A novel compound from the marine bacterium *Bacillus pumilus* S6-15 inhibits biofilm formation in Gram-positive and Gram-negative species

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Biofilm formation is a critical problem in nosocomial infections and in the aquaculture industries and biofilms show high resistance to antibiotics. The aim of the present study was to reveal a novel anti-biofilm compound from marine bacteria against antibiotic resistant Gram-positive and Gram-negative biofilms. The bacterial extract (50 μg ml⁻¹) of S6-01 (*Bacillus indicus* = MTCC 5559) showed 80–90% biofilm inhibition against *Escherichia coli*, *Shigella flexneri*, *Proteus mirabilis* and S6-15 (*Bacillus pumilus* = MTCC 5560) showed 80–95% biofilm inhibition against all the 10 tested organisms. Furthermore, they also reduced the hydrophobicity index and extracellular polymeric substances (EPS) production. Structural elucidation of the active principle in S6-15 using GC-MS, ¹H NMR, and ¹³C NMR spectral data revealed it to be 4-phenylbutanoic acid. This is the first report of 4-phenylbutanoic acid as a natural product. The purified compound (10–15 μg ml⁻¹) showed potential activity against a wide range of biofilms. This study for the first time, reports a novel anti-biofilm compound from a marine bacterium with wide application in medicine and the aquaculture industry.

**Keywords:** *Bacillus pumilus*; *Bacillus indicus*; anti-biofilm; 4-phenylbutanoic acid; hydrophobicity index; EPS production

**Introduction**

Biofilms are matrices of microbial communities surrounded by extracellular polymeric substances (EPS) (Donlan and Costerton 2002; Shirliff et al. 2002). Many reports have revealed that biofilms have a special clinical relevance (Davies et al. 1998; Davey and O’Toole 2000; Donlan and Costerton 2002; Hall-Stoodley et al. 2004). Biofilm-associated bacteria are a very serious problem in many infections because they show an innate resistance to antibiotics (Ceri et al. 1999), disinfectants (Oie et al. 1996), and clearance by host defenses (Shiau and Wu 1998; Donlan and Costerton 2002). These properties contribute to the persistence and recalcitrance to treatment of many biofilm mediated infections. A defining feature of many biofilm-forming bacteria is the secretion of EPS and it has been shown that by secreting EPS, individual bacteria can both help and harm cells in their neighborhood (Rainey and Rainey 2003; Brockhurst et al. 2006; Brockhurst et al. 2007; Xavier and Foster 2007).

Studies suggest that, after reaching a high cell density, some bacterial species activate polymer secretion, whereas others terminate polymer secretion (Li et al. 2002; Shirliff et al. 2002). EPS secretion is under quorum-sensing control in a number of bacterial species. Biofilm development by several bacteria, including *Pseudomonas aeruginosa* (Davies et al. 1998), *Burkholderia cepacia* (Huber et al. 2001, 2002), *Streptococcus mutans* (Li et al. 2002; Merritt et al. 2003) and others (Lynch et al. 2002; Steidle et al. 2002; Vance et al. 2003) is quorum sensing mediated. Many species, including the pathogen *P. aeruginosa*, activate EPS production at high cell density (Davies et al. 1998; Sakuragi and Kolter 2007). Currently, there are no therapies that effectively target microbial biofilms, due to their resistance against many antibiotics (Gilbert et al. 2002). This emphasizes the need for new antibacterial drugs active not only against planktonic bacteria but also drug resistant biofilms.

Marine bacteria are a rich source of bioactive compounds. Several studies show independently that marine bacteria are capable of producing novel anti-biofilm compound(s) that have not been isolated from terrestrial species (MikhaÁlov et al. 1995; Dobretsov et al. 2006; El-Gendy et al. 2008; Selvin et al. 2009). Recently, the present authors reported for the first time that bacteria isolated from *Acropora digitifera* possess anti-biofilm activity against *Streptococcus pyogenes* (Themmozhi et al. 2009; Nithyanand et al. 2010) and *Staphylococcus aureus* (Bakkiyaraj and Pandian 2010). Furthermore, the present authors reported for the first time...
time that bacteria isolated from Palk Bay [9° 30′ (9.5°) north latitude, 79° 15′ (79.25°) east longitude] sediment possess quorum quenching activity, antibiotic activity (Nithya et al. 2010a; Nithya and Pandian 2010b) and anti-biofilm activity (Nithya et al. 2010b; Nithya and Pandian 2010a) against P. aeruginosa and Vibrio spp.

In this study, this work has been taken a step further in that the strains S6-01 (Bacillus indicus = Microbial Type Culture Collection (MTCC) 5559) and S6-15 (Bacillus pumilus = MTCC 5560) which have shown maximum anti-biofilm activity against P. aeruginosa PA01 (Nithya et al. 2010b) were further screened against biofilm formation, hydrophobicity index (HI) and EPS production in Gram-negative (P. aeruginosa, Shigella boydii, Shigella flexneri, Proteus mirabilis, Escherichia coli, Vibrio parahaemolyticus, Vibrio alginolyticus, Chromobacterium violaceum) and Gram-positive (S. aureus, Streptococcus salivarius) species. Furthermore, purification and structural elucidation of the bioactive compound from S6-15 was undertaken.

Materials and methods

Bacterial strains and bacterial extract preparation

P. aeruginosa (American Type Culture Collection (ATCC) 10145), S. boydii (ATCC 9207), S. flexneri (ATCC 12022), P. mirabilis (ATCC 7002) and E. coli (ATCC 10536) were grown and maintained on LB agar plates at 37°C. C. violaceum (ATCC 12742) was grown and maintained on LB agar plates at 30°C. S. salivarius (ATCC 13419) and S. aureus (ATCC 11632) were grown in Todd-Hewitt agar/broth (THA/THB) and Tryptic Soy agar/broth (TSA/TSB) at 37°C, respectively. V. parahaemolyticus (ATCC 17802) and V. alginolyticus (ATCC 17749) were maintained on Zobell Marine agar/broth (ZMA/ZMB) plates at room temperature. The antibiotic resistance of the test strains is shown in Table S1 (Supplementary material [Supplementary material is available via a multimedia link on the online article webpage]). All culture media were purchased from HiMedia Laboratories, India, in the dehydrated form. The culture extracts of S6-01 and S6-15 were prepared as described in Nithya et al. (2010b).

Biofilm formation assay

The effect of marine bacterial extract on biofilm formation was measured by using the polyvinyl chloride biofilm formation assay. Briefly, overnight cultures of the test organisms (1%) were resuspended in 1 ml of fresh LB, ZMB, THB or TSB medium in the presence and absence of marine bacterial extracts (5–100 μg ml⁻¹). After 24 h incubation, prior to the crystal violet assay, the wells containing the bacterial suspension were analyzed spectrophotometrically (Hitachi-U 2800, Japan) at 600 nm to ensure that there was no antibiotic activity. In the crystal violet assay, the planktonic cells and spent media were discarded and weakly adherent cells were removed through thorough washing twice with deionized water, and allowed to air dry before being stained. The biofilms were stained with 400 μl of 0.4% crystal violet solution (w/v) for 10 min. Subsequently the dye was discarded and the wells were rinsed twice with deionized water. The wells were allowed to dry before solubilization of the crystal violet with 1 ml of absolute ethanol. The optical density was determined at 570 nm (Thenmozhi et al. 2009).

Microscopic observation

For visualization by light microscopy, the biofilms were allowed to grow on glass pieces (1 × 1 cm) placed in 24-well polystyrene plates supplemented with marine bacterial extracts (50 μg ml⁻¹) and incubated for 24 h. The biofilms were stained and inspected with a light microscope (Euromex, model: GE3045, Netherlands) at a magnification of ×400. Visible biofilms were documented with an attached digital camera (Cmx camera, model: DC5000, Netherlands) (Nithya et al. 2010a). The effect of marine bacterial extracts (50 μg ml⁻¹) on biofilm formation was monitored under a Confocal Laser Scanning Microscope (CLSM) (Model: LSM 710) (Carl Zeiss, Germany) after washing with PBS and staining with 0.1% acridine orange. The 488 nm Ar laser and a 500–640 nm band pass emission filter were used to excite and detect the stained cells. CLSM images (N = 20) were obtained from the triplicate of 24 h old control and treated biofilms and processed using Zen 2009 image software (Nithya and Pandian 2010a). The Z-stack analysis (surface topography and three-dimensional architecture) was done with the Zen 2009 software (Carl Zeiss, Germany).

Antibacterial activity

To check whether marine bacterial extracts (50 μg ml⁻¹) have any antibacterial effect against the Gram-positive and Gram-negative species, the well diffusion method was used. In LB or ZM medium or on TH or TS agar plates, bacterial species from overnight cultures were swabbed and 5 mm diameter wells were punched in the middle of swabbed plates. Marine bacterial extracts were loaded into the wells. Double distilled water alone was used as a control. The plates
were incubated overnight at 30°C for *V. alginolyticus* and *V. parahaemolyticus* and at 37°C for the other species. The plates were observed for the zones of clearance. To detect the effect of bacterial extracts on cell density, the spectroscopic method was used. Prior to the crystal violet assay, the wells containing bacterial suspensions were analyzed spectrophotometrically (Hitachi-U 2800, Japan) at 600 nm.

**Bacterial adhesion to hydrocarbon (BATH) assay**
The BATH assay was done using the general protocol described by Zhang and Miller (1992) with the following modifications. Cells were resuspended in LB, ZMB, HB or TSB medium and adjusted to an OD at 600 nm (OD_{600} = 0.8 ± 0.01). The bacterial extract and toluene (1 ml) were added to 2 ml of the adjusted cell suspension (OD_{600} = 0.8 ± 0.01) in a test tube. After vortexing for 1 min, the cell suspensions were incubated at room temperature overnight and the OD of the aqueous phase was measured (OD_{600}). The HI of microbial cells was calculated by the formula described by Serebryakova et al. 2002. The results were expressed as the proportion of the cells which were excluded from the aqueous phase, determined by the equation [(A_0 – A) A_0] × 100, where A_0 and A are the initial and final optical densities of the aqueous phase, respectively (Serebryakova et al. 2002).

**Quantification of EPS**
Determination of EPS was carried out by the total carbohydrate assay. Glass slides immersed in the culture with bacterial extract in 24-well polystyrene plates were incubated for 24 h. After incubation, the glass slides were removed and washed in 0.9% NaCl (0.5 ml). The cell suspensions in 0.9% NaCl (0.5 ml) were incubated in test tubes with an equal volume of 5% phenol (0.5 ml) to which 5 volumes of concentrated H_2SO_4 containing 0.2% of hydrazine sulphate had been added. The mixture was incubated for 1 h in the dark and after centrifugation (10,000g for 10 min), the absorbance was measured at 490 nm (Favre-Bonté et al. 2003).

**Purification and identification of the active compound**

**Thin layer chromatography (TLC)**
The crude extract showing anti-biofilm activity was partially purified by means of TLC plates (Merck, USA) using methanol: water (50:50) solvent system.

**Silica gel column**
The silica gel slurry (Silica gel 60–180 mesh, Sisco Research Laboratories, India) was packed in 20 cm long glass column having a diameter of 2 cm. The partially purified compounds were loaded onto the column. The adsorbed compound was eluted with methanol and water (50:50) under a flow rate of 4 ml min^{-1}. Fractions (1 ml each) were collected separately and tested for activity by bioassay. The active fractions were pooled and evaporated. The fractions showing anti-biofilm activity were further purified by the same column chromatography system, and purity was confirmed by TLC and bioassay. The purity of the active fraction was further confirmed by High Performance Liquid Chromatography (HPLC) and its structure was elucidated on the basis of Gas Chromatography-Mass Spectrometry (GC-MS), ^1^H Nuclear Magnetic Resonance (NMR) and ^13^C NMR spectral data.

**HPLC**
For HPLC analysis, the silica column purified bioactive compound (2 mg) was redissolved in 0.2 ml of methanol and introduced onto a SHIMADZU system (Japan) silica gel column (4.6 × 250 mm). Fractions were eluted isocratically with 50:50 methanol/water (v/v) at a flow rate of 1 ml min^{-1}.

**GC-MS**
For GC/MS, 2 mg of purified active principle from *B. pumilus* S6-15 were dissolved in methanol. A 7890A GC/MS instrument (Agilent Technologies, USA), equipped with SE-30 column (30 m by 0.25 mm inner diameter; film thickness, 0.25 mm), was used for the analysis. The GC oven was held at 150°C for 1 min and then increased to 250°C at 4°C per min. Helium was used as the carrier gas with a flow of 30 ml min^{-1}. The identification of the compounds was based on 90% similarity between the MS spectra of unknown and reference compounds in an MS spectra library.

**NMR**
The pure sample showing anti-biofilm activity was subjected to ^1^H NMR, ^13^C NMR (Bruker Biospin, DMX 500 MHz Switzerland). The sample was dissolved (3 mg for ^1^H NMR (100 mHz) and 10 mg for ^13^C NMR (25 mHz)) in 2 ml of CDCl_3 for NMR analysis.

**Statistics**
All experiments were performed independently in triplicate with pooled samples of biological replicates. Statistical analysis was performed using SPSS. Values were expressed as mean ± SD. A Duncan-ANOVA test with a *P* value of 0.05 being significant was used to compare the parameters between the groups and a
Dunnett-ANOVA test was employed to compare between the tests and control.

Results

Anti-biofilm activity

The ethyl acetate extracts of S6-01 and S6-15 were weighed and their yields were 0.014% (140 µg ml\(^{-1}\)) and 0.09% (900 µg ml\(^{-1}\)), respectively. The percentage inhibition of biofilm formation was calculated for the control and the test bacterial species at extract concentrations of 5–100 µg ml\(^{-1}\). Their biofilm inhibitory concentrations (BICs) were found to be 40–50 µg ml\(^{-1}\) resulting in a significant anti-biofilm activity (\(P < 0.05\)) in the spectroscopic assay (Table 1 and Figure 1). Hence, a BIC of 50 µg ml\(^{-1}\) for S6-01 and S6-15 was used in all assays. In the spectrophotometric assay, S6-01 and S6-15 showed maximum anti-biofilm activity against \(P. \) mirabilis (88%) and \(S. \) flexneri (91%), respectively (Figure 1). Both the extracts showed anti-biofilm activity against all the 10 Gram-positive and Gram-negative species tested, based on light microscopy. CLSM was exclusively used to analyze the surface topography and the three-dimensional architecture of the biofilm formed by the bacterial species in the presence of bacterial extracts S6-01 and S6-15. Both extracts inhibited biofilms at a minimum concentration of 50 µg ml\(^{-1}\) (Figure 2a–c). A significant reduction in biofilm thickness was observed in several species treated with S6-15 extract and in two species for S6-01, and the results are given in Table 2.

Antibacterial activity

In the well diffusion assay, no zone of clearance was observed in plates treated with the bacterial extracts (data not shown). In liquid culture also (after 24 h incubation), no significant difference was observed between the OD values of the control (bacterial suspension without extract) and the treated cells (Figure 3).

BATH assay

The HI is a major determinant of biofilm formation. The bacterial extract S6-01 significantly (\(P < 0.05\)) reduced the cell surface hydrophobicity of Gram-positive species only (\(S. \) aureus and \(S. \) salivarius). However, S6-15 reduced the HI of both Gram-positive and Gram-negative species. It showed maximum reduction against \(P. \) aeruginosa, followed by \(Vibrio\) spp., \(E. \) coli and \(S. \) aureus (Figure 4A).

Quantification of EPS

The extract of S6-01 significantly (\(P < 0.05\)) reduced EPS production of both the Gram-positive and Gram-negative species. The maximum reduction was observed in \(P. \) aeruginosa treated with S6-01 (Figure 4B).

Table 1. Biofilm inhibitory concentration (BIC) of marine bacterial extracts S6-01 and S6-15 against various Gram-negative and Gram-positive species.

| Bacterium            | BIC (µg ml\(^{-1}\)) |
|----------------------|----------------------|
|                      | Treated with S6-01   | Treated with S6-15 |
| \(C. \) violaceum    | 49 ± 0.57            | 42 ± 0.57          |
| \(E. \) coli         | 41 ± 1.00            | 44 ± 0.00*         |
| \(P. \) aeruginosa    | 48 ± 0.57            | 46 ± 0.00*         |
| \(P. \) mirabilis     | 47 ± 1.00            | 43 ± 1.00          |
| \(S. \) aureus        | 42 ± 0.57            | 40 ± 0.00*         |
| \(S. \) boydii        | 43 ± 0.57            | 41 ± 0.57          |
| \(S. \) flexneri      | 44 ± 0.57            | 42 ± 0.00*         |
| \(S. \) salivarius    | 46 ± 1.00            | 40 ± 0.57          |
| \(V. \) alginolyticus | 43 ± 0.00*           | 45 ± 1.00          |
| \(V. \) parahaemolyticus | 50 ± 0.57      | 40 ± 0.00*         |

Note: *indicates statistically significant values (\(P < 0.05\)).
S6-15 reduced EPS production only in *V. alginolyticus* and *V. parahaemolyticus* and did not show any effect against Gram-positive species.

Purification and identification of the anti-biofilm compound from S6-15

The anti-biofilm active compound was partially purified by ethyl acetate extraction of culture supernatant from S6-15 and 500 mg of the crude active principle were obtained. The crude compound was purified by TLC. The bands were extracted by ethyl acetate (100 mg) and checked for their anti-biofilm activity. Further purification, using a silica column, resulted in the recovery of 50 mg upon fraction collection and evaporation. TLC was carried out once again to check the Rf values and homogeneity. In HPLC, the active fraction displayed a single peak with a retention time of 3.087 min (Figure 5A). In the

| Bacterium               | Untreated control | Treated with S6-01 | Treated with S6-15 |
|-------------------------|-------------------|--------------------|--------------------|
| *C. violaceum*          | 32.5 ± 0.27       | 18.5 ± 0.19        | 11.3 ± 0.15        |
| *E. coli*               | 27.3 ± 0.14       | 15.3 ± 0.14        | 12.6 ± 0.09*       |
| *P. aeruginosa*         | 25.9 ± 0.19       | 17.6 ± 0.11*       | 14.8 ± 0.08*       |
| *P. mirabilis*          | 28.7 ± 0.36       | 13.6 ± 0.15        | 12.8 ± 0.10*       |
| *S. aureus*             | 35.8 ± 0.25       | 16.4 ± 0.23        | 10.4 ± 0.05*       |
| *S. boydii*             | 29.4 ± 0.21       | 14.3 ± 0.13        | 13.7 ± 0.17        |
| *S. flexneri*           | 22.2 ± 0.19       | 13.4 ± 0.12        | 9.9 ± 0.94         |
| *S. salivarius*         | 19.8 ± 0.10       | 7.2 ± 0.05*        | 5.2 ± 0.10         |
| *V. alginolyticus*      | 26.6 ± 0.23       | 12.9 ± 0.11        | 7.5 ± 0.05*        |
| *V. parahaemolyticus*   | 23.5 ± 0.22       | 11.7 ± 0.14        | 8.4 ± 0.04*        |

Note: *indicates statistically significant values (*P* < 0.05).
GC analysis too, a predominant single peak was observed with a retention time of 13.957 min having a relative abundance of 85% (Figure 5B). In the MS analysis, the peaks corresponded to a molecular formula of C\textsubscript{10}H\textsubscript{12}O\textsubscript{2}, i.e. a carboxylic acid derivative (4-phenylbutanoic acid) (Figure 5C). Furthermore, the \textsuperscript{1}H NMR spectra (11.36, 7.24, 2.66, 2.39 and 1.95) and the \textsuperscript{13}C NMR spectra (180.06, 141.20, 128.26, 126.46, 34.00, 33.48 and 26.45) confirmed the structure of the purified active principle as 4-phenylbutanoic acid (Figure 6).

Anti-biofilm activity of the purified compound

The anti-biofilm activity of the purified compound was tested against both Gram-positive and Gram-negative species as per the protocol used for crude extracts. The BIC was determined as 10–15 µg ml\textsuperscript{-1}. The antibacterial activity of the purified compound was tested against both Gram-positive and Gram-negative species at the BIC. No antibacterial activity was observed (data not shown).

Discussion

Marine bacteria are a rich source of bioactive compounds and well known for their antibiotic activity. However, reports are scanty for their anti-biofilm activity (Thenmozhi et al. 2009; Bakkayaraj and Pandian 2010; Nithya et al. 2010b; Nithya and Pandian 2010a; Nithyanand et al. 2010). Furthermore, marine bacterial extracts are yet to be tapped for their anti-biofilm activity against \textit{C. violaceum}, \textit{P. mirabilis}, \textit{S. boydii} and \textit{S. flexneri}. In this study, the anti-biofilm property of S6-01 and S6-15 extracts was evaluated against both Gram-positive and Gram-negative biofilms. When bacteria adhere to a surface, growth occurs rapidly. During the first few hours the adhesion is reversible (Marshall 1994; Hööby et al. 2001). Hence, preventing bacterial adhesion at the preliminary stage itself can reduce the risk of biofilm formation. In the current study, the marine bacterial extracts reduced biofilm formation up to 90% at a concentration of 50 µg ml\textsuperscript{-1}. The bacteria once adhered, begin to form microcolonies and bacterial genes are activated by
both the adhesion process and local environment factors (Costerton et al. 1994). From the biofilm inhibition assay and microscopic observation, it is clear that the marine bacterial extracts S6-01 and S6-15 reduced and dispersed the microcolonies. Another important step in biofilm development is the formation of the characteristic biofilm architecture (You et al. 2007). It is obvious from the microscopic observation that the bacterial extracts (S6-01 and S6-15) disturbed the architecture of the biofilm by loosening the microcolonies. Inhibition and dispersion of biofilms provide an attractive anti-biofilm strategy against all Gram-positive and Gram-negative species, most of which are resistant to multiple antibiotic agents. The architecture of all the biofilms observed by CLSM further confirmed that the bacterial extracts substantially reduced the thickness of the biofilms.

Quorum sensing (QS) and biofilm formation are largely interconnected for bacterial social life (Nadell et al. 2008). Biofilm formation is the foremost factor responsible for drug resistance in many pathogens including Gram-positive bacteria (Weigel et al. 2007). Furthermore, biofilm forming bacteria develops resistance to antibacterials more commonly than other pathogens (Weigel et al. 2007; Bakkiyaraj and Pandian 2010). This study mainly focused on the compounds that had no effect on the growth of bacteria but effectively controlled their virulence by acting against biofilm formation and QS. In the antibacterial activity tests, neither of the bacterial extracts (S6-01 and S6-15) exhibited any antibacterial activity. From this study, it is envisaged that the bacterial extract interfered in one or more of the steps involved in biofilm formation but did not inhibit the growth of the bacterial species at the

Figure 5. (A) HPLC analysis of silica column purified compound of *B. pumilus* S6-15 marine bacterial extract; (B) GC-spectra of a purified antibiofilm compound of *B. pumilus* S6-15; (C) by means of mass spectral analysis, the compound was identified as 4-phenylbutanoic acid.

Figure 6. $^1$H NMR (A) and $^{13}$C NMR (B) spectra of antibiofilm compound from *B. pumilus* S6-15.
tested concentration (50 µg ml⁻¹). The biofilm inhibiting activity of the bacterial extracts observed in the current study without any antibacterial activity may have an impact on medical applications. Interfering with biofilms is expected to overcome resistance because the effect is not lethal to the bacterium and would only restore sensitivity to antibiotics or allow clearance by the host immune system.

Marine bacterial extracts S6-01 and S6-15 showed 69–89% biofilm inhibition against the Gram-positive bacteria *S. aureus* and *S. salivarius*. The result obtained in this study is very comparable to the previous study wherein a marine actinomycete (CAA-3) reduced biofilm formation in *S. aureus* by 80% at a concentration of 100 µg ml⁻¹ (Bakkiyaraj and Pandian 2010). Barboza et al. (2005) reported that fluorides inhibit biofilm formation of *S. salivarius* at a concentration of 0.1–0.5 mM in pH dependent manner. A chemically diverse library of TAGE-triazole conjugates have been found to possess the ability to inhibit biofilm formation by *S. aureus* (Huggiens et al. 2009) and gentamycin with farnesol as an adjuvant therapeutic agent prevents biofilm-related infections in *S. aureus* (Jabra-Rizk et al. 2006). However, none of these studies have quantified the biofilm inhibition.

*Shigella* has recently been implicated in food borne outbreaks, specifically *S. boydii*. However, as yet, no biofilm inhibitors have been reported against *Shigella*. This is the first report where the bacterial extract S6-15 inhibited biofilm formation by *S. boydii* up to 88%, and *S. flexneri* up to 91%. The marine bacterial extracts S6-10 and S6-15 also inhibited biofilm formation in *V. parahaemolyticus* and *V. alginolyticus*. The results obtained in the present study are in consonance with a previous report where the marine actinomycete (*Streptomyces* sp. at 2.5% v/v) inhibited biofilm formation by *Vibrio* spp. up to 99.3% (You et al. 2007). To date, there are only very few reports of the inhibition of biofilm formation by *Vibrio* spp. (You et al. 2007; Nithya and Pandian 2010a). The multifunctional marine bacterial extracts S6-01 and S6-15 with promising anti-biofilm activity could be used in marine aquaculture to inhibit the formation and dispersion of *Vibrio* biofilm.

Cell surface charge and cell surface hydrophobicity play a crucial role in bacterium–host cell interactions (Swiatlo et al. 2002). There are several reports regarding plant extracts interfering with the hydrophobicity of Gram-negative bacteria (Türi et al. 1997; Annuk et al. 1999) and Gram-positive bacteria (Nostro et al. 2004; Razak et al. 2006) and thereby inhibiting biofilm formation. In the present study also the bacterial extracts S6-15 and S6-01 reduced the HI of both Gram-negative and Gram-positive bacteria at a concentration of 50 µg ml⁻¹. These results agree well with the previous report where sub-BICs of coral associated bacterial extracts were found to be optimal for inhibition of hydrophobicity in *S. pyogenes* (Thenmozhi et al. 2009). Biofilms can produce cyclic glucose polymers, known as periplasmic glucans that can bind to antibiotics, such as tobramycin, sequestering them in the periplasm and preventing their antimicrobial action (Mah et al. 2003). Hence, inhibition of the HI will expose the bacterial biofilm to antibiotics and that in turn will facilitate the eradication of the biofilm.

According to a recent report, EPS leads to alterations in biofilm architecture that correlate with an increased resistance of the cells to osmotic and oxidative stresses as well as killing by biocides such as chlorine (Yildiz and Visick 2009). In the present study, the bacterial extracts inhibited EPS production by both Gram-positive and Gram-negative species by 80–95%. To date, no bacterial extract is reported to have inhibited the production of EPS by Gram-positive bacteria, which is very important for biofilm formation. Capsular polysaccharides are the major reason for methicillin resistance in *S. aureus* (Smith et al. 2010). Hence, reducing EPS production may make the biofilm more vulnerable to antibiotics and the bacterial extracts from the present study may serve as potential compounds to inhibit methicillin resistant *S. aureus* (MRSA). Even though S6-01 and S6-15 showed anti-biofilm activity against all the Gram-positive and Gram-negative biofilms tested, S6-15 showed >80% anti-biofilm activity against Gram-positive biofilms and reduced the HI of both Gram-positive and Gram-negative biofilms. Furthermore, it reduced the EPS production by *Vibrio* sp., which is an aquaculture pathogen. Hence the extract of S6-15 was taken for further purification and characterization.

To the authors’ knowledge, this is the first report showing the production of phenylbutanoic acid, an aromatic short chain fatty acid with the functions of a molecule chaperon by *B. pumilus*. A phenylbutanoic acid concentration ranging from 10 to 15 µg ml⁻¹ was needed to inhibit biofilm formation by both Gram-positive and Gram-negative species. The purified compound showed promising broad spectrum anti-biofilm activity. Furthermore, it disrupted the biofilm architecture by reducing the HI and EPS production. Although different biological tests were applied, no antibiotic activity was detected. In a previous report, a link between oncogene-mediated suppression of transcription and recruitment of histone deacetylases (HDAC) into a nuclear complex has been established. 4-phenylbutyric acid (PBA) reverses this suppression by specific inhibition of HDAC activity, leading to histone hyperacetylation, chromatin relaxation, and enhanced transcription (Lea et al. 1999). In a recent
report (Yao et al. 2010), intra-peritoneal injection of PBA, an aromatic short chain fatty acid with the functions of a molecule chaperon, protected rats from electron convulsion (ECS)-induced memory deficits. From these studies it was clear that PBA was not toxic to rats. In the light of the above mentioned studies, the PBA isolated from the marine bacterial isolate B. pumilus S6-15 with broad spectrum anti-biofilm activity could serve as a valuable tool for industrial and medical applications.

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