Anti-MRSA Properties of Prodigiosin from *Serratia* sp. PDGS 120915

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Prodigiosin, a member of natural red pigment family, is produced by *Serratia marcescens*, and characterized by a common pyrrolypyrromethane skeleton. This pigment has been reported with the effects of anticancer, immunosuppressant, antifungal, and algicidal activities. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of hospital infections. In this study, anti-MRSA properties of prodigiosin isolated from *Serratia* sp. PDGS 120915 were investigated. We identified and purified prodigiosin using high performance liquid chromatography (HPLC) and evaluated anti-MRSA activity. Purified prodigiosin inhibited the growth of MRSA. The minimum inhibitory concentrations (MICs) of prodigiosin were determined to 32 μg/ml against the MRSA strains. Fractional inhibitory concentration (FIC) indices of ampicillin and penicillin were indicated synergistic effects of prodigiosin on MRSA.

Key words: Anti-MRSA, prodigiosin, red pigment, *Serratia*

Introduction

When antibiotics began making their way into clinical use, they were hailed as miracle drugs; however, miracles did not last forever [20]. Various microorganisms have survived for many years through adapting to antimicrobial agents [13]. Since penicillin was first introduced in 1943, 40% of hospital isolated *S. aureus* showed penicillin resistance in 1950, and increased by 80% in 1960 [4, 6, 12]. The penicillin-resistant strain was overcome with penicillinase-stable penicillins, including methicillin, oxacillin, cloxacillin and flucloxacillin, but only two year later, methicillin-resistant *Staphylococcus aureus* (MRSA) was reported in England [10]. MRSA became the most problematic gram-positive bacterium in public health because of its wide-resistance of antibiotics except few antibiotics [9, 22]. The genus *Serratia*, a member of the Enterobacteriaceae, is comprised of a group of bacteria that are related both phenotypically and by DNA sequence. Some species and biotypes of *Serratia* produce a nondiffusible red pigment, prodigiosin, or 2-methyl-3-amyl-6methoxyprogidiosene [21]. MRSA is a major pathogen that is associated with nosocomial infection. Vancomycin and teicoplanin have been used for the treatment of MRSA-mediated infections; however, vancomycin-intermediate and resistant *S. aureus* (VISA and VRSA) have been reported. Thus, development of new drugs or alternative therapies is required for regulation of antibiotics-resistance pathogens.

The purpose of this study is to identify and isolate bacteria which produce anti-MRSA substances, and to investigate the efficacy of novel compounds on the negative regulation of MRSA.

Materials and Methods

Bacterial strains and medium

Clinical MRSA isolates were generously provided by the Dong-A University Hospital (Busan, Korea). All strains were aerobically cultivated at 37°C in Mueller-Hinton broth (MHB; Difco, USA).

Disk diffusion assay

The antibacterial activity was evaluated by disc diffusion assay described by the Clinical and Laboratory Standards Institute [5]. In briefly, bacterial strains were cultured in PPES-II and LB at 25°C until the cell concentrations reached to about 0.5 of optical density at 600 nm. 1 ml of bacterial culture containing approximately 10⁸ CFU/ml was spread on MHA (Mueller-Hinton Agar; Difco, USA) plate and a paper disc (6 mm in diameter) containing 1 mg of each extract was then placed on the plate. After incubating 24 hr at 25°C, the diameter of inhibition zone was measured. The experiment was done three times and the means values were...
presented.

**Measurement of minimum inhibitory concentration (MIC)**

MIC means the lowest concentration of antimicrobials that needs to inhibit the growth of microorganisms after overnight incubation [8]. MICs were performed by a two-fold serial dilution method in MHB, as described by the National Committee for Clinical Laboratory Standards [15]. The MIC assays were conducted in triplicates.

**Isolation and identification of anti-MRSA substance-producing microorganisms**

In order to isolate a bacterium producing anti-MRSA substance, sea and stream water were collected. The samples were smeared on a PPES-II agar medium (0.2% polypeptone, 0.1% proteose peptone, 0.1% yeast extract, 0.1% soytone, 0.001% ferric citrate and 2.5% NaCl) [18] and Luria-Bertani agar medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) [14], respectively and incubated at 25°C for 5 days. The anti-MRSA activity was estimated by a growth inhibition assay. MRSA were spread on MHA plate and a paper disc containing a cell-free culture broth of each isolated bacterium was then placed on the plate. After 2 days at 25°C, a strain, which made a clear zone indicating the MRSA growth inhibition, was selected. The isolated strain was named “PDGS 120915” and stored at -70°C.

The identification of the isolated strain was carried out by conventional biochemical test and 16S rRNA gene sequence analysis. Biochemical test was carried out using the API 20NE kit (Bio-Merieux, France). DNA was extracted from the organism and the 16S rRNA gene was amplified by PCR using the universal primers 8F (AGAGTRTTGATCCTGGCTCAG) and 1492R (CGGTACCTTGTTACGACTT). The PCR product was sequenced using capillary DNA Sequencer. The analysis of nucleotide sequence was performed using the BLASTN at the National Center for Biotechnology Information.

**Optimum culture conditions of isolated strain**

Temperature, pH and NaCl concentration were examined to determine the optimum culture condition in LB medium. For the temperature, cells were aerobically incubated in LB broth medium at the following temperatures; 4, 15, 20, 25, 28, 30, 37 and 42°C. The pH range for growth was determined by incubating cells in LB broth at the range of pH 4-14. NaCl tolerance was tested on LB broth medium supplemented with NaCl 0-10% (w/v).

**Pigment extraction and characterization**

50 ml of isolated PDGS 120915 was pre-cultured for 24 hr at 25°C, and then re-inoculated in 200 ml LB broth. 200 ml of bacterial culture was centrifuged at 10,000× g for 20 min at 4°C. The supernatant was discarded and equal volume of ethyl acetate : acetone (1 : 1) or ethanol : HCl (9.5 : 0.5) was used to re-suspend the pellet. The mixture was shaken for 2 hr vigorously, centrifuged at 10,000× g for 20 min at 4°C. Thus extracted pigment then dried and used for further analysis. Absorption spectra were measured using a spectrophotometer at room temperature in a wavelength range from 240 to 600 nm. *Serratia marcescens* recognized as prodigiosin biosynthesis strain was used for comparative analysis. A high performance liquid chromatography (HPLC) analysis was performed using XTerra MS C18 reverse-phase column (125Å, 2.5 µm, 2.1 mm × 20 mm, Waters) with Bio-Rad HPLC system (Bio-Rad, USA). Also, peaks of two samples were compared by the concentration gradient method used 0.3 ml/min flow rate, 100% methanol and distilled water.

**Synergistic effect between extract and β-lactams against MRSA**

The interaction between extract and β-lactam, including ampicillin, penicillin and oxacillin against MRSA were assessed by the checkerboard method [16]. The synergy between extract and the antibiotics was evaluated as a fractional inhibitory concentration (FIC) index. The FIC was calculated as the MIC of an antibiotic or extract in combination, divided by the MIC of the antibiotic or extract alone, as follows. The FIC was then summed to derive the FIC index, which indicated synergy when the index values were: = 0.5, synergistic; > 0.5 to = 1, additive; > 1 to = 2, independent and > 2, antagonistic.

\[
\text{FIC}_A = \frac{\text{MIC}_A}{\text{MIC}_A} \\
\text{FIC}_B = \frac{\text{MIC}_B}{\text{MIC}_B} \\
\text{FIC Index} = \text{FIC}_A + \text{FIC}_B
\]

**Results and Discussion**

**Isolation and identification of an anti-MRSA substance producing microorganism**

A bacterial strain PDGS 120915, which exhibits an an-
Table 1. Biochemical characteristics of *Serratia* sp. PDGS 120915 and other *Serratia* species

| Characteristic       | 1 | 2 | 3 |
|----------------------|---|---|---|
| Spore                | - | - | + |
| Motility             | + | + | + |
| Anaerobic growth     | + | + | + |
| Utilization of       |   |   |   |
| Arabinose            | - | + | - |
| Cellobose            | - | - | - |
| Citrate              | + | + | + |
| Fructose             | + | + | + |
| Galactose            | + | + | + |
| Glucose              | + | + | + |
| Lactose              | - | - | - |
| Maltose              | + | + | + |
| Mannitol             | + | + | + |
| Mannose              | + | + | + |
| Melibiose            | - | + | - |
| Raffinose            | + | + | + |
| Sorbitol             | + | + | + |
| Sucrose              | + | + | + |
| Trehalose            | + | + | + |
| Xylose               | - | - | - |
| Hydrolysis of        |   |   |   |
| Gelatin              | + | + | + |
| Urea                 | - | - | + |
| Casein               | + | + | + |
| Starch               | - | - | - |
| β-hemolysis          | + | + | + |
| Production of        |   |   |   |
| Acetoin              | - | + | + |
| H2S                  | - | - | - |
| Indole               | + | + | + |
| Mixed acid           | - | - | - |
| Gas                  | - | - | - |

Strains: 1, *Serratia* sp. PDGS 120915; 2, *Serratia marcescens*; 3, *Serratia marcescens* subsp. *sakuensis*.

Table 2. Enzymatic activity features of *Serratia* sp. PDGS 120915 and other *Serratia* species

| Characteristic              | 1 | 2 | 3 |
|----------------------------|---|---|---|
| Arginine dihydrolase       | - | - | - |
| Lysine decarboxylase        | + | + | + |
| Ornithine decarboxylase     | + | + | + |
| Cytochrome oxidase          | - | + | + |
| Catalase                    | + | + | + |
| Alkaline phosphatase        | - | - | - |
| Esterase (C4)               | + | + | + |
| Esterase Lipase (C8)        | + | + | + |
| Lipase (C14)                | - | - | - |
| Leucine arylamidase         | - | - | - |
| Valine arylamidase          | - | - | - |
| Cystine arylamidase         | - | - | - |
| Trypsin                     | - | - | - |
| α-chymotrypsin              | - | - | - |
| Acid phosphatase            | + | + | + |
| Naphthol-AS-βI-phosphohydrolase | + | + | + |
| α-galactosidase             | - | - | - |
| β-galactosidase             | - | - | - |
| β-glucuronidase             | - | - | - |
| α-glucosidase               | - | - | - |
| β-glucosidase               | - | - | - |
| N-acetyl-β-glucosaminidase  | + | + | + |
| α-mannosidase               | - | - | - |
| α-fucosidase                | - | - | - |

Strains: 1, *Serratia* sp. PDGS 120915; 2, *Serratia marcescens*; 3, *Serratia marcescens* subsp. *sakuensis*.

1 Data are from Grimont and Grimont (1978).

1 Data are from Ajithkumar Bindu et al. (2003).

The isolated strain PDGS 120915, 16S rRNA gene sequencing was carried out as described above. The 16S rRNA sequences of strain PDGS 120915 was aligned by comparison with available sequences from GENBANK. According to the 16S rRNA analysis, strain PDGS 120915 was 98% similar to *Serratia marcescens* subsp. *sakuensis KRED*<sup>7</sup>. The 16S rRNA sequence was submitted in GENBANK and the accession number is KC007128 (Fig. 1).

Based on these results, the isolated PDGS 120915 was identified to be *Serratia* sp.

**Optimum culture conditions of *Serratia* sp. PDGS 120915**

To verify the optimum culture condition of *Serratia* sp. PDGS 120915, effects of culture conditions on growth rate and amount of pigment production were examined. Related to growth rate and amount of pigment production, bacterial growth was observed after 8 hr, but pigment biosynthesis was not continued after 12 hr (Fig. 2A). Our results were consistent with a previous study demonstrating the biosyn-
thesis of prodigiosin through quorum sensing [19]. However, results from bacterial growth and pigment biosynthesis were different with other studies. Isolated Serratia sp. PDGS 120915 grew in broad range at pH 5-12. Optimal growth of Serratia sp. PDGS 120915 was observed at pH 5, but optimal prodigiosin production of the bacteria was observed at pH 7. (Fig. 2B). These results consider that suppressed or non-expressed synthesis of prodigiosin are inhibition of enzyme and membrane transport protein activities. Growth rate and pigment production were measured in the range of 4~42℃. In case of Serratia sp. PDGS 120915, optimal temperature for growth and pigment production were 25℃ (Fig. 2C). However, other bacteria such as Zooshikella ganghwensis and Hahella chejuensis were 30℃, and Serratia marcescens grew at 28℃ [11]. These results might be caused by the habitat difference such as marine, soil and stream water. However, a complete block in prodigiosin production was observed at 37℃, showing that the similar result from the previous study [17]. To investigate effects of NaCl concentration on bacterial growth and secondary metabolites biosynthesis, the growth rate and amount of pigment production were measured at the range of 0-10% NaCl concentration. Bacterial growth indicated 1.0 or more OD value at the range of 0-6% NaCl concentration. Optimal growth condition was observed at 1%. In contrast with growth condition, maximum pigment was produced at 0%, and the production was reduced sharply as the concentration increased (Fig. 2D). Collectively, it is considered that Na⁺ was not necessary for stability of cell membrane and enzyme activity at the growth of Serratia sp. PDGS 120915. Furthermore, this is considered that the Serratia sp. PDGS 120915 was adapted in stream water after originated from soil.

Characteristics of pigment

The absorption spectrum of the pigment extract from Serratia sp. PDGS 120915 and Serratia marcescens was given in Fig. 3. According to the recent study, prodigiosin production was observed at a wavelength at 535-540nm [1]. Maximum absorbance of pigment from Serratia sp. PDGS 120915 and Serratia marcescens was observed at 534 nm and 536 nm, respectively. From the results HPLC, each extracted pigments were showed peak at 39.5min and 39.8min (Fig. 4).
Anti-MRSA activity of Serratia sp. PDGS 120915

*Serratia* sp. PDGS 120915 exhibited the anti-MRSA activity as shown in Fig. 5. Previous studies with *S. marcescens* demonstrated that secondary metabolite production was inhibited at higher temperatures [2, 3]. When *Serratia* sp. PDGS 120915 spotted on the lawns of MRSA isolates and incubated at 30℃, the inhibitory zone was produced. In order to elucidate the anti-MRSA activity according to bacterial growth phase, the strongest anti-MRSA activity was observed after stationary phase of growth. These results indicated that the anti-MRSA compound was produced by quorum sensing, and was highly produced after stationary phase.

Synergic effects between prodigiosin and β-lactams against MRSA

The β-lactams group of antibiotics includes enormous variety of natural and semi-synthetic compounds, which inhibit several enzymes associated with the final step of peptidoxyglycan synthesis [7]. As shown in Table 3, the MICs of ampicillin against two standard MRSA strains (KCCM 40510 and 40511) were significantly reduced from 512 to 0.5 μg/ml when the MRSA strains were incubated with 32 μg/ml of prodigiosin. The synergistic effect against MRSA was veri-
Fig. 4. HPLC profile of prodigiosin from *Serratia* sp. PDGS 120915 (A) and *Serratia marcescens* (B).

Fig. 5. Anti-MRSA activity of prodigiosin from *Serratia* sp. PDGS 120915. (A) MRSA (KCCM 40510), (B) MRSA (KCCM40511).

Table 3. Minimum inhibitory concentration (MIC) of prodigiosin and β-lactams against methicillin-resistant *S. aureus* (MRSA)

| Strain           | Source        | MIC (μg/ml) | Prodigiosin | Ampicillin | Penicillin | Oxacillin |
|------------------|---------------|-------------|-------------|------------|------------|-----------|
| MRSA (KCCM40510)| Standard strain| 128         | 512         | 512        | 128        |
| MRSA (KCCM40511)| Standard strain| 128         | 512         | 256        | 128        |

Table 4. MICs and FIC indices of prodigiosin in combination with β-lactams against MRSA

| Strain      | Ampicillin | Penicillin | Oxacillin |
|-------------|------------|------------|-----------|
|             | MIC (μg/ml) | FIC       | MIC (μg/ml) | FIC       | MIC (μg/ml) | FIC       |
|             | A          | B          | b          | A          | B          | b          | A          | B          | b          | A          | B          | b          |
| MRSA40510   | 512        | 16         | 0.251      | 512        | 16         | 0.258      | 128        | 128        | 1.250      |
| MRSA40511   | 512        | 16         | 0.251      | 256        | 32         | 0.258      | 128        | 128        | 1.250      |

A, without prodigiosin; B and b, prodigiosin at 32μg/ml

The FIC was calculated as the MIC of prodigiosin or each antibiotic in combination divided by MIC of prodigiosin of each antibiotic alone. The FIC index was obtained by the sum of FICs. The FIC index indicated synergy: 0.5, synergic; >0.5 to 1, additive; >1 to 2, independent; >2, antagonistic.

fied in combination with prodigiosin and β-lactams group. The synergy was evaluated in terms of a FIC index, as described in Materials and Methods. FIC indices of ampicillin, penicillin and oxacillin were 0.251, 0.258 and 1.250, respectively (Table 4). These results indicated the synergistic effects of prodigiosin-ampicillin and prodigiosin-penicillin combinations on MRSA growth suppression. However, prodigiosin-oxacillin combination did not exhibit the synergistic effect on MRSA growth. It is hypothesized that prodigiosin may synergize the activity of β-lactams, which inhibit cell
wall synthesis. Differences in the synergistic effects of anti-MRSA substance and β-lactams group combinations were also reported in epigallocatechin gallate (EGCG) and dieckol. EGCG showed synergy effects with penicillin and oxacillin. Similarly, Dieckol exhibited synergistic effects on MRSA growth in combination with penicillin and ampicillin.

In this study, it is expected that Serratia sp. PDGS 120915-derived prodigiosin can be used as potential antibiotics, and contributes to the development of alternative antibiotic agents against MRSA.

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References

1. Allen, E. G. 1967. Conditions of the colour change of prodigiosin. Nature 216, 929-931.
2. Bar-Ness, R. et al. 1988. Increased cell surface hydrophobicity of a Serratia marcescens NS 38 mutant lacking wetting activity. J. Bacteriol. 170, 4361-4364.
3. Blizzard, J. L. and Peterson, G. E. 1963. Selective inhibition of proline-induced pigmentation in washed cells of Serratia marcescens. J. Bacteriol. 85, 1136-1140.
4. Chambers, H. F. 2001. The changing epidemiology of Staphylococcus aureus? Emerg. Infect. Dis. 7, 178.
5. CLSI, 2009. Clinical and Laboratory Standards Institute (CLSI) Performance Standards for Antimicrobial Disc Diffusion Susceptibility Tests 19th ed. Approved standard. Linical and Laboratory Standards Institute, Wayne, PA.
6. Finland, M. 1979. Emergence of antibiotic resistance in hospitals. Rev. Infect. Dis. 1, 4-21.
7. Foster, T. J. 2004. The Staphylococcus aureus "superbug". J. Clin. Invest. 114, 1693-1696.
8. Grierson, D. S. and Afolayan, A. J. 1999. Antibacterial activity of some indigenous plants used for the treatment of wounds in the Eastern Cape, South Africa. J. Ethnopharmacol. 66, 103-106.
9. Isanasetyo, A., Cui, L., Hiramatsu, K. and Kamei, Y. 2003. Antibacterial activity of 2, 4-diacetylphloroglucinol produced by Pseudomonas sp. AMSN isolated from a marine alga, against vancomycin-resistant Staphylococcus aureus. Int J. Antimicrob. Agents 22, 545-547.
10. Jevons, M. P and Parker, M. T. 1964. The evolution of new hospital strains of Staphylococcus aureus. J. Clin. Pathol. 17, 243-250.
11. Kim, J. S., Kim, M. C., Lee, K. J. and Heo, M. S. 2009. Isolation and optimal culture conditions of prodigiosin-like pigment produced by Zooshikella sp. JE34. Kor. J. Microbiol. Biotechnol. 37, 219-225.
12. Lee, D. S. 2009. Isolation and Characterization of Anti-MRSA (Methicillin-Resistant Staphylococcus aureus) Substances from Marine Organisms. Microbiology. Pukyong National University, Busan, Korea.
13. Levy, S. B. 1998. The challenge of antibiotic resistance. Sc. Am. 278, 32-39.
14. Miller, H. 1987. Practical aspects of preparing phage and plasmid DNA: growth, maintenance, and storage of bacteria and bacteriophage. Methods Enzymol. 152, 145-170.
15. National Committee for Clinical Laboratory Standards. 2004. Method for dilution antimicrobial susceptibility testing for bacteria that grow aerobically. Apporved standard M7-A6, 7th ed. National Committee for Clinical Laboratory Standards, Wayne, PA, USA.
16. Norden, Carl, W., Heidi, W. and Eva, K. 1979. Comparison of techniques of measurement of in vitro antibiotic synergism. J. Infect. Dis. 140, 629-633.
17. Pryce, L. H. and Terry, F. W. 2000. Spectrophotometric assay of gene expression: Serratia marcescens pigmentation. Bioscience 26, 3-13.
18. Taga, N. 1968. Some ecological aspects of marine bacteria in the Kuroshio. Cur. Bull. Misak. Mar. Biol. Kyoto Univ. 12, 65-76.
19. Thomson, N. R., Crow, M. A., McGowan, S. J., Cox, A. and Salmond, G. P. 2000. Biosynthesis of carbapenem antibiotic and prodigiosin pigment in Serratia is under quorum sensing control. Mol. Microbiol. 36, 539-556.
20. Travis, J. 1994. Reviving the antibiotic miracle? Science 264, 360-362.
21. Williams, R. P and Qadri, S. M. H. 1980. The pigment of Serratia. pp. 31-79. In: Con Graevenitz A, Rubin SJ. (Eds) The genus of Serratia. Boca Raton, CRC Press.
22. Witte, W. 1999. Antibiotic resistance in gram-positive bacteria: epidemiological aspects. J. Antimicrob. Chemother. 44, 1-9.
초록: *Serratia* sp. PDGS 120915가 생산하는 prodigiosin의 항 MRSA 특성에 관한 연구

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천연의 붉은 색소인 prodigiosin은 *Serratia marcescens*에 의해서 생산되며 이는 pyrrolylpyrromethane 골격으로 구성되어 있다. 이 색소는 그 분자가 가진 특성 때문에 넓은 범위에서 활용되고 있다. 또한 항암제, 면역억제제, 항진균제, 살조제 등 다양한 분야의 효과가 보고되고 있다. Methicillin-resistant *Staphylococcus aureus* (MRSA)는 세계적으로 가장 주요한 원인 감염균으로서, 미국에서 해마다 HIV로 사망하는 수보다 더 많은 사망률을 보이고 있다. 그러므로 MRSA에 대한 새로운 치료제의 개발이 매우 중요하며 급박한 문제이다. 본 연구에서는 중증오염 도를 가진 하천수로부터 *Serratia* sp. PDGS 120915를 품리하였으며 항 MRSA 활성을 가진 prodigiosin에 대하여 연구하였다. 본 연구를 위하여 HPLC를 이용하여 물질을 정제하였으며, 분리된 물질의 항 MRSA 활성을 확인하였 다. Prodigiosin의 MRSA에 대한 최소억제농도(Minimal inhibitory concentration; MIC)는 32 μg/ml 이었으며, 분 할저해농도(Fractional inhibitory concentration; FIC)는 ampicillin과 penicillin에서 상승작용이 있는 것으로 나타 났다.