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# Production of acylated homoserine lactone by a novel marine strain of *Proteus vulgaris* and inhibition of its swarming by phytochemicals

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A marine strain of *Proteus vulgaris* capable of activating multiple acylated homoserine lactone (AHL)-based reporter cultures was isolated. The cognate signal molecule was characterized as octanoyl homoserine lactone (OHL) and its production was observed to be growth dependent, with maximum production (5.675 μg $l^{-1}$) at 24 h growth. The strain exhibited swarming, but its motility was not affected upon addition of pure OHL or culture supernatant. Phytochemicals such as quercitin and berberine chloride inhibited OHL production and reduced swarming. FliA, the predominantly upregulated protein during swarming, was considered as a possible target for these inhibitors, and docking of the two most active and two least active inhibitors to this protein suggested preferential binding of the former set of compounds. Apart from adding new evidence to AHL production in *Proteus vulgaris*, active inhibitors shortlisted from this study could help in identifying lead compounds to act against this opportunistic pathogen of the respiratory and gastrointestinal tract.

## INTRODUCTION

Quorum sensing (QS), the phenomenon of microbial communication mediated through chemical signals, has been observed among different types of bacteria. The most widely studied of these information systems is the acylated homoserine lactone (AHL)-based QS that is found predominantly in Gram-negative bacteria (Ng & Bassler, 2009). Over the years, AHLs and related QS phenotypes have been reported in several different ecological niches, from marine snow (Dobretsov *et al.*, 2009) to microbial mats (Montgomery *et al.*, 2013).

Members of the genus *Proteus* are widely distributed in nature (Armbruster & Mobley, 2012) and have also been isolated from sea water (Zhao & Dang, 2012). *Proteus vulgaris* was one of the first species of the genus *Proteus* to be reported (O'Hara *et al.*, 2000). *Proteus vulgaris*, similar to *Proteus mirabilis*, is a member of the human gut flora and a urinary tract pathogen, although the latter is a much more prolific and well-studied urinary tract infection bacterium (Manos & Belas, 2006; Gul *et al.*, 2013). QS has been implicated widely in *Proteus mirabilis*, with reports of varied phenotypical responses to externally added AHL (Stankowska *et al.*, 2012). However, studies have failed to connect these observations to the presence of a functional AHL-based system with innate autoinducer production (Armbruster & Mobley, 2012).

Swarming motility is defined as the chemotaxis-induced migration of bacteria over a solid surface, mediated by flagellar motion. This surface translocation event is known to be a typical QS-controlled phenotype, which is highly regulated and involves the interplay of several proteins (Daniels *et al.*, 2004). FliA, the alternate $\sigma$ factor, is involved in the control of several swarming-associated genes and its expression is upregulated majorly in swarm cells of *Proteus mirabilis* (Pearson *et al.*, 2010). The protein itself is regulated negatively by the anti-$\sigma$ factor, FlgM. Binding of FlgM prevents FliA from interacting with RNA polymerase and hence prevents the untimely activation of swarming-associated genes (Ding *et al.*, 2009). FliA is also known to be involved in QS regulatory effects in *Escherichia coli* (Clarke & Sperandio, 2005), making it an effective target for consideration, in the absence of any other validated targets, for analysis of QS-related activity.

Apart from communication, an emerging aspect of QS is also its interference. Phytochemicals are recognized as potent QS antagonist (Vattem *et al.*, 2007; Kalia, 2013; Nazzaro *et al.*, 2013) and have been used at sub-MIC concentrations to affect QS-associated phenotypes. For example, eugenol was found to decrease elastase production in *Pseudomonas aeruginosa* (Zhou *et al.*, 2013), and

Abbreviations: AHL, acylated homoserine lactone; LC, liquid chromatography; OHL, octanoyl homoserine lactone; QS, quorum sensing.

Five supplementary figures are available with the online version of this paper.

 

curcumin reduced QS-mediated bioluminescence and biofilm maturation in *Vibrio* spp. (Packiavathy *et al.*, 2013). Swarming has been characterized as a major virulence factor in *Proteus*, aiding colonization and subsequent infection (Wang *et al.*, 2006). There are also several reports of widespread antibiotic resistance in *Proteus* spp. (Yah *et al.*, 2007; Pandey *et al.*, 2013). Hence, compounds with a different mode of antibacterial action are needed and, in this regard, the anti-QS activity of phytochemicals makes them ideal lead molecules for the design of novel drugs.

In this paper, *Proteus vulgaris* strain BNW, a novel strain isolated from marine waters, whose extract was found to activate multiple AHL reporter strains, is reported. The molecule responsible for the activation was identified using various analytical tools. The inhibition of the AHL production and swarming motility of the bacteria by phytochemicals was also investigated experimentally and theoretically.

## METHODS

**Materials.** Chemicals used for the experiments were procured from Sigma Aldrich, HiMedia and SRL. Solvents used for extraction (analytical reagent grade) and HPLC analysis were purchased from Merck. Lyophilized culture media were procured from Difco and HiMedia. The different phytochemicals (Table 1) were procured from either Sigma Aldrich or TCI Fine Chemicals.

**Strain and culture conditions.** Bacterial strains were isolated from surface sea water from the Bay of Bengal (Chennai, South India) after serial dilution and growth in Zobell marine agar. Maintenance of the culture and experiments were carried out in Luria–Bertani (LB) agar and broth.

The initial screening (to identify potential QS bacteria) involved the use of a replica-plate-based reporter assay (Bruhn *et al.*, 2004). In this method, bacterial colonies growing in the master plates were transferred by a sterile surface onto plates containing QS reporter bacteria, *Chromobacterium violaceum* CV026 (procured from National Collection of Type Cultures, UK). Colonies positive for reporter culture induction were then selected and recultured from the master plate, and reported for induction of two other QS reporter strains, *E. coli* JM109 (psb1075) and *Agrobacterium tumefaciens* A136. The details of the various bioassays adopted are listed in Table 2.

**Table 1.** Phytochemicals used in this study

| Code | Phytochemical |
|---|---|
| N1 | 2,4,5-Trimethoxy cinnamic acid |
| N2 | 3,4-Dimethoxy cinnamic acid |
| N3 | Berberine chloride |
| N4 | Caffeic acid |
| N6 | Cinnamic acid |
| N7 | Eugenol |
| N8 | Ferulic acid |
| N9 | *p*-Coumaric acid |
| N10 | Quercitin |
| N11 | 2,6-Dimethoxy benzoic acid |
| N12 | 2,3-Dimethoxy benzoic acid |
| N13 | 4-Hydroxy-3-methoxy benzyl amine hydrochloride |
| N15 | 4-Hydroxy-3-methoxy benzoic acid |
| N20 | 2,5-Dimethoxy benzoic acid |

**Identification of the isolated bacterium.** A partial biochemical study of the isolated organism was performed using the HiAssorted biochemical test kit KB002 (HiMedia). The salt tolerance level of the culture was determined by checking for its viability with resazurin dye after growth in LB media containing different concentrations (1–10 %) of NaCl (Sarker *et al.*, 2007).

The HiPurA bacterial genomic DNA isolation kit (HiMedia) was used for extracting genomic DNA. PCR analysis of 16S rDNA was then performed using universal primers (forward 5′-GAGTTTGATCC-TGGCTCA-3′ and reverse 5′-ACGGCTAACTTGTTACGACT-3′). The purified PCR product was sequenced with the help of an external agency (Chromous Biotech Lab) and identified by the SeqMatch program of the Ribosomal Database Project (http://rdp.cme.msu.edu/).

**Structural identification of the inducer.** After 24 h of growth, the culture supernatant was extracted twice, with equal volumes of dichloromethane (Steidle *et al.*, 2002), and then the solvent was evaporated to dryness in a Rotavapor (Buchi) and finally reconstituted to 1/200 times its volume with Milli-Q water.

Liquid chromatography (LC-MS) analysis of the crude extract was performed (Central Instrumentation Facility, IIT Guwahati) with a Waters Q-ToF Premier mass spectrometer equipped with an Acquity UPLC C18 RF 1.7 μm, 2.1 × 50 column. For this analysis, the extracts were reconstituted with HPLC-grade methanol and filtered through a 0.2 μm nylon membrane (Sartorius).

A Shimadzu HPLC system equipped with a C18 reverse-phase (Phenomenex, Luna 5 μm, 100 Å, 150 × 4.6 mm) column was used for estimating the quantity of AHL produced by *Proteus vulgaris* BNW. The column was eluted initially with 8 % acetonitrile at 2 ml $min^{-1}$ to reach 46 % acetonitrile in 75 min and then another gradient to reach 98 % acetonitrile in 10 min, and this concentration was maintained for an additional 10 min (Teplitski *et al.*, 2003). The concentration of the sample in the extract was determined using pure AHL as standard.

Alkaline conditions cause AHL hydrolysis and the effect can be reverted by returning to acidic pH (Decho *et al.*, 2011). This knowledge was used to further ascertain the nature of the inducer. NaOH (2 M) was added to the supernatant of the *Proteus vulgaris* BNW culture to cause lactonolysis. One-half of this solution was extracted with dichloromethane, and HCl (2 M) was added to the other half and extracted with dichloromethane. Both the solutions were then reconstituted separately. The QS potentials of the generated extracts were then determined with the reporters, *C. violaceum* CV026 and *E. coli* JM109.

**Growth kinetics.** The *Proteus vulgaris* BNW culture was grown in LB broth and samples were collected at different time intervals. The culture supernatant was then extracted as mentioned earlier. An aliquot of 50 μl of these extracts was then added to 1 ml of fresh reporter culture, *E. coli* JM109, and luminescence was measured (Enspire multimode plate reader; Perkin Elmer) after overnight incubation (Biswa & Doble, 2013). The relative light unit values obtained were converted to concentration of AHL in the culture supernatant using a standard graph with pure AHL standard.

**Swarming.** LB plates were prepared with 0.5 % agar to check for the swarming motility phenotype in the isolated culture. The plates were also supplemented with standard AHL or culture extract, followed by point inoculation of the bacteria and 24 h incubation. The extent

**Table 2.** Summary of the different AHL reporters and bioassay methods used in the study

Note that *C. violaceum* CV026 was used in the initial screening of bacteria: replica plate method.

| Method and observation | Reporter culture | | | |
|---|---|---|---|---|
| | ***E. coli* JM109(psb1075)** | ***C. violaceum* CV026** | | ***Agrobacterium tumefaciens* A136** |
| **Method followed** | Extract of suspect culture is added to the reporter and grown for 12 h | LB agar embedded with reporter culture; filter paper discs are placed or wells plunged on it; extract added to these | TLC of extract in 60 : 40 methanol: water; overlaid with reporter culture containing LB agar | X-Gal supplemented with LB media used for co-culturing test strain |
| **Observation** | Luminescence measured in relative light units | Violacein pigment formed; comparison of retention factor of extract and standard AHL from TLC | | Blue-coloured degradation product of X-Gal with active extracts |

of swarming was then quantified, with the help of ImageJ analysis software (http://imagej.nih.gov/ij/), from the images of the swarm plates. Swarming potential was represented as the percentage area swarmed with respect to the control (Caiazza *et al.*, 2007).

**Biofilm formation.** The ability of the isolated microbe to form a biofilm, a well-studied and relevant QS phenotype, was estimated using crystal violet (Christensen *et al.*, 1985). Initially, the isolate was grown in a 24-well plate for 48 h, and then the plate was rinsed gently twice with 0.7 % saline and air-dried. The plate was then incubated with 0.1 % crystal violet for 10 min, rinsed twice with 0.7 % saline and incubated for another 10 min with 30 % acetic acid. Absorbance was measured at 540 nm (Enspire multimode plate reader; Perkin Elmer).

**QS inhibition assays.** The MICs of the 14 phytochemicals (Table 1) toward the isolate were determined in 96-well plates using resazurin dye (Palomino *et al.*, 2002). The QS inhibition potential of these compounds at concentrations below their MIC values was determined as described below.

Each of these phytochemicals was added to 75 ml sterilized LB medium to reach a final concentration of 50 μM (sub-MIC), and then inoculated with overnight culture of the bacterial isolate and allowed to grow at 30 °C and 180 r.p.m. After 24 h, the broth was extracted as mentioned in the previous section and reconstituted into a smaller volume (extract of 75 ml supernatant in 250 μl Milli-Q water). The extracts were later analysed with the help of the reporter assays, as indicated in Table 2. Inhibition of AHL production was estimated from the photographs of *C. violaceum* CV026 pour plates treated with the culture extracts (generated with the different phytochemicals) by measuring the area of the violacein halo formed and represented as percentage with respect to control (untreated extract). The effect of these phytochemicals on swarming inhibition (at 50 μM) was also quantified.

**Docking with FliA.** The upregulation of the FliA gene in swarming bacteria and its role in the regulation of several swarm-associated genes have been reported (Pearson *et al.*, 2010). This protein was therefore considered as a potential target for modelling the action of the inhibitors. The FliA protein from *Proteus mirabilis* (UniProt ID: PMI1618) was homology modelled using the *Aquifex aeolicus* FliA protein (Protein Data Bank ID: 1RP3) as the template, with the help of Swiss-Prot (http://swissmodel.expasy.org/), and the generated structure was validated by using the functions PROCHECK and Verify_3D, available in the Structure Analysis and Verification Server (http://nihserver.mbi.ucla.edu/SAVES/).

The two best and the two worst inhibitors of swarming, as determined from experimental observation, were selected for docking with this modelled protein. Initially, their structures were sketched in ChemDraw and the resultant 3D structures were then appropriately energy minimized using the MOE (Molecular Operating Environment) 2011 program, with the help of the MMFF94X force field. For docking, the binding site selected was Lys190–Leu210, which is the region encompassing Lys195 and Ile205 in the protein FliA. This was based on the known interaction of FlgM (a natural FliA inhibitor) with the corresponding amino acids, Lys195 and Ile209, in the template 1RP3 (Lys195 and Ile205 in our model) (Sorenson *et al.*, 2004). Docking was performed using the program GOLD (Genetic Optimization for Ligand Docking) suite 5.2. The best GOLD scores for each of the inhibitors were selected and the corresponding interacting amino acid residues in the binding site were determined with the MOE software.

## RESULTS

### Isolation and identification

A microbe was isolated from the surface sea waters using the replica plate method based on the positive response it showed in three different bioassays. The 16S rDNA analysis revealed that the isolate belonged to the *Proteus* genus and shared 99 % similarity with *Proteus vulgaris* (Fig. S1, available in the online Supplementary Material).

The isolate was designated *Proteus vulgaris* BNW (16S ribosomal RNA gene, partial sequence available under GenBank accession number JF915893.1). The colonies were off-white, made up of Gram-negative, rod-shaped cells, ~3 μm in length. Biochemical analysis showed positive results for phenylalanine deamination, glucose utilization, urease and indole production, and nitrate reduction, as well as presence of catalase and oxidase; the results were negative for the utilization of citrate, lysine, ornithine, lactose, arabinose, adonitol and sorbitol, and $H_2S$ production. Based on the above biochemical characteristics, the organism was further confirmed as *Proteus vulgaris*. The microbe was able to survive salt concentrations of up to 5 % and hence was classified as moderately halophilic

(Ollivier *et al.*, 1994). Growth was observed only in mannitol agar plates, hinting at the Gram-negative nature of the microbe.

## Structural elucidation of the inducer molecule

*Agrobacterium tumefaciens* A136, when co-streaked with *Proteus vulgaris* BNW, in X-Gal (HiMedia)-supplemented LB medium, generated a blue coloration, indicating that the isolate produced AHL (Fig. S2). A significant increase in the luminescence of *E. coli* JM109, with respect to the control, was observed when *Proteus vulgaris* BNW extract was added to it. *Proteus vulgaris* BNW and its extract induced violacein production in *C. violaceum* CV026.

LC-MS data showed the presence of a molecular ion $(M+H)^+$ peak at *m/z* 229 and a McLafferty rearranged fragment peak at *m/z* 144, indicating that the QS molecule was octanoyl homoserine lactone (C8 HSL, OHL) (Morin *et al.*, 2003) (Fig. 1). TLC-based bioassay with *C. violaceum* CV026 suggested similar retention factor values for standard OHL (Sigma-Aldrich) and the QS molecule isolated from the *Proteus vulgaris* BNW extract (Fig. S3). Spiking *Proteus vulgaris* BNW extract with standard OHL in HPLC further confirmed its presence. The amount of AHL produced by the culture at different times during growth was estimated by using commercial OHL as standard.

Alkali de-lactonized *Proteus vulgaris* BNW culture extract did not show any QS activity with *C. violaceum* CV026 or *E. coli* JM109 (Fig. S4), whereas the activity returned on acidification (lactonization) of the same extract, indicating the presence of a lactone group in the QS molecule produced by *Proteus vulgaris*.

## Growth kinetics and AHL production

The increase in biomass and production of OHL with time is shown in Fig. 2. The QS reporter activity was initiated at the onset of the stationary phase (~8–10 h) and it reached a maximum ~24 h (stationary phase). The production of OHL was thus adjudged to be growth-associated. At 24 h, the *Proteus vulgaris* BNW culture produced 5.67 μg OHL $l^{-1}$ (5.9 μM), which is in accordance with values reported in the literature (Charlton *et al.*, 2000).

## Swarming and biofilm formation

Swarming and biofilm formation are two commonly occurring phenotypes in the genus *Proteus* (Jones *et al.*, 2004), the presence of which has been investigated in *Proteus vulgaris* BNW. Although swarming is observed (Fig. S5) in *Proteus vulgaris* BNW, the exogenous addition of commercial OHL or culture extract did not seem to have

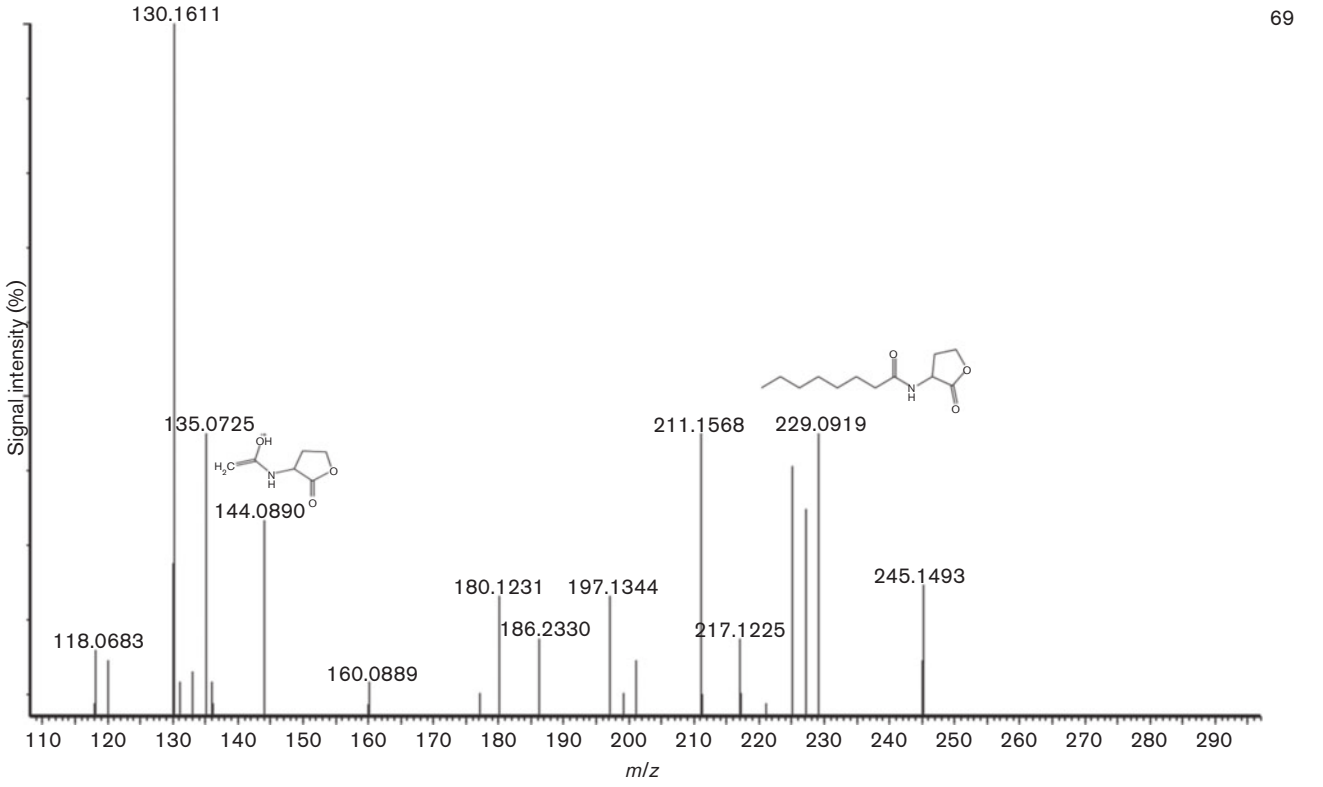


**Fig. 1.** LC-MS of the *Proteus vulgaris* BNW extract shows the *m/z* 144 McLafferty fragment characteristic of AHL and a molecular ion peak at *m/z* 229, suggesting the AHL to be OHL.

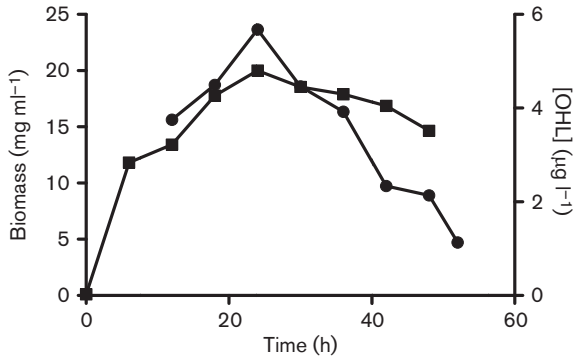


**Fig. 2.** Production kinetics of OHL (●) and biomass (■) in *Proteus vulgaris* BNW. Luminescence values for *E. coli* JM109 in response to *Proteus vulgaris* BNW extracts collected over various time points were converted to corresponding amounts of OHL from the HPLC calibration curve prepared with the standard. Growth-associated production of OHL was observed with *Proteus vulgaris* BNW.

any significant effect on the swarming phenotype. Biofilm formation was not observed for this strain.

### Inhibition of QS by phytochemicals

The growth of *Proteus vulgaris* BNW was not inhibited at phytochemical concentrations of 50 μM, and hence their effect on swarming and OHL production was measured at this value. Quercitin (N10) significantly interfered with OHL production and swarming (81 % reduction in OHL production and 75 % reduction in swarming) (Fig. 3). The second best compound was berberine chloride (N3), which reduced OHL production by 28 % and swarming by 31 %. A negative correlation was observed between swarming inhibition and the molecular mass of the phytochemicals (correlation coefficient –0.64) and the number of hydrogen bond donors (correlation coefficient –0.66). A positive correlation (correlation coefficient 0.68) was also observed between swarming inhibition and a decrease in OHL production.

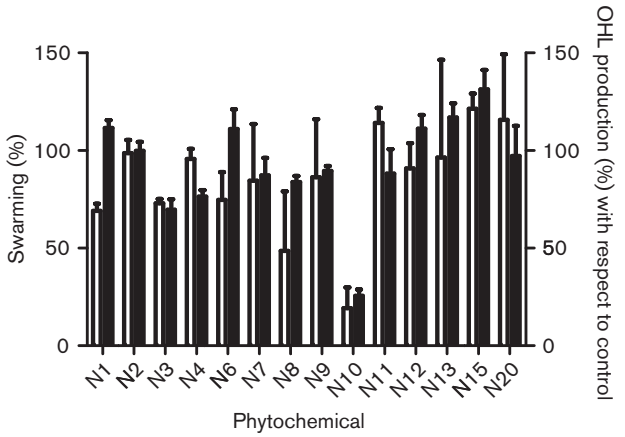


**Fig. 3.** Comparison between percentage swarming (solid columns) and percentage OHL production (open columns) in *Proteus vulgaris* BNW culture treated with different phytochemicals with respect to control (100%). A direct correlation (correlation coefficient 0.68) was seen between swarming and OHL production.

### Docking with FliA

FliA, a highly upregulated protein in *Proteus mirabilis* swarmer cells, was considered as a possible target for these inhibitors. Its structure was modelled using a template protein. Two phytochemicals with the best (N3 and N10) and worst OHL-inhibitory and anti-swarming activities (N2 and N15) were selected for docking at the binding site of FliA interaction with its innate repressor, FlgM. Docking suggested that compounds N3 and N10 had better binding ability (indicated by their GOLD scores) to the FliA binding site when compared with N2 and N15.

## DISCUSSION

AHL-based QS has been studied widely in different Gram-negative bacteria, yet its existence and growth-dependent production have never been reported among the genus *Proteus* (Armbruster & Mobley, 2012). Studies in *Proteus mirabilis* have shown that it responds to AHLs through changes in expression of virulence factors, including biofilm formation, swarming motility, and urease and haemolysin production (Stankowska *et al.*, 2008, 2012; Czerwonka *et al.*, 2014). However, these responses were never associated with an operational QS system.

In the present study, a novel AHL-producing strain of *Proteus vulgaris* (designated *Proteus vulgaris* BNW), isolated from sea water and capable of inducing several AHL-based reporters, is reported. LC-MS indicated that the inducer molecule is OHL, which was further confirmed with TLC and HPLC. OHL production was found to be growth-associated – the hallmark of several known QS systems. Although the presence of such AHL-producing *Proteus* strain has been described (Stankowska *et al.*, 2012), the authors failed to shed light on its chemical structure, amount produced and the association of signal production with growth.

Induction of the reporter cultures has been studied previously with compounds other than AHLs, including diketopiperazines that are reported to be produced in *Proteus mirabilis* (Holden *et al.*, 1999). *Proteus vulgaris* BNW extract could induce these reporters at even low concentrations (<6 μM OHL), unlike the diketopiperazines (whose detection range is ~0.3 mM) (Holden *et al.*, 1999). The pH-based inactivation–reactivation confirmed that the resultant QS activation by *Proteus vulgaris* BNW extract was due to OHL and not due to additive contributions from diketopiperazine contaminants.

Exogenous addition of OHL to *Proteus vulgaris* BNW did not affect swarming. This type of non-responsiveness towards addition of cognate AHL on swarming has also been observed with *Pseudomonas aeruginosa* (Kamatkar & Shrout, 2011). The behaviour was rationalized by stating that QS on surfaces was controlled in a manner that was not only dependent on the swarming population, but also controlled by the physical properties of the interacting surface.

QS inhibitors, including furanones, *p*-nitrophenyl glycerol and tannic acid, have been shown to reduce swarming and biofilm formation in *Proteus mirabilis* (Gram *et al.*, 1996; Jones *et al.*, 2009). In the current study, plant-based compounds were analysed for their effect on signal and swarm movements in *Proteus vulgaris* BNW. A direct correlation between OHL production and swarming was observed, suggesting QS control of swarming. Eugenol (Zhou *et al.*, 2013) and caffeic acid (Borges *et al.*, 2014) have been reported to inhibit QS, whereas no such behaviour was observed here. Other proven QS antagonists such as ferulic acid (Borges *et al.*, 2014) and quercitin (Vikram *et al.*, 2010) were active even in this study. This difference in results between this study and others may be due to the selective sensitivity of different bacteria to various phytochemicals. This study has also identified berberine chloride as an inhibitor of swarming and AHL production in *Proteus vulgaris* BNW.

Berberine chloride (N3) and quercitin (N10) exhibited better binding (higher GOLD scores) than 3,4-dimethoxy cinnamic acid (N2) and 4-hydroxy-3-methoxy benzoic acid (N15) with the modelled FliA protein. All four compounds shared the common interacting residues, Tyr197 and His221, but did not interact with the residues Lys195 and Ile205, which are known to be important for FlgM-based inhibition of FliA (Fig. 4). The fact that all four phytochemicals selected for docking studies target the same residues in the binding site indicates that they share a common mode of action, and might affect the subsequent

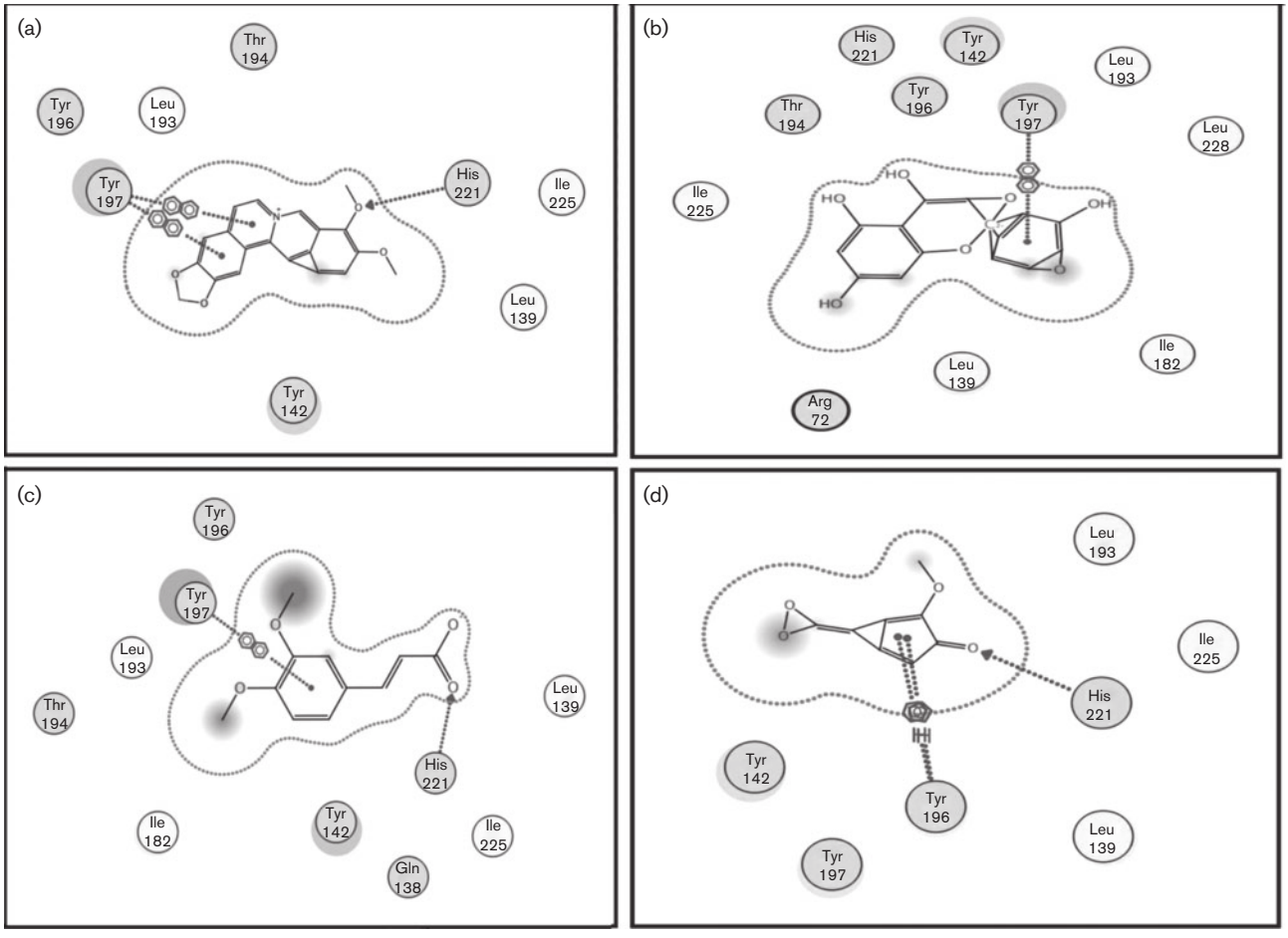


**Fig. 4.** Docking results against FliA. Protein used: homology model of FliA in *Proteus mirabilis*. Binding pocket selected for docking: Lys190–Leu210 (100 poses). (a) N3: GOLD score 70; interacting residues Tyr197 and His221. (b) N10: GOLD score 63; interacting residues Tyr197. (c) N2: GOLD score 35; interacting residues Tyr197 and His221. (d) N15: GOLD score 36.4; interacting residues Tyr196 and His221.

interaction between FliA and FlgM, thereby influencing swarming. Hence, these results hint at the possible involvement of FliA in the swarming inhibition by the phytochemicals. A negative correlation between swarming inhibition and molecular mass of the compounds also suggested that larger molecules are more potent QS inhibitors than smaller molecules. The use of phytochemicals in this study served a dual purpose: (1) it elucidated a direct relation between swarming and OHL production, and (2) it helped to identify compounds that could control the nosocomial pathogen *Proteus vulgaris* by interfering with its QS system.

Although the current investigation was hampered severely by lack of genomic data for *Proteus vulgaris*, the experimental and theoretical findings from this study hint at an active, OHL-mediated communication system in *Proteus vulgaris* BNW. The study with phytochemicals indicates the possibility of designing inhibitors that could decrease the production of the QS signal molecule as well as swarming of this pathogen. This could pave the way for discovering novel anti-bacterial compounds that target the communication system. Such an approach could be an effective alternative to counteract the emergence of multidrug-resistant strains in the opportunistic pathogen *Proteus*.

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