A predicted S-type pyocin shows a bactericidal activity against clinical *Pseudomonas aeruginosa* isolates through membrane damage

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**Abstract**

The nucleic acid sequence at the positions 1067817–1066321 of *Pseudomonas aeruginosa* PAO1 genome was predicted to encode a novel S-type pyocin, designated S5, based on the genome sequence. However, its antimicrobial spectrum, activity and mechanism have not been investigated. Herein, we report that pyocin S5 has an antimicrobial activity against seven clinical *P. aeruginosa* isolates (DWW3, InA, InB, In3, In4, In7, and In8). Among them, DWW3 is most sensitive with a minimum inhibitory concentration of 12.6 µg/ml and a killing percentage of 95.7% at 225 µg/ml. Further, we demonstrated that the antimicrobial mechanism of pyocin S5 is membrane damage, evidenced by the leakage of intracellular materials, the increase of membrane permeability, and cell surface disruption.

**1. Introduction**

Bacteriocins are antimicrobial peptides or proteins produced by strains of diverse bacterial species [1]. *Pseudomonas aeruginosa* is a widely-spread environmental bacterium and an opportunistic pathogen causing acute and chronic infection in immune-compromised individuals, such as cystic fibrosis (CF) patients [2]. More than 90% of *P. aeruginosa* strains produce bacteriocins, called pyocins, which are classified into three types: R-, F- and S-type. Ecologically, pyocin production may ensure the predominance of a producing strain in a bacterial niche upon exposure to mutagenic agents that provoke DNA damage, such as mitomycin C, UV, and H2O2 [3–5]. The killing activity of pyocins is initiated by the binding of pyocins to the specific outer membrane receptors of the susceptible cells. Most pyocinogenic strains are insensitive to their own pyocins mainly because of immunity proteins, which helps preserve the initial predominance of pyocinogenic bacteria against pyocin-sensitive cells [6].

The R-type (rod-like) pyocins, related to P2 phage and required for competitive growth advantage [7], bind their tail fibers to targeted bacterial surface receptors and then kill by forming pores in the target cell membrane [8]. The F-type (flexible and non-contractible) pyocins are related to P2 phage with similar structural and serological properties but different receptor specificities [7]. The S-type (soluble) pyocins, including S1, S2, S3, and AP41, possess the structures and the modes of action similar to those of colicins, bacteriocins produced by *Escherichia coli* [4,9]. The S-type pyocins have an effector and an immunity component, with the effector component exerting a killing activity including DNase activity [10–12]. In general, an effector component of the S-type pyocins, which has a range of 498–776 amino acids, consists of a receptor binding domain for receptor binding, a translocation domain for translocation across the membrane, and a killing domain for a killing activity. The immunity component, which has 87–153 amino acids, renders pyocinogenic strains immune to their produced pyocins. Recently, a novel pyocin from *P. aeruginosa* JJ692 and 6077 exoU-containing clinical isolates was shown to exhibit colicin M (ColM)-like hydrolase activity towards peptidoglycan precursors [9].

The antimicrobial activity and mechanisms of pyocins S1, S2, S3, and AF41 have been reported. The availability of *P. aeruginosa* genome sequences enabled the prediction of additional pyocin genes, including the nucleic acid sequence at positions 1067817–1066321 (PA0985) in *P. aeruginosa* PAO1 genome. This predicted pyocin, designated S5, contains translocation-receptor binding-killing domains (Fig. 1A), and the killing domain shares 46% homology with that of colicin Ia, a pore-forming bacteriocin [13]. In this study, for the first time, we experimentally demonstrated the antimicrobial spectrum, activity and mechanism of pyocin S5.
2. Materials and methods

2.1. Bacterial strains and growth conditions

*E. coli* BL21 (DE3) and Top10 were grown in Luria–Bertani (LB) broth at 37°C with shaking at 250 rpm. Fifteen *P. aeruginosa* strains (In1–8 [14]; InA–E [14]; DWW1, DWW3 [5] (clinical isolates)) were kindly provided by Dr. R.D. Waite at Queen Mary University of London. *P. aeruginosa* strains were grown in Mueller–Hinton broth (MHB) or Tryptone soya broth (TSB) medium at 37°C. Solid media were prepared by adding 1.5% (wt/vol) agar to the broth. Tryptone soya agar (TSA) plates and soft agar (1.0% peptone and 0.5% agar) were used for the overlay assay [5].

2.2. Secondary structure of pyocin S5

The Hierarchical Neural Network (HNN) method [15] and the SOSUI method [16] reportedly provide a high accuracy of protein structure and trans-membrane protein prediction. The HNN and SOSUI methods were employed to predict the secondary structure and transmembrane region of pyocin S5, respectively. The secondary structure of colicin Ia (GenBank Acc. No. ZP_02783400.1) was analyzed by the HNN method for comparison with pyocin S5.

2.3. Overexpression and purification of pyocin S5 in *E. coli* BL21 (DE3)

Pyocin S5 gene (*pyoS5*, PA0985, 1497 bp, encoding 498 aa) was synthesized (GeneArt, Germany) and introduced into pET30b (+) (Merck, Germany) by EcoRI/HindIII double digestion, ligation, and transformation into *E. coli* BL21 (DE3). Overexpression of pyocin S5 was induced for 3 h by adding 1.0 mM IPTG into the cell culture (OD600 = 0.5–1.0) from a single colony and then, the recombinant plasmid (pET30b-pyoS5) was harbored. The total protein was analyzed by SDS–PAGE.

To purify pyocin S5, after induction, the cells were resuspended in 1× PBS containing 10 mM imidazole (pH 7.4), and lysed by digital Sonifier® ultrasonic cell disrupter S-450 (Branson, USA). The cell debris was separated by centrifugation and filtration. The cell-free lysate was used for purification by His Trap™ HP columns (GE Healthcare, USA) through Amersham Akta fast protein liquid chromatography (FPLC) (GE Healthcare). Five-milliliters of HiTrap™ column (GE Healthcare) was employed to desalt. Finally, the protein was eluted into the protein sample buffer (10 mM PO₄⁻, 20% glycerol, pH 6.0). The purity and concentration were measured by SDS–PAGE and Bradford Assay Kit (Bio-Rad, CA, USA), respectively.

2.4. Antimicrobial spectrum and bactericidal activity

The antimicrobial spectrum of pyocin S5 was determined based on the overlay assay as previously described [5,17]. Briefly, the overlay assay employs bacteria-seeded soft agar as a top layer covering a bottom layer of agar, with antimicrobial compounds spotted onto the bottom layer, followed by a subsequent determination of inhibition zones. In our study, TSA plates were covered by 2.5 ml soft agar containing 100 µl cell cultures at a log phase (approximately 10⁷ cells/ml) of each of 16 *P. aeruginosa* strains (PA01, In1–8, InA–E, DWW1, and DWW3), three *E. coli* strains (BL21 (DE3), W3110, and ATCC15597) and *Staphylococcus aureus* ATCC6538. Ten microliters of pyocin S5 protein with twofold serial dilution was spotted onto the strain lawns. After overnight incubation at 30°C, inhibition zones on the bacterial lawns were examined. Minimum inhibition concentrations (MICs) were determined as the lowest concentration of purified pyocin S5 that induced a clear inhibition zone on the bacterial lawns [17,18].

To elucidate the effects of pyocin S5 on the viability of *P. aeruginosa* DWW3, the cells at a middle-log phase were washed three times and adjusted to an OD600 of 1.0. An aliquot of the cells resuspended in sterile 0.9% NaCl was mixed with pyocin S5 at a range of concentrations (3.5, 14, 56, 225 µg/ml). The mixtures were incubated at 37°C with a shaking at 250 rpm for 20 min, 40 min, or 60 min. The cell aliquots from each time point were collected and used for the colony forming unit (CFU) counting.
2.5. Antimicrobial mechanisms

To detect the release of the nucleic acids from the DWW3 cells, the absorbance at 260 nm (A260) in the cell-free supernatant was measured after treatment with pyocin S5 (225 μg/ml) by NanoDrop (Thermo, USA). ATP release from the cells in the supernatant was analyzed by BacTiter-Glo™ Microbial Cell Viability Assay Kit and GloMax™ 20/20 Luminometer (Promega, USA). The control cells were prepared in the same way but without pyocin S5 treatment.

Furthermore, the cells after the treatment were stained by LIVE/DEAD® BacLight Bacterial Viability Kit (Invitrogen, CA, USA), and thereafter observed under Carl Zeiss fluorescence microscope Axio A1 (Zeiss, Germany).

The field emission electron scanning microscopy (FESEM) produces clearer, less electrostatically distorted images compared to the conventional SEM and has widely been used to observe details of the cell surface. The cells were washed with 0.1 M sodium cacodylate (pH 7.4) after 1 h incubation with pyocin S5 (225 μg/ml). The first fixation was performed with 2.5% glutaraldehyde (GTA) at 4°C for overnight, followed by washing and the second fixation with 1.0% osmium tetroxide at room temperature for 1.5 h. The cells were dehydrated by ethanol (37%, 67%, 95%, and 100%) and loaded onto silicon slides. After air dry, the cells were observed using JOEL FESEM JSM-6700F (JOEL, Japan).

3. Results and discussion

3.1. Overexpression and purification of pyocin S5

Based on Pseudomonas aeruginosa PAO1 genome sequence ([http://www.pseudomonas.com/](http://www.pseudomonas.com/)), the nucleic acid sequences at positions 1067817–1066321 (the gene number: PA0985) were predicted to encode a novel pyocin, designated S5[13], with 498 amino acids, the killing domain of which shares 46% homology with that of colicin Ia. In this study, we found 23 alpha-helixes in the secondary structure of pyocin S5 using the Hierarchical Neural Network (HNN) method [15], whereas colicin la has 24 alpha-helixes (Fig. 1B). Further, the trans-membrane region prediction performed by the SOSUI program [16] indicates that pyocin S5 has two trans-membrane helixes close to C-terminus, with the trans-membrane region of PFFVKIETLAAGASWLVGIAF and ATAPGILGFALVMVGTAMID. As mentioned above, similarly, colicin la has 24 alpha-helixes, which include two trans-membrane helixes near C-terminus (Fig. 1B). This similar secondary structure and trans-membrane region, together with the 46% homology of the killing domains, suggests pyocin S5 as a pore-forming bacteriocin.

Pyocin S5 is reportedly produced under stress conditions caused by H2O2 [3] and ceftazidime [19] and in biofilm under aerobic and anaerobic condition [5]. In this study, to express and purify pyocin S5, pyoS5 gene was chemically synthesized, introduced into pET30b (+), and the recombinant construct was subsequently transformed into the host cell E. coli BL21 (DE3). As shown in Fig. 2A, pyocin S5 was efficiently expressed by induction with IPTG. To facilitate the purification, His tag-S tag (around 4.8 kDa) was fused onto the N-terminus of pyocin S5 (MW 57.6 kDa). After purification by FPLC, 2.16 mg/ml of pyocin S5 protein was obtained and showed a single band at MW 62.4 kDa (Fig. 2A, lane 4).

3.2. Antimicrobial spectrum and kinetics of bactericidal activity against P. aeruginosa

To determine the antimicrobial spectrum of pyocin S5 and identify sensitive P. aeruginosa strains, we examined the susceptibility of sixteen P. aeruginosa strains (PAO1, In1–8, InA–E, DWW1, and DWW3 [5]), three E. coli strains (BL21 (DE3), W3110, and ATCC15597) and S. aureus ATCC6538. Table 1 shows that seven P. aeruginosa strains (DWW3, In7, InA, InB, In4, In3, and In8) displayed sensitivity to pyocin S5, which showed clear inhibition zones with diffused boundaries. P. aeruginosa PAO1, E. coli (BL21 (DE3), W3110, and ATCC15597) and S. aureus ATCC6538 were insensitive to pyocin S5 even at 2.16 mg/ml. This insensitivity of pyocin S5 might stem from that: (i) those strains have no specific receptor on the cell surface and/or no translocation system for

<table>
<thead>
<tr>
<th>Strains</th>
<th>DWW3</th>
<th>In7</th>
<th>InA</th>
<th>InB</th>
<th>In4</th>
<th>In3</th>
<th>In8</th>
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<tr>
<td>MIC (μg/ml)</td>
<td>12.6</td>
<td>50.5</td>
<td>50.5</td>
<td>151.6</td>
<td>202.2</td>
<td>404.4</td>
<td>808.8</td>
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Fig. 2. SDS–PAGE analysis of overexpressed and purified pyocin S5, and the killing kinetics activity against P. aeruginosa DWW3. (A) SDS–PAGE analysis. Lanes 1–4: protein standard (Invitrogen), E. coli BL21 (DE3) (host cell) – harboring pET30b-S5 without and with IPTG induction, and pyocin S5 after purification. (B) The kinetics of killing activity of pyocin S5 against P. aeruginosa DWW3.
pyocin S5, and/or (ii) the immunity protein expressed in the insensitive strains might inactivate pyocin S5 action by binding to the C-terminus of pyocin S5. S5 immunity protein (108 amino acids) encoded by PA0984 potentially resides in the inner membrane and enables specific intramembrane helix–helix interactions with pyocin S5 to perform immunity. The antimicrobial activity is initiated by binding of pyocin S5 to its receptor on the sensitive cell membrane. Although several receptors for pore-forming colicins and other pyocins have been characterized (e.g. Cir, BtuB, FpvA, and lipopolysaccharide) [9,20–22], the receptor for pyocin S5 remains unknown. To evaluate the antimicrobial activity of pyocin S5 against different sensitive strains, the MICs were measured. Table 1 shows that among the seven sensitive strains (DWW3, In3, In4, In7, In8, InA, and InB), DWW3, which is a clinical isolate from cystic fibrosis patients [5], possessed the highest sensitivity to pyocin S5, with a MIC of 12.6 μg/ml (0.2 μM), which is more than 60 times lower than that against In8 (Table 1). At the MICs of pyocin S5 against these sensitive strains, clear inhibition zones with 10–15 mm diameters and diffused boundaries were observed. To further study the killing rate of pyocin S5 against DWW3, colony forming units (CFUs) were counted during time course (i.e. 20, 40, and 60 min) at 3.5, 14, 56, and 225 μg/ml. Fig. 2B shows that the killing percentage of pyocin S5 reached 58.9 ± 1.76% after 20 min treatment at 14 μg/ml and was increased to 95.7 ± 0.87% after 60 min treatment at 225 μg/ml. Additionally, pyocin S5 at 3.5 μg/ml displays at least three times lower killing efficacy than at 225 μg/ml against DWW3. During the time course, the killing activity showed a general trend of increase except for that at 3.5 μg/ml of pyocin S5.

3.3. Membrane leakage and permeability

Tokuda et al. [23] proposed that colicin Ia binds to a specific outer membrane receptor and is subsequently translocated to the
cytoplasmic membrane. Since pyocin S5 shows 46% homology to colicin la in the killing domain [13], we hypothesized that the mode of action of pyocin S5 might be cytoplasmic membrane damage, which causes the leakage of intracellular materials, the increase of membrane permeability, and membrane disruption.

To investigate the leakage of intracellular materials such as nucleic acids and ATP from DWW3 cells upon exposure to 225 μg/ml of pyocin S5, absorbance at 260 nm and ATP release in cell-free supernatant were measured. The control cells were prepared in the same way but without pyocin S5 treatment. As shown in Fig. 3A, after exposure to pyocin S5, absorbance at 260 nm is more than three times higher than that of the control. Since a purine or pyrimidine base shows characteristic absorbance at 260 nm, this increase in absorbance at 260 nm indicates the leakage of nucleic acids from DWW3 cells into the supernatant after exposure to pyocin S5. Similarly, ATP assay shows that the relative luminescence unit (RLU) in the supernatant after incubation with pyocin S5 for 1 h is more than two times higher than that of the control (Fig. 3B). The luminescence signal represents luminescent light intensity after ATP-luciferin reaction catalyzed by luciferase in the presence of Mg2+, and molecular oxygen. Thus, the increased RLU in the supernatant represents ATP release from the cells through membrane damage after exposure to pyocin S5.

Besides the leakage of intracellular materials, a change in membrane permeability is another characteristic of membrane damage. Fig. 3 shows that most DWW3 cells used as a control are stained with SYTO9 (green) (Fig. 3D). Fluorescent nucleic acid dye SYTO 9 penetrates and labels the bacteria with intact and damaged membrane; however, PI can only penetrate damaged membrane. Therefore, the DWW3 cells stained with PI (red) represent the cells with damaged membrane.

3.4. Membrane damage observed under FESEM

To further characterize membrane damage caused by pyocin S5, the cell morphology of DWW3 was examined upon exposure to pyocin S5 by FESEM. Fig. 3E and F shows that: (i) more than 60% of the cells shrunk to a smaller size after the exposure, likely due to the leakage of intracellular materials; (ii) the shape of the majority of the cells became irregular or distorted; and (iii) the cell surface turned very rough with observable protuberances and concaves. Moreover, the majority of the DWW3 cells exhibited shrinkage, rough surface and/or distortion after 1 h exposure. This difference in the cell morphology demonstrates that pyocin S5 caused membrane damage to DWW3 cells.

To summarize, this study has provided the first experimental evidence that the nucleic acid sequences at positions 1067817–1068321 (PA0985) encode a bacteriocin protein. We have shown that this bacteriocin, previously designated pyocin S5, is effective against seven clinical P. aeruginosa strains including DWW3, a clinical isolate from cystic fibrosis patients. Against DWW3, the MIC of pyocin S5 was 12.6 μg/ml (0.2 μM) and the killing percentage was 95.7% at 225 μg/ml after 60 min exposure. Furthermore, we have demonstrated that pyocin S5 causes membrane damage to DWW3, which was evidenced by (i) the increased leakage of intracellular materials, nucleic acids and ATP; (ii) the enhancement of PI penetration through membrane; and (iii) the shrinkage and distortion of the cell shape, and the rough cell surface. P. aeruginosa poses a serious health threat to the immunocompromised patients and also to the public because of its resistance to many synthetic antibiotics. Given the significance of the discovery of antimicrobial molecules effective against clinical isolates of P. aeruginosa, this study may suggest the potential use of pyocin S5 for P. aeruginosa treatment. Further, a specific binding capability of pyocins to their receptors, and the killing mechanism of pyocins can be exploited for developing a novel treatment regimen that has a low propensity to cause antimicrobial resistance and specifically targets P. aeruginosa. Alternatively, this narrow antimicrobial spectrum can be tailored for broader applications by developing chimeric pyocins.

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References