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Antibacterial Activity of an Ethyl Acetate Extract of *Pseudomonas* sp. UJ-6 against Methicillin-Resistant *Staphylococcus aureus*

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Abstract

In an effort to discover an alternative antibiotic for treating infections with methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas* sp. UJ-6, a marine bacterium that exhibited antibacterial activity against MRSA, was isolated. The culture broth and its ethyl acetate extract exhibited bactericidal activity against MRSA. The extract also exhibited antibacterial activity against gram-negative bacteria, which were not susceptible to vancomycin. The treatment of MRSA with the extract resulted in abnormal cell lysis. The extract retained >95% of its anti-MRSA activity after heat treatment for 15 min at 121°C. Thus, although most antibiotics are unstable under conditions of thermal stress, *Pseudomonas* sp. UJ-6 produces a heat-stable anti-MRSA substance. The results of this study strongly suggest that *Pseudomonas* sp. UJ-6 can be used to develop a novel, heat-stable, broad-spectrum antibiotic for the treatment of MRSA infections.

Key words: Antibacterial substance, Marine bacterium, Anti-MRSA activity, *Pseudomonas* sp. UJ-6

Introduction

The emergence and increasing spread of antibiotic-resistant microorganisms, including nosocomial and community-acquired infections with *Staphylococcus aureus*, has become a serious public health problem (Schaberg et al., 1991; Witte, 1999; Levy, 2005). Since it was first detected in 1961 (Jevons, 1961), methicillin-resistant *S. aureus* (MRSA) has been considered to be a serious pathogen due to its resistance to almost all commercial antibiotics, high morbidity rate, and high mortality rate. Until recently, glycopeptide antibiotics such as vancomycin and teicoplanin were used as the last resort for the treatment of MRSA infections; however, glycopeptide-resistant strains have emerged in several countries (Eom et al., 2012). Currently, several antibiotics, including linezolid, daptomycin, tigecycline, and quinupristin/dalfopristin, have been shown to possess anti-MRSA activity, but strains that are resistant to these antibiotics have been reported (Woodford, 2005). In view of these problems, the development of new anti-MRSA agents is urgently needed and many researchers have searched for alternative antibiotics against MRSA infections (Hiramatsu et al., 1997; Hanaki et al., 1998; Witte, 1999; Micek, 2007; Lee et al., 2008b; Eom et al., 2011).

Microorganisms are a source of antibacterial compounds; however, most are derived from terrestrial actinomycetes. Marine microbial metabolites provide the opportunity to produce novel antibiotics with unique chemical features, as compared to terrestrial ones, because marine microorganisms can live

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under harsh conditions such as high pressures, low food availability, total darkness, and extreme cold (Rahman et al., 2010; Abad et al., 2011). Indeed, several antibacterial compounds from marine microorganisms such as thiomarinols from *Alteromonas rava* (Shiozawa et al., 1997), bogorol A and loloatins from *Bacillus* sp. (Gerard et al., 1999; Barsby et al., 2001), agrochelin and sesbanimides from *Agrobacterium* (Acebal et al., 1998, 1999), pelagiomicins from *Pelagiobacter variabilis* (Imamura et al., 1997), δ-indomycinone and marinopyrroles from a *Streptomyces* sp. (Biabani et al., 1997; Hughes et al., 2008), MC21-A and -B from *Pseudoalteromonas phenolica* (Isnansetyo and Kamei, 2003, 2009), abyssomicin from *Verrucosispora* sp. (Keller et al., 2007), 2,4-diacetylphloroglucinol and 1-acetyl-beta-carboline from *Pseudomonas* sp. (Kamei and Isnansetyo, 2003; Lee et al., 2013), marinomycins and lynamicins from *Marinispora* sp. (Kwon et al., 2006; McArthur et al., 2008), kocurin from *Kocuria palustris* (Martín et al., 2013), dihydrophencomycin methyl ester from a streptomycete (Pusecker et al., 1997), and lipoxazolidinones from an actinomycete (Macherla et al., 2007), have been reported. Therefore, marine microorganisms have attracted great attention as potential sources of novel and effective compounds with antibacterial activity. The present study was conducted to investigate the antibacterial activity of an ethyl acetate extract against MRSA.

Materials and Methods

Microorganisms and media

Pseudomonas sp. UJ-6 (GenBank accession no. GQ988399) exhibiting antibacterial activity against MRSA was isolated from seawater and incubated at 25°C in PPES-II medium (0.2% polypeptone, 0.1% proteose peptone, 0.1% yeast extract, 0.1% soytone, and 0.001% ferric citrate, initial pH 7.6) The bacterial strains tested for antibacterial activity were purchased from the Korean Culture Center of Microorganisms (KCCM; Seoul, Korea) or the Korean Collection for Type Cultures (Daejeon, Korea); 13 clinical isolates of MRSA were provided by Donga-A University Hospital (Busan, Korea). The pathogenic bacteria were cultivated at 37°C in Mueller-Hinton broth (Difco Laboratories, Detroit, MI, USA) for minimum inhibitory concentration (MIC) testing and on Mueller-Hinton agar plates (Difco Laboratories) for disk diffusion assays.

Optimum culture conditions for *Pseudomonas* sp. UJ-6

The optimal temperature, pH, and NaCl concentration were determined for the culture of *Pseudomonas* sp. UJ-6 in PPES-II medium. To determine the optimal temperature, cells were incubated aerobically in PPES-II broth medium (pH 7, 2% NaCl) at different temperatures (4, 15, 20, 25, 30, 37, and 50°C). The pH range for growth was determined by incubating cells in PPES-II broth medium (2% NaCl, 25°C) at pH values ranging from 4-10. The salt tolerance of the cells was tested on PPES-II broth medium (pH 7, 25°C) supplemented with 0-10% NaCl (w/v).

Relationship between *Pseudomonas* sp. UJ-6 cell growth and anti-MRSA activity

Broth from a *Pseudomonas* sp. UJ-6 culture grown at 25°C in PPES-II medium was concentrated using a rotary vacuum evaporator and then mixed with Muller-Hinton broth containing MRSA strain KCCM 40510 at an estimated cell density of 10^4 CFU/mL. The cell growth of UJ-6 was monitored using the turbidity method at 640 nm. The anti-MRSA activity in the tube was evaluated based on viable cell counts of the MRSA strain after 24 h of incubation.

Crude isolation of the anti-MRSA substance from a *Pseudomonas* sp. UJ-6 culture

Isolated UJ-6 was cultured in PPES-II broth medium at 25°C with shaking at 150 rpm for 48 h, after which the cell-free supernatant was obtained by centrifugation (15,000 *g* at 4°C) and filtration (0.2-μm pore size membrane filter). The cell-free supernatant was partitioned by extraction with several organic solvents at a 1:1 (v/v) ratio according to their polarity, and the crude extracts were then concentrated using a rotary evaporator. The anti-MRSA activity of each fraction was tested, and the active fraction (*i.e*., the ethyl acetate fraction) was used as a crude antibiotic for further study.

Measurement of the MIC

The two-fold serial dilution method was used to determine the MIC of the extract as described by the National Committee for Clinical Laboratory Standards (2004). The MIC of the crude extract was defined as the lowest concentration without growth after incubation at 37°C for 24 h.

Effect of the crude extract on MRSA cell morphology

To compare the effects of the crude extract on MRSA cell morphology, MRSA cells were incubated at 37°C for 24 h in the presence or absence of the extract and then observed using a transmission electron microscope (JEM 1200EX-II; JEOL, Tokyo, Japan) at Pusan Paik Hospital (Busan, Korea).

General characteristics of the crude extract

To investigate the thermal stability of the crude extract, the extract was incubated at several temperatures (4, 25, 50, 75, and 100°C) for 1 h. It was also autoclaved at 121°C for 15

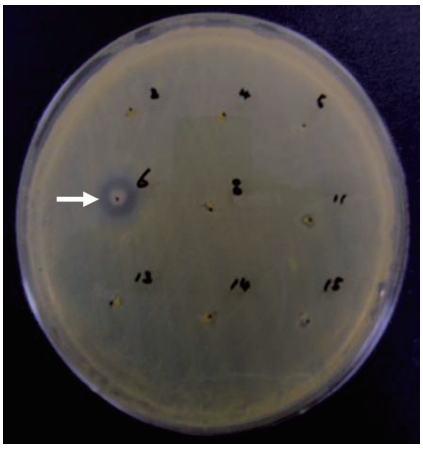

Fig. 1. Isolation of an bacterium exhibiting antibacterial activity against methicillin-resistant *Staphylococcus aureus*. Arrow indicated the isolated strain UJ-6.

min. To determine its pH stability, the crude extract was suspended in 0.1 M citrate phosphate buffer at a pH of 3 to 7 or 0.1 M Tris-HCl buffer at a pH of 8 to 10 for 30 min. After treatment, the anti-MRSA activity of the extract was estimated by the disk diffusion method.

Results and Discussion

Culture characteristics of *Pseudomonas* sp. UJ-6

The anti-MRSA activity of *Pseudomonas* sp. UJ-6 is shown in Fig. 1. To determine the optimal culture conditions for *Pseudomonas* sp. UJ-6, cells were incubated at different temperatures, pH values, and NaCl concentrations. *Pseudomonas* sp. UJ-6 was able to grow at temperatures ranging from 4 to 40°C, but not above 50°C. Also, the strain grew well between pH values of 5.0 and 9.0, but its growth was inhibited below pH 4.0 and above pH 10.0. A high concentration of NaCl (>4%) resulted in growth retardation or no growth (>8% NaCl). Thus, the most favorable growth of *Pseudomonas* sp. UJ-6 was observed in medium containing 1% NaCl, adjusted to pH 7.0, and incubated at 25°C (Fig. 2). However, there was no significant difference in anti-MRSA activity between different culture conditions (data not shown).

Anti-MRSA activity of *Pseudomonas* sp. UJ-6

The supernatant of cultured *Pseudomonas* sp. UJ-6 showed bactericidal activity against MRSA, indicating that the strain produces an anti-bacterial substance. The strongest activity was observed after the stationary phase of growth (Fig. 3). To elucidate the mechanism underlying the observed anti-MRSA activity and to purify the active compound from strain UJ-6,

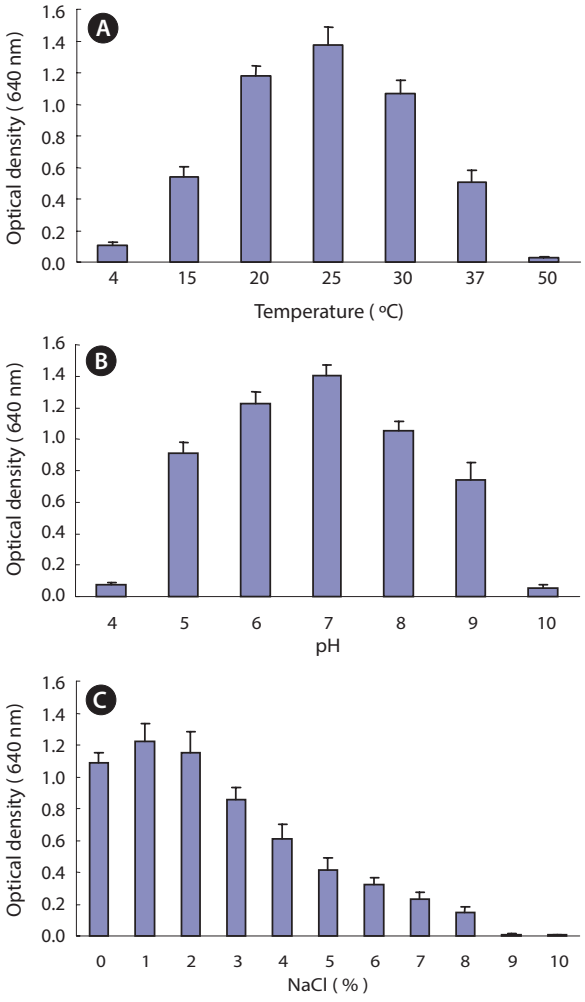

Fig. 2. Effects of temperature (A), pH (B), and NaCl concentration (C) on the growth of *Pseudomonas* sp. UJ-6 in PPES-II medium.

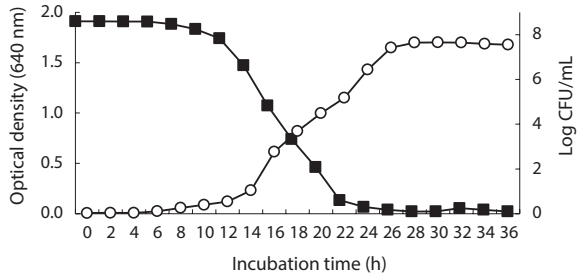

Fig. 3. Relationship between cell growth of *Pseudomonas* sp. UJ-6 and anti-MRSA activity. ○, *Pseudomonas* sp. UJ-6; ■, MRSA, methicillin-resistant *Staphylococcus aureus*.

a culture was extracted with several organic solvents, including ether, hexane, chloroform, methylene chloride, and ethyl acetate. Among these, only the ethyl acetate extract showed significant antibacterial activity against all of the tested gram-positive species, including MRSA strains, and all tested gram-

negative species. The MICs of the ethyl acetate extract against the MRSA strains and other bacteria are shown in Table 1. The ethyl acetate extract showed antibacterial activity against the tested MRSA strains with MIC values ranging from 160 to 320 μg/mL. The extract also exhibited antibacterial activity against gram-negative bacteria, although it was less effective against gram-negative bacteria and *Streptococcus iniae* than against other Gram-positive bacteria. However, vancomycin was not effective against gram-negative bacteria (Totsuka et al., 1999; Lee et al., 2008b), suggesting that the anti-MRSA effect of the substance produced by UJ-6 differs from that of vancomycin. These results are similar to those reported for other marine bacteria producing an anti-MRSA substance (Isnansetyo and Kamei, 2003).

Table 1. Antibacterial activity of the ethyl acetate extract of *Pseudomonas* sp. UJ-6 culture

Strains	MIC (μg/mL)*	
	Ethyl acetate extract	Vancomycin
MRSA KCCM 40510	320	2
MRSA KCCM 40511	320	2
MRSA DH 70503	320	2
MRSA DH 70504	320	2
MRSA DH 70505	320	1
MRSA DH 70506	160	2
MRSA DH 70508	640	2
MRSA DH 70510	320	1
MRSA DH 70512	640	2
MRSA DH 70513	640	1
MRSA DH 70514	640	2
MRSA DH 70517	320	1
MRSA DH 70518	320	2
MRSA DH 70519	640	2
MRSA DH 70520	640	2
Staphylococcus aureus KCTC 1621	320	1
Staphylococcus aureus KCTC 1927	160	0.5
Staphylococcus aureus KCTC 1928	320	1
Bacillus subtilis KCTC 1028	160	0.5
Streptococcus iniae KCTC 3657	640	4
Escherichia coli KCTC 1682	640	NA
Pseudomonas aeruginosa KCTC 1637	640	NA
Salmonella typhimurium KCTC 1925	640	NA
Vibrio parahaemolyticus KCTC 2729	1280	NA
Klebsiella pneumoniae KCTC 2242	640	NA

Pseudomonas sp. UJ-6 was cultured and extracted as described in Material and Methods.
MRSA, methicillin-resistant *Staphylococcus aureus;* NA, not active.
*Minimum inhibitory concentration (MIC) of crude extract and vancomycin was determined by the two-fold serial dilution method in Mueller-Hinton broth.

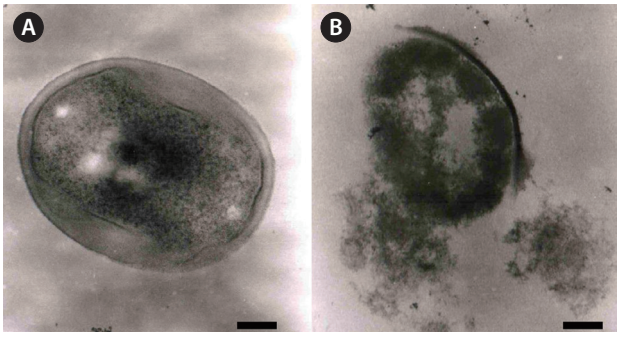

Fig. 4. Abnormal cell morphology of methicillin-resistant *Staphylococcus aureus* (MRSA) caused by the ethyl acetate extract of *Pseudomonas* sp. UJ-6 culture. A MRSA strain (KCCM 40510, 10^5 CFU/mL) was inoculated in a Mueller-Hinton broth in the absence or presence of the ethyl acetate extract (320 μg/mL). The culture was incubated at 37°C for 24 h and the cell morphology was observed with a transmission electron microscopy. (A) Normal cell of the MRSA (B) abnormal cell lysis of the MRSA grown with the ethyl acetate extract. Scale bars: A, B = 100 nm.

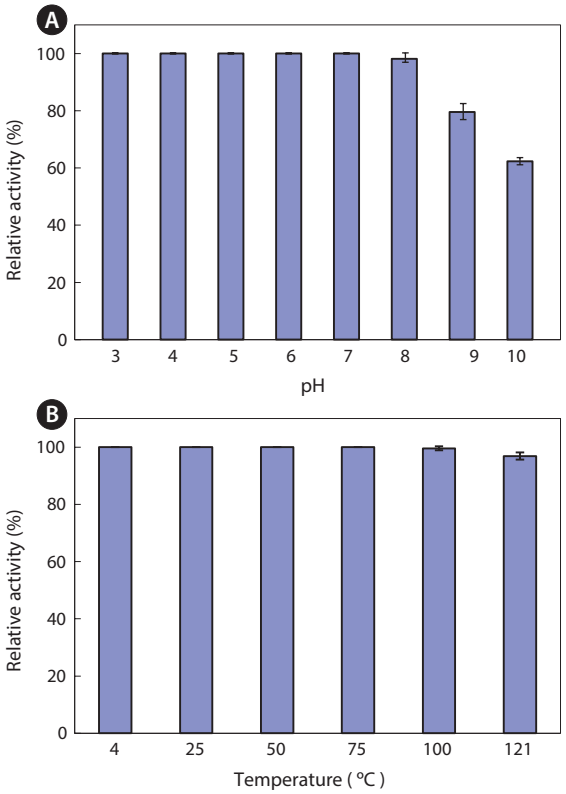

Fig. 5. The pH (A) and thermal stability (B) of the ethyl acetate extract of *Pseudomonas* sp. UJ-6 culture. For pH stability, the extract was suspended in 0.1 M citrate phosphate buffer for the range of pH 3 to 7 and 0.1 M Tris-HCl buffer for pH 8 to 10, and then kept in each buffer for 30 min. For thermal stability, the extract was incubated at an indicated temperature (4, 25, 50, 75, and 100°C) for 1 h or at 121°C for 15 min. After treatment, the anti-methicillin-resistant *Staphylococcus aureus* activity was estimated by the disk diffusion method. All assays were done in triplicate.

Effect of the ethyl acetate extract on MRSA cell morphology

We also investigated the morphology of MRSA cells exposed to the ethyl acetate extract using transmission electron microscopy. As shown in Fig. 4, MRSA cell lysis was observed following growth at 37°C for 24 h with the ethyl acetate extract (320 μg/mL). Several antibiotics, including penicillin and vancomycin, interfere with cell wall synthesis, leading to cell lysis (Barna and Williams, 1984). Based on our results, we propose that *Pseudomonas* sp. UJ-6 produces a substance that interferes with MRSA cell wall synthesis. However, we strongly believe that the anti-MRSA mechanism of *Pseudomonas* sp. UJ-6 differs from that of vancomycin since vancomycin was not effective against gram-negative bacteria (Lee et al., 2008a).

Thermal and pH stability of the ethyl acetate extract

The thermal stability and pH stability of the ethyl acetate extract were also investigated. The extract maintained >95% activity at pH 3.0-8.0, but it exhibited about 80% and 60% activity at pH 9.0 and 10.0, respectively, when the activity at pH 7.0 was defined as 100% (Fig. 5A). As shown in Fig. 5, the extract was highly resistant to thermal stress. The extract retained >95% of its activity after heat treatment for 15 min at 121°C (Fig. 5B). This result suggests that *Pseudomonas* sp. UJ-6 produces a heat-stable antibiotic, even though most known antibiotics are heat-labile. To further address this issue, the structure of the anti-MRSA compound from the crude extract should be determined. We have isolated several bioactive metabolites from *Pseudomonas* sp. UJ-6 and reported the anti-MRSA activity of 1-acetyl-beta-carboline, a compound isolated from *Pseudomonas* sp. UJ-6 (Lee et al., 2013). Currently, we are working to determine the structure of the remaining isolates.

From these results, we anticipate that *Pseudomonas* sp. UJ-6 can be used to develop a novel, heat-stable, broad-spectrum antibiotic for the treatment of MRSA infections.

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