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Anti Methicillin-Resistant *Staphylococcus aureus* (MRSA) Activity by Linolenic Acid Isolated from the Marine Microalga *Chlorococcum* HS-101

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Algae are the primary producers of organic matter in the sea. Although bacteria use algae as a food source, the algae-bacteria relationship is not always unilateral. Antibiotic or allelopathic substances derived from algae also play an important role in marine ecology. The release of antibacterial substances by marine phytoplankton has been known for a long time (Waksman et al.1937; Steeman-Nielsen 1955), and the production of antibacterial substances by a freshwater Chlorella was reported by Pratt in 1942. Since then, many antibiotic substances have been isolated from various algae (Bruce and Duff 1967; Higgs 1981; Mason et al. 1982; Rosell and Stivastava 1987). However, relatively little is known about the antibacterial substances of marine green microalgae (Lustingman 1988).

In recent years, the mass extensive use of antibiotics by medical institutions in Japan has led to outbreaks of iatrogenic methicillin-resistant Staphylococcus aureus (MRSA)infections, against which existing antibiotics are often ineffective. When patients in an immunosuppressed state in the hospital become infected with MRSA, they developed fever and diarrhea, followed by respiratory difficulty, damage to internal organs and sometimes death. Therfore, a substance that inhibits the growth of MRSA is needed.

In this paper, we report the isolation and identification of an anti-MRSA substance in the marine microalga Chlorococcum HS-101, which exhibited the highest antibiotic activity among 68 strains of isolated marine microalgae examined.

MATERIALS AND METHODS.

For this study, the green algae Chlorococcum HS-101 and Chlorella HS-569, and the blue-green alga Synechococcus HS-329 which exhibited antibiotic activity in their crude extracts, were selected from 68 marine algae collected in the coastal areas of western Japan (Ohta et al. 1993). As bacteria for antibiotic activity testing, wild type Staphylococcus aureus ATCC 25923 was

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obtained from American Type Culture Collection, and seven strains of methicillin-resistant Staphylococcus aureus (MRSA) were isolated from clinical specimens obtained at Kobe Institute Hospital. Growth conditions of algae and bacteria were as described in our previous paper (Ohta et al. 1993).

Each algal culture was centrifuged, and the algal concentration was adjusted to 0.1 g fresh wt/mL by the addition of methanol to the pellets. The algal suspension then was sonicated on ice by using an ultrasonic generator and the homogenate was centrifuged. The supernatant was filtered through a 0.22-μm Milipore filter. The filtrate was designated as the methanol extract. The methanol extract was examined for activity against wild-type S. aureus and seven strains of MRSA by observing inhibition of their growth at 25 °C on nutrient-broth (Difco Laboratories) agar plates for 24 hr as follows. The methanol extracts were freeze-dried and taken up in dimethyl sulfoxide (DMSO). An aliquot of this solution (40 μL, equivalent to 0.1 g fresh wt/mL) was transferred to a 10-mm paper disk, which was then applied to a plate seeded with a test strain. After incubation, the resulting zone of inhibition was measured. No inhibition zone was observed with any of the control disks containing DMSO alone. The antibiotic susceptibility of seven strains of MRSA to twelve kinds of authentic antibiotics shown in Table 1 was similarily investigated. The coagulase type of these MRSA strains was classified by using an immune serum test kit for S. aureus (Denka Seiken Co.Ltd., Japan).

To purify the antibiotic substance in Chlorococcum HS-101, the methanol extract was partitioned with hexane. The activity was transferred mainly to the methanol layers. This fraction was concentrated and the residue was subjected to chromatography on a column of silica(Merck), with elution by a step were gradient of methanol-chloroform mixture solution(each 300 mL; 0-100, 1-99, 5-95, 50-50 or 100-0%). Then, the 5% methanol-95% chloroform fraction, in which the activity was observed, was subjected to gel permeation chromatography on a column of Sephadex LH-20 (Pharmacia), with elution of methanol. The elutes were monitored for antibiotic activity as described above. Finally, the active substance was purified by thin-layer chromatography(Kieselgel $60F_{254}$, Merck), with developing solvent of 5% methanol-95% chloroform.

The chemical structure of the antibiotic substance in this alga was analyzed by ^{1}H-,^{13}C-NMR (JEOL GX-400) and GC-MS (Hewlett-Parkard 5890J·GC-JEOL SX-102·MS), and the spectra were compared with those of various standards. Further, the composition of fatty acids in three strains, which exhibited antibiotic activity among 68 strains of isolated marine algae, was also investigated. Total lipids in algal cells were extracted according to the methods of Bligh and Dyer (1959) after addition of heptadecanoic acid ($C_{17:0}$) as an internal standard. Fatty acids were purified according to our previous paper(Ohta et al. 1992). Purified samples of fatty acids from the algae were methylated with ethereal diazomethane, and was subjected to GC analysis. Fatty-

Table 1. Antibiotic activity against S. aureus (wild type) and methicillin-resistant S. aureus by methanol extracts from the marine green alga Chlorococcum HS-101

Strain	Inhibition zone (mm)	Strain	Inhibition zone (mm)
S. aureus	28.1 ± 2.0	MRSA #10	28.3 ± 1.7
MRSA #3	20.9 ± 1.2	MRSA #13	20.3 ± 2.1
MRSA #4	20.5 ± 1.4	MRSA #14	19.8 ± 1.8
MRSA #9	20.2 ± 1.8	MRSA #20	17.7 ± 1.5

Algal methanol extracts(40 μL, 0.1 g fresh weight/mL) were examined. Values are the mean ± s.d. of three or more experiments.

acid methyl esters were quantified on a Supelco WAX10 (0.32 mm x 30 m, 0.25 μm film thickness) column using a Hewlett-Packard 5890A GC equipped with a hydrogen flame-ionization detector. Fatty-acid methyl esters were identified by comparing the retention times and mass spectra with those of standards in GC-MS. Samples with a high recovery of at least 90% were used for data collection.

In an additional experiment on possible inhibitory effects of linolenic acid, MRSA #10 was grown with shaking at 37 °C in a 100-mL Erlenmeyer flask containing 10 mL of nutrient-broth medium. When the bacterial culture was in the late-log phase, 0.9 mL was pipetted into a test tube containing 0.1 mL of linolenic acid solution (final concn. 5, 50, or 500 μg/ml) in 50 % DMSO in distilled water. A 0.1 mL aliquot of bacterial culture was withdrawn from each test tube just before and at 2 hr after addition, and diluted stepwise with the fresh medium. Each dilution (0.2 mL) was sprayed on an agar plate. After incubation at 37 °C for 24 hr, surviving cells were counted on the agar plates. The 50 % DMSO solution alone was used as a control.

RESULTS AND DISCUSSION

We showed previously that the marine green alga Chlorococcum HS-101 exhibited strong activity against wild-type S. aureus (Ohta et al. 1993). In Table 1, antibiotic activity of the algal methanol extract against wild type S. aureus and seven strains of MRSA is summarized. Interestingly, each test bacterium had a different susceptibility: the inhibition zones for S. aureus and MRSA #10 were much larger than that for MRSA #20. We therefore conducted antibiotic susceptibility tests of these seven strains of MRSA with twelve kinds of authentic antibiotic standards, and their coagulase types also were examined (Table 2). Habekacin (ABK) and vancomycin(Va) completely inhibited the growth of all MRSA, whereas gentamicin(GM), ampicillin(AB) and oxytetracyclin(OX) were

Table 2. Antibiotic susceptibility testing of methicillin resistant S. aureus (MRSA) isolated from clinical specimens obtained at Kobe Institute Hospital

MRSA	Antibiotic[1]												Co[2]
	GM	AK	CE	ABK	AB	Va	OX	EM	CMZ	FF	IPM	Mi	
#3	R	I	R	S	R	S	R	S	I	S	R	I[3]	VII
#4	R	I	I	S	R	S	R	S	S	S	S	S[3]	VII
#9	R	I	R	S	R	S	R	S	R	R	R[3]	I	VII
#10	R	S	R	S	R	S	R	R	I	R	R	S	II
#13	R	I	R	S	R	S	R	S	I	I	R	I	I
#14	R	I	I	S	R	S	R	S	I	S	I	I	VII
#20	R	R	R	S	R	S	R	S	R	R	R	R	II

(1)Antibiotic: GM; gentamicin (10 μg/disk), AK; amikacin (30 μg/disk), CE; cephalosporin (30 μg/disk), ABK; habekacin (30 μg/disk), AB; ampicillin (10 μg/disk), Va; vancomycin (30 μg/disk), OX; oxytetracyclin (1 μg/disk), EM; erythromycin (15 μg/disk), CMZ; cefmetazole (30 μg/disk), FF; fosfomycin (50 μg/disk), IPM; imipenem (10 μg/disk), Mi; minomycin (30 μg/disk).

(2)Co;Coagulase type.

(3)R; resistant, I; intermediate (inhibition zone,12-18mm), S; sensitive (inhibition zone,19 mm≧).

completely ineffective. The other seven substances showed various patterns of action. Based on these results, strain #10 was selected as the standard strain for testing of anti-MRSA activity.

It is well known that S. aureus can be classified into eight types according to the kind of coagulase which this bacterium releases into the medium. However, we could find no relationship between the antibiotic susceptibility and coagulase type among MRSA; for example, strain #10 and #20 had the same coagulase type (Type II), but their susceptibility to amikacin was completely different (strain # 10 was sensitive and strain #20 was resistant). Thus, we concluded that antibiotic susceptibility of MRSA is unrelated to coagulase type.

The anti-MRSA substance in the methanol extract of Chlorococcum HS-101 was purified by bioassay-directed fractionation employing silica, Sephadex LH-20 and thin layer chromatographies. From the analysis of the ^{1}H-, ^{13}C-NMR and GC-MS spectra in comparison with those of various standard samples, we concluded that the anti-MRSA substance is linolenic acid ($C_{18:3}$, all-cis). This is extreamly interesting because linolenic acid is not an unusual fatty acid in marine phytoplankton. Therefore, we examined the fatty acid compositions and antibiotic activities of three of the marine algae, Synechococcus HS-364, Chlorella HS-569 and Chlorococum HS-101, which showed antibiotic activity among our algal isolates (Ohta et al. 1993).

Table 3 shows the activities against MRSA #10 of the algal methanol extracts, and the fatty-acid composition of these marine algae. Fatty acids of the C_{16} and C_{18} groups were major components in all strains. The linolenic acid content was 2.1% per algal dry weight and 33% per total fatty acids in Chlorococcum HS-101, and this alga had a high anti-MRSA activity. Conversely, Synechococcus HS-364 had a lower linolenic acid content and a weaker activity. The close corelation between the content of the fatty acid and the anti-MRSA activity strongly supported the conclusion that linolenic acid itself is the anti-MRSA substance. Stephen and Walker (1989) investigated the antibiotic activities of 132 marine microalgae, and found activities against S. aureus in 28 of them, but none was active against E. coli. Since this was in good accord with our previous results (Ohta et al. 1993), the activities in their strains could have been due to linolenic acid. McCracken et al (1980) also found antialgal substances in the culture medium of the green alga Chlamydomonas reinhardtii, and identified linoleic acid ($C_{18:2}$) and linolenic acid ($C_{18:3}$) as the active substances. Further, Kakisawa et al.(1988) purified and identified octadecatetraenoic acid ($C_{18:4}$) having antialgal activity as an allelopathic substance from the brown alga Cladosiphon okamuranus. Therefore, linolenic acid may play an important role in the survival strategy of various marine microrganisms in the marine environment.

Table 3. Size of inhibition zone (mm) against MRSA #10 by three algal methanol extracts and fatty acid compositions of marine algae

	Synecococcus HS-329	Chlorella HS-569	Chlorococcum HS-101
Inhibition zone (mm)	12.8 ± 2.1	16.8 ± 2.7	26.5 ± 3.2
	Fatty acid content(μg/mg dry wt.)		
$C_{14:0}$	0.7 ± 0.2	2.1 ± 0.5	4.4 ± 0.6
$C_{14:1}$	0.3 ± 0.4	0.8 ± 0.3	ND
$C_{16:0}$	21.3 ± 1.3	23.4 ± 5.3	17.5 ± 5.2
$C_{16:1}$	6.2 ± 0.4	1.2 ± 0.3	2.7 ± 0.4
$C_{16:2}$	0.2 ± 0.1	4.8 ± 0.5	2.1 ± 0.6
$C_{16:3}$	ND	0.3 ± 0.2	0.9 ± 0.3
$C_{18:0}$	2.1 ± 0.6	2.9 ± 0.5	1.3 ± 0.5
$C_{18:1}$	9.3 ± 0.7	5.1 ± 1.4	10.3 ± 1.1
$C_{18:2}$	5.1 ± 1.3	19.6 ± 2.4	4.8 ± 1.3
$C_{18:3}$*	7.8 ± 0.6	10.1 ± 1.9	21.2 ± 3.9
$C_{20:4}$	ND	ND	ND
$C_{20:5}$	ND	ND	ND
Total	53.0 ± 6.3	70.3 ± 7.8	65.2 ± 7.0

Algal methanol extracts (40 μL, 0.1 g fresh weight/mL) were used for antibiotic activity testing. $C_{18:3}$ is linolenic acid. Values are the mean ± s.d. of three or more experiments.

Table 4. Growth-inhibitory effect of various concentrations of authentic linolenic acid on MRSA #10

Linolenic acid concn.(μg/mL)	Number of MRSA cell (Cells/mL)
Before addition (0 hr)	2.1×10^{12}
2 hr after addtion	
0	5.2×10^{12}
5	2.1×10^{8}
50	7.2×10^{5}
500	6.5×10^{2}

A 0.1 mL aliquot of linolenic acid solution (final concn. 5, 50 or 500 μg/mL) in 50% DMSO was added to 0.9 mL of the medium. Values are the mean of two experiments.

As shown in Table 4, the growth-inhibitory effect of authentic linolenic acid on MRSA #10 was dose-dependent, and when 500 μg of linolenic acid was added to the medium, MRSA cells were completely killed. Since linolenic acid as an unsaturated fatty acid is considerd to have low toxicity to humans, it could be administered to infected patients as a dietary treatment such as a linolenic acid-rich meal, by analogy with the a dietary supplement of n-3 fatty acids for prevention of recurrence of stenosis after coronary angioplasty (Dehmer et al. 1988). It was also observed that γ-linolenic acid, an essential fatty acid, for human causes growth-inhibition of MRSA (data not shown). Further work is to clarify the biochemical mechanisms of anti-MRSA activity by linolenic acid.

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