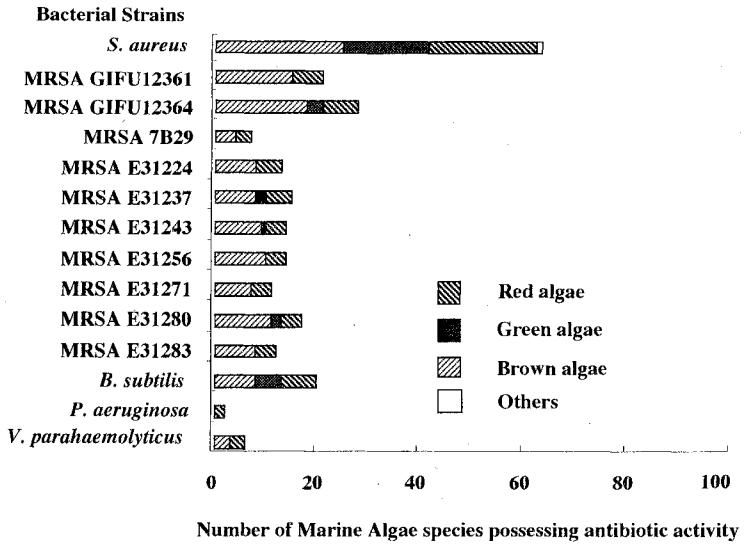
Dose Response of Algal Extracts

The positive algal extracts determined as possessing strong anti-MRSA activity were further examined for assessing the minimum concentration for antibacterial activity against the 10 clinical isolates of MRSA using a dose response assay. Approximated minimum concentrations were assessed using paper disks, which were absorbed with crude MeOH-extracts at concentrations ranging from 1.25 to 1000 μg per disk.

Isolation and Characterization of Anti-MRSA Substances

Anti-MRSA substances first were extracted with MeOH from wet *Laurencia brongniartii*, partitioned with two volumes of water and three volumes of *n*-hexane/chloroform (7 : 3), and then purified by silica gel column and thin layer chromatographies eluting with

Fig. 1. Marine algae showed antibacterial activity.



serial gradients of *n*-hexane/chloroform. Four anti-MRSA substances which were finally purified with HPLC were subjected to mass spectrometry and elemental analyses.

Results

A total of 308 marine algae species were first pre-screened for antibacterial activity against MSSA and then rescreened against 10 strains of MRSA. We found anti-MSSA activity from the MeOH-extracts of 64 species. The incidence of detection was 30.9% in the green algae (17 of 55 species), 27.5% in the brown algae (25 of 91 species), and 14.3% in the red algae (22 of 154 species) (Figure 1). After rescreening for anti-MRSA activity, we found anti-MRSA activity in many species of marine algae (29.4 % of green, 84% of brown, and 55% of red algae) which showed positive anti-MSSA activity. However, the potency of anti-MRSA activity was variable depend on which strains was tested. Significant anti-MRSA activity was found more in the brown algae than in the green or red algae. Antibacterial activity was also found against the Gram-negative bacteria, *Pseudomonas aeruginosa* and *Vibrio parahaemolyticus*. The MeOH-extracts derived from 6 brown algae, *Gelidium elegans*, *Dictyopteris undulata*, *Ishige okamurae*, *Ecklonia kurome*, *Dictyota spinulosa*, and *Sargassum horneri* and 5 red algae, *Asparagopsis taxiformis*, *Laurencia okamurae*, *Laurencia brongniartii*, *Odonthalia corymbifera*, and *Rhodomela teres* found in Japan exhibited relatively strong anti-MRSA activity against all MRSA strains tested (Table 1). Then when we attemped to isolate the anti-MRSA active substances from *Laurencia brongniartii*, we succeeded in four potent anti-MRSA substances, MC-5, MC-6, MC-7, and MC-8. The molecular weights of MC-5, MC-6, MC-7, and MC-8 were 397, 365, 475, and 443 and the elemental analysis indicated that these molecular formulas were $C_9H_6Br_3NS$, $C_{10}H_9Br_2NS_2$, $C_9H_5Br_4NS$, and $C_{10}H_8Br_3NS_2$, respectively.

Discussion

To the best of our knowledge, this paper is the first report about anti-MRSA activity *in vitro* from marine algae located on Japan's coasts. Previously, other reports showed that some distinct seaweeds contain antimicrobial substances against Gram-positive and -negative bacteria[3,4,11,12]. However, most of the previous papers described antibacterial activity from seaweeds against only one strain of *Staphylococcus aureus*, and there were no mention of anti-MRSA activity which might have chemotherapeutic value for nosocomial infection.

In the present investigation, we found antibacterial activity *in vitro* in 38 of 308 marine algae species which were collected from Japan's coasts, and most of these antibacterial activities were not only specific against MSSA strains, but also against MRSA strains. Brown algae were found to have the most potential for developing new anti-MRSA agents. Furthermore, this is the first time to detect antimicrobial activity from the

Table 1. Approximated minimum concentrations for antibacterial activity from marine algal extracts.

Marine algae		MRSA strains										S. a.[a]
		12361	12364	7B29	E 31224	E 31237	E 31243	E 31256	E 31271	E 31280	E 31283	
(Brown algae)												
GELIDIALES	*Gelidium elegans*	1000	1000	1000	1000	>1000	1000	1000	>1000	1000	>1000	1000
DICTYOTALES	*Dictyopteris undulata*	62.5	31.3	62.5	31.3	7.81	31.3	15.6	62.5	15.6	31.3	15.6
CHORDARIALES	*Ishige okamurae*	250	500	1000	250	1000	250	1000	500	250	500	250
LAMINARIALES	*Ecklonia kurome*	250	250	125	500	500	250	250	250	500	500	250
DICTYOTALES	*Dictyota spinulosa*	31.3	1000	1000	1000	>1000	1000	1000	1000	1000	>1000	1000
FUCALES	*Sargassum horneri*	>1000	>1000	>1000	>1000	250	500	>1000	>1000	>1000	>1000	500
(Red algae)												
NEMALIALES	*Asparagopsis taxiformis*	1000	1000	1000	1000	>1000	>1000	>1000	>1000	1000	>1000	1000
CERAMIALES	*Laurencia okamurae*	62.5	62.5	62.5	31.3	15.6	31.3	31.3	62.5	31.3	62.5	31.3
	Laurencia brongniartii	250	500	250	250	250	125	250	250	125	250	125
	Odonthalia corymbifera	125	62.5	31.3	62.5	62.5	62.5	62.5	31.3	62.5	62.5	31.3
	Rhodomela teres	500	1000	500	500	500	1000	500	500	250	500	500

Approximated minimum inhibitory concentrations of antibacterial activity were estimated with a paper disk penetrated each crude MeOH-extract from the marine algae. Numbers in the table represents μg order of crude extract per paper disk.

[a] A methicillin-sensitive *Staphylococcus aureus* (IFO 15035).

brown algae such as *Ishige sinicola*, *Dictyota spinulosa*, and *Sargassum horneri* possessing relatively high anti-MRSA activity, suggesting that novel antimicrobial compounds might be contained in these seaweeds. In fact, we succeeded in isolation of four anti-MRSA substances from one of these algae, *Laurencia brongniartii*, which might be bromindoles by judging from molecular formula and molecular weight analyses. These bromindole compounds are known as some of secondary metabolites in this alga[13]. However, such an antimicrobial activity described here has never been reported. Some algae exhibited different antibacterial potency according to location and time of year when the collection was performed (data not shown). It appears that the algae produce different bioactive substances in accordance with the environment and stage of life cycle, or there may be a difference in the amount of antibacterial substances produced. This seasonal variation in the antibacterial activity of seaweeds has been previously reported[14].

Since the availability of soil microorganisms may have reached its limit as resource for antibacterial agents, this paper describes that marine algae, which is a reliable resource, may be candidate for the development of new anti-MRSA agents.

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