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# Expression of Quorum Sensing and Virulence Factors Are Interlinked in *Pseudomonas aeruginosa:* an *in vitro* Approach

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## Abstract

Quorum sensing (QS) has been shown to be important for the pathogenesis of *Pseudomonas aeruginosa* in number of infections and for the expression of various virulence genes. Although QS has been shown to regulate the expression of virulence factors such as elastase, protease and rhamnolipid but link to others such as lipopolysaccharide (LPS) or exo-polysaccharide has not been studied in detail yet. Present study investigated the contribution of QS to the phenotypic expression of multiple virulence factors in vitro. Multiple virulence factors were estimated among wild type *P. aeruginosa* and its isogenic QS mutant strains by standard methods. Results indicated that QS also regulates the expression of LPS, haemolysin, siderophores and polysaccharides in addition to well-known virulence factors such as elastase, protease and rhamnolipids. Role in biofilm formation and motilities was further established. It may be concluded that QS regulates the expression of multiple virulence factors in *P. aeruginosa.* It also depicts that QS may regulates more than it is speculated. Therefore, role of QS signal molecules can also be explored further for other regulation possibilities, as futuristic drug target for the development of preventive strategies and important virulence factor per se.

**Keywords:** *Pseudomonas aeruginosa*, Quorum Sensing, Biofilm, Virulence Factors.

## 1. Introduction

*Pseudomonas aeruginosa*, an extremely versatile gram negative bacterium is responsible for number of infections such as eye, burns, wounds, cystic fibrosis, respiratory tract and urinary tract (UTI) which leads to significant morbidity and mortality [1]. Virulence is usually attributed to number of cellular and extracellular virulence factors like pilli, flagella, lipopolysaccharide, elastase, alkaline proteases, pyocyanin, pyoverdin, haemolysins, phospholipase C, rhamnolipids and biofilm [2]. In

  

number of in vivo studies, it has been speculated that expression of these multiple virulence factors and biofilm formation are under the regulation of quorum sensing (QS) operative via responding to small diffusible signalling molecules, acyl homoserine lactones (AHLs). In *P. aeruginosa* QS network is operative through two distinctive, interdependent systems, *las*R/*las*I and the *rhl*R/*rhl*I [2, 3]. Both these components operate in a hierarchal cascade. Nearly 5% of the *P. aeruginosa* total genome (over 300 genes) is under the regulation of QS [4], out of which, functions of many genes is still not understood well.

Importance of QS to establish a successful infection have been shown in number of infection model studies such as acute pulmonary infection, burn wound infection, microbial keratitis, chronic lung infection and UTI by employing QS deficient strains. In these studies, inability of QS deficient strains to induce successful infection was proposed to be linked with decreased production of protease, elastase and rhamnolipid [5-11]. Few clinical studies have also suggested that QS are fully functional during infections within infected tissues like sputum from cystic fibrosis patients and renal tissue in UTI [11-12]. In addition, QS deficient strains have also been reported to be associated with various infections like wound, cystic fibrosis and UTI since these mutants were not found to be completely avirulent. It indicated that there may be certain other virulence factors which may not be stringently regulated by QS [13]. However, very limited literature is available where role of QS has been shown to be associated with regulation of virulence factors other than elastase, protease and rhamnolipid etc. Moreover, report on in vitro phenotypic expression of various virulence factors among QS competent and deficient strains is also lacking. More or less, all the speculations have been made on the basis of in vivo studies only. Keeping this in view, present study was carried out to evaluate in vitro expression of various cell associated and extracellular virulence factors among wild type *P. aeruginosa* and its isogenic QS deficient strains for better understanding of inter-linkage and definitive role of individual QS component to the regulation of these virulence factors.

## 2. Materials and methods

### 2.1 Bacterial strains

Wild type *P. aeruginosa* PA01 and its isogenic single mutants JP1 (Δ*LasI*), R1 (Δ*LasR*) and isogenic double mutant strains JP2 (Δ*LasI*Δ*RhlI*) and JP3 (Δ*LasR*Δ*RhlR*) were used. These mutants were generated by inactivating the respective quorum sensing genes either by gene replacement or transposon insertion in these genes. Tetracycline (50 µg/ml) and mercuric chloride (7.5 µg/ml) were added to the Luria broth for the growth of double mutant strains. Tetracycline (50 µg/ml) was used for single mutant strains.

### 2.2 Test supernatant and cells

Cultures were grown in Luria broth at 37$^{o}$C at 120 rpm for 16-18hrs. After incubation, cultures were centrifuged at 10,000g at 4$^{o}$C for 15 min. Cell free supernatants and cell pellets were separated and used in various experiments.

### 2.3 Protease production

Proteolytic activity was estimated according to the method of Visca et al., [14]. Briefly, 0.5 ml of culture supernatant was diluted in 10mM Tris buffer (pH 7.5) and incubated with 15mg hide powder azure (Sigma Chemicals, USA) at 37$^{o}$C for 1hr. Absorbance was measured at 595nm and results were expressed in units per liter (U/L).

### 2.4 Elastase activity

Elastase activity was measured following the method of Visca et al., [14]. 15mg of substrate (elastin-congo red; Sigma Chemicals, USA) was suspended in 1 ml of culture supernatant mixed with 1ml of 100mM Tris-Succinate buffer (pH 7.0) supplemented with 1mM $CaCl_2$. Tubes were kept at 37$^{o}$C for 2hrs under shaking conditions. Reaction was stopped by adding 1ml of 0.7M sodium phosphate buffer (pH 6.0) and was centrifuged at 4000g for 5min at 4$^{o}$C. Absorbance was taken at 495nm and results were expressed in units per liter (U/L).

### 2.5 Hemolysis estimation

  

Both cell free and cell bound hemolysin was estimated following the method of Linkish and Vogt [15].

**2.6 Cell bound hemolysins**

1.5ml of 2% human RBC's suspension was mixed with 1.5ml of biofilm cells and incubated at 37℃ for 2hrs. Mixture was centrifuged at 8000g for 15min. Supernatant was collected and absorbance was read at 545nm. The amount of hemolysin (mg/ml) was calculated by using lyophilized hemoglobin as reference.

**2.7 Cell free hemolysins**

1.5ml of 2% human RBC's suspension was mixed with 1.5ml of cell free supernatant and incubated at 37℃ for 2hrs. Assay mixture was centrifuged at 5000g for 5min and absorbance was taken at 545nm.

**2.8 Pyocyanin estimation**

Pyocyanin was measured according to the method of Huerta et al., [16]. Briefly, 3ml of culture supernatant was mixed with 1.2ml of chloroform. Mixture was incubated for 30 minutes at room temperature. Absorbance of chloroform layer was measured at 690nm. Concentration of pyocyanin was expressed as µg/ml.

**2.9 Pyoverdin estimation**

Estimation was done by the method of Ankenbauer et al., [17]. 1ml of culture supernatant was diluted with 50mM Tris-HCl (pH 7.4) and fluorescence was measured at 460nm by exciting the samples at 400nm in Gibson Spectro-glowflourometer.

**2.10 Pyochelin estimation**

Quantitation of pyochelin was done by the method of Arnow, [18]. 1ml supernatant, 1ml each of 0.5N HCl, Nitrite molybdate reagent and 1N NaOH were mixed and the final volume was made to 5ml with DW. Absorbance was taken at 510nm.

**2.11 Rhamnolipid estimation**

Rhamnolipid was quantified by Orcinol method [19]. Briefly, strains were grown overnight in chemically defined media and culture supernatant was extracted with diethyl ether.100µl of this extract was diluted with 1:10 in freshly prepared orcinol reagent (7.5 volume of 60% $H_2SO_4$ and 1 volume of 1.6% (w/v) orcinol in distilled water) and mixture was heated in water bath at 80$^oC$ for 30min. Absorbance was measured at 421nm. L-Rhamnose (Sigma chemicals, USA) was used to standardize the assay.

**2.12 LPS estimation**

LPS was determined by following the method of Morrison and Leive [20]. Briefly, 200µl of culture was mixed with 100µl of $H_2SO_4$ and heated in boiling water bath for 15min. 200µl periodic reagent (0.125N sodium periodate in 0.125N $H_2SO_4$) was added to this and heated at 55$^oC$ for 22min. 400µl of Arsenite solution (2% Sodium arsenite in 0.5N HCl) and 1.6ml of thio-barbituric acid (0.71% in TBA with 0.7ml of 1N NaOH) was added to this mixture and was incubated in boiling water bath for 12min. Absorbance was measured at 532nm. Purified KDO was used to standardize the assay.

**2.13 Total polysaccharide estimation**

Total polysaccharide content was measured by use of the phenol-sulphuric acid assay [21]. 5 ml of 0.5% phenol and 2.5 ml of concentrated $H_2SO_4$ were added to each sample and left to cool for 30 min. Samples were centrifuged (3,000g for 15 min), and supernatant was measured spectrophotometrically at 485 nm. $H_2O$ blanks were treated as above. Total carbohydrate was expressed in mg using glucose as reference.

**2.14 Protein estimation**

Protein content in the samples was estimated by using modified method of Lowry et al., [22]. Briefly, 2.5ml working solution (98ml of 2% $Na_2CO_3$ in 0.1 N NaOH + 1ml of 1% $CuSO_4.5H_2O$+1ml of 2% Sodium Potassium Tartarate) was mixed with protein samples and incubated at room temperature for 10 min. After this, 1N Folin-Ciocateu's reagent was mixed and incubated at 37$^oC$ for 30min at room temperature. Optical density was taken at 660 nm. Blank containing distilled water was also processed simultaneously. Bovine serum albumin was used as reference.

  

**2.15 Motility assays**

Swimming, swarming, and twitching motilities were assayed on agar plates containing specific media [23].

**2.16 Swimming motility**

Plates containing 1% tryptone, 0 5% NaCl and 0 3% agarose were prepared and were point inoculated from an overnight LB culture. Plates were incubated at 30 ℃ for 24hr and motility was assayed as the radius of the circular expansion of bacterial growth from the point of inoculation.

**2.17 Swarming motility**

Plates of nutrient agar containing bacteriological agar (0.5% w/v) and glucose (5g/liter) were prepared. Plates were allowed to dry at room temperature. Cells were point inoculated from overnight culture and incubated at 37 ℃ for 24hr.

**2.18 Twitching motility**

The overnight culture was stabbed through agar of LB plates (1% agar) to the bottom of the Petri dish and incubated for 48hr at 37$^{o}$C. After removal of agar, attached cells were stained with crystal violet (1% w/v) and the radius of growth expansion was determined.

**2.19 Statistical analysis**

All the experiments were repeated three times to validate the reproducibility of experiments. Results were analysed statistically by Student's *t* test using SPSS 11.05 to calculate *p* values and $p<0.05$ were taken as significant.

**3. Results and Discussion**

QS play an important role in the regulation of cell physiology in *P. aeruginosa*. QS system, consisted of inducer and regulator proteins of las and rhl components which works interdependently in an hierarchal manner to regulate the expression of various genes including virulence ones [4, 24]. In the present study, effect of individual QS inducer or regulator component on phenotypic expression of various virulence factors was observed. Both these components of QS cascade have been implicated in the pathogenesis of various infections. Culture supernatants were evaluated for the production of extracellular factors such as elastase, protease, rhamnolipid, cell free hemolysin, pyochelin, pyocyanin and pyoverdin while cell pellet was used for the estimation of cell associated factors as cell bound hemolysin, total polysaccharide, protein and LPS content. Overnight grown cultures were used for the motilities assay.

Secretion of extra cellular enzymes is an important aspect of pathogenicity which helps in combating adverse conditions and tissue colonization inside the host. Elastase and protease are such two enzymes in *P. aeruginosa*. In the present study, significantly reduced secretion of both enzymes was observed among QS deficient strains as compared to the wild type ($p \leq 0.001$, Table 1). Inability of these QS deficient strains to produce elastase and protease has been linked with their avirulence and non-invasive in different experimental model studies [10]. Rumbaugh et al., [6] also pointed avirulence of QS deficient strains due to non-production of elastase and protease. QS deficient clinical isolates have also been reported to produce low level of elastase and protease [25]. Less production of protease and elastase by clinical isolates from different infections have also been related with deletion of QS components [26]. Various other workers have also documented low levels of elastase and protease among QS deficient strains [27-31].

Siderophores and haemolysin are another aspect of *P. aeruginosa* virulence which aids to virulence of this pathogen by chelating bound tissue iron during infections [32]. Importance of siderophores and haemolysin has been documented in the pathogenesis of respiratory tract, corneal, burn wound and urinary tract infections [14, 33]. Significantly reduced production of iron mopping agents were observed in QS deficient strains indicating that production of these agents are also regulated by QS ($p \leq 0.001$, Table 1). Arevalo et al., [34] have also demonstrated significantly reduced growth of QS deficient strains in the presence of haemoglobin as sole source of iron in comparison to wild type. Reduction in growth was restored to normal by supplementing the media with QS signal molecules, indicating that iron acquiring

  

mechanism are definitely under the regulation of QS. Deziel et al., [35] have also observed that QS deficient strains express siderophores in late phase of growth after complementation with another QS mechanism. Regulation of siderophores by QS has also been reported by various other workers [28, 31].

| Strains | Protease (IU/L) | Elastase (IU/L) | Rhamnolipid (mg/ml) | Cell Free Haemolysin (mg/ml) | Pyochelin ($0D_{510nm}$) | Pyocyanin (μg/ml) | Pyoverdin (nm) |
|---|---|---|---|---|---|---|---|
| PAO1 | 36.7 ± 0.7 | 17.0 ± 0.6 | 1.0 ± 0.08 | 4.1 ±0.5 | 0.3 ±0.01 | 8.55 ±0.16 | 2920 ±25.0 |
| JP1 | 0.7 ± 0.05<br>p ≤0.0001 | 0.28 ± 0.05<br>p ≤0.0001 | 0.36 ± 0.02<br>p ≤0.01 | 2.1 ±0.5<br>p ≤0.01 | 0.04 ±0.01<br>p ≤0.0001 | 3.26 ±0.25<br>p ≤0.0001 | 1219 ±12.6<br>p ≤0.0001 |
| R1 | 0.33 ±0.05<br>p ≤0.0001 | 1.4 ± 0.05<br>p ≤0.0001 | .026 ± 0.03<br>p ≤0.0001 | 3.2 ±0.2<br>p ≤0.03 | 0.07 ±0.01<br>p ≤0.0001 | 4.43 ±0.15<br>p ≤0.0001 | 1351 ±12.6<br>p ≤0.0001 |
| JP2 | 0.09 ±0.01<br>p ≤0.0001 | 0.1 ± 0.07<br>p ≤0.0001 | 0.14 ± 0.01<br>p ≤0.001 | 0.98 ±0.09<br>p ≤0.0005 | 0.02 ±0.01<br>p ≤0.0001 | 3.4 ±0.05<br>p ≤0.0001 | 1092 ±5.2<br>p ≤0.0001 |
| JP3 | Not Detectable | 0.25 ± 0.05<br>p ≤0.0001 | 0.20 ± 0.01<br>p ≤0.0001 | 1.47 ±0.1<br>p ≤0.0006 | 0.05 ±0.01<br>p ≤0.0001 | 3.0 ±0.20<br>p ≤0.0001 | 956 ±4.6<br>p ≤0.0001 |

**Table 1:** Production of different extra cellular virulence factors as estimated in culture supernatants of *Pseudomonas aeruginosa* wild type PAO1 and its isogenic QS single and double mutant strains.

LPS, another important aspect of gram negative pathogens virulence, has not been studied in detail in relation to QS in *P. aeruginosa*. Limited literature is available on this aspect. LPS is important for organism's pathogenicity and immunogenicity. In the present study, LPS was also produced in significant reduced amount by QS deficient strains ($p \leq 0.001$, Table 2). It indicates that probably production of this factor is also under the regulation of QS either directly or via some other regulators. Although possible relationship between QS and LPS has also been suggested earlier [36-37] but our study reports direct effect of QS competence on the production of LPS. Nakamuru et al., [37] have shown that amount of LPS secreted in the culture supernatant was directly related with the ability of QS. However, more thorough study may throw more light on the mechanism of regulation of LPS by QS. Polysaccharides are important for initial attachment and later biofilm formation. Present study showed reduced amount of polysaccharide in QS deficient strains ($p \leq 0.001$, Table 2). Shih and Huang, [38] have also shown immature and thinner biofilm formation among QS deficient strains due to low exo-polysaccharide (eps) production among these strains.

Motilities have also been shown to be inversely related with the amount of eps [39]. *P. aeruginosa* displays swarming, swimming and twitching motilities which helps in initial attachment and later in re-location of biofilm from one site to another [5, 40]. Motilities were also observed to be significantly arrested in QS mutant strains as compared to wild type PAO1 ($p \leq 0.001$, Table 2, Figure 1). It has also been demonstrated that both las and rhl QS systems contributes equally to motilities. It has also been indicated that QS is essential for normal motilities and loss of any of QS component affects the motilities [5, 41]. Importance of motilities has been showed in biofilm formation since it has been observed that QS deficient strains lacking motilities forms thin and disperse biofilm [42-43]. Normal motility has also been showed to be important for the pathogen dissemination in burn wound infection model [44]. Rhamnolipid, a bio-surfactant, helps the organism in motility and biofilms dispersion from the infection site [45-46]. In the present study, QS deficient strains also produced significantly low level of rhamnolipid as compared to wild type (p

  

≤0.001, Table 1). Although rhamnolipid has been established as important virulence factor in lungs of CF patients, [47] which also participates in stimulation of phagocytosis and inflammatory responses [48-49] but very few reports are available on relation of QS and rhamnolipid production. It has been documented that QS regulates the production of rhamnolipid [30-31].

| Strains | Polysaccharide /protein (μg/μg) | LPS (μg/ml) | Cell Bound Haemolysin (mg/ml) | Swarming (mm) | Swimming (mm) | Twitching (mm) |
|---|---|---|---|---|---|---|
| PAO1 | 0.6 ± 0.02 | 8.62 ±0.15 | 8.0 ±0.5 | 11 ±0.6 | 10.1 ±0.1 | 8.5 ±0.3 |
| JP1 | 0.36 ± 0.02<br>p ≤0.0006 | 3.46 ±0.28<br>p ≤0.0001 | 1.9 ± 0.4<br>p ≤0.0001 | 4 ±0.15<br>p ≤0.0001 | 5.5 ±0.15<br>p ≤0.0001 | 1 ±0.06<br>p ≤0.0001 |
| R1 | 0.3 ± 0.02<br>p ≤0.0003 | 4.26 ±0.25<br>p ≤0.0001 | 2.4 ±0.09<br>p ≤0.0001 | 5 ±0.015<br>p ≤0.0001 | 4.5 ±0.3<br>p ≤0.0001 | 1.5 ±0.06<br>p ≤0.0001 |
| JP2 | 0.29 ±0.02<br>p ≤0.0002 | 1.58 ±.26<br>p ≤0.0001 | 1.18 ±0.01<br>p ≤0.0001 | 1 ±0.06<br>p ≤0.0001 | 3 ±0.15<br>p ≤0.0001 | 0.5 ±0.02<br>p ≤0.0001 |
| JP3 | 0.27 ±0.02<br>p ≤0.0001 | 1.69 ± .02<br>p ≤0.0001 | 1.4 ±0.1<br>p ≤0.0001 | 2 ±0.07<br>p ≤0.0001 | 4 ±0.2<br>p ≤0.0001 | 1.5 ±0.06<br>p ≤0.0001 |

**Table 2:** Production of different cell associated virulence factors and motilities (as zone of growth expansion) as estimated in cell form of *Pseudomonas aeruginosa* wild type PAO1 and its isogenic QS single and double mutant strains.

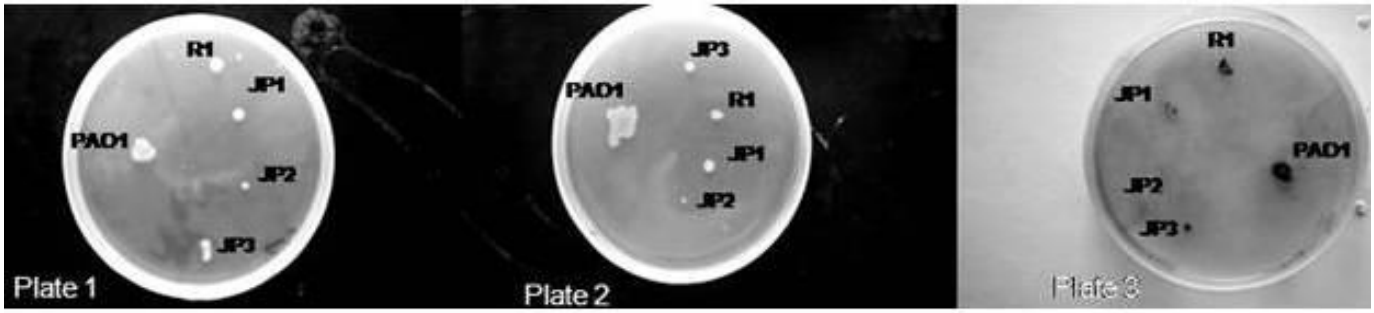


**Figure 1:** Photograph showing swimming motility (Plate 1), swarming motility (Plate 2) and twitching motility (Plate 3) as shown by standard strain of *P. aeruginosa* PAO1 and its isogenic single and double mutants.

Number of infection model studies have demonstrated that QS play important role in the pathogenesis of *P. aeruginosa*. However most of these observations were more or less on the basis of outcome of infection in infection model studies. Direct estimation of phenotypic expression of virulence factors with respect to QS has been reported in very few studies. Moreover, elastase, protease and biofilm were most extensively studied parameters [50]. In the present study, we demonstrated reduced level of multiple virulence factors in QS mutant strains which explain their avirulence in different infection model studies. Results also indicates that both las and rhl QS not only regulates the phenotypic expression of known virulence factors but there are number of other phenotypic characters which are linked with QS since reduced lavel of expression of these factors was observed among QS deficient strains. Although both the components (las and rhl) contributes equally to the regulation of various factors but strains deficient in both the components showed more significant reduction as compared to loss of single component. To the best of our knowledge, this study is first of its kind to show that QS regulates multiple virulence factors

  

in vitro including those which are not studied so far to be linked with QS. Various other studies have also shown that expression of multiple virulence factors and biofilm formation is under the regulation of QS and reduction in quorum either by means of quorum sensing inhibition or loss of one or another QS component results in reduced level of virulence factors [27-31, 35, 51]. These findings highlight the importance of other virulence factors besides elastase and protease in pathogenesis of these infections [6-7]. On the basis of present in vitro and previous in vivo studies, it may be concluded that phenotypic expression of various virulence factors of *P. aeruginosa* is definitely linked with QS. Therefore QS cascade can be explored as a futuristic attractive drug target for the development of novel therapeutics for the prevention and control of *Pseudomonas* infections.

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**Conflict of Interest:** None.

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