

## ORIGINAL ARTICLE

**Attenuation of *Pseudomonas aeruginosa* virulence by marine invertebrate-derived *Streptomyces* sp.**D.N. Naik<sup>1</sup>, S. Wahidullah<sup>1</sup> and R.M. Meena<sup>2</sup><sup>1</sup> Bio-Organic Chemistry Laboratory, CSIR-National Institute of Oceanography, Dona Paula, Goa, India<sup>2</sup> Biological Oceanography Division, CSIR-National Institute of Oceanography (CSIR), Dona Paula, Goa, India

**Significance and Impact of the Study:** This is the first report not only on marine sponge-associated *Streptomyces* for anti-QS in *Ps. aeruginosa* but also on cinnamic acid and proline-derived linear dipeptides proline-glycine as QS inhibitors. The results reveal that marine-derived actinomycetes may not only play a role in the defensive mechanism of their host but also lead to new molecules useful in the development of novel antivirulence drugs.

**Keywords**

actinomycetes, cinnamic acid, linear dipeptides, marine invertebrates, *Pseudomonas aeruginosa*, quorum sensing.

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**Abstract**

The study aimed to discover quorum sensing (QS) inhibitors from marine sponge-derived actinomycetes and analyse its inhibitory activities against QS-mediated virulence factors in *Pseudomonas aeruginosa*. Seventy-two actinomycetes isolated from marine invertebrates collected from the western coast of India were screened against the QS indicator strain *Chromobacterium violaceum* CV12472. Methanol extracts of 12 actinomycetes showing inhibition of violacein production were accessed for downregulation of QS-mediated virulence factors like swarming, biofilm formation, pyocyanin, rhamnolipid and LasA production in *Ps. aeruginosa* ATCC 27853. The isolates NIO 10068, NIO 10058 and NIO 10090 exhibited very good anti-QS activity, with NIO 10068 being the most promising one. Mass spectrometric analysis of NIO 10068 methanol extract revealed the presence of cinnamic acid and linear dipeptides proline-glycine and N-amido- $\alpha$ -proline in the active extract. Detailed investigation suggested that although linear dipeptide Pro-Gly is to some extent responsible for the observed biological activity, cinnamic acid seems to be the main compound responsible for it. Marine-derived actinomycetes are a potential storehouse for QS inhibitors.

**Introduction**

Infectious diseases are still a major scourge of human life, and recent emergence of multidrug resistance to antibiotics by bacteria due to genetic mutation or gene acquisition has left physicians with little recourse for the treatment of what were once routine infections. A number of novel strategies have been developed to tackle such situation, and one such novel development in this field is 'Quorum Quenching (QQ) or Quorum sensing Inhibition (QSI)'. QS is a bacterial cell-cell communication strategy used by both Gram-positive and Gram-negative bacteria, which help them to regulate diverse physiological processes (Miller and Bassler 2001). Targeting QS signifies blocking intercellular communication, which will attenuate virulence and not kill the microbe. This feature should

greatly reduce the emergence of resistance phenotypes often associated with the overuse of antibacterial agents.

In this work, *Pseudomonas aeruginosa* (ATCC 27853) has been used for virulence inhibition assays as the reference strain. The nosocomial human pathogen *Ps. aeruginosa* is the major cause of ventilator-associated pneumonia (VAP) in intubated patients, chronic lung infections such as cystic fibrosis (CF) and urinary tract infections in immunocompromised hosts. These pathological effects of *Ps. aeruginosa* are attributed to its various characteristics including virulence factors such as motility, biofilm formation, pyocyanin pigment formation, production of enzymes such as elastases and proteases all controlled by QS (Smith and Iglewski 2003).

Marine organisms and their associated bacteria are known to inhibit QS-mediated virulence factors in

various monitor strains (Teasdale et al. 2009; Kanagasabhapathy et al. 2009; Nithya and Pandian 2010; Nithya et al. 2010). The aim of this investigation was to study actinomycetes associated with marine invertebrate for potential QSI against *Ps. aeruginosa*. Till date, only inhibition of biofilm formation in *Vibrio* sp. by *Streptomyces albus* from marine sediments (You et al. 2007) and inhibition of QSI in *Chromobacterium violaceum* ATCC 12472 and biofilm formation in *Staphylococcus* sp by *S. akiyoshiensis* from mucus of the coral *Acropora digitifera* (Bakkiyaraj and Pandian 2010) are reported. To the best of our knowledge, this is the first report on marine invertebrate-associated *Streptomyces* for QSI in *Ps. aeruginosa*. Chemical investigation of NIO 10068 indicated that cinnamic acid and proline-derived linear dipeptides proline-glycine and N-amido- $\alpha$ -proline are constituents of the active fraction. This is also the first report on cinnamic acid and linear dipeptide as QS inhibitors.

**Results**

Results of the above-mentioned study indicated that of the three promising extracts selected, pronounced activity was observed in NIO 10068. Chemical investigation of NIO 10068 led to the identification of cinnamic acid and linear dipeptides Pro-Gly and N-amido- $\alpha$ -proline on the basis of tandem mass spectra.

**Violacein inhibition assay**

In the study, a panel of 72 actinomycetes isolated from marine invertebrates collected from the western coast of India were screened for their ability to inhibit violacein pigment production in the QS indicator strain *C. violaceum* CV12472 using disc diffusion assay. Twelve actinomycetes extracts exhibited QSI, as indicated by the inhibition of pigmentation around the disc. Among them, the effect of methanol extracts of NIO 10058, NIO 10068 and NIO 10090 was most pronounced with violacein pigment inhibition zones of 15, 28 and 21 mm diameter, respectively, as compared to no inhibition zone in pigmentation exhibited by the control disc (Supporting Information). The active extracts showing QSI were further evaluated for their potential inhibition of QS-mediated virulence factors (motility, pyocyanin pigmentation, biofilm formation, rhamnolipid production and LasA protease) in *Ps. aeruginosa* (ATCC 27853) (Table 1).

**Motility inhibition assay**

In this investigation, methanol extract of NIO 10068 and NIO 10058 inhibited swarming motility in *Ps. aeruginosa* by 90 and 85% ( $P < 0.0005$ ), respectively. NIO 10090

**Table 1** Effect of crude extracts of strains NIO 10058, NIO 10068, NIO 10090, cinnamic acid, Pro-Gly and cinnamic acid + Pro-Gly on QS-mediated virulence factors (swarming, pyocyanin pigmentation, biofilm formation, rhamnolipid production and LasA) in *Pseudomonas aeruginosa* and violacein production in *Chromobacterium violaceum* CV12472

Strain	Virulence parameters (% inhibition) at 0.1 mg ml <sup>-1</sup>						Effect on the growth of test organisms		
	Swarming motility	Pyocyanin pigmentation	Biofilm formation	Rhamnolipid production	LasA	Effect on violacein production in <i>C. violaceum</i> CV12472 (diameter of zone of inhibition in mm)	<i>C. violaceum</i> CV12472	<i>Ps. aeruginosa</i>	
NIO 10058	85	39	47	52	37	15	-	-	
NIO 10068	90	45	61	71	43	28	-	-	
NIO 10090	65	34	37	47	26	21	-	-	
Cinnamic acid	77	95	30	76	31	17	-	-	
Pro-Gly	27	20	15	12	14	4	-	-	
Cinnamic acid + Pro-Gly	51	62	9	26	20	10	-	-	

extract inhibited swarming by 65% ( $P < 0.0005$ ) (Supporting Information). NIO 10068 reduced swimming and twitching motilities by 20 and 15%, respectively. Extracts NIO 10058 and NIO 10090 did not show any significant inhibition in swimming and twitching motilities (Fig. 4c).

#### Pyocyanin inhibition assay

We tested the effect of actinomycete extracts on pyocyanin pigment production in *Ps. aeruginosa*. Data suggest that NIO 10068 extract showed inhibition of pyocyanin pigment formation by 45% ( $P < 0.005$ ), whereas at the same concentration NIO 10058 and NIO 10090 showed inhibition of 39 and 34% ( $P < 0.005$ ), respectively (Table 1).

#### Static biofilm inhibition assay

The effect of actinomycete methanol extracts on biofilm formation in *Ps. aeruginosa* was assessed via static biofilm inhibition assay. NIO 10068 extract was the most effective with an inhibition of 67% ( $P < 0.005$ ) biofilm formation in *Ps. aeruginosa*, whereas NIO 10058 and NIO 10090 showed 47 and 37% ( $P < 0.005$ ) inhibition.

#### Rhamnolipid inhibition assay

Reduction in the rhamnolipid production by actinomycete extracts was observed in *Ps. aeruginosa*. NIO 10068 extract showed inhibition of rhamnolipid production by

45% ( $P < 0.005$ ), whereas at the same concentration, NIO 10058 and NIO 10090 showed inhibition of 32 and 28% ( $P < 0.005$ ), respectively.

#### LasA staphylolytic assay

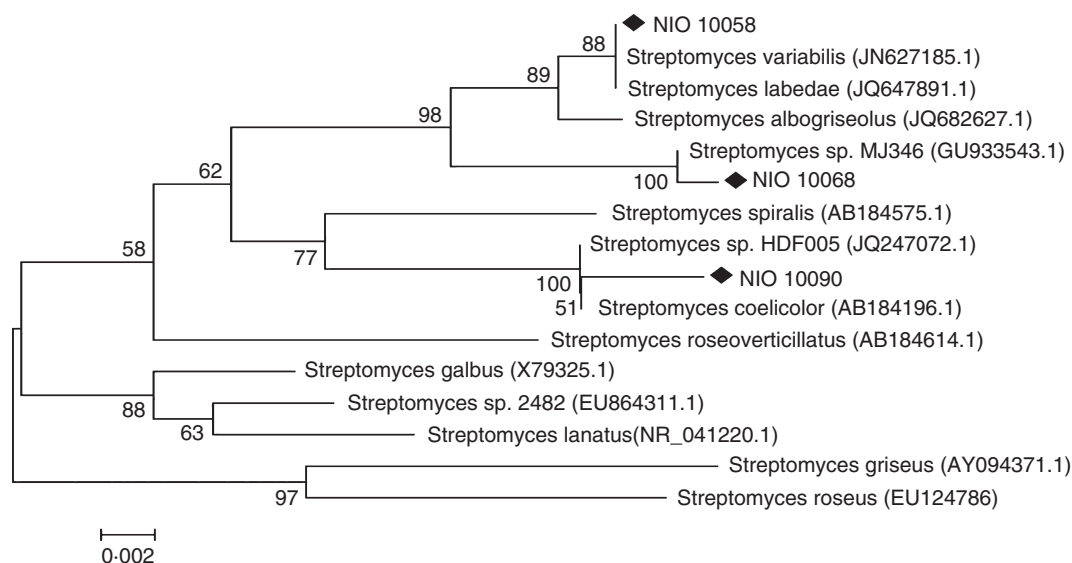
Inhibition of LasA protease in *Ps. aeruginosa* by actinomycetes extract was assessed via LasA staphylolytic assay. NIO 10068 extract exhibited inhibition of LasA protease production by 43% ( $P < 0.005$ ), whereas at the same concentration NIO 10058 and NIO 10090 showed inhibition of 34 and 37% ( $P < 0.005$ ), respectively.

#### Phylogeny of active isolates

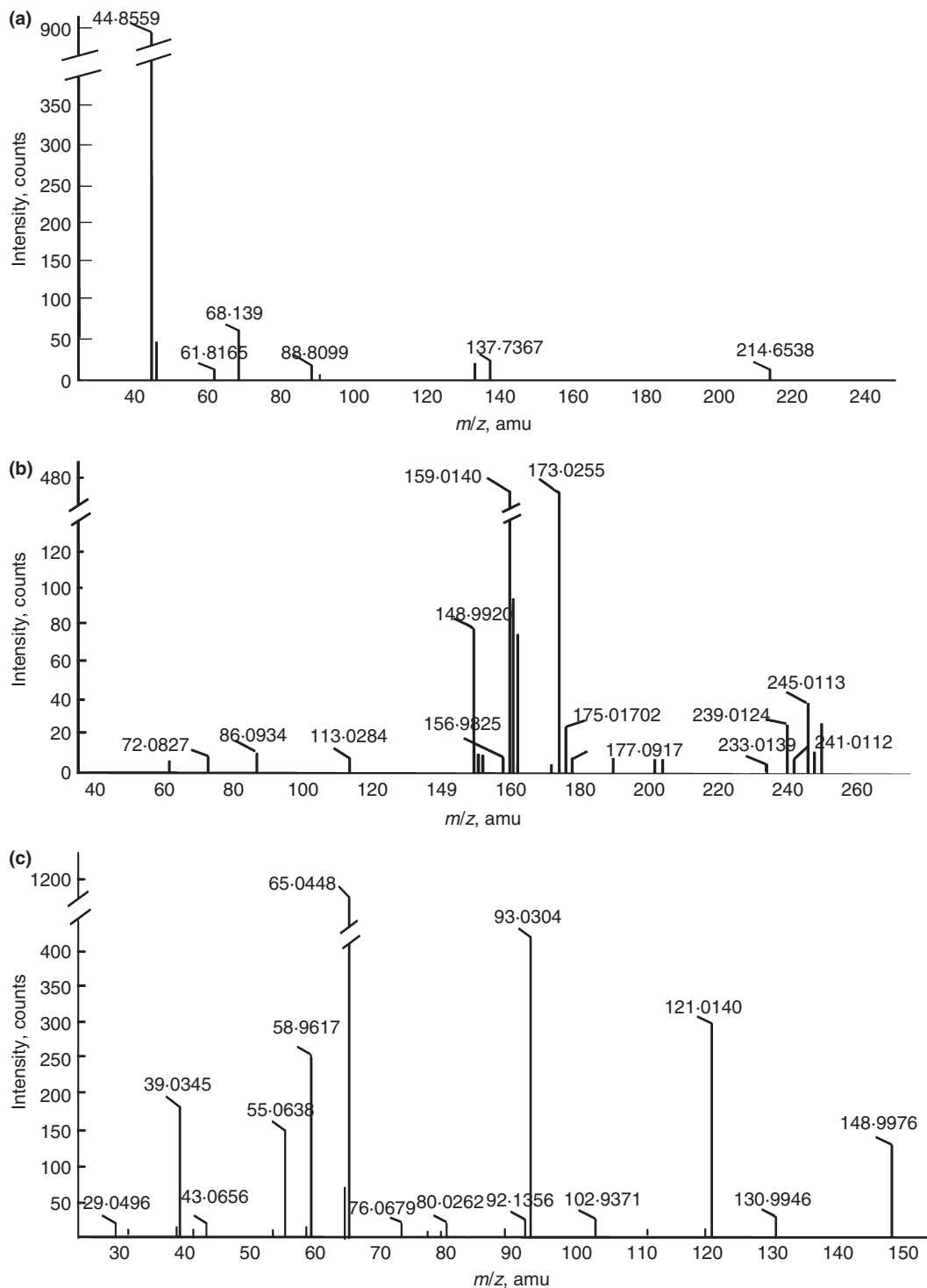
Seventy-two isolates were used in this study, but only the most potent strains were subjected to phylogenetic analysis. These include isolates NIO 10058, NIO 10068 and NIO 10090, which were assigned to different species of the genus *Streptomyces*. Neighbour-joining phylogenetic tree shows maximum identity of NIO 10058 towards *S. variabilis* and *S. labedae*, NIO 10068 to *Streptomyces* sp. MJ346 and NIO 10090 to *S. coelicolor* (Fig. 1).

#### ESI-MS analysis of NIO 10068

The most promising isolate (NIO 10068) was further investigated for the identification of active principle using ESI-MS, a highly sensitive technique for the identification



**Figure 1** Phylogenetic tree showing the relationship among actinobacterial 16S ribosomal RNA gene sequences isolated from marine invertebrates with reference sequences obtained through BLAST analysis. The tree includes three actinomycetes sequences (NIO 10058, NIO 10068 and NIO 10090) and 13 reference sequences. The actinomycetes sequences are shown by NIO codes which are marked and GenBank sequence are shown by species followed by a GenBank accession number in parentheses. Scale bar represents the number of changes per base position.



**Figure 2** (a) Negative ionization ESI-MS of crude extract of NIO 10068 indicating presence of salicylic acid anions at  $m/z$  137. (b) Positive ionization ESI-MS of methanol fraction of NIO 10068. (c) Positive ionization ESI-MS/MS of cinnamic acid.

of molecules present in traces in crude biological mixtures without prior purification. ESI-MS profile of the most active extract (NIO 10068) in both positive and negative

ionization mode showed it to be a mixture of multiple components. Negative ESI-MS profile (Fig. 2a) displayed a deprotonated molecule  $[M-H]^-$  at  $m/z$  137.7. Although

this molecular mass is close to that of salicylic acid, it was found to be a different molecule as the actual molecular mass of deprotonated molecule is 137.0775, and the fragment at  $m/z$  93.0719 resulting from its decarboxylation is also not observed. This ruled out presence of salicylic acid. The anion  $[M-H]^-$  at  $m/z$  45 observed in Fig. 2a has been assigned to formic acid (HCOOH), probably originated from the carboxylic acid group. Same fraction in positive ionization mode (Fig. 2b) displayed several signals of which we could arrive at the structure of three components having protonated ions  $[M+H]^+$  at  $m/z$  173,  $m/z$  159 and  $m/z$  149. Tandem mass analysis of the signal at  $m/z$  173 yielded fragments that led to the identification of the molecule as linear dipeptide proline–glycine (Fig. 3a). Similarly, fragmentation observed for the peak at  $m/z$  159 was well in agreement with the N-amido- $\alpha$ -proline structure assigned to the molecule (Fig. 3b), and the compound with molecular mass of 148 was identified as cinnamic acid (Fig. 2c). Identified as cinnamic acid (Fig. 2c) by TLC comparison with standard (aluminium-backed silica gel plates 60 F254; petrol:ethyl acetate:acetic acid 75 : 25 : 1 solvent system and visualization under UV at 254 nm). ESI-MS fragmentation observed for cinnamic acid as well as Pro–Gly dipeptide was well in agreement with the data for commercially available reference standards, cinnamic acid (Aldrich) and Pro–Gly (Bachem).

## Discussion

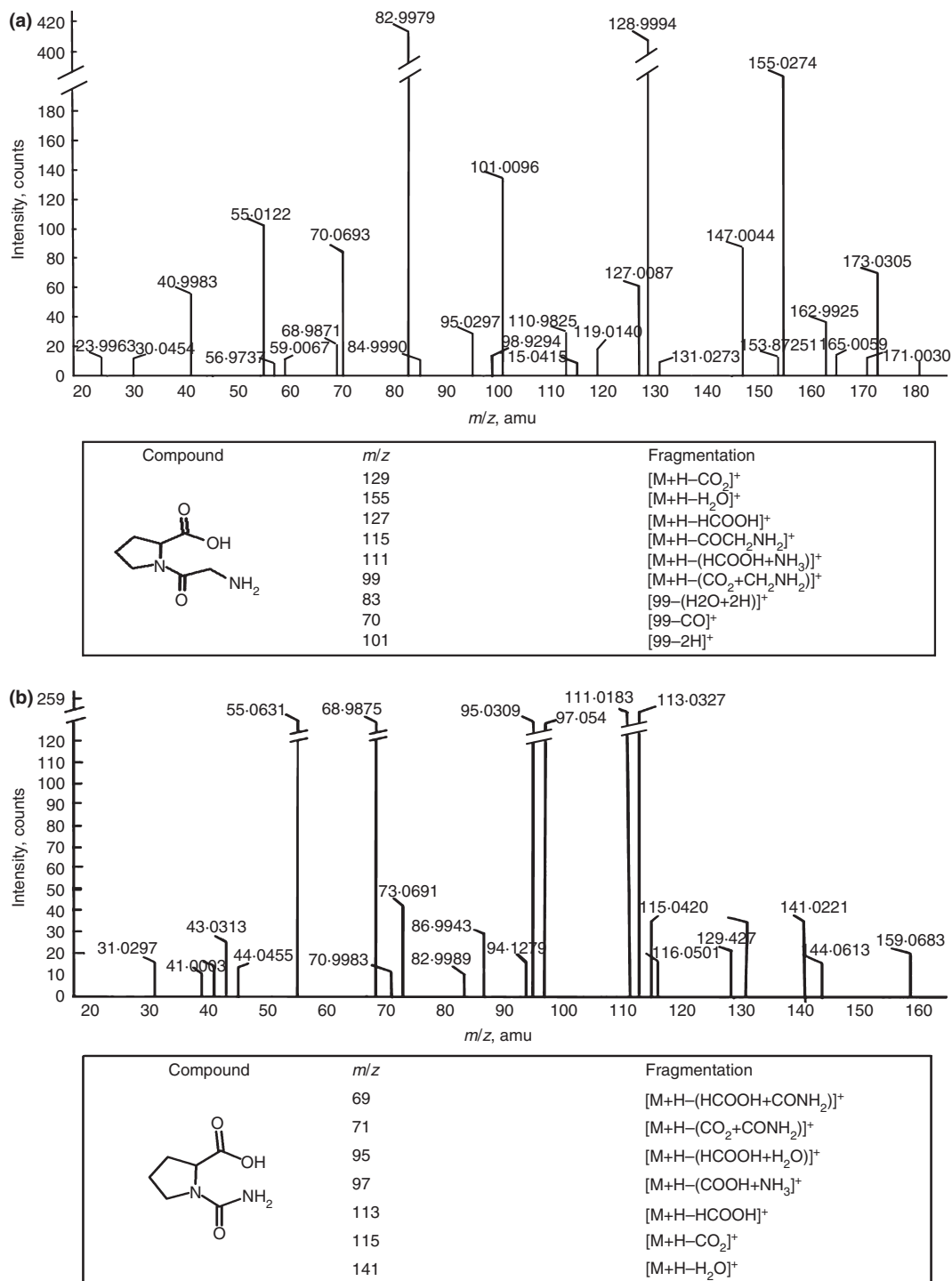
In this study, 72 marine actinomycetes were isolated and screened for anti-QS activities against the *C. violaceum*. Twelve promising candidates (Fig. 4a) were selected for further anti-QSI activities against *Ps. aeruginosa* ATCC 27853. *Streptomyces* sp. NIO 10058, NIO 10068 and NIO 10090 were found to exhibit significant activity with NIO 10068 being the best one in inhibiting virulence factors in reference strain *Ps. aeruginosa*. Numerous studies address the importance of bacterial motility in initial contact and biofilm formation on a surface. *Pseudomonas aeruginosa* exhibits three types of motilities: swarming, swimming and twitching. Swarming and swimming motilities are mediated by flagella, whereas twitching motility is mediated by type IV pili. Swarming is a specialized state where cells differentiate into elongated hyperflagellated swarmer cells. It is associated with production of wetting agents, which enables them to migrate smoothly on viscous surfaces and results in the formation of a monolayer in early stages of biofilm development (Shrout *et al.* 2006). Swarming has been reported to confer resistance against a variety of antibiotics in *Salmonella enterica* serovar Typhimurium (Kim *et al.* 2003). Recent reports also suggest that like biofilms, swarm cells also

show a higher degree of resistance to antibiotics than planktonic cells (Lai *et al.* 2009). In the present study, methanol extracts of NIO 10058, NIO 10068 and NIO 10090 effectively reduced swarming motility and biofilm formation in the reference strain as indicated (Table 1).

Rhamnolipids have been detected in sputum from patients with cystic fibrosis and are known to lyse polymorphonuclear leucocytes (PMNs) and monocyte-derived macrophages, resulting in necrotic cell death (McClure and Schiller 1992; Jensen *et al.* 2007). They also play a role in maintenance of mushroom-shaped macrocolonies and open channels in a mature biofilm. Rhamnolipids also play a role in overcoming surface tension and allow swarming motility of *Ps. aeruginosa* (Caiazza *et al.* 2005). Extract of NIO 10068 reduced rhamnolipid production to the extent of 71%. Extracts of other two actinomycetes also showed significant reduction in this wetting agent production.

LasA (Elastase A), also known as staphylolysin, is one of the most abundant of at least four secreted endopeptidases of *Ps. aeruginosa*. The mature LasA is a 20-kDa protein both with low elastolytic and high staphylolytic activities. LasA increases significantly the elastolytic activity of other proteases, including that of LasB in the establishment of a *Ps. aeruginosa* infection. In addition to the protease activity of LasA against substrates like elastin, the protease possesses staphylolytic activity. Besides the mentioned activities, LasA has a particular influence on the shedding process in the host tissue which is of particular importance to enhance pathogenesis of *Ps. aeruginosa* (Hoge *et al.* 2010). The LasA activity was reduced by all the extracts screened, with considerable reduction (43%) being exhibited by NIO 10068. NIO 10058 and NIO 10090 reduced LasA activity by 37 and 26%, respectively.

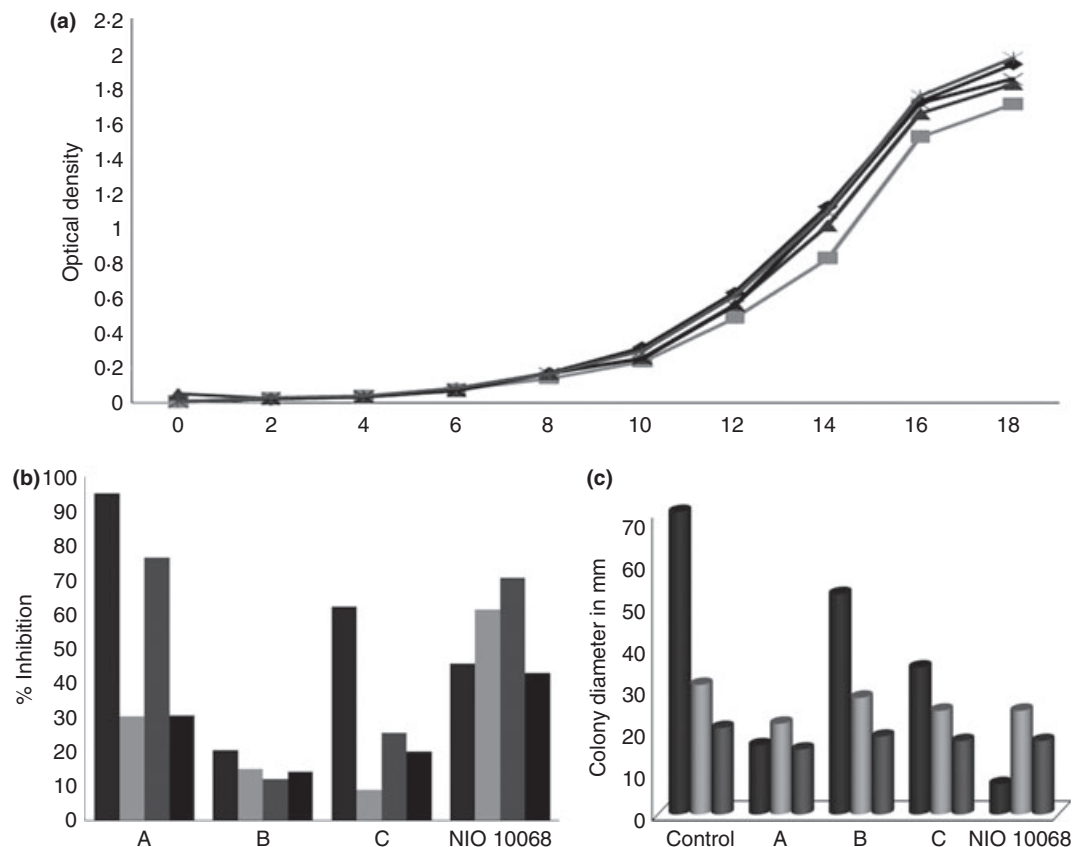
Earlier reports indicate the importance of pyocyanin in *Ps. aeruginosa* virulence. Pyocyanin is a blue-coloured pigment produced by *Ps. aeruginosa* found in the sputum of patients suffering from cystic fibrosis, suggesting its role in pulmonary tissue damage observed with chronic lung infections (Hassett *et al.* 1992). The pigment also confers a selective advantage to *Ps. aeruginosa* as it is bactericidal for many species and helps in elimination of competing micro-organisms (Stephen and Hawkey 2006). Pyocyanin is also involved in upregulation of QS-controlled genes such as mexGHI-opmD, which are involved in efflux and iron acquisition (Dietrich *et al.* 2006). The active extracts (NIO 10058, 10068, 10090) significantly reduced pyocyanin pigment production in *Ps. aeruginosa* (Table 1). Both swarming and pyocyanin are produced and regulated by the *rhl* system in *Ps. aeruginosa* PAO1. Therefore, collectively inhibition of *Ps. aeruginosa* swarming motility and pyocyanin production suggests the presence of *rhl* inhibitor.



**Figure 3** (a) Positive ionization ESI-MS/MS of the proline-glycine with [M+H]<sup>+</sup>- 173. (b) Positive ionization ESI-MS/MS of N-amido-α-proline with [M+H]<sup>+</sup>- 159.

As mentioned earlier, chemical analysis of the most effective extract of *Streptomyces* sp. NIO 10068 using ESI-MS/MS led to the identification of linear dipeptides, proline-glycine and N-amido-α-proline, and the aromatic

acid, cinnamic acid, as the active constituents. In the current study, cinnamic acid, at subinhibitory concentration (<0.5 mg ml<sup>-1</sup>), was found to affect the QS-related virulence factors in *Ps. aeruginosa* and was antibacterial at



**Figure 4** (a) Effect of crude extract of NIO 10068, Cinnamic acid (A), Pro-Gly (B) and Cinnamic acid + Pro-Gly (C) on the growth of *Pseudomonas aeruginosa*. (b) Effect of crude extract of NIO 10068, Cinnamic acid (A), Pro-Gly (B) and Cinnamic acid + Pro-Gly (C) on QS mediated virulence factors (Pyocyanin pigmentation, Biofilm formation, Rhamnolipid production and Las A) in *Ps. aeruginosa*. (c) Effect of crude extract of NIO 10068 and pure compounds, Cinnamic acid (A), Pro-Gly (B) and Cinnamic acid + Pro-Gly (C) on motility (swarming, swimming and twitching) in *Ps. aeruginosa*. (◆) Control; (■) A; (▲) B; (◄) NIO 10068. (■) Pyocyanin; (◄) Biofilm; (■) Rhamnolipid; (■) Las A. (■) Swarming; (■) Swimming; (■) Twitching.

MIC ( $\geq 0.5 \text{ mg ml}^{-1}$ ). Skindersoe *et al.* (2008) report anti-QS activity of antibiotics at subinhibitory concentration in *Ps. aeruginosa*. Cinnamic acid is reported to inhibit the growth of several bacteria (Burt 2004), in particular *Escherichia coli* O157:H7 (Wells *et al.* 2005), and is an active component in medicinal plants with anti-*Helicobacter pylori* (Bae *et al.* 1998) or anti-tuberculosis (Barnes *et al.* 2003) activity. It also enhances the activity of anti-tuberculosis drugs isoniazid or rifampin (Rastogi *et al.* 1998). Biosynthetically, phenylalanine is converted to cinnamic acid via phenylalanine ammonia lyase (PAL). These enzymes are present in various plants, fungi and yeast but in only two prokaryotes marine-derived *Streptomyces maritimus* (Xiang and Moore 2002) and nematode symbionts *Photobacterium luminescens* (Williams *et al.* 2005). In analogy with literature reports, as the active strain of the present investigation is cinnamic acid producer, it is quite likely that it is biosynthesized via PAL. Thus, this is the second report of PAL from *Streptomyces*.

There is a solitary reference in the literature (Bodini *et al.* 2009) on the antagonist activity of p-coumaric acid against *Chromobacterium*, *Agrobacterium* and *Pseudomonas* QS and inhibition of swimming motility of *E. coli* ATCC by cinnamaldehyde (Niu *et al.* 2006). These two compounds, cinnamaldehyde and p-coumaric acid, are reduction product and phenolic analogue, respectively, of cinnamic acid. Although cinnamic acid is known to be bactericidal, in the present study, it was found to possess previously undescribed QS antagonist properties.

Cyclic dipeptides such as cyclo-L-proline-L-tyrosine isolated from *Bacillus cereus* D28 (Teasdale *et al.* 2011) are known for inhibition of bacterial quorum sensing. In our earlier studies, we also have observed QS swarming in cyclic dipeptides with proline as one of the amino acid constituents (Prabha Devi, Ciavatta M.L., Wahidullah S., Naik, D.N., Nuzzo G., Manzo, E., D'Souza L., unpublished data). In the present study also, we observe that proline-derived linear dipeptides, proline-glycine and N-amido- $\alpha$ -proline are

constituents of the active fraction, and so far, there are no reports on the bacterial quorum-sensing activity exhibited by them. Detailed anti-QS investigation of commercially available linear dipeptide (Table 1) has indicated that it does possess effectiveness against the QS system, but it is less significant as compared to cinnamic acid. As both these compounds are chemical constituents of the active crude extract of NIO 10068, it was thought desirable to study a combination of cinnamic acid/Pro–Gly (1 : 1), and it was found that cinnamic acid alone is more potent than linear dipeptide (Table 1, Fig. 4b,c).

In summary, this work describes three sponge-associated actinomycetes belonging to genus *Streptomyces* having different but significant effect on virulence factors in *Ps. aeruginosa* at 0.1 mg ml<sup>-1</sup> concentration. The most effective candidate on further chemical studies based on mass spectrometry revealed the presence of linear dipeptides and cinnamic acid as the constituents of active fraction. These compounds are being reported for the first time as anti-QS molecules. Research is underway in this laboratory to study their mechanism of action.

The results reveal that marine-derived actinomycetes may not only play a role in the defensive mechanism of their host but also lead to new molecules useful in the development of novel antivirulence drugs.

## Materials and methods

### Bacterial strains

In this study, *C. violaceum* (ATCC 12472) and *Ps. aeruginosa* (ATCC 27853) were used. *C. violaceum* was used as a QS indicator strain for preliminary QSI assays and was obtained from MTCC (India). *Pseudomonas aeruginosa* (ATCC 27853) was obtained as a generous gift from Goa Medical College.

### Isolation of marine actinomycetes

Actinomycetes (72) were isolated from marine invertebrates collected from the western coast of India (Supporting Information). Briefly, the invertebrate samples were washed in sterile sea water and pretreated using moist heat treatment at 56°C for 30 min. The pretreated samples were homogenized, diluted to 10<sup>-5</sup> with sterile seawater and 100 µl of each dilution plated on actinomycetes isolation agar (AIA) (Lechevalier 1975), International Streptomyces Medium 4 (Shirling and Gottlieb 1966), aqueous sponge/ascidian extract agar (Sipkema *et al.* 2011), chitin agar (Bredholt *et al.* 2008) and M1 agar medium (Mincer *et al.* 2002). The agar media were supplemented with 20 µg ml<sup>-1</sup> of naldixic acid and 100 µg ml<sup>-1</sup> each of nystatin and

cyclohexamide. The plates were incubated for 3–4 weeks, and individual actinomycetes colonies were selected based on their morphology and purified on International Streptomyces Medium 2 (Shirling and Gottlieb 1966).

### Preparation of actinomycete extracts

The isolates were fermented in 500-ml conical flasks containing FM17 medium (Hong *et al.* 2009) (100 ml) for 7 days, and the whole broth was centrifuged and the supernatant was lyophilized. This was followed by extraction of the residue with methanol. The solvent was removed on a rotary vacuum evaporator under reduced pressure and at room temperature to obtain crude methanolic extract.

### Evaluation of anti-quorum sensing activity

**Violaecin inhibition assay.** Overnight culture of *C. violaceum* CV12472 (0.1 ml) (Mcfarland standard 0.5) was mixed with 5 ml warm Luria–Bertani medium (1% peptone, 0.5% yeast extract, 0.5% NaCl) solidified with 1% agar, and overlaid over a plate containing LB medium solidified with 1.6% agar. Paper discs, 6 mm in diameter, containing 0.1 mg per disc of the extract were placed at the centre of the above LB plates and incubated overnight at 28°C. Inhibition of pigment production near the edge of the disc indicated QSI. Extracts showing QSI were further studied for downregulation of QS-related parameters such as motility (swarming, swimming and twitching), pyocyanin, biofilm, rhamnolipid and LasA production in *Ps. aeruginosa* ATCC 27853.

**Motility inhibition assay.** Swarm agar (0.8% nutrient broth, 0.5% glucose, 0.5% agar), swim agar (1% tryptone, 0.5% NaCl, 0.3% agar) and twitch agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1% agar) media were prepared and air-dried for 20 min before use (Inoue *et al.* 2008). Actinomycete extracts and pure compounds cinnamic acid (1), linear dipeptide Pro–Gly (2) and 1 : 1 combination of cinnamic acid+ Pro–Gly (3) dissolved in methanol were added to each agar medium. For swarm and swim assays, the bacterial cells were gently inoculated using a toothpick at the centre of the agar plate, and for twitch assay, the cells were stabbed at the centre of the agar plate using a nichrome wire. After incubation for 24 h at 37°C, the movement of the colony on the agar surface was assessed.

**Pyocyanin inhibition assay.** Pyocyanin was extracted from *Ps. aeruginosa* culture supernatant and measured by the method as described by Ra'ooof and Latif (2010). Briefly, a 5-ml sample of culture grown for 72 h, in *Pseudomonas*



broth medium, containing the testing material, was centrifuged, and the supernatant extracted with 3 ml of chloroform and then re-extracted into 1 ml of 0.2 mol l<sup>-1</sup> HCl to give a pink to deep red solution. The absorbance of this solution was measured at 520 nm. Concentrations expressed as pyocyanin produced in µg ml<sup>-1</sup> of culture supernatant were determined by multiplying the optical density at 520 nm (OD<sub>520</sub>) by 17.072.

**Static biofilm inhibition assay.** The static biofilm assay was performed as outlined by Burton *et al.* (2007). Briefly, *Pseudomonas* strain grown overnight in LB medium was diluted 1 : 100 with fresh LB medium containing the testing material. The inoculated culture (150 µl) was transferred to a 96-well polystyrene microtitre plate and incubated at 37°C for 24 h. The planktonic cells were removed from the wells after incubation and the wells washed thrice with milli-Q water. This was followed by the staining of the wells with 0.1% crystal violet for 10 min and washing the unbound stain thrice with milli-Q water. The cell-bound dye was extracted with 300 µl of 95% ethanol, and the absorbance of the solution was measured using a microplate reader at 595 nm (BMG Lab, Ortenberg, Germany).

**Rhamnolipid inhibition assay.** *Pseudomonas aeruginosa* was grown at 30°C for 72 h in MSM medium containing the testing material. The bacterial cells were separated by centrifugation, and the supernatant was acidified to pH 2 with concentrated HCl. Rhamnolipids were extracted twice from the acidified supernatant with chloroform/methanol (2 : 1). The lower organic phases were pooled and evaporated to dryness. Dried extract was resuspended in methanol and was analysed by TLC on silica gel 60 F254 plate (Merck, Darmstadt, Germany) using chloroform/methanol/acetic acid (70 : 28 : 2) as developing solvent. Rhamnolipid spots were visualized by 2% anthrone in H<sub>2</sub>SO<sub>4</sub> as yellow-coloured spots.

The rhamnolipid concentration in the broth was estimated by anthrone method. Briefly, anthrone (20 mg) dissolved in a minimum amount of ethanol was diluted to 100 ml with 70% (v/v) sulphuric acid. A mixture of 1 ml of a test sample and 4 ml of anthrone solution was vortexed for 1 min, and the tubes placed in a boiling water bath for 7 min and allowed to cool in the dark for 30 min. The absorbance was measured at 625 nm using a UV/vis spectrophotometer (Shimadzu, Kyoto, Japan). The amount of rhamnolipid in the test sample was subsequently calculated in terms of g l<sup>-1</sup> of rhamnose using a calibration curve of standard rhamnose in the concentration range of 0–100 mg l<sup>-1</sup>.

**LasA staphylolytic assay.** LasA protease activity was measured by determining the ability of *Ps. aeruginosa* culture supernatants to lyse boiled *Staphylococcus aureus* cells.

Briefly, a 50-ml volume of an overnight culture of *Staph. aureus* was boiled for 10 min and then centrifuged for 10 min at 10 000 g. The resulting pellet was resuspended in 10 mmol l<sup>-1</sup> Na<sub>2</sub>PO<sub>4</sub> (pH 4.5) to an OD<sub>600</sub> of approximately 0.8. An overnight grown culture of *Ps. aeruginosa* containing testing material was centrifuged, 100 µl aliquot of supernatant was then added to 900 µl of *Staph. aureus* suspension, and the OD<sub>600</sub> was determined after 0, 5, 10, 15, 20, 25 and 30 min.

All the above-mentioned experiments were conducted in triplicates with the crude extracts (0.1 mg ml<sup>-1</sup>) and the compounds cinnamic acid (7 mmol l<sup>-1</sup>) and Pro–Gly (6 mmol l<sup>-1</sup>). *Pseudomonas aeruginosa* without extracts and the compounds was used as control.

### PCR and sequencing analysis

Molecular identification of the isolates was performed by means of PCR. Briefly, fresh actinomycete colonies were grown in ISP 2 medium, and DNA was extracted using Hipura DNA Extraction kit (Himedia, Mumbai, India) according to the manufacturer's instructions. Amplification of 16S rRNA gene was performed by PCR using the universal primers 27f (5' AGA GTT TGA TCC TGG CTC AG 3') and 1385r (5' CGG TGT GTA CAA GGC CC 3') (Thakur *et al.* 2004). Sequencing of the 16S rDNA PCR product was performed using 3130xl Genetic analyzer (Applied Biosystems, Carlsbad, CA, USA) at the National Institute of Oceanography, Goa, India. The 16S rDNA forward sequences (700 bp approximately) were used to search the GenBank database with the BLAST N algorithm to reveal closest matches to the 16S rDNA sequences for known species. Sequences were aligned with representative actinomycete 16S rDNA sequences, and a phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis (MEGA, Tempe, AZ, USA) software, ver. 4.0.

### Electrospray ionization–mass spectrometric (ESI-MS) analysis

The active methanol fraction was subjected to ESI-MS analysis. Both positive- and negative-mode QTOF mass spectra were acquired by direct infusion using a QTOF-XL MS/MS, Applied Biosystem instrument equipped with the MDS Sciex Analyst software (AB MDS Sciex, Concord, ON, Canada). Full scan data acquisition was performed, scanning from *m/z* 0 to 500 in the profile mode and using a cycle time of 1 s. MS/MS values were recorded at different collision energies in the range 10–40 V so as to obtain optimum fragmentation as evidenced by the decrease in the intensity of the molecular ion signal. The identity of compounds was based on the fragmentation pattern observed and comparison with those of commercial authentic standard, cinnamic acid (Aldrich, MO, USA) and Pro–Gly (Bachem).

### Statistical analysis

All the experiments were performed in triplicates, and the *P* values were calculated statistically by Student's *t*-test.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** List of invertebrates used in the study with collection sites.

**Figure S1.** Representative image of QS inhibition of *Chromobacterium violaceum* in the presence of methanolic extracts of NIO 10058, NIO 10068 and NIO 10090. Figure 4b: Representative image of swarming motility inhibition of *Pseudomonas aeruginosa* in the presence of methanolic extracts of NIO 10058, NIO 10068, NIO 10090 and control plate without extract.