The Effect of \textit{pstS} and \textit{phoB} on Quorum Sensing and Swarming Motility in \textit{Pseudomonas aeruginosa}

Inna Blus-Kadosh\textsuperscript{1,2}, Anat Zilka\textsuperscript{1,2}, Gal Yerushalmi\textsuperscript{1,2}, Ehud Banin\textsuperscript{1,2,*}

\textsuperscript{1}The Institute for Nanotechnology and Advanced Materials, Bar-Ilan University, Ramat Gan, Israel, \textsuperscript{2}The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat Gan, Israel

Abstract

\textit{Pseudomonas aeruginosa} is an opportunistic pathogen that can cause a wide range of infections and inflammations in a variety of hosts, such as chronic biofilm associated lung infections in Cystic Fibrosis patients. Phosphate, an essential nutrient, has been recognized as an important signal that affects virulence in \textit{P. aeruginosa}. In the current study we examined the connection between phosphate regulation and surface motility in \textit{P. aeruginosa}. We focused on two important genes, \textit{pstS}, which is involved in phosphate uptake, and \textit{phoB}, a central regulator that responds to phosphate starvation. We found that a mutant lacking \textit{pstS} is constantly starved for phosphate and has a hyper swarming phenotype. Phosphate starvation also induced swarming in the wild type. The \textit{phoB} mutant, on the other hand, did not express phosphate starvation even when phosphate was limited and showed no swarming. A double mutant lacking both genes (\textit{pstS} and \textit{phoB}) showed a similar phenotype to the \textit{phoB} mutant (i.e. no swarming). This highlights the role of \textit{phoB} in controlling swarming motility under phosphate-depleted conditions. Finally, we were able to demonstrate that PhoB controls swarming by up-regulating the Rhl quorum sensing system in \textit{P. aeruginosa}, which resulted in hyper production of rhamnolipids: biosurfactants that are known to induce swarming motility.

Introduction

\textit{Pseudomonas aeruginosa} is a versatile bacterium that can adapt to a variety of niches and grows well in soil, water, plants and animals \cite{1}. \textit{P. aeruginosa} is also an opportunistic pathogen that can cause a variety of infections such as chronic infection of the lungs in Cystic Fibrosis (CF) patients \cite{2}, urinary tract infections resulting from catheterization \cite{3}, burn wounds \cite{4} and Keratitis \cite{5}. An important aspect of the \textit{P. aeruginosa} ‘life style’ is its ability to switch from a planktonic to a sessile mode of growth, depending on nutrient availability and its surrounding bacterial community. When nutrient availability is scarce, \textit{P. aeruginosa} uses swarming motility, a coordinated movement on a surface, using flagella, biosurfactants \cite{6} and Type-IV pili \cite{7}, in order to find an optimal niche \cite{8}. In this mode of growth, planktonic cells differentiate to elongated and hyper-flagellated cells that spread across the surface. In order to facilitate the movement, \textit{P. aeruginosa} secretes rhamnolipids \cite{6}, glycolipids that act as a biosurfactant and are produced by the \textit{rhlAB} operon \cite{9}. Rhamnolipid production is regulated by Quorum Sensing (QS) \cite{10–12}, a social behavior in which bacteria sense and respond to its surrounding population by producing and receiving signal molecules. There are three known QS systems in \textit{P. aeruginosa} – Las and Rhl, each consisting of an auto-inducer molecule from the acyl-homoserine lactone (AHL) family (synthesized by the LasI and RhlI enzymes) and a response regulator (LasR and RhlR) \cite{13}, and PQS (\textit{Pseudomonas} Quinolone Signal), a secondary metabolite that functions as a QS molecule \cite{14}. In addition to rhamnolipid production, QS in \textit{P. aeruginosa} affects many bacterial processes, such as virulence \cite{15} and biofilm formation \cite{16}. While searching for irregular swarming phenotypes using a transposon mutant library, we came upon a hyper-swarming mutant. The mutated gene was mapped to \textit{pstS}, a key component in the phosphate specific transport system (Pst) \cite{17}. Phosphate is an essential nutrient, used in the assembly of ATP, LPS, nucleic acids and other cell components. \textit{P. aeruginosa} has two phosphate uptake systems – Pst, a low-affinity, constitutively operating channel \cite{18}, and Pst – a high-affinity ABC transporter. The Pst system is encoded by the \textit{pst} operon, containing the genes \textit{pstA}, \textit{pstB}, \textit{pstC}, \textit{pstS} and \textit{phoB}. \textit{pstA}, \textit{pstB} and \textit{pstC} encode the transporter proteins, while \textit{pstS} encodes a periplasmic phosphate-binding protein that transfers the phosphate to the bacterial cytoplasm through the transporter. The system is regulated by PhoB/R – a two-component system activated by phosphate depletion \cite{19}. When phosphate levels in the bacteria become low, PhoB phosphorylates PhoB, which in turn acts as a transcription factor and activates genes that have the consensus sequence ‘Pho Box’ in their promoters, such as the \textit{pst} operon. In recent years, studies concerning the Pst system have uncovered a connection between phosphate depletion and virulence – over-expression of \textit{PstS} has been found in ampicillin-resistant \textit{Streptococcus pneumoniae} \cite{20}, a possible correlation between phosphate depletion and \textit{P. aeruginosa} in a gut mouse model has also been suggested \cite{21}, and it has been shown that deletion of PhoB affects swarming \cite{22}. Furthermore, the Pst system has been connected to virulence in many bacteria \cite{23}. For example, in clinical strains of \textit{P. aeruginosa},
PstS forms extracellular appendages that increase the strain’s virulence in a mouse model [24]. In this study, we establish the intricate connection between swarming, QS and phosphate availability in order to better understand the molecular mechanisms that regulate surface motility in P. aeruginosa. We show that in the absence of phosphate, PhoB up-regulates rhlR expression, which results in rhamnolipid production that promotes hyper swarming.

Materials and Methods

Bacterial Strains, Plasmids, and Media

The bacterial strains and plasmids used in this study are shown in Table 1. For Alkaline Phosphatase and Real-time assays, strains were grown on M9 minimal medium (20 mM NH4Cl; 12 mM Na2HPO4; 22 mM KH2PO4; 8.6 mM NaCl; 1 mM MgSO4; 1 mM CaCl2; 11 mM Dextrose) supplemented with 50 μM FeCl3. For swarming assays, we used M9 minimal medium or M9 depleted of phosphate (20 mM NH4Cl; 8.6 mM NaCl; 1 mM MgSO4; 11 mM Dextrose), both supplemented with 0.5% Casamino acids, FeCl3, and carbenicillin if necessary

Table 1. Strains used in this study.

| Strain or plasmid | Description | Source or reference |
|-------------------|-------------|---------------------|
| P. aeruginosa strains | | |
| PAO1 | Wild type | [36] |
| ΔpstS | PAO1 with an unmarked deletion of pstS | This study |
| ΔphoB | PAO1 with an unmarked deletion of phoB | This study |
| ΔphoBΔpstS | PAO1 with an unmarked deletion of pstS and phoB | This study |
| ΔrhlA | PAO1 with an unmarked deletion of rhlA | This study |
| ΔrhlR | PAO1 with an unmarked deletion of rhlR | This study |
| ΔrhlAΔrhlR | PAO1 with an unmarked deletion of pstS and rhlA | This study |
| ΔrhlAΔrhlR | PAO1 with an unmarked deletion of pstS and rhlR | This study |
| E. coli strains | | |
| D317 (λpir) | recA derivative of E. coli 294 (F thi pro hsdR) carrying a modified derivative of IncP plasmid pR104 (Aps Tcs Kms) integrated in the chromosome, Tpr; lysogenized with bacteriophage λpir | Y. Irie and M. R. Parsek |
| S17.1 λpir | F′endA1 hsdR17 supF44 thi-1 recA1 gyrA relA1 ΔlacZYA-argF U169 deoR (880 dioCZ-M15 recA1) | [37] |
| Plasmids | | |
| pUCP18Ap | A broad-host range cloning vector. Cbr/AmpR | [38] |
| pEX18GmGW | pEX18Gm containing the Gateway (GW) destination cloning site. GmR | Nan Fulcher and Matthew Wolfgang |
| phoB | pUCP18Ap containing the phoB gene for complementation | This study |
| pstS | pUCP18Ap containing the pstS gene for complementation | This study |
| rhlA | pUCP18Ap containing the rhlA gene for complementation | This study |
| rhlR | pUCP18Ap containing the rhlR gene for complementation | This study |
| pECP61S | Contains a rhlA-lacZ fusion, used for C4-HSL detection | [11] |

Alkaline Phosphatase Assay

Alkaline Phosphatase (AP) is a protein whose expression is enhanced under phosphate starvation [29]. We utilized AP activity in order to assay a strain’s phosphate starvation levels. AP activity was measured by sampling strains grown in a liquid culture or on agar plates. Strains were grown overnight in M9 medium supplemented with Casamino acids, FeCl3, and carbenicillin if necessary. Afterwards, the bacteria were diluted 1:10 into fresh medium and grown for an additional three hours in order to reach the logarithmic growth phase. 2.5 μl from each culture was plated in the middle of a swarming plate, which contained M9 medium or M9 depleted of phosphate, supplemented with 0.5% Casamino acids, 50 μM FeCl3 and carbenicillin if necessary, and solidified with 0.5% Bacto Agar. Plates were incubated at 37°C for 24 hours.

Effect of Phosphate on Swarming & Quorum Sensing

Construction of Strains and Plasmids

Deletion mutants of pstS, phoB, pstSΔphoB, rhlA and rhlR were constructed as previously described [25]. Overlap extension PCR using the primers specified in Table S1 was used in order to generate a fragment containing the upstream and downstream regions of each gene. Each fragment was cloned into the allelic exchange vector DB3.1 pEX18GmGW [26] using BP-Clonase (Invitrogen). Each deletion was introduced to PAO1 or ΔpstS using bi-parental mating [27]. Deletions were generated using a standard method for two-step allelic exchange as described by Schweizer and Hoang [28] and were confirmed by PCR.

Swarming Motility Assay

Strains were grown overnight in M9 medium supplemented with Casamino acids, FeCl3, and carbenicillin if necessary. Afterwards, the bacteria were diluted 1:10 into fresh medium and grown for an additional three hours in order to reach the logarithmic growth phase. 2.5 μl from each culture was plated in the middle of a swarming plate, which contained M9 medium or M9 depleted of phosphate, supplemented with 0.5% Casamino acids, 50 μM FeCl3 and carbenicillin if necessary, and solidified with 0.5% Bacto Agar. Plates were incubated at 37°C for 24 hours.

Table 1.

Strains used in this study.

AmpR – Ampicillin resistance for E. coli Cbr. GmR – Gentamicin resistance.
To measure AP from a liquid culture, strains were grown overnight in M9 medium supplemented with FeCl3, and carbenicillin if necessary. Afterwards, bacteria were diluted to an O.D595nm of 0.04 into 50 ml of fresh M9 medium, containing 50 μM FeCl3 and grown for an additional 10 hours. Then, 6 ml from each strain were taken and centrifuged for 10 minutes at 2,200 g (Centrifuge 5418; Eppendorf). The pellet was resuspended with 20 ml of Chloroform. After 15 minutes of incubation at room temperature, 20 ml of 0.01 M Tris-HCl (pH = 8) were added to each sample, and the samples were centrifuged for 10 minutes at 6,000 g. Following centrifugation, 30 μl from each sample’s supernatant were added to a 96-well plate, containing the reaction buffer (5 μl of 0.5 mM MgCl2 and 10 μl of 1 M Tris (pH = 9.5)). 5 μl of 500 mM p-Nitrophenyl Phosphate (PNPP, NEB) were added to each well and the reaction was read at 405 nm in an ELISA plate reader (Synergy™ Multi-Detection Microplate Reader; Biotech). Results were normalized to each samples’ total protein concentration using Bradford assay (Thermo).

When measuring AP activity from swarming plates, bacteria grown for 24 hours on swarming plates were scraped off and suspended in 2 ml of M9, then submitted to the same procedure as the samples taken from liquid culture.

RNA Extraction, RT-PCR and Real-Time PCR Analysis

Strains were grown overnight in M9 medium supplemented with FeCl3. Then, bacteria were diluted to an O.D595nm of 0.04 into 50 ml of fresh M9 medium, containing FeCl3 and grown for an additional 48 hours. Afterwards, 2 ml from each culture was incubated with 4 ml of RNAprotect Bacteria Reagent (Qiagen). RNA extraction was performed using RNeasy Mini Kit (Qiagen). cDNA was synthesized from 500 ng of RNA using GoScript™ Reverse Transcription System (Promega). Real-time PCR was performed using CFX-96 Touch Real-Time PCR detection system (Bio-Rad). Results were normalized to the expression of PA1769 [30].

C4–HSL Quantification

The level of C4-HSL was measured using a bio-reporter strain as previously reported [31]. Briefly, strains were grown overnight in M9 medium supplemented with FeCl3. Then, bacteria were diluted to an O.D595nm of 0.04 into 50 ml of fresh M9 medium, supplemented with FeCl3 and grown for an additional 48 hours. Afterwards, 5 ml from each culture was extracted twice with an equal volume of ethyl acetate containing 0.1% glacial acetic acid, as previously described [32]. Ethyl acetate was evaporated using nitrogen gas. Overnight culture of DH5α harboring pECP61.5 was diluted into an O.D595nm of 0.1 into modified A medium [33] with ampicillin and 1 mM IPTG. 500 μl of the diluted bacteria was added to each sample and grown at 30°C for 5.5 hours. Cells were then lysed with chloroform and the β-galactosidase activity was measured using Tropix-Galacton kit according to the manufacturer guidelines (Applied Biosystems).

Statistical Analysis

Statistical analysis was carried out using unpaired t-test and Turkey’s Post Hoc test. P<0.05 was considered as significant.

Results and Discussion

Phosphate Starvation Promotes phoB-mediated Hyper Swarming

While scanning a transposon mutant library for irregular swarming phenotypes, we found that transposon insertion in the pstS gene promoted hyper-swarming. In order to ensure that the phenotype is indeed caused by the disruption of the gene, we generated a clean deletion of pstS in PAO1. The mutant showed a hyper-swarming phenotype both in standard M9 minimal medium representing phosphate-repleted conditions (20 mM Pi) and in M9 medium with phosphate depleted conditions (0.2 mM Pi). The wild type, on the other hand, showed swarming ability only under phosphate-depleted conditions (Fig. 1). Complementation of the ΔpstS strain with a plasmid encoding pstS restored the wild type phenotype.
Next, we generated a clean deletion of *phoB* in PAO1, in order to see how deletion of a protein with a regulatory function in the phosphate uptake system affects the bacteria’s swarming pattern. We saw that deletion of *phoB* caused the bacteria to lose all swarming ability, even under phosphate depleted conditions. A similar result was demonstrated previously by Bains *et al.* [22], who showed that a *phoB* mutant loses its ability to swarm when grown on a BM2 medium with low (0.2 mM) phosphate concentration. Complementation of our Δ*phoB* strain with a plasmid encoding *phoB* restored the wild type phenotype (Fig. 1). We then wanted to see which one of the two genes – *pstS* or *phoB* – is responsible for controlling the swarming phenotype under phosphate depleted conditions. To do so, we generated a double mutant, lacking both *pstS* and *phoB* in PAO1. We assumed that if PstS controlled swarming in the presence or lack of phosphate, the double mutant would show the hyper-swarming phenotype presented by the *pstS* mutant, and if PhoB controlled swarming under the tested conditions, the double mutant would act like the *phoB* mutant and show a non-swarming phenotype. Plating the double mutant on swarming plates containing M9 or M9 with low levels of phosphate, generated a non-swarming phenotype under both conditions (Fig. 1), which led us to conclude that PhoB is responsible for controlling *P. aeruginosa*’s swarming patterns in the presence or lack of phosphate. When we complemented the double mutant with a plasmid containing the *pstS* gene, we received the phenotype presented by *phoB*, and when we complemented the double mutant with a plasmid containing the *phoB* gene (*pphoB*), we received the phenotype presented by *pstS* (Fig.S1A). From these experiments we can conclude that while both *pstS* and *phoB* have impact on swarming motility on media containing low levels of phosphate, PhoB is the protein that regulates swarming under the specified conditions.

The *pstS* Mutant is Constantly Starved for Phosphate, while the *phoB* Mutant cannot sense the Bacteria’s Phosphate Levels

The results from the swarming experiments led us to hypothesize that deletion of *pstS* causes *P. aeruginosa* to be constantly starved for phosphate, whereas deletion of *phoB* causes the bacteria to lose its ability to sense its intra-cellular phosphate levels. Because PhoB is activated under low phosphate levels, we assumed that deletion of *phoB* causes the bacteria to act as if they
were in a constant state of phosphate saturation. To test this hypothesis, we plated PAO1, ΔpstS, ΔphoB and ΔpstSΔphoB on either phosphate depleted or repleted swarming plates in order to measure the Alkaline Phosphatase (AP) activity in each strain, as described in Materials and Methods. AP levels increase when intra-cellular phosphate levels are low, thus, AP levels point to the degree of phosphate starvation in bacteria. PAO1 was used as a reference – as expected, when grown on standard M9 plates, AP levels were low, and when grown on phosphate-depleted M9, AP levels increased dramatically (p < 0.05) (Fig. 2). AP levels in the pstS mutant were higher than in PAO1 in both media (p < 0.05), and AP levels in ΔphoB and ΔpstSΔphoB were similar to those of PAO1 grown on standard M9 plates, but did not increase under phosphate depleted conditions (Fig 2). Complementation of the deletion strains, with plasmids encoding pstS or phoB, reverted the phenotypes (Fig. S1B). The results suggest that absence of both pstS and phoB disrupts P. aeruginosa’s ability to respond to its intra-cellular phosphate levels, but in different ways. Deletion of pstS, the gene that encodes the phosphate-carrying protein, causes the bacteria to lose its ability to acquire phosphate through the Pst system, which puts the bacteria in a state of constant phosphate starvation. On the other hand, deletion of phoB causes P. aeruginosa to lose the regulatory part of the phosphate uptake system. Since PhoB/R are activated only when phosphate levels are low, bacteria with a phoB deletion will lose their ability to sense when there is a decrease in intra-cellular phosphate levels and therefore will always have a false sense of being in a phosphate saturated environment, hence the lack of swarming and the low AP levels in ΔphoB and ΔpstSΔphoB grown in media containing low levels of phosphate.

*rhlR and rhlA are Essential for Swarming Under Phosphate-deplete Conditions*

After determining how deletion of pstS and phoB affects P. aeruginosa’s ability to sense phosphate, we were interested to study the molecular pathway that leads from the bacteria’s reaction to phosphate depletion to swarming motility. It was previously shown that the rhlR promoter contains a Pho Box, that rhlR transcription can be activated under phosphate-limiting conditions [34] and that RhlR controls rhamnolipid production [10]. Therefore, we wanted to see if up-regulation of rhlR, and in turn, up-regulation of rhlA and increase in rhamnolipid production, is the cause for hyper-swarming under phosphate-depleted conditions. In order to do so, we measured the transcription levels of rhlR and rhlA in PAO1, ΔpstS, ΔphoB and ΔpstSΔphoB grown in M9 medium, using Real-Time PCR. Results were normalized to those of PAO1, the
reference strain (Fig. 3). Only in ΔpstS did we see a significant rise in transcription levels of rhlA and rhlR (2.5 and 3.9 fold elevation in gene transcription as opposed to the WT, respectively, p<0.05). Because ΔpstS remains constantly starved for phosphate, PhoB is activated in this strain, which strengthens our hypothesis that PhoB is indeed responsible for setting in motion the cascade that ultimately results in swarming in the absence of phosphate. This result is further supported by microarray studies done by Bains et al., showing that rhlA and rhlR transcription increases under minimal phosphate conditions in P. aeruginosa [22]. To further corroborate this data, we measured the C4-HSL levels in each of the strains (Fig. 4). Coinciding with the RT-PCR results, C4-HSL levels in ΔpstS were significantly higher than those of the other strains (p<0.05), which shows that the response to phosphate starvation involves all factors of the Rhl system.

Next, in order to further prove that rhlR and rhlA are responsible for hyper-swarming when phosphate level is low, we generated clean deletions of rhlR and rhlA in PAO1 and in ΔpstS, and plated them on swarming plates containing M9 with either low or high levels of phosphate. The results (Fig. 5) clearly show that deletion of each of these genes abolished the swarming completely in both strains and in both media, which proves that these genes are crucial for phoB-mediated swarming when phosphate is low. Complementation of rhlR and rhlA restored the phenotypes of both PAO1 and ΔpstS (data not shown).

All of these results combined piece together the molecular mechanism that is initiated under phosphate-deplete conditions (Fig. 6) - PhoB is activated upon phosphorylation by PhoR, acts as a transcription factor and binds to promoters containing a Pho Box motif. One of these promoters is the promoter for rhlR. RhlR undergoes activation, and activates transcription of the rhlAB operon, causing rhamnolipid production, which results in swarming. Interestingly, we have previously shown in P. aeruginosa that iron depletion can also cause increased motility by QS-mediated rhamnolipid production [35]. The current study further links between environmental cues and QS, and sheds light on the complicated regulatory network in P. aeruginosa that makes this pathogen so versatile and highly adjustable to a variety of niches.

Supporting Information

Figure S1 Complementation of pstS and phoB restores the wild type swarming phenotype.
(TIF)
Table S1 Primers used in this study.
(DOCX)

Author Contributions
Conceived and designed the experiments: IBK GY EB. Performed the experiments: IBK AZ. Analyzed the data: IBK AZ GY EB. Wrote the paper: IBN GY EB.
References

1. tover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, et al. (2000) Complete genome sequence of Pseudomonas aeruginosa PA01, an opportunist pathogen. Nature 406: 959–964.

2. Kirov SM, Webb JS, O’May C Y, Reid DW, Woo JK, et al. (2007) Biofilm differentiation and dispersal in mucoid Pseudomonas aeruginosa isolates from patients with cystic fibrosis. Microbiology 153: 3264–3274.

3. Mittal R, Aggarwal S, Sharma S, Chhibber S, Harjai K (2009) Urinary tract infections caused by Pseudomonas aeruginosa: a minireview. J Infect Public Health 2: 101–111.

4. Church D, Elayed S, Reid O, Winston B, Lindsay R (2006) Burn wound infections. Clin Microbiol Rev 19: 403–454.

5. Hazlett LD (2004) Corneal response to Pseudomonas aeruginosa infection. Proc Retin Eye Res 23: 1–30.

6. Caiazza NC, Shanks RM, O’Toole GA (2005) Rhamnolipids modulate swarming motility patterns of Pseudomonas aeruginosa. J Bacteriol 187: 7351–7361.

7. Kohler T, Curty LK, Barja F, van Delden C, Pechere JC (2000) Swarming of Pseudomonas aeruginosa is dependent on cell-to-cell signaling and requires flagella and pili. J Bacteriol 182: 5990–5996.

8. Verstraeten N, Bracken K, Debakker B, Fauvart M, Franauer J, et al. (2008) Living on a surface: swarming and biofilm formation. Trends Microbiol 16: 496–506.

9. Deziel E, Lepine F, Milot S, Villeneuve R (2005) rhlA is required for the production of a novel biosurfactant promoting swarming motility in Pseudomonas aeruginosa: 3-(3-hydroxyalkanoyloxy)alkanoic acids (HAA), the precursors of rhamnolipids. Microbiology-Sgm 149: 2005–2013.

10. Daniels R, Vanderleyden J, Michiels J (2004) Quorum sensing and swarming motility patterns of Pseudomonas aeruginosa. J Bacteriol 186: 3985–3994.

11. Lamarche MG, Warner BL, Carpins S, Harel J (2009) The phosphate regulon and bacterial virulence: a regulatory network connecting phosphate homeostasis and pathogenesis. FEMS Microbiol Rev 32: 461–473.

12. Venturi V (2006) Regulation of quorum sensing in Pseudomonas aeruginosa. FEMS Microbiol Rev 30: 274–291.

13. Pesci EC, Milbank JB, Pearson JP, Gray KM, Passador L, Tucker KD, Eberhard A, et al. (1994) Identification of a major protein upon phosphate starvation of Pseudomonas aeruginosa PA01. Journal of Basic Microbiology 34: 277–287.

14. Parsek MR, Carpins S, Harel J, Pearson JP (1995) A second N-acetylmuramoyl-l-alanine amidase is expressed in a rhlA mutant of Pseudomonas aeruginosa. J Bacteriol 177: 5756–5767.

15. Pearson JP, Carpins S, Harel J, Pearson JP (2005) Production of rhamnolipids by Pseudomonas aeruginosa. Appl Microbiol Biotechnol 68: 718–725.

16. de Kievit TR (2009) Quorum sensing in Pseudomonas aeruginosa biofilms. Environmental Microbiology 11: 279–288.

17. Mathustsdudhan KT, McBain RM, Komori N, Matsumoto H (2003) An essential role for the CheA protein in contact regulation and swarming motility in Pseudomonas aeruginosa. Mol Microbiol 48: 1549–1559.

18. Segall JH, Graae SH, Blazquez M, Matsumoto H (2003) Quorum sensing in Pseudomonas aeruginosa: differences between PA01 and PA14. FEMS Microbiol Rev 27: 361–371.

19. Eberhard A, Gray KM, Passador L, Tucker KD, Carpins S, Eberhard A, et al. (1994) Identification of a major protein upon phosphate starvation of Pseudomonas aeruginosa PA01. Journal of Basic Microbiology 34: 277–287.

20. O’Sullivan J, Crook V, Menard F, Pampapthoulou B, Weiss K, et al. (2005) A proteomic analysis of penicillin resistance in Staphylococcus pneumoniae reveals a novel role for PsS, a subunit of the phosphate ABC transporter. Molecular Microbiology 58: 1430–1440.

21. Long J, Zaborina O, Holbrook C, Zaborin A, Alveryd J (2008) Depletion of intestinal phosphate after operative injury activates the virulence of P. aeruginosa causing lethal gut-derived sepsis. Surgery 144: 189–197.

22. Bains M, Fernandez L, Hancock RE (2012) Phosphate starvation promotes swarming motility and cytotoxicity of Pseudomonas aeruginosa. Appl Environ Microbiol 78: 6762–6768.

23. Schweizer HP (1991) Activation of quorum-sensing systems in control of elastase and rhamnolipid biosynthesis in Pseudomonas aeruginosa: a minireview. J Infect Public Health 2: 101–111.

24. Rylake MT, Borlee BR, Muraikami K, Iriy H, Hentzer M, et al. (2012) Expression of the rhlRABC operon in a rhlA mutant of Pseudomonas aeruginosa: a tool for identifying differentially regulated genes. Genome Res 15: 583–589.

25. Schweizer HP, Hoang TT (1995) An improved system for gene replacement and qRT analysis reporter in Pseudomonas aeruginosa. Gene 158: 15–22.

26. Hou CI, Galloway AF, Campbell JJ (1996) Influence of phosphate starvation on cultures of Pseudomonas aeruginosa. J Bacteriol 192: 851–855.

27. Rossen MS, Matthews WJ, Jr, Kang Y, Nguyen DT, Hoang TT (2007) In vivo evidence of Pseudomonas aeruginosa nutrient acquisition and pathogenesis in the lungs of cystic fibrosis patients. Infect Immun 75: 5313–5324.

28. Parsák MR, Singh PK (2003) Bacterial biofilms: an emerging link to disease pathogenesis. Annu Rev Microbiol 57: 677–701.

29. Pearson JP, Carpins S, Harel J, Pearson JP (1995) A second N-acetylmuramoyl-l-alanine amidase is expressed in a rhlA mutant of Pseudomonas aeruginosa. Proc Natl Acad Sci U S A 92: 1499–1494.

30. Pearson JP, Gray KM, Passador L, Tucker KD, Eberhard A, et al. (1994) Structure of the autoinducer required for expression of Pseudomonas aeruginosa virulence genes. Proc Natl Acad Sci U S A 91: 197–201.

31. Jensen V, Lons D, Zaoui C, Bredenbruch F, Meissner A, et al. (2006) RhlR regulates expression of rhlA, rhlR, and rhlS in Pseudomonas aeruginosa. J Bacteriol 188: 8501–8506.

32. Glick R, Gilmore C, Tremblay J, Satomura S, Aviad O, et al. (2010) Deficiency in rhizobial symbiosis under iron-limited conditions influences surface motility and biofilm formation in Pseudomonas aeruginosa. J Bacteriol 192: 2973–2980.

33. Holloway BW, Krishnapillai V, Morgan AF (1979) Chromosomal genetics of Pseudomonas aeruginosa. Microbiol Rev 43: 73–102.

34. Woodcock DM, Crowther PJ, Doherty J, Jefferson S, DeCruz E, et al. (1989) Construction of a mini-Tn5-luxCDABE mutant library in Pseudomonas aeruginosa. Proc Natl Acad Sci U S A 86: 8606–8610.

35. Glick R, Gilmore C, Tremblay J, Satomura S, Aviad O, et al. (2010) Deficiency in rhizobial symbiosis under iron-limited conditions influences surface motility and biofilm formation in Pseudomonas aeruginosa. J Bacteriol 192: 2973–2980.

36. Schweizer HP (1991) Activation of quorum-sensing systems in control of elastase and rhamnolipid biosynthesis in Pseudomonas aeruginosa: a minireview. J Infect Public Health 2: 101–111.