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Activity of Pyocin S2 against *Pseudomonas aeruginosa* Biofilms

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In cystic fibrosis patients, chronic lung infection with *Pseudomonas aeruginosa* and the associated decline in lung function are the major cause of mortality. In this report, we show that pyocin S2 displays potent activity against *P. aeruginosa* biofilms, thus representing a potentially improved therapeutic option. Using an invertebrate model of *P. aeruginosa* infection, we also show that pyocin S2 is highly active *in vivo*.

Due to the increasing prevalence of multidrug-resistant pathogenic bacteria and the poor efficacy of existing treatments against chronic bacterial infection, there is a critical requirement for the development of novel classes of antibiotics (4, 14). This is exemplified in cystic fibrosis (CF) patients, for whom, despite aggressive antibiotic therapy, chronic lung infection with *P. aeruginosa* and the concomitant intense inflammatory response leads to a progressive loss of lung function and is the major proven cause of mortality among this group of patients (8, 11).

An alternative strategy for antibiotic discovery is to utilize the narrow-spectrum antibiotics used by bacteria for intraspecies competition. In Gram-negative bacteria, these often take the form of high-molecular-weight protein antibiotics known as bacteriocins (6, 7, 13). In this report, we show that pyocin S2 displays potent activity *in vitro* against clinical isolates of *P. aeruginosa* growing in the biofilm state. Further to this, pyocin S2 is highly active in an invertebrate model of *P. aeruginosa* infection.

P. aeruginosa grows predominantly as a biofilm in the CF-infected lung, and this state is associated with high levels of resistance to small-molecule antibiotics (6, 9). To determine if pyocins display potent activity against *P. aeruginosa* growing in the biofilm state, we first cloned the genes for pyocin S2 (2) and its immunity protein ImS2 into an *Escherichia coli* expression vector and expressed and purified the protein in complex with its immunity protein.

The pyocin S2-ImS2 complex was isolated by nickel affinity chromatography by virtue of an engineered C-terminal His_6 tag on the immunity protein (Fig. 1a). The purified pyocin S2-ImS2 complex was highly active against *P. aeruginosa* growing on LB

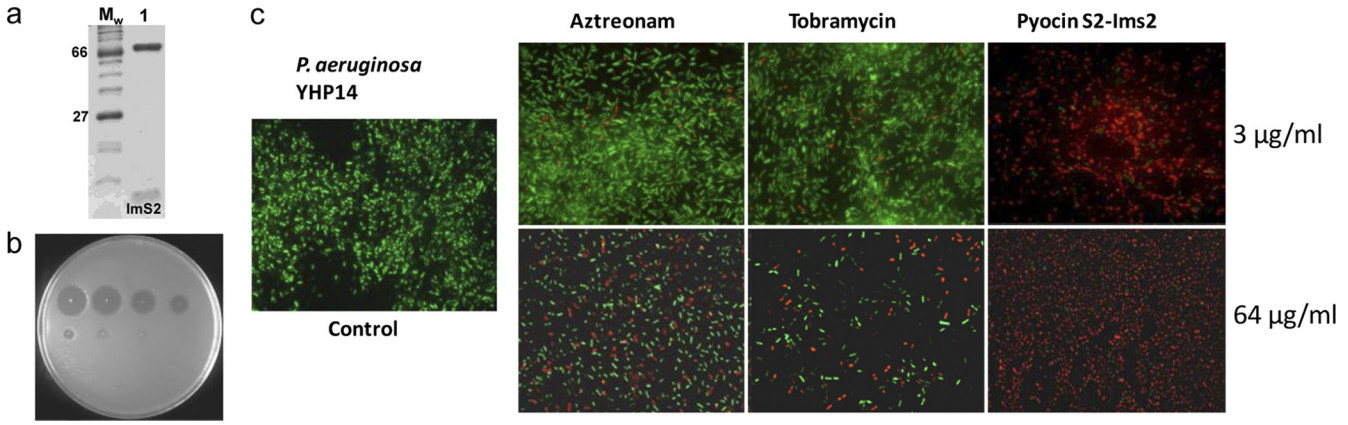

FIG 1 Purification of S-type pyocins and killing activity of the pyocin S2-Im2 complex against *P. aeruginosa*. (a) SDS-PAGE of purified pyocin S2-ImS2 complex (lane 1). (b) Activity of purified pyocin S2-ImS2 against *P. aeruginosa*. A 5-fold serial dilution (starting concentration, 2 mg/ml [top left]) of pyocin S2-ImS2 was spotted onto a lawn of growing *P. aeruginosa* strain YHP17 which was grown overnight at 37°C. Clear zones indicate cell death. (c) Activity of pyocin S2-ImS2 against *P. aeruginosa* biofilms. Biofilms (24 h) of *P. aeruginosa* YHP14 were grown on poly-L-lysine glass cover slides, treated with a pyocin S2 at 3 μg/ml and 64 μg/ml for 1 h, and visualized by fluorescence microscopy using LIVE/DEAD cell viability staining. Red, dead cells; green, live cells.

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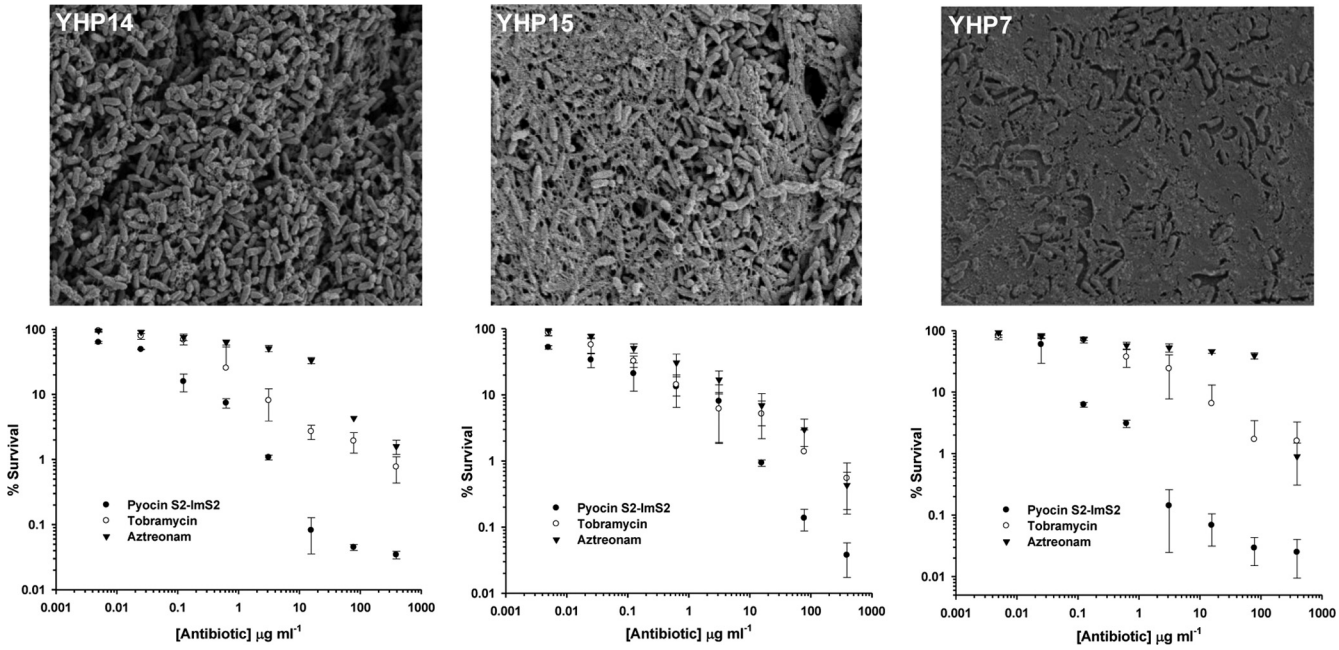

FIG 2 Biofilm-associated survival of *P. aeruginosa* strains YHP14, YHP15, and YHP7 after treatment with pyocin S2, aztreonam, or tobramycin. Upper panels show 24-h biofilms from each strain visualized by scanning electron microscopy. Lower panels show the percent survival after a single treatment with pyocin S2-ImS2, aztreonam, or tobramycin (0.001 μg/ml to 390 μg/ml) for 1 h. Error bars represent the standard deviations between replicate samples ($n = 6$). All *P. aeruginosa* clinical isolates were collected from sputum samples from children with cystic fibrosis at the Royal Hospital for Sick Children (Yorkhill, Glasgow, United Kingdom). *P. aeruginosa* biofilms were formed on an MBEC 96-peg plate platform (Innovotech, Edmonton, Canada), as previously described (1).

agar (Fig. 1b). We initially tested the activity of pyocin S2 against biofilms formed by *P. aeruginosa* isolate YHP14 treated with a single dose of pyocin S2 for 1 h at 3 μg/ml and 64 μg/ml and visualized by fluorescence microscopy using LIVE/DEAD cell viability staining (Fig. 1c). Parallel biofilm killing assays were also performed for tobramycin and aztreonam, approved antibiotics for treatment of chronic CF lung infection by inhalation (12). From the relative number of live (green) and dead (red) cells, it is apparent that pyocins are highly active against *P. aeruginosa* in the biofilm state, killing a larger proportion of cells than either tobramycin or aztreonam at equivalent concentrations (Fig. 1c).

To obtain quantitative data on the ability of pyocins to kill *P. aeruginosa* biofilms, we treated YHP14 24-h biofilms, grown on the plastic pegs of the MBEC biofilm cultivation system, with pyocin S2, tobramycin, and aztreonam (0.001 to 390 μg/ml) for 1 h (Fig. 2). Cells were removed by sonication, and colonies were counted by plating serial dilutions of recovered cells on solid media. The ability of YHP14 to form structured multilayered biofilms

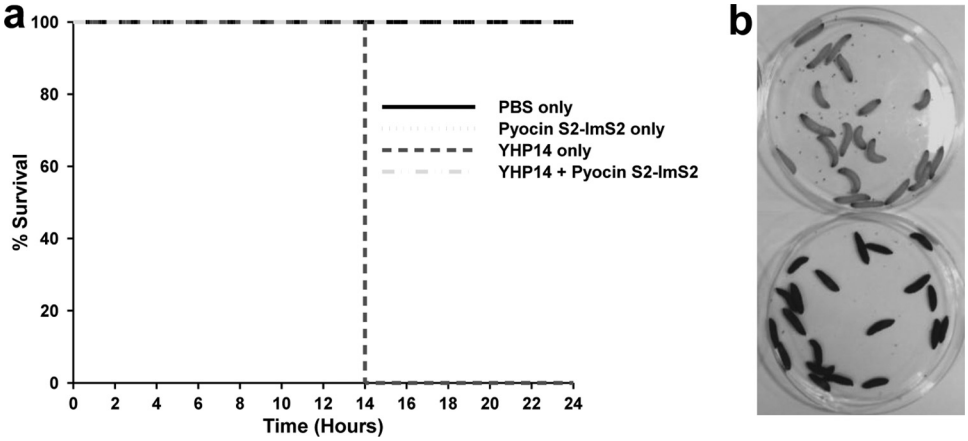

FIG 3 Ability of pyocin S2-ImS2 to provide protection against a lethal *P. aeruginosa* infection in the *Galleria mellonella* caterpillar. (a) Survival plot for groups of larvae infected with *P. aeruginosa* YHP14 and treated with pyocin S2-ImS2. Groups of 20 larvae were not infected or were inoculated with 10^4 CFU of *P. aeruginosa* YHP14 and after 3 h were injected with either PBS (control) or pyocin S2 (27 mg/kg). (b) Lethal infection by *P. aeruginosa* is accompanied by the generation of melanin in larvae leading to a distinct change in color from cream to dark brown in larvae close to death. Shown are larvae infected with YHP14 and treated with pyocin S2-ImS2 (top) or with PBS (bottom). Infection of *Galleria mellonella* larvae (Livefood, United Kingdom) was performed as previously described (5).

under these conditions was first confirmed by electron microscopy (Fig. 2). In this assay, pyocin S2 was found to kill *P. aeruginosa* in the biofilm state considerably more effectively than aztreonam or tobramycin, with S2-treated biofilms showing a reduction of close to 4 log units in cell survival at the highest concentration of antibiotic tested.

Since clinical isolates of *P. aeruginosa* are phenotypically diverse, in particular in terms of the level of production and incorporation of extracellular polysaccharide into the biofilm matrix, we compared the activity of pyocin S2 against isolates with different levels of mucoidy (Fig. 2). Despite different levels of extracellular polysaccharide production, the level of killing of pyocin S2 against YHP15 and YHP7 biofilms was remarkably similar to that shown against YHP14 and in all cases was greater than the level of killing shown by tobramycin and aztreonam. At an antibiotic concentration of 3 μg/ml, cell survival of pyocin S2-treated YHP7 biofilms was 0.3%, with tobramycin- and aztreonam-treated biofilms showing $\geq$100-fold increased survival rates of 30% and 60%, respectively.

To determine if pyocins are active during infection, we tested the ability of pyocin S2 to provide protection against a lethal *P. aeruginosa* infection in a whole animal. For this purpose, we chose a nonvertebrate host, the *Galleria mellonella* caterpillar, in which *P. aeruginosa* has been shown to be highly virulent and in which pathogenesis is dependent on similar virulence factors that are essential for pathogenesis in mammals (10). Groups of 20 larvae were inoculated with 10^4 CFU of YHP14 and after 3 h injected with either phosphate-buffered saline (PBS) (control) or pyocin S2 (27 mg/kg). The larvae were monitored every 2 h between 10 and 72 h. Those in the control group (PBS injected after inoculation) died 12 to 14 h postinfection, while those treated with pyocin S2 postinfection survived until the experiment was stopped at 72 h (Fig. 3a). Lethal infection by *P. aeruginosa* is accompanied by the generation of melanin in larvae, leading to a distinct change in color from cream to dark brown in larvae close to death (note light and dark larvae in Fig. 3b). Larvae in additional control groups that were not infected with *P. aeruginosa* but were treated with PBS or pyocin also survived until the end of the experiment, indicating that pyocin S2 is not toxic to the host in the absence of infection (Fig. 3a). Colony counts from larvae confirmed that the PBS-only and pyocin-only controls contained no *P. aeruginosa*. The group that was infected with strain YHP14 contained between 5×10^8 and 1×10^9 CFU at time of death. The infected group treated with pyocin contained between 10 and 40 CFU.

In this report, we have shown that pyocin S2 has potent activity against *P. aeruginosa* growing in the biofilm state and is capable of protecting against a lethal *P. aeruginosa* infection. In infections where the microbiology of infection is closely monitored, species-specific protein antibiotics may provide a useful therapeutic option.

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