RESEARCH LETTER

Production of acylated homoserine lactone by Gram-positive bacteria isolated from marine water

Pramal Biswa & Mukesh Doble

Bioengineering and Drug Design Lab, Department of Biotechnology, Indian Institute of Technology (IIT) Madras, Chennai, Tamil Nadu, India

Correspondence: Mukesh Doble, Bioengineering and Drug Design Lab, Department of Biotechnology, Indian Institute of Technology (IIT) Madras, Chennai 600036, Tamil Nadu, India. Tel.: +91 (44) 2257 4107; fax: +91 (44) 2257 4102; e-mail: mukeshd@iitm.ac.in

Received 16 January 2013; revised 1 March 2013; accepted 4 March 2013. Final version published online 2 April 2013.

DOI: 10.1111/1574-6968.12123

Editor: Kendra Rumbaugh

Keywords
acylated homoserine lactone; quorum sensing; Gram positiv; *Exiguobacterium*.

Abstract

Acylated homoserine lactone (AHL)-based quorum sensing (QS) has been reported to be present only in Gram-negative microorganisms. Isolation of a novel Gram-positive microorganism from sea water, capable of producing AHL, is reported here. The isolate (GenBank: JF915892, designated as MPO) belonging to the *Exiguobacterium* genera is capable of inducing the AHL bioreporters, namely *Chromobacterium violaceum* CV026, *Agrobacterium tumefaciens* A136, and *E. coli* JM 109(psb1075). This inducer is characterized as C3-oxo-octanoyl homoserine lactone (OOHL), and its production reaches a maximum of 15.6 μg L^{-1}, during the stationary growth phase of the organism. MPO extract when exogenously added inhibits the formation of biofilm for the same organism and lowers the extracellular polymeric substances, indicating an AHL-associated phenotypic trait. The isolated sequence of a probable LuxR homolog from MPO (designated as ExgR) shows similar functional domains and contains conserved residues in LuxR from other known bacterial QS LuxR regulators. Also present immediately downstream to ExgR was found a sequence showing homology to known LuxI synthase of *Pseudomonas putida*. qPCR analysis suggests an increment in *exgR* mRNA on addition of AHL, further proving the role of ExgR as a QS regulator.

Introduction

Quorum sensing (QS) involves cell density-dependent production and perception of chemical signals which regulates gene expression leading to particular phenotypes including biofilm formation, swarming motility, antibiotic production, etc (McClean *et al.*, 1997a). Unlike their terrestrial counterparts, very little is known about the QS repertoire of marine habitats although such microorganisms have been found in both freely living bacteria and bacteria associated with other marine organisms (Dobretsov *et al.*, 2009). The sea-surface microlayer has been shown to resemble a gelatinous biofilm (Wurl & Holmes, 2008) with possibility of wide range of ecological processes including QS (Cunliffe & Murrell, 2009).

There are two distinct and most widely acknowledged groups of major signaling molecules divided among the two different classes of bacteria. They are short peptide signals produced by Gram-positive bacteria and acyl homoserine lactones (AHL) produced by the Gram-negative bacteria (Taga & Bassler, 2003). However, signaling paths collide resulting in interspecies communication. This is evident in LuxS signaling leading to an universal language among the Gram-positive and Gram-negative microorganisms, the inducing molecule in such a case being
autoinducer-2 (AI2) (Taga & Bassler, 2003). The presence of Gram-negative bacteria producing peptide signal has already been predicted (Dirix *et al.*, 2004) and proved (Han *et al.*, 2011). The existence of Gram-positive bacteria producing and responding to AHL is thus a valid probability.

Recently, AHLs have been reported in Archae (Zhang *et al.*, 2012) as well as in cyanobacteria (Sharif *et al.*, 2008), further expanding the role of AHL-based QS and making it an almost universally occurring phenomenon. The only group missing from the picture thus remains the Gram-positive bacteria.

The genera *Exiguobacterium* comprises of a group of Gram-positive, orange- to yellow-pigmented bacteria,

FEMS MICROBIOLOGY LETTERS

placed in the class *Incertae sedis*, which consists of organisms having ambiguous taxonomic identification (Ludwig *et al.*, 2009). Species belonging to this genus have been isolated from surface sea water as well as from marine sediments (Gontang *et al.*, 2007; Wang *et al.*, 2008).

In this paper, a novel strain of *Exiguobacterium*, whose extract is found to induce multiple AHL reporter strains, is reported. The molecule responsible for the induction of these reporters is characterized as an AHL. Identification of a suspect LuxR homolog and genetic studies on the same have been performed.

Materials and methods

Materials

All the chemicals used for the experiments were procured from Sigma-Aldrich (Bangalore, India), HiMedia Laboratories Pvt. Ltd (Mumbai, India), and SRL (Mumbai, India). Solvents used for extraction (AR grade) and HPLC analysis were supplied either by Rankem (New Delhi, India) or by Merck (Mumbai, India). Powdered culture media were procured from Difco (Haryana, India) and Himedia.

Strain and culture conditions

A bacterial strain was isolated from surface sea water of Mamallapuram (Chennai, South India) after serial dilution and growth in Zobell marine agar. After isolation, the culture was maintained and used at 30 °C in Luria-Bertani (LB) agar and broth for all future experiments.

The reporter strains used in the study were *Chromobacterium violaceum* CV026 (procured from National Collection of Type Cultures, UK), *E.coli JM109* (psb 1075), and *Agrobacterium tumefaceins* A136. Bioassay with these reporters included their growth in solid or liquid medium and then checking for a particular phenotypical interpretation (McClean *et al.*, 1997a; McLean *et al.*, 1997b; Middleton *et al.*, 2002) with the isolated MPO culture or its extracts, as described in Table 1.

Identification of the isolated bacterium

A partial biochemical analysis of the isolated organism was performed (HiAssorted™Biochemical test kit KB002, Himedia Mumbai) along with test for catalase and oxidase activity (*in vitro* diagnostic, Becton Dickinson and Company, USA). To rule out the possibility of contamination, the isolated strain was serially diluted and grown in MacConkey and Mannitol agar for selective growth of Gram-positive and Gram-negative cultures, respectively. The salt tolerance level of the culture was determined by growing it in LB media with different concentrations of NaCl and checking for viability with resazurin assay (Sarker *et al.*, 2007).

Genomic DNA was isolated from overnight cultures using HiPurA™ bacterial genomic DNA isolation kit (Himedia). 16S rDNA PCR analysis was then performed using universal primers(Forward 5′-GAGTTTGATCC TGGCTCA-3′ and reverse 5′-ACGGCTAACTTGTTACGA CT-3′), and the purified PCR product was sequenced in two different private organizations (Chromous Biotech Lab, Bangalore and Sci-Genomics, Cochin, India). Sequence identification was performed using the SeqMatch function of the Ribosomal database project [RDP (http://rdp.cme.msu.edu/)].

Structural identification of the inducer

After 48 h of growth of the MPO, the culture supernatant was extracted twice with equal volume of dichloromethane (Steidle *et al.*, 2002) and reconstituted to 1/200th times its volume in MiliQ water, after the solvent was totally evaporated with the help of Rotavapor® (BUCHI, Switzerland: Generally, 200 mL of the culture supernatant was prepared and reconstituted in 1 mL of acetonitrile/sterile MiliQ water).

Three different chromatographic techniques were used to identify the structure of the inducer produced by the MPO culture.

Gas chromatography–mass spectral (GCMS) analysis of the crude extract was performed (Sophisticated Analytical

Table 1. A summary of the different AHL reporters and bioassay methods used in the study

Reporter culture	Phenotype on induction	Method	References
E. coli JM109(psb1075)	Luminescence	Overnight growth of reporter with extract followed by luminescence measurement	(Middleton *et al.*, 2002)
A. tumefaceins A136	Blue colouration due to X-Gal degradation	Co-culture of reporter strain and isolated bacteria in LB media supplemented with X-gal	(McLean *et al.*, 1997b)
Chromobacterium violaceum CV026	Induction Violet pigment formation Inhibition Halo formed on Violacein plates	Induction: Test extract added to wells plunged in LB agar with embedded reporter culture Inhibition: Test extract added to wells plunged in LB agar(+ octanoyl homoserine lactone) with embedded reporter culture	(McClean *et al.*, 1997a)

Instrumentation Facility, IIT Madras), according to the parameters provided in literature (Huang *et al.*, 2008) with a JEOL GCMATE II GC-MS system equipped with a fused silica capillary column HP-5 (30 m × 0.25 mm I.D0.25-μm film thickness).

Liquid chromatography–mass spectral (LCMS) analysis of the crude extract was performed (Central Instrumentation Facility, IIT Guwahati) with a Waters Q-TOF Premier & Waters' Q-ToF Premier Mass Spectrometer equipped with an ACQUITY UPLC C18 RF 1.7 μm, 2.1 × 50 column.

For the analysis, the extracts were reconstituted in HPLC grade methanol and filtered through a 0.2-μ Nylon membrane (Sartorius, USA).

HPLC analysis was performed on a Shimadzu system equipped with C18 reverse phase (Phenomenex, Luna 5u 100A, 150 × 4.6 mm) column using a binary gradient program. The column was initially eluted with 10% acetonitrile (ACN) at 1 mL min^{-1} for 10 min followed by a linear gradient to reach 100% ACN in 65 min and an additional 15 min at 100% ACN. This procedure is a slight modification to the reported method (Teplitski *et al.*, 2003).The quantity of the reporter in the extract was determined from a standard graph prepared with pure C-3-oxo-C8 HSL (Sigma-Aldrich).

Growth kinetics and inducer activity

The MPO culture was grown and extracts were collected at different time points. 50 μL of these was then added to 1 mL of fresh reporter culture *E. coli* JM109 (psb1075), and the luminescence was measured after overnight incubation (LSIRIUS V3.1, tube format luminometer). The relative light unit values obtained were converted to concentration of AHL in the culture supernatant with the help of the standard AHL calibration curve.

Biofilm analysis

The formation and development of biofilm has been found in many cases to be under the control of QS (Parsek & Greenberg, 2005). The biofilm forming ability of MPO at varying AHL concentrations was investigated.

Different concentrations of the standard C-3-oxo-C8 HSL (Sigma) was added into each well of a 24-well microtitre plate, and the biofilm formation capability of MPO was analyzed after incubating at 30 °C under static condition for 48 hrs with the help of Crystal violet assay (Christensen *et al.*, 1985). The cells adhering to the microtitre plates were stained (after rinsing) with 0.1% crystal violet for 10 min, rinsed twice, and then incubated for another 10 min with 30% acetic acid, and the absorbance was measured at 540 nm (JASCO V-550, UV-Vis Spectrophotometer). Similarly, the effect of MPO extract on the biofilm was studied. For this study, 2 day-old culture (400 mL) was extracted with dichloromethane and reconstituted in 2 mL ultrapure water and used in different concentration.

Scanning electron microscopy (SEM) analysis of biofilms was performed by incubating sterilized PET (polyethylene terephthalate) polymers of 1×1 mm^2 dimensions with bacterial culture, with and without standard AHL (1 mL of 80 μM AHL in 100 mL of LB media) and incubated at 30 °C for 72 h under static condition. The attached cells were fixed onto the polymers with 3% glutaraldehyde treatment and dehydrated by serial alcohol wash (Sivakumar *et al.*, 2012). Images were then captured with Quanta 200 FEG scanning electron microscope (USA). In addition, the polymers were also stained with a LIVE/DEAD bacterial viability kit (BacLight™, Invitrogen, Germany) containing SYTO-9 and propidium iodide dyes from Molecular Probes (Invitrogen) and visualized using a fluorescence microscope (Leica DM5000, Germany).

Exopolysaccharide from 72-h static cultures of MPO, MPO + AHL(80 μM), and MPO + MPO extract was extracted separately by adding three volumes of chilled ethanol and kept overnight at 4 °C (Bramhachari & Dubey, 2006) [The MPO extract, to be added, was pre-extracted for extracellular polymeric substances (EPS) by the same protocol to avoid miscalculation in the observed values]. The ethanol-extract solution was then centrifuged at 13 000 ***g*** for 20 min, and the amount of sugar in the recovered EPS was estimated by phenol sulfuric acid method (Dubois *et al.*, 1956) using glucose as the standard. The resulting values were then compared.

Antimicrobial activity of the MPO extracts (prepared as mentioned above) and the standard AHL on MPO, as well as *Escherichia coli (NCIM 2931)* and *Proteus vulgaris (NCIM 2813),* was determined by well diffusion method (Rojas *et al.*, 2006) and resazurin-based microtitre plate assay (Sarker *et al.*, 2007).

Sequence analysis for probable LuxR and LuxI homolog (ExgR and ExgI)

Primers were designed from the predicted LuxR and its immediately downstream located N-acetyltransferase GCN5 sequence individually (sequence obtained from the closely related *Exiguobacterium* spcs AT1b (RefSeq ID: NCBI-YP_002886724.1 and YP_002886725.1) and are listed in Table S1.

Sequence analysis of the corresponding PCR product obtained was performed by a suite of online bioinformatics functions, namely ORF Finder (Open Reading Frame Finder) (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) for ORF prediction, PROSITE (http://prosite.expasy.org) for

the identification of functional domain of the predicted protein, DELTA-BLAST (Domain Enhanced Lookup Time Accelerated BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) for finding the nearest homologs of the protein, and ClustalW(http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html) for Multiple Sequence Alignment (MSA). [Detailed parameters for MSA and BLAST have been mentioned in Supplementary information.]

Primer designing and sequencing for the above genes were performed at Xcelris Lab Ltd, Ahmadabad, India.

qPCR analysis for *exgR* and *exg I* mRNA on AHL addition

RNA was extracted from control and OOHL (80 μM, added at 24th h) supplemented MPO cultures at the end of 28th and 32nd hour. RNA Protect® Bacterial Reagent was used for RNA stabilization prior to extraction.

Total RNA was isolated following manufacturer guidelines (Qiagen RNAeasy mini-Kit), after pretreating the cell pellet with lysozyme (Sigma) and proteinase K (Qiagen). On-column DNA removal was also performed as a part of the RNA purification process.

cDNA was synthesized from freshly extracted 1 μg of RNA sample using QuantiTect® Reverse Transcription kit. qPCR was performed using Mastercycler® ep realplex (Eppendorf AG, Germany) and QuantiTect SYBR Green PCR Kit (Qiagen Inc., USA). The housekeeping gene Gyrase (Rodrigues & Tiedje, 2007) was used as an internal reference for data normalization. Relative quantification of the generated data was performed directly using the Mastercycler® ep realplex analysis software. The primers used for qPCR are mentioned in Table S2.

Statistical analysis

Two sample *t*-tests were performed using MiniTab ver 14.0 (USA).

Results

Isolation and identification

The microorganism isolated from the surface sea water (initially selected based on its ability to induce response in AHL reporters cultures) based on 16S rRNA gene analysis was found to share 99% similarity with *Exiguobacterium* sp AT1b (GenBank CP001615.1). The isolate was identified as a novel *Exiguobacterium* strain and designated as *Exiguobacterium MPO* (MPO, GenBank accession Number: JF915892). The bacterial colonies were Gram-positive (Supporting information Fig. S1), orange-colored, rod-shaped, and about 0.83 μm in size.

The description of MPO based on partial biochemical analysis was as follows: positive for phenylalanine deamination, catalase and oxidase and glucose and adonitol utilization, whereas negative for utilization of citrate, lysine, ornithine, lactose, arabinose and sorbitol, urease production, nitrate reduction, and H_2S production.

The microorganism was found to survive salt concentrations of up to 10% and hence could be classified as a moderately halophilic isolate (Ollivier *et al.*, 1994), which was an indication of its marine existence. Growth was observed only in Mannitol agar, and MPO failed to grow in MacConkey agar, thus suggesting its Gram-positive nature.

Induction of AHL reporter strains

Blue coloration was observed when *A. tumefaceins* A136 was co-cultured with MPO in LB medium supplemented with X-gal (Himedia), thus indicating the isolate produced AHL (Fig. S2). Another bioassay involving CV026 failed to generate violacein with MPO extract. However, when the extract was used in an inhibition assay (McClean *et al.*, 1997a), clear halos were observed on violacein plates (Fig. S3). Significant increase in the luminescence of *E. coli* JM109 was also observed, with respect to control, when MPO extract was added to it.

Structure of the inducer molecule

GC-MS chromatogram contains a molecular ion peak at *m/z* 241 and a peak at *m/z* of 102, which are characteristics of the lactone fragment (Fig. 1). LC-MS data also contain a molecular ion $(M+H)^+$ peak at *m/z* 242 and lactone fragment peak at the *m/z* of 102 (Morin *et al.*, 2003) (Fig. S4 and Data S1). Based on these two chromatograms, the QS molecule is identified as c-3-oxo-c8 HSL (OOHL). The extracts from the MPO strain, although Gram Positive, was found to produce AHL-based QS molecule.

The presence of OOHL was further confirmed by spiking the MPO extract with standard OOHL in HPLC to obtain a combined peak at a retention time of 21.6 min. Also, calibration curve was prepared with the help of the standard OOHL, and with the help of this graph, its amount in the extract at various time points was estimated.

Growth and AHL production

Figure. S2 shows the buildup of OOHL and biomass as a function of time. The QS-reporter activity is initiated at the onset of stationary phase (around 8–10 h) and reaches a maximum around the 32nd hour (stationary phase). The production of OOHL can thus be adjudged to be nongrowth associated. The 32nd-hour-old MPO culture was found to produce 15.6 μg L^{-1} of OOHL

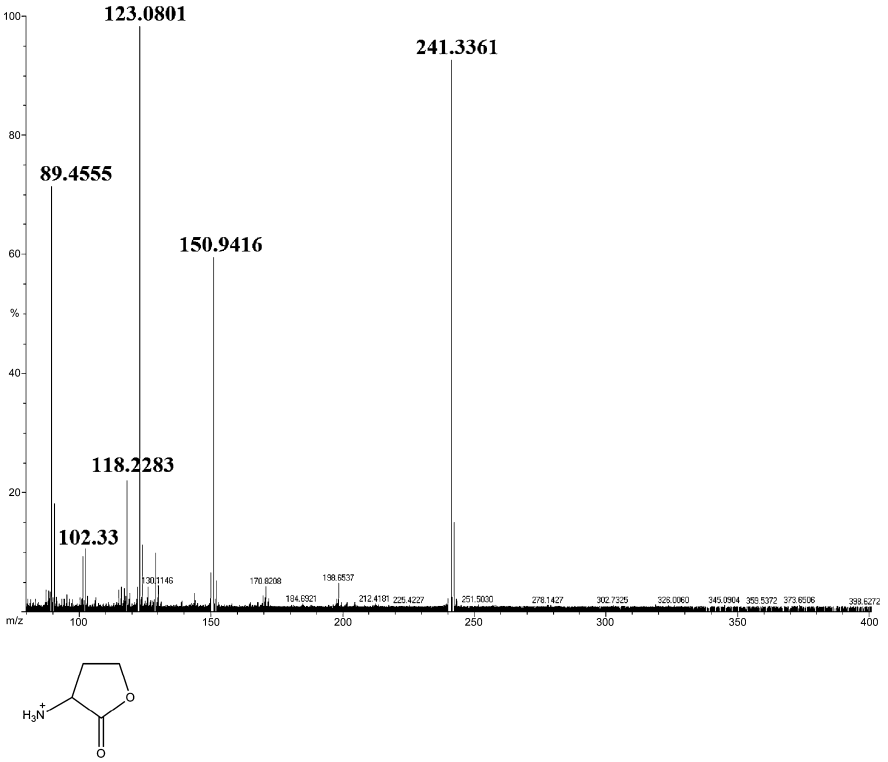

Fig. 1. GCMS data for MPO extract indicating the presence of a characteristic lactone fragment at m/z of 102 and the molecular ion peak at m/z of 241, suggesting the AHL to be C3-oxo-octanoyl Homoserine lactone (OOHL).

(corresponds to about 64.66 μM), which was higher than the values reported in the literature (Charlton *et al.*, 2000).The EC_{50} of MPO extract for the induction of luminescence in the *E. coli* reporter strain was found to be 50 μM (Fig. S5).

Biofilm analysis

The crystal violet assay confirms that MPO forms biofilm on solid surfaces. Exogenous addition of standard OOHL is found to inhibit the formation of this biofilm, and the percentage inhibition increases with increasing concentration of OOHL. 100 μM of the standard OOHL inhibits 61% of the biofilm when compared with the control (Fig. S3a). Around 30% biofilm inhibition is observed when 25% (containing around 15 μM OOHL) of MPO extract is added (Fig. S3b).

Scanning electron microscopy images indicate decrease in cellular aggregates or clumps in the biofilm formed by MPO supplemented with standard OOHL, in comparison with the nonsupplemented control, which suggests its antibiofilm effect on MPO. (Fig. S4a). The same was also observed when the polymers were stained with cellular-viability dyes (attachment of MPO to the polymer was less on OOHL-supplemented samples Fig. S4b). Live cells are seen as green and the dead ones as red.

Extracellular polymeric substances in OOHL (80 μM)-supplemented and extract (100%)-supplemented MPO culture was found to be 30% and 16% less, respectively (Fig. S6), than in the control MPO culture as estimated from their total sugar content. Being an important component of biofilms (Sutherland, 2001), EPS decrease could be attributed to the corresponding reduction in biofilm by OOHL.

The antibacterial activity of standard OOHL and MPO extracts was tested using well-diffusion assays and plate assays. MPO extracts and OOHL (even at 100 μM concentration) did not show any antibacterial activity against MPO, *S. aureus,* or *P. vulgaris* organisms, indicating that the reduction in the MPO biofilm observed here is not due to cell death.

Sequence analysis of suspected LuxR and LuxI homolog in MPO

Interestingly, protein belonging to the Lux R family had been predicted in fully sequenced genome of closely related *Exiguobacterium* spcs AT1b (Vishnivetskaya *et al.*, 2011).

Primers based on LuxR *E* .spcs AT1b of this protein generated a 0.7-kb fragment (GenBank ID **JX126482**) in MPO.

Immediately downstream of the LuxR gene in *Exiguobacterium* spAT1b was located a sequence which was predicted to be a gene for a GCN5 acetyltransferase (GNATs), not

only do these proteins belong to the same superfamily as acyl homoserine lactone synthase [acyl-CoA N-acyltransferases (Nat), http://supfam.cs.bris.ac.uk/SUPERFAMILY/cgi-bin/scop.cgi?sunid=55729] but are also known to share structural features with other LuxI synthases (Watson *et al.*, 2002). Furthermore, the fact that this sequence was strategistically positioned just following a LuxR sequence demanded further probing.

Primers for this protein resulted in a 0.540-kb fragment (GenBank KC577445) in MPO.

The resulting proteins were confirmed by PROSITE to contain:

A) For LuxR primer-based product (designated ExgR): An HTH domain with an internal DNA binding region characteristic of the LuxR superfamily of proteins was found. Multiple sequence alignment with autoinducer binding regions of LuxR, LasR, and TraR QS regulators suggested that the proposed protein showed preservation of the previously mentioned strictly conserved amino acids, W and D (Nasser & Reverchon, 2007) (Fig. S6).

B) For the GCN5 acetyltransferase primer-based product: The *in silico* translated protein was found to contain a single domain belonging to Gcn5-related N-acetyltransferase (GNAT) family. BLAST p search (parameters mentioned in supplementary) suggested the closest homolog to be the 4101 protein [acyl homoserine lactone synthase, *Pseudomonas putida* WCS358, GenBank accession No.: CAF32981.1]. They shared 17% identity/30% similarity and were of similar size < 200 amino acids.

PCR with the forward primer of ExgR and reverse primer of ExgI generated a single fragment of 1.2Kb validating that the genes are adjacent to each other in MPO genome (data not shown).

qPCR analysis for *exgR* and *exgI* mRNA on OOHL addition

qPCR showed a 0.8 ± 0.18- and 2.5 ± 0.5-fold increase in *exgR* mRNA (in OOHL-supplemented MPO cultures) of the 28th- and 32nd-hour sample, respectively, when compared with the control.

However, *exgI* mRNA for control of 28th hour was observed only at 38th cycle (data not shown), suggesting very low level of expression for the gene. No mRNA was observed in control 32nd-hour, OOHL-supplemented 28th- and 32nd-hour samples.

Data reported here are representative of duplicates from three independent experiments.

Discussion

A novel strain of *Exiguobacterium* (designated MPO) was isolated from marine environment based on its ability to induce AHL-based reporter cultures. The fact that multiple AHL bioreporters are induced by MPO, and its extract, suggested that the QS inducer in MPO is an AHL, against the possibility of other molecules including diketopiperazines (DKPs) which are also known to activate *lux*-based AHL biosensors (Tommonaro *et al.*, 2012).

Spectral data (GCMS and LCMS) confirmed the inducer to be an AHL, a C3-oxo-octanoyl homoserine lactone (OOHL), and the amount of OOHL in MPO was found to be higher than the amounts reported in literature.

The involvement of AHLs in biofilm sloughing and dispersion has been shown before (Arevalo-Ferro *et al.*, 2005; Rice *et al.*, 2005). The fact that the formation of biofilm is considerably reduced with the addition of exogenous OOHL suggests a phenotypical role for its production in this strain. Reports suggesting the response of Gram-positive *S. aureus* to AHLs (Qazi *et al.*, 2006) have mentioned that there is associated inhibition of growth, even at 30 μM of the AHL, indicating the foreign nature of the compound in the *S. aureus* system. However, in the current study, inhibition of MPO is not observed even when 100 μM of standard OOHL or 100% of MPO extract is added (data not shown), suggesting that these molecules might be well recognized in the MPO system.

Reports of LuxR proteins in *Exiguobacterium* spAT1b and close proximity of MPO to the strain made us to search for probable LuxR protein in this strain. Isolation and sequencing revealed that the LuxR protein (ExpR) has features similar to the known homologs of QS-regulating proteins from various other bacteria. In addition to this, immediately downstream of the mentioned LuxR gene an ORF was found which translated into a protein sequence exhibited domain features similar to proteins belonging to the same superfamily as LuxI synthases and had a BLAST homolog match with *Pseudomonas putida* WCS358 acyl homoserine lactone synthase. Both proteins were also smaller (< 200 amino acids) than what is supposed to be the average length of acyl homoserine synthases.

qPCR analysis suggested that the addition of OOHL significantly increased *exgR* transcription, indicating its role for the regulation of the gene by inherent OOHL concentration and its possible involvement in the proposed quorum-sensing events in MPO. This result is in agreement with the positive regulatory roles of the AHL on expression of cognate LuxR (Shadel & Baldwin, 1992).

On the other hand, very low expression of the exgI mRNA was observed for control and no exgI mRNA was found in the OOHL-supplemented samples. This could be indicative of a possible inherent negative regulatory mechanism that affects transcription of AHL synthase gene at higher AHL concentration as seen in the RsaL

systems of *Pseudomonas aeruginosa and Pseudomonas putida* (Venturi *et al.*, 2011).

AHL-based QS Signals, until now, had been viewed to be distributed only among the Gram-negative microorganisms. This study is the first one to suggest the presence of an AHL-based communication process in Gram-positive bacteria, which is confirmed by bioassays with multiple bioreporters. The methods used here have been used extensively for the identification and elucidation of QS systems in other Gram-negative bacteria.

The fact that putative LuxR and LuxI homolog were found adjacent to each other in MPO further strengthens the presence of an AHL-based QS system.

The genera of *Exiguobacterium* with its anomalous classification and known Gram variable nature (Kim *et al.*, 2005) could thus be the perfect candidate for the presence of such a strain. The isolation of such a unique strain further warrants the search for new microorganisms from the less-studied niche of marine water.

Reports that indicate the presence of AHLs in Gram-positive bacteria are nonexistent, and if available Gram-positive strains are tested with known AHL bioassay systems, then one might possibly find new Gram-positive microorganisms that may have an AHL-based communication system.

Acknowledgements

Pramal Biswa would like to thank the Council of Scientific and Industrial Research (CSIR), India, for financial support. The QS-reporter strains, *E. coli JM109* (psb 1075) and *Agrobacterium tumefaceins* A136, were kindly gifted by Prof Paul Williams of Nottingham University, UK, and Dr Scott A.Rice, University of New South Wales, Australia, respectively. Support from Sophisticated Analytical and Instrumentation Facility (SAIF) at Indian Institute of Technology Madras, for GCMS data, is acknowledged.

GenBank accessions

16S rDNA MPO: JF915892, ExqR (probable LuxR protein from MPO): JX126482 ExgI(Probable LuxI protein from MPO): KC577445

References

Arevalo-Ferro C, Reil G, Gorg A, Eberl L & Riedel K (2005) Biofilm formation of *Pseudomonas putida* IsoF: the role of quorum sensing as assessed by proteomics. *Syst Appl Microbiol* **28**: 87–114.

Bramhachari PV & Dubey SK (2006) Isolation and characterization of exopolysaccharide produced by *Vibrio harveyi* strain VB23. *Lett Appl Microbiol* **43**: 571–577.

Charlton TS, De Nys R, Netting A, Kumar N, Hentzer M, Givskov M & Kjelleberg S (2000) A novel and sensitive method for the quantification of N-3-oxoacyl homoserine lactones using gas chromatography-mass spectrometry: application to a model bacterial biofilm. *Environ Microbiol* **2**: 530–541.

Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM & Beachey EH (1985) Adherence of coagulase-negative *staphylococci* to plastic tissue culture plates: a quantitative model for the adherence of *staphylococci* to medical devices. *J Clin Microbiol* **22**: 996–1006.

Cunliffe M & Murrell JC (2009) The sea-surface microlayer is a gelatinous biofilm. *ISME J* **3**: 1001–1003.

Dirix G, Monsieurs P, Dombrecht B, Daniels R, Marchal K, Vanderleyden J & Michiels J (2004) Peptide signal molecules and bacteriocins in Gram-negative bacteria: a genome-wide *in silico* screening for peptides containing a double-glycine leader sequence and their cognate transporters. *Peptides* **25**: 1425–1440.

Dobretsov S, Teplitski M & Paul V (2009) Mini-review: quorum sensing in the marine environment and its relationship to biofouling. *Biofouling* **25**: 413–427.

Dubois M, Gilles KA, Hamilton JK, Rebers PA & Smith F (1956) Colorimetric method for determination of sugars and related substances. *Anal Chem* **28**: 350–356.

Gontang EA, Fenical W & Jensen PR (2007) Phylogenetic diversity of gram-positive bacteria cultured from marine sediments. *Appl Environ Microbiol* **73**: 3272–3282.

Han SW, Sriariyanun M, Lee SW, Sharma M, Bahar O, Bower Z & Ronald PC (2011) Small protein-mediated quorum sensing in a gram-negative bacterium. *PLoS ONE* **6**: e29192.

Huang Y-L, Ki J-S, Lee OO & Qian P-Y (2008) Evidence for the dynamics of Acyl homoserine lactone and AHL-producing bacteria during subtidal biofilm formation. *ISME J* **3**: 296–304.

Kim IG, Lee MH, Jung SY, Song JJ, Oh TK & Yoon JH (2005) *Exiguobacterium aestuarii* sp. nov. and *Exiguobacterium marinum* sp. nov., isolated from a tidal flat of the Yellow Sea in Korea. *Int J Syst Evol Microbiol* **55**: 885–889.

Ludwig W, Schleifer KH & Whitman WB (2009) Revised road map to the phylum *Firmicutes*. *Syst Bacteriol* **3**: 1–13.

McClean KH, Winson MK, Fish L *et al.* (1997a) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. *Microbiology* **143**: 3703–3711.

McLean RJC, Whiteley M, Stickler DJ & Fuqua WC (1997b) Evidence of autoinducer activity in naturally occurring biofilms. *FEMS Microbiol Lett* **154**: 259–263.

Middleton B, Rodgers HC, CÃmara M, Knox AJ, Williams P & Hardman A (2002) Direct detection of N-acylhomoserine lactones in cystic fibrosis sputum. *FEMS Microbiol Lett* **207**: 1–7.

Morin D, Grasland B, Vallee-Rehel K, Dufau C & Haras D (2003) On-line high-performance liquid chromatography-mass spectrometric detection and quantification of N-acylhomoserine lactones, quorum sensing signal molecules, in the presence of biological matrices. *J Chromatogr A* **1002**: 79–92.

Nasser W & Reverchon S (2007) New insights into the regulatory mechanisms of the LuxR family of quorum sensing regulators. *Anal Bioanal Chem* **387**: 381–390.

Ollivier B, Caumette P, Garcia J-L & Mah RA (1994) Anaerobic bacteria from hypersaline environments. *Microbiological reviews* **58**: 27–38.

Parsek MR & Greenberg EP (2005) Sociomicrobiology: the connections between quorum sensing and biofilms. *Trends Microbiol* **13**: 27–33.

Qazi S, Middleton B, Muharram SH *et al.* (2006) N-acylhomoserine lactones antagonize virulence gene expression and quorum sensing in *Staphylococcus aureus*. *Infect Immun* **74**: 910–919.

Rice SA, Koh KS, Queck SY, Labbate M, Lam KW & Kjelleberg S (2005) Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues. *J Bacteriol* **187**: 3477–3485.

Rodrigues DF & Tiedje JM (2007) Multi-locus real-time PCR for quantitation of bacteria in the environment reveals Exiguobacterium to be prevalent in permafrost. *FEMS Microbiol Ecol* **59**: 489–499.

Rojas J, Ochoa V, Ocampo S & Munoz J (2006) Screening for antimicrobial activity of ten medicinal plants used in Colombian folkloric medicine: a possible alternative in the treatment of non-nosocomial infections. *BMC Complement Altern Med* **6**: 2.

Sarker SD, Nahar L & Kumarasamy Y (2007) Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the *in vitro* antibacterial screening of phytochemicals. *Methods* **42**: 321–324.

Shadel GS & Baldwin TO (1992) Positive autoregulation of the *Vibrio fischeri* luxR gene. LuxR and autoinducer activate cAMP-catabolite gene activator protein complex-independent and -dependent luxR transcription. *J Biol Chem* **267**: 7696–7702.

Sharif DI, Gallon J & Smith CJ (2008) Quorum sensing in Cyanobacteria: N-octanoyl-homoserine lactone release and response, by the epilithic colonial cyanobacterium Gloeothece PCC6909. *ISME J* **2**: 1171–1182.

Sivakumar PM, Cometa S, Alderighi M, Prabhawathi V, Doble M & Chiellini F (2012) Chalcone embedded polyurethanes as a biomaterial: synthesis, characterization and antibacterial adhesion. *Carbohydr Polym* **87**: 353–360.

Steidle A, Allesen-Holm M, Riedel K, Berg G, Givskov M, Sr Molin & Eberl L (2002) Identification and characterization of an N-acylhomoserine lactone-dependent quorum-sensing system in *Pseudomonas putida* strain IsoF. *Appl Environ Microbiol* **68**: 6371–6382.

Sutherland IW (2001) Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology* **147**: 3–9.

Taga ME & Bassler BL (2003) Chemical communication among bacteria. *P Natl Acad Sci USA* **100**: 14549–14554.

Teplitski M, Eberhard A, Gronquist MR, Gao M, Robinson JB & Bauer WD (2003) Chemical identification of N-acyl homoserine lactone quorum-sensing signals produced by *Sinorhizobium meliloti* strains in defined medium. *Arch Microbiol* **180**: 494–497.

Tommonaro G, Abbamondi G, Iodice C, Tait K & De Rosa S (2012) Diketopiperazines Produced by the *Halophilic archaeon*, *Haloterrigena hispanica* activate AHL bioreporters. *Microb Ecol* **63**: 490–495.

Venturi V, Rampioni G, Pongor Sn & Leoni L (2011) The virtue of temperance: built-in negative regulators of quorum sensing in *Pseudomonas*. *Mol Microbiol* **82**: 1060–1070.

Vishnivetskaya TA, Lucas S, Copeland A *et al.* (2011) Complete genome sequence of the thermophilic bacterium *Exiguobacterium* sp. AT1b. *J Bacteriol* **193**: 2880–2881.

Wang L, Qiao N, Sun F & Shao Z (2008) Isolation, gene detection and solvent tolerance of benzene, toluene and xylene degrading bacteria from nearshore surface water and Pacific Ocean sediment. *Extremophiles* **12**: 335–342.

Watson WT, Minogue TD, Val DL, von Bodman SB & Churchill MEA (2002) Structural basis and specificity of acyl-homoserine lactone signal production in bacterial quorum sensing. *Mol Cell* **9**: 685–694.

Wurl O & Holmes M (2008) The gelatinous nature of the sea-surface microlayer. *Mar Chem* **110**: 89–97.

Zhang G, Zhang F, Ding G *et al.* (2012) Acyl homoserine lactone-based quorum sensing in a methanogenic archaeon. *ISME J* **6**: 1336–1344.

Supporting Information

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

Fig. S1. Gram staining (positive) of *Exiguobacterium* MPO cells viewed at 40× magnification.

Fig. S2. Cross-feeding bioassay of *A. tumefaceins* A136 bioreporter responding to MPO culture by producing a blue colouration due to X-gal degradation, indicative of the presence of AHL.

Fig. S3. Results for inhibition plate assay with CV026 bioreporter: Positive control A-10 μM OOHL,B-100 μM OOHL,C-Octanoyl homoserine lactone(just to check activation of CV026), Negative control D-methanol, E-MPO extract in methanol.

Fig. S4. LCMS data for MPO indicating OOHL with the presence of the characteristic lactone fragment at *m/z* 102 and the molecular ion peak $(M+H)^+$ 242.

Fig. S5. EC50 for the MPO extract luminescence-inducing activity in the *E.coli* JM109 (psb1075) reporter.

Fig. S6. 30% and 16% reduction in EPS in OOHL and extract supplemented cultures of MPO with respect to control is observed respectively.(P value = 0.04).

Table S1. List of primers used in sequencing.

Table S2. List of the primers used in the qPCR study.

Data S1. Details of the parameter used in the *in silico* analysis of ExgR and ExgI.