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SHORT COMMUNICATIONS

Inhibition of quorum-sensing-dependent phenotypic expression in *Serratia marcescens* by marine sediment *Bacillus* spp. SS4

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Abstract The role of quorum sensing (QS) is well known for bacterial virulence, antibiotic resistance and biofilm production. Consequently, inhibition of QS may reduce the risk of microbial pathogenicity in systemic and local infections. Hence, in the present study, the marine sediment bacterial isolate *Bacillus* spp. SS4 (GenBank accession number GU471751) isolated from the Point Calimere coastal region was assessed for its anti-quorum-sensing (anti-QS) activity against acyl homoserine lactone (AHL)-dependent prodigiosin, protease production and biofilm formation in *Serratia marcescens* (FJ584421). The ethyl acetate extract obtained from SS4 exhibited a concentration-dependent (50–200 μg/ml) reduction in prodigiosin production in *S. marcescens* to a level of 40–87%. The azocasein-degrading proteolytic activity of *S. marcescens* was also reduced by up to 60%, when treated with the SS4 extract at a final concentration of 200 μg/ml. Light- and confocal laser scanning-microscopic (CLSM) analysis further confirmed the reduction in the biofilm-forming ability of *S. marcescens* when treated with this extract. In an antibiotic susceptibility assay, cells of *S. marcescens* showed increased susceptibility towards antibiotics in the presence of SS4 extract. In addition, SS4 extract by itself showed no growth inhibitory effect on *S. marcescens*. Thus, the results of present study reveal the anti-QS potential of *Bacillus* spp. SS4 by notably reducing AHL-dependent behaviors in *S. marcescens*.

Keywords Marine bacteria · *Bacillus* spp · *Serratia marcescens* · Acyl homoserine lactone · Biofilm · Anti-quorum-sensing activity

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Introduction

Serratia marcescens is a Gram-negative bacterium, recognized as an important opportunistic pathogen that causes pneumonia, bloodstream infections, central nervous system infections, urinary tract infections, and conjunctivitis (Hejazi and Falkiner 1997; Morohoshi et al. 2007; Maragakis et al. 2008). It produces a range of secreted virulence factors, including proteases, nucleases, lipases, chitinases, and hemolysin (Hejazi and Falkiner 1997; Morohoshi et al. 2007). There is also increasing evidence that the emergence of *S. marcescens* strains resistant to multiple antibiotics signify a growing public health risk (Maragakis et al. 2008). Thus, it is necessary to search for novel treatment techniques to prevent *Serratia* infections.

Bacteria have been found to coordinate their behavior through a cell-to-cell communication system known as quorum sensing (QS), mediated by small signaling molecules called autoinducers (Fuqua et al. 1994; Rasmussen and Givskov 2006). A well known autoinducer molecule in Gram-negative bacteria is acyl homoserine lactone (AHL), which regulates various phenotypic characters such as conjugation, antibiotic production, biosurfactant synthesis, bioluminescence, virulence factor production and biofilm formation. The role of AHL on virulence factor production and biofilm formation has now suggested a new strategy to combat bacterial infection (Williams 2002; Rasmussen and Givskov 2006). It has also been shown that *Serratia* strains utilize QS molecules like *N*–butanoyl homoserine lactone (C4–HSL) and *N*–hexanoyl homoserine lactone (C6–HSL) for the regulation of genes encoding extracellular virulence factors and biofilm formation (Labbate et al. 2004; Rice et al. 2005; Morohoshi et al. 2007). Therefore, quenching the AHL system in bacterial pathogens will pave the way to preventing emerging bacterial infectious diseases. Hence, in

the present study, an attempt was made to investigate the anti-QS activity of marine bacteria *Bacillus* spp. SS4 in inhibiting AHL-mediated prodigiosin, protease production and biofilm formation in *S. marcescens*.

Materials and methods

Culture conditions and polar extraction

The marine bacterial culture *Bacillus* spp. SS4 (GU471751) was cultivated in marine Luria-Bertani (mLB) broth consisting of 1% tyrptone, 0.5% yeast extract and 2% NaCl (pH 7.5±0.2) and incubated for 24 h at 30°C. The cell-free supernatant from SS4 was obtained by centrifugation at 10,000 rpm twice; the pH was reduced to 7 and the supernatant filtered through 0.22 μm membrane filter. The filtered supernatant was then extracted with an equal volume of ethyl acetate; the solvent layer was separated and evaporated to dryness. The dried extract was weighed and used for anti-QS bioassays. The target pathogen *S. marcescens* used in this study (FJ584421) was isolated from a patient with urinary tract infection. It was cultivated in Luria-Bertani (LB) broth (pH 7.2±0.2) at 150 rpm in a shaker overnight at 37°C. For experimental analysis, *S. marcescens* was sub cultured to reach an OD of 0.4 at 600 nm. *Chromobacterium violaceum* CV026 (OD of 0.2 at 600 nm) was used to confirm the AHL interfering activity of SS4 extract. CV026 is an AHL-negative strain, and requires external addition of AHL to produce violacein.

Prodigiosin assay

One percent of *S. marcescens* cells (OD of 0.4 at 600 nm) was inoculated into 2 ml fresh LB medium, cultivated without and with SS4 extract at a final concentration of 50–200 μg/ml (w/v), and incubated for 18 h at 30°C. During late stationary phase, the culture from each tube was collected in a 2-ml sterile tube and centrifuged at 13,000 rpm for 10 min to precipitate cells along with prodigiosin. Prodigiosin from the cell pellet was extracted in an acidified ethanol solution (4% 1 M HCl in ethanol), and absorbance of the extracted prodigiosin was measured at 534 nm using a UV-visible spectrophotometer (HITACHI U-2800) (Morohoshi et al. 2007).

Total proteolytic activity

Serratia marcescens cells were treated with SS4 extract as in the prodigiosin assay and incubated for 18 h. After incubation, the total proteolytic activity of cell-free supernatant of *S. marcescens* culture cultivated in the absence and presence of SS4 extract was determined by an azocasein assay (Kessler et al. 1993). Briefly, 150 μl of both treated and untreated *S. marcescens* culture supernatant was added separately to 1 ml 0.3% azocasein (Sigma, St. Louis, MO) in 0.05 M Tris-HC1, 0.5 mM $CaCl_2$ (pH 7.5) and incubated at 37°C for 15 min. The reaction was stopped by adding trichloroacetic acid (10%, 0.5 ml) followed by centrifugation, and the absorbance was read at 400 nm in a UV–visible spectrophotometer.

Confocal laser scanning- and light-microscopic analysis of biofilm prevention

The target pathogen was inoculated into 1 ml fresh LB broth in 24-well microtiter plates (MTP) and cultivated in the absence or presence of SS4 extract at a final concentration of 200 μg/ml with glass slides (1×1 cm). Plates were incubated for 18 h at 37°C. Planktonic cells and spent medium from the MTP was discarded, and adherent cells in the glass slides were rinsed gently with deionized water and allowed to air dry before being stained. The biofilms were stained with 0.4% crystal violet (for light microscopic analysis) and 0.1% acridine orange solution (w/v) [for confocal laser scanning microscopy (CLSM) analysis] separately for 1 min, after which the glass slides were again rinsed twice gently with deionized water. The stained biofilms were then air dried and visualized under a light microscope (Nikon Eclipse *Ti*, Japan) or CLSM (Model LSM 710, Zeiss, Germany) (You et al. 2007).

Effect of SS4 extract on antibiotic susceptibility of *S. marcescens*

The antibiotic susceptibility pattern of *S. marcescens* in the presence and absence of SS4 extract was assessed by following the modified method of Brackman et al. (2008). Briefly, *S. marcescens* cells were exposed to erythromycin (10 μg) and chloramphenicol (10 μg) in the presence and absence of SS4 extract at a final concentration of 200 μg/ml in MTP containing 1 ml LB broth and incubated for 18 h. The antibiotic susceptibility of extracts treated and untreated cultures was compared by measuring the cell density of each culture set up at 600 nm using a UV-visible spectrophotometer.

Effect of SS4 extract on bacterial QS

The mode of action of SS4 extract in exhibiting anti-QS activity was assessed by performing the following experiment. *Serratia marcescens* was cultivated in the presence and absence of SS4 extract at a final concentration of 200 μg/ml and incubated at 30°C for 14 h. After incubation, AHL was extracted with acidified ethyl acetate by following the method of Morohoshi et al. (2007). The

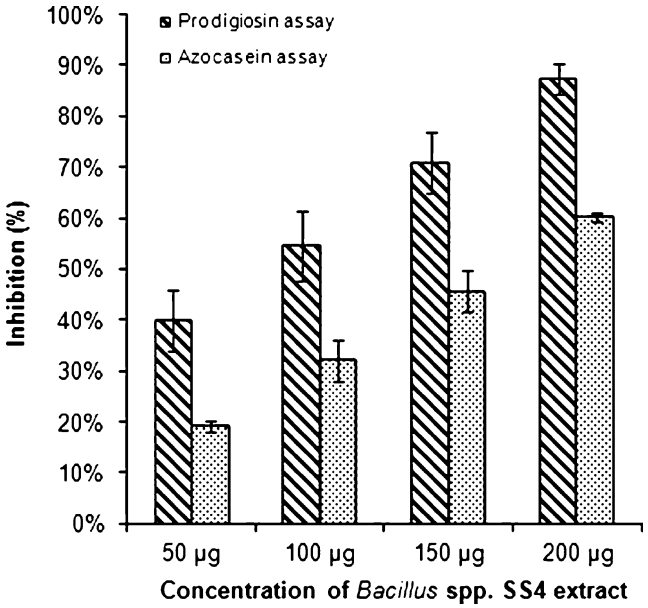

Fig. 1 Effects of *Bacillus* spp. SS4 extract on prodigiosin and protease production in *Serratia marcescens*. The results were reproduced in three independent experiments. *Error bars* Standard deviation

extracted AHL from both treated and untreated samples were inoculated in 2 ml LB broth containing CV026 and incubated for 16 h at 30°C. After incubation, a violacein quantification assay was performed following the method of Choo et al. (2006).

Results and discussion

Owing to the importance of QS during bacterial pathogenesis, much research has been focused on inhibiting QS using various bacterial models (Choo et al. 2006; You et al. 2007; Adonizio et al. 2008). In this study, we used *S. marcescens* (Morohoshi et al. 2007) as a target pathogenic model to study the anti-QS activity of marine SS4 extract. The influence of SS4 ethyl acetate extract was assessed for its ability to inhibit protease production and biofilm formation in *S. marcescens*. In addition, we tested the production of prodigiosin, a red pigment whose biosynthesis is regulated by QS (Morohoshi et al. 2007). A concentration-dependent reduction in the prodigiosin pigment production was observed with increasing concentration of SS4 extract. A minimum of 40% inhibition was observed at 50 µg/ml and the maximum of 87% at 200 µg/ml (Fig. 1). Our results are consistent with the findings of Morohoshi et al. (2007), who reported that a synthetic compound of AHL analog 4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone showed a reduction in AHL-dependent prodigiosin production in *S. marcescens* AS-1. In addition to prodigiosin, *S. marcescens* is also known to produce various secreted virulence factors, including proteases, that contribute to the pathogenecity to host (Wei and Lai 2006). Therefore, we tested the efficacy of ethyl acetate extract in inhibiting protease production by *S. marcescens*. As expected, the extract from SS4 showed a considerable reduction in protease production, with inhibition levels of 19%, 32%, 45% and 60% when treated with 50, 100, 150 and 200 µg/ml SS4 extract, respectively (Fig. 1).

Biofilms are high density, matrix-encased populations attached to surfaces, and are recognized as the predominant form of growth in the environmental life cycle of bacteria. Biofilms are more resistant to environmental stresses and to host-mediated responses (Rice et al. 2005). It is also well proven that QS plays a vital role in regulating the formation of biofilms in variety of bacterial pathogens including *S. marcescens* (Labbate et al. 2004; Rice et al. 2005). In order to analyze the antibiofilm efficiency of the SS4 extract in inhibiting biofilm formation, in the present study, *S. marcescens* cells were allowed to grow in an MTP-containing cover glass in the absence and presence of SS4 extract and the results were visualized under a light microscope. As revealed by the light microscopic analysis, only meager aggregations of *S. marcescens* biofilm were observed in the presence of SS4 extract compared with biofilm formation seen in the control (Fig. 2). This result indicates the efficiency of marine bacterial SS4 extract in

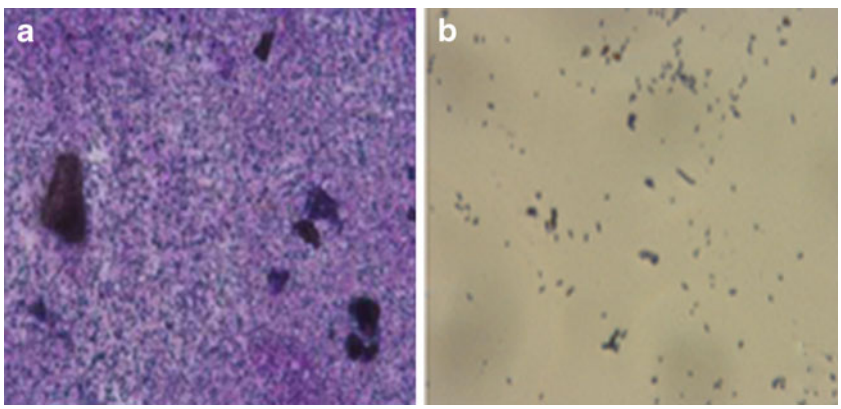

Fig. 2 Light microscopic images of *S. marcescens* biofilms grown in the absence (**a**) and presence (**b**) of *Bacillus* spp. SS4 extract

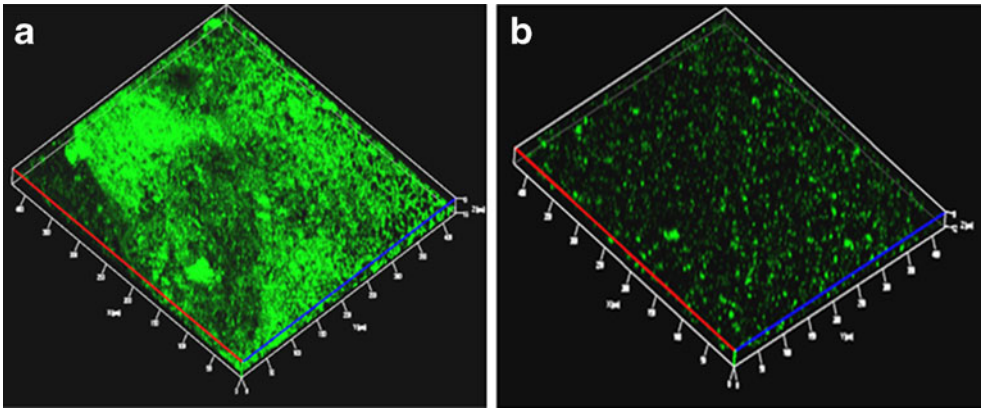

Fig. 3 Confocal laser scanning microscopy (CLSM) images of *S. marcescens* biofilms grown in the absence (**a**) and presence (**b**) of *Bacillus* spp. SS4 extract

preventing biofilm development of *S. marcescens* in cover glasses. This result is in agreement with previous findings by You et al. (2007), in which *Streptomyces albus* strain A66 showed antibiofilm activity against a *Vibrio* biofilm on cover glass. Recently, the antibiofilm activity of coral-associated bacterial and actinomycetes isolates was also reported to prevent the biofilm formation of *Streptococcus pyogenes* and *Staphylococcus aureus*, respectively (Thenmozhi et al. 2009; Bakkiyaraj and Pandian 2010).

CLSM analysis was also carried out to determine the efficiency of SS4 extract in reducing/inhibiting the biofilm architecture of *S. marcescens*. As shown in Fig. 3, CLSM analysis revealed the loosened architecture of *S. marcescens* biofilms when treated with SS4 extract. Further, the thickness of *S. marcescens* biofilms formed in control slides and the reduction in thickness in treated slides were also assessed. The 16-μm thick *S. marcescens* biofilm in the control slide was reduced to 12 μm when treated with SS4 extract. In support of our findings, extracts of coral-associated actinomycetes isolated from the coral *Acropora digitifera* also reportedly reduce the thickness of *S. aureus* biofilm in CLSM analysis (Bakkiyaraj and Pandian 2010).

Many bacterial pathogens including *S. marcescens* use biofilms as a barrier to protect themselves from host immune mechanisms as well as against the action of antimicrobials. Further, the biofilm mode of growth weakens antibiotic therapy even in the case of non-resistant strains (Thenmozhi et al. 2009). Since the biofilm formation of *S. marcescens* depends on QS, disruption of biofilm by means of anti-QS compounds will pave the way to enhancing the antibiotic sensitivity of bacterial cells. In this context, we assessed the anti-QS potential of SS4 extract in enhancing the susceptibility of *S. marcescens* towards the tested antibiotics. The results revealed an increased susceptibility of *S. marcescens* towards erythromycin and chloramphenicol when cultivated in the presence of SS4 extract compared with that of the control (Fig. 4). This result

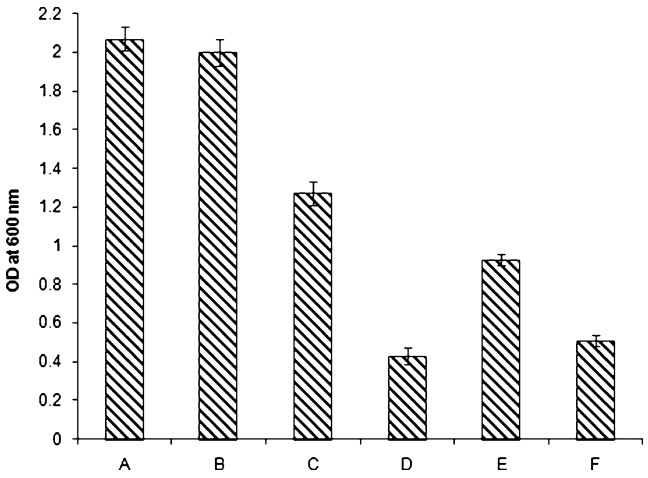

Fig. 4 Effect of *Bacillus* spp. SS4 extract in enhancing the antibiotic susceptibility of *S. marcescens* towards antibiotics. *(A) S. marcescens* control; *(B) S. marcescens* cultivated with SS4 extract; *C*, *D S. marcescens* exposed to erythromycin in the absence (*C*) or presence (*D*) of SS4 extract. *E*,*F S. marcescens* exposed to chloramphenicol in the absence (*E*) or presence (*F*) of SS4 extract

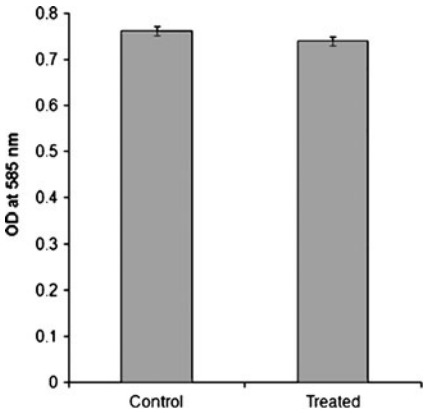

Fig. 5 Mode of action of *Bacillus* spp. SS4 extract involving anti-QS activity against *S. marcescens*. Induction of violacein production in CV026 by acyl homoserine lactone (AHL) extracted from *S. marcescens* culture grown in the absence (control) or presence (treated) of *Bacillus* spp. SS4 extract

clearly reveals the anti-biofilm as well as the anti-QS properties of metabolites present in the SS4 extract in enhancing the antibiotic susceptibility of *S. marcescens*. In an earlier report by Brackman et al. (2008), an increased antibiotic susceptibility in *Vibrio vulnificus* was observed upon treatment with cinnamaldehyde, which also supports the results obtained in the present study.

An AHL extraction assay was performed to reveal the mode of action of SS4 extract in exhibiting anti-QS activity. AHL extracted from *S. marcescens* cultures either treated or untreated with SS4 extract induced the development of violacein production in CV026 without any significant variation (Fig. 5). These results revealed that SS4 extract did not inhibit AHL synthesis in *S. marcescens*. However, the SS4 extract reduced prodigiosin production in *S. marcescens* to a considerable level (Fig. 1). Thus, from the results obtained, it is envisaged that, since intracellular AHL concentrations are similar in treated and untreated cells, the extract might inhibit the binding of AHL to a LuxR-like transcription regulator in *S. marcescens*.

Regarding antibacterial activity, *S. marcescens* cells either treated or untreated with SS4 extract showed no significant variation in growth curve values, indicating that SS4 extract had no effect on the growth of *S. marcescens* (data not shown). As revealed in the present study, SS4 extract led only to a reduction in the pigment production of *S. marcescens* without affecting growth. Further, the extract also exhibited a reduction in the C4–HSL- and C6–HSL-dependent protease and biofilm formation in *S. marcescens*. This result implies that compounds with the ability to inhibit pigment production, virulence and biofilm formation without affecting bacterial growth can be considered as promising QS inhibitors (Choo et al. 2006; Morohoshi et al. 2007; Musthafa et al. 2010). Thus, in conclusion, it is envisaged that the active principle from *Bacillus* spp. SS4 extract involved in the reduction of prodigiosin, protease production and biofilm formation could be developed as a potential antipathogenic compound for use in preventing emerging *S. marcescens* infection.

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