EVALUATION OF ANTI-QUORUM SENSING ACTIVITY OF N-Hexadecanoic ACID PRODUCED BY PSEUDOMONAS STUTZERI SJ4 – A MARINE EPIBIONT BACTERIUM

Siddhi D. Shah1, Nikita Vadodartya2, Bhakti Bajpai*

Address(es): Ashok & Rita Patel Institute of Integrated Study and Research in Biotechnology and Allied Sciences (ARIBAS), ADIT campus, New vallabh Vidyanagar, Anand-388121, Gujarat, India.

*Corresponding author: bhaktibajpai@aribas.edu.in

INTRODUCTION

The discovery of antibiotics was a boon but its excessive use and decreased efficiency turned it into a bane. The pathogens Which are become completely or partially resistant to one or more antibiotics/antimicrobial compounds are referred to as Multi-Drug Resistant (MDR) microbes. This global issue drew attention of scientific community to search for a novel approach which can combat pathogenic infections without triggering selection pressure which leads to development of drug resistance (World Health Organization, 2017; Reina et al., 2019).

Many pathogenic bacteria, have mechanisms which control the expression of virulence genes such as pigmentation, antibiotic resistance, extracellular proteases and biofilm formation termed as Quorum sensing (QS) (Whitehead et al., 2001; Quinones et al., 2005; Jayaraman and Wood, 2008; Garg et al., 2014; Papenfort et al., 2016; Whiteley et al., 2017). It is a cell population dependent process where cells communicate, coordinate and express genes leading to the production of signalling molecules called as autoinducers in the surrounding environment (Fuqua et al., 1994; Fuqua et al., 2001). The concentration of signalling molecules inducing characteristic phenotypes is directly proportional to the cell density. Gram positive bacteria produce oligopeptides and Gram negative bacteria produce N-Acyl Homoserine Lactones (AHLs) as QS signal molecules (Ng WL et al., 2009; Parke et al., 2009). QS regulation plays vital role in establishment of infection within the host by several pathogens such as P. aeruginosa, Staphylococcus aureus, Escherichia coli, Vibrio cholerae and Vibrio harveyi, therefore the disruption or silencing of virulence gene expression can be a novel strategy to combat MDR. (Ng WL et al., 2009; Kalia et al., 2011; Nathra et al., 2011; Lasre et al., 2013; Grandclement et al., 2016; Deoirdrt et al., 2018; Kalia et al., 2019; Zhao et al., 2019).

P. aeruginosa PAO1 is an infectious strain associated with the urinary tract infections (UTIs) and hospital acquired infections. It is a Gram-negative, motile organism and becoming resistant towards conventional antibiotics. QS inhibitory approach can be an effective way to control MDR clinical strain P. aeruginosa PAO1. The four QS systems of P. aeruginosa PAO1 are divided broadly into two groups, (i) AHL-based signalling system namely Las/LasR and Rhl/RhlR system and (ii) non-AHL or quinolone based such as Pseudomonas quinolone signal (PQS) and integrated QS signal (IQS) (Rutherford and Bassler 2012; Pérez-Pérez et al., 2017; Li et al., 2018; Hennmati et al., 2020). Las and rhl encode a signal synthetase which synthesizes the required signal molecules, lasR and rhlR code for a receptor protein that binds to the signal molecule and initiate transcription of QS genes. The LasIR system uses 3-oxo-C12-HSL as a cognate signal molecule, and RhlR utilizes C4-HSL. These two systems work in a hierarchical manner, as the las-encoded system affects the expression of the rhl-encoded system (Hentzer and Givskov 2003; Mostafa et al., 2020). The PQS system is monitored by 2-alkyl-quinolones while IQS is controlled by 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (Lasarre & Federle 2013; Li et al., 2018).

Many natural and synthetic compounds are known to function as quorum sensing inhibitors (QSI). QSI is non-enzymatic compounds, which can be cyclic or linear peptides, amides, AHL analogs. Further, inactivation of QS signal molecules by enzymes (Acylases, AHL Lactonases, and AHL Reductase) are termed as Quorum Quenching (QQ) or Quorum sensing inhibitors (QQIs). QQIS are non-enzymatic or enzymatic in nature termed as Quorum Quenching (QQ) or Quorum sensing inhibitors (QQI) and reduced activity against other QSIs respectively. We used marine epibiotic bacteria as a source to obtained novel bacterial strain as QSI producer. One of the potent isolate, SJ4 was identified as Pseudomonas stutzeri SJ4, it is a short rod, gram negative bacterium. The ethyl acetate extract from P. stutzeri SJ4, showed highest QSI activity against monitor strain Chromobacterium violaceum (MTCC 5526). The extracted compound was tested against P. aeruginosa PAO1 at minimum inhibitory concentration (MIC) and sub-MIC to study the effect on virulence factors. The significant inhibition of pyocyanin pigment, EPS production, rhamnolipid production and reduced swimming and swarming motility was observed. In addition, biofilm formation was notably inhibited which was confirmed by staining and spectometric method. Based on this observation, QS interruption by extract which contain QSI markedly reduced the virulence of pathogen hence, can be use as therapeutic agents. The Thin Layer Chromatography (TLC) and Gas chrmoatography-Mass Spectrometry (GC-MS) identified major compound as n-Hexadecanoic acid. However, further research is required on purification of compound and its potential applications for the treatment of infections.

Keywords: Quorum sensing, Quorum sensing inhibition, marine bacteria, Pseudomonas stutzeri, Pseudomonas aeruginosa

ABSTRACT

Quorum sensing (QS) mechanism is cell communication that plays vital role in the development of infection by many pathogenic microorganisms. It controls multiple virulence factors such as pigmentation, biofilm formation, swarming motility, resistance towards antibiotics, extracellular polysaccharide production (EPS) and expression of several collective traits. The disruption of quorum sensing mechanism can be a solution to the emerging problem of multi-drug resistance among pathogenic bacteria. The effector molecule for Quorum sensing inhibition may be enzymatic or non-enzymatic in nature termed as Quorum Quenching (QQ) or Quorum sensing inhibitors (QQIs)/anti-QS compound, respectively. We used marine epibiotic bacteria as a source to obtained novel bacterial strain as QSI producer. One of the potent isolate, SJ4 was identified as Pseudomonas stutzeri SJ4, it is a short rod, gram negative bacterium. The ethyl acetate extract from P. stutzeri SJ4, showed highest QSI activity against monitor strain Chromobacterium violaceum (MTCC 5526). The extracted compound was tested against P. aeruginosa PAO1 at minimum inhibitory concentration (MIC) and sub-MIC to study the effect on virulence factors. The significant inhibition of pyocyanin pigment, EPS production, rhamnolipid production and reduced swimming and swarming motility was observed. In addition, biofilm formation was notably inhibited which was confirmed by staining and spectometric method. Based on this observation, QS interrption by extract which contain QSI markedly reduced the virulence of pathogen hence, can be use as therapeutic agents. The Thin Layer Chromatography (TLC) and Gas chrmoatography-Mass Spectrometry (GC-MS) identified major compound as n-Hexadecanoic acid. However, further research is required on purification of compound and its potential applications for the treatment of infections.

https://doi.org/10.55251/jmbfs.5644
organism like algae, sponges and seaweeds, for QSI activity. Isolates were screened for the anti-QS activity using C. violaceum (MTCC 5526) as a monitor strain. Total 20 isolates showed anti-QS activity. Isolate showing highest anti-QS activity was selected for further studies on clinical strain of Pseudomonas aeruginosa PAO1.

MATERIALS AND METHODS

Sample Collection
The marine seaweeds, algae and sponges were collected in sterile sample bottles from coastal site of Olka, Gujrat, India. (22°28’38.7’’N 69°04’51.7’’E). The samples were kept in the sea water at 4°C until use.

Isolation of marine epibiotic bacteria
A 1 gram sample (marine seaweeds, algae and sponges) was washed thrice with sterile sea water. The washings were collected (Total 30 mL) and treated as samples. All the samples were diluted up to 10 times; 100 µL was spread on Zobell Marine Agar (ZMA, Himedia, Mumbai India) and incubated at 37°C for 5-7 days. Morphologically distinct colonies were transferred to the ZMA plates to obtain pure colonies (Singh et al., 2020).

Screening of epibiotic bacteria for anti-QS activity
Isolates were screened for anti-QS activity by soft agar overlay method using C. violaceum (MTCC 2656) as a monitor strain (McLean et al., 2004). The secondary screening was done by quantification of violacein pigment. The bacterial isolates (1×10^6 CFU/mL cells) and C. violaceum (1×10^6 CFU/mL cells) were inoculated in Luria broth (LB) and incubated at 37°C for 24 h under shaking condition. Next day, 1 mL sample was withdrawn and centrifuged at 10,000 rpm for 15 min., pellet was dissolved in DMSO and centrifuged again at 10,000 rpm for 15 min. Absorbance of the supernatant was measured at 585 nm. Cell density was also measured at 600 nm to check the growth of isolates (Cho et al., 2006; Musthafa et al., 2011).

Extraction of Anti-QS compounds
The select marine isolates were grown in ZMB at 37°C for 24 h under shaking condition. The cultures were centrifuged and the supernatant was extracted with equal volume of organic solvents of varying polarity like hexane, chloroform, ethyl acetate, n-butanol, isopropanol and methanol. The extract was concentrated by evaporation and the residue was dissolved in 500 µL of respective solvent. The compound was used for C. violaceum based bioassay by well diffusion method (Nithya and Pandian SK., 2009; Nithya et al., 2010).

Morphological characterization of bacterial isolate
Isolate SJ4 showing highest QSI inhibition, was gram stained and its morphologies were examined at 100X magnification using Magnus MLM light microscope.

Biochemical characterization of bacterial isolate
Biochemical characterization of selected bacteria was done, which included tests such as catalase, oxidase and sugar utilization (lactose, glucose, sucrose, mannitol, rhamnose and arabinose).

Molecular identification of bacterial isolate
Genotypic characterization of SJ4 was by sequencing its 16S rDNA gene. The extracted genomic DNA was shipped to Eurofins Scientific India Pvt Ltd. (Bangalore, India) and served as a template for PCR amplification of the nearly complete 16S rDNA gene using the universal oligonucleotide primer pair 27F (5’-AGA GTT TGA TCC TGG CAC G-3’) and 1492R (5’-TAC GGT TAC CTT GTC AGC T-3’), as recommended by Lane (Lane,1991).

Testing QSI activity of extract
QSI activity of the extract was determined using well diffusion method. LB agar plates were inoculated with 100 µL of C. violaceum 50 µL of extract was added into the wells. The plates were then incubated at 37°C for 24 h. Extract showing zone of pigment inhibition in C. violaceum were shortlisted and then tested against P. aeruginosa PAO1.

Determination of Minimum Inhibition Concentration (MIC) of QSI extract
The MIC was determined by broth dilution method using P. aeruginosa PAO1 as a test strain. The extract was serially diluted to make different concentrations from 1 mg/mL to 0.098 mg/mL. Test strain was inoculated in LB broth and incubated at 37°C for 18 h. The control was prepared without extract. The tube showing no turbidity was considered as MIC. Consequently, sub-MICs were selected for further studies (CLSI, 2012).

Inhibition of virulence factors in P. aeruginosa
Pyocyanin inhibition
Pyocyanin inhibition was assayed by growing P. aeruginosa overnight with QSI extract (MIC and sub-MIC) and without extract at 37°C for 24 h. After incubation, 5.0 mL culture supernatant was extracted with 2.5 mL of chloroform and was centrifuged at 3000 rpm for 2 min for separation of chloroform layer. 500µL of 0.2N HCL was added to the separated layer and vortexed vigorously which produced a red colored thick ring. The ring was carefully removed, the absorbance was measured at 520 nm and inhibition percentage was calculated (Ganesh and Rai, 2016).

Inhibition of Exopolysaccharide (EPS)
EPS was quantified from supernatant of P. aeruginosa. EPS was precipitated by three volumes of iso-propyl-alcohol (100%) and incubated for 24 h at room temperature. The precipitated EPS was pelleted by centrifugation (10000 rpm, 15 min) and dissolved in distilled H2O. EPS was quantified using the phenol-sulfuric acid method, wherein 1 mL of 5% cold phenol and 5 mL of conc. H2SO4 were mixed with 1 mL of EPS suspension, which was measured spectrophotometrically at 490 nm (Packiaratnam et al., 2014).

Rhamnolipid inhibition
A 1 mL cell free supernatant was extracted with twice the volume of ethyl acetate and dried. The dried extract was suspended in 900 µL of 0.19% orcinol solution (0.19% orcinol dissolved in 53% (v/v) sulfuric acid). The mixture was incubated for 30 min at 80°C and quantified at 421 nm spectrophotometrically (Packiaratnam et al., 2014).

Biofilm inhibition
The test strain was mixed with QSI extract (MIC and sub-MIC) and incubated for 24 h at 37°C; a control was prepared without QSI extract. After incubation, the wells were washed with sterile phosphate-buffered saline (PBS) to remove unadhered cells. The biofilm was stained with 1% crystal violet for 5 min and again washed with sterile PBS to remove excess stain. The crystal violet stained biofilm was dissolved with 33% acetic acid and was quantified by absorbance at 595 nm (Luo et al., 2016).

Motility inhibition
Swimming and swarming was studied as described by Rashmi et al. (2018). Growth media used for swimming assay comprised nutrient broth along with extract or without extract. Actively growing culture was point inoculated and plates were incubated at 37°C for 24-48 h. In Swarming, test strain was inoculated in nutrient agar with or without QSI extract. Actively growing culture was point inoculated and plates were incubated at 37°C for 24-48 h.

Identification of active compound(s) in E/A extract of P. stutzeri SJ4
Thin layer chromatography (TLC) was performed to partially purify active compound (QSI) from Ethyl acetate crude extract of P. stutzeri SJ4 using chloroform: methanol (10:1) as solvent system (Basaran et al., 2020). The eluted bands were checked for the anti-QS activity. The band showing highest activity was analysed by Gas chromatography. The chromatogram was matched with NIST library database in order to predict the compound.

Anti-QS activity of n-Hexadecanoic acid
QSI inhibitory activity of different concentrations of commercially available n-hexadecanoic acid (HDA) (Himedia, Mumbai, India) was compared with the crude extract from P. stutzeri SJ4 against C. violaceum MTCC 2656. The concentration showing highest decolorization zone was used for further studies.

RESULTS AND DISCUSSION

Epicobic bacterium P. stutzeri SJ4 as a QSI producer
Total 60 bacteria having distinct morphology were isolated from the surfaces of 8 samples of seaweed, algae and sponges. All isolates were screened for QSI activity using C. violaceum MTCC 2656 as a monitor strain. 20 isolates showed QSI activity by inhibiting the violacein pigment production. SJ4 showing highest activity was selected for further study (figure 1).
The colony of SJ4 was small, reddish brown, dry, and irregular in shape. It was gram negative, short rod, arranged singly (figure 2 a&b). SJ4 was catalase negative, oxidase positive, fermented lactose, glucose, sucrose, mannitol, rhamnose and arabinose sugars. Apart from morphological and biochemical characterisation, molecular identification was carried out by 16S rDNA sequencing, SJ4 was identified as P. stutzeri SJ4. The nucleotide sequences were submitted to Genebank (Accession No. MW547117) and analysed using NCBI Blast tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was done using MegAlign (Downloaded from https://www.megasoftware.net/ ) software. The phylogenetic tree was constructed by Neighbor-Joining method with the bootstrap value of 1000 (figure 2c).

**Extraction of QSI Substances**

The supernatant was extracted with different organic solvents such as hexane, chloroform, ethyl acetate, n-butanol, isopropanol and methanol. The extracts were checked for the QSI activity against C. violaceum by well diffusion method. The zone of pigmentation inhibition was highest in ethyl acetate extract compared to other solvents (Table 1). Teasdale et al., (2009), also found highest anti-QS activity of Halobacillus salinus C42 in the ethyl acetate extract. On the other hand, Abudoleh et al., (2017), reported more anti-QS activity from three isolates in butanol, chloroform and hexane extracts. Hence, further virulence assays of P. aeruginosa were performed using ethyl acetate (EA) extract.

**Table 1** QSI activity of compound extracted with various solvents

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Solvent</th>
<th>Diameter of Zone of Pigment Inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Hexane</td>
<td>5</td>
</tr>
<tr>
<td>2.</td>
<td>Chloroform</td>
<td>7</td>
</tr>
<tr>
<td>3.</td>
<td>Ethyl acetate</td>
<td>15</td>
</tr>
<tr>
<td>4.</td>
<td>n-Butanol</td>
<td>10</td>
</tr>
<tr>
<td>5.</td>
<td>Isopropanol</td>
<td>4</td>
</tr>
<tr>
<td>6.</td>
<td>Methanol</td>
<td>2</td>
</tr>
</tbody>
</table>

**Inhibition of virulence factor in P. aeruginosa**

Pyocyanin is a green, fluorescent compound produced by P. aeruginosa which plays a vital role in the infection. To investigate, the effect on pyocyanin and other virulence factors, the MIC of QSI extract was tested and it was found to be 6.25 mg.mL⁻¹ and the sub-MIC was 3.125 mg.mL⁻¹. These two concentration were used for the study. QSI extract inhibited pyocyanin production up to 79% and 34% at 6.25 and 3.125 mg.mL⁻¹ concentration respectively (figure 3).

In a previous study, Venkatramanam et al., (2020) reported 2 mg.mL⁻¹ and 1mg.mL⁻¹ as MIC and sub-MIC respectively, 75.8% inhibition of violacein pigment in C. violaceum was observed. Busetti et al. (2015) have reported significant decrease in pyocyanin production (60%) at 1 mg.mL⁻¹ concentration from Pseudalteromonas sp. EPS is a fundamental component of the biofilm formation and it is crucial to maintain integrity of biofilm. EPS production was reduced by 77% at MIC while 19.23% at sub-MIC (figure 3). Mishra et al., (2018) studied effect of extract of Alternaria alternata on EPS production of P. aeruginosa PAO1; 66.6% reduction was observed.

Rhamnolipid is one of the important aspects in the formation of biofilm. In the present study, QSI reduced rhamnolipid production upto 45% at MIC; while 29% reduction was observed at sub-MIC (figure 3). Rajkumari et al., (2018) observed 16.51% reduction in rhamnolipids of P. aeruginosa when cinnamic acid was used as QSI.
Effect of EA extract of *P. stutzeri* SJ4 on Biofilm formation of *P. aeruginosa*

Biofilm formation is most important factor responsible for the virulence of any pathogenic bacteria. It majorly contributes in antibiotic resistance and development of severe infection. Our study showed 69% reduction in biofilm formation in comparison with control. The staining with crystal violet showed marked difference in the color intensity in the presence and absence of EA extract (figure 4). In a study by Christiaen *et al.* (2014), *Diaphorobacter* sp. and *Delftia* sp., had reduced *P. aeruginosa* PAO1 biofilm formation by 20–30% whereas marine derived strain *Rhzobium* sp. NA01, reduced biofilm formation by 72.3% in *P. aeruginosa* PAO1 (Chang *et al.*, 2017).

**Figure 4** Biofilm inhibition of *P. aeruginosa* PAO1 after staining with crystal violet (a) control (without extract) (b) at 6.25 mg.mL\(^{-1}\) (MIC) (c) at 3.125 mg.mL\(^{-1}\), (Sub-MIC).

Motility helps in attachment to host cell surface and responsible for the movement. *P. aeruginosa* is a highly motile strain so swimming and swarming patterns of mobility were studied in the absence and presence of QSI. A significant decrease in the colony diameter was noticed in the presence of QSI compound (figure 5). According to earlier studies, aspirin inhibited the swimming motility of *P. aeruginosa* by 34% (El-Mowafy *et al.*, 2014).

**Figure 5** Inhibition of Motility in *P. aeruginosa* PAO1 (a) Swimming (b) Swarming (1a & 1b) control, (2a&2b) extract concentration 6.25 mg.mL\(^{-1}\) (3a &3b) extract concentration 3.125 mg.mL\(^{-1}\).

Characterization of anti-QS compound of EA extract of *P. stutzeri* SJ4

A total of 4 prominent bands were observed on TLC plate under the UV light. QS inhibition was observed in only two eluted bands, one band with highest anti-QS activity was selected for the GC-MS analysis. The chromatogram of partially purified extract showed 15 peaks with different retention time (RT) (figure 6); 3 peaks were selected for MS based on the percentage of area. According to NIST library, major compound was n-hexadecanoic acid (18.47%) at 23.307 RT followed by Undecanoic acid (12.18%) at 21.451 RT and Hexadecane (7.82) at 25.903 RT (table 2). Basaran *et al.* (2020), obtained 7 bands after TLC separation of EA extract of *Natriema versiforme*. Venkatramanam *et al.*, (2020) also reported n-hexadecanoic acid as the main QSI compound after GC-MS analysis of *Passiflora edulis* extract.

**Figure 6** Chromatogram of Gas Chromatography showing 15 peaks at different retention time

**Table 2** List of compounds at 23.307 RT obtained after searching in NIST library

Comparative study of crude and standard compound

Different concentrations (1 mg.mL\(^{-1}\), 0.5 mg.mL\(^{-1}\), and 0.25 mg.mL\(^{-1}\) of HDA was tried, figure 7a represents that crude extract showed 12 mm zone of inhibition and HDA showed 9 mm zone. Further, quantification was done using 1 mg.mL\(^{-1}\) HDA where as the concentration of crude extract was 6 mg.mL\(^{-1}\). As shown in the figure 7b, the percentage production of violacein pigment was reduced to 71.06% and 90.32% with HDA and crude extract respectively. A significant difference in the activity of the two compounds was observed, however the concentrations were also different. Since the purity of the standard and test compound is different their potency can not be compared. Chemmugil and co-workers have also reported the anti-QS activity of HDA against *Staphylococcus aureus* using qualitative method. They observed 12 mm zone of inhibition which is similar to our results (Chemmugil *et al.*, 2019).
CONCLUSION
A total of 60 marine epibiotic bacteria were screened for the anti-QS activity. *Pseudomonas stutzeri* S14 significantly inhibited the virulence factors which are associated with the quorum sensing of clinical strain of *Pseudomonas aeruginosa* PAO1. The active anti-QS compound present in the crude extract is tentatively identified as n-hexadecanoic acid. Further investigations regarding the mechanism of inhibition and genetic level expression is required.

Conflict of interest: The authors declare that there is no potential conflict of interest.

Acknowledgments: Authors are grateful to Charutar Vidya mandal (CVM), Vallabhi Vidyanagar, Gujarat, India for providing facilities to conduct the research work. We are thankful to education department, Government of Gujarat, India for providing scheme for developing high research quality (SHODDD) fellowship.

REFERENCES
https://doi.org/10.2147/IDR.S263196

Luo, J., Kong, J. L., Dong, B. Y., Huang, H., Wang, K., Wu, L. H., ... & Chen, Y. Q. (2016). Bacilaien attenuates the quorum sensing-controlled virulence factors of *Pseudomonas aeruginosa* and relieves the inflammatory response in *P. aeruginosa*-infected macrophages by downregulating the MAPK and NfKβ signal-transduction pathways. Drug design, development and therapy, 10, 183. https://doi.org/10.2147/DDDT.S97221


Reina, J. C., Pérez-Victoria, I., Martín, J., & Llamas, I. (2019). A quorum-sensing inhibitor strain of vibrio alginolyticus blocks Qs-controlled phenotypes in *Chromobacterium violaceum* and *Pseudomonas aeruginosa*. Marine Drugs, 17(9). https://doi.org/10.3390/md17090494


