



Anti-biofilm activity of the marine bacterium *Pseudoalteromonas ruthenica* KLPp3 against *Serratia marcescens* and *Vibrio alginolyticus*

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ABSTRACT

Aims: *Pseudoalteromonas ruthenica* KLPp3 is the marine Gram-negative strain isolated from the surface of mud crab at Pulau Perhentian Malaysia. In this work, the anti-biofilm activity of *P. ruthenica* supernatant was examined on *Serratia marcescens* and *Vibrio alginolyticus*.

Methodology and results: The crude extract of *P. ruthenica* KLPp3 was obtained using ethyl acetate. The sub-minimum inhibitory concentration (MIC) of the crude extract was determined using the minimum inhibitory test. The sub-MIC crude extract was tested against two of the *S. marcescens* virulence factors, which are the swarming ability and production of prodigiosin. The crystal violet assay was used to test the anti-biofilm activity of the sub-MIC crude extract against *S. marcescens* and *V. alginolyticus*. The productions of prodigiosin were reduced by 72%. The swarming area was reduced by 56.06%. It inhibits 26.9% and 48.5% of biofilm production in *S. marcescens* and *V. alginolyticus* respectively. The crude extract was heat stable.

Conclusion, significance and impact of study: Besides combating the *S. marcescens* virulence factor, *P. ruthenica* KLPp3 crude extract in sub-MIC reduces the formation of biofilm of *S. marcescens* and *V. alginolyticus*, which may find applications in biofilm inhibition and prevention.

Keywords: Anti-microbial, sub-MICs, crude extract, Anti-biofilm, *Pseudoalteromonas ruthenica*

INTRODUCTION

Biofilms are the predominant mode of growth for bacteria in most environments (Bendaoud *et al.*, 2011). Biofilms are more resistant to environmental stresses, which can cause problems in medical and industrial settings. In the medical settings, biofilms are the cause of persistent infections implicated in 80% or more of all the microbial cases releasing harmful toxins and even obstructing in dwelling catheters (Epstein *et al.*, 2012) or causing orthopedic implant infections (Drago *et al.*, 2013). Biofilms also contaminate a wide range of infrastructure elements such as water and air purification systems (Sublette *et al.*, 2006), optical sensors (Kerr *et al.*, 1998) and industrial equipment (Holmstrom *et al.*, 2002).

Given the increasing relevance of the bacterial biofilms, the development of new approaches for the prevention and treatment of adhesion and biofilm formation capabilities has amplified. Marine bacteria belong to the genus *Pseudoalteromonas* produce

compounds of biotechnological interest, including the anti-biofilm molecules (Klein *et al.*, 2011). *Pseudoalteromonas ruthenica* KLPp3 is a gram-negative strain isolated from the surface of mud crabs at Pulau Perhentian, Malaysia. GenBank accession number is KT071710. Ivanova *et al.* (2002) first isolated the *P. ruthenica* strain KMM290 from marine invertebrates that are known to harbor antibiotic-producing bacteria.

In this work we examined the anti-biofilm compounds of *P. ruthenica* crude extract against the *Serratia marcescens* SM-11 and *Vibrio alginolyticus* GmPp2. *Vibrio alginolyticus* GmPp2 was isolated from sea cucumber at Pulau Perhentian while *S. marcescens* SM-11 was isolate from sea mussel at Sungai Merbuk. *Pseudoalteromonas ruthenica* KLPp3 secretes anti-microbial and anti-biofilm activity which impair the biofilm development of *V. alginolyticus*. It also prevents the production of pigment *S. marcescens*.

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MATERIALS AND METHODS

Bacterial strains and culture conditions

Bacterial strains used in this work were *P. ruthenica* K LPp3, *V. alginolyticus* GmPp2, *S. marcescens* SM-11. The bacteria were grown in LB broth (Difco) at 30 °C.

Minimum Inhibitory Concentration (MIC)

MIC assay was performed according to CLSI guidelines (2006). All bacterial strains were subjected to the antimicrobial tests by using the disc diffusion method on the Mueller-Hinton (MH) agar. An aliquot of 0.1 mL of 0.5 McFarland equivalents, approximately from an exponentially growing culture was spread on the agar for the development of a bacterium strain lawn at 30 °C. Next, on the lawn-agar of each plate, eight discs of crude extracts with concentration of 0.1 mg/mL, 0.2 mg/mL, 0.5 mg/mL and 1 mg/mL were placed individually at equal distances from one another. The plates were incubated for 18 h at 30 °C and were examined for size-measurements of inhibition zones around each disc.

Effect of the crude extract on prodigiosin production in *S. marcescens*

Prodigiosin assay in *S. marcescens* was performed following a method used by Morohoshi *et al.* (2007). Prodigiosin from the cell pellet was extracted with acidified ethanol solution (4% 1 M HCl in ethanol) and the absorbance of prodigiosin in ethanol was measured at 534 nm by using a UV-visible spectrophotometer.

Swarming assay

The swimming and swarming motility assays were performed by following the method of Packiavathy *et al.* (2012) with slight modification. Assays were performed on LB medium with 0.5% agar for swarming motility assays. Solidified swarming agar was overlaid with a swarming agar supplemented with 0.1 mg/mL of crude extract. Overnight cultures of *S. marcescens* (2 µL) were inoculated in the centre of the agar and incubated for 48 h at 25 °C.

Preparation of the *P. ruthenica* KLPp3 crude extract

Pseudoalteromonas ruthenica KLPp3 was grown by shaking for 90 h, and was extracted using ethyl acetate. After the incubation, the bacterial culture was centrifuged at 4000 rpm for 15 min and the supernatant was filtered through a 0.22 µm membrane. The supernatants were

extracted with the equal volumes of ethyl acetate. The extraction process was repeated three times and then the extract was concentrated in a rotary evaporator. The crude extracts were stored at 4 °C.

Biofilm formation of *V. alginolyticus* PPGm2, *S. marcescens* SM-11

The biofilm formation of *V. alginolyticus* PPGm2 and *S. marcescens* SM-11 was evaluated in the presence of the crude extract. The quantification of the *in vitro* biofilm production was based on the method previously reported (Artini *et al.*, 2011). Briefly, the wells of a sterile 96-well flat-bottomed polystyrene plate were filled with 100 µL of the appropriate medium. A 1/100 dilution of overnight bacterial culture was added into each well. The first row contained the untreated bacteria, while the second row contained 0.1 mg/mL of the crude extract. The plates were incubated for 24 h at 30 °C. After rinsing with 0.9% saline, the adhered cells were stained with 0.1% crystal violet, rinsed twice with saline and thoroughly dried as previously described (Christensen *et al.*, 1985). The dye bound to the adherent cells was resolubilized with 80% (v/v) ethanol per well. The OD of each well was measured at 590 nm.

Heat sensitivity of the anti-biofilm compound

Heat sensitivity of the anti-biofilm compound was performed by following the method of Papa *et al.* (2013). To examine the heat sensitivity of the crude extract, it was incubated for 1 h at 50 °C and cooled on ice. For each of the above tests, the anti-biofilm activities of treated and untreated cultured crude extract were compared using the microtiter plate assay against *S. marcescens* SM-11.

RESULTS

Minimum Inhibitory Concentration (MIC)

Preliminary experiments were carried out to assess the effects of crude extract on the growth of *V. alginolyticus* GmPp2 and *S. marcescens* SM-11 (Table 1). The bacterial cultures were separately treated with the crude extract at a concentration of 0.1 mg/mL, 0.2 mg/mL, 0.5 mg/mL and 1 mg/mL. The inhibitory effect started at the concentration of 0.5 mg/mL. The MIC of the crude extract was 0.5 mg/mL. There were no inhibitory effects at the concentration of 0.1 mg/mL and 0.2 mg/mL. This result has shown that the concentration of 0.1 mg/mL and 0.2 mg/mL did not kill the bacteria. The sub-MIC, 0.1 mg/mL, was chosen for further tests.

Table 1: Minimum inhibitory concentration of crude extracts.

Tested bacteria	Inhibition (mm)				Negative Control	Positive control
	0.1 mg/mL	0.2 mg/mL	0.5 mg/mL	1 mg/mL		
<i>S. marcescens</i>	6	6	7	7	6	13 (streptomycin)
<i>V. alginolyticus</i>	6	6	7	7	6	11 (streptomycin)

Effects of the crude extract on the prodigiosin production of *S. marcescens*

The production of prodigiosin by *S. marcescens* is one of the quorum sensing (QS) dependent behaviours (Morohoshi *et al.*, 2007). The concentration reduction in prodigiosin pigment production of *S. marcescens* was observed with treatment (Figure 1). A maximum of 72% inhibition in prodigiosin production was observed when treated with the crude extract.

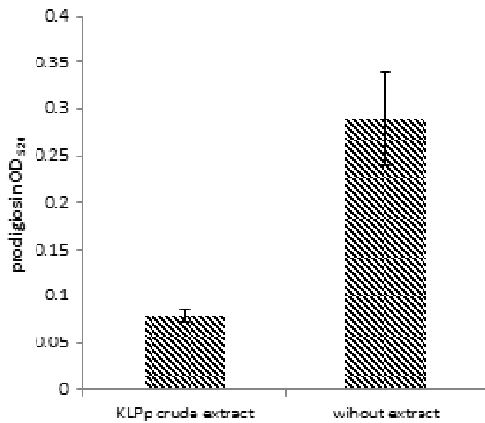


Figure 1: Effect of crude extract on prodigiosin production of *S. marcescens*.

Swarming assay

Concentrations of 0.1 mg/mL *P. ruthenica* KLPp3 crude extract were seeded into the swarming agar. The average diameters of swarming area in untreated agars were 8.7 cm while the average diameters of swarming area in treated agars were 3.8 cm (Table 2). There was 55.06% of reduction in the area of swarming.

Table 2: The diameters of swarming area.

Bacteria	Diameter of swarming area (cm)
<i>S. marcescens</i> treated	8.7
<i>S. marcescens</i> untreated	3.8

Effects of *P. ruthenica* KLPp3 crude extract on the biofilm formation

The effects of sub-MIC crude extract of *P. ruthenica* KLPp3 on different bacteria species were examined. It inhibited 26.9% and 48.5% of the biofilm production in *S. marcescens* and *V. alginolyticus* respectively (Figure 2). The crude extract had a good anti-biofilm effect on *V. alginolyticus* PPGm2 and *S. marcescens* SM-11.

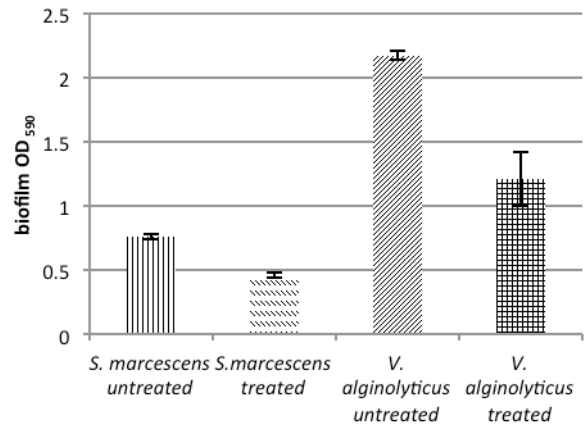


Figure 2: Effect of *P. ruthenica* KLPp3 crude extract on biofilm formation.

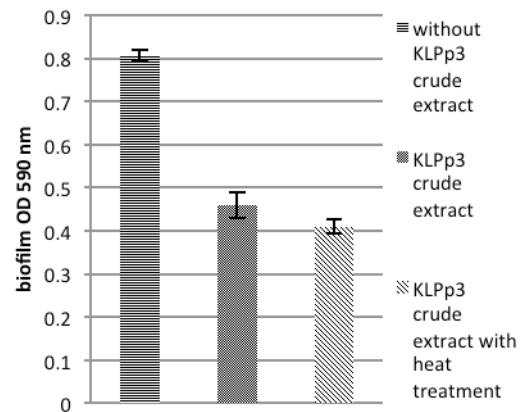


Figure 3: Effect of heat treatment on crude extract anti-biofilm activity.

Heat sensitivity of the anti-biofilm compound

The biofilm-inhibiting material was heat stable and retained about 90% of its inhibitory activity after 1 h at 50 °C (Figure 3).

DISCUSSION

Most of the known anti-biofilm molecules are also antibacterial (bactericidal or bacteriostatic). This can lead to the appearance of resistant mutants. This problem can be overcome by using sub-MICs. By definition, sub-MIC antibiotic concentrations allow susceptible strains to continue growing. Studies show that the sub-MICs of antibiotic can reduce the ability of the biofilm production without killing the targets (Henriques *et al.*, 2005; Wojnicz and Tichaczek-Goska, 2013).

The activity of the crude extract was first assessed against the prodigiosin production and swarming ability of *S. marcescens* as both are considered as the major

virulence factors (Liu and Nizet, 2009) and involved in the biofilm formation. The two signal molecules N-butanoylhomoserine lactone and HHL are shown to regulate the production of prodigiosin (Morohoshi *et al.*, 2007). Therefore, any interference with these QS systems leads to a reduction in the prodigiosin production. Swarming refers to the bacterial surface translocation which is QS-dependent and it requires flagella and pili (Sharma and Anand, 2002). Generally, there are three stages of the swarming process, firstly, the differentiation of vegetative cells into swarmer cells, followed by the migration of swarmer cell populations and finally the consolidation (Kearns, 2011). Here, a reduction in the swarming motility of the *S. marcescens* by the crude extract was observed with a marked change in the area of swarming. *S. marcescens* spread throughout the agar in the untreated control (8.7 cm in diameter) but only filled half of the plate (3.8 cm in diameter) in the presence of 0.1 mg/mL concentration of the crude extract. The extract of *P. ruthenica* KLPp3 might be exerting its inhibition of swarming during the migration of swarmer cells or causing biofilm dispersal, which may in turn, lead to the development of immature or reduced biofilm of *S. marcescens*. This may have important applications in preventing the biofilm. The results of the swarming assay and prodigiosin assay might suggest that the culture crude extract inhibited the biofilm formation by disrupting the bacterial communication. Subsequently, as both processes are QS-regulated traits, further studies can be done by using the mutants which are deficient in QS.

The anti-biofilm activities of sub-MIC crude extract of *P. ruthenica* KLPp3 were investigated. Results obtained demonstrate that the crude extract of *P. ruthenica* KLPp3 reduced the biofilm of *V. alginolyticus* GmPp2 and *S. marcescens* SM-11. Wojnicz and Tichaczek-Goska (2013) reported that the sub-MICs of ciprofloxacin, amikacin and colistin reduced the ability of *E. coli* strains to form biofilm. *Candida dubliniensis* biofilm formations were also reduced by sub MIC of antifungal agent, without affecting the growth of the *Candida* strains (Henriques *et al.*, 2005). The present work concluded that the *P. ruthenica* KLPp3 crude extract in sub-MIC reduces the formation of biofilm. Since this compound is used at its sub-MIC concentration, it is less expected to impose any selective pressure for the development of antibiotic resistance.

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