Identification of potential common cyclic-di-GMP regulated biomarkers among three gram negative bacteria via transcriptomic and metabolomic profiling

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NANYANG
TECHNOLOGICAL
UNIVERSITY

**IDENTIFICATION OF POTENTIAL COMMON CYCLIC-DI-GMP
REGULATED BIOMARKERS AMONG THREE GRAM NEGATIVE
BACTERIA VIA TRANSCRIPTOMIC AND METABOLOMIC PROFILING**

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Interdisciplinary Graduate School

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2016

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List of Abbreviations and Symbols

Terms	Abbreviation
<i>Pseudomonas aeruginosa</i>	<i>P. aeruginosa</i>
<i>Burkholderia cenocepacia</i>	<i>B. cenocepacia</i>
<i>Klebsiella pneumoniae</i>	<i>K. pneumoniae</i>
Cystic fibrosis	CF
Extrapolymetric matrix	EPS
Bis-(3'-5')-cyclic dimeric GMP	c-di-GMP
High Performance Liquid Chromatography	HPLC
Liquid Chromatography-Mass Spectrometry	LC-MS
Ribonucleic Acid	RNA
Small RNA	sRNA
RNA sequencing	RNA-seq
Extracellular deoxyribonucleic Acid	eDNA
Phosphodiesterase	PDE
Diguanylate cyclase	DGC
2-heptyl-3- hydroxy-4-quinolone	PQS

List of Publications

1. **Zhao Cai**, Yang Liu, Yicai Chen, Joey Kuok Hoong Yam, Su Chuen Chew, Song Lin Chua, Ke Wang, Michael Givskov and Liang Yang (2016) *RpoN Regulates Virulence Factors of Pseudomonas aeruginosa via Modulating the PqsR Quorum Sensing Regulator*, International journal of molecular sciences, 2015, 16, 28311–28319
2. Yang-Chun Yong , **Zhao Cai** , Yang-Yang Yu , Peng Chen , Rongrong Jiang , Bin Cao , Jian-Zhong Sun, Jing-Yuan Wang, Hao Song (2013) *Increase of riboflavin biosynthesis underlies enhancement of extracellular electron transfer of Shewanella in alkaline microbial fuel cells*; Bioresource Technology, 130 (2013) 763–768
3. Victor Bochuan Wang, Song-Lin Chua, **Zhao Cai**, Krishnakumar Sivakumar, Qichun Zhang, Staffan Kjelleberg, Bin Cao, Say Chye Joachim Loo, Liang Yang (2014) *A stable synergistic microbial consortium for simultaneous azo dye removal and bioelectricity generation*; Bioresource Technology, 155 (2014) 71–76
4. Song Lin Chua, Yichen Ding, Yang Liu, **Zhao Cai**, Jianuan Zhou, Sanjay Swarup, Daniela I. Drautz-Moses, Stephan Christoph Schuster, Staffan Kjelleberg, Michael Givskov and Liang Yang (2016) *Reactive oxygen species drive evolution of pro-biofilm variants in pathogens by modulating cyclic-di-GMP levels* *Reactive oxygen species drive evolution of pro-biofilm variants in pathogens by modulating cyclic-di-GMP levels*; Open Biology, 6: 160162

Book Chapter

1. Yang-Chun Yong, Jing-Xian Wang, **Zhao Cai** and Jian-Zhong Sun. (2013) Chapter 5 Quorum sensing for clean environment and green bioenergy. Advances in Environmental Research. Volume 28. pp. 129-148. ISBN: 978-1-62417-738-5.

Summary

Bacterial cells can switch between two life styles, namely planktonic and biofilm, depending on different environmental cues. Biofilms are involved in almost 80% of microbial infections. Biofilm infections can evade host immune attack and develop into chronic conditions, which cannot be efficiently eradicated by conventional antimicrobial treatments. In most Gram-negative bacteria, biofilm formation can be regulated by a secondary messenger, bis-(3'-5')-cyclic dimeric guanosine monophosphate, c-di-GMP.

Variations in intracellular c-di-GMP levels may change cellular metabolic profile and lead to the expression of specific biomarkers. The identification of such biomarkers modulated by c-di-GMP might provide alternative methods to diagnose biofilm-related infections. In this study, we applied RNA-sequencing (RNA-seq) and High Performance Liquid Chromatography (LC/MS) techniques to analyse the transcriptome and metabolome of *Pseudomonas aeruginosa*, *Burkholderia cenocepacia* and *Klebsiella pneumoniae* cells which are genetically modified to have either excessive or reduced intracellular c-di-GMP levels, in hope to discover possible common cross-species biomarkers.

At transcriptional level, one common gene, *metE* that encodes 5-methyl-tetrahydropteroyl-triglutamate-homocysteine S-methyltransferase was found to be significantly upregulated in the presence of excessive amount of c-di-GMP in both *P. aeruginosa* PAO1 and *B. cenocepacia* H111 strains. At metabolic level, 2 common metabolites were found to be significantly overproduced in both *P. aeruginosa* and *K. pneumoniae*

under high intracellular c-di-GMP content, whereas 15 of that were found in both *P. aeruginosa* and *B. cenocepacia*.

Transcriptomics analysis also indicated that global regulators such as c-di-GMP, quorum sensing and alternative sigma factors coordinately have regulatory capability on the production of virulence factors in *P. aeruginosa*. We therefore took a step further to investigate the regulation of virulence factor secretion by alternative sigma factor RpoN by both genotypic and phenotypic analysis. The results indicated that RpoN modulates virulence secretion through a PQS quorum sensing regulator, *pqsR*.

This study provides evidence for the possible detection and diagnosis of biofilm infections in clinical prospective using c-di-GMP regulated metabolites; it also demonstrates the complex connections and regulations among global regulators at the same time.

Chapter 1. Introduction

1.1. Biofilms

A bacterial biofilm is defined as surface attached groups of microorganisms that is embedded and protected by self-produced extracellular polymeric substances including exopolysaccharides, eDNA and proteins. Bacteria can live either in planktonic form or as biofilm under the influence of physiological and environmental changes, such as cell density, availability of nutrition, stress and so on. Due to the influence of these changes, planktonic cells and biofilm cells exhibit significant differences in their morphology, pattern of gene expression, and physiology. Studies of biofilms reveal the mode of bacterial survival and communication in the community; the problems caused in industrial, clinical and medical settings because of its antimicrobial resistance; biocide treatment and host immune responses. [1-6]

Development of biofilms from bacterial cells undergo several distinctive stages (Figure 1.1). The initiation of biofilm starts from reversible attachment of planktonic cells to an inert surface which can be counteracted upon activity of polar flagellar [7]. Irreversible attachment is then achieved upon longitudinal attachment by bacterial cells and develops into microcolonies. These microcolonies further develop into mature biofilms and form a thick sheet of embedded bacterial cells. Last stage of biofilm formation is dispersion of bacterial cells from the mature biofilm. [8]

Figure 1.1 Stages of biofilm development. (1) Reversible surface attachment; (2) Irreversible surface attachment; (3) Microcolony formation; (4) Mature biofilm formation; (5) Dispersion. Figure is adapted from[9]

Infections caused by such bacterial biofilm constitute almost upto 80% of total microbial infections according to the US National Institute of Health (NIH) [10]. Biofilm bacteria have slow growth rate and much higher antibiotic resistance compared to their free-living counterparts as they are shield and protected by EPS, eDNA, quorum sensing molecules, rhamnolipids and etc., to escape from host immunal attacks, thus enables the development of chronic infections.[11-15] Therefore, biofilm cells can evade the actions of immune responses and cause severe damage to human host. Chronic infections caused by bacterial biofilms are extremely difficult to eradicate and often recurring even with antibiotic treatment, making it hard to diagnose in the clinic and create a huge burden to public health. [16]

1.2. Bis-(3'-5')-cyclic dimeric GMP

To discover effective treatment of chronic biofilm infections, deeper insight into mechanisms regulating the formation of biofilm are continuously taking places in various scientific areas. One of the most significant achievements in biofilm study is

the discovery of the second messenger, c-di-GMP. This molecule was firstly discovered as cellulose synthase in *Gluconacetobacter xylinus*. [17] Its regulatory role in biofilm as intracellular second messenger was then studied extensively by different groups in both Gram-negative bacteria and Gram-positive bacteria. [18-21]

C-di-GMP is formed from two guanosine monophosphates molecules under catalyzation by diguanylate cyclases (DGC) and degraded by the c-di-GMP phosphodiesterases (PDE) intracellularly. Accumulation of c-di-GMP intracellularly will repress bacterial motility, reduce virulence and increase biosynthesis of extracellular matrix materials and thus enhance biofilm formation (Figure 1.2). In contrast, reduced level of intracellular c-di-GMP will facilitate bacterial movement and cause dispersal of biofilms. Thus, c-di-GMP might determine the capacity of bacterial pathogen to cause infection as well as the type of infection, acute or chronic. [22]

Figure 1.2 Modulation of c-di-GMP and its cellular functions. Diguanylate cyclases (GGDEF) catalyze its formation; phosphodiesterase (EAL/HD-GYP) catalyzes its degradation. C-di-GMP regulates various cellular activities. Figure is adapted from [23].

Moreover, c-di-GMP regulates over various cellular functions involved in biosynthesis of extracellular polymeric matrix which protects biofilm cells from antimicrobial attacks, stresses and clearance by host. Bacterial cells with different intracellular c-di-GMP content have distinct physiologies which could be reflected by production of those specific, secondary metabolites serving as virulence factors during the infection process. [24-26]

1.3. Quorum sensing

Besides c-di-GMP, quorum sensing is another significant mechanism taking place in the formation of chronic bacterial biofilms. Quorum sensing is the population-density-dependent intercellular communication which controls over the expression of many virulence genes included in the biosynthesis of rhamolipids, phenazine, siderophore, elastases, and proteases.[12, 27-29]

Previous studies had shown that quorum sensing regulated virulence factors alter antibiotic resistance, resistance to host immune cells and adaptation to host environment for establish chronic infections.[11, 13, 15, 30] Mutations in quorum sensing genes like *las* genes and *rhl* genes are frequently detected during its chronic colonization in respiratory system of Cystic fibrosis (CF) patients. [13, 30, 31] Variation in quorum sensing is a significant mechanism influencing bacterial biofilm formation and adaption in human host.

Quorum sensing mechanism depends on the synthesis, secretion, and sensing of autoinducers. Different bacteria possess different quorum sensing systems, like N-acylhomoserine lactones (AHLs)-dependent quorum sensing and 2-heptyl-3-hydroxy-

4-quinolone (PQS) in *Pseudomonas* species [32], autoinducer-2-dependent (AI-2) quorum sensing in Enterobacteriaceae such as *Escherichia coli* and *Klebsiella* species [33], AHL-dependent and cis-2-dodecenoic acid (BDSF)-dependent quorum sensing in *Burkholderia* species [34, 35]. Studies showed that these quorum sensing molecules control certain stages in the development of biofilm, such as surface attachment, EPS production, fluid channel formation, formation of mushroom structure and dispersal.[36, 37]

1.4. Knowledge gap

Understanding the influence on the physiology and ecology of pathogens by c-di-GMP, quorum sensing systems and other adaptation mechanisms, and interlinks among these mechanisms, is crucial for developing novel approaches for controlling and overcoming biofilm infections. Cells with differential expression of intracellular c-di-GMP or QS molecule have distinct physiologies which could be reflected by production of specific pattern of transcriptomes and metabolomes.

However, there is lack of a large scale systematic investigation of the transcriptomes and metabolomes performed in how c-di-GMP levels affect bacterial biofilm infection across different pathogenic species. Such comparative transcriptomics and metabolomics characterization of bacterial cells expressing contrasting level of c-di-GMP intracellularly will facilitate identification of biomarkers of biofilm formation, antibiotic resistance as well as efficacy of dispersal chemistry that aims at controlling c-di-GMP levels.

Moreover, there is still a knowledge gap in the control of quorum sensing systems in *P. aeruginosa*. Previous studies demonstrated that mutation in alternative sigma factor *rpoN* greatly impacts quorum sensing system in *P. aeruginosa*. [31, 38] However, how quorum sensing genes are manipulated by *rpoN* regulation and its effects on variation in the bacterial ecology in the CF lung still remain unknown.

1.5. Hypothesis and objectives

Here in this study, I hypothesize that if bacterial cells could keep expressing constantly high level of c-di-GMP, distinctive transcriptomic profile and metabolomics profile of these cells will be generated comparing to those expressing reduced amount of c-di-GMP, thus specific set of transcripts and/or metabolites positively regulated by c-di-GMP could be identified as potential biomarkers of biofilm infections.

The major objective of first part in this project is the discovery of common potential transcriptomic and metabolomic biomarkers under regulation of c-di-GMP among different Gram-negative pathogens through high throughput analysis technologies including RNA-sequencing and high performance mass spectrometry coupled with liquid chromatography. Three pathogens, *Pseudomonas aeruginosa* PAO1, *Klebsiella pneumoniae* KP-1 and *Burkholderia cenocepacia* H111, were genetically modified to express different amount of intracellular c-di-GMP to mimic either acute or chronic mode of growth.

These three pathogens are the major biofilm species in the respiratory system of CF patients. *P. aeruginosa* PAO1 is a well-studied model bacterium of biofilm formation, which leads to many different infections, such as burnt wound infection, chronic

infections in the lungs of CF patients and nosocomial infections. [39] *K. pneumoniae* which is found from environmental origins like water and soil is an opportunistic pathogen causing hospital-acquired infections, catheter-associated urinary tract infection (CAUTI), bacteremia, septicemia and biofilm infection on various medical implants.[40, 41] *B. cenocepacia* is another opportunistic Gram-negative pathogen capable of inducing long-term infections in lungs of CF patients and immunocompromised individuals. It is one of constitutive strain of the *Burkholderia cepacia* complex (Bcc). *B. cenocepacia* usually invades the CF lungs after *P. aeruginosa* infection. Extracellular matrix components synthesized by *P. aeruginosa* may alter the surface characterization of epithelial cells of CF lungs which makes the cells more prone to *B. cenocepacia* attachment. [42, 43] Identification of common transcriptomics and metabolomics features among these pathogens may give rise to a possibility of early and easy detection and diagnose of biofilm-associate infections clinically.

The major objective of the second part of this project is investigation of the regulation of PQS quorum sensing system by RpoN, the alternative sigma factor, in *P. aeruginosa* PAO1. The bacterium was genetically modified to establish *rpoN* mutants with or without expression of *pqs* genes. By comparing the transcriptomics and virulence factor synthesis of these mutants, a deeper insight in the regulation of PQS quorum sensing could be achieved.

Conclusively, this study demonstrates how c-di-GMP and quorum sensing systems interlinks in different pathogens in order to manipulate virulence factors generation and biofilm formation; and provides evidence for the possibility of detecting and

diagnosing biofilm infections causing by various pathogens in clinical prospective using common c-di-GMP regulated metabolites.

1.6. Literature Review

Mutants of pathogens, such as QS mutants and c-di-GMP mutants, used in this project, exhibited different pattern of transcription and metabolism, so it is necessary to understand the properties of such mutants. QS regulates various cellular events such as gene transcription and synthesis of secondary metabolites. The expression of PAO1 virulence factors are also regulated through QS. Many groups of researchers proved that without quorum sensing, PAO1 displays lower drug resistance and higher vulnerability to PMN attack. [13, 44]

The second messenger, c-di-GMP controls over various cellular behaviours, including biofilm formation, virulence, motility, and cell cycle.[23] From its discovery, numerous studies revealed the impact of this second messenger to cellular events and mode of survival in both of Gram positive and Gram negative bacteria. Recently, its influence on biofilm formation and biofilm infection in clinical research was intensively studied. It regulates the formation of flagella and motility of *P. aeruginosa*, expression of virulence factors, biosynthesis of extracellular polysaccharides and adhesions, and cell cycle inhibitors. Mutations in QS and c-di-GMP will cause changes in the metabolite production in bacterial cells, where QS-specific or biofilm-specific metabolite can be discovered as biomarker for clinical diagnosis.

Many studies have been done on learning the modulation of c-di-GMP and its regulation on other cellular functions. Here I will give an overview of the outstanding

achievements reached by different groups of scientists on this second messenger and its functions in bacterial cells.

1.6.1. Biosynthesis and breakdown of c-di-GMP

C-di-GMP regulates the shift between planktonic growth lifestyle to biofilm lifestyle in many bacteria species. [45] It controls over multiple cellular functions, including surface adhesins [46, 47], biosynthesis of exopolysaccharides [48-50], secretion of virulence factors, cell motility including swimming, swarming and twitching and etc, to modulate the transition between modes of cell growth [51-53].

C-di-GMP regulatory module involves a pair of enzymes responding oppositely to environmental and cellular cues for formation and degradation of c-di-GMP, an effector attaching to and regulated by c-di-GMP, and an output-generating target binding to the effector. All of the components in such regulatory module exhibits multiplicity and diversity in the bacteria making c-di-GMP regulation a very complex process. [23]

A very well studied pair of enzymes manipulating expression of intracellular c-di-GMP is diguanylate cyclases (DGC) and phosphodiesterases (PDE) (Figure 1.3). Functional DGCs contain GGDEF domain and functional PDEs carry EAL and/or HD-GYP domain. DGC contains a subunit dimer for GTP binding with the active GGDEF domain for catalysing c-di-GMP formation. EAL domain on PDE linearizes c-di-GMP to 5'-pGpG for degradation. HD-GYP domain has same function as EAL domain which degrades c-di-GMP to GMPs by cleaving its phosphodiester bond.[23, 54-57] These c-di-GMP mediating domains connect to different N-terminal sensory

domains for sensing input signals including antibiotics, oxygen, nutrients and etc. [58, 59]

Figure 1.3 Enzyme regulators and receptors of c-di-GMP. Graph illustrates major c-di-GMP-interacting domains and important receptors of c-di-GMP. Figure is adapted from [18]

GGDEF and EAL domain were found to be expressed in high copy numbers in procaryotic bacteria as many proteins containing either domain were discovered with detail functions remaining unknown previously. [51, 57, 60-65]. Later functions of many GGDEF and EAL containing proteins were discovered, especially for c-di-GMP regulation.

Weinhouse's team had demonstrated the necessity of involvement of GGDEF-containing-DGC and EAL-containing-PDE for c-di-GMP regulation in *G. xylinus*. [22] Pesci's team demonstrated that protein carrying GGDEF domain leads to induction of self-aggregation whereas protein carrying EAL domain activates cell motility repressed by *hns* in *P. aeruginosa*. [62, 65] Moreover, regulating role of GGDEF- and EAL-containing proteins on c-di-GMP expression was reported in various different bacteria such as *V. cholerae* [66] and *S. typhimurium* [57]. Different bacteria species accommodate different number of proteins carrying GGDEF, EAL and GGDEF/EAL domain.[67]

For instance, *P. aeruginosa* genome contains more than 50 regulatory proteins and effectors along c-di-GMP signalling pathway. Most of genes encoding for DGC and PDE locate in the genome of *P. aeruginosa*. [26, 67] In this bacterium, *wsp* genes encode for proteins of a chemotaxis responsive signaling pathways regulating intracellular amount of c-di-GMP. (Figure 2.2) These genes encode for a sensor protein WspA, adaptors WspD and WspB, methyl transferase WspC, histidine kinase WspE, methyl esterase WspF, and GGDEF containing WspR protein. This regulatory system controls over the expression of c-di-GMP upon detecting the variation in concentration of environmental signals. [65, 68] Moreover, AdrA containing GGDEF domain leads to elevated expression of c-di-GMP and induces expression of EAL containing protein encoded by *yhjH* gene in *Salmonella* which exhibits similar functions in *P. aeruginosa* [57]. Other c-di-GMP effectors in *P. aeruginosa* include GGDEF-containing PelD and EAL-containing FimX. [46, 49, 69]

Roc/Sad system exhibits opposite mode of regulation of c-di-GMP comparing to *wsp* signaling pathway. RocR/SadR protein contains EAL domain for degradation of c-di-GMP. Other factors in this system include RocS1/SadS as sensor and RocA1/SadA as response regulator (Figure 1.4). Through manipulating c-di-GMP concentration, this system possesses regulating capability on the transcription of *cup* fimbriae genes, virulence, type III secretion system and biofilm formation upon receiving environmental cues. [68, 70, 71] There are also many proteins carrying enzymatically inactive PDE or DGC domains. Different bacteria contain different numbers of c-di-GMP mediating proteins. [19,61,62]

Figure 1.4 Regulation of c-di-GMP regulated by WspR and Roc/Sad system. GGDEF-containing WspR and EAL-containing RocR/SadR regulate c-di-GMP in opposite manner upon receiving signals. Figure is adapted from[68].

DGC and PDE regulation on c-di-GMP is localized and target-directing. Distinct phenotypes were observed by creating mutation in different DGCs and PDEs [67] These mutations take place for adaptation and escape from clearance by host immune

system, such as the formation of small colony variants (SCVs) during chronic infections caused by *P.aeruginosa* in CF lungs. *yfiBNR* gene operon regulates c-di-GMP synthesis in SCVs where *yfiN* functions as a DGC under regulation of YfiR. *yfiR* mutant overexpressed virulence factors including pyochelin, pyoverdine and pyocyanine, suggesting that c-di-GMP regulates biosynthesis of secondary metabolites, thus pathogenesis. [26, 72-74]

Study of these GGDEF/EAL carrying protein and c-di-GMP in different bacterial strains indicated the universal role of c-di-GMP in promoting biofilm formation. [57] In the following sections, c-di-GMP regulation on different cellular activities involving in biofilm formation will be discussed.

1.6.2. Regulation by c-di-GMP

The mechanism of how changes in the expression of c-di-GMP regulate transcription of genes still remains largely unknown. However, it was revealed that PilZ domain on many proteins including several of those carrying PDE and DGC domains serves possibly as a c-di-GMP receptor for regulation.[75]

There are currently four major different kinds of c-di-GMP effectors identified including PilZ domains, I site in GGDEF domains, and two specific sites on FleQ and PelD resembling I site on GGDEF. (Figure 1.3) PilZ domains were found either as a discrete protein or as a regulatory part of a complex protein and normally linked to carboxyl terminal on c-di-GMP regulating domains and induce changes in the conformation of bounded c-di-GMP.[75]

Quite a number of PilZ effectors were identified in different bacteria. Active PilZ domains are found on Alg44 for alginate production and FlgZ for flagellar in *P. aeruginosa*, BscA for cellulose production in many Gram-negative bacteria, DgrA for flagellar in *C. crescentus*, Plz for virulence in *V. cholerae*, YcgR for flagellar and Pel production in *Salmonella* species and *E.coli*. Other effector proteins of c-di-GMP include GGDEF/EAL domain-containing PelD and FimX proteins, transcriptional regulatore PA4395, FleQ and BrlR. C-di-GMP-binding I site effector on GGDEF was found on PopA of *C. crescentus*. Moreover, FleQ and VpsT contain noval motifs specifcally for c-di-GMP binding in *V. cholera* and *P.aeruginosa*. [49, 69, 75-83]

1.6.2.1. C-di-GMP regulation on biosynthesis of exopolysaccharides

Exopolysaccharides (EPS) are produced either intracellularly and secreted into extracellular space or extracellularly by membrane-associated enzymes directly in bacterial cells. [26] Bacterial cells secrete EPS to maintain biofilm structure as matrix component and to establish irreversible attachment for development of biofilm infections in the host. Thus, EPS contributes to integrity and rigidity to biofilm structure, protection of bacterial cells from host immune clearance and resistance to antibiotics or other stresses.[8, 16, 26] Lacking of exopolysaccharide synthesis resulted in incapability of establishing mature biofilms.[84]

C-di-GMP manipulates the biosynthesis of exopolysaccharide in different bacteria transcriptionally and post-transcriptionally, and eliminates cell motility to enhance biofilm formation. [26, 85] It promotes production of cellulose in *Acetobacter xylinus* [86]; expression of the glucose-rich Pel polysaccharide, the mannose-rich Psl

polysaccharide and alginate in *P. aeruginosa* PAO1 [84, 87]; synthesis of Pel polysaccharide in *P. aeruginosa* PA14 [49].

As *P.aeruginosa* is one of the most well studied organisms for c-di-GMP regulation on EPS and is also key organism used in this project, the following discussion will focus on this bacterium. Mature *P. aeruginosa* biofilms are formed by macrocolonies grown from microcolonies. Formation of these structures needs EPS including alginate, Pel and Psl, as major constitutive factors of extracellular matrix. [49] Pel and Psl are major matrix component in non-mucoid biofilms of *P. aeruginosa* while alginate contributes to mucoid phenotype for chronic infection. [18]

Increased biosynthesis of Pel and Psl associates with rugose SCV phenotype in chronic CF infection. [88] Either of these two polysaccharides is able to enhance irreversible attachment.[89] Variation in concentration of intracellular c-di-GMP contributes to biofilm formation by performing as a cofactor for synthesis of these three EPS.[90]

Figure 1.5 Pel,Psl and alginate gene operons. Pel opreon consists of 7 consecutive genes *pelABCDEFG*, Psl operon consists of 12 consecutive genes *pslABCDEFGHIJKL*, while 12 genes encoding for aliginate include *algD*,

alg8, alg44, algK, algE, algG, algX, algL, algI, algJ, algF, algA. Figure is adapted from[91].

Pel is encoded by a highly conserved gene operon consisting of 7 genes, *pelABCDEFG*. (Figure 1.5) FleQN complex blocks Pel synthesis by binding to *pelA* promoter and stopping *pel* transcription.[92] Pel is assembled by PelF activity and translocated into extracellular space by activity of PelBCDEG. (Figure 1.6) PelD containing a PilZ domain and an enzymatically inactive GGDEF domain binds to c-di-GMP for post-translational upregulation of Pel synthesis. [49, 77, 87, 92-95] C-di-GMP possesses transcriptional regulation of Pel through binding with regulator protein FleQ which imposes inhibitory effect on Pel synthesis. C-di-GMP binds with FleQ and removes it from promoter of *pelA* to enhance expression of *pel* genes for Pel synthesis. [77]

Figure 1.6 Biosynthesis of Pel polysaccharide. C-di-GMP interacts with PelD for Pel synthesis. PelF contributes to Pel assembly. Assembled Pel is translocated to

extracellular space upon the activity of PelBCDEG. Figure is modified from [91].

As c-di-GMP imposes its regulation of Pel, diguaylate cyclases and phosphodiesterases manipulate the expression of Pel by varying c-di-GMP concentration. For example, Removal of PDE-encoding *bifA* in *P. aeruginosa* PA14 induced synthesis of Pel and thus leads to increased biofilm formation without altering *pel* expression [96]. Mutation in DGC-encoding *roeA* gene resulted in lower c-di-GMP concentration without changing *pel* expression in *P. aeruginosa*. RoeA protein probably interacts with PelD-bounded c-di-GMP for increasing Pel synthesis as mutated RoeA leads to reduction in Pel-mediated exopolysaccharide synthesis. [96, 97] Another DGC protein, SadC, also regulates the synthesis of exopolysaccharides [8]. These DGC proteins are found at different location intracellularly indicating that they may produce different localized pools of c-di-GMP for Pel synthesis. [96]

The regulatory cascade from quorum sensing system to c-di-GMP via *pel* operon was also discovered in PA14 where tyrosine phosphatase TpbA interacts with *pelD* and downregulates biofilm formation. TpbA represses biosynthesis of c-di-GMP by intracting with a DGC, TpbB [85]. Mutation in TpbA was demonstrated to elevate EPS secretion through regulation of *pel* genes. Mutation in TpbB and PelABDEG restored the phenotypic changes induced by loss of TpbA indicating TpbA represses c-di-GMP synthesis. Since TpbA production is under positive regulation of *las* quorum sensing, c-di-GMP expression is negatively regulate by *las* quorum sensing through regulating *pel* genes [85]. Elevated Pel production in some CF isolates depends on TpbB-induced c-di-GMP overexpression. [74] This indicates the importance of

quorum sensing in c-di-GMP regulation. Quorum sensing systems will be discussed in the later section.

Malfunction in these *pel* genes leads to serious reduction in formation of biofilm and pellicles, and antibiotic resistance as Pel contributes to resistance to aminoglycosides [77, 87, 98]. PA14 possesses only *pel*-encoding exopolysaccharide while PAO1 possesses two types of exopolysaccharides, *pel*-encoding and *psl*-encoding [87, 94]. In the following paragraphs, regulation of c-di-GMP on Psl will be discussed.

Genes required for Psl expression involve *pslACDEFGHIJKL* whereas expression of *pslBMNO* genes has insignificant influence on Psl synthesis. (Figure 1.5) PslB may acts mainly for Psl precursor synthesis; PslCFHI may mainly integrate individual Psl-building sugar molecule into repeating structure; polymerization of Psl structure is suggested to be catalysed by PslAEJKL; while polymerized Psl is exported into extracellular space by PslDEG [91] (Figure 1.7). This EPS is expressed in free-moving cells whereas *psl* gene expression localised in the center of microcolonies in the biofilm. [94, 99, 100] However, it promotes biofilm formation by enhancing cell transition from free-moving to sessile lifestyle and upholding biofilm structure. [8] It contributes to mushroom structure formation and cell-cell interaction during biofilm development.[89, 101] A trace of Psl left along the track of migration by PAO1 on the surface facilitates further surface attachment for microcolony development.[102]

Figure 1.7 Biosynthesis of Psl polysaccharide. Precursors of Psl integrated by PslCFHI (black arrow) and polymerized by PslAEJKL before translocating by PSLDEG (red arrow). Figure is modified from [91].

PAO1 possesses post-transcriptional regulation of Psl while PilZ protein is not necessary for its production unlike PilZ-requiring Pel. However, the mechanism of c-di-GMP regulation on this polysaccharide post-transcriptionally is still remaining unclear.[8] High level of c-di-GMP induces Psl production whereas Psl polysaccharide itself in turn promotes c-di-GMP expression as positive feedback through mediation by SadC and SiaD both carrying GGDEF domain. [103] C-di-GMP upregulates Psl synthesis transcriptionally by binding with FleQ as FleQ also inhibits transcription of *psl* genes. FleQN plays a similar role in *psl* gene expression as in *pel* gene expression. [77] RsmA regulated by small RNA *rsmZ* and *rsmY* inhibits Psl translation by interacting with mRNA ribosomal binding site. *rsmY* and *rsmZ* were demonstrated to play a apart in the biosynthesis of c-di-GMP. [104, 105] Moreover, high c-di-GMP

level upon deletion of *wspF* gene in *P. aeruginosa* PAO1 stimulates transcription of *cdrA* gene. CdrA binds to Psl and contributes to auto-aggregation and biofilm formation. [106]

Synthesis of both Pel and Psl relies on c-di-GMP which is under regulation by several proteins such as GGDEF-containing WspR. WspR is a part of chemotaxis-like system in *P. aeruginosa* for upregulation of EPS and formation of biofilm by boosting c-di-GMP synthesis. WspR system is activated upon surface attachment and results in wrinkled colony formation as accumulation of c-di-GMP activates *pel* and *psl* gene expression and thus enhances synthesis of Pel and Psl. [72, 107, 108]

P. aeruginosa also produces alginates to support cell structure, form mucoid phenotype and contributes to biofilm formation during infection of CF lungs. Overexpression of alginate was frequently discovered from *P. aeruginosa* isolates obtained from CF patients. [39, 109, 110] Alginate is encoded by *algD* operon consisting of 12 genes located at different parts along its genome. [111] (Figure 1.5) It is a key factor for biofilms in CF lungs but not for *in vitro* biofilms although upregulation of some of the alginate-encoding genes such as *algU*, *algC* and *algD* was seen in *P. aeruginosa* upon surface attachment. [8, 112, 113] Production of alginate is controlled by the transcriptional regulator, AlgR, in *P. aeruginosa*. It is also involved in the synthesis of other virulence factors as a transcriptional regulator such as type IV pili, and also possesses positive regulation over the expression of *mucR* through direct promoter binding. [110] MucR containing both GGDEF domain and EAL domains acts as diguanylate cyclase during biofilm formation and as phosphodiesterase during free-moving growth to create intracellular pool of c-di-GMP. [114, 115] Thus, *algR*

regulates the expression of c-di-GMP through *mucR*. Alg44, an inner-membrane anchored protein, was observed to bind with these c-di-GMP molecules synthesized by MucR.[76, 116] C-di-GMP binds with PilZ domain on N-terminal of Alg44 protein to enhance polymerization of alginate components thus upregulating its synthesis. [116] However, MucR-dependent alginate synthesis is strain-specific as in *P. aeruginosa* PAO1, MucR upregulates Pel/Psl expression instead of alginate. [18, 117] eDNA also plays a part in surface attachment of *P. aeruginosa* and promotes cell aggregation.[118] No correlation has been found between eDNA and c-di-GMP yet. Besides EPS, c-di-GMP also possesses regulatory function over bacterial motility.

1.6.2.2. Regulation of motility by c-di-GMP

C-di-GMP exhibits regulatory effects on different types of motility including swarming, twitching, swimming, and gliding in different bacteria. [119-121] Flagellar and non-flagellar surface appendages are involved in specific motility respectively to fulfill different cellular functions. Flagellar and pili/fimbriae motility have great impact on biofilm formation in *P. aeruginosa*. [84, 87, 122]

Migration of *P. aeruginosa* cells over surface after irreversible attachment is mediated by twitching and swarming. *P.aeruginosa* cells perform initial reversible contact to surfaces by employing swimming motility driven by flagellar in order to resist to surface repulsion as the initiation of biofilm formation. [122, 123] Once irreversible attachment is established on the surface, *P. aeruginosa* cells can extend over the surface utilizing both swarming by flagellar and twitching by pili, or form stable longitudinal attachment to the surface. By doing so, a thin monolayer of biofilm will be formed over the surface. [8]

The complete flagellar structure consists of basal body, flagellar hook, motor, filamentous structure and anchoring ring-structures of the complex to cell membrane. These flagellar components are encoded by 41 genes found on three discontinuous locations on *P. aeruginosa* genome.[8, 124] Location I consists of *flgBCDE* and *flgFGHIJKL* encoding for basal body rod, hook and cap, *fliCfleL* and *fliDS* encoding for flagellin and filament, *fliEFGHIJ* encoding for motor, ring and hook components, *fleQ* encoding for σ^{54} dependent transcriptional regulator and *fleSR* as two-component system. Location II consists of *fliK* encoding for hook length regulator, *fliLMNOPQRflhB* and *flhA* encoding for motor/switch and export apparatus, *flhFfleN* encoding for flagellar site determinant and number regulator, *fliA* encoding for σ^{28} sigma factor FliA, *cheAB*, *cheYZ*, *cheW* and *motAB* genes encoding for chemotaxis proteins and motor. Location III consists of *flgMN* and *flgA* encoding for antisigma factor and export apparatus, and *cheVR* encoding for chemotaxis regulators. [124]

High level of c-di-GMP induces repression of flagellar gene expression by c-di-GMP/FleQ complex during initial phase of biofilm establishment to regulate swimming motility. [8] Expression of flagellar components starts from binding of the transcriptional regulator FleQ to promoter of *flhA* gene and induce downstream gene transcription cascade for flagellar synthesis.[125] Upon binding, c-di-GMP leads to changes in FleQ conformation and prevents it from binding to *flhA* promoter. FleQ was also demonstrated to be under the control of FleN and c-di-GMP together. These two regulatory molecules impede the FleQ-dependent induction of flagellar genes by repressing its ATPase activity. FleQ also exhibits regulational capability over c-di-GMP since the absence of FleQ resulted in lower c-di-GMP concentration in *P. aeruginosa* cells.[77, 126]

Flagellar rotating activity is essential for approaching to inert substrate and overcoming repulsive force at liquid-surface junction for surface attachment. [18] High level of c-di-GMP in *E.coli* also induces modulation in flagellar rotation regulated by c-di-GMP/YcgR complex. [8] YcgR protein carrying PilZ-domain in *E.coli* controls flagellar activity after assembly. It reduces swimming motility by interacting with the motor protein FliG and the stator protein MotA when there is accumulation of c-di-GMP. [8, 62, 80, 127-131] FlgZ also carries a c-di-GMP-interacting PilZ domain and interacts with flagellar unit. [131, 132] Overexpression of c-di-GMP and repression of flagella rotation were observed where YcgR functions as c-di-GMP receptor binding with FliG and FliM for regulating flagellar motor. Another study indicated that flagellar activities could be blocked upon interaction between c-di-GMP/YcgR complex and MotA. [53, 133] *flgZ* gene in *Pseudomonas* strains was identified as a potential homolog of *ycgR* gene. [8]

Nonflagellar surface appendages, pili/fimbriae, are also regulated by c-di-GMP. There are different types of fimbriae including type IV fimbriae and *cup* (chaperone/usher-dependent pathway) fimbriae which are essential for biofilm formation. [18, 117] Such fimbriae are key elements in irreversible surface attachment and mushroom cap structure formation. [119]

CupABCDE gene cluster encodes for Cup fimbriae which function as surface adhesion and factor for *P. aeruginosa* biofilm formation. This gene cluster is under transcriptional upregulation of c-di-GMP. Such c-di-GMP pool is generated by a diguanylate cyclase YfiN and reduced by a phosphodiesterase MorA respectively in *P. aeruginosa* small colony variance isolates. [73, 134] *cupBC* clusters are under

coregulation by three-component Roc1 system which EAL domain containing RocR represses *cupC* transcription and RocR-phosphorylated RocS2 regulates *cupBC* expression. [70]

Type IV pili which is partially under c-di-GMP regulation is essential for twitching motility. [135, 136] Type IV pili is also essential for 3D structure during biofilm maturation. Mutation in type IV pili impairs biofilm forming capability in *P.aeruginosa*. [122] FimX localizing in cell pole containing a GGDEF-EAL dual domains interacts with c-di-GMP as an active PDE through EAL domain to downregulate type IV pili expression. [52, 137] Removal of FimX leads to induction of type IV pili formation upon release of c-di-GMP. Another c-di-GMP interacting protein PilZ exerts positive regulation on twitching motility. [8, 127] PilZ-PilB interaction enables polymerization of Type IV pili and malfunction in PilZ impairs type IV pili assembly and restricts twitching motility. [76, 119, 122, 138] Non-fimbrial adhesins in *P. aeruginosa* such as the β -helix adhesion encoded by *cdrAB* operon is also under positive transcriptional regulation of c-di-GMP generated by active YfiN as a DGC. [85, 106]

Other DGC and PDE controlling synthesis of c-di-GMP for motility regulation involve SadC and RoeA as DGCs, and BifA as PDE. SadC and RoeA regulate synthesis of exopolysaccharides and swarming motility. [8] SadC and BifA regulate c-di-GMP oppositely to modulate surface behaviour in *P.aeruginosa* PA14. SadC promotes c-di-GMP synthesis and inhibit swarming while BifA degrades c-di-GMP and induces swarming and twitching to promote planktonic lifestyle. [97, 121] Mutation in *sadC* in *P. aeruginosa* cells leads to enhancement in swarming motility and initiation in

chronic surface attachment for biofilm establishment, whereas mutation in *bifA* reduces the directional reversal. [97, 121] Besides *Pseudomonas*, c-di-GMP has great impact on motility in other bacteria such as, *K.pneumoniae*, another key bacterium used here.

K. pneumoniae is able to cause catheter-associated infections, respiratory infection and nosocomial infections. Two different adhesins, type 1 fimbriae and type 3 fimbriae, are produced in *K. pneumoniae*, which are involved in biofilm formation. Type 1 fimbriae formation was repressed in biofilm cells. Most of the clinical isolates possess type 3 fimbriae which associate with infection development. [139-142]

In *K. pneumoniae*, type 1 fimbriae are cognate to those found in *E.coli*. [143] FimK which carries an EAL domain and functions as a phosphodiesterase regulates type 1 fimbriae expression in *K. pneumoniae*. Removal of FimK elevates the expression of fimbriae and promotes *in vivo* surface colonization. [143, 144] Type 3 fimbriae in this bacterium enhances cell attachment and formation of biofilm on inert surface and human host proteins, thus involved in onset of chronic biofilm infection in respiratory system and nosocomial infections. C-di-GMP modulated by YfiN (DGC) and MrkJ (PilZ, PDE), stimulates the expression of type 3 fimbriae transcriptionally. [143, 145, 146]

Mrk gene cluster containing *mrkABCDF* genes encodes for type 3 fimbriae components where *mrkA* is responsible for expression of major subunits of fimbriae. The shaft of type 3 fimbriae is formed by polymerization of MrkA peptide-constituted subunits whereas its attachment to extracellular matrix is mediated by MrkD protein.

Removal of MrkD promotes biofilm formation on inert surface by inducing non-adhesive fimbriae expression. A three gene cluster locates next to *mrk* gene cluster, consisting of *mrkJIH* genes encoding for regulator and sensor proteins of c-di-GMP. *MrkJ* gene encodes for a EAL-containing phosphodiesterase and *mrkIH* genes encode for a PilZ-containing MrkH protein and a probably transcriptional regulator MrkI. [68, 143, 145, 147-149]

C-di-GMP regulating genes in *K. pneumoniae* cells demonstrated regulatory role on type 3 fimbriae biosynthesis. *MrkJ* gene residing in proximity to *mrk* gene cluster regulates type 3 fimbriae expression through manipulating the availability of intracellular c-di-GMP. Removal of *mrkJ* elevated expression of type 3 fimbriae and increased biofilm formation due to accumulation of c-di-GMP. MrkH functioning as a transcriptional activator binds to c-di-GMP forming MrkH/c-di-GMP complex and attaches to promoter region of *mrkA* gene for activation of type 3 fimbriae synthesis. [145, 150]

1.6.2.3. Quorum sensing

Quorum sensing is cell communication mechanism depending on cell density through diffusible autoinducers to modulate cell behavior in the community. Once the accumulation of autoinducer extracellularly hits the threshold, bacteria cells will start to modify its gene transcription as quorum sensing effects. Bacterial cells talk to one another through quorum sensing and organise cellular behaviour based on it. [151, 152]

Cellular activities under regulation of quorum sensing system include biofilm formation and biosynthesis of exopolysaccharides. [85] Quorum sensing also controls

over other cellular activities including antibiotic resistance, synthesis of virulence factors and secondary metabolites. [153, 154] Moreover, architecture of *P.aeruginosa* biofilms on inert substrates is also under regulation of quorum sensing system. [20]

There are three identified quorum sensing systems interconnecting in a hierarchical manner in *P. aeruginosa* (Figure 1.8), being *las* quorum sensing utilizing LasRI-generated N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) as autoinducer [155]; *rhl* quorum sensing utilizing RhlRI-generated N-butyryl homoserine lactone (C4-HSL) as autoinducer [156]; and *pqs* quorum sensing utilizing 2-heptyl-3-hydroxy-4-quinolone (PQS) as autoinducer [36]. Among the three systems, *las* system locates at the top in the hierarchy which activates the expression of the other two systems. [157, 158]

A new QS signal was later identified and named as integrated quorum sensing, IQS. IQS is under the positive control of *las* in rich medium and is also regulated by the availability of phosphate. IQS system was also demonstrated to enhance the expression of *rhl* and *pqs* quorum sensing and biosynthesis of virulence factors in the absence of *las* system. Thus, IQS may serve as interconnection between *las* system and the downstream *rhl* and *pqs* systems. *ambABCDE*, the gene cluster which previously known to be responsible for the production and translocation of antimetabolites AMB, was discovered to encode for IQS which in turn controls the production of AMB via *rhl* and *pqs* systems. [159, 160]

However, a recent research done by another group indicated from molecular structural aspect that *ambABCDE* encodes for the production of AMB using amino acids l-Ala

and l-Glu as substrates, but is not responsible for the biosynthesis of IQS which may utilize salicylate and cysteine as substrate. [161] Therefore, the mechanism of expression of IQS is still controversial and thus further deeper research is needed for a confirmation. This does not make any shade on the significant discovery of IQS which accomplish the network of quorum sensing systems.

Figure 1.8 Hierarchical relationship among three quorum sensing systems. Autoinducers and their precursors are indicated by black dots; regulators are indicated by oval; synthesis reaction and activation are indicated by arrow; inhibition is indicated by flathead arrow. Figure is adapted from [155].

Las quorum sensing system consists of *lasRI* where, LasR, a homolog of LuxR, is the key global regulator of various virulence-related genes, including *lasAB*, *aprA*, *toxA*, and the *luxI* homolog, *lasI*, is responsible for the synthesis of autoinducer, 3-oxo-C12-HSL. [155, 160, 162-164] The autoinducer binds to LasR to form a functional

complex and attaches to the conserved sites of target genes to promote the production of various virulence factors including exotoxin A, alkaline protease, elastase, pyocyanin and LasA protease. [162, 164-166] This forms the first autoinducing cycle in the top of the quorum sensing hierarchy. It was discovered that *las* autoinducer, 3-oxo-C12-HSL, is also involved in the differentiation of biofilm structures. [20] Deficiency in *las* quorum sensing system leads to impaired biofilm structure which is more vulnerable to antibiotic attack. [20]

Previous study had discovered that *lasR* mutation frequently arises during chronic respiratory infection in the CF patients. *LasR* encoding for the transcriptional regulator regulates the expression of various virulence factors. Disfunction of *lasR* enhances β -lactamase activity in *P. aeruginosa* and thus is more resistant to antibiotics such as ceftazidime. Thus, *lasR* is able to serve as a genetic marker of chronic *P. aeruginosa* infection in CF patients. [167]

Rhl quorum sensing system consists of *rhlRI* where *rhlI* regulates the formation of autoinducer, C4-HSL. *RhlR* gene is activated by LasR complex to express RhlR. The autoinducer binds to and activates RhlR to form the second autoinducing cycle in the hierarchy. The functional complex controls the synthesis of virulence factors such as pyocyanin, LasA protease, elastase, rhamnolipids, siderophores, hydrogen cyanide, and lectins. Rhamnolipids regulated by *rhl* quorum sensing also contributes to biofilm structure. [27, 168-172] RhlR was also demonstrated to enhance the transcription of *lasI*. [32, 173] Both *lasRI* and *rhlRI* is regulated by GacA positively. [174-176]

To regulate these two quorum sensing systems and compensate the positive feedback loop of autoinduction, several repressive mechanisms take place to cease the autoinduction loop. QscR associates with the autoinducer complexes to interfere their interaction with DNA promoter and represses the expression of *lasI* and *rhlI* to decrease the expression of virulence factors. [176-178] LasR is able to activate the biosynthesis of RsaL, which is the repressor of *lasI*, and binds with it to inhibit the expression of *lasRI* genes to cease the positive feedback loop of autoinducer expression.[179]

Previous study analyzing CF sputum samples had demonstrated that both of *las* and *rhl* autoinducers are secreted in *P. aeruginosa* biofilms colonizing the lungs of CF patients indicating that quorum sensing is essential for the establishment of microbial biofilm infections in human hosts. More C4-HSL is synthesized by biofilm cells in CF lungs comparing to 3-oxo-C12-HSL.[180] Both of *las* and *rhl* quorum sensing also participate in the regulation of *pel* operons for exopolysaccharide expression. [181]

These two quorum sensing systems functions in a hierarchical form where *las* system possesses regulation over *rhl* system. [168-172] *Rhl* quorum sensing system in turn possesses regulatory function over *pqs* quorum sensing system as it inhibits the synthesis of PQS molecule by inhibiting the expression of *pqsABCDE* and *pqsR* genes. [182, 183] PQS molecule is synthesized from its precursor anthranilate which is involved in tryptophan biosynthesis. [184] *TrpEG* genes for tryptophan biosynthesis and *phnAB* genes for pyocyanin expression were recognized as anthranilate synthase gene pairs which TrpEG and PhnAB catalyse the formation of anthranilic acid from chorismic acid. [185, 186] (Figure 1.9) *pqsABCDE* gene operon encodes for PQS

where bioynthesis of PQS starts at the activation of anthranilic acid to form anthranilate-CoA catalyzed by anthranilate coenzyme A ligase which is regulated by *pqsA*. Condensation of anthranilate-CoA and active beta-ketodecanoate is catalysed by *pqsBCD* enzymes to form HHQ, giving out water and carbon dioxide. *PqsH* and *pqsL* are also essential for PQS expression. *PqsH* encodes for the enzyme, FAD-dependent-monooxygenase, which catalyses the conversion from HHQ to PQS. (Figure 1.9) *pqsL* represses biosynthesis of PQS. The transcriptional regulator, PqsR binds with PQS molecule and initiates autoinduction of *pqsABCDE* operon through attaching to *pqsA* promoter. Specific function of PqsE remains unclear but its involvement in cellular response to PQS was confirmed. [36, 65, 184, 187-189]

Figure 1.9 Genetic regulation and formation of PQS molecule. Gene operon for PQS synthesis is indicated on the upper part while reaction cascade of PQS from shikimic acid is indicated on the lower part of the figure. Figure is adapted from [34]

Malfunction in any of these *pqs* genes will lead to inhibition of PQS synthesis. Removal of *pqsR* gene leads to inhibition of both PQS production and pyocyanin expression. Except that, removal of *pqsE* gene has negligible impact on synthesis of HHQ and PQS but reduces elastase production and abolishes the expression of pyocyanin and PA-1L lectin. [157, 158, 190] *Pqs* quorum sensing is controlled by both *las* and *rhl* quorum sensing systems; meanwhile, it also possesses the capability to regulate *las* quorum sensing through *lasB* expression. [183] *Rhl* quorum sensing represses the transcription of *pqsABCDE* operon upon upregulation of C4-HSL expression. LasR works in an opposite manner to RhlR and activates *pqsR* expression. There is a concentration-dependent competition for PQS regulation between *las* and *rhl* quorum sensing. [182] However, PQS synthesis is only partially regulated by *las* system as removal of *lasR* was unable to cause complete inhibition of PQS expression. [32, 158]

Furthermore, PQS synthesis and some PQS-regulated phenotypes are also positively regulated by genes encoding for efflux pump, *mex* and *opmD*. Mutation in this efflux pump greatly impacted on growth, *lecA* transcription, synthesis of PQS and AHL-autoinducers, antibiotic resistance, and AHL-dependent virulence factors such as rhamnolipids, pyocyanine, and elastase. Transcription of *pqsABCDE* operon is totally inhibited upon removal of *mexI* or *opmD* genes in early growth stage. [169, 191, 192]

Pqs quorum sensing system was discovered to be able to stimulate *lasB* without the presence of *lasR* gene which expression of *lasB* is inhibited without *lasR*. [157, 183] Besides, PQS induces *rhlI* gene to regulate *rhl* quorum sensing system while

combination of *rhl* and *pqs* autoinducers has greater capability in regulation of *lasB* gene expression. It was demonstrated that maximal level of PQS is synthesized at late log to early stationary growth stage. Elevated expression of PQS in late stationary phase was seen in the absence of *lasR* gene indicating PQS expression is *lasR*-independent. [193, 194] PQS stimulates expression of *rhl* quorum sensing during late stationary growth. Rhamnolipid regulated by *rhl* system was also proved to be under regulation of PQS. [32]

PQS was found in the sputum and respiratory fluid samples of *P. aeruginosa* infected CF patients. [195] Level of PQS synthesized during CF infection exhibits an age-dependent, cell-density-dependent and growth-dependent manner. [195, 196] *Pqs* quorum sensing has innegligible impact on cellular functions, such as cells with excessive production of PQS tend to have higher chance to get autolysis. [65] Adding PQS externally into *P. aeruginosa* culture possibly increases expression of PA-IL lectin and enhances formation of biofilm. [32] Malfunction of *fimL* gene promotes autolysis further in *lasR* mutant regardless of PQS level but probably due to excessive HHQ synthesis.

Moreover, PQS plays a key role in the expression of virulence factors in *P. aeruginosa* as loss of *pqsR* or other *pqs* genes resulted in significant reduction in death rate of nematode and mice during *in vivo* killing study. [158, 188] However, the mechanism of quorum sensing imposes its effect on biofilm formation still largely remains unknown. One known example is that *las*-regulating tyrosine phosphatase TpbA in *P. aeruginosa* PA14 represses biosynthesis of c-di-GMP, thus controls its biofilm development. [85]

Similar to *P. aeruginosa*, *B. cenocepacia* also retains AHL-dependent quorum sensing system, known as *cep* quorum sensing. *Cep* quorum sensing system consists of *cepR* and *cepI* genes for synthesis of N-acylhomoserine lactones as signalling molecules (Figure 1.10). *CepI* as AHL synthase encodes for production of N-octanoylhomoserine lactone (C8-HSL) being the major output and N-hexanoylhomoserine lactone (C6-HSL) being a minor output, whereas *cepR* regulates transcription of target genes upon binding with C8-HSL. [197, 198]

In *B. cenocepacia*, various cellular functions are mediated by *cep* quorum sensing system (Figure 1.10). It was demonstrated to be required in maturation of *B. cenocepacia* H111 biofilm, and regulation of its swarming motility probably via synthesis of biosurfactant. [199] Mutation of *cepR* genes leads to diminished biosynthesis of AHLs, as well as impairs the ability of *B. cenocepacia* cells in swarming motility and generates much less differentiated microcolonies thus less matured biofilms. [199] Mutation in *cep* system also results in overproduction of siderophore and repression of activities of secreted protease and lipase. [198] A screening analysis revealed that nearly ninety promoters are under regulation of CepR in *B. cenocepacia*. [200] Surface protein, AidA which is essential for *in vivo* virulence of *B. cenocepacia* is under regulation of *cepRI*. [201]

A second quorum sensing system, *cciRI*, is possessed by *B. cenocepacia* producing homoserine lactone as autoinducer while a non-AHL-synthase-gene-encoded *cepR2* functions as an independent regulator. (Figure 1.10) These quorum sensing systems performs some overlapping but usually opposite regulation as that of *cepRI* system. [202, 203] *Brucella* species also contain homologous to *pqsABCDE* operon, *pqsR*

gene and *phnAB* operon, and are able to synthesize certain 4Q signalling molecules such as HHQ. [36]

Figure 1.10 Cep and Cci quorum sensing in *Burkholderia cenocepacia*. *cepRI* system is indicated by blue, *cciRI* system is indicated by green while *cepR2* is indicated by red. Activation is illustrated by arrows and inhibition is illustrated by flathead arrows. Figure is adapted from [188].

Moreover, a non-AHL molecule known as *Burkholderia* diffusible signalling factor (BDSF), cis-2-dodecenoic acid, has also been identified in *B. cenocepacia* as signalling molecule and is involved in motility, *in vivo* virulence and biofilm formation. [34, 204] It binds to PAS domain on RpfR to stimulate its EAL domain and PDE function to degrade c-di-GMP, thus regulates the development of biofilm of the

strain. [205] This molecule is also probably required for both cross-species and cross-kindom communication. [204]

Precious study indicated that *cepRI* may play a part in communication with *P. aeruginosa*. [198] Expression of virulence factors in both *P. aeruginosa* and *B. cenocepacia* is under regulation of AHL-producing quorum sensing systems to ensure a population-dependent mode of secretion to invade host tissues. [29, 198] As sharing the same quorum sensing signals, co-existence of *P. aeruginosa* and *B. cenocepacia* enable them to synergistically increase the virulence of other strain. [206] However, quorum sensing and c-di-GMP interaction mainly follows a single direction which variation in the level of autoinducers regulates c-di-GMP formation and degradation. [18, 205]

1.6.2.4. C-di-GMP regulation on biofilm dispersion

Biofilm dispersion releases biofilm cells into surrounding as free-moving cells upon triggering by various signals. Nitrous oxide (NO) was demonstrated to induce biofilm dispersion from matured biofilm. The NO donor, sodium nitroprusside (SNP) also significantly reduced biovolume of established biofilm at sub-MIC concentration by dose inducing dispersion. Addition of SNP activated PDE function which leads to significant reduction of intracellular c-di-GMP to trigger dispersion. [207-209]

Amount of c-di-GMP in dispersed cells was demonstrated to be similar to that of planktonic cells. [210] Dispersion by SNP/NO was found to be possibly regulated by two PDEs, encoded by *dipA* and *rbdA* in *P. aeruginosa* strains after screening various mutants. Removal of GGDEF-EAL-containing *dipA* and *rbdA* genes upregulates c-di-

GMP biosynthesis, and thus enhances the production of EPS at post-transcriptional level. [210, 211] *BdlA* gene contributes to biofilm dispersion in a c-di-GMP-dependent way since it lacks of c-di-GMP regulating domains. However, variation in the concentration of c-di-GMP regulated by DipA and GcbA impacts BdlA protein cleavage. Thus DipA reduces level of c-di-GMP in order to initiate biofilm dispersion through BdlA cleavage. [8, 212, 213]

1.6.2.5. C-di-GMP and virulence

C-di-GMP may either upregulate or downregulate virulence in different pathogens at different path and phase of infection. [18] Its effect on virulence was first discovered in *V. cholera* for upregulation of secretion of cholera toxin by reducing c-di-GMP concentration. However, c-di-GMP plays a minor role in acute infections by many bacteria species. [214, 215]

C-di-GMP was demonstrated to suppress *in vivo* virulence of *P. aeruginosa* whereas mutation in certain PDEs and a DGC, SadC, was also found to repress its virulence. [67] Repression of type III secretion system has been attained by elevated c-di-GMP level resulted from upregulation of diguanylate cyclases while repression of effector ExoS has been achieved by downregulation of HD-GYP containing phosphodiesterase in *P. aeruginosa*. Activation of type IV secretion system has also seen with elevated c-di-GMP level. [105, 216, 217]

An example of such regulation is the signaling cascade consists of RetS/LadS/GacS controlling the transition between acute infection and chronic infection. Deletion of *ladS* leads to onset of chronic infection where repressed type III secretion system

expression and elevated type IV secretion system expression was achieved by diguanylate cyclase activity of WspR and PA0290. [105] RsmA, a secondary metabolism regulator in *P. aeruginosa* cells, is involved in regulation of components in chronic and acute infection in an opposite manner. C-di-GMP inversely controls type III/IV secretion systems which are targets of RsmA. [105] C-di-GMP also regulates multiple phenotypes including cytotoxicity, attachment and invasion to host cells, immune response regulation, and oxidative stress resistance. [18, 49, 66, 67]

Currently, identification of biomarkers of biofilm infections is still mostly based on detecting/visualizing exopolysaccharides such as EPS staining and Psl antibody, and mostly significant biomarkers for biofilm detection are still biofilm matrix components. Previous studies had demonstrated that *Hippeastrum* Hybrid (Amaryllis)/mushroom lectin MOA and fluorophore conjugate binds with mannosyl components of Psl specifically for detecting biofilms [218, 219] while CDy11 as fluorescent probe labels functional amyloids, which is an essential structural component of biofilm matrix, for *in vivo* biofilm detection. [220] Antibodies targeting specific epitopes of Psl were also identified for biofilm detection and a measure for defeating bacterial infections. [221] These methods, however, either could detect only *in vitro* biofilms or requires invasive sample collecting procedure.

Up to the present date, clinical measurements for biofilms are still based on staining and visualization of infected tissue samples using microscopy. Detection of biofilm infection is also based on traditional culturing techniques to recover bacteria cells on agar plates. These methods excluded the possibility to discover the infections caused by viable but non-culturable microorganisms. [222] The advantages and disadvantages of each method are listed in Table 1.1 below.

Approaches		Advantages	Disadvantages
EPS staining	HHA/MOA Lectin	Direct visualization of biofilm; Suitable for only <i>in vitro</i> models; Complicate and time-consuming staining and labeling process;	<i>In vivo</i> detection is limited; Not suitable for viable but non-culturable bacteria; Detection at mid- or late-stage of biofilm growth
	CDy11	Direct visualization of infection site; Suitable for both <i>in vitro</i> and <i>in vivo</i> models; Complicate and time-consuming staining and labeling process	Invasive sampling method; Not suitable for viable but non-culturable bacteria; Detection at mid- or late-stage of biofilm growth
Antibody Targeting	Anti-Psl monoclonal antibodies	Very specific target-focused approach; Antibody library readily available Clinically relevant; Suitable for both <i>in vitro</i> and <i>in vivo</i> models	Invasive sampling method; Not suitable for viable but non-culturable bacteria
Omics	LCMS/NMR	Non-invasive sampling method(blood/body fluid); Small sample size; Suitable for both <i>in vitro</i> and <i>in vivo</i> models; High sensitivity	Limitation in database; Identification of unknown molecules

Table 1.1 Comparison table of advantages and disadvantages of current biomarker identification approaches. [218-221]

Chapter 2. Identification of signature genes regulated by c-di-GMP through transcriptomics analysis

Transcriptomics analysis and profiling is very often used in studying changes in cellular activities and the impact of environmental cues on bacteria cells through quantification of transcripts. Previous studies utilized transcriptomics profiling to investigate the signature genes of different growth states of bacterial cells, including planktonic cells, biofilm cells and dispersed cells respectively of a sole organism [223, 224]; or of comparative analysis of one organism under different stresses such as variation in the availability of iron, copper, oxygen and quorum sensing [225]. These studies provide valuable information on the specific genetic signatures in the testing organism under various different conditions, especially during biofilm formation.

However, there is a lack in the cross-strain or cross-species study of discovering common genetic signatures of biofilms among different bacteria due to the highly diversified and complex regulation systems utilized by different organisms. However, although c-di-GMP signalling network is vastly different in bacterial species, the enzymes that regulate c-di-GMP are highly conserved across species firstly, all containing either functional GGDEF domain for synthesis or functional PDE domain for degradation. C-di-GMP receptor domain, PilZ, is also highly conserved across species. [48, 57, 80, 226] Secondly, similar traits are controlled by c-di-GMP across strain such as secretion of EPS, formation of surface appendages and motility. [18, 45, 227] Its regulation over EPS production in different strain reveals its capability in change of carbon response in different bacteria. Thirdly, after transforming c-di-GMP regulating genes into the strains, similar phenotypes are generated such as high c-di-

GMP strains show decreased swimming and swarming motility. Thus, we assume that at metabolic level, there should be also certain degree of similarity across different bacteria species that regulated by c-di-GMP.

Therefore, here in this project, comparative transcriptomic profiling of mutants expressing different levels of c-di-GMP of three Gram-negative pathogens, *P. aeruginosa* PAO1, *B. cenocepacia* H111 and *K. pneumonia* KP-1, were carried out in order to find common c-di-GMP regulated genetic signatures across the three pathogens. As c-di-GMP is an important regulator of biofilm formation in all of these strains, the common genetic signature discovered may also functions as a common genetic marker of biofilms.

One common gene, *metE*, was identified to be upregulated in both *P. aeruginosa* and *B. cenocepacia* mutants expressing excessive c-di-GMP. However, same gene in *K. pneumonia* mutant was downregulated which indicated the difference and complexity of genetic regulation in different bacteria. Overall, this project provides the possibility and feasibility of identification common biofilm genetic markers across different pathogens by transcriptomics profiling.

2.1. Materials and methods

2.1.1. ABTG/ABTGC minimal media

1.5 mM of ammonium sulphate, 3.4 mM of sodium sulphate, 2.2 mM of potassium dihydrogen phosphate, 5 mM of sodium chloride, 1mM of magnesium chloride hexahydrate, 100 µM of calcium chloride anhydrous, 1 µM of iron chloride, 0.2%

glucose was dissolved in 1L of Milli-Q water. [228] ABTGC medium was prepared with ABTG minimal medium supplemented with 2 grams of casamino acids per liter.

2.1.2. Plasmid transformation

P. aeruginosa, *B. cenocepacia* and *K. pneumoniae* were inoculated from overnight culture and grown to mid-log phase (OD₆₀₀ 0.3-0.4) in 10 mL of LB media at 37°C with 200 rpm shaking. Cells were harvested by centrifugation at 4°C and washed twice with ice-cold 300 mM sucrose. Cells were then resuspended in 100 µl of ice-cold 300 mM sucrose and kept on ice for 30 mins before electroporation. 50 µl of cells were mixed with 5 µl of either *YedQ*₂ plasmid or *YhjH* plasmid and loaded to pre-chilled electroporation 0.2 cm cuvette. Electroporation was carried out with the conditions of 25 uF, 200Ω and 18kV/cm. The electroporated cells were then recovered in 1 mL LB for 1 hour at 37°C with 200 rpm shaking. The recovered cells were plated on LB agar plates with respective antibiotics, gentamicin (60 µg/mL for *P. aeruginosa*, 120 µg/mL for *B. cenocepacia* and 20 µg/mL for *K. pneumoniae*), and tetracycline (60 µg/mL for *P. aeruginosa*, 120 µg/mL for *B. cenocepacia* and 20 µg/mL for *K. pneumoniae*).

2.1.3. Strains and cultural conditions

For transcriptomics, c-di-GMP mutants of three different Gram negative bacteria were included, being *P. aeruginosa* PAO1, *K. pneumoniae* KP-1 and *B. cenocepacia* H111, respectively. All strains used here are listed in Table 4.1.

P. aeruginosa/pYhjH and *P.aeruginosa/pYedQ2*, *K. pneumoniae/pYhjH* and *K. pneumoniae/pYedQ2*, *B. cenocepacia/pYhjH* and *B. cenocepacia/pYedQ2*, were recovered at 37°C on LB agar plate. Single colony was inoculated into fresh LB medium [229] with antibiotics and incubated for overnight at 37°C, 200 rpm shaking. The overnight liquid cultures were diluted in ABTG medium to OD₆₀₀ ~0.1 and cultured to respective early stationary phase. 60 µg mL⁻¹ gentamicin (GM) and 60 µg mL⁻¹ tetracycline were supplemented to media for plasmid maintenance when necessary.

Duplicates of each strain at early stationary phase were cultured. 0.4-0.8 mL of each culture were collected and mixed immediately with 2 to 4 volumes of RNeasy Protect® Bacteria Reagent (Qiagen). After 5 to 20 minutes of incubation at room temperature, samples were centrifuged at 10,000 g for 5 min at 4°C for pellet isolation. Collected pellets were then kept until use at -80 °C.

2.1.4. Sample preparation for RNA-sequencing transcriptome analysis

Total RNA was purified using miRNeasy Mini Kit (Qiagen). RNase-free DNase Set (Qiagen) was applied to remove DNA contaminant through on-column DNase digestion. The integrity and concentration of total RNA and DNA contamination were examined by an Agilent 2100 Bioanalyzer (Agilent Technologies) and Qubit® 2.0 Fluorometer (Invitrogen). Analysis of gene expression was carried out via Illumina RNA sequencing (RNA-Seq). Libraries were produced using an Illumina TruSeq RNA Sample Prep Kit. The libraries were sequenced using the Illumina HiSeq 2500 platform with a paired-end protocol and read lengths of 100 nt. Gene annotation was done using either GO annotation or RAST annotation. [230-232]

Analysis of the RNA-seq data was performed as previous described. [233] Briefly, the sequence reads were mapped onto their respective genome using the “RNA-Seq and expression analysis” application of CLC genomics Workbench 6.0 (CLC Bio, Aarhus, Denmark). The transcript count table was subjected to DESeq package [234] of R/Bioconductor [235] for statistical analysis. The transcript counts were normalized to the effective library size. The differentially expressed genes were identified by performing a negative binomial test. Transcripts were stringently determined as differentially expressed when having a fold change larger than 4 and an adjusted p-value smaller than 0.05. Hierarchical clustering analysis was performed and a heatmap was drawn for the differentially expressed genes, using heatmap.2 package of R/Bioconductor. [235] Principle component analysis (PCA) was plotted using online web tool provided on <http://biit.cs.ut.ee/clustvis/>. [236]

2.1.5. Quantitative reverse transcriptase PCR (qRT–PCR) analysis

RNA samples of *P. aeruginosa*/pYhjH, *P. aeruginosa*/pYedQ2, *B. cenocepacia*/pYhjH and *B. cenocepacia*/pYedQ2 were prepared as described in the previous paragraph. RNA of three biological replicates of each mutant was collected while gene expression level of three technical replicate of each biological replicates were measured. Primer sequences could be found from Table 4.1 while primer efficiency is illustrated in Figure 7.3.2. KAPA SYBR®FAST qPCR kit (KAPAbiosystems) were used for qPCR analysis. StepOnePlus™ Real-Time PCR Systems was used to run qRT-PCR programs. *gyrB* and *trpB* genes were used as housekeeping genes in *B. cenocepacia* mutants [237], while *gyrB* and *rpoD* genes were used as housekeeping genes in *P. aeruginosa* mutants.

2.2. Results

In order to discover the variation in gene expression under the influence of increased c-di-GMP content in *P. aeruginosa*, *B. cenocepacia* and *K. pneumoniae* respectively, transcriptomics profiles of *pYedQ2* and *pYhjH* mutant pair of each bacteria were compared and analysed. Differently expressed genes in *P. aeruginosa* antithetical mutant pair were annotated by GO annotation and matching on *Pseudomonas* website. Those of *B. cenocepacia* antithetical mutant pair and *K. pneumoniae* antithetical mutant pair were annotated by RAST annotation and matching with NCBI reference genomes.

2.2.1. Strain construction and validation

The plasmids containing *yedQ* or *yhjH* gene were transformed into each pathogen constructing a pair of mutants with opposite c-di-GMP synthesising capability (Figure 2.1). *YedQ* gene encodes for a diguanylate cyclase catalysing c-di-GMP biosynthesis whereas *yhjH* gene encodes for a phosphodiesterase for c-di-GMP degradation. [226, 238] As these genes are connected to a *lac* promoter, continuous gene expression maintains the level c-di-GMP consistently in the cells.

Figure 2.1 Construction of c-di-GMP mutants of all three pathogens. A. DGC-encoding *yedQ* gene was transformed into each strain creating an excessive

pool of c-di-GMP; B. PDE-encoding *yhjH* gene was transformed into each strain breaking c-di-GMP into GMPs.

To validate c-di-GMP concentrations in each mutant, intracellular c-di-GMP was extracted from each mutant by the approach described in the following chapter. As c-di-GMP is expressed in picomolar to nanomolar range intracellularly, LC/MS which provides great sensitivity down to nanomolar range is an ideal approach for analysis of these trace metabolites.[239-243]

With commercial c-di-GMP standard provided, targeted quantification of intracellular c-di-GMP was easily quantified by LC/MS to validate the differential expression of c-di-GMP induced by either *pYedQ2* or *pYhjH* gene (Table 2.1). Absolute c-di-GMP concentration was calculated by normalization with protein quantity. Significant difference can be seen between antithetical mutant pair of each pathogen. *P. aeruginosa/pYedQ2* produces almost 90 times more c-di-GMP than *P. aeruginosa/pYhjH* (Figure 2.2A). *B. cenocepacia/pYedQ2* produces around 74 times more than *B. cenocepacia/pYhjH* (Figure 2.2B). Although *K. pneumoniae/pYedQ2* produces relatively lower concentration of c-di-GMP comparing to the other two bacteria, it still expressed much more of it than *K. pneumoniae/pYhjH* which synthesizes non-detectable level of c-di-GMP (Figure 2.2C).

Strains	Amount of c-di-GMP (pmol/mg protein)
<i>P. aeruginosa/pYedQ2</i>	295.38
<i>P. aeruginosa/pYhjH</i>	3.32
<i>K.pneumoniae/pYedQ2</i>	1.26

<i>K.pneumoniae/pYhjH</i>	Non-detectable
<i>B.cenocepacia/pYedQ2</i>	134.67
<i>B.cenocepacia/pYhjH</i>	1.82

Table 2.1 C-di-GMP concentrations of all mutants measured by LC/MS. All concentrations (in picomole) are mean values of triplicates normalized by total protein (in milligram) of 1 ml cells. All mutants carrying *pYedQ2* plasmid generated significantly higher level of c-di-GMP comparing to mutants carrying *pYhjH* plasmid.

Figure 2.2 C-d-GMP concentrations in mutants of three pathogens. A. c-di-GMP concentration in *P. aeruginosa/pYedQ2* and *P. aeruginosa/pYhjH*; B. c-di-GMP concentration in *B. cenocepacia/pYedQ2* and *B. cenocepacia/pYhjH*; C.

c-di-GMP concentration in *K. pneumoniae/pYedQ2* and *K. pneumoniae/pYhjH*. Mean values of triplicates were calculated with standard deviation.

Beside the change in c-di-GMP level, there are phenotypic changes of the strains observed. High c-di-GMP strain of *P. aeruginosa* produces more aggregates in liquid culture and smaller colonies on agar plate. Both low c-di-GMP strains of *P. aeruginosa* and *B. cenocepacia* show greater capability in swarming (average zone sizes = 12.9 mm and 21.3 mm) and swimming (average zone sizes = 27.2 mm and 21.6 mm) compare to swarming (average zone sizes = 2.0mm and 2.3 mm) and swimming (average zone sizes = 15.5 mm and 13.3 mm) of their high c-di-GMP strains. All zone sizes are mean values of triplicates.

2.2.2. Statistical analysis of transcriptomic data of *P. aeruginosa* mutants

Prior to analyse differentially expressed genes, integrity of RNA-seq data were validated by performing quality-control tests including box plot and Principle Component Analysis (PCA). Similarity in the means of normalized gene expression values between duplicates of either *P. aeruginosa/pYedQ2* strain or *P. aeruginosa/pYhjH* strain illustrated by box plot indicated good reproducibility of the duplicates. (Figure 2.3)

Figure 2.3 Box plot of genes regulated in *P. aeruginosa/pYedQ2* and *P. aeruginosa/pYhjH*. No outliers were seen; negligible variations were seen from the maximum, minimum and median of the transcriptomics data between mutants and between replicates of each mutant. n=2.

On PCA plot (Figure 2.4), each dot represents the total transcripts of one sample. Principle Component 1 accounts for 67.6% of total variance where Principle Component 2 accounts for 21.6 of total variance.

Figure 2.4 PCA plot of genes in *P. aeruginosa/pYedQ2* and *P. aeruginosa/pYhjH* mutants. Fold change ≥ 4 , p-value ≤ 0.05 , n=2

Clear separation could be seen between replicates of each mutant along PC1 (67.6% of total variance). *P. aeruginosa/pYedQ2* replicates showed greater variation along PC2 while *P. aeruginosa/pYhjH* replicates showed greater variation along PC1. Replicates of *P. aeruginosa/pYhjH* mutant were more spreaded over PC1 while replicates of *P. aeruginosa/pYedQ2* mutants were more spreaded over PC2. Proximity between two replicates of same mutant indicated low variation in the transcriptomics profile of the duplicates. Clear separation between the locations of replicates of two mutants on the graph inferred the difference in transcriptional expression of two mutants under the influence of regulated c-di-GMP concentration. Genes expressed differentially in two mutants were visualized by heatmap plotted with Z-scores. (Figure 2.5)

Figure 2.5 Heatmap of genes regulated in *P. aeruginosa/pYedQ2* and *P. aeruginosa/pYhjH*. Similar pattern could be seen between replicates while distinct difference in pattern could be seen between mutants. Fold change ≥ 4 , p-value ≤ 0.05 , n=2. Brown: downregulated genes; Blue: upregulated genes.

Parallel comparison took place between each mutant pair to find c-di-GMP regulated genes while vertical comparison among same mutant of different bacteria achieved the aim of identification of common c-di-GMP regulated genes. With fold change ≥ 4 and p-value ≤ 0.05 as the selection criteria, 57 genes were upregulated (Table 7.1.1) while 65 genes were downregulated in *P. aeruginosa/pYedQ2* (Table 7.1.2). Among all upregulated genes in *P. aeruginosa/pYedQ2*, most of them are involving in fimbriae biosynthesis and virulence. For genes involving in fimbriae biosynthesis, five members of fimbrial-related *cupA* operon, *cupA1-5*, upregulated for at least 50 times, as well as 2 *cupA* gene regulators, *cgrA* and *cgrC*, boosted up for 4.9 and 6.5 folds respectively, under the influence of high c-di-GMP level. Expressions of two lectin

encoding genes, *lecA* and *lecB*, were increased for 5.6 folds and 4.7 folds. Besides, *cdrA* was also upregulated for 2.5 folds.

For genes involving in generation of virulence factors, expression of *phzA2*, *phzB2* and *phzC2* encoding for phenazine biosynthesis proteins were increased for at least 8 folds. Genes of type III secretion system including *popB* encoding for translocator protein PopB; *popD* encoding for outermembrane translocator PopD precursor protein; *popN* encoding for outermembrane protein for Type III secretion; *pcrG* encoding for Type III regulator; *pcrV* encoding for type III secretion protein PcrV; PA1697 encoding for ATP synthase in type III secretion system were upregulated for 4 to 8 times. Transcription of *spcS*, *pcrH*, *exsC* encoding for chaperones were boosted up significantly. *pelC* gene which contributes to biosynthesis of exopolysaccharide, an important component of extracellular polymeric matrix, was also highly activated.

Other genes upregulated in *P. aeruginosa/pYedQ2* mutant including *pfpI*, an anti-mutator gene; PA2133 encoding for cyclic-guanylate-specific phosphodiesterase; *pcrI* and *pcr3* genes; *bapF* encoding for beta-peptidyl aminopeptidase; *osmC* encoding for osmotically inducible protein OsmC; *lptF* encoding for Lipotoxon F, LptF; *exsB* and *exsC* encoding for exoenzyme S synthesis protein B and exoenzyme S synthesis protein C precursor; *dhcA* encoding for dehydrocarnitine CoA transferase subunit A, DhcA; and *aphA* encoding for acetylpolyamine aminohydrolase.

Most attention-attracting finding here is the significant upregulation of *metE* gene which encoding for an enzyme, 5 methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase. This enzyme acts in the final reaction of methionine synthesis

pathway which leads to the formation of L-methionine. Upregulation of *metE* gene was also seen in *B. cenocepacia/pYedQ2* strain which infers its possibility to be a common biomarker between these two bacteria.

Most of the downregulated genes in *P. aeruginosa/pYedQ2* are involved in motility, surface attachment, chemotaxis, and virulence factors synthesis. For genes involving in motility and virulence, two component system, *fleSR*, positively regulates the expression of genes involving in flagellar biosynthesis were underexpressed. [244] With downregulated *fleSR*, expression of flagellar genes were highly reduced including genes encoding flagellar structures, *flgBCDFGHJKLM*; genes encoding for flagellar biosynthesis proteins, *flhA* and *flhF*; flagellin encoding genes, *fliCDEFI*. Previous study demonstrated that level of c-di-GMP exhibited very slight modulating effect on the expression of *fleR*. [77] However, our RNA-seq results reached a conclusion that with elevated level of c-di-GMP, expression of *fleR* gene is significantly reduced up to more than 9 folds in *P. aeruginosa/pYedQ2* under experimental conditions in this study. C-di-GMP repressed transcription of *amrZ* which encodes for alginate and motility regulator Z. This gene was found to inhibit Psl biosynthesis. [245]

A nearly 10 folds of reduction in the transcription of *motY* gene that contributes to swimming motility was seen. Interestingly, *gcbA* which encodes for a DGC protein expressed at reduced level. This diguanylate cyclase was demonstrated to enhance the formation of biofilm in *P. aeruginosa* through modulating swimming motility. [246] It was then proved by same group that it possesses biofilm-dispersing capability which overwrites its DGC function in a concentration dependent manner of c-di-GMP. [247]

For genes involving in surface attachment, type IV fimbrial precursor *pilA* was also transcribed at decreased level with elevated c-di-GMP concentration. Psl and pyoverdine operon regulator, *ppyR*, downregulated for almost 6 times, which lead to the repression of *psl* genes even with downregulation of *amrZ* gene. Induction of *ppyR* gene promotes biofilm formation by regulating Psl and pyoverdine synthesis. As *ppyR* is transcribed together with *fhp* and *nnrS* as an operon [248], transcription of *ppyR* reduced with down-regulation of *fhp* even with expression of elevated c-di-GMP. However, the regulation of *ppyR* is still remaining unknown.

Bacteraoferritin involved in iron metabolism, *bfrB*, downregulated for more than 35 folds, while flavohemoprotein, *fhp*, was reduced up to nearly 14 folds. Fhp was shown to be regulated by three genes, *fhpR*, *asrA* (PA0779) and PA3697 as demonstrated by Koskenkorva and coworkers. [249] As *asrA* positively regulates the expression of *fhp* and level of *asrA* transcripts decreased for more than 3 folds in *P. aeruginosa/pYedQ2*, reduction in *fhp* can be explained. Furthermore, underexpression of *asrA* lead to downregulation of heat shock proteins [250], this explained the reduction in transcriptional level of genes carrying heat shock proteins, *grpE* and *htpG*.

Other genes downregulated including *bexR*, a bistable expression regulator, reduced its expression up to more than 4 folds; *himD* encoding for integration host factor beta subunit; PA2652, PA2654 and PA2788 encoding for methyl-accepting chemotaxis protein and probable chemotaxis transducer; and *pctB* encoding for chemotactic transducer PctB.

2.2.3. Statistical analysis of transcriptomic data of *B. cenocepacia* mutants

Good reproducibility of replicates was seen from similarity between sample means illustrated in box plot. (Figure2.6)

Figure 2.6 Box plot of genes regulated in *B.cenocepacia/pYedQ2* and *B.cenocepacia/pYhjH*. No outliers were seen; negligible variations were seen from the maximum, minimum and median of the transcriptomics data between mutants and between replicates of each mutant. n=2.

PC1 represents 79.1% of total variance while PC2 accounts for 13.4% of total variance. PC1 and PC2 account for almost all of the total variance of the samples. Clear separation can be seen between the replicates of *B. cenocepacia/pYedQ2* mutants and those of *B. cenocepacia/pYhjH* mutants. Two replicates of *B. cenocepacia/pYedQ2*

mutants exhibited a greater variation along PC1 while replicates of *B. cenocepacia/pYhjH* exhibited greater variation along PC2. (Figure 2.7)

Figure 2.7 PCA plot of genes in *B.cenocepacia/pYedQ2* and *B.cenocepacia/pYhjH* replicates. Clear separation could be seen between replicates of each mutant along PC1 (79.1% of total variance). *B.cenocepacia/pYedQ2* replicates showed greater variation along PC2 while *B.cenocepacia/pYhjH* replicates showed greater variation along PC1. Fold change ≥ 4 , p-value ≤ 0.05 , n=2

Similar to *P. aeruginosa/pYedQ2*, genes up- or down- regulated in *B. cenocepacia/pYedQ2* were picked with the selection criteria of at least 4 folds change and p-value less than 0.05. Transcription of 57 genes was highly induced (Table 7.1.3) while expression of 97 genes was significantly repressed in this mutant (Table 7.1.4). Genes regulated for more than 4 folds and less than 4 folds were illustrated using heatmap. (Figure 2.8)

Figure 2.8 Heatmap of genes regulated in *B. cenocepacia/pYedQ2* and *B. cenocepacia/pYhjH*. Similar pattern could be seen between replicates while distinct difference in pattern could be seen between mutants. Fold change ≥ 4 , p-value ≤ 0.05 , n=2. Brown: downregulated genes; Blue: upregulated genes.

Most of genes upregulated belongs to few function groups including surface appendages (fimbriae and pili), oxidases in aerobic respiration chain, transcriptional regulators, type II/IV secretion systems and dehydrogenases. For surface appendages, *fimA* encoding for Type I fimbriae major subunit was upregulated for almost 10 folds under the regulation of elevated c-di-GMP, as well as I35_1589 and I35_1590, the genes encoding for fimbriae usher protein StfC and periplasmic fimbrial chaperone StfD. Both of *pilA* and *pilA_2* were upregulated for more than 100 folds and thus lead to increase in translation of pilus assembly protein, pilin Flp. Other pilus related genes overexpressed including I35_7316 encoding for Flp pilus assembly protein, pilin Flp;

I35_7317 encoding for Type IV prepilin peptidase TadV/CpaA; I35_7319 encoding for Flp pilus assembly protein RcpC/CpaB, I35_7327 encoding for Flp pilus assembly protein TadB; I35_7323 and I35_7324 encoding for proteins similar to TadZ/CpaE, which associated with Flp pilus.

For oxidases in aerobic respiration chain, *cyoA*, *cyoB* and *cyoC* which are encoding for cytochrome O ubiquinol oxidase subunits I, II, III, respectively, were all upregulated under the regulation of c-di-GMP for 8 to 10 folds. Other genes relevant to cytochromes include I35_6568 and I35_6569 encoding for putative Cytochrome bd2, subunit I and subunit II, respectively, which upregulated for 28-30 folds.

For transcriptional regulators, *uspA* in CRP family was upregulated for almost 14 folds; I35_4027 in PadR family were elevated for 7 folds. For dehydrogenases, glycolate dehydrogenase encoding genes, *glcD_2*, *glcE_2*, *glcF_2* and *glcG* were increased for 4 to more than 50 folds; *ykgE*, *ykgF* and *ykgG* genes encoding for putative L-lactate dehydrogenase Fe-S oxidoreductase subunit, putative L-lactate dehydrogenase Iron-sulfur cluster-binding subunit and putative L-lactate dehydrogenase hypothetical protein subunit, respectively, were elevated for 7 to more than 20 folds. In addition, *glcA* encoding for L-lactate permease was also upregulated for almost 9 times.

For genes involving type II/IV secretion systems, I35_7317 encoding for Type IV prepilin peptidase TadV/CpaA; I35_7320 encoding for Type II/IV secretion system secretin; I35_7325 encoding for Type II/IV secretion system ATPase; I35_7326 encoding for Type II/IV secretion system ATP hydrolase; and I35_7328 encoding for Type II/IV secretion system protein, were overexpressed for 12 to more than 40 times.

Conversely, genes downregulated in *B. cenocepacia/pYedQ2* mutants mostly including those related to lectin expression, transcriptional regulator, flagellar biosynthesis, and chemotaxis. For genes involved in lectin expression, *bclA* and *bclC* were deactivated by overexpression of c-di-GMP for 4 to 9 folds. For transcriptional regulator, *cepS* in AraC family was down-regulated for more than 4 folds.

Similar to that of *P. aeruginosa/pYedQ2* mutant, the ability to synthesize flagellar-forming subunits in *B. cenocepacia/pYedQ2* were significantly impaired through downregulation of flagellar-associated genes. These genes involving in flagella biosynthesis include *motA_1* and *motB_1* encoding for flagellar motor rotation protein MotA and MotB; *flgK*, *flgL* and *fliD1* encoding for flagellar hook-associated protein FlgK, FlgL and FliD; *flgM* encoding for negative regulator of flagellin synthesis; *flgN*, *fliC*, *fliS* and *fliT* encoding for flagellar biosynthesis protein FlgN FliC, FliS and FliT. Folds reduced in these genes ranged from 5 to 10 times.

For chemotaxis related genes, *cheB_1* encoding for chemotaxis response regulator protein-glutamate methyltransferase CheB; *cheD* encoding for chemotaxis protein CheD; *cheR* encoding for chemotaxis protein methyltransferase CheR; *cheW* encoding for positive regulator of CheA protein activity; *cheY1* encoding for chemotaxis regulator-transmits chemoreceptor signals to flagellar motor components CheY; I35_0136 encoding for Signal transduction histidine kinase CheA; I35_2905, I35_5428 and I35_5683 encoding for methyl-accepting chemotaxis protein I (serine chemoreceptor protein); I35_5857 and I35_6270 encoding for methyl-accepting chemotaxis protein; were repressed for at least 5 folds.

2.2.4. Statistical analysis of transcriptomic data of *K. pneumoniae* mutants

Good reproducibility was also seen between replicates of *K. pneumoniae* mutants by plotting the quality controlling box plot (Figure 2.9).

Figure 2.9 Box plot of genes regulated in *K. pneumoniae/pYedQ2* and *K. pneumoniae/pYhjH*. No outliers were seen; negligible variations were seen from the maximum, minimum and median of the transcriptomics data between mutants and between replicates of each mutant. n=2

Difference in gene expression between mutants was illustrated on PCA plot (Figure 2.10). For *K. pneumoniae* mutants, PC 1 accounts for 42.3% of total variance where PC2 accounts for 39.8% of total variance.

Figure 2.10 PCA plot of genes in *K. pneumoniae/pYedQ2* and *K. pneumoniae/pYhjH* mutants. Clear separation could be seen between replicates of each mutant along both PC. *K.pneumoniae/pYhjH* replicates showed greater variation along both PC. Fold change ≥ 4 , p-value ≤ 0.05 , n=2.

Two replicates of *K. pneumoniae/pYhjH* mutants varied to a greater extent along both PC1 and PC2 comparing to those of *K. pneumoniae/pYedQ2* mutants. Clear separation of replicates of different mutants along PC1 indicated difference in gene expression of the two mutants. Those significantly regulated genes were visualized by heatmap. (Figure 2.11)

Figure 2.11 Heatmap of genes regulated in *K.pneumoniae/pYedQ2* and *K.pneumoniae/pYhjH*. Similar pattern could be seen between replicates while distinct difference in pattern could be seen between mutants. Fold change ≥ 4 , $p\text{-value} \leq 0.05$, $n=2$. Brown: downregulated genes; Blue: upregulated genes.

Among those genes, 12 genes were found to be increasingly expressed (Table 7.1.5) while 37 genes were expressed at highly repressed level in *K.pneumoniae/pYedQ2* (Table 7.1.6). *HisG* encoding for ATP phosphoribosyltransferase were overexpressed for more than 5 folds under regulation of elevated c-di-GMP. Two transcriptional regulators, encoding by KLP1_06700 and KLP1_14575, were upregulated for 248 folds and 5 folds.

Other upregulate genes include KLP1_04290 encoding for 1-(5-phosphoribosyl)-5-((5-phosphoribosylamino) methylideneamino) imidazole-4-carboxamide isomerase;

KLP1_04295 encoding for Imidazole glycerol phosphate synthase cyclase subunit; KLP1_04300 encoding for phosphoribosyl-ATP pyrophosphatase; KLP1_06690 encoding for integrase; KLP1_06705 encoding for DNA cytosine methyltransferase; DNA cytosine methyltransferase encoding for relaxase; KLP1_14580 encoding for ribose ABC transporter permease; KLP1_15410 encoding for anaerobic ribonucleoside triphosphate reductase and KLP1_22450 encoding for stress-induced protein.

Conversely, genes under-expressed with elevated concentrations of c-di-GMP in *K. pneumoniae* cells were mostly involved in autoinducer related protein synthesis, ABC transporter biosynthesis, glucose transporters, transcription regulators, and heat shock proteins. For autoinducer related genes, KLP1_09505 encoding for autoinducer-2 (AI-2) modifying protein LsrG; KLP1_09515 encoding for AI-2-binding protein lsrB; KLP1_09520 encoding for AI-2 import system permease LsrD; and KLP1_09535 encoding for transcriptional regulator LsrR, were reduced for at least 4.7 folds. Moreover, AraC family transcription regulator encoded by KLP1_16265/22420 was also expressed at a significantly reduced level.

For ABC transporter biosynthesis, KLP1_09525 encoding for ABC transporter permease; KLP1_09530 encoding for ABC transporter ATP-binding protein; KLP1_02210 encoding for spermidine/putrescine ABC transporter permease; and KLP1_02215 encoding for iron ABC transporter substrate-binding protein were repressed for at least 6 folds.

For genes involved in glucose transporter, KLP1_00195 encoding for PTS glucose transporter subunit IIBC and KLP1_12520 encoding for PTS alpha-glucoside transporter subunit IICB were underexpressed for 4.2 and 33 folds. For genes involving in heat-shock protein synthesis, significant downregulation of KLP1_12540 encoding for heat-shock proteins IbpB and KLP1_12545 encoding for heat-shock protein IbpA were observed.

Reduction in transcription was also observed in other genes including *gcvT* encoding for glycine cleavage system protein T; *lacY* encoding for galactoside permease; KLP1_03135 encoding for cell envelope biogenesis protein OmpA; KLP1_00190/12515 encoding for 6-phospho-beta-glucosidase and 6-phospho-alpha-glucosidase; KLP1_02425 encoding for shikimate 5-dehydrogenase; KLP1_02430 encoding for D-galactonate transporter DgoT; KLP1_02435 encoding for 4-hydroxyphenylpyruvate dioxygenase; KLP1_08770 encoding for cupin; KLP1_13065 encoding for alpha-galactosidase; KLP1_16920 encoding for malate permease; KLP1_18000 encoding for carbon starvation induced protein; KLP1_22400 encoding for gamma-glutamylputrescine synthetase; and KLP1_24060 encoding for 3-oxoacyl-ACP reductase.

Most attention-attracting difference is the repression of KLP1_02575/KLP1_02575 both encoding for 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase, the enzyme catalysing final reaction in L-methionine synthesis. This result is totally opposite to the case of *P. aeruginosa/pYedQ2* and *B. cenocepacia/pYedQ2*, where *metE* expression was significantly induced. This gene found upregulated commonly in *P. aeruginosa/pYedQ2* and *B. cenocepacia/pYedQ2*, encodes for the

2.2.5. qRT-PCR validation of *metE* expression in PAO1 and H111 mutants

To validate the expression level of *metE* gene in *P. aeruginosa* and *B. cenocepacia* mutants, quantitative RT-PCR analysis was carried out to measure relative expression level of *metE* in these mutants using housekeeping genes as reference. *rpoD* and *gyrB* were used as housekeeping genes of *P. aeruginosa* while *trpB* and *gyrB* were used as housekeeping genes of *B. cenocepacia*. Sequences of primers of housekeeping genes used are listed in (Table 4.1). Results indicated that *metE* expression was almost 5 times higher in *B. cenocepacia/pYedQ2* mutant than in *B. cenocepacia/pYhjH* mutant (Figure 2.13B). More significantly, *metE* expression in *P. aeruginosa/pYedQ2* mutant upregulated for around 40 folds comparing to that in *P. aeruginosa/pYhjH* mutant (Figure 2.13A).

Figure 2.13 qPCR measurements of expression of *metE* gene in two different pathogens. A. Expression of *metE* gene in *P. aeruginosa* mutants. *metE* expression in *P. aeruginosa/pYhjH* was taken as reference. Mean of triplicates were taken with standard deviation. B. Expression of *metE* gene in *B.*

cenoepectia mutants. *metE* expression in *B. cenoepectia/pYhjH* was taken as reference. Mean of triplicates were taken with standard deviation.

2.3. Discussion

The three pathogens tested in this study are of the top infectious bacteria in nosocomial infections, catheter-associated infections, respiratory infections, and especially in the chronic lung infections of CF patients. There is always association between the development of such chronic infections and bacterial biofilm formation. The initiation of these bacterial biofilms requires the attachment of cells to a substrate and further regulation for establishment of mature biofilm. The establishment of such biofilms are under control of several factors such as quorum sensing, sRNA and c-di-GMP. Quorum sensing systems such as AHL (*las*, *rhl*) and PQS in *P. aeruginosa*, AHL (*cep*, *cciI*) and BDSF in *B. cenoepectia*, and AI-2 in *K. pneumoniae* play major part in virulence and biofilm formation in these pathogens. C-di-GMP functions as a global regulator of biofilm formation in these bacterial species. This transcriptomics study focuses on discovery of common genes under c-di-GMP regulation among all the three pathogens tested.

2.3.1. Transcriptomics analysis of *P. aeruginosa* mutants

2.3.1.1. Analysis of surface appendage relevant genes in *P. aeruginosa* mutants

From the results obtained from RNA-seq analysis, the most regulated cellular functions by excessive c-di-GMP include flagellar and non-flagellar surface appendages, synthesis of exopolysaccharides and virulence in *P. aeruginosa*; flagellar

and non-flagellar surface appendages, virulence and quorum sensing in *B. cenocepacia*; and quorum sensing in *K. pneumoniae*.

Gram negative bacteria adapt five different pathways for assembly of extracellular pili structure, including chaperone-usheer pathway (*cup*), biosynthesis of curli, formation of type IV pili, assembly of type III secretion needle and formation of type IV secretion pili. [253] In *P. aeruginosa*, extracellular filamentous structures like flagellum, *cup* fimbriae, and type IV fimbriae play important role for surface adherence. [122]

Upregulation of c-di-GMP alters these surface appendages in different ways in *P. aeruginosa* in order to approach to and attach to the surface for biofilm formation. Genes encoding for flagellar components were downregulated while genes encoding for non-flagellar surface appendages were upregulated in *P. aeruginosa* under the regulation of c-di-GMP.

Expression of flagellar by *P. aeruginosa* cells serves as a double-edged sword which facilitates cell motility and also acts as a ligand for recognition by host cells. Flagellar is required and is essential in establishing infection in lungs and respiratory tracts, especially in CF patients.

Previous study demonstrated that the capability of *P. aeruginosa* causing pneumonia and long-term infection in CF patients was significantly reduced without the expression of *fliC*, the gene encoding for flagellin. [254, 255] Moreover, ability of spreading of the infection in pneumonia lungs was also impaired in *fliC* mutant. *P. aeruginosa* cells adopted such mutation in escaping from clearance by macrophages

and adapting to the environment in either pneumonia patients or CF lungs for chronic infection. [254, 255] During chronic infection associated with *P. aeruginosa* in CF patients, *fliC* was inactivated by a mechanism that is independent from any known flagella-regulating network. [255] Inactivation of *fliC* by c-di-GMP indicated that active *fliC* expression is required for surface approaching while it is silenced upon formation of biofilm.

Movement of flagellum in *P. aeruginosa* cells is driven by rotary motors encoded by 5 genes, *motAB*, *motCD* and *motY*. MotAB serve as torque generator to rotate the flagella while *motCD* and *motY* contributes to motor function. Loss of any of these genes would impair swimming motility, swarming motility and translocation.[256] c-di-GMP was previously shown to interfere with localization of MotCD and impair swarming motility. [257] Here, transcriptomic analysis revealed that the expression of *motCD* (fold change = -3.15 and -2.33 respectively) and *motY* are also inhibited by c-di-GMP.

Downregulation of flagellum and flagellum relevant genes expression induced by c-di-GMP contributes to surface attachment, disease establishment and progression and resistance to host immune clearance in *P. aeruginosa* cells. *GcbA* encoding for a DGC, GcbA, in *P. aeruginosa* represses motility generated by flagellar by reducing flagellar reversal rate. GcbA contributes to the biosynthesis of excessive intracellular c-di-GMP and promotes longitudinal surface attachment but not required for biofilm formation. [246] As intracellular c-di-GMP biosynthesis is manipulated to express continuously upon transformation of plasmid carrying *yedQ* genes, there is abundance of c-di-GMP which inhibits the expression of *gcbA*. Similar phenomenon is seen from the

upregulation in gene expression of PA2133 encoding for a cyclic-guanylate-specific phosphodiesterase in *P. aeruginosa*/pYedQ2 cells in order to break down the excessive c-di-GMP formed.

Besides flagellar, *P. aeruginosa* possesses different types of non-flagellar surface appendages including *cup* fimbriae and type IV pili which also contribute to motility and surface attachment. The filamentous surface structure, type IV pili are essential for twitching motility and biofilm formation. It is the major adhesin for surface sensing in *P. aeruginosa* cells. Upon attaching with surface, signal will be transduced through Chp chemosensory system which regulates the expression of different virulence genes and cAMP. It works as a positive feedback signal transduction loop which enhances pili attachment further once attaching to an inert surface to initiate biofilm formation. PilA, which is the major subunit of type IV pilin, interacts with PilJ, the chemosensor, to activate this mechanochemical pathway to regulate production of virulence factor, motility and thus biofilm formation. [258] High c-di-GMP concentration inhibits the transcription of *pilA* gene in *P. aeruginosa* cells under the experimental conditions in this study.

Besides type IV pili, *cup* fimbria is also under the regulation by c-di-GMP in *P. aeruginosa* cells. *Cup* fimbriae consist of two parts, the periplasm chaperon protein subunits and the pore-forming, membrane-located usher protein. Chaperon promotes pilus-folding and translocate to usher which serves as assembly location. Usher then coordinates the assembly of pili and their subsequent release into extracellular space. [253, 259]

Previous works demonstrated the extensive involvement and regulation of *cup* gene clusters in the biosynthesis of *cup* fimbriae for surface attachment besides type IV pili. Three *cup* gene clusters were identified as *cupA*, *cupB* and *cupC* where *cupA* genes regulate the adhesive capability of bacterial cells to surfaces through fimbriae formation and explicitly relate to biofilm formation. *CupBC* play a relatively minor role comparing to *cupA*. [259, 260]

Expression of *cupA* genes are positively modulated by *cgrABC* regulators, which in turn repressed by *mvaT* and induced by *anr*. Such positive regulation of *cupA* genes by *cgr* genes was seen to display a phase-variable pattern in anaerobic environment. [261] *Cgr* genes locate at upstream of *cupA* genes while CgrC binds firstly to the promotor of *cupA* gene and recruit CgrA to the complex through direct binding with CgrC for initiation of *cupA* transcription. [262]

Expression of all 5 genes in *cupA* gene, *cupA1-5*, as well as the 2 *cupA* gene regulators, *cgrA* and *cgrC*, were highly upregulated in *P. aeruginosa/pYedQ2*. Previous study demonstrated that expression of *cupA* is influenced by the quantity of c-di-GMP available in *P.aeruginosa* cells. Increase in *cupA* fimbriae expression was seen simultaneously with elevated c-di-GMP biosynthesis in *P. aeruginosa* small colony variant (SCV). [73] The results obtained here perfectly matched with previous studies where the expression of *cupA* genes boosted up with elevated c-di-GMP expression. As *cgrA* and *cgrC* genes were also upregulated, c-di-GMP also exerts positive regulation of *cgrA* and *cgrC* genes.

Another group demonstrated that upregulation of two genes encoding functional GGDEF domain, PA1120 and *morA*, promotes the expression of *cupA* fimbriae in *P. aeruginosa*, and conversely upregulation of EAL domain encoding gene, *pvrR*, inhibits the expression of *cupA* fimbriae. [73] As EAL and GGDEF domains are responsible for the formation and degradation of intracellular c-di-GMP, the formation of *cupA* fimbriae is regulated by changes in the level of c-di-GMP. This validates the correctness of our results and also proves the genotypic changes when expression of c-di-GMP upregulates.

Besides *cup* fimbriae, the surface adhesin, lectin, also participates as a key factor in biofilm formation and initiating infection by *P. aeruginosa* cells through regulating cell interaction and attachment to host cells. Two sugar-binding lectins, LecA and LecB, were demonstrated by *in vivo* study to contribute to chronic *P. aeruginosa* lung infection by enhancing cell adhesion, increasing cytotoxicity and inducing barrier permeability of murine lung alveolar. [263]

They were also demonstrated to induce infection in human respiratory tracts, especially those with CF lungs. LecB locates on outer membrane through association with an outer membrane protein OprF and promotes bacterial biofilm formation. [264] Expression of LecB positively regulates twitching motility through mediating formation of type IV pili structure. [265]

The *rhl* autoinducer, C4-HSL, directly induced transcription of *lecA* gene while lectin expression would be delayed by the absence of *lasR*. [171] Under the experimental conditions of this study, transcription of both *lecA* and *lecB* genes were under positive

regulation of c-di-GMP. As the expression of these two lectins was also found to be under the positive regulation of both *rhl* quorum sensing systems and RpoS [171], it reflects an interlinking modulation of both quorum sensing and c-di-GMP on cell surface adhesion in *P. aeruginosa* cells.

2.3.1.2. Analysis of EPS relevant genes in *P. aeruginosa* mutants

Moreover, c-di-GMP exerts regulating activity on biosynthesis of exopolysaccharides in *P. aeruginosa* cells. Three different types of exopolysaccharides are produced in *P. aeruginosa* including Pel, Psl and alginate depending on non-mucoid or mucoid type of growth. These exopolysaccharides contribute to the structure of exopolymeric matrix of *P. aeruginosa* biofilms and results in the formation of cell aggregation.

Mutation in either *pel* or *psl* gene impaired biofilm forming capacity in *P. aeruginosa* cells. At beginning of biofilm attachment, Pel and Psl polysaccharides functions as adhesin to facilitate surface attachment. [8] *Pel* genes encode for an extracellular matrix component rich in glucose while *psl* genes encode for matrix component rich in mannose. The expression of either gene cluster enables the formation of mature biofilm. [84, 266] Mucoid cells taken from CF patients acquiring chronic *P. aeruginosa* infection produced excessive amount of alginate and diminished expression of Psl polysaccharide. [245] Correlation between c-di-GMP and Pel biosynthesis was discovered long ago. [267] The expression of *pel* genes were all upregulated with elevated level of c-di-GMP for excessive secretion of Pel polysaccharide. However, expression of *psl* genes was downregulated under the same conditions. Variation in the expression of *pel* genes may be regulated by several of other genes under the influence of increase in c-di-GMP.

Expression of *ppyR* gene, encoding for *psl* and pyoverdine operon regulator, is involved in exopolysaccharide production and pyoverdine synthesis, and biofilm formation in *P. aeruginosa*. Malfunction of *ppyR* causes downregulation of *psl* operon and pyoverdine genes. [268] The results obtained indicated that c-di-GMP negatively regulates *ppyR* gene expression where it downregulated under elevated c-di-GMP level. The reduction of *psl* genes found most probably due to reduction in *ppyR* gene. Moreover, *Fhp* gene and its regulator *fhpR* which serve as nitric oxide sensor and detoxifying system are also under regulation of *ppyR* and *nnrS* genes. [248, 249] Expression of *nnrS* was also downregulated in this study in high c-di-GMP PAO1 mutant, thus reduction in *fhp* was also seen. Moreover, underexpression of *ppyR* repressed transcription of *lasB* gene and *pqs operon* thus leads to inactivation of elastase synthesis and PQS quorum sensing system. Inversely, this gene promotes the reduction in both of swimming and swarming of *P. aeruginosa* cells. [268] Such results reflected the interlinking relationship between c-di-GMP and quorum sensing systems in *P. aeruginosa*.

Another gene, *amrZ*, regulates the expression of *pslA* gene in a negative manner by blocking its promoter. Conversely, *amrZ* activates the expression of alginate through upregulating *algD* and twitching motility to promote biofilm formation. [245] *AmrZ* also represses the expression of *ppyR* and other pyoverdine and pyochelin genes. This gene directly regulates the transcription of many genes for virulence including upregulating *algD* and *cdrA*; and downregulating chemotaxis *pctC*, flagellar genes, type VI secretion system, *rhlR* and rhamnolipid. [269] Downregulation of *amrZ* and *adcA* were seen in the presence of high level of c-di-GMP here. As *amrZ* represses the expression of *adcA* gene encoding for diguanylate cyclase in *P. aeruginosa*, it

indirectly reduces the biosynthesis of c-di-GMP. [269] As there is constant accumulation of c-di-GMP in the cells probably, expression of the diguanylate cyclase reduced which may result in the slight reduction in *amrZ* gene expression. C-di-GMP may exhibit its regulation on *ppyR* gene oppositely to that of *amrZ* as a dose-dependent competition, which may provide an explanation to the downregulation of *ppyR* and upregulation of *psl* gene with reduction of *amrZ* expression.

Besides *pel* and *psl* genes, alginate genes *algU* was also induced by c-di-GMP. AlgU functions as a sigma factor to induce alginate biosynthesis and *ppyR* gene expression. It also induces *psl* gene expression through *ppyR* gene, and contributes to twitching motility by indirectly enhancing expression of *lecAB*. [270] Thus, c-di-GMP has impact on the biosynthesis of all three exopolysaccharide to different extend with the influence of multiple other genes.

2.3.1.3. Analysis of virulence relevant genes in *P. aeruginosa* mutants

Virulence is another major factor regulated by c-di-GMP. Type III secretion associated genes were also upregulated by increased c-di-GMP concentration. The virulence of *P. aeruginosa* to host cells is largely mediated by its type III secretion system which consists of a hollow type III secretion needle directly injecting toxins into host cytoplasm. While type III secretion serves as a virulent factor invading host cells, it is in turn be recognized by host immune system. Structure of type III secretion machinery for toxin transportation is complicated and expression of T3SS is well-regulated. [271, 272]

Translocation of exoenzymes from *P.aeruginosa* cells to host cells by type III secretion system needs the presence of PcrV, PopB/D proteins. Expression of PcrG, PcrV and PopN repress biosynthesis of exoenzyme S (ExoS) and direct polarized translocation of effectors into host cells. [271, 273] PcrV is a component of type III secretion translocation apparatus which participates in pore formation and contact-dependent translocation and serves as a target for development of strategies against virulence of *P. aeruginosa*. [273, 274] Previous work had indicated that addition of synthetic c-di-GMP switches the activation from type III secretion system to type VI secretion system through action of WspR, RsmY and RsmZ. High c-di-GMP produced by WspR repressed expression of PcrV. [105]

However in this study, *Pcr* genes including *pcrG*, *pcrH* and *pcrV*; *pop* genes including *popB*, *popD* and *popN* and *spcS* gene were upregulated in by high concentration of c-di-GMP. These type III secretion genes in *P. aeruginosa* were triggered by c-di-GMP giving opposite effect to the results obtained by previous studies. This may due to specific intracellular localization of the pool of c-di-GMP generated by *yedQ* gene introduced. As previous study had demonstrated, different DGCs could give rise to localized c-di-GMP in proximity to its target. [67] Thus, c-di-GMP synthesized by *yedQ* gene may localize at intracellular space where it cannot exert its regulating function over T3SS.

2.3.1.4. Analysis of mutation relevant genes in *P. aeruginosa* mutants

P. aeruginosa cells tend to mutate spontaneously to adapt to its residential environment such as in the CF lungs while expression of mismatch repair genes such as *mutS* and *mutL* compensates such tendency of mutation or repairs DNA damage to

maintain the integrity of its genome. [275] Besides these anti-mutator gene, *pfpI* gene which belongs to DJ-1/ThiJ/PfpI superfamily, was proved to shield its genome by reducing rate of spontaneous mutation and protect DNA damage induced by oxidative stress. Expression of *pfpI* positively regulates bacteriophage Pf1 and negatively regulates iron metabolism. More importantly, *pfpI* gene plays a critical role in biofilm formation as the removal of *pfpI* lead to reduction in biofilm formed by *P. aeruginosa* PAO1 cells. It was claimed that biofilm maturation was closely related to the upregulation of bacteriophage Pf1. [254] Here in this study, RNA-seq results indicated that *pfpI* gene overexpressed in *P. aeruginosa* PAO1 mutant producing excessive amount of c-di-GMP. This shows a high possibility that *pfpI* function in biofilm formation is regulated by c-di-GMP.

Under the influence of excessive c-di-GMP, downregulation of *bexR* gene encoding for a bistable switch modulating the transcription of virulence genes was seen in *P. aeruginosa* cells. *BexR* is under regulation of a LysR-type transcription regulator and also self-regulated in a positive feedback loop for auto-upregulation. Expression of *bexR* leads to diverse expression of its target genes in subpopulation of *P. aeruginosa* cells to adapt to different environments. [276] This repression of *bexR* by c-di-GMP may be involved in regulating virulence through manipulating bistable expression regulator.

2.3.1.5. Analysis of c-di-GMP regulating genes in *P. aeruginosa* mutants

The final stage in biofilm formation is dispersion of biofilm cells. Dispersed cells were proved to be different from the cells from all other stages. [233] Dispersion requires

the expression of phosphodiesterases (PDEs) which catalyses the biodegradation of c-di-GMP and under the regulation of BdlA, a chemosensory protein. [247] C-di-GMP enhances expression of BdlA through post-translational modifications and cleavage during biofilm maturation stage. GcbA was proved to give great contribution to cleavage of BdlA after initial surface attachment, thus promotes biofilm dispersal to respond to various cues. GcbA functions depending on level of c-di-GMP and growth status, where repressed expression of c-di-GMP by phosphodiesterase leads to activation of GcbA expression. BdlA modulates activity of phosphodiesterase, DipA and RbdA, which are involved in biofilm dispersion and thus regulates c-di-GMP expression. [247]

Since the testing cells were manipulated to express c-di-GMP upon transformation of *yedQ* gene and were grown as planktonic cultures, they are different from biofilm cells while signals for biofilm dispersion may not be generated and received as cells kept growing with constant c-di-GMP production. Thus, expression of *dipA* and *rbdA* remains unchanged while *bdlA* gene expression was slightly downregulated, but significant reduction of *gcbA* gene expression was seen as there is excessive c-di-GMP accumulated in *P. aeruginosa* cells.

2.3.2. Transcriptomics analysis of *B. cenocepacia* mutants

Similar to that of *P. aeruginosa*, the expression of flagellar-related genes were downregulated in *B. cenocepacia* cells. Genes encoding for flagellar motor rotation proteins, *motA_I* and *motB_I* were also repressed to minimize flagellar activities. More interestingly, quorum sensing genes and virulence genes in *B. cenocepacia* were also repressed by c-di-GMP. Virulence factors in *B. cenocepacia* include ornibactin

siderophore, LPS, AidA, HtrA protease, type III/IV/VI secretion systems, ZmpA/B proteases, ShvR regulator, flagellar, fimbria/pili, OpcI outer membrane porin, MgtC and phenylacetic acid catabolic pathway. [204, 277-281] Many of these virulence factors are under regulation of quorum sensing systems. *B. cenocepacia* cells possesses two types of quorum sensing systems, N-acyl-homoserine lactone (AHL) and cis-2-dodecenoic acid (BDSF), to regulate gene to modulate motility, virulence and biofilm formation. [282]

All *Burkholderia* strains retain AHL quorum sensing system where some strains possess only CepI/R quorum sensing system utilizing C8-HSL as autoinducer and other strains accommodate multiple LuxI/R for AHL synthesis. [35] *CepI* encodes for majorly octanoylhomoserine lactone (OHL) and slightly hexanoylhomoserine lactone (HHL). *CepR* encodes for a transcriptional regulator whereas *cepR2* is another lux-like transcriptional regulator working independently and is able to function in the absence of AHL. *CepR* possesses regulatory capability on the expression of both *cepI* and *cepR2* genes. [283, 284] *CepR2* exhibits negative self-regulation and controls expression of several virulence factors. [203] *Cep* quorum sensing system is involved in the pathogenicity of *B. cenocepacia* H111 cells. [285]

Expression of *cepR2* and *cepS* genes was all found downregulated in the presence of high level of c-di-GMP in this study. *CepR* acts as a repressor and antagonist of *cepS* gene which encodes for AraC family transcriptional regulator to deactivate *cepS* expression. [284] *CepIR* quorum sensing also upregulates lectin-encoding genes *bclABC* and biofilm-promoting BapA protein. *BapA* gene was found to be under regulation of both AHL and BDSF quorum sensing systems with the cofunction of

bapR gene for achieving ultimate *bapA* transcription and thus boosting biofilm formation. [282]

The expression of *bapR* gene increased while that of *cepR2*, *cepS*, *bapA* and *bclABC* decreased with excessive c-di-GMP. As there was insignificant change in *cepIR* gene expression, variation in *bapA*, *cepS* and lectin encoding genes might due to the reduction in *cepR2* gene. As *bapR* is neither self-regulated nor regulated by quorum sensing [282], the increase in *bapR* expression is most probably due to c-di-GMP. Such results suggest that c-di-GMP and quorum sensing are interlinked with a complex regulatory system influencing many other cellular functions and especially for biofilm formation.

Besides the genes mentioned above, *cepR2* in *B. cenocepacia* H111 cells negatively regulates the expression of *zmpA*, *zmpB* and *aidA* gene for defence mechanism which is opposite to the regulation of *cepR* on the same genes. [203, 283, 284] However, there was reduction in *zmpA*, *zmpB* and *aidA* gene expression even though the expression of *cepR2* was decreased in the presence of excessive c-di-GMP in *B. cenocepacia* H111 cells. This suggests a possibility in which c-di-GMP also impose its regulation on these genes besides *cep* quorum sensing systems.

Secretion systems are other measures for virulence control in *B. cenocepacia* cells. Previous study demonstrated that type II, type IV and type VI secretion system of *B. cenocepacia* are involved in virulence and infection of host cells. Type II proteins could be secreted into host cytoplasm upon membrane disruption mediated by Type VI secretion system. [286] Moreover, type IV secretion system was found to be involved

in survival and proliferation in host cells during infection. *B. cenocepacia* cells became more vulnerable for lysosomal degradation in host cells without a functional Type IV secretion system. [287] Besides, type III secretion system was demonstrated to be essential for survival of *B. cenocepacia* cells during *in vivo* infection. Malfunction in type III secretion system resulted in a higher mortality rate in host tissues. [288]

Two zinc metalloproteases are expressed and secreted by *B. cenocepacia*, being ZmpA and ZmpB respectively. ZmpA and ZmpB are secreted by Type II secretion and are essential for interleukin activation or inflammation induction in host cell and intra-host bacterial survival with the aid of Type VI secretion. In addition, these zinc metalloproteases are able to break down immunoglobulins, type IV collagen and antimicrobial peptides. [286, 289-291] ShvR, a Lys-like transcription regulator determining the shiny colony morphology variant (shv) phenotype of *B. cenocepacia* cells, negatively regulates transcription of *zmpA* and *zmpB* to repress type II secretion system. ShvR self-regulates in a negative feedback manner and modulates virulence, quorum sensing, biofilm formation and inflammation in chronic infection in animal models caused by *B. cenocepacia*. It inhibits activation of *cepI/R* and *cciI/R* genes of quorum sensing system. [292, 293]

Overexpression of c-di-GMP promotes downregulation of *zmpA* and *zmpB* expression in *B. cenocepacia* cells. This enhances the escape of *B. cenocepacia* cells from host recognition and clearance. However, genes involved in type II/IV secretion system were upregulated with excessive c-di-GMP whereas *shvR* gene expression was not affected. As discussed previously, such activation of type II/IV gene might due to

specific localization of c-di-GMP molecules synthesized by *yedQ* gene in the cells. As these c-di-GMP molecules are restricted in certain region, they may not be able to impose its impact on these virulent secretion systems. Such phenomenon was also observed from the upregulation of genes involved in type III secretion system in *P. aeruginosa* mutant overexpressing c-di-GMP.

Besides *cep* quorum sensing, cis-2-dodecenoic acid (BDSF) quorum sensing system also regulates virulence, motility and other cellular functions in *B. cenocepacia* cells. BDSF was demonstrated to be related with c-di-GMP as it activates expression of RpfR, a phosphodiesterase containing EAL domains to degrade intracellular c-di-GMP and thus leads to phenotypical changes in swarming motility, virulence and development of biofilm. BDSF also controls the expression of genes encoding for chemotactic proteins including *cheR*, *cheD* and methyl-accepting chemotaxis proteins where removal of BDSF gene increased chemotaxis gene expression. [205, 294] These chemotaxis genes and also *cheB_1*, *cheW*, and *cheY_1* genes were also repressed by c-di-GMP as obtained from RNA-seq results here. This suggests there is interlink between BDSF and c-di-GMP in their functions regulating cellular activities.

Another virulence determinant, *hppD*, encoding for 4-hydroxyphenylpyruvate dioxygenase in *B. cenocepacia*, was downregulated with excessive c-di-GMP. [152] Mutation in *hppD* gene gives rise to a phenotype which is more prone to attack by hydrogen peroxide. HppD enzyme also shields *B. cenocepacia* cells from reactive nitrogen species. [295] Here the result suggests that c-di-GMP may impact the transcription of *hppD* and leads to a less virulent phenotype.

2.3.3. Transcriptomics analysis of *K. pneumoniae* mutants

Besides *P. aeruginosa* and *B. cenocepacia*, variation in intracellular c-di-GMP level in *K. pneumoniae* KP-1 also induced some significant changes in its surface-attaching ability and quorum sensing system. In *K. pneumoniae* cells, type III fimbriae are the major surface appendages promoting surface attachment to host cells such as urinary bladder cells and respiratory cells, and initiating biofilm formation through enhancing surface hydrophobicity. [149, 296-298] Previous study had shown type III fimbriae genes are partially under the regulation of intracellular c-di-GMP. [299]

This type of fimbriae is encoded by *mrkABCDF* genes where malfunction in these genes impairs the capability of biofilm formation. There are several other genes involved in the regulation of these fimbriae. *yfiN* encodes for GGDEF containing diguanylate cyclase while *mrkJ* gene encodes for EAL containing phosphodiesterase, which regulates c-di-GMP expression in an antithetical manner. *MrkIH* genes function as transcriptional regulator of type III fimbriae and biofilm formation, where *mrkH* encodes for a protein containing PilZ domain for c-di-GMP binding and *mrkI* encodes for LuxR-type transcriptional regulator. *mrkH* activates *mrkA* gene and also *mrkHI* genes by binding to the regulatory part in its promotor and recruiting RNA polymerase for transcription in the presence of c-di-GMP. [146, 227]

It was found that mutation in *yadH* gene impairs fimbriae-synthesizing and biofilm-forming capability with retained *in vivo* virulence in *K. pneumoniae* 43816. Since *yadH* encodes for a membrane-associated factor of ABC transporter system in *E. coli*, it is possible that changes in ABC transporter system in *K. pneumoniae* KP-1 will affect its fimbriae biosynthesis. [145] From the result obtained here, genes encoding

for components of ABC transporter system were inhibited by c-di-GMP. This may suggest that regulation of c-di-GMP on type III fimbriae formation is possibly through altering ABC transporter system.

K. pneumoniae possesses non-homoserine lactone as quorum sensing autoinducer, autoinducer 2 (AI-2). AI-2 quorum sensing is *luxS* dependent as constitutive expression of *luxS* gene was found to be highly correlated with expression of AI-2 during exponential stage of growth. [300-302] Variation in the level of autoinducer 2 secreted regulates its biofilm formation in *K. pneumoniae*. AI-2 is secreted into extracellular space by transporter TqsA and taken up by Lsr cassette importer encoded by *lsrACDBFGE* operon. *lsrR* and *lsrK* encode for transcriptional regulator where kinase LsrK phosphorylates and activates intracellular AI-2, and these activated AI-2 enhances transcription of both *lsr* operon and *lsrR* gene and leads to inhibition of transcriptional repressor LsrR. Mutation in *tqsA* decreases the extracellular concentration of AI-2 whereas removal of *lsrCD* results in extracellular accumulation of AI-2. Both of the mutants enhance biofilm formation with impaired architecture. [33, 303] The results obtained in this study indicated that the expression of *lsrBDG* genes and *lsrR* gene were repressed by c-di-GMP in *K. pneumoniae* KP-1. It suggests that c-di-GMP may induce changes in quorum sensing through altering *lsr* genes in *K. pneumoniae*. In addition, c-di-GMP and quorum sensing may both impose its regulation on lipopolysaccharide synthesis via *lsr* genes as mutation in *lsrCD* genes induced transcription of LPS-related genes in *K. pneumoniae* biofilm cells. [303]

It was indicated that pH and nutrient availability such as glucose and glycerol could modify AI-2 expression and *lsr* gene transcription in different bacteria including *E.coli*

and *Salmonella*. [33, 304-306] Modification from nutrient could be eliminated in this study as glucose was used as the only carbon source and culture conditions were maintained constant for both low and high c-di-GMP mutants. Thus, the changes in *lsr* genes were not induced by these factors but most possibly by c-di-GMP. Besides *lsr* genes, there are several other genes, such as gene encoding for PTS alpha-glucoside transporter subunit IICB, genes encoding for ABC transporter proteins, and gene encoding for outer membrane protein OmpA, expressed at altered manner when level of c-di-GMP increases.

Phosphoenolpyruvate transferase system, PTS, functions as extracellular hexose sugars importer to transport hexoses into bacterial cells, and thus involves in carbohydrate metabolism and chemotactic response. PTS consists of one sugar translocating enzyme II complex, and other two enzymes I and histidine carrier protein. [307] Gene encoding for PTS subunits IICB responsible for glucose import was downregulated in the presence of high level of c-di-GMP. As testing cells were collected in their early stationary phase and growth rates of both mutant cells are not significantly different, such reduction is most probably induced by c-di-GMP solely to slow down glucose uptake.

ATP-binding cassette transporters, also known as ABC transporters in *K.pneumoniae*, are powered by ATP for cross-membrane molecule translocation. *ycjV* regulated by ABC transporter upregulated greatly in a resistant clinical isolate of *K. pneumoniae*, indicating an increase in the activity of efflux mechanism. [308] c-di-GMP reduced the expression of genes encoding for ABC transporter permease and ATP-binding protein.

These results suggest that c-di-GMP is also involved in membrane transport mechanism in this bacteriae to regulate uptake or export of suger and other molecules. Outer Membrane Protein A, OmpA, was demonstrated to induce host inflammatory effect through interacting with immune cells and thus promotes host removal of *K. pneumoniae* cells. [309] OmpA is also involved in resistance to antimicrobial peptides where removal of OmpA resulted in a more prone phenotype to antimicrobial peptide attack. [310] Reduction in expression of gene encoding for OpmA in *K. pneumoniae* under the impact of c-di-GMP indicated that c-di-GMP helps cells evading from host clearance by reducing host-cell interaction and host recognition.

Among all of these results, the most interesting finding in this study is the upregulation of *metE* gene in both of *P. aeruginosa* PAO1 and *B. cenocepacia* H111 cells with excessive c-di-GMP synthesized. This gene encodes for the enzyme, 5-methyltetrahydropteroyltrigluta- mate-homocysteine methyltransferase, catalyzing the final reaction in L-methionine biosyntythesis. qPCR results further confirmed *metE* gene upregulation in these two mutants. This demonstrated that c-di-GMP could induce common changes in different pathogen at tanscriptomic level. To monitor the level of L-methionine synthesized in these two strains and investigate if such genetic changes could be reflected on metabolomics level to generate possible common cross-species biomarkers, metabolic profiles of c-di-GMP mutants of all three pathogens were plotted by LC/MS and will be discussed in the following chapter.

Chapter 3. Identification of signature metabolites regulated by c-di-GMP through metabolomics profiling

Besides its regulation of transcriptomics profiles in the testing pathogenic strains, variations in the intracellular levels of c-di-GMP due to catalytic enzymes may also change cellular metabolic profile and lead to expression of specific biomarkers of the biofilm mode of growth. Identification of biomarkers regulated by c-di-GMP may provide alternative methods to diagnose biofilm-associated chronic infections.

As demonstrated previously, three pathogens, *P. aeruginosa* PAO1, *K. pneumoniae* KP-1 and *B. cenocepacia* H111, were genetically modified to express different intracellular levels of c-di-GMP to mimic either acute or chronic mode of growth. They showed diversified transcriptomic profiles which could lead to the discovery of common biofilm gene marker regulated by c-di-GMP in different pathogenic bacteria. This gave a possibility in the discovery of common c-di-GMP-modulating biofilm biomarkers among these pathogens. Using the same bacterial mutants, their metabolomics profiles were generated by Liquid Chromatography-Mass Spectrometer (LC/MS) and statistically analysed and visualized to identify common signature metabolites under same conditions of growth.

Among three high c-di-GMP generating strains, we have found 2 common signature metabolites between *P. aeruginosa* and *K. pneumonia*, while 15 were found between *P. aeruginosa* and *B. cenocepacia*. Among three low c-di-GMP generating strains, 1 common feature were found among all the strains, while 19 were found between *P. aeruginosa* and *K. pneumonia*, and 7 were found between *P. aeruginosa* and

B.cenocepacia. This study provides evidence for the possibility of detecting and diagnosing biofilm infections in clinical prospective using c-di-GMP regulated metabolites.

3.1. Materials and methods

3.1.1. Strains and cultural conditions

For metabolomics study, same c-di-GMP mutant strains used as transcriptomics study which included *P. aeruginosa* PAO1, *K. pneumoniae* KP-1 and *B. cenocepaci* H111. *P.aeruginosa/pYhjH* and *P. aeruginosa/pYedQ2*, *K. pneumonia/pYhjH* and *K. pneumonia/ pYedQ2*, and *B. cenocepacia/pYhjH* and *B. cenocepacia/pYedQ2*, were recovered on LB agar plate at 37°C. Single colony was inoculated into LB medium [229] with antibiotics and incubated for overnight at 37°C with 200 rpm shaking. The overnight liquid cultures were diluted to OD₆₀₀ 0.1 in ABTG medium and allowed to grow to respective early stationary phase. 60 µg mL⁻¹ gentamicin (GM) and 60 µg mL⁻¹ tetracycline were supplemented to media for plasmid maintenance when necessary. All strains used here can be found in Table 4.1.

3.1.2. Metabolite extraction for LC/MS analysis

5 replicates of each strain at early stationary phase were cultured. 10 ml of each sample were harvested and centrifuged at 13,000 g for 3 min. The cell pellets were washed twice with 1 mM ice-cold ammonium acetate. Cells were re-suspended in ice-cold acetonitrile /methanol/water (4:4:2) solution and sonicated at frequency of 37 Hz, 100% power for 30 min in ice-water sonicator. Supernatants containing metabolites extracted were collected and dried by temperature controlled speedvac concentrator.

The dried metabolites were then dissolved in 1 mM ammonium acetate for LC/MS analysis.

Cell pellets were washed with ice-cold 1 mM ammonium acetate and were re-suspended in 1 mL of ice cold Acetonitrile/Methanol/Water (2:2:1) solution. Cells were further lysed by sonication in ice-filled water sonicator (brand) of 37 Hz frequency and 100% power. Supernatants containing metabolites were collected and dried in temperature controlled Vacuum dryer at 4°C. Dried metabolites were dissolved in ice-cold 1 mM ammonium acetate for LC/MS analysis. The extracted metabolites from c-di-GMP mutants were analyzed by LC/MS as described below.

3.1.3. Liquid Chromatography-Mass Spectrum (LC/MS) analysis

All metabolite samples described above were analysed using an Accela 1250 Quaternary LC system (Thermo Fisher) adjoining to an LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Scientific), supplied with electrospray ionization (ESI) source. Both positive and negative modes were performed to analyze the samples.

Reconstituted metabolite samples were first separated chromatographically through a Phenomenex - Kinetex® C18 reverse phase core-shell column (2.0 x 50mm, 1.3-micron) using gradient elution with two mobile phases. For positive mode of detection, mobile phase A was 0.1 % formic acid in de-ionized water while mobile phase B was 0.1 % formic acid in LC/MS grade acetonitrile. For negative mode of detection, mobile phase A was 1mM ammonium fluoride while mobile phase B was LC/MS grade acetonitrile. Gradient program used for both modes of detection was 1 % B at 1 min, 98 % B at 12 min to 17 min, 1% B at 17.5 min, 0% B at 20 min, with flow rate of

0.3 mL/min and injection volume of 15 μ L. Temperatures of column compartment and auto-sampler were kept at 45°C and 6°C, respectively. Profiling analyses were carried out with 60-1200 m/z as mass range, 3.6kV source voltage with resolution of 60,000. Temperature of source heater and capillary were kept at 300 °C with flow rate of sheath gas at 45, auxiliary gas at 15 and sweep gas at 1 arbitrary unit. Data were obtained and processed preliminarily using Xcalibur v2.2 software package with list of peak intensities as results. Peak intensities obtained for each sample were normalized by total protein of the cells for facilitating comparison.

Metabolic data were analysed using comprehensive tools suite provided on <http://www.metaboanalyst.ca/>. [311] Raw data were filtered based on relative standard deviation (SD/mean) and were then transformed to log2 values. Principle Component Analysis and dendrogram were plotted for clustering samples. Student T-test was done between each pair of mutants with filtering threshold of fold change $FC \geq 2$ and p-value ≤ 0.05 . Volcanic plot were drawn based on same threshold values. Heatmap of top 100 features were plotted based on random forest analysis to visualise difference between each mutant pairs.

The selected significant LC/MS features were searched and matched using metabolite database provided on <http://ecmdb.ca/>. [312, 313] Matching criteria were set as positive ionization mode and M+H adduct type with molecular weight tolerance of 0.05 Da based on the m/z value of each feature. Features with no hit were indicated as 'No match' while one match with smallest difference between adduct molecular weight and compound molecular weight was chosen from features with multiple hits. Compounds with best hit were matched with KEGG pathway and retrieved with a

KEGG compound number for pathway matching using KEGG mapping tool. [251, 252]

3.1.4. Protein quantification

1 mL of cell pellet from each sample were collected and re-suspended in 1 mL of 0.1M sodium hydroxide. Cell suspensions were incubated at 95°C for 10 mins. Protein concentration was measured using Qubit® 2.0 Fluorometer (Invitrogen).

3.2. Results

3.2.1. Metabolomics profiling

LC/MS analysis was carried out for global metabolic profiling using these c-di-GMP mutants. The comparison was carried out between strains producing high and low level of c-di-GMP. Raw LC/MS peak intensities obtained were normalized by unit protein concentration and then transformed to log₂ values before filtering by relative standard deviation (SD/mean).

3.2.1.1. Statistical analysis of metabolic profile of *P. aeruginosa* strains

PCA plot was drawn with the pre-processed peak values of five replicates of each mutant (Figure 3.1). PC1 accounts for 31.1% of total variance whereas PC2 accounts for 15.5% of total variance. Replicates of *P. aeruginosa/pYedQ2* clustered together along PC2 while replicates of *P. aeruginosa/pYhjH* clustered along PC1.

Figure 3.1 PCA plot of metabolites in *P.aeruginosa/pYedQ₂* and *P.aeruginosa/pYhjH* mutant. Clear separation could be seen between replicates of each mutant along PC1 (31.1% of total variance). *P. aeruginosa/pYedQ₂* replicates showed greater variation along PC1 while *P. aeruginosa/pYhjH* replicates showed slightly greater variation along PC2. *P. aeruginosa/pYedQ₂* replicates are denoted by “High” with red triangles; *P. aeruginosa/pYhjH* replicates are denoted by “Low” with green crosses. Fold change ≥ 2 , p-value ≤ 0.05 , n=5

Clear separation can be seen between replicates of *P. aeruginosa/pYedQ₂* and those of *P. aeruginosa/pYhjH* where *P. aeruginosa/pYedQ₂* replicates varied more along PC1 and *P. aeruginosa/pYhjH* varied more along PC2. Differentially expressed metabolites were selected with criteria of fold change ≥ 2 and p-value ≤ 0.05 . 172 metabolites were upregulated and 151 metabolites were downregulated in *P. aeruginosa/pYedQ₂*

selected based on these criteria. 100 of most significantly regulated metabolites in each mutant were visualized by constructing heatmap (Figure 3.2).

Figure 3.2 Metabolites regulated in *P. aeruginosa/pYedQ2* and *P. aeruginosa/pYhjH* mutants Only top 100 most significantly regulated metabolites illustrated. Similar pattern could be seen among replicates while distinct difference in pattern could be seen between mutants. Fold change ≥ 2 , p-value ≤ 0.05 , n=5 Blue: downregulated genes; Red: upregulated genes.

Volcano plot was constructed to visualize and identify all differentially expressed metabolites where each dot represents one metabolite (Figure 3.3). Pink dots illustrate those significantly modulated metabolites and black dots illustrate the unchanged ones.

Figure 3.3. Volcano plot of total metabolites in *P. aeruginosa/pYedQ2*. Each dot represents an individual metabolite. Significantly regulated metabolites in *P. aeruginosa/pYedQ2* comparing to those in *P. aeruginosa/pYhjH* are illustrated by pink dots. Fold change ≥ 2 for upregulated metabolites, Fold change ≤ -2 for downregulated metabolites, p-value ≤ 0.05 . n=5

The further the location of dots from origin zero point is, the more significant changes in level of metabolic expressed. Full list of metabolites upregulated or downregulated can be found in Table 7.2.1 & 7.2.2. Primary matching based on the molecular weight of these compounds with the ECMDB database was carried out to obtain a first-step understanding of the possible identities of these compounds. After the primary database matching, all compounds with a most suitable hit were selected for KEGG compound search and pathway mapping. All the following results and discussion are based on these primary matching results and certainly further analysis is needed for confirmation of these ‘identified’ metabolites.

Among all the upregulated metabolites in *P. aeruginosa/pYedQ2*, 691.0772 eluted at 4.12 min is possibly c-di-GMP based on database matching results. Such match proves the validity of the matching results. Other compounds with a hit in the database and assigned with a KEGG compound code are listed in the table below. (Table 3.1)

M/Z	RT	Possible match	KEGG code
355.108	0.84m	5-Amino-6-(5'-phosphoribosylamino)uracil	C01268
152.112	4.13m	N-Methyltyramine	C02442
717.531	4.12m	2,3-Bis-O-(geranylgeranyl)glycerol 1-phosphate	C04638
613.148	2.23m	Glutathione disulphide	C00127
135.086	4.13m	Deoxyribose	C01801
183.15	3.59m	Geosmin	C16286
146.148	1.16m	Spermidine	C00315
413.351	11.86m	Isofucosterol	C08821
231.093	0.85m	Thioredoxin	C00342
179.103	0.85m	Phenanthrene	C11422
129.121	1.16m	3-Isopropyl-3-butenic acid	C11950
		Cyclohexane-1-carboxylate	C09822
355.109	2.23m	5-Amino-6-(5'-phosphoribosylamino)uracil	C01268
300.272	10m	Sphingosine	C00319
298.094	0.98m	5'-Methylthioadenosine	C00170
355.103	13.29m	5-Amino-6-(5'-phosphoribosylamino)uracil	C01268
135.086	4.41m	1-Deoxy-D-xylulose	C11437
413.352	11.72m	Isofucosterol	C08821
92.9709	11.17m	Enzyme N6-(dihydrolipoyl)lysine	C15973
413.35	11.5m	Isofucosterol	C08821
163.116	2.69m	Carnitine	C00487
180.105	1.76m	7-Aminomethyl-7-carbaguanine	C16675
184.061	8.54m	3,5-Dihydroxy-phenylglycine	C12026
190.965	0.58m	3-Sulfocatechol	C06336
127.062	8.54m	5-Amino-4-imidazolecarboxamide	C04051
561.396	12.16m	2-Hexaprenyl-3-methyl-6-methoxy-1,4-benzoquinone	C05804
415.365	12.17m	beta-Sitosterol	C01753
903.188	4.53m	Coenzyme F420-3	C00876
243.138	4.06m	Thymidine	C00214
135.086	4.64m	Deoxyribose	C01801
560.078	3.44m	Adenosine diphosphate ribose	C00301
112.105	0.98m	Histamine	C00388

Table 3.1 List of upregulated metabolites in *P. aeruginosa/pYedQ2* with a hit in the database. Each matched metabolite was assigned with a KEGG compound code. Only hits with highest similarity are illustrated here based on molecular weight of each metabolite. Adduct type was set as M+H; tolerance of molecular weight was set as 0.05 Da.

Multiple metabolites are found to be involved in same pathway. Such as seven compounds, histamine, 5-Amino-6-(5'-phosphoribosylamino) uracil, beta-Sitosterol, 2-hexaprenyl-3-methyl-6-methoxy-1,4-benzoquinone, isofucosterol, 1-deoxy-d-xylulose 5-phosphate and geosmin are involved in biosynthesis of secondary metabolites. Two compounds, 3-sulfocatechol and cyclohexane-1-carboxylate, are involved in benzoate degradation; thymidine and thioredoxin are involved in pyrimidine metabolism; glutathione disulphide and spermidine are involved in glutathione metabolism; spermidine and carnitine are for ABC transporters; 1-deoxy-d-xylulose 5-phosphate and 3,5-dihydroxy-phenylglycine are involved in biosynthesis of antibiotics; ADP-ribose and 5-amino-4-imidazolecarboxamide are for purine metabolism.

One metabolite is found participating in multiple pathways. Such as, spermidine is also involved in other pathways including arginine and proline metabolism and beta-Alanine metabolism; 2-hexaprenyl-3-methyl-6-methoxy-1,4-benzoquinone is also included in ubiquinone and other terpenoid-quinone biosynthesis; 5-amino-6-(5'-phosphoribosylamino) uracil is also involved in riboflavin metabolism; enzyme N6-(dihydrolipoyl)lysine plays multiple roles in citrate cycle, glycolysis/gluconeogenesis, valine, leucine and isoleucine degradation, propanoate metabolism, and pyruvate

metabolism. 1-deoxy-d-xylulose 5-phosphate is also found in terpenoid backbone biosynthesis.

Other metabolites are involved in a single pathway. Such as N-methyltyramine is involved in tyrosine metabolism; carnitine is involved in lysine degradation; 7-aminomethyl-7-carbaguanine is involved in folate biosynthesis. Coenzyme F420, sphingosine, 5'-methylthioadenosine, 1-deoxy-d-xylulose 5-phosphate, 2,3-bis-o-(geranylgeranyl)glycerol 1-phosphate, 3-isopropyl-3-butenic acid, phenanthrene, histamine, and deoxyribose are involved in methane metabolism, sphingolipid metabolism, cysteine and methionine metabolism, thiamine metabolism, glycerophospholipid metabolism, limonene and pinene degradation, degradation of aromatic compounds, histidine metabolism, and pentose phosphate pathway, respectively. A summary of individual metabolic pathways affected are listed in Table 3.2.

KEGG map NO.	Pathways involved	Number of compounds involved
pae00362	Benzoate degradation	2
pae00480	Glutathione metabolism	2
pae00240	Pyrimidine metabolism	2
pae01130	Biosynthesis of antibiotics	2
pae02010	ABC transporters	2
pae00230	Purine metabolism	2
pae00270	Cysteine and methionine metabolism	1
pae00740	Riboflavin metabolism	1
pae00010	Glycolysis / Gluconeogenesis	1
pae00020	Citrate cycle (TCA cycle)	1
pae00600	Sphingolipid metabolism	1
pae00130	Ubiquinone and other terpenoid-quinone biosynthesis	1
pae01220	Degradation of aromatic compounds	1
pae00030	Pentose phosphate pathway	1

pae00680	Methane metabolism	1
pae00900	Terpenoid backbone biosynthesis	1
pae00280	Valine, leucine and isoleucine degradation	1
pae00330	Arginine and proline metabolism	1
pae00730	Thiamine metabolism	1
pae00903	Limonene and pinene degradation	1
pae00620	Pyruvate metabolism	1
pae00790	Folate biosynthesis	1
pae00564	Glycerophospholipid metabolism	1
pae00640	Propanoate metabolism	1
pae00410	beta-Alanine metabolism	1
pae00350	Tyrosine metabolism	1
pae00340	Histidine metabolism	1
pae00310	Lysine degradation	1

Table 3.2. Pathways involving upregulated metabolites in *P. aeruginosa/ pYedQ2*.

Pathways assigned are based on compounds listed in Table 3.1. *P. aeruginosa* PAO1 (pae) was chosen as reference strain.

Among all downregulated metabolite in *P. aeruginosa/pYedQ2*, feature with m/z value of 213.1384 eluted at 0.71 min was matched with Pyocyanine. Other downregulated metabolites are listed in Table 3.3.

M/Z	RT	Possible match	KEGG code
454.2471	2.62m	1-Acyl-sn-glycero-3-phosphoglycerol (N-C14:1)	C18126
130.1047	0.71m	4-Guanidinobutanal	C02647
221.0514	0.76m	(-)-threo-Iso(homo)2-citrate	C16597
241.1306	0.71m	Homocarnosine	C00884
298.1324	3m	5'-Methylthioadenosine	C00170
119.1041	7.92m	Betaine	C00719
625.397	14.15m	2,2'-Diketospirilloxanthin	C15885
274.2038	10.97m	(2R,3R)-3-Methylglutamyl-5-semialdehyde-N6-lysine	C20279
91.1076	11.37m	(R,R)-Butane-2,3-diol	C03044
152.1185	13.43m	N-Methyltyramine	C02442
91.1078	7.93m	(R,R)-Butane-2,3-diol	C03044

119.0899	1.95m	Betaine	C00719
145.1065	7.92m	4-Guanidinobutanamide	C03078
277.1465	0.71m	N6-(L-1,3-Dicarboxypropyl)-L-lysine	C00449
385.3263	14.15m	7-Dehydrocholesterol	C01164
327.1163	10.86m	cis-beta-D-Glucosyl-2-hydroxycinnamate	C05839
591.1339	0.71m	UDP-4-amino-4,6-dideoxy-N-acetyl-beta-L-altrosamine	C19961
137.1004	1.35m	(-)-Limonene	C00521
166.0937	7.92m	D-Phenylalanine	C02265
369.1559	10.97m	Curcumin	C10443
111.0995	7.92m	Imidazole-4-acetaldehyde	C05130
274.204	11.2m	(2R,3R)-3-Methylglutamyl-5-semialdehyde-N6-lysine	C20279
265.1537	7.93m	Formyl-N-acetyl-5-methoxykynurenamine	C05642
563.2636	12.44m	Protoporphyrin	C02191
263.1389	7.92m	1,6-Dimethoxypyrene	C18260
226.1181	7.92m	4-Amino-4-deoxychorismate	C11355
180.1343	11.2m	7-Aminomethyl-7-carbaguanine	C16675
241.1717	12.43m	Homocarnosine	C00884
369.1587	12.77m	Curcumin	C10443
130.1422	1.65m	4-Guanidinobutanal	C02647
275.1698	10.01m	4-Hydroxy-3-polyprenylbenzoate	C05848
383.1803	7.92m	2-cis,6-trans-Farnesyl diphosphate	C19760
331.1682	9.25m	Gibberellin A7	C00859
373.1729	9.24m	Biocytin	C05552

Table 3.3 Downregulated metabolites in *P. aeruginosa/pYedQ2* with a hit in the database. Each matched compound was assigned with a KEGG compound code. Only hits with highest similarity are illustrated here based on molecular weight of each metabolite. Adduct type was set as M+H; tolerance of molecular weight was set as 0.05 Da.

Multiple metabolites are involved in same metabolic pathway. Such as seven metabolites, N6-(L-1,3-dicarboxypropyl)-L-lysine, (-)-limonene, gibberellin A1, protoporphyrin, cis-beta-D-glucosyl-2-hydroxycinnamate, 4-hydroxy-3-polyprenylbenzoate and curcumin are involved in biosynthesis of secondary metabolites; three

compounds, homocarnosine, 4-guanidinobutanal and 4-guanidinobutanamide are involved in Arginine and proline metabolism; two compounds, N6-(L-1,3-dicarboxypropyl)-L-lysine and (2R,3R)-3-methylglutamyl-5-semialdehyde-N6-lysine are for lysine biosynthesis; 4-amino-4-deoxychorismate and 7-aminomethyl-7-carbaguanine are for folate biosynthesis.

One compound is found to be involved in multiple pathways. Such as N6-(L-1,3-dicarboxypropyl)-L-lysine is also involved in lysine degradation, biosynthesis of amino acids and biosynthesis of antibiotics; betaine is involved in ABC transporters and glycine, serine and threonine metabolism. 4-hydroxy-3-polyphenylbenzoate is involved in ubiquinone and other terpenoid-quinone biosynthesis.

Other compounds included in single pathway include (-)-threo-iso(homo)2-citrate which is involved in 2-oxocarboxylic acid metabolism and methane metabolism. Biocytin, protoporphyrin, (R,R)-butane-2,3-diol, imidazole-4-acetaldehyde, (-)-limonene, UDP-4-amino-4,6-dideoxy-N-acetyl-beta-L-altrosamine, (2Z,6Z)-farnesyl diphosphate, formyl-N-acetyl-5-methoxykynurenamine, and D-phenylalanine are involved in biotin metabolism, porphyrin and chlorophyll metabolism, butanoate metabolism, histidine metabolism, limonene and pinene degradation, amino sugar and nucleotide sugar metabolism, terpenoid backbone biosynthesis, tryptophan metabolism and phenylalanine metabolism, respectively.

A summary of individual metabolic pathways involving downregulated metabolites in *P. aeruginosa/pYedQ2* is illustrated in Table 3.4.

However, two compounds, N-methyltyramine in tyrosine metabolism and 5'-methylthioadenosine in cysteine and methionine metabolism also appeared in upregulated metabolites probably due to the similarity in m/z value of the features. Thus, this match will be considered as wrong matching and further analysis is needed.

KEGGmap No.	Pathway involved	Number of compounds involved
pae00330	Arginine and proline metabolism	3
pae00300	Lysine biosynthesis	2
pae00790	Folate biosynthesis	2
pae00340	Histidine metabolism	1
pae01130	Biosynthesis of antibiotics	1
pae00903	Limonene and pinene degradation	1
pae00360	Phenylalanine metabolism	1
pae00860	Porphyrin and chlorophyll metabolism	1
pae02010	ABC transporters	1
pae00780	Biotin metabolism	1
pae00130	Ubiquinone and other terpenoid-quinone biosynthesis	1
pae00900	Terpenoid backbone biosynthesis	1
pae00260	Glycine, serine and threonine metabolism	1
pae01210	2-Oxocarboxylic acid metabolism	1
pae00310	Lysine degradation	1
pae00380	Tryptophan metabolism	1
pae00650	Butanoate metabolism	1
pae00350	Tyrosine metabolism	1
pae00564	Glycerophospholipid metabolism	1
pae00270	Cysteine and methionine metabolism	1
pae00680	Methane metabolism	1
pae01230	Biosynthesis of amino acids	1
pae00520	Amino sugar and nucleotide sugar metabolism	1

Table 3.4. Pathways involving downregulated metabolites in *P. aeruginosa/pYedQ2*.

Pathways assigned are based compounds listed in Table 3.3. *P. aeruginosa* PAO1 (pae) was chosen as reference strain.

3.2.1.2. Statistical analysis of metabolic profile of *B. cenocepacia* strains

Similarly to *P. aeruginosa* mutants, PCA plot of *B. cenocepacia* mutant was also generated with pre-processed peak values of 5 replicates of each mutant (Figure 3.4). PC1 accounts for 23.5% of total variation and PC2 accounts for 18% of total variation.

Figure 3.4 PCA plot of metabolites in *B.cenocepacia/pYedQ2* and *B.cenocepacia/pYhjH*. Clear separation could be seen between replicates of each mutant. *B.cenocepacia/pYedQ2* replicates showed greater variation along both PC. *B.cenocepacia/pYedQ2* replicates are denoted by ‘High’ with red triangles; *B.cenocepacia/pYhjH* replicates are denoted by ‘Low’ with green crosses. Fold change ≥ 2 , p-value ≤ 0.05 , n=5

B. cenocepacia/pYedQ2 replicates demonstrated greater variation along both PC1 and PC2 comparing to *B. cenocepacia/pYhjH* replicates which locate in nearer proximity

to each other. This shows more variations found among *B. cenocepacia*/pYedQ2 replicates but higher similarity among *B. cenocepacia*/pYhjH replicates. With same selection criteria, 75 metabolites were upregulated while 136 metabolites were downregulated under overexpression of c-di-GMP.

Figure 3.5 Metabolites regulated in *B.cenocepacia*/pYedQ2 and *B.cenocepacia*/pYhjH.

Only top 100 most significantly regulated metabolites are illustrated. Slight variation among replicates of each mutant was seen. Similar pattern could be still seen among replicates while distinct difference in pattern could be seen between mutants. Fold change ≥ 2 , p-value ≤ 0.05 , n=5. Blue: downregulated genes; Red: upregulated genes.

100 of most significantly regulated metabolites in each mutant were visualized by constructing heatmap (Figure 3.5). Most differentially expressed metabolites were identified and viewed on Volcano plot illustrating by pink dots (Figure 3.6).

717.531	4.12m	2,3-Bis-O-(geranylgeranyl)glycerol 1-phosphate	C04638
697.57	12.48m	2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinone	C05814
563.283	12.43m	Protoporphyrin IX	C02191
206.078	7.21m	5-Methoxyindoleacetate	C05660

Table 3.5 List of upregulated metabolites in *B.cenocepacia/pYedQ₂* with a hit in the database. Each matched metabolite was assigned with a KEGG compound code. Only hits with highest similarity are illustrated here based on molecular weight of each metabolite. Adduct type was set as M+H; tolerance of molecular weight was set as 0.05 Da.

2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinone is involved in biosynthesis of secondary metabolites and ubiquinone and other terpenoid-quinone biosynthesis. Protoporphyrin is involved in both of biosynthesis of secondary metabolites and porphyrin and chlorophyll metabolism. 5-methoxyindoleacetate, 3,4-dehydrorhodopin, N-methyltyramineis and 2,3-bis-O-(geranylgeranyl)glycerol 1-phosphate are involved in tryptophan metabolism, carotenoid biosynthesis, tyrosine metabolism, glycerophospholipid metabolism, respectively. Summary of individual metabolic pathway involving upregulated metabolites in *B.cenocepacia/pYedQ₂* are listed in Table 3.6 below.

KEGG map No.	Pathway involved	No. of compounds involved
bceo00906	Carotenoid biosynthesis	1
bceo00380	Tryptophan metabolism	1
bceo00860	Porphyrin and chlorophyll metabolism	1
bceo00130	Ubiquinone and other terpenoid-quinone biosynthesis	1
bceo00564	Glycerophospholipid metabolism	1
bceo00350	Tyrosine metabolism	1

Table 3.6 Pathways involves upregulated metabolites in *B. cenocepacia/pYedQ2*.

Pathways assigned are based on the compounds listed in Table 3.5. *B. cenocepacia* H111 (bceo) was chosen as reference strain.

For downregulated metabolites in *B. cenocepacia/pYedQ2*, one feature has been matched as same compound, N-Methyltyramine, with one upregulated compound due to the similarity in their molecular weight. Further differentiation is needed using both molecular weight and retention time. All metabolites with database hit and KEGG match are listed in Table 3.7.

M/Z	RT	Possible match	KEGG code
119.0901	0.72m	Betaine	C00719
167.0081	1.17m	Phthalate	C01606
232.0864	0.64m	N-Acetyl-L-2-amino-6-oxopimelate	C05539
268.1497	1.94m	Butirosin	C17585
152.111	2.57m	N-Methyltyramine	C02442
151.1412	0.64m	(-)-Carvone	C01767
136.0697	2.78m	Sinapyl alcohol	C02325
94.0936	3.14m	Aniline	C00292
214.9689	0.5m	3,4,6-Trichloro-cis-1,2-dihydroxycyclohexa-3,5-diene	C12832
136.1164	3.14m	Sinapyl alcohol	C02325
348.1055	3.44m	Adenine 5'-phosphate	C00020
119.0897	3.13m	Betaine	C00719
198.9928	0.5m	2,4,5-Trichloro-2,5-cyclohexadiene-1-ol	C06598
298.1383	3.14m	5'-Methylthioadenosine	C00170
189.106	2.81m	cis-2,3-Dihydro-2,3-dihydroxybiphenyl	C06589
163.0967	3.14m	N6-Hydroxy-L-lysine	C01028
140.9723	0.5m	Sulfoacetate	C14179
124.9777	0.58m	Sulfoacetaldehyde	C00593
170.9831	0.5m	3-Sulfolactate	C16069
186.976	0.5m	3-Phospho-D-glycerate	C00197
121.1065	2.8m	Acetophenone	C07113
560.0779	3.44m	Adenosine diphosphate ribose	C00301
109.0844	1.67m	Benzyl alcohol	C00556
786.1219	3.59m	FAD	C00016
190.9649	0.58m	3-Sulfocatechol	C06336

261.0883	1.67m	Biotin sulfoxide	C20386
296.1245	1.69m	Prunasin	C00844
344.126	1.69m	Coenzyme B	C04628
561.4627	12.44m	2-Hexaprenyl-3-methyl-6-methoxy-1,4-benzoquinone	C05804
243.0788	1.66m	2-Hydroxy-2H-benzo[h]chromene-2-carboxylate	C11425
191.1206	2.8m	5-Methoxytryptamine	C05659
123.1105	1.15m	(S)-1-Phenylethanol	C11348
255.1392	1.48m	Lactenocin	C01723
587.2729	2.62m	(3Z)-Phycoerythrobilin	C05912

Table 3.7 Downregulated metabolites in *B. cenocepacia/pYedQ2* with a hit in the database. Each matched metabolite was assigned with a KEGG compound code. Only hits with highest similarity are illustrated here based on molecular weight of each metabolite. Adduct type was set as M+H; tolerance of molecular weight was set as 0.05 Da.

Eight metabolites, FAD, AMP, 3-phospho-D-glycerate, prunasin, (-)-carvone, sinapyl alcohol, N-acetyl-L-2-amino-6-oxopimelate and 2-hexaprenyl-3-methyl-6-methoxy-1,4-benzoquinone, are involved in biosynthesis of secondary metabolites. Seven metabolites, benzyl alcohol, phthalate, cis-2,3-dihydro-2,3-dihydroxybiphenyl, acetophenon, (S)-1-phenylethanol, 2-hydroxy-2H-benzo[h]chromene-2-carboxylate and 3,4,6-trichloro-cis-1,2-dihydroxycyclohexa-3,5-diene are involved in degradation of aromatic compounds. Four metabolites, AMP, 3-phospho-D-glycerate, N-acetyl-L-2-amino-6-oxopimelate and butirosin A, are involved in biosynthesis of antibiotics. Three metabolites, 3-phospho-D-glycerate, sulfoacetaldehyde and coenzyme B are involved in methane metabolism.

2,4,5-trichloro-2,5-cyclohexadiene-1-ol and 3,4,6-trichloro-cis-1,2-dihydroxycyclohexa-3,5-diene are involved in chlorocyclohexane and chlorobenzene degradation. Sulfoacetaldehyde and sulfoacetate are involved in taurine and hypotaurine

metabolism. 3-phospho-D-glycerate and N-acetyl-L-2-amino-6-oxopimelate are involved in biosynthesis of amino acids. Betaine and phthalate are for ABC transporters while 3-phospho-D-glycerate and coenzyme B are also for carbon metabolism. AMP and ADP-ribose are involved in purine metabolism. Phthalate and 2-hydroxy-2H-benzo[h]chromene-2-carboxylate are also for polycyclic aromatic hydrocarbon degradation.

For those single metabolites involved in multiple pathways, 3-phospho-D-glycerate and betaine are involved in glycine, serine and threonine metabolism. 3-Phospho-D-glycerate is also involved in Pentose phosphate pathway, Glyoxylate and dicarboxylate metabolism, Glycolysis /Gluconeogenesis and Glycerolipid metabolism.

5'-Methylthioadenosine is involved in Cysteine and methionine metabolism. Other pathways affected including Lysine degradation, Limonene and pinene degradation, Monobactam biosynthesis, Tryptophan metabolism, Tyrosine metabolism, Cyanoamino acid metabolism, Riboflavin metabolism, Ubiquinone and other terpenoid-quinone biosynthesis, Benzoate degradation, Lysine biosynthesis, Porphyrin and chlorophyll metabolism, Toluene degradation, Dioxin degradation, Biotin metabolism, and Aminobenzoate degradation, contain N6-Hydroxy-L-lysine, (-)-Carvone, N-Acetyl-L-2-amino-6-oxopimelate, 5-Methoxytryptamine, N-Methyltyramine, Prunasin, FAD, 2-Hexaprenyl-3-methyl-6-methoxy-1,4-benzoquinone, 3-Sulfocatechol, N-Acetyl-L-2-amino-6-oxopimelate, (3Z)-Phycoerythrobilin, Benzyl alcohol, cis-2,3-Dihydro-2,3-dihydroxybiphenyl, Biotin sulfoxide and Aniline, respectively. List of pathways containing downregulated metabolites in *B. cenocepacia/pYedQ2* are illustrated in Table 3.8 below.

KEGG map No	Pathway involved	No. of compounds involved
bceo01220	Degradation of aromatic compounds	7
bceo01130	Biosynthesis of antibiotics	4
bceo00680	Methane metabolism	3
bceo00624	Polycyclic aromatic hydrocarbon degradation	2
bceo00430	Taurine and hypotaurine metabolism	2
bceo02010	ABC transporters	2
bceo00260	Glycine, serine and threonine metabolism	2
bceo00361	Chlorocyclohexane and chlorobenzene degradation	2
bceo00230	Purine metabolism	2
bceo01200	Carbon metabolism	2
bceo01230	Biosynthesis of amino acids	2
bceo00780	Biotin metabolism	1
bceo00561	Glycerolipid metabolism	1
bceo00621	Dioxin degradation	1
bceo00460	Cyanoamino acid metabolism	1
bceo00710	Carbon fixation in photosynthetic organisms	1
bceo00130	Ubiquinone and other terpenoid-quinone biosynthesis	1
bceo00261	Monobactam biosynthesis	1
bceo00362	Benzoate degradation	1
bceo00030	Pentose phosphate pathway	1
bceo00270	Cysteine and methionine metabolism	1
bceo00350	Tyrosine metabolism	1
bceo00310	Lysine degradation	1
bceo00010	Glycolysis / Gluconeogenesis	1
bceo00380	Tryptophan metabolism	1
bceo00627	Aminobenzoate degradation	1
bceo00903	Limonene and pinene degradation	1
bceo00623	Toluene degradation	1
bceo00740	Riboflavin metabolism	1
bceo00630	Glyoxylate and dicarboxylate metabolism	1
bceo00300	Lysine biosynthesis	1
bceo00860	Porphyrin and chlorophyll metabolism	1

Table 3.8 Pathways involving downregulated metabolites in *B. cenocepacia/pYedQ2*.

Pathways assigned are based on the compounds listed in Table 3.7. *B. cenocepacia* H111 (bceo) was chosen as reference strain.

3.2.1.3. Statistical analysis of metabolic profile of *K. pneumoniae* strains

Same data processing method and selection criteria were carried out to analyze metabolic profiles of *K. pneumoniae* KP-1 mutants as the other two bacteria. Pre-processed peak values of 5 replicates of each *K. pneumoniae* mutant were used to construct PCA (Figure 3.7).

Figure 3.7 PCA plot of metabolites in *K. pneumoniae/pYedQ2* and *K.pneumoniae/pYhjH*. Clear separation could be seen between replicates of each mutant along PC1. *K. pneumoniae /pYhjH* replicates showed greater variation along both PC. *K. pneumoniae/pYedQ2* replicates are denoted by 'High' with red triangles; *K. pneumoniae/pYhjH* replicates are denoted by 'Low' with green crosses. Fold change ≥ 2 , p-value ≤ 0.05 , n=5

PC1 accounts for 30.4% of total variance while PC2 accounts for another 16.5% of total variance. Replicates of *K. pneumoniae/pYhjH* are more diversified comparing to those of *K. pneumoniae/pYedQ2* mutant as the replicates are more scattered along both PC1 and PC2. Replicates of *K. pneumoniae/pYhjH* clustered together and varied more along PC2 comparing to PC1. This indicates a greater variation among *K. pneumoniae/pYhjH* replicates and a higher similarity among *K. pneumoniae/pYedQ2* replicates. 232 metabolites were found to be over-expressed while 150 were found to be under-expressed in *K. pneumoniae/pYedQ2* mutant. 100 of most significantly regulated metabolites in each mutant were visualized by constructing heatmap (Figure 3.8).

Figure 3.8 Metabolites regulated in *K. pneumoniae/pYedQ2* and *K. pneumoniae/pYhjH*. Only top 100 most significantly regulated metabolites are illustrated. Similar pattern could be seen among replicates while distinct

M/Z	RT	Possible match	KEGG code
788.2747	3.12m	5,10-Methenyltetrahydromethanopterin	C04330
533.2461	3.09m	Galactan	C05796
589.1516	3m	GDP-4-amino-4,6-dideoxy-alpha-D-mannose	C20638
837.3028	3.83m	Uroporphyrinogen III	C01051
389.2421	0.8m	Ubiquinol-3	C00390
191.1564	3.06m	5-Methoxytryptamine	C05659
992.2624	3m	3-Oxotetradecanoyl-CoA	C05261
582.2438	2.76m	Streptomycin	C00413
401.3022	3.34m	26-Hydroxycholest-4-en-3-one	C05455
557.2537	2.62m	3-Vinylbacteriochlorophyllide d	C18157
163.1165	0.7m	Carnitine	C00487
587.2603	3.12m	(3Z)-Phycocyanobilin	C05786
128.126	3.06m	O-Phosphoseryl-tRNA(Cys)	C17022
143.1215	0.5m	2-Methylnaphthalene	C14098
257.0939	0.5m	Nicotinate D-ribonucleoside	C05841
851.2847	3.09m	Precorrin-1	C02463
228.1378	1.11m	Deoxycytidine	C00881
591.1086	3.01m	UDP-2-acetamido-4-amino-2,4,6-trideoxy-alpha-D-glucose	C04630
863.2484	2.94m	Sirohydrochlorin	C05778
119.0901	0.72m	Betaine	C00719
180.1295	0.79m	7-Aminomethyl-7-carbaguanine	C16675
751.5968	2.79m	Plastoquinol-9	C16695
180.1329	0.97m	7-Aminomethyl-7-carbaguanine	C16675
156.1333	2.97m	Deisopropylhydroxyatrazine	C06557
155.1375	3.12m	(S)-(-)-Citronellal	C11384
255.1447	1.11m	Lactenocin	C12003
128.1255	1.1m	O-Phosphoseryl-tRNA(Cys)	C17022
136.117	0.72m	2-Phenylacetamide	C02505
168.1213	3m	3-Methoxytyramine	C05587
176.157	0.67m	Carboxynorspermidine	C18174
159.1474	3.15m	Protamine	D02224
191.1559	1.1m	5-Methoxytryptamine	C05659
152.1116	3.36m	N-Methyltyramine	C02442
387.2912	2.94m	Ubiquinone-3	C00399
146.0904	0.69m	(S)-5-Amino-3-oxohexanoic acid	C03656
94.0936	0.73m	Aniline	C00292
585.3202	3.72m	Bilirubin	C00486
138.1105	3m	2,4,6-Triaminotoluene	C16400
201.1398	0.68m	3,6,8-Trimethylallantoin	C16362
186.1304	3m	Ecgonine	C10858

91.1078	3.15m	(R,R)-Butane-2,3-diol	C03044
455.2684	3.41m	Ribostamycin	C01759
94.0933	1.94m	Aniline	C00292
142.1209	3.15m	Tropine	C00729
226.1204	3m	4-Amino-4-deoxychorismate	C11355
180.1347	3.14m	7-Aminomethyl-7-carbaguanine	C16675
118.1202	3.15m	5-Aminopentanoate	C00431
97.0825	3m	Fluorobenzene	C11272
146.1155	3.15m	6-Amino-2-oxohexanoate	C03239
373.203	0.68m	Biocytin	C05552
170.1151	3.15m	L-Noradrenaline	C00547
188.1245	3.15m	8-Amino-7-oxononanoate	C01092
191.1299	0.55m	5-Methoxytryptamine	C05659
143.1283	3.15m	2-Methylnaphthalene	C14098
110.0896	1m	2-Aminophenol	C01987
275.1143	8.54m	N2-Succinyl-L-arginine	C03296
91.03	0.54m	L-Glyceraldehyde	C02426
897.4133	13.78m	Precorrin 6Y	C06319
130.1047	0.71m	4-Guanidinobutanal	C02647
136.1169	0.85m	2-Phenylacetamide	C02505
240.1471	0.87m	6-Lactoyl-5,6,7,8-tetrahydropterin	C04244

Table 3.9 List of upregulated metabolites in *K.pneumoniae/pYedQ2* with a hit in the database. Each matched metabolite was assigned with a KEGG compound code. Only hits with highest similarity are illustrated here based on molecular weight of each metabolite. Adduct type was set as M+H; tolerance of molecular weight was set as 0.05 Da.

Six metabolites are involved in porphyrin and chlorophyll metabolism including precorrin 6Y, coproporphyrin III, sirohydrochlorin, (3Z)-phycocyanobilin, bilirubin and 3-vinylbacteriochlorophyllide. Four metabolites are involved in biosynthesis of secondary metabolites, including ubiquinone, tropine, precorrin 6Y, and sirohydrochlorin. Four metabolites are involved in arginine and proline metabolism, including 5-aminopentanoate, 4-guanidinobutanal, N2-succinyl-L-arginine and carboxynorspermidine. Four metabolites involved in lysine degradation, including 5-

aminopentanoate, carnitine, 6-amino-2-oxohexanoate and (S)-5-amino-3-oxohexanoic acid.

Two metabolites are involved in biosynthesis of antibiotics, including streptomycin and ribostamycin. Three are involved in ABC transporters including carnitine, betaine, protamine sulfate (JP17/USP/INN); while three are involved in tyrosine metabolism including L-noradrenaline, N-methyltyramine and 3-methoxytyramine. 6-lactoyl-5,6,7,8-tetrahydropterin, 4-amino-4-deoxychorismate, and 7-aminomethyl-7-carbaguanine are for folate biosynthesis. 2-aminophenol and 5-methoxytryptamine are for tryptophan metabolism. Ubiquinone and plastoquinol-9 are for ubiquinone and other terpenoid-quinone biosynthesis while fluorobenzene and 2-Methylnaphthalene are for degradation of aromatic compounds. Ubiquinol, ubiquinone and L-noradrenaline are involved in oxidative phosphorylation and two-component system. UDP-2-acetamido-4-amino-2,4,6-trideoxy-alpha-D-glucose and GDP-4-amino-4,6-dideoxy-alpha-D-mannose are involved in amino sugar and nucleotide sugar metabolism. Aminobenzoate degradation includes aniline and 2-aminophenol while biotin metabolism includes 8-amino-7-oxononanoate and biocytin.

For those single metabolites involved in multiple pathways, 3-oxotetradecanoyl-CoA is involved in fatty acid metabolism and serine and threonine metabolism. 5,10-methenyl-tetrahydromethanopterin is involved in carbon metabolism and methane metabolism.

Other metabolites upregulated including deoxycytidine, L-glyceraldehyde, O-phosphoseryl-tRNA(Cys), nicotinate D-ribonucleoside, 2-methylnaphthalene, strepto-

mycin, (S)-(-)-citronellal, 2,4,6-triaminotoluene, fluorobenzene, galactan, 2-phenylacetamide, (R,R)-butane-2,3-diol, protamine sulfate (JP17/USP/INN), deisopropylhydroxyatrazine, betaine, and 2-phenylacetamide, are involved in pyrimidine metabolism, pentose and glucuronate interconversions, aminoacyl-tRNA biosynthesis, nicotinate and nicotinamide metabolism, naphthalene degradation, streptomycin biosynthesis, geraniol degradation, nitrotoluene degradation, fluorobenzoate degradation, galactose metabolism, styrene degradation, butanoate metabolism, cationic antimicrobial peptide (CAMP) resistance, atrazine degradation, glycine, fatty acid degradation, and phenylalanine metabolism. Individual metabolic pathways containing upregulated metabolites in *K. pneumoniae/pYedQ2* are listed in Table 3.10.

KEGG map No.	Pathways involved	No. of compounds involved
kpt00860	Porphyrin and chlorophyll metabolism	7
kpt00310	Lysine degradation	4
kpt00330	Arginine and proline metabolism	4
kpt00350	Tyrosine metabolism	3
kpt02010	ABC transporters	3
kpt00790	Folate biosynthesis	3
kpt02020	Two-component system	2
kpt00380	Tryptophan metabolism	2
kpt00190	Oxidative phosphorylation	2
kpt00627	Aminobenzoate degradation	2
kpt00520	Amino sugar and nucleotide sugar metabolism	2
kpt00780	Biotin metabolism	2
kpt00130	Ubiquinone and other terpenoid-quinone biosynthesis	2
kpt01212	Fatty acid metabolism	1
kpt00680	Methane metabolism	1
kpt00633	Nitrotoluene degradation	1
kpt00040	Pentose and glucuronate interconversions	1
kpt00626	Naphthalene degradation	1
kpt00240	Pyrimidine metabolism	1
kpt00071	Fatty acid degradation	1

kpt01200	Carbon metabolism	1
kpt00521	Streptomycin biosynthesis	1
kpt00360	Phenylalanine metabolism	1
kpt00052	Galactose metabolism	1
kpt00970	Aminoacyl-tRNA biosynthesis	1
kpt01503	Cationic antimicrobial peptide (CAMP) resistance	1
kpt00760	Nicotinate and nicotinamide metabolism	1
kpt00281	Geraniol degradation	1
kpt00643	Styrene degradation	1
kpt00260	Glycine, serine and threonine metabolism	1
kpt00650	Butanoate metabolism	1
kpt00791	Atrazine degradation	1
kpt00364	Fluorobenzoate degradation	1

Table 3.10 Pathways involving upregulated metabolites in *K. pneumoniae/pYedQ2*.

Pathways assigned were based on the compounds listed in Table 3.9.

For all downregulated metabolites in *K. pneumoniae/pYedQ2*, one metabolite was matched with pyocyanine due to its similar in molecular weight to that of *P. aeruginosa/pYedQ2*. However, the retention times of the two metabolites were different. Further analysis is needed to confirm their identity. Other downregulated metabolites with database match and KEGG matches are listed in Table 3.11.

M/Z	RT	Possible match	KEGG code
327.1163	10.86m	cis-beta-D-Glucosyl-2-hydroxycinnamate	C05839
177.1402	0.99m	N(pi)-Methyl-L-histidine	C01152
457.1249	3.72m	FMN	C00061
196.1136	3.9m	2-Amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine	C01300
217.1555	3.05m	Gamma-glutamyl-gamma-aminobutyraldehyde	C15700
155.1364	1.15m	(S)-(-)-Citronellal	C11384
560.0779	3.44m	Adenosine diphosphate ribose	C00301
231.1477	2.89m	Thioredoxin	C00342
428.0603	3.53m	ADP	C00008
231.1477	1.15m	Thioredoxin	C00342
327.1585	3.74m	6,7-dimethyl-8-(D-ribityl)lumazine	C04332

340.1007	2.92m	5-carboxyamino-1-(5-phospho-D-ribose)imidazole	C15667
284.1429	2.57m	Guanosine	C00387
157.0981	2.75m	(1R,6S)-1,6-Dihydroxycyclohexa-2,4-diene-1-carboxylate	C06321
245.1077	2.75m	1,2-Bis(4-hydroxyphenyl)-2-propanol	C13629
116.0722	2.24m	L-Proline	C00148
100.0756	2.75m	(2R)-2-Hydroxy-2-methylbutanenitrile	C18796
142.0874	2.75m	L-Histidinol	C00860
144.0667	2.75m	5-(2-Hydroxyethyl)-4-methylthiazole	C04294
116.0713	2.74m	L-Proline	C00148
76.0731	2.74m	Dihydrobiopterin	C00268
170.0816	2.75m	Pyridoxine	C00314
203.1009	2.75m	Indolepyruvate	C00331
162.0766	2.75m	o-acetyl-l-homoserine	C01077
		D-Alanyl-(R)-lactate	C19694
275.1149	2.75m	N2-Succinyl-L-arginine	C03296
118.0868	2.75m	L-Valine	C00183
221.1101	2.75m	5-Hydroxy-L-tryptophan	C00643
245.1611	0.72m	2,2-Bis(4-hydroxyphenyl)-1-propanol	C13631
146.0826	2.75m	(S)-5-Amino-3-oxohexanoic acid	C03656
185.1461	3.33m	(3R)-3-Isopropenyl-6-oxoheptanoate	C11405
213.1381	3.33m	Pyocyanine	
585.281	3.56m	15,16-Dihydrobiliverdin	C11630
387.2226	9.5m	Ubiquinone-3	C00399
373.1729	9.24m	Biocytin	C05552
685.3472	4.46m	Ferrichrome minus Fe(III)	C06228
437.3849	12.22m	2-Phytyl-1,4-naphthoquinone	C13309
411.3744	12.04m	5-Dehydroavenasterol	C15783
451.3983	13.21m	Phylloquinone	C02059

Table 3.11 Downregulated metabolites in *K.pneumoniae/pYedQ2* with a hit in the database. Each matched metabolite was assigned with a KEGG compound code. Only hits with highest similarity are illustrated here based on molecular weight of each metabolite. Adduct type was set as M+H; tolerance of molecular weight was set as 0.05 Da.

Thirteen molecules are involved in biosynthesis of secondary metabolites, including ADP, FMN, L-proline, L-valine, ubiquinone, L-histidinol, phylloquinone, 6,7-

dimethyl-8-(D-ribityl)lumazine, cis-beta-D-glucosyl-2-hydroxycinnamate, 2-phytyl-1,4-naphthoquinone, 5-carboxyamino-1-(5-phospho-D-ribosyl)imidazole, 5-dehydroavenasterol, and (2R)-2-Hydroxy-2-methylbutanenitrile. Six metabolites are involved in biosynthesis of antibiotics including ADP, FMN, L-proline, L-valine, O-acetyl-L-homoserine and 5-carboxyamino-1-(5-phospho-D-ribosyl)imidazole. Four metabolites are involved in purine metabolism including ADP, ADP-ribose, guanosine and 5-carboxyamino-1-(5-phospho-D-ribosyl) imidazole.

Three metabolites are involved in biosynthesis of amino acids including L-proline, L-valine and L-histidinol and three are involved in arginine and proline metabolism including L-proline, N2-succinyl-L-arginine and gamma-glutamyl-gamma-amino-butyraldehyde. L-proline, L-valine and ferrichrome are for ABC transporters. Ubiquinone and other terpenoid-quinone biosynthesis includes ubiquinone, phyloquinone and 2-phytyl-1,4-naphthoquinone while oxidative phosphorylation utilizes ADP, FMN and ubiquinone. Indolepyruvate and 5-hydroxy-L-tryptophan are involved in tryptophan metabolism while L-histidinol and N(pi)-methyl-L-histidine are involved in histidine metabolism.

L-valine and (2R)-2-hydroxy-2-methylbutanenitrile are involved in cyanoamino acid metabolism while dihydrobiopterin and 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine are involved in folate biosynthesis. Two metabolites involved in riboflavin metabolism are FMN and 6,7-dimethyl-8-(d-ribityl)lumazine while two metabolites, L-proline and L-valine, are involved in aminoacyl-tRNA biosynthesis. (1R,6S)-1,6-dihydroxy-cyclohexa-2,4-diene-1-carboxylate and (3R)-3-isopropenyl-6-oxoheptanoate are involved in degradation of aromatic compounds. L-valine is

involved in 2-oxocarboxylic acid metabolism, pantothenate and CoA biosynthesis, valine, leucine and isoleucine degradation and biosynthesis. L-proline is involved in novobiocin biosynthesis and carbapenem biosynthesis.

Other metabolites downregulated in this strain include biocytin, (S)-(-)-citronellal, D-alanyl-(R)-lactate, (S)-5-amino-3-oxohexanoic acid, O-acetyl-L-homoserine, pyridoxine, ubiquinone, 5-(2-hydroxyethyl)-4-methylthiazole, (1R,6S)-1,6-dihydroxycyclohexa-2,4-diene-1-carboxyl-ate, 15,16-dihydrobiliverdin, thioredoxin and (3R)-3-isopropenyl-6-oxoheptanoate which are involved in biotin metabolism, geraniol degradation, vancomycin resistance, lysine degradation, cysteine and methionine metabolism, vitamin B6 metabolism, two-component system, thiamine metabolism, benzoate degradation, porphyrin and chlorophyll metabolism, pyrimidine metabolism and limonene and pinene degradation. Individual metabolic pathways containing downregulated metabolites in *K. pneumoniae/pYedQ2* are listed in Table 3.12 below.

KEGG map No.	Pathways involved	No. of compounds involved
kpt00230	Purine metabolism	4
kpt00130	Ubiquinone and other terpenoid-quinone biosynthesis	3
kpt02010	ABC transporters	3
kpt00330	Arginine and proline metabolism	3
kpt01230	Biosynthesis of amino acids	3
kpt00190	Oxidative phosphorylation	3
kpt00340	Histidine metabolism	2
kpt00380	Tryptophan metabolism	2
kpt00460	Cyanoamino acid metabolism	2
kpt00790	Folate biosynthesis	2
kpt00970	Aminoacyl-tRNA biosynthesis	2
kpt00740	Riboflavin metabolism	2
kpt01220	Degradation of aromatic compounds	2
kpt00401	Novobiocin biosynthesis	1
kpt00903	Limonene and pinene degradation	1

kpt00770	Pantothenate and CoA biosynthesis	1
kpt00290	Valine, leucine and isoleucine biosynthesis	1
kpt00281	Geraniol degradation	1
kpt00750	Vitamin B6 metabolism	1
kpt02020	Two-component system	1
kpt00270	Cysteine and methionine metabolism	1
kpt00860	Porphyrin and chlorophyll metabolism	1
kpt01502	Vancomycin resistance	1
kpt00310	Lysine degradation	1
kpt00730	Thiamine metabolism	1
kpt00240	Pyrimidine metabolism	1
kpt00332	Carbapenem biosynthesis	1
kpt00280	Valine, leucine and isoleucine degradation	1
kpt00780	Biotin metabolism	1
kpt01210	2-Oxocarboxylic acid metabolism	1
kpt00362	Benzoate degradation	1

Table 3.12 Pathways involving downregulated metabolites in *K. pneumoniae/pYedQ2*.

Pathways assigned are based on the compounds listed in Table 3.11.

3.2.1.4. Comparison among mutants of different bacteria

Through comparison between either three high c-di-GMP mutants or three low c-di-GMP mutants, quite a number of metabolites were found upregulated in high c-di-GMP strains. 2 common upregulated metabolites were discovered between *P. aeruginosa/pYedQ2* and *K. pneumoniae /pYedQ2* (Table 3.13). Both of these two metabolites could not be matched to any compound in the database.

Feature	Possible Match	Strain	Fold change	p-value
591.2555 / 2.6m	No match	<i>P. aeruginosa/pYedQ2</i>	30.6	0.02293
		<i>K. pneumoniae/pYedQ2</i>	558.4	1.71E-09
644.2727 / 3.03m	No match	<i>P. aeruginosa/pYedQ2</i>	62.8	0.013508
		<i>K. pneumoniae/pYedQ2</i>	28.563	0.000724

Table 3.13 Metabolites upregulated in *P. aeruginosa/pYedQ2* and *K. pneumoniae/pYedQ2*. Feature is denoted by m/z value/retention time. Metabolites without database hit are listed as ‘No match’. Fold change ≥ 2 , p-value ≤ 0.05 .

15 common upregulated featured were found between *P. aeruginosa/pYedQ2* and *B.cenocepacia/pYedQ2* (Table 3.14). Six out of fifteen metabolites have database hits and match to only four KEGG compounds. Two metabolites were recognized as same compound due to the similarity in their molecular weight. N-methyltyramine is involved in tyrosine metabolism whereas 2,3-bis-O-(geranylgeranyl)glycerol 1-phosphate is involved in glycerophospholipid metabolism. However, demethylmenaquinol is not involved in any metabolic pathway.

Feature	Possible Match (KEGG code)	Strain	Fold change	p-value
691.0772/4.12m	Cyclic di-3',5'- Guanylate (C16463)	<i>P. aeruginosa/pYedQ2</i>	4931.8	1.02E-07
		<i>B. cenocepacia/pYedQ2</i>	11193	0.004002
152.1122/4.13m	N-Methyltyramine (C02442)	<i>P. aeruginosa/pYedQ2</i>	239.82	1.09E-05
		<i>B. cenocepacia/pYedQ2</i>	1607.8	0.003989
718.0299/4.13m	No match	<i>P. aeruginosa/pYedQ2</i>	570	4.96E-08
		<i>B. cenocepacia/pYedQ2</i>	722.2	0.004044
718.5299/4.14m	No match	<i>P. aeruginosa/pYedQ2</i>	226	3.31E-08
		<i>B. cenocepacia/pYedQ2</i>	255.4	0.004071
703.0527/4.13m	No match	<i>P. aeruginosa/pYedQ2</i>	207.6	1.46E-07
		<i>B. cenocepacia/pYedQ2</i>	248.2	0.004157
540.0564/4.13m	No match	<i>P. aeruginosa/pYedQ2</i>	166.4	4.03E-08
		<i>B. cenocepacia/pYedQ2</i>	228.6	0.004154

135.0859/4.13m	1-Deoxyxylonojirimycin	<i>P. aeruginosa/pYedQ2</i>	113	2.74E-08
		<i>B. cenocepacia/pYedQ2</i>	221.5	0.004938
717.5305/4.12m	2,3-Bis-O-(geranylgeranyl)glycerol 1-phosphate (C04638)	<i>P. aeruginosa/pYedQ2</i>	186	2.31E-07
		<i>B. cenocepacia/pYedQ2</i>	138.6	0.004139
1036.0382/4.12m	No match	<i>P. aeruginosa/pYedQ2</i>	196.8	0.00192
		<i>B. cenocepacia/pYedQ2</i>	135.8	0.004502
744.9827/4.15m	No match	<i>P. aeruginosa/pYedQ2</i>	53.6	5.01E-05
		<i>B. cenocepacia/pYedQ2</i>	105.17	0.005163
710.0463/4.13m	No match	<i>P. aeruginosa/pYedQ2</i>	104.4	5.97E-06
		<i>B. cenocepacia/pYedQ2</i>	101.8	0.004156
705.5502/12.27m	2-Demethylmenaquinol 8 (C19847)	<i>P. aeruginosa/pYedQ2</i>	10.4	0.041488
		<i>B. cenocepacia/pYedQ2</i>	65.667	0.040799
756.4498/13.03m	No match	<i>P. aeruginosa/pYedQ2</i>	13.1	0.002013
		<i>B. cenocepacia/pYedQ2</i>	35.8	0.032979
705.5781/12.2m	2-Demethylmenaquinol 8 (C19847)	<i>P. aeruginosa/pYedQ2</i>	74.878	0.001214
		<i>B. cenocepacia/pYedQ2</i>	9.1781	0.017308
752.4726/12.17m	No match	<i>P. aeruginosa/pYedQ2</i>	3.9308	0.017516
		<i>B. cenocepacia/pYedQ2</i>	4.1905	0.045628

Table 3.14 Common metabolites upregulated in *P. aeruginosa/pYedQ2* and *B. cenocepacia/ pYedQ2*. Feature is denoted by m/z value/retention time. Metabolites with database hit are listed as “Compound (KEGG code). Metabolites without database hit are listed as ‘No match’. Fold change ≥ 2 , p-value ≤ 0.05 .

1 common downregulated featured identified among *P. aeruginosa/pYedQ2*, *K. pneumoniae/pYedQ2* and *B.cenocepacia/pYedQ2*. (Table 3.15)

Feature	Possible Match	Strain	Fold change	p-value
219.2278/ 13.95m	No match	<i>P. aeruginosa/pYedQ2</i>	24.83361478	0.008954
		<i>K. pneumoniae/pYedQ2</i>	12.28577	0.000309
		<i>B. cenocepacia/pYedQ2</i>	4.181826	0.030487

Table 3.15 Common metabolite downregulated among *P. aeruginosa/pYedQ2*, *B. cenocepacia/pYedQ2* and *K. pneumoniae/pYedQ2*. Feature is denoted by mz value/retention time. Metabolotes without database hit are listed as ‘No match’. Fold change ≥ 2 , p-value ≤ 0.05 .

3.2.1.5. Relative quantification of L-methionine

The relative amount of L-methionine was calculated by normalizing the LC/MS peak values with total protein concentration of 1 mL of sample cells (Figure 3.10). Levels of methionine synthesized by *B. cenocepacia* mutants were similar while same phenomenon was observed in *K. pneumoniae* mutants. However, amount of methionine synthesized by *P. aeruginosa /pYedQ2* was found to be lower than that synthesized in *P. aeruginosa/pYhjH*. Such result is different from what observed from transcriptomics analysis and will be discussed in the following section.

Figure 3.10. Relative methionine level in mutants of three pathogens. Methionine concentrations were calculated relatively by LC/MS peak values per total protein of 1 mL cells. A. Relative methionine level in *P. aeruginosa*/pYhjH and *P. aeruginosa*/ pYedQ2. B. Relative methionine level in *B. cenocepacia*/pYhjH and *B. cenocepacia*/pYedQ2. C. Relative methionine level in *K. pneumoniae*/pYhjH and *K. pneumoniae*/pYedQ2. Mean value of triplicates were taken and error bar was calculated based on standard deviation.

3.3. Discussion

Metabolomics analysis is a powerful approach to learn metabolic fingerprints in all organisms by using high throughput analysis technologies such as NMR or LC/MS. It is frequently utilized in retrieving the metabolic profiles of biofilms formed by different bacteria. Many previous studies applied either targeted or non-targeted

metabolomics analysis in discovering disease biomarkers such as identification of biomarkers of nonalcoholic fatty liver disease [314], chronic obstructive pulmonary disease (COPD) [315], and lung cancer [316]; and also different metabolic patterns between planktonic cells and biofilm cells of *P. aeruginosa* [317] and other bacteria like *S. aureus* [318]. However, current limitation in LC/MS is its small size of database for data matching and identification for non-targeted metabolomics analysis. [239-243]

To discover the common metabolites regulated by c-di-GMP, metabolic profiles were obtained here by LC/MS illustrating peak values, m/z values and retention time. Primary database matching was done using molecular weight of metabolites while matched metabolites were mapped to respective pathways. All pathway mappings in this study were done based on KEGG pathway mapping database.

3.3.1. Metabolomics analysis of *P. aeruginosa*

Based on the primary matching results of all metabolites regulated in *P. aeruginosa/pYedQ2*, duplicate matches were removed and other matched metabolites with KEGG compound code were analysed. Duplicated matches include 5-Amino-6-(5'-phosphoribosylamino)uracil, N-Methyltyramine, Deoxyribose, Isofucosterol, 5'-Methylthioadenosine, 7-Aminomethyl-7-carbaguanine, 4-Guanidinobutanal, Homocarnosine, Betaine, (2R,3R)-3-Methylglutamyl-5-semialdehyde-N6-lysine, (R,R)-Butane-2,3-diol and curcumin. These duplicated matches may due to similarity in their molecular weights. Such mismatches could induce higher degree of uncertainty in pathway mapping and will not be considered.

For other matched metabolites with higher degree of accuracy, most significant finding is upregulation of histamine and downregulation of Imidazole-4-acetaldehyde along histidine metabolism pathway in *P. aeruginosa/pYedQ2*. Histamine is formed from L-histidine catalysed by histidine decarboxylase. Histamine can be transformed into three different compounds depending different functional enzymes, being imidazole-4-acetaldehyde catalysed by histaminase, 4-(beta-Acetylaminoethyl) imidazole catalysed by unidentified enzyme and N-methylhistamine catalysed by histamine N-methyltransferase. As Imidazole-4-acetaldehyde was found to be downregulated, histamine was possibly transformed into the other two compounds, 4-(beta-Acetylaminoethyl) imidazole and N-methylhistamine. Notably, the formation of N-methylhistamine also requires another reactant S-adenosyl-L-methionine, which is the product of L-methionine degradation (Figure 3.11)

Moreover, L-methionine is encoded by the initiation codon of mRNA and is the starting amino acid translated in polypeptides. [319] Thus, excessive L-methionine produced in high c-di-GMP strains may be used up by other cell metabolic reactions such as histamine formation and protein synthesis, thus, reduced level of L-methionine in *P. aeruginosa/pYedQ2* observed could be explained. Previous study also revealed that *P. aeruginosa* and several other Gram negative bacteria synthesize clinically significant level of histamine as inflammatory inducer to enhance their pathogenicity during respiratory infections. [320] This makes histamine a potential biomarker of biofilm infections caused by *P. aeruginosa*.

Figure 3.11 Formation of S-adenosyl-L-methionine from L-methionine. L-methionine transforms to S-adenosyl-L-methionine is indicated by red arrow; S-adenosyl-L-methionine transforms to AHL and S-methyl-5'-thioadenosine is indicated by blue arrow. Figure is modified from KEGG pathway of cysteine and methionine metabolism.[251, 252]

Besides histamine, spermidine, which is involved in arginine and proline metabolism and glutathione metabolism, was also found to be upregulated in *P. aeruginosa/pYedQ2*. Spermidine is formed from arginine with agmatine and putrescine as intermediates along arginine and proline metabolism pathway while spermidine could be formed from both glutathione and putrescine derived from arginine biosynthesis pathway. Formation of spermidine from putrescine is catalysed by SpeE or gene product of PA4774 which did not show significant change in expression level as indicated by RNA-seq analysis. Thus, the increase in spermidine may be resulted from arginine or the other reaction from glutathione or due to wrong database matching. However, previous study showed that spermidine residing on cell surface enhances membrane resistance to antibiotic treatment and peroxide attack of *P.*

aeruginosa. [321] Further analysis is necessary to confirm the expression level of spermindine in high c-di-GMP mutant in order to consider it as a potential biofilm biomarker.

Another interesting finding is the downregulation of both 4-Amino-4-deoxychorismate along folate biosynthesis pathway and formyl-N-acetyl-5-methoxykynurenamine along tryptophan metabolism. 4-Amino-4-deoxychorismate is derived from its precursor, chorismate. Thus, downregulation of 4-Amino-4-deoxychorismate might result in accumulation of chorismate. Chorismate could be integrated into tryptophan biosynthesis as the precursor of anthranilate. However, formyl-N-acetyl-5-methoxykynurenamine, an end-product of tryptophan metabolism, was down-regulated. There are several different pathways that tryptophan could adapt to and generates different final products. Tryptophan may degrade into other compounds or there might be low level of tryptophan synthesized. With accumulation of chorismate and less tryptophan biosynthesis, more anthranilate might be produced. As anthranilate is precursor of PQS [184] (Figure 1.9), *pqs* quorum sensing might be affected under such circumstances.

From overall database matching and pathway mapping results of *P. aeruginosa* strains, majority of the pathways are related to amino acid metabolism. Such as along arginine and proline metabolism pathway, downregulation of 4-guanidinobutanamide, which is formed from reaction of L-arginine and oxygen with carbon dioxide and water as side products, was seen. Along lysine biosynthesis/degradation pathway, carnitine was upregulated and N6-(L-1,3 dicarboxyprop-yl)-L-lysine was downregulated. Carnitine is one of the two end-products of degradation of peptidyl-L-lysine. N6-(L-1,3-

dicarboxypropyl)-L-lysine is one of immediate products of L-lysine which leads to the formation of acetyl-CoA for citrate cycle for lysine degradation. This compound is produced from L-2-Aminoadipate 6-semialdehyde and gives rise to L-lysine with 2-Oxoglutarate from citrate cycle and acetyl-CoA as starting materials for lysine biosynthesis. Moreover, along thiamine metabolism, 1-deoxy-D-xylulose 5-phosphate was upregulated. 1-deoxy-D-xylulose 5-phosphate is formed from pyruvate and glyceraldehyde 3-phosphate with catalysation by product of *dxs* (PA4044) gene for thiamine synthesis. Along phenylalanine metabolism pathway, D-phenylalanine was downregulated. D-phenylalanine may be derived reversibly from L-phenylalanine or phenylpyruvate by product of PA5084 or PA5304 (*dadA*). Decrease of D-phenylalanine may be caused by decrease of these two compounds or their precursors and may lead to decrease in N-Acetyl-D-phenylalanine production.

There are a number of other pathways affected including glutathione metabolism, terpenoid backbone biosynthesis pathway, benzoate degradation pathway, pyrimidine metabolism, purine metabolism, ubiquinone and other terpenoid-quinone biosynthesis, biotin metabolism and 2-Oxocarboxylic acid metabolism. Upregulated metabolites involving in these pathways include glutathione disulphide, 1-deoxy-D-xylulose 5-phosphate, 3-sulfocatechol, cyclohexane-1-carboxylate, thymidine, thioredoxin, ADP-ribose and 5-amino-4-imidazole-carboxamide, 2-hexaprenyl-3-methyl-6-methoxy-1,4-benzoquinone and biocytin. While, downregulated metabolites include (2Z,6Z)-farnesyl diphosphate, 4-Hydroxy-3-polyprenyl-benzoate and (-)-threo-Iso(homo)2-citrate.

3.3.2. Metabolomics analysis of *B. cenocepacia*

For all metabolites regulated in *B. cenocepacia/pYedQ2*, duplicate matches including N-methyltyramine, 3, 4-dehydrorhodopin, betaine, and sinapyl alcohol were removed for more precise pathway mapping. Other matched metabolites with KEGG compound code were mapped to respective pathway for further analysis. Similar to what has been observed from *P. aeruginosa* strain, there was a number of metabolites along several different amino acid metabolism pathways regulated in *B. cenocepacia/pYedQ2*. Among all, downregulation of 5'-methylthioadenosine along cysteine and methionine metabolism was found relevant to degradation of L-methionine. 5'-methylthioadenosine is derived from L-methionine with S-adenosyl-L-methionine and S-adenosylmethioninamine as intermediates, in the presence of functional enzymes MetK, SpeD, CepI and gene products of I35_0485/5927/7500. However, expression of genes encoding for these enzymes changed insignificantly based on RNA-seq results. Reduction in 5'-methylthioadenosine might due to less L-methionine synthesized.

Gene product of *metE* catalyzes L-methionine synthesis from L-homocysteine and S-adenosyl-L-homocysteine. There are reversible reactions from L-homocysteine and L-serine to L-cystathionine where L-homocysteine reacts with L-serine to form L-cystathionine, and L-cystathionine could also function as precursor of L-homocysteine reversely. As database matching results indicated that 3-phospho-D-glycerate, an intermediate of serine formation, was downregulated, level of L-serine synthesized might be reduced. Thus, synthesis of L-homocysteine and L-methionine was possibly affected under such situation. This may give a clue on the insignificant change in L-methionine synthesis in *B. cenocepacia/pYedQ2* strain although there was upregulated *metE* expression.

Moreover, S-adenosyl-L-methionine could give rise to both 5'-methylthioadenosine and N-acyl-L-homoserine lactone (AHL) directly (Figure 3.11). Lactonized S-adenosyl-L-methionine results in AHL formation with catalyzation of CepI.[322] Such observation may indirectly indicate the regulation of quorum sensing molecules by c-di-GMP in *B. cenocepacia*.

3-phospho-D-glycerate is also involved in both of pentose phosphate pathway and glycolysis/gluconeogenesis pathways. As the sole carbon source used in this study is glucose, glycolysis/gluconeogenesis pathway might be affected directly in *B. cenocepacia/pYedQ2*. As 3-phospho-D-glycerate could be formed from several starting compounds including D-glucose 6-phosphate, D-glucose and D-glucosamine along both pathways, the reduction of 3-phospho-D-glycerate might be caused by multiple reactions along these paths.

Similar to what observed from *P. aeruginosa/pYedQ2*, amino acid metabolism including tryptophan metabolism and lysine degradation were also affected in *B. cenpcepacia/pYedQ2*. Along tryptophan metabolism, 5-methoxyindoleacetate was upregulated while 5-methoxytryptamine was downregulated. Similar to that of *P. aeruginosa*, 5-methoxyindoleacetate is one of the products of typtophan metabolism through the formation of serotonin. 5-Methoxytryptamine is another product of the same pathway. It may suggest that *B. cenocepacia/pYedQ2* prefers the tryptophan metabolism pathway generating 5-methoxyindoleacetate rather than that generating 5-methoxytryptamine. This may due to different enzymes regulated in *B. cenocepacia/pYedQ2*. Along Lysine degradation, N6-Hydroxy-L-lysine was

downregulated. N6-Hydroxy-L-lysine is derived from L-lysine in the presence of gene product of I35_7041, and leads to formation of aerobactin.

Another interesting observation is the downregulation of flavin denine dinucleotide (FAD) along riboflavin metabolism. FAD is derived from riboflavin with FMN as intermediate. Riboflavin plays different roles in different organisms, such as functioning as shuttle for extracellular electron transport in *Shewanella oneidensis* MR-1 [323] and reducing iron in *Helicobacter pylori* [324]. Riboflavin was also proved to trigger LasR receptors in bacterial quorum sensing system, such as that in *P. aeruginosa* [325]. Thus, although there is no specific report on riboflavin and quorum sensing in *B. cenocepacia*, it is possible that variation in riboflavin and its derivatives induced by c-di-GMP may be able to lead to changes in quorum sensing in *B. cenocepacia* since this bacterium functions similar to *P. aeruginosa* in many aspects.

Other pathways possibly regulated in *B. cenocepacia/pYedQ2* include ubiquinone and other terpenoid-quinone biosynthesis, polycyclic aromatic hydrocarbon degradation, taurine and hypotaurine metabolism, chlorocyclohexane and chlorobenzene degradation, biotin metabolism, dioxin degradation, cyanoamino acid metabolism, monobactam biosynthesis, benzoate degradation and aminobenzoate degradation.

Along cyanoamino acid metabolism, Prunasin was downregulated. Prunasin is an intermediate along L-Phenylalanine metabolism pathway which is formed from mandelonitrile catalysed by gene product of I35_2480 or I35_5177. I35_5177 encoding for beta-glucosidase was found to be downregulated for nearly three folds by

RNA-seq. Decrease in prunasin might due to the reduction in gene expression of I35_5177.

3.3.3. Metabolomics analysis of *K. pneumoniae*

For all metabolites regulated in *K. pneumoniae/pYedQ2*, duplicate matches were removed and other matched metabolites with KEGG compound code were analysed. Duplicate matches include 5-methoxytryptamine, O-phosphoseryl-tRNA(Cys), 2-methylnaphthalene, 7-aminomethyl-7-carbaguanine, (S)-(-)-citronellal, 2-phenylacetamide, ubiquinone-3, (S)-5-amino-3-oxohexanoic acid, aniline, biocytin, N2-succinyl-L-arginine, thioredoxin and L-proline.

Unlike the case observed in *P. aeruginosa/pYedQ2* and *B. cenocepacia/pYedQ2*, *metE* expression downregulated in *K. pneumoniae/pYedQ2*. However, downregulated ones were left out as the main target of this study is to discover upregulated metabolites as potential biomarkers. Thus, measurement of expression of *metE* in *K. pneumoniae/pYedQ2* was excluded during qPCR analysis. However, metabolomics analysis could give a clue on L-methionine synthesis in this mutant. LC/MS quantification indicated that there was insignificant difference in L-methionine level between two *K. pneumoniae* mutants.

By mapping the matched metabolites to their respective pathways, it was observed that L-methionine has been involved in various reactions along different pathways. Such as along tyrosine metabolism, L-noradrenaline, N-methyltyramine and 3-methoxytyramine were upregulated. L-noradrenaline is synthesized from tyrosine through the formation of dopamine. 3-methoxytyramine is another product synthesized from

dopamine requiring S-adenosyl-L-methionine as another reactant. N-methyltyramine is synthesized from tyrosine through the formation of tyramine in the presence of S-adenosyl-L-methionine which is a derivative of L-methionine. Upregulation of N-methyltyramine may requires more S-adenosyl-L-methionine thus utilising more L-methionine.

However, along ubiquinone and other terpenoid-quinone biosynthesis, phyloquinone and 2-phytyl-1,4-naphthoquinone were downregulated. Phyloquinone is derived from phytyl diphosphate with 2-phytyl-1,4-naphthoquinone as intermediate. Conversion from 2-phytyl-1,4-naphthoquinone to phyloquinone requires the presence of S-adenosyl-L-methionine. Decrease in both of phyloquinone and 2-phytyl-1,4-naphthoquinone thus might reduce the requirement of L-methionine.

Moreover, along glycine, serine and threonine metabolism, betaine was upregulated. Betain is derived from choline through the formation of betaine aldehyde. It could be transformed to dimethylglycine and L-methionine in the presence of L-homocystein in a reversible manner. Increase in betain might lead to increase in L-methionine production. Beside this, L-histidinol and N (pi)-methyl-L-histidine was downregulated along histidine metabolism. L-histidine reacts with S-adenosyl-L-methionine to form N(pi)-methyl-L-histidine and S-adenosyl-L-homocysteine in a reversible manner. In addition, O-acetyl-L-homoserine was downregulated along cysteine and methionine metabolism. O-acetyl-L-homoserine leads to formation of L-homocystein which could be also evolved from L-methionine through a series of reactions. Therefore, such complex reactions might explain the negligible variation in L-methionine level between two *K. pneumoniae* mutants observed.

Similar to *P. aeruginosa/pYedQ2* and *B. cenocepacia/pYedQ2*, various other amino acid metabolism pathways were also affected by increase in c-di-GMP level in *K. pneumoniae*. Such as upregulation of 2-aminophenol, and downregulation of indolepyruvate and 5-hydroxy-L-tryptophan, was observed along tryptophan metabolism. 2-aminophenol is derived from anthranilate along tryptophan metabolism, with 3-hydroxyanthranilate as intermediate and gives rise to isophenoxazine. 5-Hydroxy-L-tryptophan is derived from tryptophan directly along a different path comparing to 2-aminophenol. It could give rise to three different molecules, being serotonin, 5-hydroxyindolepyruvate and 5-hydroxy-N-formylkynurenine.

Along lysine degradation, 5-aminopentanoate, carnitine and 6-amino-2-oxohexanoate were upregulated. 5-aminopentanoate could be formed through three paths from lysine degradation, either the formation of 6-acetamido-2-oxohexanoate, the formation of 5-aminopentanamide or that of cadaverine, and leads to the synthesis of acetyl-CoA. Carnitine is the endproduct of degradation of protein-lysine.

Along arginine and proline metabolism, 5-aminopentanoate, 4-guanidinobutanal, and carboxynorspermidine were upregulated. 5-aminopentanoate is the product of D-proline degradation. 4-guanidinobutanal is an intermediate synthesized from arginine through the formation of 2-oxoarginine for production of homocarnosine. Carboxynorspermidine is derived from putrescine and leads to the formation of norspermidine. Along arginine and proline metabolism, gamma-glutamyl-gamma-aminobutyraldehyde was downregulated. This molecule is evolved from putrescine through the formation of gamma-L-glutamylputrescine.

Same as that in *B. cenocepacia/pYedQ2*, metabolites along riboflavin metabolism were regulated in *K. pneumoniae/pYedQ2*. FMN and 6,7-Dimethyl-8-(D-ribityl)lumazine was downregulated. FMN is derived from riboflavin while 6,7-Dimethyl-8-(D-ribityl)lumazine is the direct precursor of riboflavin. Thus, synthesis of riboflavin should also be decreased. Besides riboflavin, ubiquinol which interacts with cytochrome bc1 complex was upregulated along oxidative phosphorylation. Ubiquinol is oxidized by donating one electron to cytochrome bc1 complex upon interaction.[326] As previous study indicated that riboflavin and cytochrome bc1 complex are both part of extracellular electron transport [323, 326], electron transport might be also modified by c-di-GMP in *K. pneumoniae*. Other regulated pathways in *K. pneumoniae/pYedQ2* include thiamine metabolism, folate biosynthesis, biotin metabolism, amino sugar and nucleotide sugar metabolism, pentose and glucuronate interconversions, purine metabolism, pyrimidine metabolism, cyanoamino acid metabolism, and etc.

All information obtained from primary database matching and pathway mapping of all metabolites regulated by c-di-GMP in three pathogens could give a glance of changes induced in their metabolic profiles. It is possible to consider those upregulated metabolites as potential biofilm biomarkers under c-di-GMP modulation. However, further confirmation test and more precise identification will be done based on these primary results to validate the identities of these upregulated metabolites.

These two chapters on comparison of transcriptomes and metabolomes of c-di-GMP mutants of the three pathogens give an overview of possibility of discovery of c-di-GMP regulated genetic and metabolic biomarkers across species for easy detection and diagnosis of bacterial biofilm infections. To wrap up with these two chapters, a

comparison of advantages and disadvantages of ‘-omics’ work done in these two chapters is illustrated in Table 3.16 as reference for future studies.

“-omics” works	Techniques	Advantages	Disadvantages
Transcriptomics	RNA-sequencing for RNA profiling	Easy mapping Large database	Invasive method for collection of samples e.g. organ/tissue from host
Metabolomics	LCMS for intracellular metabolites profiling	Non-invasive and easy collection of samples e.g. blood/body fluid Sensitive to nM range	Limitation in the size of database Difficulty in identification

Table 3.16 Comparison of advantages and disadvantages of Omics works done in this study.

Chapter 4. RpoN (σ^{54}) controls virulence factors by modulating PqsR in *pqs* quorum sensing of *P. aeruginosa*

P. aeruginosa cells are able to survive under various environmental conditions due to its adaptive capability. A previous study utilized mRNA profiling and CHIP-seq analysis revealing that alternative sigma factors RpoN regulating the expression of various genes in *P. aeruginosa* in order to adapt to different environmental conditions. [327] RpoN impacts greatly on the global gene expression in *P. aeruginosa*, where 680 genes related to metabolism, virulence and motility are under the direct or indirect regulation of RpoN. [327]

Thus, RpoN serves as a global regulator, besides c-di-GMP, in *P. aeruginosa*. It was discovered long ago that *P. aeruginosa* cells evolve *rpoN* mutants to escape from host immune system and thus lead to establishment of chronic biofilm infection in CF lungs.[31, 328] RpoN is reported to be involved in antibiotic resistance and regulating quorum sensing systems in *P. aeruginosa*. Though it is clear that RpoN has effect on the regulation of *pqs* quorum sensing system, it still remains unknown how such regulation works.

Here in this project, we demonstrated that RpoN plays its role in *P. aeruginosa* virulence through regulation of PqsR in PQS quorum sensing system. PqsR functions as a QS receptor which regulates the biosynthesis of pyocyanin, a phenazine. Without functional *rpoN*, activation of PqsR was ceased and thus silenced PQS quorum sensing even with normal expression of HHQ, the precursor of PQS signal. RpoN deficiency also resulted in reduction in both expressions of pyocyanin and anti-staphylococcal

agents. Complementation of *pqsR* gene restored all of these abrogated functions in $\Delta rpoN$ mutant to normal cellular expression level. Thus, RpoN regulates *pqsR* function to modulate virulence in *P. aeruginosa* cells.

4.1. Materials and methods

4.1.1. Strains and cultural conditions

For analysis of RpoN regulations, all maintenance and manipulations of DNA and plasmid were done using *Escherichia coli* DH5a lab strain. *E. coli* cells were cultivated in LB medium supplemented with 100 µg/mL ampicillin (Ap), 15 µg/mL gentamicin (Gm), 15 µg/mL tetracycline (Tc), 8 µg/mL chloramphenicol (Cm) for maintenance of plasmids when necessary. *P. aeruginosa* cells were cultured in ABTGC medium with supplementation of 30 µg/mL Gm, 50 µg/mL Tc, 200 µg/mL carbenicillin (Cb) for marker selection at 37 °C. *P. aeruginosa* were cultivated in King's medium A (Sigma-Aldrich) for pyocyanin quantification,. *S. aureus* were cultivated in Tryptic Soy Broth (TSB) medium (BD Biosciences), at 37 °C, with 10 µg/mL chloramphenicol (Cm) for plasmid maintenance. Strains used can be found in Table 4.1.

Strain(s) or plasmid	Relevant characteristic(s)	Source or reference
<i>P. aeruginosa</i> strains		
PAO1	Prototypic wild-type strain	[38]
$\Delta rpoN$	Gm ^r ; <i>rpoN</i> derivative of PAO1 constructed by allelic exchange	[38]
$\Delta rpoN$ COM	Gm ^r ;Tc ^r ; $\Delta rpoN$ carrying the pME6031- <i>rpoN</i> vector	This work
$\Delta rpoN$ /pME6032- <i>pqsR</i>	Gm ^r ;Tc ^r ; $\Delta rpoN$ carrying the pME6032- <i>pqsR</i> vector	This work
$\Delta rpoN$ /pME6032-	Gm ^r ;Tc ^r ; Carb ^r ; $\Delta rpoN$ /pME6032- <i>pqsR</i> carrying the P _{<i>pqsA</i>} -	This work

<i>pqsR</i> /P _{<i>pqsA-gfp</i>}	<i>gfp</i> vector	
Δ <i>rpoN</i> COM/P _{<i>pqsA-gfp</i>}	Gm ^r ;Tc ^r ; Carb ^r ; Δ <i>rpoN</i> COM carrying the P _{<i>pqsA-gfp</i>} vector	This work
Δ <i>rpoN</i> /pME6032	Gm ^r ;Tc ^r ; Δ <i>rpoN</i> carrying the pME6032 vector	This work
Δ <i>pqsR</i>	<i>pqsR</i> derivative of PAO1 constructed by allelic exchange	[329]
<i>pYedQ2</i>	Gm ^r ; wild type carrying the pBBR1-MCS5 vector containing <i>yedQ2</i> gene from <i>E. coli</i> cloned behind <i>lacOP</i>	[226, 330]
<i>pYhjH</i>	Tc ^r ; wild type carrying the pBBR1-MCS3 vector containing <i>yhjH</i> gene from <i>E. coli</i> cloned behind <i>lacOP</i>	[238]
<i>Staphylococcus aureus</i>		
15981	Prototypic wild-type strain	[331]
15981/pSB2019	Chl ^r ; 15981 carrying the pSB2019 <i>gfp</i> -expressing vector	[331]
<i>Klebsiella pneumoniae</i> strains		
KP-1	Prototypic wild-type strain	[332]
<i>pYedQ2</i>	Gm ^r ; wild type carrying the pBBR1-MCS5 vector containing <i>yedQ2</i> gene from <i>E. coli</i> cloned behind <i>lacOP</i>	This work
<i>pYhjH</i>	Tc ^r ; wild type carrying the pBBR1-MCS3 vector containing <i>yhjH</i> gene from <i>E. coli</i> cloned behind <i>lacOP</i>	This work
<i>Burkholderia cenocepacia</i> strains		
H111	Prototypic wild-type strain	[333]
<i>pYedQ2</i>	Gm ^r ; wild type carrying the pBBR1-MCS5 vector containing <i>yedQ2</i> gene from <i>E. coli</i> cloned behind <i>lacOP</i>	[333]
<i>pYhjH</i>	Tc ^r ; wild type carrying the pBBR1-MCS3 vector containing <i>yhjH</i> gene from <i>E. coli</i> cloned behind <i>lacOP</i>	[333]
<i>Plasmids</i>		
pME6031	Tc ^r ; Broad-host-range cloning vector	[190]
pME6031- <i>rpoN</i>	Tc ^r ; pME6031 carrying the <i>rpoN</i> gene	[31]
pME6032	Tc ^r ; broad host range vector	[190]
pME6032- <i>pqsR</i>	Tc ^r ; pME6032 carrying the <i>pqsR</i> gene	[329]
P _{<i>pqsA-gfp</i>}	Gm ^r ;Carb ^r ; pUCP22 carrying the <i>pqsA-gfp</i> transcriptional fusion	[334]
<i>pYedQ2</i>	Gm ^r ; wild type carrying the pBBR1-MCS5 vector containing <i>yedQ2</i> gene from <i>E. coli</i> cloned behind <i>lacOP</i>	[226, 330]
<i>pYhjH</i>	Tc ^r ; wild type carrying the pBBR1-MCS3 vector containing <i>yhjH</i> gene from <i>E. coli</i> cloned behind <i>lacOP</i>	[238]
<i>Primers</i>		
PAO1_ <i>metE</i> -F	5'-TGACCCATTCGCTGACCTTC-3'	This work
PAO1_ <i>metE</i> -R	5'-GAACAGGGTATGCAGGGTCG-3'	This work
H111_ <i>metE</i> -F	5'-CGTGTGGATCTGCGTATCGT-3'	This work
H111_ <i>metE</i> -R	5'-GCAGTGGAGCGAATACCTGA-3'	This work
H111_ <i>gyrB</i> _F	5'-AGTGCTGAACGTCGAGAAGG-3'	This work
H111_ <i>gyrB</i> _R	5'-ATGATGCGGTGATAGCGGAG-3'	This work
H111_ <i>trpB</i> _F	5'-ATCAACAACGTGATCGGCCA-3'	This work

H111_ <i>trpB</i> _R	5'-TAGACGACGCATTCCATCCC-3'	This work
PAO1_ <i>rpoD</i> _F	5'-GGGGCTGTCTCGAATACGTT-3'	This work
PAO1_ <i>rpoD</i> _R	5'-GGGATACCTGACTTACGCGG-3'	This work
PAO1_ <i>gyrB</i> _F	5'-CACGTACGACTCTTCCAGCA-3'	This work
PAO1_ <i>gyrB</i> _R	5'-GAACACCATGTGGTGCAGAC-3'	This work

Table 4.1 Bacterial strains, plasmids and primers used in this study. *P. aeruginosa* is denoted as 'PAO1', *B. cenocepacia* is denoted as 'H111' while *K. pneumon-iae* is denoted as 'KP-1'.

4.1.2. RNA-sequencing transcriptome analysis

P. aeruginosa strains were cultivated in ABTGC medium at 37°C, 200 rpm, to early stationary phase. Cultures were harvested and mixed immediately with 2 volumes of RNeasy Protect® Bacteria Reagent (Qiagen). After 5 minutes of incubation at room temperature, cells were pelleted by centrifugation at 10,000 g for 5 min at 4°C. Collected pellets were then kept at -80 °C until use.

Total RNA was purified by RNeasy Protect Bacteria Mini Purification Kit (Qiagen). Removal of DNA contamination was done using RNase-free DNase Set (Qiagen) by on-column DNase digestion. The integrity of total RNA and DNA contamination were examined with an Agilent 2100 Bioanalyzer (Agilent Technologies) and Qubit® 2.0 Fluorometer (Invitrogen).

Gene expression analysis was carried out by Illumina RNA sequencing (RNA-Seq technology). RNA-Seq was conducted for two biological replicates of each sample. Libraries were produced using an Illumina TruSeq RNA Sample Prep Kit. The libraries were sequenced using the Illumina HiSeq 2500 platform with a paired-end protocol and read lengths of 100 nt. Analysis of the RNA-seq data was performed as

previous described.[233] Briefly, the sequence reads were mapped onto the *P.aeruginosa* PAO1 genome using specific application of CLC genomics Workbench 6.0 (CLC Bio, Aarhus, Denmark). The transcript count table was subjected to DESeq package [234] of R/Bioconductor [235] for statistical analysis. The transcript counts were normalized to the effective library size. The differentially expressed genes were identified by performing a negative binomial test. Transcripts were stringently determined as differentially expressed when having a fold change larger than 4 and an adjusted p-value smaller than 0.01. Hierarchical clustering analysis was performed and a heatmap was drawn for the differentially expressed genes, using heatmap.2 package of R/Bioconductor [235].

4.1.3. Quantitative reverse transcriptase PCR (qRT–PCR) analysis

qRT-PCR was performed using a two-step method. First-strand cDNA was reverse transcribed using SuperScript III First-Strand Synthesis SuperMix kit (Invitrogen) from total RNA. Newly synthesized cDNA was taken as template for qRT–PCR with a SYBR Select Master Mix kit (Applied Biosystems by Life Technologies) on an Applied Biosystems StepOnePlus Real-Time PCR System. 16s rRNA gene was taken as an endogenous control. Verification of specific single-product amplification was done via melting curve analysis.

4.1.4. Quantification of HHQ

Triplicates of each *P. aeruginosa* strains were cultured at 37 °C with 200 rpm shaking in 25 mL ABTGC for 8 hours to its early stationary phase. 20 mL of supernatants free of cells were obtained by membrane-filtration with Hydrophilic Cartridge Filters (0.22 µm, Millipore, Singapore) after centrifuging at 10,000× g for 10 min. Supernatants

containing HHQ was mixed with acidified ethyl acetate for HHQ extraction for three times, 10 mL each time. The HHQ-containing part was isolated and instantly dried. The leftover was reconstituted into 200 μ L of isopropanol as described previously[335]. High Performance Liquid Chromatography (HPLC) analysis was conducted to measure HHQ level with a C18 Targa reverse-phase column (5 μ m, 4.6 mm $\times$ 150 mm) using gradient program with 10 mM ammonium acetate in water (solvent A)/methanol(solvent B). The gradient program applied was 30%, 70%, 100%. 100% , 20% ,and 20% of solvent B at a timeline of 0, 3, 29, 36, 40, and 42 min at a flow rate of 0.3 mL/min and 20 μ L of injection volume at detection wavelength of 314 nm. Retention time of HHQ using this program was detected as 22.5 min. All of HPLC-determined HHQ concentrations were normalized by total protein content.

4.1.5. Quantification of pyocyanin

Testing strains were cultured in 10 mL of King's medium A at 37 °C with 200 rpm shaking for one day. 5 ml of pyocyanine-containing supernatants were collected by centrifugation and membrane-filtration as described above to get rid of cells. 1 mL of chloroform was then mixed with these clean supernatants as well as medium control in a new tube and was allowed to settle for several minutes. Chloroform layer residing at bottom of the tubes was carefully drawn and transferred to a new tube. Pyocyanin was then extracted into 200 μ L of 0.2 M HCl by continuous high-speed vortexing. Relative pyocyanin concentration was determined by measuring its absorbance at OD520 nm. Quantity of pyocyanin was normalized by their respective absorbance values at OD600 nm.

4.1.6. Mixed-species biofilm assay

In order to establish mixed-species biofilms, *S. aureus* 15981- /pSB2019 were co-cultured respectively with *P. aeruginosa* PAO1 wild-type strain, $\Delta rpoN$ knockout mutant, *rpoN* complemented strain $\Delta rpoN$ COM, and $\Delta rpoN$ mutant with plasmid pME6032-*pqsR*, as described previously[336]. *S. aureus* cells expresses *gfp* and thus emitted green fluorescence while *P. aeruginosa* cells were stained red by CYTO62SYTO62, and thus emitted red fluorescence. Zeiss LSM780 Confocal Laser Scanning Microscope (CLSM) configured with a 63 $\times$ / 1.4 objective was applied to visualize and image the biofilms. Simulated 3D images were generated and biovolumes of biofilms were calculated by Imaris software package (Bitplane AG, Zürich, Switzerland).

4.1.7. *Staphylococcus aureus* inhibitory assay

Overnight cultures of *S. aureus*, *P. aeruginosa* PAO1 wild-type strain, $\Delta rpoN$ knockout mutant, *rpoN* complemented strain $\Delta rpoN$ COM, and $\Delta rpoN$ mutant with plasmid pME6032-*pqsR* were washed and reconstituted in PBS to absorbance value at OD_{600 nm} of 0.1. 100 μ L of reconstituted *S. aureus* cells was spread to dry over LB agar plates evenly. 20 μ L of each reconstituted *P. aeruginosa* mutant was taken and spotted onto corresponding filter paper discs placed previously over the lawn of *S. aureus* cells. Cultures on these LB agar plates were then incubated at 37 °C for overnight. The diameters of inhibition zones (mm) were then measured for determination of *S. aureus* inhibitory effect caused by each *P. aeruginosa* strain.

4.1.8. Induction assay of *P_{pqsA}-gfp* fusion

P. aeruginosa/*p_{pqsA}-gfp*, $\Delta rpoN$ /*p_{pqsA}-gfp*, $\Delta rpoN$ /pME6032-*pqsR*/*p_{pqsA}-gfp* and $\Delta rpoN$ COM/ *p_{pqsA}-gfp* strains were allowed to grow for overnight in LB medium with

respective antibiotics. Overnight cultures were then washed and reconstituted into ABTGC medium to OD_{600 nm} ~ 0.01. External synthesized PQS signaling molecule [329] was added to cell culture of $\Delta rpoN/p_{pqsA}\text{-}gfp$ strain. Six replicates of 200 μ L of diluted culture of each mutant were drawn and expelled into corresponding wells of a 96-well microplate. OD_{600 nm} and GFP with 485 nm as excitation wavelength and 535 nm as emission wavelength of all cell cultures were monitored in a Magellen Tecan® Infinite M200 PRO plate reader for 24 hours.

4.1.9. Killing assay of *Caenorhabditis elegans*

Cell suspension of *P. aeruginosa* mutants were evenly spread over on top of PGS agar solidified in a 6-well plate (Nunc) and allowed to form a lawn at 37 °C for overnight. Triplicates of overnight culture plate were prepared where 20 hermaphrodite *C. elegans* strain N2 (Bristol) at L3-stage were placed on top of each replicate [337]. Plates seeded with worms were then incubated for 24 h at 25 °C, allowing the worms to survive over the lawn of *P. aeruginosa* strains. Numbers of living and dead worms were counted and percentage of death was tabulated.

4.2. Results

4.2.1. Transcriptomic analysis of *rpoN* regulon in *P. aeruginosa* strains

In order to obtain a sophisticated overview of the *rpoN* regulon of *P. aeruginosa*, we firstly compared the transcriptomes from the *P. aeruginosa* PAO1 wild-type strain, $\Delta rpoN$ mutant and *rpoN* complementary strain $\Delta rpoN$ COM by RNA-sequencing. Using a negative binomial test with a FDR adjusted P-value cut-off of 0.01 and a fold-change cut-off of 4, we found that 140 genes (Table 7.3.1) were upregulated and 247

genes (Table 7.3.2) were downregulated in $\Delta rpoN$ mutant compared with wild-type strain. $\Delta rpoN$ COM has a similar transcriptome to the *P. aeruginosa* PAO1 wild-type. Heatmap of the genes that were differently expressed among these three strains is illustrated in Figure 4.1.

Figure 4.1 Gene expression analysis of PAO1 wild-type, $\Delta rpoN$ and $\Delta rpoN$ COM mutants. Red: upregulated genes; blue: downregulated genes. Differences in gene expression (fold change > 4, adjusted p-value < 0.01, n=2) between (i) PAO1 and $\Delta rpoN$, (ii) PAO1 and $\Delta rpoN$ COM and (iii) $\Delta rpoN$ and $\Delta rpoN$ COM were identified based on negative binomial test using the DESeq package of R/Bioconductor.

As we expected, expression of the well-known *rpoN* regulated genes such as flagellum biosynthesis genes (PA1077-PA1087, PA1452, PA1453, PA1092, PA1094, PA1100) and type IV fimbriae biosynthesis gene (PA4525) was largely downregulated in the $\Delta rpoN$ mutant comparing to wild-type strain (Table 7.3.2). The expression of a prophage operon (PA0616-PA0648) and the MexGHI-OpmD multidrug efflux pump

genes (PA4205-PA4206) were found to be downregulated in $\Delta rpoN$ mutant comparing to wild-type strain (Table 7.3.2). Two important regulatory small non-coding RNAs, *crcZ* and *amrZ*, were found to be downregulated in $\Delta rpoN$ mutant comparing to wild-type strain by 111.4 fold and 14.8 fold, respectively (Table 7.3.2). RT-PCR analysis confirmed the reduction in the expression of selected genes including *fliC*, *fliD* and *crcZ* in $\Delta rpoN$ mutant comparing to wild-type strain and $\Delta rpoN$ COM complementary strain (Figure 4.2).

Figure 4.2 qPCR analysis of selected genes in PAO1 wild-type, $\Delta rpoN$ and $\Delta rpoN$ COM. Gene expression level in PAO1 was taken as reference.

Mutation in the *rpoN* gene induces the transcription of a wide spectrum of genes with known metabolic functions such as the *dhcA* and *dhcB* for amino acid biosynthesis and metabolism, and *liuABCDE* operon for carbon compound catabolism (Table 7.3.1). Strikingly, we observed that the expression of the *pqsABCDE* operon, which encodes the *Pseudomonas* quinolone signaling molecules, was induced in $\Delta rpoN$ mutant comparing to the wild-type strain. RT-PCR analysis confirmed the upregulation of

pqsA gene in $\Delta rpoN$ mutant (Figure 4.2). This result appears not in accordance with the low virulence and pyocanine production phenotypes of the $\Delta rpoN$ mutant, as it is well known that the *pqs* quorum sensing positively regulates pyocanine production [32].

4.2.2. Regulation of *pqs* quorum sensing signalling by RpoN through *pqsR*

Pqs quorum sensing system directly regulates biosynthesis of pyocyanin which could be abolished upon mutation in *rpoN* gene [187]. Multiple genes, *pqsABCDE* operon, *pqsH* and *pqsR*, are responsible for biosynthesis of PQS signals. *PqsABCDE* regulates the expression of 2-heptyl-4-quinolone (HHQ) which is precursor of PQS. [36]. With the presence of PQS, PqsR interacts directly to promoter of *pqsA* and promotes auto-induction of the operon [187]. Here we demonstrated how *rpoN* regulates the mechanism of *pqs* quorum sensing system.

Transcription of *pqsA* in wild type, $\Delta rpoN$ and its complementation strain $\Delta rpoN$ COM, was measured by monitoring fluorescence intensity of P_{pqsA} -*gfp* containing the *pqsA* promoter *gfp* fusion transformed into these testing strains. A significant reduction in P_{pqsA} -*gfp* expression was seen in *rpoN* knockout mutant comparing to wild type and complementation strain (Figure 4.3).

Figure 4.3 Regulation of *pqs* genes by *rpoN* at post-transcriptional level. Measurement of GFP fluorescence upon inducing transcriptional fusion P_{pqsA} -*gfp* in wild type PAO1, $\Delta rpoN$, $\Delta rpoNCOM$, $\Delta rpoN/pME6032-pqsR$ and $\Delta rpoN$ with external PQS. Mean GFP of triplicates normalized by OD600nm are shown with standard deviation in relative fluorescence units (RFU).

Later it was proved by HPLC measurement that biosynthesis of HHQ in *rpoN* knockout mutant was similar to that in wild type strain (Figure 4.4) which validated the normal functionality of the *pqsABCDE* operon in *rpoN* knockout mutant.

Figure 4.4 HPLC measurement of level of HHQ synthesized. HHQ synthesized by PAO1 wild type, $\Delta rpoN$, $\Delta rpoNCOM$, $\Delta rpoN/pME6032-pqsR$, $\Delta rpoN/pME6032$ and $\Delta pqsR$ were quantified. Means of HHQ concentrations of triplicates normalized by unit protein are shown with standard deviation.

Presence of external PQS had no effect on restoration of $P_{pqsA-gfp}$ expression in $rpoN$ knockout mutant (Figure 4.3). Transformation of a plasmid carrying $pqsR$ gene under the constitutively expressed *lac* promoter into $rpoN$ knockout mutant recovered its ability to activate *pqs* quorum sensing signals (Figure 4.3). Similar results were obtained using another PAO1 strain with different background indicating such regulation is not strain-specific (Figure 7.3.1).

4.2.3. Regulation of virulence factors production and interspecies interactions by RpoN via pqs signaling

Virulence gene transcription, biofilm formation and interaction among different bacterial species were all under regulation of *pqs* quorum sensing system [329, 334,

338, 339]. Level of expression of pyocyanin in all testing strains was measured to validate if *pqs* exhibits its regulation on these phenotypic traits in *rpoN*-dependent manner. Significant reduction in pyocyanin production was observed in $\Delta rpoN$ mutant comparing to that of wild type strain (Figure 4.5).

Figure 4.5 Quantification of pyocyanin production. Pyocyanine synthesized by PAO1 wild-type, $\Delta rpoN$, $\Delta rpoNCOM$, $\Delta rpoN/P_{pqsR-his}$ and $\Delta rpoN/pME6032$ were extracted by chloroform. Absorption at OD520nm was measured and normalized by cell density at OD600nm. Mean of triplicates are shown with standard deviation. Differences between groups are calculated using Student's t-test with * p-value ≤ 0.05 .

Such reduction could be recovered by complementation of *rpoN* gene and excessive transcription of *pqsR* gene (Figure 4.5). Similar level of pyocyanin biosynthesis to that of wild type PAO1 strain was observed in $\Delta rpoN$ mutant carrying an empty pME6032

vector indicated that pyocyanin production was not influenced by the control vector (Figure 4.5).

Biofilm infection in CF patients involves coexistence and interaction of multiple bacterial species, such as the dominating *P. aeruginosa* and *S. aureus*. Interactions between these bacterial species are key determinant of disease progression during infections. Inhibition in proliferation of *S.aureus* cells by *P.aeruginosa* was found to be dependent on *pqs* signaling system [340, 341]. Effect of mutation in *rpoN* gene on interspecies interaction between these two bacteria was tested by performing growth inhibition assay on plate (Figure 4.6A and 4.6B). Smaller zone of inhibition indicated a much weaker inhibiting capability on *S.aureus* growth exhibited by $\Delta rpoN$ mutant comparing to PAO1 wild type strain (Figure 4.6A and 4.6B).

Figure 4.6 Growth inhibition assay. (A) Inhibition of *S. aureus* 15981 on agar plate by PAO1 wild type, $\Delta rpoN$, $\Delta rpoNCOM$ and $\Delta rpoN/pME6032-pqsR$ was illustrated respectively through zone of inhibition indicated by white arrows. (B) Average of inhibition zone size (mm). n=3. Mean values of triplicates were taken with standard deviation; *p-value ≤ 0.05 .

Similar to that of pyocyanin production, *S. aureus* growth inhibition capability of $\Delta rpoN$ mutant was recovered after complemented with *rpoN* gene and over-expression of *pqsR* gene (Figure 4.7). Beside this on-plate growth inhibition assay, mixed-species biofilm formation by *P.aeruginosa* and *S.aureus* strains were established for further interspecies interaction analysis. Survival rate of *S. aureus* cells co-cultured with $\Delta rpoN$ mutant was much higher than co-cultured with PAO1 wild type strain (Figure 4.8).

Figure 4.7 Mix-species biofilm assay. *S. aureus* 15981/pSB2019 was cultured with PAO1 wild type, $\Delta rpoN$, $\Delta rpoN$ COM and $\Delta rpoN$ /pME6032-*pqsR*, respectively. Green: *S. aureus* 15981/pSB2019 cells with GFP expression; red: *P. aeruginosa* cells with red stain. Scale bar: 20 μm .

Complementation of *rpoN* gene into $\Delta rpoN$ mutant brought back its capacity to inhibit *S. aureus* to wild type level in biofilm. Nonetheless, only partial restoration of its

inhibition capability was observed in $\Delta rpoN$ mutant with overexpression of *pqsR* gene (Figure 4.7&4.8).

Figure 4.8 Survival rate of *S. aureus* in mix-species biofilm with *P. aeruginosa* strains. Survival rate is indicated by biomass ratios calculated by Imaris and displayed by means of triplicates with standard deviation. Differences between groups are calculated using Student's t-test with * $p \leq 0.05$.

Such observation suggests that interspecies competition between these two bacteria in biofilm co-culture might be regulated by not only *pqsR* but also other factors dependent on *rpoN* regulation.

4.2.4. Mortality of *Caenorhabditis elegans* regulated by RpoN-dependent *pqs* signalling in *P. aeruginosa*

Virulence factors produced by *P. aeruginosa* in *rpoN*-dependent manner increase the mortality rate of *C. elegans* [337] In this study, the involvement of *pqs* signalling in

such *C.elegans* killing was demonstrated to perform in *rpoN*-dependent manner. Significant higher number of survived *C.elegans* was observed in $\Delta rpoN$ mutant comparing to that in PAO1 wild type strain (Figure 4.9).

Figure 4.9 Killing of *C. elegans* by different *P. aeruginosa* strains on agar plates.

Killing rate is indicated by death rate of *C. elegans* displayed by histogram of means of six replicates with standard deviation. Variation among groups are tested by One-way ANOVA with * p-value ≤ 0.05 .

Mortality rates of *C.elegans* in $\Delta rpoN$ mutant complemented with *rpoN* gene and overexpression of *pqsR* gene were brought back to only slightly lower level than that in PAO1 wild type strains (Figure 4.9). Control plasmid itself has insignificant effect on virulence factor expression in $\Delta rpoN$ mutant. All of the results obtained matched perfectly and suggesting that virulence of *P. aeruginosa* is regulated by *rpoN* via *pqsR*.

4.3. Discussion

Alternative sigma factor, RpoN, is a global regulator which is involved in modulation of multiple cellular behaviors such as virulence, metabolism, cell motility, nutrient transport and quorum sensing in many different bacteria. [38, 176, 342-346] Alternative sigma factors are able to associate with RNA polymerase and enhancer binding proteins, and bind with DNA promoter to initiate gene transcription.[347, 348]. 135 DNA binding sites of alternative sigma factors were discovered. Many of these binding sites are conserved across bacterial species which locates at -12 to -24 from transcription start site. [344, 347, 349]

Mutation in RpoN occurs during chronic infection by *P.aeruginosa* cells to adapt to the environment in CF lungs [31]. RpoN was demonstrated to be essential for positive regulation of pili and flagella formation thus *rpoN* mutation may leads to less bacterial attachment [122, 176, 350]. Such evolutionary selection takes place to repress motility in *P. aeruginosa* cells in order to evade from clearance by host immunal defence [350, 351]. 22 proteins are predicted to have σ^{54} ATP-binding region, including FleQR for flagellin formation. [124, 344] *RpoN* associates with FleQ to regulate the expression of *fleSR* and *fliD* genes while the expression of *fleQ* appears to be RpoN-independent.[124, 352-354]. RpoN also associates with PilR to regulate the transcription of *pilA* for the formation of type 4 fimbriae [355, 356]. Such regulation could be seen from our results where *fleSR*, *fliD*, *pilA* and other flagella-related genes are downregulated in *rpoN* mutant.

Beside its effect in motility reduction, malfunction in RpoN leads to lower degree of virulency in *P.aeruginosa* cells which is another shield for the cells from being

phagocytosed, and thus promotes chronic infection in CF lungs [357, 358]. It was previously proved that RpoN positively regulates *RhlI* [38], while negatively regulates *lasRI* in low cell population and *rhlIR* at log phase [176]. However, RpoN regulation on *P. aeruginosa* virulence remains unknown upto now. It is proved in this study that *rpoN* controls the virulence in *P. aeruginosa* cells through regulating the expression of *pqsR* in *pqs* quorum sensing system. This result matches with the research done by Schulz's group showing interaction between RpoN and *pqsR* sequence by CHIP-seq study [327]. Another group had also demonstrated that *rpoN* regulates the expression of *pqsAEH* negatively in rich medium. [359] This also indirectly indicates the validity of our results.

Pqs quorum sensing system is somewhat specific to *Pseudomonas* consisting of *pqsABCDE* operon which is in charge for expression of 2-alkyl-4-quinolone (AHQs) such as HHQ; *pqsH* encoding for PqsH which converts HHQ to PQS; and *pqsR* encoding for PqsR which is activated by both of HHQ and PQS [360]. Activated PqsR in turn interact with *pqsA* promoter leading to transcription of *pqsABCDE* operon, the process known as autoinduction [360]. *PqsR* transcription is activated by *las* quorum sensing and repressed by *rhl* quorum sensing. In addition, *pqs* quorum sensing is capable to be activated independently from *las* quorum sensing and has effect in controlling *rhl* quorum sensing in *P. aeruginosa* cells [32].

Recent studies demonstrated that *pqs* quorum sensing could be regulated post-transcriptionally by variation in nutrient supply through PqsR. Anr functioning as a transcriptional regulator triggers the transcription of PhrS, a non-coding small RNA under restricted supply of oxygen, which in turn activates the expression of *pqsR* to onset *pqs* quorum sensing system [361]. However, insignificant difference was

detected in the transcriptional levels of *anr* and *phrS* between PAO1 wild type and *rpoN* knockout mutant by RNA-seq analysis. This indicates that regulation of *pqsR* by *rpoN* is unlikely via *phrS*.

Transcription of non-coding small RNA, CrcZ, was reduced in $\Delta rpoN$ mutant comparing to its transcription in PAO1 wild type strain. CrcZ is involved in segregation of RNA-binding Crc and Hfq proteins in *Pseudomonas* [362, 363]. This small non-coding RNA was demonstrated to regulate *pqs* quorum sensing system in a reverse manner [339]. The capability of Hfq to interact with RsmY, another non-coding small RNA, gives rise to abolishment of the function of RsmA as a post-transcriptional regulator to downregulate quorum sensing effect in *P. aeruginosa* [364]. This indicates that reduction in Crcz expression represses *pqs* quorum sensing through Crc alone or Hfq via RsmA. A deeper insight into RpoN regulation on *pqsR* and PhrS/CrcZ effect on RpoN regulation could be done for future study.

In summary, we used RNA-seq based transcriptomic analysis to investigate the regulatory role of RpoN on the virulence of *P. aeruginosa*. We provided evidence that RpoN modulates the *pqs* quorum sensing through PqsR. The $\Delta rpoN$ mutant reduces *pqs* quorum sensing regulated virulence factors, which can be restored by overexpressing the *pqsR* in the $\Delta rpoN$ mutant. Our study opens a new direction for developing novel strategies for modulating *P. aeruginosa* virulence.

Chapter 5. Conclusion and Future Plan

5.1. Conclusion

Global regulators, including c-di-GMP, quorum sensing autoinducers and alternative sigma factors regulates transition between planktonic life style to biofilm life style, virulence, cell behavior and cellular fuctions in a great variety of bacteria species. Study of these global regulators in different bacteria may contribute in understanding the development of biofilm infections, in promoting the establishment of detection methods of these infections, and in discovering cures to defeat the infections.

Here in this study, expression of c-di-GMP were manipulated genetically in three different pathogenic bacteria, *P. aeruginosa* PAO1, *B. cenocepacia* H111 and *K. pneumonia* KP-1, for discovering common biomarkers regulated by c-di-GMP among these strains applying transcriptomic analytic technique, RNA-seq, and metabolomic analytic technique, LC/MS.

Transcriptomic analysis revealed that 57 genes were upregulated while 65 genes were downregulated significantly in *P. aeruginosa/pYedQ2*, 57 genes were upregulated while 97 genes were downregulated significantly in *B.cenocepacia/pYedQ2*, and 12 genes were upregulated while 37 genes were downregulated significantly in *K.pneumoniae/pYedQ2*.

Between all the upregulated genes in *P. aeruginosa/pYedQ2* and *B.cenocepacia/pYedQ2*, one gene, *metE*, was found in common. 3 common genes were found to be downregulated in both of *P. aeruginosa/pYedQ2* and

B.cenocepacia/pYedQ2, being *flgK*, *fliS* and *flgL*, respectively. *metE* encodes an enzyme, 5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase, catalyzing the formation of L-methionine.

One common gene was found to be downregulated in both of *K.pneumoniae/pYedQ2* and *B.cenocepacia/pYedQ2* encoding for an enzyme, 4-hydroxyphenylpyruvate dioxygenase. No common genes found either upregulated or downregulated among three testing bacteria under regulation of c-di-GMP under experimental conditions in this study.

Furthermore, metabolomics analysis of the intracellular extracts of same strains was carried out to investigate if the changes in transcriptomes could be reflected at metabolic level. 172 metabolites were upregulated and 151 metabolites were downregulated significantly in *P. aeruginosa/pYedQ2*, 75 metabolites were upregulated while 136 metabolites were downregulated significantly in *B.cenocepacia/pYedQ2*, and 232 metabolites were upregulated while 150 were downregulated significantly in *K.pneumoniae/pYedQ2*.

As *metE* was found to be upregulated in *P. aeruginosa/pYedQ2*, influence of such change in this gene could be reflected on metabolomics level. It directly alters the synthesis of L-methionine along cysteine and methionine metabolism pathway (Figure 5.1.1). Among all upregulated metabolites in *P. aeruginosa/pYedQ2*, histamine along histidine metabolism pathway was found to be under regulation of S-adenosyl-L-methionine which is the direct derivative of L-methionine.

Figure 5.1.1 *metE* and its regulation on metabolic pathways. Gene product of *metE* (EC2.1.1.14) is indicated in red box. Histamine and its derivative N-methylhistamine are indicated in red. Histamine and S-adenosyl-L-methionine as two reactants of N-methylhistamine are indicated by blue circles, while direction of reaction is indicated by blue arrows. Pathways are adapted from KEGG pathway. [251, 252]

Furthermore, 15 common upregulated metabolites were found between *P. aeruginosa/pYedQ2* and *B.cenocepacia/pYedQ2*. 2 common upregulated metabolites were discovered between *P. aeruginosa/pYedQ2* and *K.pneumoniae/pYedQ2*. 1 common downregulated metabolite identified among *P. aeruginosa/pYedQ2*, *B.cenocepacia/pYedQ2* and *K.pneumoniae/pYedQ2*.

However, level of L-methionine synthesized in different mutants did not show significant difference although *metE* expression was found to be significantly upregulated in *P. aeruginosa/pYedQ2* and *B.cenocepacia/pYedQ2*. L-methionine might be used up by other cellular metabolic reactions resulting in non-detectable variation in level of L-methionine synthesized in different strains.

Beside discovery of biomarkers across bacteria species, transcriptomics and metabolomics analysis done also gave much information on the interlink between c-di-GMP and quorum sensing systems on regulation of virulence factor secretion. Since alternative sigma factor, RpoN, was also known to regulate virulence factors, investigation on how RpoN controls the secretion of virulence in *P. aeruginosa* PAO1 was carried out while the results indicated that RpoN modulate PQS quorum sensing regulator, *pqsR*, in order to regulate the virulency of the strain.

Such results provide evidences in mainly two aspects: firstly, the possibility and feasibility of identification of common biomarker among different pathogens using transcriptomics and metabolomics analysis for easy detection and diagnosis of biofilm infections; and secondly, the interlinks among global regulators c-di-GMP, quorum sensing and alternative sigma factors, in modulating virulence and other cell functions.

5.2. Future Plan

Current studies on biomarker discovery focuses on single species analysis, findings in this study give a glance of the possibility to discover common biomarkers across multiple bacterial species. As biofilms in the environment and especially in human hosts are usually formed by co-existence of multiple bacterial species, this study leads to a new direction of biomarker discovery of biofilm infections.

However, this study is based on planktonic genetically modified pathogenic cells, it is important to find out if cross-species common biomarkers could be also found in biofilm samples. Therefore, future work should focus on analysis of transcriptomics

and metabolomics of biofilm samples such as static biofilm samples in microplates or flow-cell biofilm samples of both single species and mixed species biofilms.

Furthermore, the ultimate target of this study is easy and fast detection and diagnosis of biofilm infections through identification of cross-species biomarkers. To test the results obtained from *in vitro*, *in vivo* biofilm infections should be established in animal implant models for discovery of biomarkers in the blood in further works. Such model could allow detection of specific biomarkers based on the results obtained from *in vitro* analysis by plotting metabolomics profiles of blood plasma.

Although identification of metabolites have been done in this study by primary database matching and KEGG pathway mapping, the information could be retrieved from the results is still limited as specific identification is difficult for non-targeted metabolomics study due to the small size of metabolomics databases. Targeted metabolomics analysis based on the information obtained from this study could help to generate deeper understanding on metabolic profiles and more specific identification of possible biomarkers. Moreover, more bacterial species, both Gram-negative and Gram positive bacteria, should be included in the future.

Besides biomarker discovery and identification, deeper understanding on the interlink and regulation among biofilm regulating global regulators including c-di-GMP, quorum sensing and alternative sigma factors in next stage of work could give more clues on modulation of biofilm formation, virulence factor secretion and other cellular functions, thus leads to establishing effective measures of biofilm control.

Chapter 6. References

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Chapter 7. Appendix

7.1. Transcriptomics data

Genes upregulated in <i>P. aeruginosa</i> /pYedQ2				
NCBI locus tag	Gene	Protein	Fold change	FDRcorrected p-value
PA1409	<i>aphA</i>	acetylpolysamine aminohydrolase	5.40	1.95E-13
PA2128	<i>cupA1</i>	fimbrial subunit CupA1	357.27	5.20E-84
PA2129	<i>cupA2</i>	chaperone CupA2	294.68	6.16E-78
PA2130	<i>cupA3</i>	usher CupA3	51.03	1.65E-53
PA2131	<i>cupA4</i>	fimbrial subunit CupA4	89.49	4.84E-63
PA2132	<i>cupA5</i>	chaperone CupA5	129.62	2.03E-71
PA1999	<i>dhcA</i>	DhcA, dehydrocarnitine CoA transferase, subunit A	4.16	1.28E-02
PA1712	<i>exsB</i>	exoenzyme S synthesis protein B	4.41	2.31E-14
PA1710	<i>exsC</i>	ExsC, exoenzyme S synthesis protein C precursor	4.33	1.37E-10
PA2570	<i>lecA</i>	LecA	5.59	4.77E-09
PA3361	<i>lecB</i>	fucose-binding lectin PA-III	4.66	1.06E-08
PA3692	<i>lptF</i>	Lipotoxon F, LptF	4.04	3.98E-06
PA1927	<i>metE</i>	5-methyltetrahydropteroyltrimethylglutamate-homocysteine S-methyltransferase	7.53	1.31E-03
PA0059	<i>osmC</i>	osmotically inducible protein OsmC	6.28	2.04E-09
PA1168		hypothetical protein	677.58	9.65E-77
PA1169		probable lipoxygenase	99.05	3.32E-64
PA1170		conserved hypothetical protein	12.29	9.91E-28
PA1323		hypothetical protein	5.34	2.36E-08
PA1324		hypothetical protein	5.03	2.36E-08
PA1410		probable periplasmic spermidine/putrescine-binding protein	4.37	2.53E-13
PA1486	<i>bapF</i>	beta-peptidyl aminopeptidase	5.39	2.14E-14
PA1697		ATP synthase in type III secretion system ATPase	4.73	4.34E-09
PA1699	<i>pcr1</i>	Pcr1	4.72	6.31E-05
PA1701	<i>pcr3</i>	Pcr3	5.05	7.98E-03
PA2021		hypothetical protein	4.02	1.35E-07
PA2069		probable carbamoyl transferase	4.53	5.80E-03
PA2125		probable aldehyde dehydrogenase	4.71	6.66E-15
PA2126	<i>cgrC</i>	cupA gene regulator C, CgrC	6.47	5.74E-20
PA2127	<i>cgrA</i>	cupA gene regulator A, CgrA	4.92	1.74E-09

PA2133		Cyclic-guanylate-specific phosphodiesterase	71.01	2.81E-61
PA2134		hypothetical protein	28.16	6.12E-49
PA2135		probable transporter	5.57	4.11E-14
PA2146		hypothetical protein	6.64	3.79E-04
PA2440		hypothetical protein	15.02	3.72E-50
PA2441		hypothetical protein	12.31	5.33E-39
PA3691		hypothetical protein	4.57	1.65E-06
PA3842	<i>spcS</i>	specific Pseudomonas chaperone for ExoS, SpcS	4.07	1.42E-07
PA4139		hypothetical protein	98.32	3.03E-63
PA4140		hypothetical protein	27.30	1.51E-51
PA4141		hypothetical protein	8.72	7.06E-09
PA4142		probable secretion protein	8.31	4.15E-09
PA4143		probable toxin transporter	7.17	2.00E-07
PA4144		probable outer membrane protein precursor	5.41	1.37E-06
PA4826		hypothetical protein	5.07	5.30E-04
PA4828		hypothetical protein	4.05	2.80E-06
PA1705	<i>pcrG</i>	regulator in type III secretion	6.30	1.03E-11
PA1707	<i>pcrH</i>	regulatory protein PcrH	4.84	2.82E-12
PA1706	<i>pcrV</i>	type III secretion protein PcrV	4.56	6.19E-15
PA3062	<i>pelC</i>	pellicle/biofilm biosynthesis outer membrane protein PelC	5.32	2.37E-07
PA0355	<i>pfpI</i>	protease PfpI	4.35	5.48E-05
PA1899	<i>phzA2</i>	probable phenazine biosynthesis protein	11.11	2.88E-05
PA1900	<i>phzB2</i>	probable phenazine biosynthesis protein	8.14	5.22E-04
PA1901	<i>phzC2</i>	phenazine biosynthesis protein PhzC	8.59	7.43E-05
PA1708	<i>popB</i>	translocator protein PopB	4.82	3.38E-12
PA1709	<i>popD</i>	Translocator outer membrane protein PopD precursor	4.22	2.31E-14
PA1698	<i>popN</i>	Type III secretion outer membrane protein PopN precursor	7.97	7.52E-12

Table 7.1.1 List of upregulated genes in *P. aeruginosa/pYedQ2* comparing to those in *P. aeruginosa/pYhjH*. Selection criteria were set as fold upregulated ≥ 4 and p-value ≤ 0.05 , n=2. NCBI gene locus tags, gene names and protein functions are displayed.

Genes downregulated in *P. aeruginosa*/pYedQ2

NCBI locus tag	Gene	Protein	Fold change	FDR corrected p- value
PA3385	<i>amrZ</i>	alginate and motility regulator Z	-4.37	7.86E-09
PA2432	<i>bexR</i>	bistable expression regulator, BexR	-4.14	3.79E-13
PA3531	<i>bfrB</i>	Bacterioferritin	-36.27	3.91E-51
PA2664	<i>fhp</i>	flavoheмоprotein	-13.89	1.06E-08
PA1099	<i>fleR</i>	two-component response regulator	-9.30	3.20E-16
PA1098	<i>fleS</i>	two-component sensor	-6.60	9.50E-12
PA1077	<i>flgB</i>	flagellar basal-body rod protein FlgB	-12.51	5.83E-44
PA1078	<i>flgC</i>	flagellar basal-body rod protein FlgC	-4.58	2.97E-13
PA1079	<i>flgD</i>	flagellar basal-body rod modification protein FlgD	-5.70	9.40E-20
PA1080	<i>flgE</i>	flagellar hook protein FlgE	-9.01	1.11E-20
PA1081	<i>flgF</i>	flagellar basal-body rod protein FlgF	-21.97	4.99E-54
PA1082	<i>flgG</i>	flagellar basal-body rod protein FlgG	-15.71	6.00E-38
PA1083	<i>flgH</i>	flagellar L-ring protein precursor FlgH	-6.59	1.47E-22
PA1084	<i>flgI</i>	flagellar P-ring protein precursor FlgI	-11.02	8.29E-37
PA1085	<i>flgJ</i>	flagellar protein FlgJ	-6.06	3.43E-18
PA1086	<i>flgK</i>	flagellar hook-associated protein 1 FlgK	-4.94	1.07E-11
PA1087	<i>flgL</i>	flagellar hook-associated protein type 3 FlgL	-4.39	4.10E-10
PA3351	<i>flgM</i>	FlgM	-5.03	1.75E-11
PA1452	<i>flhA</i>	flagellar biosynthesis protein FlhA	-7.22	4.55E-18
PA1453	<i>flhF</i>	flagellar biosynthesis protein FlhF	-4.43	4.28E-10
PA1092	<i>fliC</i>	flagellin type B	-17.64	0.00E+00
PA1094	<i>fliD</i>	flagellar capping protein FliD	-7.62	0.00E+00
PA1100	<i>fliE</i>	flagellar hook-basal body complex protein FliE	-20.70	2.29E-49
PA1101	<i>fliF</i>	Flagella M-ring outer membrane protein precursor	-9.28	4.61E-22
PA1104	<i>fliI</i>	flagellum-specific ATP synthase FliI	-4.25	5.32E-13
PA4762	<i>grpE</i>	heat shock protein GrpE	-4.00	6.34E-10
PA3161	<i>himD</i>	integration host factor beta subunit	-4.19	3.13E-08
PA1596	<i>htpG</i>	heat shock protein HtpG	-4.83	9.17E-09
PA0951a		hypothetical protein	-7.27	7.83E-15
PA1048		probable outer membrane protein precursor	-8.67	2.39E-14
PA1088		hypothetical protein	-4.74	2.97E-11
PA1089		hypothetical protein	-5.77	4.99E-14
PA1090		hypothetical protein	-4.59	3.13E-11
PA1093		hypothetical protein	-7.12	5.25E-24

PA1095		hypothetical protein	-9.29	9.61E-20
PA1096		hypothetical protein	-21.79	7.74E-38
PA1103		probable flagellar assembly protein	-4.14	3.33E-12
PA1289		hypothetical protein	-4.73	8.32E-15
PA1441		putative flagellar hook-length control protein FliK	-14.31	2.98E-23
PA1500		probable oxidoreductase	-4.48	6.01E-12
PA1503		hypothetical protein	-6.01	8.20E-14
PA1545		hypothetical protein	-6.62	1.48E-15
PA1547		hypothetical protein	-4.22	1.73E-11
PA1679		hypothetical protein	-5.09	6.91E-17
PA1967		hypothetical protein	-28.90	4.77E-53
PA2027		hypothetical protein	-5.03	7.26E-13
PA2260		hypothetical protein	-5.13	2.33E-13
PA2261		probable 2-ketogluconate kinase	-4.44	4.19E-11
PA2263		probable 2-hydroxyacid dehydrogenase	-4.10	9.15E-11
PA2652		methyl-accepting chemotaxis protein	-7.64	1.68E-23
PA2654		probable chemotaxis transducer	-9.88	1.43E-25
PA2788		probable chemotaxis transducer	-7.05	3.50E-17
PA3526	<i>motY</i>	MotY	-9.78	1.62E-25
PA3762		hypothetical protein	-6.32	4.14E-20
PA4326		hypothetical protein	-8.89	8.45E-30
PA4523		hypothetical protein	-8.41	0.00E+00
PA4633		probable chemotaxis transducer	-4.95	2.02E-11
PA4758.1	<i>ncRNA</i>	P32	-8.35	4.07E-02
PA4843	<i>gcbA</i>	GcbA	-4.11	4.54E-09
PA4310	<i>pctB</i>	chemotactic transducer PctB	-4.30	9.58E-11
PA4525	<i>pilA</i>	type 4 fimbrial precursor PilA	-7.44	4.28E-05
PA2663	<i>ppyR</i>	psl and pyoverdine operon regulator, PpyR	-5.84	4.35E-03

Table 7.1.2. List of downregulated genes in *P. aeruginosa/pYedQ2*. Selection criteria were set as fold change ≤ -4 and p-value ≤ 0.05 , n=2. NCBI gene locus tags, gene names and protein functions are displayed.

Genes upregulated in <i>B. cenocepacia/pYedQ2</i>				
NCBI Locus tag	Gene	Protein	Fold change	FDR corrected p-value
I35_2045	<i>aceA</i>	Isocitrate lyase	4.27	4.21E-04
I35_5712	<i>acnB</i>	Aconitate hydratase(EC:4.2.1.3)	7.47	7.59E-11

I35_6632	<i>cstA</i>	Carbon starvation protein A	27.71	1.20E-22
I35_2072	<i>cyoA</i>	Cytochrome O ubiquinol oxidase subunit II	9.93	1.31E-02
I35_2071	<i>cyoB</i>	Cytochrome O ubiquinol oxidase subunit I	8.25	2.16E-02
I35_2070	<i>cyoC</i>	Cytochrome O ubiquinol oxidase subunit III	10.78	1.22E-02
I35_1588	<i>fimA</i>	type 1 fimbriae major subunit FimA	9.98	1.01E-10
I35_2820	<i>glcA</i>	L-lactate permease	8.90	4.58E-14
I35_6697	<i>glcD_2</i>	Glycolate dehydrogenase, subunit GlcD	50.55	2.66E-42
I35_6698	<i>glcE_2</i>	Glycolate dehydrogenase,FAD-binding subunit GlcE	17.72	5.00E-22
I35_6699	<i>glcF_2</i>	Glycolate dehydrogenase,iron-sulfur subunit GlcF	23.43	1.38E-37
I35_6700	<i>glcG</i>	hypothetical protein GlcG in glycolate utilization operon	4.53	1.97E-09
I35_0780	<i>hemN</i>	Coproporphyrinogen III oxidase,oxygen-independent	27.78	3.46E-03
I35_1589		Fimbriae usher protein StfC	5.96	3.62E-09
I35_1590		Periplasmic fimbrial chaperone StfD	7.53	1.10E-07
I35_2720		Permeases of the major facilitator superfamily	4.32	6.78E-08
I35_4026		iron-chelator utilization protein	7.38	2.26E-04
I35_4027		transcriptional regulator, PadR family	7.08	4.08E-05
I35_4049	<i>uspA</i>	transcriptional regulator, CRP family	13.98	2.32E-03
I35_4278		Undecaprenyl-phosphate galactosephosphotransferase	5.46	2.90E-02
I35_4388		hypothetical protein	4.22	9.09E-03
I35_4786		Histidine ABC transporter, histidine-binding periplasmic protein precursor HisJ (TC 3.A.1.3.1)	5.41	1.97E-04
I35_5200		cAMP-binding proteins-catabolite gene activator and regulatory subunit of cAMP-dependent protein kinases	4.66	3.62E-09
I35_5447		Isocitrate lyase	4.20	2.14E-04
I35_6041		Protein ygiW precursor	10.42	9.16E-03
I35_6317		hemagglutinin domain-containing protein	4.21	2.47E-08
I35_6318		Outer membrane protein	5.49	5.00E-03
I35_6568		putative Cytochrome bd2, subunit I	30.96	8.26E-03
I35_6569		putative Cytochrome bd2, subunit II	28.35	3.69E-03
I35_6631	<i>cstA</i>	hypothetical small protein yjiX	22.33	2.86E-21
I35_7134		Putrescine importer	6.29	2.13E-08
I35_7135		Omega-amino acid--pyruvate aminotransferase	7.19	4.04E-12
I35_7314	<i>pilA</i>	Flp pilus assembly protein, pilin Flp	101.09	3.54E-12
I35_7316		Flp pilus assembly protein, pilin Flp	109.50	1.94E-09

I35_7317		Type IV prepilin peptidase TadV/CpaA	43.36	1.21E-11
I35_7318		putative ATPase with chaperone	33.15	4.57E-10
I35_7319		Flp pilus assembly protein RcpC/CpaB	38.43	2.15E-11
I35_7320		Type II/IV secretion system secretin	28.38	3.61E-10
I35_7321		tRNA delta(2)-isopentenylpyrophosphate	20.70	1.40E-09
I35_7322		Von Willebrand factor type A domain	28.74	1.17E-10
I35_7323		Similar to TadZ/CpaE, associated with Flp pilus	11.01	1.82E-05
I35_7324		Similar to TadZ/CpaE, associated with Flp pilus	9.40	1.95E-04
I35_7325		Type II/IV secretion system ATPase	20.03	1.48E-08
I35_7326		Type II/IV secretion system ATP hydrolase	14.10	1.15E-07
I35_7327		Flp pilus assembly protein TadB	12.61	3.94E-06
I35_7328		Type II/IV secretion system protein	13.56	7.91E-07
I35_7329		hypothetical protein	84.56	7.92E-03
I35_7330		Protein involved in meta-pathway of phenol	4.80	3.27E-03
I35_6384	<i>metE</i>	5-methyltetrahydropteroyltryglutamate--homocysteine methyltransferase	5.65	2.15E-11
I35_7315	<i>pilA_2</i>	Flp pilus assembly protein, pilin Flp	113.65	2.46E-14
I35_2817	<i>ykgE</i>	putative L-lactate dehydrogenase, Fe-S oxidoreductase subunit YkgE	20.58	3.64E-16
I35_2819	<i>ykgF</i>	putative L-lactate dehydrogenase, Iron-sulfur cluster-binding subunit YkgF	7.11	1.72E-11
I35_2818	<i>ykgG</i>	putative L-lactate dehydrogenase, hypothetical protein subunit YkgG	17.67	4.15E-07

Table 7.1.3 List of upregulated genes in *B. cenocepacia/pYedQ2*. Selection criteria were set as fold change ≥ 4 and p-value ≤ 0.05 , n=2. NCBI gene locus tags, gene names and protein functions are displayed.

Genes downregulated in <i>B. cenocepacia/pYedQ2</i>				
NCBI locus tag	Feature ID	Protein	Fold change	FDR corrected p-value
I35_1830	<i>acoA</i>	Acetoin dehydrogenase E1 component alpha-subunit	-7.63	1.66E-03
I35_1829	<i>acoB</i>	Acetoin dehydrogenase E1 component beta-subunit	-5.22	2.04E-03
I35_1828	<i>acoC</i>	Dihydrolipoamide acetyltransferase component (E2) of acetoin dehydrogenase complex	-4.39	3.54E-02

I35_6461	<i>aer</i>	Aerotaxis sensor receptor protein	-9.22	6.75E-03
I35_7309	<i>aidA</i>	nematocidal protein AidA	-7.74	0.00E+00
I35_6025	<i>bapA</i>	Large repetitive protein	-4.33	3.77E-05
I35_4184	<i>bclA</i>	lectin	-9.08	1.13E-16
I35_4183	<i>bclC</i>	lectin	-4.38	2.38E-05
I35_7538	<i>benC</i>	benzoate 1,2-dioxygenase electron transfer component	-4.93	1.12E-04
I35_1831	<i>budC</i>	Short-chain alcohol dehydrogenase associated with acetoin utilization	-5.99	7.77E-03
I35_7541	<i>catA2</i>	Catechol 1,2-dioxygenase	-4.22	3.83E-03
I35_4187	<i>cepS</i>	transcriptional regulator, AraC family	-4.57	7.96E-08
I35_0141	<i>cheB_1</i>	Chemotaxis response regulator protein-glutamate methylesterase CheB	-4.98	2.73E-05
I35_0140	<i>cheD</i>	Chemotaxis protein CheD	-7.35	2.44E-07
I35_0139	<i>cheR</i>	Chemotaxis protein methyltransferase CheR	-5.08	2.61E-05
I35_0137	<i>cheW</i>	Positive regulator of CheA protein activity (CheW)	-6.38	7.48E-07
I35_0142	<i>cheY_1</i>	Chemotaxis regulator-transmits chemoreceptor signals to flagellar motor components CheY	-5.39	3.14E-05
I35_3090	<i>flgK</i>	Flagellar hook-associated protein FlgK	-5.63	2.54E-05
I35_3089	<i>flgL</i>	Flagellar hook-associated protein FlgL	-6.01	5.74E-06
I35_3102	<i>flgM</i>	Negative regulator of flagellin synthesis	-6.54	1.09E-05
I35_3103	<i>flgN</i>	Flagellar biosynthesis protein FlgN	-6.20	1.48E-06
I35_0121	<i>fliC</i>	Flagellar biosynthesis protein FliC	-9.54	3.71E-09
I35_0120	<i>fliD1</i>	Flagellar hook-associated protein FliD	-7.28	5.27E-07
I35_3138	<i>fliS</i>	Flagellar biosynthesis protein FliS	-10.32	7.62E-09
I35_3137	<i>fliT</i>	Flagellar biosynthesis protein FliT	-9.45	1.69E-08
I35_0673	<i>hmgA</i>	Homogentisate 1,2-dioxygenase	-5.84	1.31E-05
I35_0198	<i>hppD</i>	4-hydroxyphenylpyruvate dioxygenase	-4.22	3.38E-07
I35_0119		Adenosylhomocysteinase	-4.99	6.48E-05
I35_0136		Signal transduction histidine kinase CheA	-6.34	2.72E-06
I35_0138		hypothetical protein	-6.15	3.37E-06
I35_0669		hypothetical protein	-5.87	1.59E-05
I35_0670		2-polyprenyl-6-methoxyphenol hydroxylase and	-6.14	1.91E-06
I35_0671		hypothetical protein	-8.32	1.91E-06
I35_0674		Fumarylacetoacetase	-6.35	2.92E-05
I35_0963		hypothetical protein	-4.57	9.18E-05
I35_1350		hypothetical protein	-7.11	7.10E-03
I35_2449		Ferredoxin	-4.74	1.30E-07
I35_2450		hypothetical protein	-4.63	3.41E-07

I35_2451	DNA-directed RNA polymerase specialized sigma	-4.06	3.27E-07
I35_2905	Methyl-accepting chemotaxis protein I (serine	-6.61	2.23E-04
I35_4188	Omega-amino acid--pyruvate aminotransferase	-6.09	8.21E-11
I35_4189	Enterobactin synthetase component F	-5.52	2.63E-09
I35_4191	probable remnant of a transposase gene protein	-5.47	1.42E-05
I35_4192	hypothetical protein	-5.40	4.78E-02
I35_4471	hypothetical protein	-9.56	5.55E-04
I35_4472	Sialic acid transporter (permease) NanT	-8.11	1.61E-03
I35_4473	Similar to 5-oxoprolinase and Methylhydantoinases A, B	-7.44	3.72E-03
I35_4529	Leucyl aminopeptidase (aminopeptidase T)	-4.92	5.58E-09
I35_4530	hypothetical protein	-5.83	1.23E-08
I35_4980	hypothetical protein	-4.21	4.49E-02
I35_4982	hypothetical protein	-8.01	1.13E-02
I35_5266	hypothetical protein	-4.42	2.57E-08
I35_5267	hypothetical protein	-5.51	7.74E-10
I35_5428	Methyl-accepting chemotaxis protein I (serine	-7.15	7.35E-07
I35_5429	hypothetical protein	-5.14	5.88E-03
I35_5614	hypothetical protein	-11.77	5.62E-04
I35_5615	Extracellular protease precursor	-10.45	1.59E-08
I35_5616	hypothetical protein	-4.27	1.65E-02
I35_5655	hypothetical protein	-6.40	1.30E-12
I35_5683	Methyl-accepting chemotaxis protein I (serine	-5.70	1.42E-05
I35_5857	Methyl-accepting chemotaxis protein	-8.71	3.76E-11
I35_5860	hypothetical protein	-4.25	2.26E-03
I35_5897	hypothetical protein	-8.46	2.31E-06
I35_5898	hypothetical protein	-17.18	1.88E-03
I35_6052	hypothetical protein	-6.64	1.11E-06
I35_6053	hypothetical protein	-4.77	6.97E-08
I35_6270	Methyl-accepting chemotaxis protein	-12.91	3.15E-10
I35_6271	Multimodular transpeptidase-transglycosylase	-7.93	6.33E-03
I35_6272	Lipoprotein releasing system transmembrane	-4.42	1.55E-03
I35_6273	hypothetical protein	-8.33	9.41E-08
I35_7149	hypothetical protein	-8.45	1.29E-06
I35_7278	Response regulator	-7.23	4.33E-09

I35_7279		Histidine kinase	-5.62	3.28E-08
I35_7305		hypothetical protein	-12.75	6.18E-06
I35_7306		putative oxygenase (putative secreted protein)	-8.50	5.18E-14
I35_7307		Periplasmic binding protein	-9.19	4.55E-14
I35_7308		AidA	-10.34	0.00E+00
I35_7537		1,2-dihydroxycyclohexa-3,5-diene-1-carboxylate	-5.09	1.38E-02
I35_7652		monooxygenase, putative	-4.33	1.42E-05
I35_7653		Membrane GTPase LepA	-10.37	5.07E-16
I35_7654		hypothetical protein	-9.29	1.75E-15
I35_7655		putative RebB like protein	-5.74	3.68E-10
I35_7656		DNA-directed RNA polymerase specialized sigma	-5.60	2.21E-07
I35_7658		Thioredoxin reductase	-9.19	1.52E-15
I35_7659		Thioredoxin reductase	-19.44	4.54E-22
I35_7660		Thioredoxin reductase	-20.11	1.56E-22
I35_0133	<i>motA_1</i>	Flagellar motor rotation protein MotA	-5.36	3.67E-05
I35_0134	<i>motB_1</i>	Flagellar motor rotation protein MotB	-6.37	1.80E-05
I35_0672	<i>pcaK</i>	4-hydroxybenzoate transporter	-7.37	3.41E-07
I35_6260	<i>pqqA</i>	Coenzyme PQQ synthesis protein A	-6.11	1.04E-02
I35_7447	<i>zmpA</i>	zinc metalloprotease ZmpA	-15.19	0.00E+00

Table 7.1.4 List of downregulated genes in *B. cenocepacia/pYedQ2*. Selection criteria were set as fold change ≤ -4 and p-value ≤ 0.05 , n=2. NCBI gene locus tags, gene names and protein functions are displayed.

Genes upregulated in <i>K. pneumoniae/pYedQ2</i>				
NCBI locus tag	Feature ID	Product	Fold change	FDR corrected p-value
KLP1_04265	<i>hisG</i>	ATP phosphoribosyltransferase	5.22	2.18E-04
KLP1_04290		1-(5-phosphoribosyl)-5-((5-phosphoribosylamino)methylideneamino)imidazole-4-carboxamide isomerase	4.62	1.47E-02
KLP1_04295		Imidazole glycerol phosphate synthase cyclase subunit	4.34	9.19E-03
KLP1_04300		phosphoribosyl-ATP pyrophosphatase	4.48	9.96E-03
KLP1_06690		integrase	2361.44	2.22E-21

KLP1_06695		hypothetical protein	1674.66	8.40E-16
KLP1_06700		transcriptional regulator	247.85	2.70E-04
KLP1_06705		DNA cytosine methyltransferase	7352.82	1.74E-24
KLP1_06710		relaxase	172.91	3.14E-04
KLP1_14575		transcriptional regulator	5.17	5.86E-03
KLP1_14580		ribose ABC transporter permease	4.24	7.99E-03
KLP1_15410		anaerobic ribonucleoside triphosphate reductase	4.48	8.36E-04
KLP1_15415		hypothetical protein	5.17	3.65E-02
KLP1_22450		stress-induced protein	4.61	1.46E-03

Table 7.1.5 List of upregulated genes in *K. pneumoniae/pYedQ2*. Selection criteria were set as fold change ≥ 4 and p-value ≤ 0.05 , n=2. NCBI gene locus tags, gene names and protein functions are displayed.

Genes downregulated in <i>K. pneumoniae/pYedQ2</i>				
NCBI locus tag	Feature ID	Protein	Fold change	FDR corrected p-value
KLP1_08640	<i>gcvT</i>	glycine cleavage system protein T	-5.58	1.34E-02
KLP1_00190		6-phospho-beta-glucosidase	-6.98	1.45E-04
KLP1_00195		PTS glucose transporter subunit IIBC	-4.26	5.23E-04
KLP1_01845		dehydrogenase	-4.35	8.26E-04
KLP1_02205		lipase	-4.39	6.30E-04
KLP1_02210		spermidine/putrescine ABC transporter permease	-12.70	4.62E-12
KLP1_02215		iron ABC transporter substrate-binding protein	-6.18	1.47E-03
KLP1_02425		shikimate 5-dehydrogenase	-4.36	9.87E-05
KLP1_02430		D-galactonate transporter DgoT	-5.30	2.75E-06
KLP1_02435		4-hydroxyphenylpyruvate dioxygenase	-4.56	1.53E-04
KLP1_02570		hypothetical protein	-4.15	5.98E-04
KLP1_02575		5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	-6.07	5.39E-05
KLP1_02580		hypothetical protein	-9.52	2.21E-04
KLP1_03135		cell envelope biogenesis protein OmpA	-8.01	4.02E-02
KLP1_08770		cupin	-5.51	2.35E-03
KLP1_09505		autoinducer-2 (AI-2) modifying protein LsrG	-5.17	3.99E-03
KLP1_09515		autoinducer 2-binding protein lsrB	-4.75	8.36E-04

KLP1_09520		autoinducer 2 import system permease LsrD	-4.88	4.93E-04
KLP1_09525		ABC transporter permease	-7.76	1.78E-05
KLP1_09530		ABC transporter ATP-binding protein	-7.06	1.75E-05
KLP1_09535		transcriptional regulator LsrR	-5.02	1.29E-03
KLP1_10515		50S ribosomal protein L3	-4.17	5.00E-03
KLP1_12165		hypothetical protein	-10.72	8.38E-12
KLP1_12515		6-phospho-alpha-glucosidase	-81.02	0.00E+00
KLP1_12520		PTS alpha-glucoside transporter subunit IICB	-33.17	5.70E-13
KLP1_12540		heat-shock protein IbpB	-4.72	5.39E-05
KLP1_12545		heat-shock protein IbpA	-4.12	1.45E-04
KLP1_13065		alpha-galactosidase	-9.63	7.01E-08
KLP1_13700		5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	-5.69	1.68E-03
KLP1_16265		AraC family transcriptional regulator	-4.17	1.46E-03
KLP1_16920		malate permease	-42.26	1.35E-18
KLP1_17215		hypothetical protein	-4.24	4.31E-03
KLP1_18000		carbon starvation induced protein	-10.72	1.02E-05
KLP1_22400		gamma-glutamylputrescine synthetase	-6.92	5.39E-05
KLP1_22420		AraC family transcriptional regulator	-4.44	1.27E-02
KLP1_24060		3-oxoacyl-ACP reductase	-5.77	1.46E-05
KLP1_13070	<i>lacY_I</i>	galactoside permease	-7.05	3.43E-04

Table 7.1.6 List of downregulated genes in *K. pneumoniae/pYedQ2*. Selection criteria were set as fold change ≤ -4 and p-value ≤ 0.05 , n=2. NCBI gene locus tags, gene names and protein functions are displayed.

7.2. Metabolomics data

Metabolites upregulated in <i>P. aeruginosa</i> /pYedQ2			
Feature ID (mz/retention time)	Possible Match	FC	p-value
691.0772 / 4.12m	Cyclic di-3',5'-guanylate	4931.80	1.02E-07
355.1076 / 0.84m	5-Amino-6-(5'-phosphoribosylamino)uracil	631.60	3.06E-07
718.0299 / 4.13m	No match	570.00	4.96E-08
681.5261 / 12.41m	No match	419.00	2.74E-03
484.1282 / 0.84m	(2,3-Dihydroxybenzoyl)adenylic acid	393.69	1.28E-05
664.4232 / 1.71m		384.27	3.21E-02
342.2779 / 10.74m	No match	239.91	6.87E-03
152.1122 / 4.13m	N-Methyltyramine	239.82	1.09E-05
718.5299 / 4.14m		226.00	3.31E-08
703.0527 / 4.13m	No match	207.60	1.46E-07
795.578 / 14.16m	No match	199.20	4.22E-02
637.4977 / 11.97m	No match	199.14	4.59E-07
1036.0382 / 4.12m	No match	196.80	1.92E-03
324.2757 / 10.32m	No match	194.92	5.09E-03
152.1124 / 4.64m	N-Methyltyramine or Phenylalaninol	192.67	1.70E-09
717.5305 / 4.12m	2,3-Bis-O-(geranylgeranyl)glycerol 1-phosphate	186.00	2.31E-07
540.0564 / 4.13m	No match	166.40	4.03E-08
651.5241 / 12.44m	No match	136.50	1.97E-02
298.2542 / 9.76m	No match	127.50	1.80E-02
326.2901 / 10.33m	No match	119.02	3.06E-03
833.5243 / 14.11m	No match	115.50	1.07E-02
613.148 / 2.23m	Glutathione disulphide or Oxidized glutathione	113.40	8.09E-03
135.0859 / 4.13m	1-Deoxyxylonojirimycin	113.00	2.74E-08
710.0463 / 4.13m	No Match	104.40	5.97E-06
183.1502 / 3.59m	Geosmin	100.80	4.52E-06
664.3455 / 1.71m	No Match	97.20	4.53E-02
328.2986 / 11.23m	No Match	96.00	5.42E-04
314.2501 / 10.75m	No Match	79.33	3.13E-03
705.5781 / 12.2m	2-Demethylmenaquinol 8	74.88	1.21E-03
370.1142 / 1.17m	No Match	72.67	3.24E-03
314.2508 / 10.04m	No Match	71.99	3.72E-02
286.2262 / 8.05m	No Match	69.40	2.59E-02
850.2783 / 1.15m	No Match	68.64	8.74E-03
644.2727 / 3.03m	No Match	62.80	1.35E-02

316.2688 / 10.45m	No Match	60.04	1.45E-02
499.1778 / 1.72m	No Match	59.88	1.87E-02
1051.2496 / 2.78m	No Match	59.20	4.34E-02
314.2483 / 9.6m	No Match	54.79	2.45E-02
607.5228 / 12.64m	1-(9Z,12Z-heptadecadienoyl)-2-octadecanoyl-sn-glycerol or 1-heptadecanoyl-2-(9Z,12Z-octadecadienoyl)-sn-glycerol	54.20	2.42E-02
694.2536 / 2.75m	No Match	54.00	4.62E-03
744.9827 / 4.15m	No Match	53.60	5.01E-05
750.4543 / 12.45m	No Match	48.73	9.97E-03
146.1479 / 1.16m	Spermidine	45.40	1.68E-02
298.26 / 9.24m	No Match	41.21	2.63E-02
409.1097 / 0.84m	No Match	40.00	6.66E-04
694.5772 / 12.28m	No Match	39.60	4.00E-02
441.375 / 12.44m	No Match	38.79	2.33E-02
484.1303 / 2.23m	(2,3-Dihydroxybenzoyl)adenylic acid	38.20	1.08E-02
413.3512 / 11.86m	Isofucosterol	37.00	1.10E-02
621.2941 / 12.13m	No Match	34.33	2.29E-06
231.0934 / 0.85m	Thioredoxin or Ergothioneine	34.00	2.63E-05
559.3844 / 11.71m	No Match	33.40	4.40E-03
971.4398 / 11.18m	No Match	33.10	1.65E-02
591.2555 / 2.6m	No Match	30.60	2.29E-02
179.1025 / 0.85m	3-(all-trans-diprenyl)benzene-1,2-diol	30.00	1.49E-05
489.369 / 12.8m	No Match	29.80	4.75E-03
388.1642 / 1.71m	No Match	29.17	3.15E-02
775.4978 / 12.75m	No Match	27.96	3.04E-02
298.2544 / 9.67m	No Match	27.56	8.55E-03
354.1479 / 1.17m	No Match	25.83	1.44E-02
129.1207 / 1.16m	3-Isopropyl-3-butenic acid or Cyclohexane-1-carboxylate	25.80	1.75E-02
975.3598 / 11.97m	No Match	25.40	1.45E-02
755.4098 / 12.44m	No Match	25.04	9.18E-03
693.5511 / 12.14m	No Match	24.86	1.15E-02
722.4321 / 11.86m	13-[O(2')-beta-D-Glucopyranosyl-beta-D-glucopyranosyloxy]docosanoate O(6'')-acetate	23.90	1.29E-02
525.1854 / 11.97m	No Match	23.40	4.10E-03
298.2571 / 9.98m	No Match	20.61	4.09E-02

355.1086 / 2.23m	5-Amino-6-(5'-phosphoribosylamino)uracil	20.40	4.77E-02
686.757 / 2.55m	No Match	20.21	4.40E-02
654.3102 / 11.97m	No Match	20.13	1.90E-04
720.2143 / 2.65m	No Match	19.31	1.23E-02
948.1605 / 4.65m	No Match	18.48	3.81E-02
722.433 / 11.71m	13-[O(2')-beta-D-Glucopyranosyl-beta-D-glucopyranosyloxy]docosanoate O(6'')-acetate	17.94	2.16E-02
693.7644 / 2.65m	No Match	17.64	2.89E-02
762.233 / 2.83m	No Match	17.60	4.03E-02
300.2724 / 10m	Sphingosine	16.72	1.36E-02
293.1649 / 11.5m	octyl alpha-D-mannopyranoside or octyl alpha-D-galactopyranoside or octyl alpha-D-glucopyranoside	16.38	1.14E-02
750.4575 / 11.72m	No Match	15.73	3.00E-02
161.1873 / 11.71m	No Match	15.00	9.33E-03
338.1814 / 1.16m	No Match	14.84	9.64E-03
298.0941 / 0.98m	5'-methylthioformycin		
	5'-S-methyl-5'-thioadenosine	14.83	1.94E-02
	5'-Methylthioadenosine		
639.3008 / 11.36m	No Match	14.59	1.24E-03
665.5347 / 9.79m	No Match	14.40	4.35E-02
430.9371 / 0.58m	No Match	13.23	1.79E-03
443.3921 / 13.07m	No Match	13.18	2.92E-02
137.1879 / 11.71m	No Match	13.17	6.75E-03
756.4498 / 13.03m	No Match	13.10	2.01E-03
743.3568 / 11.5m	No Match	12.00	2.91E-02
833.522 / 13.3m	No Match	11.91	3.23E-02
427.1171 / 0.98m	No Match	11.60	1.28E-02
179.1971 / 11.72m	No Match	11.40	3.23E-02
494.3369 / 10.69m	lipid	11.31	2.50E-02
705.5502 / 12.27m	2-Demethylmenaquinol 8	10.40	4.15E-02
903.5397 / 5.68m	No Match	10.22	6.43E-03
355.1031 / 13.29m	5-Amino-6-(5'-phosphoribosylamino)uracil	10.16	3.88E-02
760.2036 / 3.65m	No Match	10.00	4.97E-02
675.2352 / 11.97m	No Match	9.98	3.42E-04
293.1663 / 11.72m	octyl alpha-D-glucopyranoside or octyl alpha-D-mannopyranoside or octyl alpha-D-galactopyranoside	9.71	1.45E-02
665.5654 / 13.06m	No Match	9.27	2.11E-02

651.5502 / 12.49m	No Match	9.19	1.08E-02
1133.4922 / 11.17m	No Match	9.03	8.14E-03
581.2368 / 11.97m	No Match	8.83	2.19E-05
135.0857 / 4.41m	1-Deoxyxylonojirimycin	8.80	8.98E-03
413.3515 / 11.72m	Isofucosterol	8.42	2.23E-02
752.5027 / 13.05m	No Match	8.33	4.84E-02
92.9709 / 11.17m	Thioglycolate	7.83	1.88E-02
743.3586 / 11.72m	No Match	7.82	3.93E-02
665.5326 / 12.99m	No Match	7.70	1.82E-02
413.3496 / 11.5m	Isofucosterol	7.49	3.60E-02
724.4466 / 12.17m	No Match	7.38	2.80E-02
727.3868 / 11.72m	No Match	7.08	1.51E-02
163.1162 / 2.69m	Carnitine or 3-Hydroxy-4- trimethylammoniobutanoic acid	7.00	9.28E-04
694.2329 / 2.75m	No Match	6.88	3.16E-05
686.7336 / 2.56m	No Match	6.85	4.39E-04
180.1051 / 1.76m	(2S,3S)-3-Methylphenylalanine	6.73	1.19E-03
705.7185 / 2.65m	No Match	6.34	1.53E-03
903.1667 / 3.24m	No Match	6.18	8.80E-04
184.0607 / 8.54m	3,5-Dihydroxy-phenylglycine	6.17	1.33E-02
263.1247 / 2.69m	1,6-Dimethoxypyrene	6.17	1.73E-02
1068.4246 / 4.74m	No Match	6.00	1.83E-02
190.9649 / 0.58m	Bromobenzene-2,3-dihydrodiol or Benzo[a]pyrene-7,8-oxide or Bromobenzene-3,4-dihydrodiol	5.86	3.06E-02
727.3847 / 11.51m	No Match	5.85	5.00E-02
197.2072 / 10.73m	No Match	5.83	4.89E-02
127.0624 / 8.54m	5-Amino-4- imidazolecarboxamide	5.67	7.53E-03
561.3964 / 12.16m	2-Hexaprenyl-3-methyl-6- methoxy-1,4-benzoquinone	5.48	4.02E-02
637.2883 / 11.97m	No Match	5.21	1.08E-05
784.1135 / 3.65m	No Match	5.11	1.09E-03
444.0535 / 4.64m	Guanosine diphosphate	5.00	1.68E-02
757.4276 / 13.07m	No Match	4.97	3.43E-02
692.743 / 2.66m	No Match	4.90	4.08E-05
1068.7135 / 4.74m	No Match	4.65	1.99E-02
415.365 / 12.17m	beta-Sitosterol	4.57	1.75E-02
745.3742 / 12.16m	No Match	4.56	1.41E-02
903.1882 / 4.53m	Coenzyme F420-3	4.50	1.10E-02
138.6664 / 7.58m	No Match	4.50	4.98E-02
720.6919 / 2.65m	No Match	4.38	6.12E-05

579.1326 / 3.24m	No Match	4.25	1.61E-03
729.4013 / 12.16m	No Match	4.24	1.85E-02
542.4302 / 10.62m	No Match	4.22	4.92E-02
199.2212 / 12.17m	No Match	4.21	2.00E-02
293.1648 / 12.17m	octyl alpha-D-mannopyranoside or octyl alpha-D-galactopyranoside or octyl alpha-D-glucopyranoside	4.14	3.89E-02
691.5769 / 13.04m	No Match	4.13	3.39E-02
693.7406 / 2.65m	No Match	4.06	3.95E-04
681.5539 / 12.58m	No Match	3.96	2.60E-02
752.4726 / 12.17m	No Match	3.93	1.75E-02
181.212 / 12.17m	No Match	3.93	1.29E-02
820.9323 / 4.85m	No Match	3.90	4.11E-03
1128.3059 / 4.53m	No Match	3.88	5.42E-03
243.1378 / 4.06m	Menaquinone-1 or Thymidine	3.86	2.95E-02
930.2493 / 6.29m	No Match	3.79	1.79E-02
504.8792 / 13.09m	No Match	3.79	2.37E-02
707.5624 / 12.55m	No Match	3.70	4.33E-02
841.3614 / 11.34m	No Match	3.67	1.89E-02
553.1753 / 3.79m	No Match	3.67	2.80E-02
369.3304 / 11.2m	No Match	3.66	2.63E-02
135.0857 / 4.64m	1-Deoxyxylonojirimycin	3.60	5.71E-04
741.1518 / 3.24m	No match	3.59	2.93E-02
560.0779 / 3.44m	Adenosine diphosphate ribose or ADP-ribose or 1-(5-phosphoribosyl)-AMP or Phosphoribosyl-AMP	3.54	1.84E-02
118.1405 / 0.77m	No Match	3.50	1.65E-02
978.8228 / 5.69m	No Match	3.48	1.42E-02
217.2305 / 12.18m	No Match	3.45	2.93E-02
1084.524 / 11.53m	No Match	3.36	1.39E-02
699.2208 / 3.1m	No Match	3.27	3.96E-03
60.3003 / 13.54m	No Match	3.20	4.30E-02
761.5089 / 12.85m	No Match	3.09	1.63E-02
138.6847 / 8.93m	No Match	3.00	3.38E-02
833.4407 / 12.75m	No Match	3.00	4.52E-02
1162.9973 / 4.85m	No Match	2.95	2.58E-02
996.594 / 4.85m	No Match	2.89	3.65E-03
545.8168 / 10.17m	No Match	2.84	3.71E-02
997.0269 / 4.9m	No Match	2.74	1.14E-02
112.1048 / 0.98m	Histamine	2.67	1.66E-02

Table 7.2.1. List of upregulated metabolites in *P. aeruginosa/pYedQ2*. Selection criteria were set as fold change (FC) ≥ 2 and p-value ≤ 0.05 , n=5. Metabolites is denoted by mz value/retention time. Metabolites with dababase hit are listed as ‘Compound name’ while metabolotes without database hit are listed as ‘No match’. Only hits with highest similarity are illustrated here based on molecular weight of each metabolite.

Metabolites downregulated in <i>P. aeruginosa/pYedQ2</i>			
Feature ID (mz/retention time)	Possible Match	FC	p-value
454.2471 / 2.62m	1-Acyl-sn-glycero-3-phosphoglycerol (N-C14:1) or 2-Acyl-sn-glycero-3-phosphoglycerol (N-C14:1)	-2.80	4.00E-02
371.1321 / 13.79m	No match	-2.92	1.25E-02
84.0964 / 0.71m	No match	-3.08	2.61E-03
707.4846 / 12.56m	lipid	-3.10	4.46E-02
130.1047 / 0.71m	4-Guanidinobutanal	-3.13	3.15E-03
221.0514 / 0.76m	(-)-threo-Iso(homo)2-citrate or (R)-(Homo)2-citrate	-3.13	1.31E-02
728.4194 / 13.91m	No match	-3.14	3.85E-02
335.1152 / 3.44m	Glutathione episulfonium ion	-3.33	2.31E-02
378.2529 / 12.3m	No match	-3.40	4.11E-04
285.2849 / 8.66m	Stearic acid	-3.40	2.17E-02
311.2985 / 9.19m	No match	-3.40	2.53E-02
707.4714 / 12.46m	lipid	-3.47	3.99E-02
737.3423 / 13.28m	No match	-3.50	4.89E-02
302.2366 / 2.68m	No match	-3.52	3.53E-02
770.5553 / 13.3m	No match	-3.65	3.51E-03
241.1306 / 0.71m	Homocarnosine	-3.67	1.83E-03
298.1324 / 3m	5'-S-methyl-5'-thioadenosine or 5'-methylthioformycin or 5'-Methylthioadenosine	-3.69	3.68E-02
453.2344 / 13.3m	No match	-3.70	9.43E-03
119.1041 / 7.92m	Betaine	-3.74	1.41E-04
707.4762 / 12.39m	lipid	-3.77	2.90E-02

625.397 / 14.15m	2,2'-Diketospiriloxanthin	-3.88	6.87E-03
621.3 / 13.28m	No match	-4.01	3.53E-02
259.139 / 0.71m	Glycerophosphocholine	-4.14	1.66E-03
213.1384 / 0.71m	Pyocyanine	-4.30	4.19E-03
557.1666 / 1.15m	No match	-4.46	9.72E-03
274.2038 / 10.97m	(2R,3R)-3-Methylglutamyl-5-semialdehyde-N6-lysine	-4.54	5.95E-07
91.1076 / 11.37m	(R,R)-Butane-2,3-diol or 2,3-Butanediol or meso-2,3-Butanediol	-4.60	1.35E-02
303.1781 / 4.79m	16alpha-Hydroxyandrost-4-ene-3,17-dione	-4.62	8.86E-03
281.0969 / 13.14m	No match	-4.80	9.55E-03
152.1185 / 13.43m	N-Methyltyramine or Phenylalaninol	-4.89	1.11E-03
663.3434 / 14.15m	No match	-4.98	2.73E-03
386.0484 / 1.89m	No match	-5.09	5.05E-03
91.1078 / 7.93m	(R,R)-Butane-2,3-diol or 2,3-Butanediol or meso-2,3-Butanediol	-5.20	3.70E-04
119.0899 / 1.95m	Betaine or 2,4-Diaminobutyric acid	-5.26	1.29E-02
670.4445 / 14.15m	No match	-5.58	1.86E-02
647.3739 / 14.13m	No match	-5.62	1.23E-02
79.1064 / 7.93m	No match	-5.69	4.76E-04
105.209 / 7.93m	No match	-5.80	2.05E-02
313.3141 / 9.76m	No match	-6.03	2.36E-04
133.156 / 11.38m	No match	-6.15	2.27E-03
145.1065 / 7.92m	4-Guanidinobutanamide	-6.17	8.57E-03
482.3341 / 11.15m	2-Acyl-sn-glycero-3-phosphoethanolamine (N-C18:0)	-6.20	2.11E-02
763.5613 / 13.44m		-6.31	3.62E-02
277.1465 / 0.71m	N6-(L-1,3-Dicarboxypropyl)-L-lysine	-6.44	2.91E-03
393.4917 / 13.44m	No match	-6.52	2.43E-03
385.3263 / 14.15m	Cholest-4-en-3-one or 7-Dehydrocholesterol	-6.60	9.69E-03
638.508 / 13.37m	No match	-6.77	8.03E-04
772.5569 / 14.18m	No match	-7.03	1.76E-02
267.0467 / 13.14m	No match	-7.25	4.33E-04
327.1163 / 10.86m	cis-beta-D-Glucosyl-2-hydroxycinnamate or	-7.30	2.72E-04

	6,7-dimethyl-8-(D-ribityl)lumazine or 6,7-Dimethyl-8-(1-D-ribityl)lumazine		
196.1646 / 11.2m	No match	-7.52	1.55E-03
281.1471 / 7.92m	No match	-7.73	3.98E-03
827.2103 / 7.92m	No match	-7.77	4.31E-03
281.1462 / 5.2m	No match	-7.80	6.80E-03
282.1816 / 12.43m	No match	-8.00	2.86E-04
607.1054 / 0.71m	No match	-8.05	2.28E-03
636.4447 / 13.07m	phosphatidylethanolamine	-8.24	2.43E-03
	UDP-4-amino-4,6-dideoxy-N-acetyl-beta-L-altrosamine		
591.1339 / 0.71m	or UDP-2-acetamido-4-amino-2,4,6-trideoxy-alpha-D-glucose	-8.55	1.61E-03
267.172 / 7.92m	No match	-8.83	1.93E-02
137.1004 / 1.35m	N,N'-dimethyl-p-phenylenediamine	-8.89	3.61E-04
252.1734 / 12.43m	No match	-9.32	7.45E-04
930.2955 / 14.21m	No match	-9.56	1.26E-02
311.2987 / 8.99m	No match	-9.58	1.26E-04
256.3128 / 13.29m	No match	-9.70	3.20E-04
411.2043 / 13.44m	1-hexadecanoyl-sn-glycerol 3-phosphate	-9.73	1.78E-03
166.0937 / 7.92m	N-Benzylglycine or D-Phenylalanine or L-Phenylalanine	-10.10	6.98E-04
264.1713 / 12.43m	No match	-10.31	5.83E-04
594.4889 / 13.39m	No match	-10.58	3.96E-03
369.1559 / 10.97m	curcumin	-10.75	5.25E-04
111.0995 / 7.92m	Imidazole-4-acetaldehyde	-11.00	1.35E-05
406.1957 / 8.53m	No match	-11.00	6.32E-03
339.325 / 10.02m	No match	-11.25	4.05E-04
274.204 / 11.2m	(2R,3R)-3-Methylglutamyl-5-semialdehyde-N6-lysine	-11.56	8.31E-06
265.1537 / 7.93m	Formyl-N-acetyl-5-methoxykynurenamine	-11.80	6.17E-03
563.2636 / 12.44m	protoporphyrin	-12.18	1.50E-04
263.1389 / 7.92m	1,6-Dimethoxypyrene	-12.22	1.81E-03
441.3783 / 13.37m	No match	-12.86	7.55E-04
377.3594 / 13.45m	No match	-13.08	1.90E-02

393.2227 / 7.93m	No match	-13.09	6.77E-03
286.1405 / 7.92m	No match	-13.25	1.05E-03
226.1181 / 7.92m	2-Amino-2-deoxyisochorismate or 4-Amino-4-deoxychorismate	-13.29	1.72E-04
180.1343 / 11.2m	2S,3S)-3-Methylphenylalanine	-13.33	2.39E-06
243.1766 / 12.44m	Menaquinone-1	-13.67	1.61E-02
814.5737 / 13.28m	No match	-13.68	6.54E-06
663.345 / 13.9m	No match	-13.80	4.73E-03
346.1608 / 7.92m	No match	-13.98	3.06E-04
325.3119 / 9.57m	No match	-14.13	2.98E-03
505.3621 / 13.9m	No match	-15.17	9.34E-03
174.2356 / 4.51m	No match	-15.20	4.72E-02
293.1458 / 7.93m	No match	-15.60	4.05E-02
241.1717 / 12.43m	Homocarnosine	-15.75	1.09E-03
243.1689 / 12.43m	Menaquinone-1	-15.81	3.74E-04
670.4449 / 13.9m	No match	-16.32	5.38E-03
858.5863 / 13.26m	No match	-17.24	8.42E-04
312.367 / 14.15m	No match	-17.37	2.19E-02
489.2389 / 7.92m	No match	-17.46	2.35E-04
369.1587 / 12.77m	curcumin	-17.86	3.90E-05
540.3313 / 13.43m		-19.56	3.37E-04
558.9597 / 3.15m	No match	-19.60	6.96E-05
480.3204 / 10.02m		-21.56	1.32E-03
393.4356 / 13.45m	No match	-23.07	9.92E-05
574.2806 / 3.12m	No match	-23.20	4.00E-04
130.1422 / 1.65m	4-Guanidinobutanal	-24.00	3.70E-07
454.3105 / 9.76m	1-Acyl-sn-glycero-3-phosphoethanolamine (N-C16:0)	-24.22	5.31E-05
219.2278 / 13.95m	No match	-24.83	8.95E-03
603.3575 / 13.11m	No match	-25.00	3.87E-05
275.1698 / 10.01m	4-Hydroxy-3-polyprenylbenzoate or 2-Polyprenyl-6-methoxy-1,4-benzoquinone	-26.60	2.84E-02
723.3387 / 5.27m	No match	-27.00	6.30E-04
387.2226 / 9.5m	Ubiquinone-3	-27.60	1.97E-03
553.3331 / 11.14m	Elastin	-31.00	1.44E-03
530.3512 / 11.38m	No match	-31.67	1.53E-03
383.1803 / 7.92m	2-tetradecanoyl-sn-glycerol	-34.17	6.78E-03

	3-phosphate or 2-cis,6-trans-Farnesyl diphosphate or Farnesyl pyrophosphate		
452.2964 / 8.99m		-34.50	2.46E-06
780.8746 / 5.72m	No match	-35.63	3.14E-02
369.2027 / 7.92m	No match	-36.18	1.27E-04
222.1656 / 12.43m	No match	-37.14	5.14E-05
229.1732 / 12.43m	Myristic acid	-39.00	1.15E-06
558.6246 / 3.14m	No match	-39.00	2.76E-05
331.1685 / 9.5m	Gibberellin A7 or Gibberellin A5	-42.20	4.72E-03
495.2806 / 13.3m	No match	-42.80	5.39E-08
653.2999 / 3.12m	No match	-42.80	1.66E-04
354.2962 / 7.35m	No match	-44.00	4.01E-02
780.374 / 5.72m	No match	-44.42	3.05E-02
231.1706 / 12.43m	No match	-47.60	5.57E-10
523.2514 / 11.38m	No match	-47.71	5.46E-04
666.3516 / 13.28m	No match	-51.60	2.72E-08
507.2809 / 11.38m	No match	-53.27	2.30E-04
558.2904 / 3.15m	No match	-54.80	1.35E-05
836.8695 / 3.14m	No match	-56.20	5.74E-03
517.3059 / 3.23m	No match	-57.20	1.63E-05
406.1811 / 7.92m	No match	-62.43	4.47E-05
517.3057 / 3.17m	No match	-69.20	1.72E-04
489.2368 / 11.26m	No match	-70.89	1.04E-05
766.8393 / 3.38m	No match	-72.80	1.03E-04
601.7898 / 2.97m	No match	-74.80	6.88E-09
887.4935 / 13.44m	No match	-75.00	4.35E-03
617.7882 / 3.11m	No match	-77.60	8.12E-06
373.1725 / 9.5m	etrahydrocurcumin	-78.40	3.01E-05
642.4191 / 14.15m	No match	-116.60	3.99E-03
781.3842 / 6.06m	No match	-120.10	3.88E-02
387.2227 / 9.25m	Ubiquinone-3	-120.40	6.19E-07
902.6023 / 13.24m	No match	-123.20	1.12E-10
331.1682 / 9.25m	Gibberellin A7 or Gibberellin A5	-146.80	1.23E-06
474.2758 / 9.24m	No match	-150.40	5.38E-06
218.2565 / 4.51m	No match	-161.20	4.53E-02
732.3504 / 3.57m	No match	-192.40	6.60E-08
373.1729 / 9.24m	Tetrahydrocurcumin	-248.40	2.12E-07
1168.6696 / 10.77m	No match	-310.00	4.13E-02
429.2259 / 9.5m	No match	-385.19	1.60E-06

353.3405 / 10.68m	No match	-579.61	7.07E-03
429.2259 / 9.24m	No match	-1514.81	2.83E-08

Table 7.2.2 List of downregulated metabolites in *P. aeruginosa/pYedQ2*. Selection criteria were set as fold change (FC) ≤ -2 and p-value ≤ 0.05 , n=5. Metabolites are denoted by m/z value/retention time. Metabolites with database hit are listed as ‘Compound name’ while metabolites without database hit are listed as ‘No match’. Only hits with highest similarity are illustrated here based on molecular weight of each metabolite.

Metabolites upregulated in <i>B. cenocepacia/pYedQ2</i>			
Feature ID (m/z/retention time)	Possible Match	FC	p.value
665.5962 / 13.74m	No match	50253.00	1.27E-02
666.6016 / 13.54m	No match	14357.00	9.64E-03
691.0772 / 4.12m	Cyclic di-3',5'-guanylate	11193.00	4.00E-03
665.5972 / 13.91m	No match	7618.80	4.94E-02
681.5897 / 13.46m	No match	3425.40	4.08E-02
681.5807 / 12.6m	No match	2667.60	1.29E-02
152.1122 / 4.13m	N-Methyltyramine	1607.80	3.99E-03
589.5365 / 12.65m	No match	1453.60	1.82E-02
718.5337 / 12.08m		1272.50	5.87E-05
730.5309 / 12.65m	No match	1258.90	3.79E-03
589.5361 / 12.86m	No match	962.39	2.47E-02
575.5249 / 12.39m	No match	940.91	5.81E-04
665.5937 / 13.96m	No match	891.42	4.36E-02
578.5376 / 12.37m	No match	797.00	4.76E-03
718.0299 / 4.13m	No match	722.20	4.04E-03
523.477 / 11.1m	No match	675.00	4.90E-02
394.3919 / 12.73m	No match	652.81	2.11E-02
647.4086 / 14.14m	No match	615.83	2.24E-02
577.5384 / 12.12m	No match	592.00	3.73E-04
665.8094 / 13.82m	No match	533.83	2.06E-02
744.5434 / 12.33m	No match	506.95	5.56E-04
730.526 / 12.96m	No match	437.55	4.76E-02
603.5498 / 12.32m	No match	372.00	6.03E-03
681.7972 / 12.58m	No match	319.15	1.79E-02
718.5299 / 4.14m	No match	255.40	4.07E-03
703.0527 / 4.13m	No match	248.20	4.16E-03

716.5221 / 12.07m	No match	235.45	3.41E-03
540.0564 / 4.13m	No match	228.60	4.15E-03
553.4244 / 13.41m	3,4-Dehydrorhodopin or Oleoyl-[acyl-carrier protein] or Zeinoxanthin or 1'-Hydroxytorulene or alpha-Cryptoxanthin or beta-Cryptoxanthin	227.33	1.15E-02
135.0859 / 4.13m	1-Deoxyxylonojirimycin	221.50	4.94E-03
716.5292 / 13.38m	2-Octaprenyl-3-methyl-5- hydroxy-6-methoxy-1,4- benzoquinol	218.71	2.31E-02
681.5888 / 14.19m	No match	214.75	4.33E-02
553.4225 / 13.43m	1'-Hydroxytorulene or 3,4-Dehydrorhodopin or Oleoyl-[acyl-carrier protein] or Zeinoxanthin or alpha-Cryptoxanthin or beta-Cryptoxanthin	201.00	4.86E-02
681.5801 / 12.67m	No match	165.07	3.58E-02
717.5305 / 4.12m	2,3-Bis-O- (geranylgeranyl)glycerol 1-phosphate	138.60	4.14E-03
1036.0382 / 4.12m	No match	135.80	4.50E-03
720.5144 / 12.28m		114.12	1.56E-03
907.5058 / 9.98m	Chlorophyll b	112.54	2.40E-02
744.9827 / 4.15m	No match	105.17	5.16E-03
710.0463 / 4.13m	No match	101.80	4.16E-03
756.4479 / 14.04m	No match	95.80	2.95E-02
806.4988 / 13.03m	No match	87.44	1.72E-02
705.5601 / 12.2m	2-Demethylmenaquinol 8	77.55	3.63E-02
732.5445 / 12.08m		66.15	1.54E-02
720.5119 / 12.45m		65.86	2.83E-03
705.5502 / 12.27m	2-Demethylmenaquinol 8	65.67	4.08E-02
591.5516 / 12.09m	No match	64.46	2.40E-02
697.5701 / 12.48m	2-octaprenyl-3-methyl-6- methoxy-1,4- benzoquinone	50.93	2.23E-02
760.4739 / 11.33m		49.00	8.15E-03
515.1539 / 0.82m	S-Formylmycothiol	45.40	8.60E-03
708.5923 / 12.67m	No match	44.64	3.28E-02

734.4991 / 14.2m	Galactolipid	43.30	1.12E-02
756.4498 / 13.03m	No match	35.80	3.30E-02
758.5546 / 12.08m	No match	31.09	4.84E-02
907.4771 / 9.96m	Chlorophyll b	30.43	1.30E-02
744.5452 / 12.04m		25.40	9.29E-03
563.2826 / 12.43m	Protoporphyrin IX or protoporphyrin	24.57	4.36E-03
1179.9547 / 5.13m	No match	23.36	1.44E-02
1095.7918 / 4.98m	No match	19.88	1.76E-02
787.6938 / 5.13m	No match	19.40	5.77E-03
1018.1882 / 0.83m	No match	17.94	3.51E-02
206.0779 / 7.21m	5-Methoxyindoleacetate	17.00	1.26E-02
716.4453 / 13.87m	No match	16.62	1.42E-02
691.5035 / 12.04m	No match	15.02	1.38E-02
787.5809 / 5.12m	No match	14.52	3.66E-02
756.58 / 5.44m	No match	11.70	2.53E-02
1095.9141 / 4.98m	No match	11.58	2.59E-02
705.5781 / 12.2m	2-Demethylmenaquinol 8	9.18	1.73E-02
217.1646 / 3.29m	gamma-Glutamyl-gamma-butyraldehyde	6.33	2.52E-02
933.0884 / 5.62m	No match	4.80	4.80E-02
654.4336 / 12.93m	No match	4.50	3.10E-02
933.1887 / 5.63m	No match	4.40	4.60E-02
752.4726 / 12.17m	No match	4.19	4.56E-02
885.3807 / 13.1m	No match	3.85	3.87E-02
172.1703 / 11.17m	Decanoate (N-C10:0)	2.50	4.97E-02

Table 7.2.3 List of upregulated metabolites in *B. cenocepacia/pYedQ2*. Selection criteria were set as fold change (FC) ≥ 2 and p-value ≤ 0.05 , n=5. Metabolites are denoted by m/z value/retention time. Metabolites with database hit are listed as ‘Compound name’ while metabolites without database hit are listed as ‘No match’. Only hits with highest similarity are illustrated here based on molecular weight of each metabolite.

Metabolites downregulated in <i>B. cenocepacia/pYedQ2</i>			
Feature ID (m/z/retention time)	Possible Match	FC	p.value
614.4351 / 13.08m	No match	-2.79	4.69E-02
371.1321 / 13.79m	No match	-2.91	2.36E-02

341.3393 / 12.96m	No match	-3.04	3.75E-02
707.4846 / 12.56m		-3.04	2.67E-02
119.0901 / 0.72m	Betaine	-3.20	4.44E-02
664.4365 / 12.87m		-3.26	3.58E-02
167.0081 / 1.17m	Phthalate or 4-Hydroxyphenylglyoxylate Or 3-Formylsalicylic acid	-3.40	2.53E-02
135.0857 / 2.76m	1-Deoxyxylonojirimycin	-3.50	5.94E-03
347.8998 / 1.47m	No match	-3.50	2.64E-02
527.1651 / 0.87m	No match	-3.65	4.01E-02
134.1149 / 0.64m	No match	-3.76	1.96E-02
369.1587 / 12.77m	curcumin	-3.85	1.31E-02
282.3233 / 12.33m	No match	-3.90	1.18E-02
119.0899 / 1.95m	Betaine	-4.03	3.79E-02
282.3235 / 12.18m	No match	-4.13	1.75E-02
219.2278 / 13.95m	No match	-4.18	3.05E-02
119.0906 / 1.35m	Betaine	-4.56	2.96E-02
702.4727 / 12.72m	No match	-4.65	2.67E-02
135.1719 / 12.2m	No match	-4.75	1.48E-02
303.1793 / 4.19m	16alpha-Hydroxyandrost-4- ene-3,17-dione	-4.83	2.76E-02
232.0864 / 0.64m	N-Acetyl-L-2-amino-6- oxopimelate or N2-Succinyl-L-glutamic acid 5-semialdehyde	-5.09	1.81E-02
208.1345 / 1.04m	No match	-5.14	2.77E-02
139.1665 / 12.17m	No match	-5.25	1.65E-02
268.1497 / 1.94m	Butirosin	-5.31	3.26E-02
152.111 / 2.57m	N-Methyltyramine or Phenylalaninol	-5.44	1.81E-02
265.2989 / 12.18m	No match	-5.48	2.50E-02
151.1412 / 0.64m	Perillyl aldehyde or 1-Haloalkane or p-Cumic alcohol or (-)-Carvone	-5.50	1.02E-02
247.2902 / 12.18m	No match	-5.72	2.17E-02
170.208 / 12.17m	No match	-5.75	3.34E-02
699.5347 / 12.94m	No match	-5.90	4.17E-02
136.0697 / 2.78m	Sinapyl alcohol or 2-Phenylacetamide	-6.00	1.97E-02
94.0936 / 3.14m	Aniline	-6.00	4.34E-02
214.9689 / 0.5m	3,4,6-Trichloro-cis-1,2- dihydroxycyclohexa-3,5-	-6.06	2.15E-02

	diene		
135.085 / 2.57m	1-Deoxyxylonojirimycin	-6.10	5.24E-03
85.012 / 0.5m	3-Amino-1,2,4-triazole	-6.14	4.28E-03
603.1633 / 3.95m	No match	-6.26	1.19E-02
254.9234 / 1.54m	No match	-6.43	4.50E-02
448.3008 / 9.78m	Ubiquitin	-6.56	2.93E-02
109.1553 / 9.58m	No match	-6.63	4.61E-02
136.1164 / 3.14m	Sinapyl alcohol or 2-Phenylacetamide	-6.75	2.60E-02
163.2025 / 12.17m	No match	-6.78	7.44E-03
111.1713 / 9.58m	No match	-7.00	1.17E-02
265.2988 / 12.33m	No match	-7.21	1.57E-02
128.1619 / 12.18m	No match	-7.33	3.10E-03
135.1719 / 9.58m	No match	-7.47	6.78E-03
263.2827 / 11.54m	No match	-7.50	5.40E-03
707.4762 / 12.39m		-7.63	6.82E-03
348.1055 / 3.44m	cephalexin	-7.67	7.08E-03
745.2614 / 0.65m	No match	-7.79	5.54E-03
247.2903 / 12.33m	No match	-7.92	1.04E-02
1092.6419 / 4.85m	No match	-8.20	1.06E-02
81.1219 / 9.59m	No match	-8.25	3.54E-03
119.0897 / 3.13m	Betaine	-8.39	1.79E-02
198.9928 / 0.5m	4,5-Dihydroxyphthalate	-8.80	4.24E-02
	5'-S-methyl-5'- thioadenosine or		
298.1383 / 3.14m	5'-methylthioformycin or 5'-Methylthioadenosine	-8.91	2.89E-02
189.106 / 2.81m	Azelaic acid	-9.00	2.17E-02
310.3526 / 13.24m	No match	-9.18	1.92E-02
414.6736 / 5.3m	No match	-9.20	4.07E-02
163.0967 / 3.14m	N6-Hydroxy-L-lysine or 5-Hydroxylysine	-9.20	4.17E-02
694.5598 / 12.75m	No match	-9.44	4.31E-03
121.1559 / 9.57m	No match	-9.78	1.29E-03
1092.242 / 4.85m	No match	-9.80	3.50E-02
140.9723 / 0.5m	Sulfoacetate	-9.83	3.24E-02
124.9777 / 0.58m	Sulfoacetaldehyde	-10.11	2.95E-02
745.3742 / 12.16m	No match	-10.20	1.22E-02
782.4616 / 14.11m	No match	-10.28	4.08E-03
685.214 / 0.82m	No match	-10.58	2.92E-03
	3-Sulfolactate or		
170.9831 / 0.5m	(S)-3-Sulfolactate or (2R)-3-Sulfolactate	-10.60	1.96E-02

186.976 / 0.5m	2-Phospho-D-glyceric acid or 3-Phospho-D-glycerate Or 2-Phosphoglyceric acid Or 3-Phosphoglycerate Acetophenone	-11.20	1.23E-02
121.1065 / 2.8m	Or 2-Methylbenzaldehyde Or 3-Methylbenzaldehyde Or p-Tolualdehyde Or Phenylacetaldehyde Adenosine diphosphate	-12.20	1.91E-02
560.0779 / 3.44m	ribose or ADP-ribose or 1-(5-phosphoribosyl)-AMP Or Phosphoribosyl-AMP	-12.38	5.30E-03
84.0964 / 0.71m	No match	-12.40	7.69E-04
577.4935 / 12.7m	No match	-13.00	3.77E-03
1092.4436 / 4.85m	No match	-13.00	9.56E-03
109.0844 / 1.67m	p-Cresol or Benzyl alcohol or Ketone or 4-methylphenol Or o-Cresol	-13.00	1.03E-02
448.3006 / 9.57m	Ubiquitin	-14.00	1.17E-02
274.9184 / 0.58m	No match	-14.20	2.09E-03
346.208 / 1.01m	No match	-14.63	1.99E-02
251.2842 / 9.58m	No match	-14.73	2.18E-03
786.1219 / 3.59m	FAD or FADH(2)	-16.73	1.61E-03
321.3552 / 14.11m	No match	-17.15	9.05E-03
931.489 / 9.58m	No match	-17.74	4.94E-02
679.5368 / 12.55m	No match	-18.13	4.85E-03
577.4931 / 12.57m	No match	-20.64	2.65E-02
275.3191 / 13.11m	No match	-21.17	2.15E-02
681.5308 / 13.32m	No match	-22.03	1.72E-02
190.9649 / 0.58m	Bromobenzene-2,3- dihydrodiol or Benzo[a]pyrene-7,8-oxide Or Bromobenzene-3,4- dihydrodiol	-22.60	2.79E-02
605.2669 / 5.2m	No match	-22.92	2.10E-02
261.0883 / 1.67m	D-Biotin D-sulfoxide or Biotin sulfoxide	-23.00	8.49E-03
681.5341 / 13.66m	No match	-23.33	3.37E-02
468.2886 / 8.42m	No match	-25.80	5.92E-03
303.3472 / 14.11m	No match	-26.88	7.85E-03

296.1245 / 1.69m	Prunasin or Citalopram aldehyde	-28.20	5.38E-03
344.126 / 1.69m	Coenzyme B	-28.80	1.36E-04
559.0216 / 1.66m	No match	-29.33	1.39E-03
575.4766 / 12.43m	No match	-29.80	3.41E-02
574.9842 / 1.66m	No match	-31.67	5.52E-04
148.1154 / 0.9m	No match	-34.60	1.81E-02
561.4627 / 12.44m	2-Hexaprenyl-3-methyl-6- methoxy-1,4-benzoquinone	-38.50	4.55E-02
325.4091 / 9.58m	NO MATCH	-40.71	3.56E-02
243.0788 / 1.66m	2-Hydroxy-2H- benzo[h]chromene-2- carboxylate or thymidine	-40.80	1.52E-03
702.447 / 12.43m	No match	-41.22	2.57E-02
211.1534 / 1.48m	No match	-42.80	4.57E-02
665.5326 / 12.99m	No match	-43.88	1.16E-02
191.1206 / 2.8m	5-Methoxytryptamine (S)-1-Phenylethanol or 3-Methylbenzyl alcohol	-45.20	1.52E-02
123.1105 / 1.15m	Or 2-Methylbenzyl alcohol Or Phenylethyl alcohol Or 4-Ethylphenol	-52.89	5.78E-07
314.1326 / 1.69m	Dihydroisopteroate	-53.11	2.84E-05
255.1392 / 1.48m	Galactosylglycerol	-58.40	4.44E-02
314.1337 / 1.78m	Dihydroisopteroate	-58.80	1.90E-06
1066.0395 / 4.76m	No match	-67.00	4.84E-03
510.3235 / 9.85m	2-Acyl-sn-glycero-3- phosphoglycerol (N-C18:1)	-70.90	4.71E-02
587.2729 / 2.62m	(3Z)-Phycoerythrobilin	-80.97	1.62E-04
687.5075 / 13.34m	No match	-90.15	8.83E-03
625.5157 / 13.08m	No match	-90.80	4.52E-02
670.4152 / 14.13m	No match	-93.00	3.79E-02
480.3161 / 9.7m	No match	-93.14	6.76E-03
663.5185 / 12.38m	No match	-105.92	4.39E-02
647.5229 / 13m	No match	-115.00	1.10E-02
652.5307 / 13.06m	No match	-195.71	1.65E-02
681.529 / 13.11m	No match	-224.85	3.35E-04
625.5129 / 12.49m	No match	-241.06	1.02E-02
665.5314 / 14.22m	No match	-304.91	1.62E-02
651.5296 / 12.98m	No match	-408.50	3.33E-04
799.4605 / 14.17m	No match	-459.66	9.29E-07
667.5336 / 13.9m	No match	-478.79	3.92E-03
485.4106 / 14.17m	No match	-555.99	9.83E-04

815.3957 / 14.17m	No match	-861.99	2.09E-04
485.4305 / 14.17m	No match	-876.81	1.06E-07
794.5063 / 14.17m	No match	-913.49	4.92E-06
815.4294 / 14.17m	No match	-991.08	4.06E-07
794.4735 / 14.17m	No match	-1101.07	8.67E-05
667.5167 / 12.3m	No match	-1145.00	6.41E-03
665.5313 / 13.82m	No match	-3525.72	2.99E-04
799.4267 / 14.18m	No match	-5200.75	3.81E-05
665.5377 / 13.8m	No match	-6922.33	2.87E-03
681.5261 / 12.41m	No match	-38824.40	1.46E-03

Table 7.2.4 List of downregulated metabolites in *B. cenocepacia/pYedQ2*. Selection criteria were set as fold change (FC) ≤ -2 and p-value ≤ 0.05 , n=5. Metabolites are denoted by m/z value/retention time. Metabolites with database hit are listed as ‘Compound name’ while metabolites without database hit are listed as ‘No match’. Only hits with highest similarity are illustrated here based on molecular weight of each metabolite.

Metabolites upregulated in <i>K. pneumoniae/pYedQ2</i>			
Feature ID (m/z/retention time)	Possible Match	FC	p-value
790.531 / 3.31m	No match	659.60	8.10E-10
591.2555 / 2.6m	No match	558.40	1.71E-09
648.4698 / 3.07m	No match	518.00	1.89E-09
833.3275 / 3.44m	No match	431.60	9.97E-09
716.2846 / 2.54m	No match	414.00	8.79E-08
798.3036 / 3.09m	No match	258.44	1.21E-06
701.2691 / 3.31m	No match	257.67	1.72E-08
942.3111 / 3.12m	No match	244.40	9.67E-08
542.2619 / 3.17m	No match	232.40	9.13E-08
752.4776 / 14.02m	No match	196.08	6.87E-03
605.269 / 3.31m	No match	190.11	3.89E-06
1089.2626 / 6.5m	No match	184.40	2.49E-08
383.2236 / 2.94m	2-tetradecanoyl-sn-glycerol 3-phosphate	168.40	3.69E-11
1089.429 / 6.5m	No match	154.40	4.34E-08
740.8028 / 3.02m	No match	148.00	1.29E-06
577.7764 / 3.09m	No match	144.40	5.77E-07
605.1211 / 3m	No match	143.43	1.37E-05

788.2747 / 3.12m	5,10-Methenyltetrahydromethanopterin	122.80	1.03E-07
690.2575 / 3.73m	No match	110.00	3.17E-06
533.2461 / 3.09m	Galactan	106.33	5.77E-08
1089.5986 / 6.49m	No match	104.00	2.14E-08
589.1516 / 3m	GDP-4-amino-4,6-dideoxy-alpha-D-mannose	99.60	1.50E-09
774.3414 / 3.45m	No match	84.00	1.99E-06
532.5777 / 3.09m	No match	82.78	3.39E-05
651.228 / 1.57m	No match	82.40	1.03E-10
320.1856 / 3.06m	No match	82.25	2.36E-06
674.8528 / 3.65m	No match	79.20	8.72E-06
704.3154 / 3.09m	No match	78.40	4.84E-06
284.1695 / 2.94m	No match	77.60	7.78E-10
864.1921 / 3m	3-trans-hexenoyl-CoA or trans-2-Hexenoyl-CoA	74.80	8.07E-07
790.4457 / 3.31m	No match	74.80	1.42E-03
721.2847 / 3.09m	1,6-Anhydrous-N-Acetylmuramyl-tetrapeptide	73.60	7.03E-07
837.3028 / 3.83m	Uroporphyrinogen III or Uroporphyrinogen I	70.00	6.28E-06
987.5469 / 10.69m	No match	69.97	3.63E-02
648.3939 / 3.06m	No match	68.40	5.36E-06
651.2367 / 3.38m	No match	68.00	1.73E-05
389.2421 / 0.8m	Ubiquinol-3	67.20	7.12E-09
561.2136 / 1.09m	No match	66.00	8.44E-08
604.1141 / 3m	No match	65.33	9.85E-07
516.2388 / 3.31m	No match	63.43	9.31E-08
443.2125 / 3.12m	No match	60.36	4.93E-06
727.308 / 3.56m	No match	59.60	5.24E-03
532.912 / 3.08m	No match	58.56	8.44E-05
414.6756 / 3.31m	No match	58.00	2.73E-06
526.3292 / 3.92m	No match	56.67	2.97E-05
501.2592 / 3.12m	No match	56.40	8.83E-07
719.2743 / 3.24m	tunicamycin	54.83	3.98E-04
561.5828 / 3.09m	No match	53.20	2.02E-07
191.1564 / 3.06m	5-Methoxytryptamine	51.14	7.31E-07
992.2624 / 3m	3-Oxotetradecanoyl-CoA or 3-hydroxy-5-cis-tetradecenoyl-CoA	51.00	1.85E-05
661.2587 / 2.93m	No match	50.33	5.86E-08
674.313 / 3.63m	No match	50.33	1.55E-07
561.9174 / 3.09m	No match	48.00	3.08E-07
631.3251 / 2.79m	No match	47.89	9.75E-05

463.2217 / 3.06m	No match	47.29	5.96E-04
531.2822 / 2.81m	No match	47.00	4.09E-06
487.7545 / 2.8m	No match	44.27	6.96E-05
738.8685 / 3.6m	No match	41.94	8.22E-05
144.122 / 11.23m	Crotonobetaine or Stachydrine	41.00	3.05E-06
790.2966 / 3.31m	No match	40.57	2.93E-03
494.2413 / 3.03m	No match	40.40	1.83E-06
532.1905 / 1.72m	No match	39.60	3.10E-08
462.2375 / 3.31m	L-Alanine-D-glutamate-meso-2,6- diaminoheptanedioate-D-alanine or L-alanine-D-glutamate-meso-2,6- diaminoheptanedioate	38.00	2.38E-08
513.9071 / 3.06m	No match	37.60	1.96E-07
513.575 / 3.07m	No match	36.80	2.94E-05
484.3006 / 2.79m	1-Acyl-sn-glycero-3- phosphoglycerol (N-C16:0)	36.78	8.61E-05
462.715 / 3.12m	No match	36.00	4.83E-07
539.2119 / 2.61m	No match	35.60	7.45E-08
861.331 / 3.78m	No match	35.00	1.94E-05
645.3379 / 2.78m	No match	34.80	1.50E-06
302.1769 / 3.31m	No match	34.40	1.23E-08
322.2801 / 11.23m	No match	34.33	7.66E-05
582.2438 / 2.76m	Streptomycin	34.00	9.92E-07
575.1606 / 3m	No match	33.07	1.10E-04
471.76 / 2.68m	No match	30.80	2.09E-07
442.1805 / 3.12m	Dihydrofolic acid or Folic acid	30.40	2.60E-05
494.5758 / 3.03m	No match	30.00	4.68E-05
644.2727 / 3.03m	No match	28.56	7.24E-04
974.2561 / 3.11m	2-trans,5-cis-tetradecadienoyl-CoA or 3,5-Tetradecadienoyl-CoA	28.00	2.89E-06
559.2749 / 3.13m	No match	28.00	6.61E-06
391.3748 / 0.68m	No match	26.80	6.51E-06
401.3022 / 3.34m	4,4'-Diapolycopene	26.40	7.29E-08
520.274 / 3.03m	No match	26.40	2.12E-05
648.2556 / 3.06m	No match	26.25	1.20E-03
555.2446 / 3.03m	No match	26.00	4.42E-06
557.2537 / 2.62m	3-Vinylbacteriochlorophyllide d	25.60	9.28E-04
445.2135 / 3.31m	No match	25.20	3.99E-08
311.2137 / 3.14m	Sugar phosphate	25.00	4.83E-06
173.1467 / 3.31m	No match	24.40	3.93E-08
415.2317 / 2.68m	No match	23.33	9.51E-08

163.1165 / 0.7m	Carnitine or 3-Hydroxy-4- trimethylammoniobutanoic acid	23.20	1.49E-08
471.7189 / 3.12m	No match	23.02	1.72E-03
440.2357 / 3.17m	lipid	22.40	2.93E-07
587.2603 / 3.12m	(3Z)-Phycocyanobilin	22.40	5.83E-05
371.2361 / 2.79m	No match	22.00	2.46E-07
128.126 / 3.06m	O-Phosphoseryl-tRNA(Cys) or N-Cyclohexylformamide	20.20	5.38E-05
472.2572 / 2.94m	Secondary alcohol	18.62	5.27E-04
143.1215 / 0.5m	nonan-2-one	18.29	8.97E-05
573.1833 / 3m	No match	18.00	1.91E-07
547.2478 / 0.66m	No match	17.20	1.23E-06
562.2518 / 3.09m	No match	16.80	3.09E-04
257.0939 / 0.5m	Nicotinate D-ribonucleoside	16.67	1.54E-04
851.2847 / 3.09m	Pecorin-1	15.41	3.42E-03
228.1378 / 1.11m	Deoxycytidine	15.39	1.88E-03
781.3078 / 0.68m	No match	14.66	2.49E-03
487.262 / 2.68m	lipid	14.07	9.94E-04
343.7136 / 3.15m	No match	14.00	6.34E-05
702.1625 / 3.07m	No match	13.90	1.09E-03
591.1086 / 3.01m	UDP-2-acetamido-4-amino-2,4,6- trideoxy-alpha-D-glucose or UDP-4-amino-4,6-dideoxy-N- acetyl-beta-L-altrosamine	13.63	7.89E-04
110.1266 / 3.13m	Cyclohexyl isocyanide	12.80	1.11E-07
597.3541 / 3.79m	Astaxanthin	12.74	1.93E-03
532.2751 / 2.98m	No match	12.35	1.51E-04
672.295 / 1.25m	No match	11.94	5.64E-03
529.2506 / 3m	No match	11.87	2.25E-03
446.2444 / 1.23m	No match	11.68	1.40E-03
863.2484 / 2.94m	Sirohydrochlorin or Pecorin 2	11.60	5.52E-03
589.2818 / 3.15m	No match	10.12	1.13E-03
119.0901 / 0.72m	Betaine	9.38	2.84E-06
401.2658 / 2.94m	No match	9.33	5.83E-04
180.1295 / 0.79m	(2S,3S)-3-Methylphenylalanine	9.00	1.14E-05
276.1546 / 3m	N-Succinyl-L-citrulline	9.00	3.49E-04
752.2632 / 2.79m	No match	8.67	3.03E-04
502.2242 / 2.58m	No match	8.63	1.25E-04
751.9304 / 2.79m	No match	8.61	5.84E-04
751.5968 / 2.79m	Plastoquinol-9	8.61	7.42E-04
1037.4133 / 5.63m	No match	8.60	6.79E-03

370.2928 / 11.23m	No match	8.49	1.13E-03
619.2877 / 2.76m	No match	8.41	2.01E-03
311.2143 / 2.97m	Sugar phosphate	8.29	1.72E-05
180.1329 / 0.97m	(2S,3S)-3-Methylphenylalanine	7.67	8.24E-05
359.2294 / 1.24m	No match	7.63	8.40E-04
156.1333 / 2.97m	isoleucine tetrazole	7.60	4.80E-06
	(S)-(-)-Citronellal or		
	(-)-Linalool or		
	(-)-Borneol or		
155.1375 / 3.12m	(+)-Borneol or	7.60	4.21E-04
	(R)-(+)-Citronellal or		
	3,7-Dimethylocta-1,6-dien-3-ol or		
	(+)-Linalool or		
	Glucagon		
835.2148 / 0.68m	No match	7.50	2.14E-03
843.4374 / 11.23m	No match	7.48	4.80E-04
328.1886 / 0.68m	No match	7.44	2.20E-03
	Taurodeoxycholate or		
500.2914 / 2.88m	Taurochenodeoxycholate	7.39	3.41E-03
173.1463 / 1.1m	No match	7.35	1.31E-02
	7,12-Dimethylbenz[a]anthracene		
291.1619 / 1.1m	5,6-oxide or	7.26	6.57E-03
	trans-5,6-Dihydro-5,6-dihydroxy-		
	7,12-dimethylbenz[a]anthracene		
255.1447 / 1.11m	Galactosylglycerol	7.18	9.86E-03
561.1421 / 3.01m	No match	7.14	3.72E-03
571.3086 / 3.1m	No match	6.88	2.58E-03
	O-Phosphoseryl-tRNA(Cys) or		
128.1255 / 1.1m	N-Cyclohexylformamide	6.85	6.43E-03
318.1702 / 0.99m	No match	6.80	1.75E-04
533.1089 / 2.56m	No match	6.80	4.49E-02
	Sinapyl alcohol or		
136.117 / 0.72m	2-Phenylacetamide	6.77	5.46E-07
137.1971 / 1.35m	No match	6.73	2.96E-04
282.1629 / 3m	No match	6.67	9.53E-05
168.1213 / 3m	3-Methoxytyramine	6.67	7.02E-04
176.157 / 0.67m	Carboxynorspermidine	6.67	2.57E-03
159.1474 / 3.15m	Protamine	6.57	1.08E-04
92.0775 / 0.74m	No match	6.40	1.62E-06
191.1559 / 1.1m	5-Methoxytryptamine	6.39	2.38E-02
649.2356 / 3.4m	No match	6.33	5.17E-03
82.1172 / 1.1m	No match	6.32	1.35E-02
152.1116 / 3.36m	N-Methyltyramine or	6.13	3.30E-02

387.2912 / 2.94m	Phenylalaninol		
	Ubiquinone-3	6.12	6.54E-04
146.0904 / 0.69m	(S)-5-Amino-3-oxohexanoic acid or 6-Amino-2-oxohexanoate or L-2-Aminoadipate 6- semialdehyde	6.00	4.04E-04
669.3197 / 4m	No match	5.96	2.25E-02
431.2351 / 2.6m	No match	5.88	1.53E-02
387.2526 / 2.92m	Ubiquinone-3	5.73	4.52E-03
94.0936 / 0.73m	Aniline	5.67	3.22E-05
585.3202 / 3.72m	Bilirubin or 15,16-Dihydrobiliverdin	5.65	1.05E-02
138.1105 / 3m	2,4,6-Triaminotoluene	5.63	5.48E-04
201.1398 / 0.68m	Ethylglyoxalbis(guanyldiazide)	5.48	1.32E-03
96.0984 / 3m	No match	5.43	5.23E-03
577.0966 / 3.01m	No match	5.26	1.17E-02
186.1304 / 3m	Ecgonine or Pseudoecgonine	5.14	2.88E-03
557.2904 / 3.38m	No match	5.11	1.51E-02
734.3877 / 11.23m	No match	5.11	1.75E-02
579.0108 / 1.71m	No match	5.08	9.83E-03
573.258 / 0.79m	No match	5.03	1.47E-03
91.1078 / 3.15m	(R,R)-Butane-2,3-diol or 2,3-Butanediol or meso-2,3-Butanediol	5.00	2.00E-04
614.4528 / 11.23m	No match	4.86	4.73E-04
470.2105 / 1.15m	No match	4.67	4.36E-03
455.2684 / 3.41m	octyl beta-1,6-D-galactofuranosyl- alpha-D-glucopyranoside	4.65	2.02E-02
469.221 / 1.15m	Gentamicin C	4.63	9.22E-03
94.0933 / 1.94m	Aniline	4.40	2.43E-03
142.1209 / 3.15m	Tropine or L-histidinol	4.33	1.84E-03
559.2762 / 3.02m	No match	4.29	2.84E-03
258.054 / 3m	alpha-(2,6-anhydro-3-deoxy-D- arabino-heptulopyranosid)onate 7- phosphonate	4.26	4.20E-03
385.2847 / 2.88m	No match	4.25	9.22E-03
445.1702 / 3.09m	No match	4.20	1.36E-02
226.1204 / 3m	2-Amino-2-deoxyisochorismate or 4-Amino-4-deoxychorismate	4.17	1.10E-03
180.1347 / 3.14m	(2S,3S)-3-Methylphenylalanine	4.14	1.62E-02
144.1214 / 3m	Crotonobetaine or Stachydrine	4.13	4.32E-03
1032.71 / 5.83m	No match	4.03	2.15E-02
118.1202 / 3.15m	5-Aminopentanoate or L-Valine	4.02	2.10E-04

97.0825 / 3m	Fluorobenzene	4.00	3.43E-05
146.1155 / 3.15m	gamma-Butyrobetaine	3.93	2.18E-04
373.203 / 0.68m	Biocytin	3.92	3.53E-03
170.1151 / 3.15m	L-Noradrenaline or Pyridoxine	3.86	8.30E-04
188.1245 / 3.15m	8-Amino-7-oxononanoate	3.83	2.82E-04
144.1361 / 3.15m	Crotonobetaine or Stachydrine	3.82	2.04E-04
689.5895 / 2.74m	No match	3.79	2.96E-03
191.1299 / 0.55m	5-Methoxytryptamine	3.79	2.79E-02
117.1137 / 3.15m	No match	3.78	1.61E-03
537.2242 / 2.9m	No match	3.75	1.86E-02
69.084 / 3m	Imidazole or pyrazole	3.67	8.14E-04
647.5566 / 13.04m	No match	3.67	2.62E-02
343.649 / 3.06m	No match	3.66	1.60E-02
257.1608 / 0.68m	No match	3.63	1.30E-02
572.3265 / 3.63m	No match	3.59	4.10E-02
302.1764 / 0.68m	No match	3.59	9.07E-03
406.244 / 2.89m	3-alpha,12-alpha-dihydroxy-7-oxo-5-beta-cholanate	3.56	1.48E-02
604.2499 / 2.94m	No match	3.52	1.89E-02
528.3085 / 3.53m	No match	3.50	2.04E-02
487.2539 / 2.95m	lipid	3.49	4.46E-02
143.1283 / 3.15m	nonan-2-one	3.43	7.19E-04
82.1173 / 0.68m	No match	3.43	5.70E-03
378.9318 / 0.58m	No match	3.40	1.06E-02
320.1846 / 0.68m	No match	3.39	3.61E-03
689.9233 / 2.74m	No match	3.39	3.77E-03
110.0896 / 1m	Cyclohexyl isocyanide	3.33	4.29E-03
690.2576 / 2.74m	No match	3.31	1.68E-02
275.1143 / 8.54m	anhydro-n-acetylmuramic acid	3.27	1.22E-02
91.03 / 0.54m	Carboxymethoxylamine	3.27	1.85E-02
180.1422 / 3.18m	(2S,3S)-3-Methylphenylalanine	3.24	8.85E-03
530.2531 / 3.59m	No match	3.13	2.64E-02
929.5322 / 6.25m	No match	3.13	7.74E-03
897.4133 / 13.78m	Precorrin 6Y	3.01	2.57E-02
130.1047 / 0.71m	4-Guanidinobutanal	2.94	7.22E-03
136.1169 / 0.85m	Sinapyl alcohol or 2-Phenylacetamide	2.78	1.92E-02
708.482 / 12.72m		2.74	3.14E-02
627.3586 / 3.75m	No match	2.73	3.08E-02
444.2651 / 2.87m	No match	2.67	2.82E-02
486.269 / 2.78m	No match	2.62	3.42E-02
240.1471 / 0.87m	6-Lactoyl-5,6,7,8-tetrahydropterin	2.47	3.75E-02
1017.3018 / 4.82m	No match	2.45	3.70E-02

1165.9447 / 5.62m	No match	2.44	2.31E-02
1032.4845 / 5.84m	No match	2.28	3.42E-02
848.5489 / 5.64m	No match	2.18	2.74E-02

Table 7.2.5 List of upregulated metabolites in *K. pneumoniae/pYedQ2*. Selection criteria were set as fold change(FC) ≥ 2 and p-value ≤ 0.05 , n=5. Metabolites are denoted by mz value/retention time. Metabolites with database hit are listed as ‘Compound name’ while metabolites without database hit are listed as ‘No match’. Only hits with highest similarity are illustrated here based on molecular weight of each metabolite.

Metabolites downregulated in <i>K. pneumoniae/pYedQ2</i>			
Feature ID (mz/retention time)	Possible Match	FC	p-value
1105.45 / 11.2m	No match	-2.19	4.68E-02
897.4096 / 12.21m	Precorrin 6Y	-2.37	4.07E-02
257.2963 / 12.2m	Palmitic acid	-2.38	2.15E-02
728.1896 / 3.72m	No match	-2.45	3.53E-02
584.2845 / 3.23m	Lipid hydroperoxide	-2.50	4.05E-02
712.1987 / 3.72m	No match	-2.54	1.31E-02
311.2987 / 8.99m	No match	-2.55	4.11E-02
1156.5624 / 11.17m	No match	-2.58	3.12E-02
1096.4141 / 4.13m	No match	-2.60	1.25E-02
653.3878 / 11.11m	No match	-2.74	4.75E-02
973.4074 / 11.27m	No match	-2.92	1.38E-02
120.1357 / 1m	No match	-2.92	9.70E-03
327.1163 / 10.86m	cis-beta-D-Glucosyl-2-hydroxycinnamate or 6,7-dimethyl-8-(D-ribityl)lumazine or 6,7-Dimethyl-8-(1-D-ribityl)lumazine	-2.93	1.09E-02
177.1402 / 0.99m	N(pi)-Methyl-L-histidine or Serotonin or Canavanine	-3.00	3.46E-03
457.1249 / 3.72m	FMN or Flavin Mononucleotide Or FMNH(2)	-3.00	1.24E-02
196.1136 / 3.9m	2-Phenyl-1,3-propanediol	-3.10	2.57E-02

217.1555 / 3.05m	monocarbamate gamma-Glutamyl-gamma- butyraldehyde or Gamma-glutamyl-gamma- aminobutyraldehyde	-3.11	7.87E-04
452.2964 / 8.99m		-3.12	2.59E-02
389.2311 / 1.15m	Ubiquinol-3	-3.23	2.36E-02
155.1364 / 1.15m	(S)-(-)-Citronellal or (-)-Linalool or (-)-Borneol or (+)-Borneol or (R)-(+)-Citronellal or 3,7-Dimethylocta-1,6-dien-3- ol or (+)-Linalool or Glucagon	-3.24	1.05E-02
522.2246 / 2.78m	No match	-3.24	5.10E-03
456.6208 / 3.72m	No match	-3.32	2.09E-02
859.2181 / 3.72m	No match	-3.34	2.27E-04
560.0779 / 3.44m	Adenosine diphosphate ribose or ADP-ribose or 1-(5-phosphoribosyl)-AMP or Phosphoribosyl-AMP	-3.38	2.65E-02
231.1477 / 2.89m	Thioredoxin or Ergothioneine	-3.40	1.45E-02
231.2197 / 3.88m	No match	-3.46	2.46E-03
583.1846 / 3.72m	No match	-3.57	2.14E-03
1096.2136 / 4.13m	No match	-3.60	4.79E-02
442.1465 / 3.72m	Folic acid or Dihydrofolic acid	-3.67	4.57E-02
449.1385 / 3.72m	Quercitrin	-3.75	5.91E-04
1061.4371 / 11.23m	No match	-3.80	8.33E-03
784.1135 / 3.65m	No match	-3.80	2.39E-04
450.3649 / 12.84m	Glycochenodeoxycholate Or Glycodeoxycholate	-3.83	3.38E-02
628.4847 / 12.34m	No match	-3.89	1.68E-02
583.1858 / 3.65m	No match	-3.96	2.04E-05
621.3 / 13.28m	No match	-3.99	5.46E-03
1017.4221 / 11.25m	No match	-4.08	2.21E-02
730.2066 / 3.65m	No match	-4.08	8.32E-06
428.0603 / 3.53m	Adenosine 3',5'-	-4.21	7.95E-03

	Diphosphate or ADP or dGDP		
329.1485 / 2.85m	No match	-4.43	1.41E-02
505.3621 / 13.9m	No match	-4.51	4.21E-02
664.4365 / 12.87m		-4.57	3.64E-02
860.4591 / 8.73m	No match	-4.62	1.89E-02
627.1606 / 2.57m	No match	-4.67	1.36E-02
231.1477 / 1.15m	Thioredoxin or Ergothioneine	-4.68	3.15E-05
229.2036 / 3.33m	Myristic acid	-4.94	3.71E-04
1069.5928 / 4.35m	No match	-5.00	5.11E-03
297.1524 / 3.65m	Didemethylcitalopram	-5.00	8.20E-03
584.3486 / 10.3m		-5.20	4.91E-02
745.3742 / 12.16m	No match	-5.23	4.45E-02
285.2774 / 11.11m	Stearic acid	-5.50	1.93E-02
475.2358 / 4.03m	No match	-5.57	2.88E-02
1022.0833 / 14.2m	No match	-5.61	2.65E-02
746.1969 / 3.08m	No match	-5.95	1.81E-02
213.2106 / 0.78m	No match	-6.00	5.42E-03
327.1585 / 3.74m	6,7-Dimethyl-8-(1-D- ribityl)lumazine or 6,7-dimethyl-8-(D- ribityl)lumazine	-6.29	7.76E-04
469.1225 / 2.92m	No match	-6.43	3.64E-03
340.1007 / 2.92m	5-carboxyamino-1-(5- phospho-D-ribosyl)imidazole or 5-Amino-1-(5-phospho-D- ribosyl)imidazole-4- carboxylate	-6.67	6.40E-03
284.1429 / 2.57m	Guanosine	-6.80	1.97E-05
452.2962 / 9.18m	lipid	-6.83	2.67E-02
369.1559 / 10.97m	curcumin	-6.86	8.53E-03
118.1405 / 0.77m	No match	-6.95	9.15E-04
344.2162 / 3.33m	No match	-6.95	7.33E-06
157.0981 / 2.75m	3-Indoleacetonitrile or Acrylamide	-7.00	3.25E-04
913.1234 / 3.72m	No match	-7.08	9.06E-03
245.1077 / 2.75m	5'-amino-5'-deoxyuridine	-7.20	4.51E-04
1022.4244 / 14.2m	No match	-7.54	8.71E-03
116.0722 / 2.24m	L-Proline or D-Proline	-7.67	1.25E-02
100.0756 / 2.75m	(2R)-2-Hydroxy-2-	-7.69	3.51E-04

1069.793 / 4.35m	methylbutanenitrile No match	-8.25	1.02E-03
449.3816 / 13.04m	3-(all-trans-hexaprenyl)benzene-1,2-diol	-8.43	2.18E-02
142.0874 / 2.75m	L-Histidinol	-8.59	5.82E-04
526.1342 / 2.83m	No match	-8.84	3.70E-03
1022.1982 / 14.21m	No match	-8.85	8.48E-03
144.0667 / 2.75m	5-(2-Hydroxyethyl)-4-methylthiazole or 4-methyl-5-(2-hydroxyethyl)thiazole	-8.86	3.93E-04
1069.3918 / 4.35m	No match	-9.00	1.01E-02
116.0713 / 2.74m	L-Proline or D-Proline	-9.13	4.75E-04
732.2201 / 3.74m	No match	-9.18	2.78E-03
452.402 / 13.27m	No match	-9.20	4.17E-02
76.0731 / 2.74m	Dihydrobiopterin or (R)-1-Aminopropan-2-ol or Trimethylamine N-Oxide or 1-Amino-2-propanol	-9.33	3.68E-04
170.0816 / 2.75m	Pyridoxine or L-Noradrenaline	-9.50	2.97E-05
203.1009 / 2.75m	4-methylene DAP	-9.72	5.46E-03
162.0766 / 2.75m	Aminoadipic acid or o-acetyl-L-homoserine or D-Alanyl-(R)-lactate	-9.79	1.48E-03
275.1149 / 2.75m	anhydro-n-acetylmuramic acid	-9.88	2.32E-03
291.1091 / 2.75m	N-Succinyl-L,L-2,6-diaminopimelate or N-succinyl-L-2,6-diaminoheptanedioate	-10.33	2.01E-04
118.0868 / 2.75m	L-Valine or 5-Aminopentanoate	-10.42	1.52E-03
221.1101 / 2.75m	2',3'-Cyclic nucleotide	-10.73	4.29E-03
245.1611 / 0.72m	[Pyruvate dehydrogenase (acetyl-transferring)]	-11.00	1.29E-04
449.3622 / 13.2m	3-(all-trans-hexaprenyl)benzene-1,2-diol	-11.20	7.18E-04
872.3489 / 4.94m	No match	-11.40	1.85E-02
309.1846 / 3.34m	No match	-11.80	4.91E-03
872.3485 / 4.85m	No match	-11.90	1.68E-02
350.1367 / 2.75m	S-(Formylmethyl)glutathione	-11.90	5.09E-03

1162.9973 / 4.85m	No match	-12.00	4.80E-02
219.2278 / 13.95m	No match	-12.29	3.09E-04
	(S)-5-Amino-3-oxohexanoic acid or		
146.0826 / 2.75m	6-Amino-2-oxohexanoate or L-2-Aminoadipate 6-semialdehyde	-12.40	3.84E-04
739.2855 / 3.46m	No match	-12.95	6.18E-03
872.4743 / 4.87m	No match	-13.40	1.01E-02
610.3045 / 3.25m	No match	-13.59	2.37E-04
872.2839 / 4.86m	No match	-13.90	2.87E-02
887.4935 / 13.44m	No match	-14.00	4.27E-02
996.594 / 4.85m	No match	-14.20	2.12E-02
454.1663 / 3.72m	No match	-14.29	1.21E-04
996.9573 / 4.99m	No match	-14.40	4.06E-02
	(3R)-3-Isopropenyl-6-oxoheptanoate or		
185.1461 / 3.33m	(3S)-3-Isopropenyl-6-oxoheptanoate	-14.44	2.80E-05
697.2857 / 2.71m	No match	-14.45	4.73E-03
666.3516 / 13.28m	No match	-14.54	2.54E-02
333.1154 / 2.75m	No match	-14.80	3.56E-07
416.2009 / 1m	No match	-15.33	1.47E-04
397.1133 / 2.83m	No match	-15.43	4.95E-04
213.1381 / 3.33m	Pyocyanine	-15.75	6.36E-06
286.2232 / 12.75m	No match	-16.11	1.73E-02
720.4894 / 11.22m		-16.83	3.44E-02
930.6375 / 5.01m	No match	-17.20	4.12E-02
996.3636 / 3.81m	No match	-17.76	1.38E-04
328.1883 / 1.13m	No match	-18.43	5.04E-07
996.562 / 3.81m	No match	-18.50	8.36E-05
429.2572 / 2.87m	4,4'-Diapolycopenedial	-18.61	1.09E-03
466.2798 / 3.18m	No match	-18.98	5.35E-04
451.1157 / 2.83m	No match	-20.33	1.71E-04
	2-Acyl-sn-glycero-3-phosphoethanolamine (N-C18:1)		
477.2503 / 4.17m		-21.14	8.11E-05
995.9627 / 3.81m	No match	-28.15	5.00E-05
430.2158 / 1.34m	No match	-37.40	1.13E-02
387.2521 / 3.26m	Ubiquinone-3	-41.60	2.84E-09
530.2835 / 3.05m	No match	-52.57	1.74E-04
387.2227 / 9.25m	Ubiquinone-3	-55.20	1.76E-06
326.2096 / 3.86m	-[6(RS)-8-diamino-5,6,7,8-	-55.60	2.24E-08

	tetradexy-beta-D-ribo- octofuranosyl]-9H-purin-6- amine or 5'-[(3-aminopropyl)-amino]- 5'-deoxyadenosine		
585.281 / 3.56m	15,16-Dihydrobiliverdin Or Bilirubin	-62.00	6.54E-08
553.2976 / 3.06m	No match	-62.04	3.20E-07
474.2758 / 9.24m	No match	-64.20	4.44E-03
730.433 / 3.65m	No match	-65.20	1.47E-05
331.1682 / 9.25m	Gibberellin A7 or Gibberellin A5	-67.60	2.01E-07
661.2599 / 3.74m	No match	-69.60	2.95E-07
578.3796 / 12.23m	No match	-84.40	9.47E-05
387.2226 / 9.5m	Ubiquinone-3	-104.40	1.05E-07
552.3695 / 12.04m	lipid	-108.80	1.24E-05
373.1729 / 9.24m	Tetrahydrocurcumin	-117.60	1.31E-07
454.2471 / 2.62m	1-Acyl-sn-glycero-3- phosphoglycerol (N-C14:1)	-127.64	2.11E-07
331.1685 / 9.5m	Gibberellin A7 or Gibberellin A5	-138.80	5.13E-08
480.2621 / 2.71m		-140.67	2.42E-05
441.254 / 3.02m	3-demethylubiquinone-4	-164.40	1.09E-08
685.3472 / 4.46m	Ferrichrome minus Fe(III)	-189.60	3.91E-12
437.3849 / 12.22m	2-Phytol-1,4-naphthoquinone	-203.60	5.47E-07
556.3353 / 4.72m	lipid	-209.60	1.53E-10
411.3744 / 12.04m	5-Dehydroavenasterol	-241.40	5.55E-07
373.1725 / 9.5m	Tetrahydrocurcumin	-257.20	1.08E-08
714.4717 / 12.24m	No match	-361.40	3.39E-02
451.3983 / 13.21m	Phylloquinone	-487.33	8.51E-08
429.2259 / 9.24m	No match	-734.38	7.78E-09
429.2259 / 9.5m	No match	-1157.59	1.11E-09

Table 7.2.6 List of downregulated metabolites in *K. pneumoniae/pYedQ2*. Selection

criteria were set as fold change (FC) $\leq$ -2 and p-value $\leq$ 0.05, n=5.

Metabolites are denoted by m/z value/retention time. Metabolites with database hit are listed as ‘Compound name’ while metabolites without database hit are listed as ‘No match’. Only hits with highest similarity are illustrated here based on molecular weight of each metabolite.

7.3. Gene expression of $\Delta rpoN$ mutant

Genes upregulated of in $\Delta rpoN$ mutant comparing to PAO1 wild type				
Locus Tag	Gene Name	FC	padj	Gene Products
PA5015	<i>aceE</i>	5.1	0.000	pyruvate dehydrogenase
PA5016	<i>aceF</i>	4.2	0.000	Dihydrolipoamide acetyltransferase
PA1787	<i>acnB</i>	5.4	0.000	aconitate hydratase 2
PA0887	<i>acsA</i>	4.6	0.000	acetyl-coenzyme A synthetase
PA3686	<i>adk</i>	6.1	0.000	adenylate kinase
PA1249	<i>aprA</i>	5.9	0.000	Secreted Factors (toxins, enzymes, alginate)
PA5204	<i>argA</i>	21.4	0.000	N-acetylglutamate synthase
PA2001	<i>atoB</i>	5.6	0.000	acetyl-CoA acetyltransferase
PA3221	<i>csaA</i>	8.6	0.000	CsaA protein
PA5302	<i>dadX</i>	5.6	0.000	catabolic alanine racemase
PA1999	<i>dhcA</i>	61.7	0.000	Amino acid biosynthesis and metabolism; Carbon compound catabolism
PA2000	<i>dhcB</i>	32.7	0.000	Amino acid biosynthesis and metabolism; Carbon compound catabolism
PA1976	<i>ercS'</i>	5.7	0.000	Two-component regulatory systems
PA3333	<i>fabH2</i>	11.0	0.000	3-oxoacyl-[acyl-carrier-protein] synthase III
PA0447	<i>gcdH</i>	4.9	0.000	glutaryl-CoA dehydrogenase
PA0482	<i>glcB</i>	6.6	0.000	malate synthase G
PA1580	<i>gltA</i>	7.3	0.000	citrate synthase
PA2320	<i>gntR</i>	5.5	0.000	transcriptional regulator GntR
PA0085	<i>hcp1</i>	10.2	0.000	Secreted Factors (toxins, enzymes, alginate)
PA2623	<i>icd</i>	4.3	0.000	isocitrate dehydrogenase
PA2624	<i>idh</i>	4.1	0.000	isocitrate dehydrogenase
PA2015	<i>liuA</i>	14.0	0.000	Carbon compound catabolism
PA2014	<i>liuB</i>	7.7	0.000	Carbon compound catabolism
PA2013	<i>liuC</i>	10.8	0.000	Carbon compound catabolism
PA2012	<i>liuD</i>	5.1	0.000	Carbon compound catabolism
PA2011	<i>liuE</i>	6.0	0.000	Carbon compound catabolism
PA2016	<i>liuR</i>	29.2	0.000	Transcriptional regulators
PA0208	<i>mdcA</i>	6.1	0.000	malonate decarboxylase alpha subunit
PA1927	<i>metE</i>	25.4	0.000	5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase
PA1843	<i>metH</i>	5.0	0.000	methionine synthase
PA4640	<i>mgoB</i>	11.8	0.000	malate:quinone oxidoreductase
PA2637	<i>nuoA</i>	4.7	0.000	NADH dehydrogenase I chain A
PA2638	<i>nuoB</i>	4.7	0.000	NADH dehydrogenase I chain B
PA2640	<i>nuoE</i>	5.8	0.000	NADH dehydrogenase I chain E
PA2641	<i>nuoF</i>	4.4	0.000	NADH dehydrogenase I chain F

PA2642	<i>nuoG</i>	5.0	0.000	NADH dehydrogenase I chain G
PA2644	<i>nuoI</i>	5.2	0.000	NADH Dehydrogenase I chain I
PA2645	<i>nuoJ</i>	4.6	0.000	NADH dehydrogenase I chain J
PA2391	<i>opmQ</i>	7.2	0.000	Membrane proteins; Transport of small molecules
PA0083		7.4	0.000	Protein secretion/export apparatus
PA0086		5.9	0.000	Protein secretion/export apparatus
PA0092		6.2	0.000	Hypothetical, unclassified, unknown
PA1134		5.7	0.000	Hypothetical, unclassified, unknown
PA1199		6.0	0.000	Membrane proteins
PA1203		6.9	0.000	Hypothetical, unclassified, unknown
PA1244		4.2	0.000	Hypothetical, unclassified, unknown
PA1683		4.0	0.000	Putative enzymes
PA1853		6.9	0.000	Transcriptional regulators
PA1975		6.5	0.000	Hypothetical, unclassified, unknown
PA2062		5.8	0.000	Putative enzymes
PA2260		17.7	0.000	Hypothetical, unclassified, unknown; Carbon compound catabolism
PA2261		9.3	0.000	Carbon compound catabolism
PA2263		8.4	0.000	Putative enzymes
PA2301		4.1	0.000	Hypothetical, unclassified, unknown
PA2367		4.1	0.000	Hypothetical, unclassified, unknown
PA2368		6.1	0.000	Hypothetical, unclassified, unknown
PA2369		4.7	0.000	Membrane proteins
PA2370		4.6	0.000	Hypothetical, unclassified, unknown
PA2371		4.2	0.000	Translation, post-translational modification, degradation
PA2384		5.8	0.000	Hypothetical, unclassified, unknown
PA2393		21.5	0.000	Central intermediary metabolism
PA2402		17.4	0.000	Putative enzymes
PA2403		4.4	0.000	Membrane proteins
PA2404		4.3	0.000	Membrane proteins
PA2405		5.5	0.000	Hypothetical, unclassified, unknown
PA2411		17.0	0.000	Adaptation, Protection; Putative enzymes
PA2412		24.7	0.000	Hypothetical, unclassified, unknown
PA2427		13.8	0.000	Hypothetical, unclassified, unknown
PA2451		9.4	0.000	Hypothetical, unclassified, unknown
PA2452		8.4	0.000	Hypothetical, unclassified, unknown
PA2531		62.5	0.000	Amino acid biosynthesis and metabolism
PA2555		5.4	0.000	Putative enzymes
PA2560		5.9	0.000	Hypothetical, unclassified, unknown
PA2679		34.6	0.000	Hypothetical, unclassified, unknown
PA3094		6.1	0.000	Transcriptional regulators

PA3222		6.6	0.000	Membrane proteins
PA3271		4.7	0.000	Two-component regulatory systems
PA3327		5.4	0.000	Adaptation, Protection
PA3328		12.8	0.000	Putative enzymes
PA3329		6.4	0.000	Hypothetical, unclassified, unknown
PA3330		10.6	0.000	Putative enzymes
PA3331		7.5	0.000	cytochrome P450
PA3332		10.8	0.000	Hypothetical, unclassified, unknown
PA3334		6.9	0.000	Fatty acid and phospholipid metabolism
PA3335		5.8	0.000	Hypothetical, unclassified, unknown
PA3336		8.8	0.000	Membrane proteins; Transport of small molecules
PA3614		6.6	0.000	Hypothetical, unclassified, unknown
PA3660		4.1	0.000	Membrane proteins; Transport of small molecules
PA3988		4.8	0.000	Hypothetical, unclassified, unknown
PA4141		8.3	0.000	Hypothetical, unclassified, unknown
PA4181		9.7	0.000	Hypothetical, unclassified, unknown
PA4182		11.2	0.000	Hypothetical, unclassified, unknown
PA4183		4.9	0.000	Hypothetical, unclassified, unknown
PA4290		9.7	0.000	Adaptation, Protection; Chemotaxis
PA4438		18.2	0.000	Hypothetical, unclassified, unknown
PA4872		5.2	0.000	Hypothetical, unclassified, unknown
PA5150		4.5	0.000	Putative enzymes
PA5445		4.9	0.000	Putative enzymes
PA0423	<i>pasP</i>	11.4	0.000	Secreted Factors (toxins, enzymes, alginate)
PA1001	<i>phnA</i>	10.2	0.000	anthranilate synthase component I
PA1899	<i>phzA2</i>	8.2	0.000	Secreted Factors (toxins, enzymes, alginate)
PA1900	<i>phzB2</i>	5.6	0.000	Secreted Factors (toxins, enzymes, alginate)
PA4526	<i>pilB</i>	4.8	0.000	type 4 fimbrial biogenesis protein PilB
PA4031	<i>ppa</i>	5.4	0.000	inorganic pyrophosphatase
PA1986	<i>pqqB</i>	11.4	0.000	pyrroloquinoline quinone biosynthesis protein B
PA1987	<i>pqqC</i>	6.2	0.000	pyrroloquinoline quinone biosynthesis protein C
PA1988	<i>pqqD</i>	6.0	0.000	pyrroloquinoline quinone biosynthesis protein D
PA1989	<i>pqqE</i>	7.5	0.000	pyrroloquinoline quinone biosynthesis protein E
PA1973	<i>pqqF</i>	10.1	0.000	pyrroloquinoline quinone biosynthesis protein F
PA1990	<i>pqqH</i>	7.7	0.000	Putative enzymes
PA0996	<i>pqsA</i>	8.0	0.000	probable coenzyme A ligase

PA0997	<i>pqsB</i>	8.7	0.000	Homologous to beta-keto-acyl-acyl-carrier protein synthase
PA0998	<i>pqsC</i>	10.1	0.000	Homologous to beta-keto-acyl-acyl-carrier protein synthase
PA0999	<i>pqsD</i>	6.2	0.000	3-oxoacyl-[acyl-carrier-protein] synthase III
PA1000	<i>pqsE</i>	5.5	0.000	Quinolone signal response protein
PA0796	<i>prpB</i>	5.2	0.000	carboxyphosphoenolpyruvate phosphonmutase
PA2259	<i>ptxS</i>	6.5	0.000	transcriptional regulator PtxS
PA2386	<i>pvdA</i>	42.8	0.000	L-ornithine N5-oxygenase
PA2399	<i>pvdD</i>	20.5	0.000	pyoverdine synthetase D
PA2397	<i>pvdE</i>	4.6	0.000	pyoverdine biosynthesis protein PvdE
PA2396	<i>pvdF</i>	16.9	0.000	pyoverdine synthetase F
PA2425	<i>pvdG</i>	23.2	0.000	PvdG
PA2413	<i>pvdH</i>	8.2	0.000	"L-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase, PvdH"
PA2400	<i>pvdJ</i>	20.9	0.000	PvdJ
PA2424	<i>pvdL</i>	18.5	0.000	PvdL
PA2394	<i>pvdN</i>	10.9	0.000	PvdN
PA2395	<i>pvdO</i>	12.3	0.000	PvdO
PA2392	<i>pvdP</i>	15.3	0.000	PvdP
PA2385	<i>pvdQ</i>	11.3	0.000	PvdQ
PA2389	<i>pvdR</i>	9.9	0.000	Transport of small molecules
PA3479	<i>rhlA</i>	10.2	0.000	Secreted Factors (toxins, enzymes, alginate)
PA3478	<i>rhlB</i>	7.6	0.000	Secreted Factors (toxins, enzymes, alginate)
PA1584	<i>sdhB</i>	4.9	0.000	succinate dehydrogenase (B subunit)
PA1581	<i>sdhC</i>	11.1	0.000	succinate dehydrogenase (C subunit)
PA1582	<i>sdhD</i>	6.2	0.000	succinate dehydrogenase (D subunit)
PA5128	<i>secB</i>	4.6	0.000	secretion protein SecB
PA2991	<i>sth</i>	11.9	0.000	soluble pyridine nucleotide transhydrogenase
PA1585	<i>sucA</i>	4.2	0.000	2-oxoglutarate dehydrogenase (E1 subunit)
PA0707	<i>toxR</i>	4.3	0.000	transcriptional regulator ToxR
PA4042	<i>xseB</i>	6.2	0.000	exodeoxyribonuclease VII small subunit

Table 7.3.1 Genes upregulated in $\Delta rpoN$ mutant comparing to PAO1 wild type.

Upregulated genes were filtered based on the selection criteria of fold change ≥ 4 and p-value < 0.01 , n=2. Fold change is denoted as FC in short; adjusted p-value is denoted as padj. NCBI locus tags, gene names and protein functions are also displayed.

Genes downregulated in $\Delta rpoN$ mutant comparing to PAO1 wild type

Locus Tag	Gene Name	FC	padj	Gene Products
PA4151	<i>acoB</i>	-4.3	0.000	acetoin catabolism protein AcoB
PA4147	<i>acoR</i>	-8.2	0.000	transcriptional regulator AcoR
PA5538	<i>amiA</i>	-4.8	0.000	N-acetylmuramoyl-L-alanine amidase
PA0807	<i>ampDh3</i>	-16.7	0.000	Antibiotic resistance and susceptibility
PA3385	<i>amrZ</i>	-14.8	0.000	Transcriptional regulators
PA3719	<i>armR</i>	-12.3	0.001	Antibiotic resistance and susceptibility
PA0895	<i>aruC</i>	-19.0	0.000	N-succinylglutamate 5-semialdehyde dehydrogenase
PA0898	<i>aruD</i>	-8.2	0.000	succinylglutamate 5-semialdehyde dehydrogenase
PA0896	<i>aruF</i>	-9.8	0.000	arginine/ornithine succinyltransferase AI subunit
PA0897	<i>aruG</i>	-8.7	0.000	arginine/ornithine succinyltransferase AII subunit
PA2886	<i>atuA</i>	-8.1	0.000	Hypothetical, unclassified, unknown
PA2887	<i>atuB</i>	-11.6	0.000	Putative enzymes
PA2888	<i>atuC</i>	-10.2	0.000	Putative enzymes
PA2889	<i>atuD</i>	-6.4	0.000	Putative enzymes
PA2890	<i>atuE</i>	-8.4	0.000	Putative enzymes
PA2891	<i>atuF</i>	-7.4	0.000	Putative enzymes
PA2892	<i>atuG</i>	-8.6	0.000	Putative enzymes
PA1423	<i>bdlA</i>	-13.6	0.000	Cell wall / LPS / capsule; Chemotaxis
PA5386	<i>cdhA</i>	-7.0	0.000	Putative enzymes; Carbon compound catabolism
PA5387	<i>cdhC</i>	-5.9	0.000	Carbon compound catabolism
PA4726.11	<i>crcZ</i>	-111.4	0.000	Non-coding RNA gene; Transcriptional regulators
PA5169	<i>dctM</i>	-4.3	0.000	Membrane proteins; Transport of small molecules
PA5167	<i>dctP</i>	-5.0	0.000	Membrane proteins; Transport of small molecules
PA1911	<i>femR</i>	-4.8	0.000	Membrane proteins; Transcriptional regulators
PA4159	<i>fepB</i>	-9.0	0.000	ferrienterobactin-binding periplasmic protein precursor FepB
PA4158	<i>fepC</i>	-9.0	0.000	ferric enterobactin transport protein FepC
PA4160	<i>fepD</i>	-7.0	0.000	ferric enterobactin transport protein FepD
PA4572	<i>flkB</i>	-6.1	0.000	peptidyl-prolyl cis-trans isomerase FklB
PA1099	<i>fleR</i>	-18.1	0.000	two-component response regulator
PA1098	<i>fleS</i>	-5.9	0.000	two-component sensor
PA1077	<i>flgB</i>	-40.8	0.000	flagellar basal-body rod protein FlgB
PA1078	<i>flgC</i>	-33.8	0.000	flagellar basal-body rod protein FlgC
PA1079	<i>flgD</i>	-7.9	0.000	flagellar basal-body rod modification protein

PA1081	<i>flgF</i>	-43.4	0.000	FlgD
PA1082	<i>flgG</i>	-13.5	0.000	flagellar basal-body rod protein FlgF
PA1083	<i>flgH</i>	-12.0	0.000	flagellar basal-body rod protein FlgG
PA1084	<i>flgI</i>	-7.4	0.000	flagellar L-ring protein precursor FlgH
PA1085	<i>flgJ</i>	-5.5	0.000	flagellar P-ring protein precursor FlgI
PA1086	<i>flgK</i>	-4.7	0.000	flagellar protein FlgJ
PA1087	<i>flgL</i>	-5.3	0.000	flagellar hook-associated protein 1 FlgK
PA1452	<i>flhA</i>	-8.8	0.000	flagellar hook-associated protein type 3 FlgL
PA1453	<i>flhF</i>	-5.5	0.000	flagellar biosynthesis protein FlhA
PA1092	<i>fliC</i>	-207.0	0.000	flagellar biosynthesis protein FlhF
PA1094	<i>fliD</i>	-25.3	0.000	flagellin type B
PA1100	<i>fliE</i>	-99.7	0.000	flagellar capping protein FliD
PA4306	<i>flp</i>	-8.1	0.000	flagellar hook-basal body complex protein FliE
PA5355	<i>glcD</i>	-11.5	0.000	Motility & Attachment
PA5354	<i>glcE</i>	-25.1	0.000	glycolate oxidase subunit GlcD
PA5353	<i>glcF</i>	-25.7	0.000	glycolate oxidase subunit GlcE
PA4022	<i>hdhA</i>	-16.0	0.000	glycolate oxidase subunit GlcF
PA1546	<i>hemN</i>	-5.4	0.000	Putative enzymes
PA5384	<i>hocS</i>	-43.5	0.000	oxygen-independent coproporphyrinogen III oxidase
PA5098	<i>hutH</i>	-33.8	0.000	Carbon compound catabolism; Energy metabolism
PA5100	<i>hutU</i>	-33.1	0.000	histidine ammonia-lyase
PA2570	<i>lecA</i>	-4.4	0.000	Urocanase
PA3361	<i>lecB</i>	-8.6	0.000	Adaptation, Protection; Motility & Attachment; Cell wall / LPS / capsule
PA2862	<i>lipA</i>	-16.3	0.000	fucose-binding lectin PA-IIL
PA2863	<i>lipH</i>	-9.1	0.000	lactonizing lipase precursor
PA3692	<i>lptF</i>	-5.8	0.000	lipase modulator protein
PA4205	<i>mexG</i>	-5.2	0.000	Membrane proteins; Transport of small molecules
PA4206	<i>mexH</i>	-6.1	0.000	hypothetical protein
PA3721	<i>nalC</i>	-4.6	0.000	probable Resistance-Nodulation-Cell Division (RND) efflux membrane fusion protein precursor
PA3396	<i>nosL</i>	-10.1	0.000	Transcriptional regulators; Antibiotic resistance and susceptibility
PA3395	<i>nosY</i>	-17.2	0.000	NosL protein
PA0216	PA0216	-4.4	0.000	NosY protein
PA0451	PA0451	-10.7	0.000	Membrane proteins; Transport of small molecules
PA0452	PA0452	-16.2	0.000	Membrane proteins

PA0453	PA0453	-5.2	0.000	Hypothetical, unclassified, unknown
PA0613	PA0613	-8.4	0.000	Hypothetical, unclassified, unknown
PA0614	PA0614	-10.6	0.000	Hypothetical, unclassified, unknown
PA0615	PA0615	-6.9	0.000	Hypothetical, unclassified, unknown
PA0616	PA0616	-13.2	0.000	Related to phage, transposon, or plasmid
PA0617	PA0617	-26.9	0.000	Related to phage, transposon, or plasmid
PA0618	PA0618	-30.0	0.000	Related to phage, transposon, or plasmid
PA0619	PA0619	-21.5	0.000	Related to phage, transposon, or plasmid
PA0620	PA0620	-10.4	0.000	Related to phage, transposon, or plasmid
PA0621	PA0621	-7.2	0.000	Related to phage, transposon, or plasmid
PA0622	PA0622	-43.9	0.000	Related to phage, transposon, or plasmid
PA0623	PA0623	-61.2	0.000	Related to phage, transposon, or plasmid
PA0624	PA0624	-47.5	0.000	Related to phage, transposon, or plasmid
PA0625	PA0625	-21.6	0.000	Related to phage, transposon, or plasmid
PA0626	PA0626	-18.9	0.000	Related to phage, transposon, or plasmid
PA0627	PA0627	-12.5	0.000	Related to phage, transposon, or plasmid
PA0628	PA0628	-26.4	0.000	Related to phage, transposon, or plasmid
PA0629	PA0629	-20.7	0.000	Related to phage, transposon, or plasmid
PA0630	PA0630	-8.1	0.000	Related to phage, transposon, or plasmid
PA0631	PA0631	-10.5	0.000	Related to phage, transposon, or plasmid
PA0632	PA0632	-23.4	0.000	Related to phage, transposon, or plasmid
PA0633	PA0633	-35.3	0.000	Related to phage, transposon, or plasmid
PA0634	PA0634	-26.2	0.000	Related to phage, transposon, or plasmid
PA0635	PA0635	-22.6	0.000	Related to phage, transposon, or plasmid
PA0636	PA0636	-23.8	0.000	Related to phage, transposon, or plasmid
PA0637	PA0637	-9.9	0.000	Related to phage, transposon, or plasmid
PA0638	PA0638	-22.2	0.000	Related to phage, transposon, or plasmid
PA0639	PA0639	-19.3	0.000	Related to phage, transposon, or plasmid
PA0640	PA0640	-21.1	0.000	Related to phage, transposon, or plasmid
PA0641	PA0641	-20.6	0.000	Related to phage, transposon, or plasmid
PA0642	PA0642	-15.1	0.000	Related to phage, transposon, or plasmid
PA0643	PA0643	-6.9	0.000	Related to phage, transposon, or plasmid
PA0644	PA0644	-4.2	0.000	Related to phage, transposon, or plasmid
PA0647	PA0647	-4.7	0.000	Related to phage, transposon, or plasmid
PA0648	PA0648	-4.1	0.000	Related to phage, transposon, or plasmid
PA0713	PA0713	-20.4	0.000	Hypothetical, unclassified, unknown
PA0737	PA0737	-4.0	0.000	Hypothetical, unclassified, unknown
PA0788	PA0788	-5.8	0.000	Hypothetical, unclassified, unknown
PA0809	PA0809	-4.4	0.000	Membrane proteins; Transport of small molecules
PA0826	PA0826	-4.6	0.000	Hypothetical, unclassified, unknown
PA0878	PA0878	-10.3	0.000	Hypothetical, unclassified, unknown
PA0881	PA0881	-6.2	0.000	Hypothetical, unclassified, unknown

PA0908	PA0908	-4.1	0.000	Hypothetical, unclassified, unknown
PA0909	PA0909	-5.7	0.000	Related to phage, transposon, or plasmid; Membrane proteins
PA0910	PA0910	-8.4	0.000	Hypothetical, unclassified, unknown
PA0911	PA0911	-7.8	0.000	Hypothetical, unclassified, unknown
PA1093	PA1093	-59.7	0.000	Hypothetical, unclassified, unknown
PA1095	PA1095	-13.3	0.000	Hypothetical, unclassified, unknown
PA1096	PA1096	-22.5	0.000	Hypothetical, unclassified, unknown
PA1111	PA1111	-4.9	0.000	Hypothetical, unclassified, unknown
PA1260	PA1260	-8.4	0.000	Transport of small molecules
PA1414	PA1414	-7.6	0.000	Hypothetical, unclassified, unknown
PA1418	PA1418	-4.8	0.000	Transport of small molecules
PA1441	PA1441	-21.1	0.000	Motility & Attachment
PA1465	PA1465	-4.1	0.000	Hypothetical, unclassified, unknown
PA1514	PA1514	-4.5	0.000	Hypothetical, unclassified, unknown
PA1519	PA1519	-4.9	0.000	Membrane proteins; Transport of small molecules
PA1545	PA1545	-66.7	0.000	Hypothetical, unclassified, unknown
PA1547	PA1547	-4.5	0.000	Membrane proteins
PA1549	PA1549	-5.4	0.000	Membrane proteins; Transport of small molecules
PA1550	PA1550	-4.4	0.000	Hypothetical, unclassified, unknown
PA1608	PA1608	-7.3	0.000	Adaptation, Protection; Chemotaxis
PA1617	PA1617	-12.4	0.000	Putative enzymes
PA1679	PA1679	-8.0	0.000	Hypothetical, unclassified, unknown
PA1692	PA1692	-6.6	0.019	Protein secretion/export apparatus
PA1728	PA1728	-7.1	0.000	Hypothetical, unclassified, unknown
PA1759	PA1759	-5.7	0.000	Transcriptional regulators
PA1760	PA1760	-5.1	0.000	Transcriptional regulators
PA1761	PA1761	-5.1	0.000	Hypothetical, unclassified, unknown
PA1784	PA1784	-5.9	0.000	Hypothetical, unclassified, unknown
PA1870	PA1870	-4.1	0.000	Hypothetical, unclassified, unknown
PA1887	PA1887	-8.2	0.000	Hypothetical, unclassified, unknown
PA1888	PA1888	-10.9	0.000	Hypothetical, unclassified, unknown
PA1913	PA1913	-7.6	0.000	Hypothetical, unclassified, unknown
PA1967	PA1967	-17.5	0.000	Hypothetical, unclassified, unknown
PA2021	PA2021	-4.2	0.000	Hypothetical, unclassified, unknown
PA2024	PA2024	-6.2	0.000	Putative enzymes
PA2027	PA2027	-33.2	0.000	Hypothetical, unclassified, unknown
PA2092	PA2092	-4.9	0.000	Membrane proteins; Transport of small molecules
PA2134	PA2134	-4.3	0.000	Hypothetical, unclassified, unknown
PA2137	PA2137	-6.2	0.000	Hypothetical, unclassified, unknown

PA2140	PA2140	-8.9	0.001	Central intermediary metabolism
PA2141	PA2141	-13.2	0.000	Hypothetical, unclassified, unknown
PA2143	PA2143	-5.1	0.000	Hypothetical, unclassified, unknown
PA2146	PA2146	-18.0	0.000	Hypothetical, unclassified, unknown
PA2150	PA2150	-4.5	0.000	Hypothetical, unclassified, unknown
PA2159	PA2159	-5.1	0.000	Hypothetical, unclassified, unknown
PA2166	PA2166	-21.5	0.000	Hypothetical, unclassified, unknown
PA2169	PA2169	-4.3	0.000	Hypothetical, unclassified, unknown
PA2170	PA2170	-6.7	0.003	Hypothetical, unclassified, unknown
PA2171	PA2171	-4.3	0.000	Hypothetical, unclassified, unknown
PA2187	PA2187	-5.0	0.000	Hypothetical, unclassified, unknown
PA2283	PA2283	-4.1	0.000	Hypothetical, unclassified, unknown
PA2286	PA2286	-4.0	0.000	Membrane proteins
PA2313	PA2313	-7.3	0.000	Membrane proteins
PA2422	PA2422	-8.2	0.000	Hypothetical, unclassified, unknown
PA2567	PA2567	-5.2	0.000	Hypothetical, unclassified, unknown
PA2654	PA2654	-20.2	0.000	Adaptation, Protection; Chemotaxis
PA2747	PA2747	-22.2	0.000	Hypothetical, unclassified, unknown
PA2777	PA2777	-7.7	0.000	Membrane proteins
PA2778	PA2778	-12.8	0.000	Hypothetical, unclassified, unknown
PA2779	PA2779	-12.9	0.000	Hypothetical, unclassified, unknown
PA2867	PA2867	-7.6	0.000	Adaptation, Protection; Chemotaxis
PA3259	PA3259	-5.4	0.000	Hypothetical, unclassified, unknown
PA3260	PA3260	-4.7	0.000	Transcriptional regulators
PA3273	PA3273	-5.8	0.000	Hypothetical, unclassified, unknown
PA3274	PA3274	-12.8	0.000	Hypothetical, unclassified, unknown
PA3307	PA3307	-6.6	0.000	Hypothetical, unclassified, unknown
PA3323	PA3323	-8.4	0.000	Hypothetical, unclassified, unknown
PA3324	PA3324	-4.1	0.000	Putative enzymes
PA3341	PA3341	-12.3	0.000	Transcriptional regulators
PA3350	PA3350	-5.5	0.000	Hypothetical, unclassified, unknown
PA3353	PA3353	-4.5	0.000	Hypothetical, unclassified, unknown
PA3362	PA3362	-4.3	0.000	Membrane proteins
PA3369	PA3369	-6.7	0.000	Membrane proteins
PA3370	PA3370	-7.1	0.000	Membrane proteins
PA3390	PA3390	-11.4	0.000	Hypothetical, unclassified, unknown
PA3410	PA3410	-8.7	0.000	Transcriptional regulators
PA3414	PA3414	-6.5	0.000	Hypothetical, unclassified, unknown
PA3415	PA3415	-5.5	0.000	Energy metabolism
PA3416	PA3416	-6.3	0.000	Energy metabolism
PA3417	PA3417	-5.6	0.000	Energy metabolism
PA3441	PA3441	-8.7	0.000	Transport of small molecules
PA3526	PA3526	-16.5	0.000	Membrane proteins

PA3600	PA3600	-21.1	0.000	Translation, post-translational modification, degradation
PA3601	PA3601	-17.9	0.000	Translation, post-translational modification, degradation
PA3662	PA3662	-8.5	0.000	Hypothetical, unclassified, unknown
PA3677	PA3677	-4.2	0.000	Transport of small molecules
PA3691	PA3691	-6.4	0.000	Hypothetical, unclassified, unknown
PA3762	PA3762	-9.7	0.000	Hypothetical, unclassified, unknown
PA3986	PA3986	-8.2	0.000	Hypothetical, unclassified, unknown
PA4093	PA4093	-5.5	0.000	Hypothetical, unclassified, unknown
PA4148	PA4148	-6.8	0.000	Putative enzymes
PA4152	PA4152	-10.6	0.000	Carbon compound catabolism
PA4153	PA4153	-9.4	0.000	"2,3-butanediol dehydrogenase"
PA4155	PA4155	-6.3	0.000	Hypothetical, unclassified, unknown
PA4156	PA4156	-4.0	0.000	Transport of small molecules
PA4323	PA4323	-4.6	0.000	Hypothetical, unclassified, unknown
PA4324	PA4324	-5.7	0.000	Hypothetical, unclassified, unknown
PA4326	PA4326	-14.1	0.000	Hypothetical, unclassified, unknown
PA4341	PA4341	-5.5	0.000	Transcriptional regulators
PA4499	PA4499	-7.1	0.000	Transcriptional regulators
PA4500	PA4500	-8.5	0.000	Transport of small molecules
PA4520	PA4520	-8.6	0.000	Adaptation, Protection; Chemotaxis
PA4575	PA4575	-6.6	0.000	Hypothetical, unclassified, unknown
PA4633	PA4633	-17.4	0.000	Adaptation, Protection; Chemotaxis
PA4634	PA4634	-5.7	0.000	Hypothetical, unclassified, unknown
PA4680	PA4680	-7.7	0.000	Hypothetical, unclassified, unknown
PA4681	PA4681	-8.5	0.000	Hypothetical, unclassified, unknown
PA4682	PA4682	-7.2	0.000	Hypothetical, unclassified, unknown
PA4683	PA4683	-5.7	0.000	Hypothetical, unclassified, unknown
PA4702	PA4702	-5.1	0.000	Hypothetical, unclassified, unknown
PA4738	PA4738	-28.0	0.000	Hypothetical, unclassified, unknown
PA4739	PA4739	-31.9	0.000	Hypothetical, unclassified, unknown
PA4837	PA4837	-9.2	0.000	Membrane proteins; Transport of small molecules
PA4843	PA4843	-15.9	0.000	Transcriptional regulators; Two-component regulatory systems
PA4911	PA4911	-4.2	0.000	Membrane proteins; Transport of small molecules
PA4912	PA4912	-4.1	0.000	Membrane proteins; Transport of small molecules
PA4929	PA4929	-7.5	0.000	Membrane proteins
PA5072	PA5072	-4.0	0.000	Adaptation, Protection; Chemotaxis
PA5096	PA5096	-8.4	0.000	Transport of small molecules

PA5097	PA5097	-19.2	0.000	Membrane proteins; Transport of small molecules
PA5348	PA5348	-5.9	0.000	DNA replication, recombination, modification and repair
PA5352	PA5352	-19.3	0.000	Hypothetical, unclassified, unknown
PA5383	PA5383	-16.7	0.000	Hypothetical, unclassified, unknown
PA5391	PA5391	-4.5	0.000	Hypothetical, unclassified, unknown
PA5446	PA5446	-11.3	0.000	Hypothetical, unclassified, unknown
PA5460	PA5460	-19.6	0.000	Hypothetical, unclassified, unknown
PA5469	PA5469	-5.1	0.000	Membrane proteins
PA5481	PA5481	-51.3	0.000	Hypothetical, unclassified, unknown
PA5482	PA5482	-71.3	0.000	Membrane proteins
PA0235	<i>pcaK</i>	-4.0	0.000	4-hydroxybenzoate transporter PcaK
PA4309	<i>pctA</i>	-5.5	0.000	chemotactic transducer PctA
PA4310	<i>pctB</i>	-6.5	0.000	chemotactic transducer PctB
PA0355	<i>pfpI</i>	-5.0	0.000	protease PfpI
PA4525	<i>pilA</i>	-20.3	0.000	type 4 fimbrial precursor PilA
PA0195	<i>pntAA</i>	-6.1	0.000	Energy metabolism; Transport of small molecules
PA0612	<i>ptrB</i>	-9.9	0.000	Transcriptional regulators
PA1947	<i>rbsA</i>	-7.1	0.000	ribose transport protein RbsA
PA1946	<i>rbsB</i>	-4.9	0.000	binding protein component precursor of ABC ribose transporter
PA5531	<i>tonB1</i>	-4.7	0.000	Transport of small molecules

Table 7.3.2 Genes downregulated in $\Delta rpoN$ mutant comparing to PAO1 wild type.

Downregulated genes were filtered based on the selection criteria of fold change ≤ -4 and p-value < 0.01 , $n=2$. Fold change is denoted as FC in short; adjusted p-value is denoted as padj. NCBI locus tags, gene names and protein functions are also displayed.

Figure 7.3.1 Regulation of *pqs* genes by *rpoN* at post-transcriptional level. Measurement of GFP fluorescence upon inducing transcriptional fusion $P_{pqsA-gfp}$ in mPAO1 wild type, $\Delta rpoN$, $\Delta rpoNCOM$, $\Delta rpoN/pME6032-pqsR$ and $\Delta rpoN$ with PQS. Mean GFP of triplicates normalized by OD600nm are shown with standard deviation in relative fluorescence units (RFU).

Figure 7.3.2 Primer efficiency test for *metE* gene of *P. aeruginosa* PAO1 strain and *B. cenocepacia* H111 strain. Standard curve obtained from triplicates. (A) Primer

efficiency test of *metE* gene of *P. aeruginosa* PAO1, efficiency=100.479%,
 $R^2 = 0.999$, slope=-3.31, y-intercept =16.583; (B) Primer efficiency test of
metE gene of *B. cenocepacia* H111, efficiency=108.288%, $R^2 = 0.962$,
slope=-3.138, y-intercept = 24.107.