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Direct Antagonistic Method for Screening Anti-methicillin-resistant *Staphylococcus aureus* (MRSA) Substance-Producing Marine Bacteria

Metode Antagonistik Langsung untuk Skrining Bakteri Laut Penghasil Senyawa Anti-methicillin-resistant *Staphylococcus aureus* (MRSA)

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Abstrak

Metode yang berbasis antagonistik langsung antara bakteri laut dan *Staphylococcus aureus* resistan metisilin (Methicillin-resistant *Staphylococcus aureus*, MRSA) berhasil digunakan untuk skrining bakteri laut penghasil senyawa anti-MRSA. Metode ini menggunakan medium agar lapis ganda untuk mendukung pertumbuhan bakteri laut dan MRSA. Beberapa bakteri laut penghasil senyawa anti-MRSA berhasil diisolasi dengan metode ini. Suatu bakteri laut, strain O-BC30 merupakan bakteri yang paling kuat menghambat pertumbuhan MRSA. Isolat-isolat bakteri yang didapat menunjukkan aktivitas yang stabil terhadap MRSA dan aktivitas ini juga konsisten dengan aktivitas ekstrak yang diperoleh dari sel bakteri yang dikultur pada medium agar. Metode ini sangat berguna dan efisien untuk skrining bukan hanya bakteri penghasil senyawa anti-MRSA tetapi juga bakteri penghasil antibiotik.

Kata kunci : Antagonistik, Senyawa Anti-MRSA, Methicillin-resistant *Staphylococcus aureus* (MRSA)

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Introduction

The National Nosocomial Infections Surveillance reports an increase trend of MRSA by 40% in 1999 comparing to 1994-1998 and occupies 52.3% of *S. aureus* nosocomial infection (NNIS, 2000). MRSA are well known gram-positive bacteria that are able to produce β-lactamase (Williams, 1999). This enzyme encoded by the gene *BlaZ*, hydrolyses β-lactam antibiotics and greatly diminishes or even eliminates their biological activity. MRSA also produces penicillin-binding protein, PBP 2a that attributes to the resistance of this bacterium to methicillin and others penicillinase-resistant penicillin. Penicillin binding proteins (PBPs) as the bacterial target of β-lactam antibiotics, are enzyme transpeptidase that maintain the structural integrity of the bacterial polysaccharides cell wall. MRSA is able to produce new transpeptidase, PBP 2a (Williams, 1999; Archer and Bosilevac, 2001; Zhang *et al*., 2001).

MRSA is one of the leading causative agents of deadly nosocomial infection. The mortality for bacteremia caused by MRSA is 2-fold higher than that caused by Methicillin-sensitive *Staphylococcus aureus* (MSSA) (Selvey *et al*., 2000). MRSA also causes longer hospital stay and 3-fold higher hospital cost (Abramson and Sexton, 1999). Vancomycin is the only commercially available antibiotic to control MRSA infection. However, recently,

the susceptibility of MRSA to vancomycin has become reduced and rather vancomycin-intermediumte (VISA) and -resistant *S. aureus* (VRSA) are found in several countries. The above evidences have made the currently available antibiotics and chemotherapeutics ineffective, necessitating to search alternative antibiotics and chemotherapeutics. Therefore, this study were conducted to develop screening method and to find potent anti-MRSA substance-producing marine bacteria as new resources of antibiotics, especially new anti-MRSA substances.

Materials and Methods

Bacterial strains

A clinical isolate (7B29) and a reference strain (ATCC 33291) of MRSA were used in this study. These strains were stocked in TSB containing 20% of glyserol at -80°C.

Seawater samples

Seawater samples were collected from Ogasawara Island (Tokyo prefecture), Iki Island and Shimabara (Nagasaki Prefecture), and Kashiwa Jima Island (Saga Prefecture), Japan in spring and summer 1998 – 1999. The samples were kept in a cool box and transported to laboratory.

Sample preparation, antagonistic assay and isolation of bacterial strains

Seawater samples were serially diluted in sterile ASW if necessary, and plated onto ZoBell agar medium [polypepton (Katayama), 5 gr/l; yeast extract (Nihon Seiyaku), 1 gr/l; glucose (Katayama), 2 gr/l; KBr, 100 mg/l dissolved in 75% ASW] and incubated at 25^{o}C for 18 – 24h until bacterial colony grown enough. The bacterial colony grew on the plate then were overlaid with MRSA-inoculated 0.8% agar of Trypticase Soy Agar (TSA) medium (Difco Laboratories, Detroit MI, USA) at final concentration 1 x 10^{6} cell/ml, and incubated at 25^{o}C for 24 h. The antagonistic activity of colonies against MRSA was observed after 24 h incubation, and active colonies were isolated on ZoBell agar medium. This activity was used to guide the isolation of anti-MRSA substance producing marine bacteria. Further the anti-MRSA activity of bacterial isolates was confirmed on double layer plate as described above. After the agar medium have solidified, marine bacteria isolates were stab-inoculated onto the double layer agar by a straight needle.

Production and isolation of anti-MRSA substance

To produce anti-MRSA substance, the marine bacterial isolates were cultured on ZoBell agar medium plate (10 × 14 cm) at 25°C for 5 days. Bacterial cells were harvested by flooding MeOH onto the agar lawn and scrapping gently by a glass spreader. MeOH volume in each bacterial cell suspension then was adjusted to be 10 ml/g of wet weight cell, homogenized with a homoginizer (Polytron PT 3000; Kinematica, Tokyo) at 10,000 rpm for 15 min. Then MeOH extracts were obtained by centrifugation at 3,000 x *g* for 20 min. This extraction was carried out twice in the same volume of MeOH (10 ml MeOH/g cell). Homogenization was conducted by the same procedure as used in the first extraction. Finally, these MeOH extracts were filtered through 0.2 μm of filter paper (Millipore, Tokyo, Japan).

To explore anti-MRSA substance from intracellular rather than cell wall-bounded substance, cell pellets obtained from the second MeOH extraction was added with 10 ml MeOH/1 g cell, and sonicated (Branson Sonifier 250; Sonic Power Co., Dunbury CT, USA) for 15 min on ice bath. The sonicated cell suspensions were subsequently centrifuged at 5,000 *g* for 15 min, and supernatants were filtered through the same filter paper as described above.

Evaluation of anti-MRSA activity of MeOH extracts

Anti-MRSA activity of MeOH extracts from each bacterial strain were evaluated by paper disk diffusion method on double layer agar of Trypticase Soy Agar (TSA) medium

(Difco Laboratories, Detroit MI, USA) as described previously (Horikawa *et al*., 1999; Isnansetyo *et al*., 2001) after 10-fold concentrated. Sterile paper disks (ø 8 mm, Advantec) was impregnated with 50 µl of each MeOH extract, and dried at 25°C. Vancomycin at 2 µg/disk was used as a positive control.

Melted 0.8% agar of TSA medium incubated in water bath at 48°C was inoculated with an overnight culture of MRSA (ATCC 33591) to give an initial bacterial density of 10^6 cells/ml, and overlaid onto 1.5% TSA medium plate. After the medium had solidified, dried MeOH extracts-impregnated paper disks were placed on the plates and incubated at 37°C for 24 h. The intensity of anti-MRSA activity was considered by measuring clear zone around the paper disks. Then the most active MeOH extracts were partitioned with $CHCl_3$ after being added with the same volume of water.

Determination of Minimum inhibitory concentrations (MICs) of the CHCl3 extracts

The minimum inhibitory concentrations (MICs) of the $CHCl_3$ extracts from the most potent anti-MRSA substance-producing marine bacteria were determined by the standard microdilution method described by the National Committee for Clinical Laboratory Standards (1997), using cation-adjusted Muller Hinton Broth (CAMHB) medium (Difco Laboratories, Detroit, MI, USA) containing 20 µg/ml Ca^{++} and 10 µg/ml Mg^{++}. The final volume of CAMHB containing the $CHCl_3$ extracts was 100 µl per well to give a starting inoculum density of MRSA at 5×10^5 cells/ml.

Results and Discussion

The screening purposes in this report are to discover potent anti-MRSA substance-producing marine bacteria as new resources of antibiotics, and to develop the efficient detecting method by direct antagonistic assay. This assay significantly improves the efficiency of screening process because the anti-MRSA strains are easily detected to isolate. The anti-MRSA activity-guided isolation also drastically reduces the time-consuming than the conventional isolation method. By this direct antagonistic assay, large amount of bacterial colonies could be screened for anti-MRSA activity.

Marine bacteria originated from a total of 39 seawater samples were evaluated for anti-MRSA activity by direct antagonistic assay. All colonies possessing potent anti-MRSA activity (Figure 1) were obtained from seawater samples collected in Ogasawara Island, Tokyo, but not from the other sampling points. The total colony tested for anti-MRSA activity was approximately 50,000 colonies.

Ten isolates of anti-MRSA substance-producing marine bacteria were successfully isolated by this new screening procedure. In the final step of the isolation, the isolates were evaluated for their anti-MRSA activities on double layer agar of ZoBell and TSA medium (Figure 2).

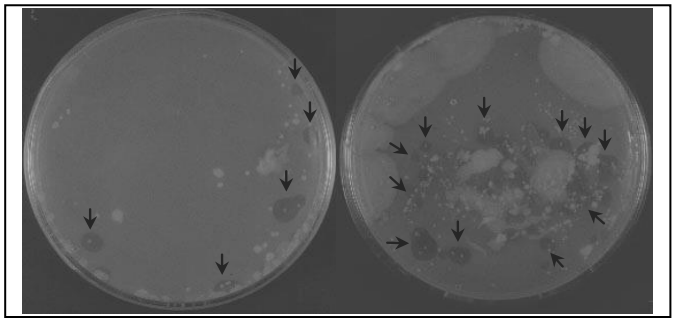

Figure 1. Direct antagonistic method for detecting anti-MRSA substance-producing marine bacteria by overlying MRSA-suspended soft agar onto marine bacterial colonies. Arrow heads indicate the anti-MRSA activity by the marine bacterial colonies, demonstrating the clear zones surrounding the colonies.

Figure 2 shows that ten of the isolates exhibited potent anti-MRSA activity indicating clear zones around the growth colonies of these marine bacteria. The antibacterial potency of each isolate might be predicted by the intensity of the inhibition zones.

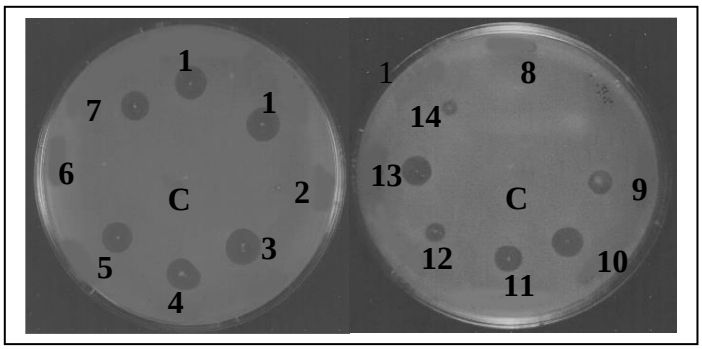

Figure 2. Anti-MRSA activities of marine bacterial isolates.
1, strain O-BC30; 2, strain O-BCX1; 3, strain O-BC40(B); 4, strain O-BCX3; 5, strain O-BC12; 6, strain O-WC12; 7, strain O-BC20; 8, strain O-GC20; 9, strain O-GC14; 10, strain O-TCX1; 11, strain O-GC13; 12, strain O-TC11; 13, strain O-GC16; C, negative control (*Vibrio parahaemolyticus* V-7).

Table 1. Anti-MRSA activity of the MeOH extract from newly isolated anti-MRSA marine bacteria

Strain	MeOH extracts from cell pellets	MeOH extracts from sonicated cells
O-BC12	-	-
O-BC20	++	-
O-BC30	++	-
O-BCX3	++	-
O-BC40(B)	++	-
O-GC13	-	-
O-GC14	-	-
O-GC16	-	-
O-TCX1	+	-
O-TC11	-	-

Note : (-) not inhibition (+) low inhibition (++) strong inhibition

To confirm the above results and to produce anti-MRSA substance, each bacterial strain was cultured on ZoBell agar medium to determine the most potent anti-MRSA substance-producing marine bacteria among the ten marine bacterial isolates. The MeOH extracts from each bacterial cell were evaluated for the anti-MRSA activity, and the result is presented in Table 1. This second screening was able to determine four most potent isolates, strains O-BC20, O-BC30, O-BCX3, and O-BC40 (B), and a bacterial isolate O-TCX1 also exhibited moderate anti-MRSA activity. However, such an activity was not detected even in 10-fold concentrated MeOH extracts of both bacterial cell pellets and sonicated cells from other five bacterial isolates. Undetectable anti-MRSA activity in these extracts might be due to low concentration of active substances at sub MIC even though these samples were 10-fold concentrated. This hypothesis was proved by extraction of these MeOH extracts with $CHCl_3$. The anti-MRSA activity of these five strains was barely detected at 500 µg/disk of the $CHCl_3$ extracts.

The third screening was conducted to determine the most potent anti-MRSA substance-producing marine bacterium among strains O-BC20, O-BC30, O-BCX3, and O-BC40 (B). In this final screening, samples were prepared by $CHCl_3$ extraction and the anti-MRSA activity was evaluated by microplate

dilution method to determine the MIC. Strain O-BC30 was the most potent anti-MRSA bacterium. MIC of its $CHCl_3$ extract against MRSA was 6.25 µg/ml, 2-fold lower than the same extracts from the other three bacterial strains O-BC20, O-BCX3, and O-BC40 (B). This result indicated that strain O-BC30 is the most potent anti-MRSA substance producing marine bacterium found in this screening.

Then strain O-BC30 was characterized and identified by morphological, biochemical, physiological and genetic methods. The results indicated that this bacterium is a new species in the genus *Pseudoalteromonas*. When anti-MRSA substance produced by this strain was purified and their chemical structures were elucidated, phenolic anti-MRSA substances were found. Therefore, *Pseudoalteromonas phenolica* sp. nov. was proposed name for this novel bacterium (Isnansetyo and Kamei, 2003a, 2003b).

Thus, although massive efforts have been continued to discover chemically new structural antibiotics, more than 90% of all bioactive compounds from terrestrial microorganism are reported to be already known compounds (Fenical, 1993). This direct antagonistic method may be used widely to discover antibiotic-producing marine bacteria as a new resources for antibiotics.

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