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Transcriptional Profiling Analysis Likely to Indicate that c-di-GMP is Involved in Iron Uptake in *P. aeruginosa* PAO1

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Cyclic dimeric guanosine monophosphate (c-di-GMP), a small secondary messenger, regulates various bacteria physiological processes at transcriptional, post-transcriptional and post-translational levels. *P. aeruginosa* PAO1 genome contains multiple c-di-GMP metabolizing genes. Transposon mutant of PA0861, one of the c-di-GMP metabolizing genes, showed increased biofilm formation. Siderophores, mainly pyoverdine and pyochelin from *P. aeruginosa,* are involved in iron uptake. In current study, mainly microarray and RT-PCR analysis were applied to compare the expression profile between wild type and mutant strains of P. aeruginosa. Our transcriptional profiling analysis indicated that pyoverdine synthesis was upregulated in PA0861 mutant. Through spectrophotometrically comparison, it was further demonstrated that pyoverdine production was increased in PA0861 mutant strain. Our results raise the possibility that c-di-GMP regulates siderophore synthetic genes, and affects biofilm formation. Further investigations will be needed to elucidate the mechanisms.

Keywords: Biofilm formation; cyclic dimeric guanosine monophosphate (c-di-GMP); *P. aeruginosa*; pyoverdine; siderophores.

Firstly identified as an allosteric activator of *Gluconacetobacter xylinus* cellulose[1], cyclic dimeric guanosine monophosphate (c-di-GMP) has become a central player in the signaling networks in bacteria. As a unique second messenger, c-di-GMP has been demonstrated to be involved in a plethora of physiological functions, including motility, biofilm formation, quorum sensing, virulence, and cell cycle, at the transcriptional, post-transcriptional and post-translational levels [2].

c-di-GMP is synthesized from two GTP molecules by diguanylate cyclases (DGCs), usually containing GGDEF domain[3]; while its degradation is catalized by phosphodiesterases (PDEs) of either the EAL[4] or HD-GYP domain [5]. Diguanylate cyclases (DGCs) and phosphodiesterases (PDEs), or the c-di-GMP-metabolizing proteins, are multidomain/modular proteins with N-terminal

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signaling domain(s) and C-terminal catalytic domains [6]. Additionally, some degenerate/ catalytically inactive GGDEF and EAL domain could bind to c-di-GMP and sense cellular concentration of this small molecule. GGDEF and EAL domain-containing proteins are therefore involved in both turnover and regulation of the c-di-GMP signaling pathway [2].

Many bacteria genomes, however, contain multiple copies of c-di-GMP-metabolizing proteins,[7,8] *eg. E. coli* K12 contains 19 proteins with GGDEF domain and 17 with EAL domain, whereas *Vibrio vulnificus* genome encodes 66 proteins with the GGDEF domain and 33 with the EAL domain[7]. This multiplicity of DGCs and PDEs raises the question always puzzling: how could the regulatory specificity be achieved? As c-di-GMP, a small cyclic di-nucleotide, is soluble and presumed to diffuse freely in solution.

With more and more accumulated data[2], it has been realized that there might exist respective c-di-GMP regulatory pathways/circuits, *i.e.*, 'individual c-di-GMP signaling components are controlled by specific environmental and intracellular stimuli, and that they often serve specific targets'[2]. In a detailed quantitative analysis[9], individual DGCs are shown to correlate directly to c-di-GMP production and c-di-GMP–mediated phenotypes, while there is no correlation between the global c-di-GMP concentration and biofilm formation. Reasonably, dissection of individual c-di-GMP-metabolizing proteins signaling pathway will help to deepen our understandings of c-di-GMP signaling mechanisms.

Pseudomonas aeruginosa, an opportunistic pathogen affecting cystic fibrosis and immunologically impaired patients, has been applied as a model organism in research labs. *P. aeruginosa* PAO1 genome encodes 17 GGDEF domain-containing proteins, 5 EAL ones, and 16 with both of them[10]. Quite a few of these proteins/ genes have been analyzed and elucidated in detail, including WspR, a response regulator with DGC activity[11]; RocR, a response regulator with PDE activity[12]; and LapD, a c-di-GMP effector, with both its GGDEF and EAL domains losing catalytic activity[13]; and some other c-di-GMP metabolizing genes/proteins, such as MorA, FimX, BifA, SiaD, YfiN, DipA, NbdA et al[14-20].

PA0861 or RbdA[21] from *P. aeruginosa* PAO1, a modular protein containing PAS-PAC-GGDEF-EAL multidomains, was characterized as a PDE and involved in regulation of a number of physiological processes, such as bacterial motility, production of rhamnolipids, negative regulation of exopolysaccharides production, and finally, biofilm dispersal[21]. However, the detailed signaling circuit of this molecule is not clear. What is the mechanism for PA0861 to affect the phenotype of biofilm formation?

In current study, DNA microarray analysis was applied to compare gene expression pattern between wild type PAO1 and PA0861 mutant. From the transcriptional profiles obtained, we attempt to explore, especially at the molecular level, the information and mechanisms related to the physiological functions and signaling of PA0861.

MATERIALS AND METHODS

Bacteria biofilm analysis

The PA0861 mutant and wild type *P. aeruginosa* were requested from Manoil lab[22].

For biofilm analysis, overnight cultures were diluted to O.D.(600nm) 0.1 with fresh LB broth. The diluted cultures (1 ml, in Falcon 352057 tube) were incubated at 37°C, 100 rpm for the indicated periods of time.

Bacterial cultures were carefully removed, cells bound to the walls of the tubes (biofilms) were stained with 0.2% crystal violet (Sigma) for 20 min at room temperature. The tubes were then rinsed with water, air dried and photographed.

For quantification, biofilms were suspended in 5 ml of 95% ethanol, and measured with a spectrophotometer at 570nm. Each experiment was repeated at least three times.

Microarray analysis

Overnight culture of both *P. aeruginosa* wild type and PA0861 mutant was used as seed for 1:100 inoculation. The cells were collected after 6 hours of growth, for RNA extraction, cDNA making and hybridization.

Microarray analysis was applied by the Capitalbio Company (http://cn.capitalbio.com/). Briefly, an aliquot of 2 μg of total RNA was used to synthesize double-stranded cDNA, and produce biotin-tagged cRNA using the MessageAmpTM II aRNA Amplification Kit. The resulting bio-tagged

cRNA were fragmented to strands of 35–200 bases in length according to the protocols from Affymetrix. The fragmented cRNA was hybridized to GeneChip®Pseudomonas aeruginosa

Genome Array (Affymetrix), which contains probe sets for over 5,500 ORFs from the PAO1 strain of *P. aeruginosa,* was applied. Hybridization was performed at 45! with rotation for 16 h (Affymetrix GeneChip Hybridization Oven 640). The GeneChip arrays were washed and then stained (streptavidin-phycoerythrin) on an Affymetrix Fluidics Station 450 followed by scanning on a GeneChip Scanner 3000.

The hybridization data were analyzed using GeneChip Operating software (GCOS 1.4). The scanned images were first assessed by visual inspection then analyzed to generate raw data files saved as CEL files using the default setting of GCOS 1.4. An invariant set normalization procedure was performed to normalize the different arrays using DNA-chip analyzer [dChip].

In a comparison analysis, we applied a two class unpaired method in the Significant Analysis of Microarray software (SAM) to identify significantly differentially expressed genes between PA0861 mutant and *P. areuginosa* PAO1 wild type groups. Over 2700 genes were determined to be significantly differentially expressed with a selection threshold of false discovery rate, FDR<5% , fold changeÿ2.0 , and Wilcoxon Rank-Sum test significance level at 0.05 ($P < 0.05$) in the SAM output result.

The microarray data were analyzed by research staff from Capitalbio Company. Online resources of GO (http://www.geneontology.org/), WEGO (http://wego.genomics.org.cn/cgi-bin/wego/index.pl) were applied to group of the differentially expressed genes. KEGG (http://www.genome.jp/kegg/) and DAVID (http://david.abcc.ncifcrf.gov/) were applied for analysis of the pathways affected

RT-PCR analysis

A 2 ml culture was collected for RNA extraction. Total RNA was isolated (CWbiotech, China) and cDNA was synthesized using the SuperScript b! 1st strand cDNA synthesis kit (Invitrogen) . cDNA was normalized and applied for semi-quantitative RT-PCR analysis (Taq DNA polymerase, Applied Biosystem).

PCR reaction conditions are: 94!,3min; 94!, 30s; 57-62!, 30s; 72!, 1min; 30-40 cycles, followed by 72!, 7min, in 50ul reaction system.

Pyoverdine measurement

The pyoverdine production in both wild type PAO1 and PA0861 mutant was estimated spectrophotometrically. Modified King B medium (acid-hydrolyzed casein 20g/l, Dipotassium hydrogen phosphate 1.5g / l, Magnesium sulphate Heptahydrate 1.5g / l, glycerol. 15ml / l) was applied for the reason of iron depletion. 6 ml of bacteria culture were centrifuged at 15000 rpm for 5 minutes, and the supernatant was collected and added with 500 microliters of 0.1 molar Tris-HCl, PH 8.0.In order to normalize for differences in cell density, pyoverdine production was represented as OD405/OD600. Each experiment was repeated at least three times.

RESULTS

Phenotypic comparison between wild type and PA0861 mutant

A few *P. aeruginosa* transposon mutants were requested from Manoil Lab[22]. Out of these mutants, one with transposon harboring on gene PA0861 (or *rbdA*[21]) was verified by PCR (data not shown) and selected for further analysis.

Firstly, biofilm formation was compared between wild type PAO1 and this mutant. Quantification of the crystal violet-stained biofilms is shown in Fig. 1. After growth for 12 h in LB medium, the mutant exhibited enhanced biofilm formation (approximately two-fold) over that of the wild type. The mutant grows at a rate indistinguishable from that of the wild type PAO1 (data not shown), suggesting the accumulated biofilm mass should not be due to increased growth rate. This observation shows a similar trend to what has been reported by Zhang *et al* in 2010[21].

Recent years have witnessed that c-di-GMP, a small secondary messenger, shows versatile effects on bacteria physiology. PA0861 or RbdA in *P aeruginosa* has been characterized as a PDE, with both the PAS and GGDEF domains important for its biological functions [21]. For the attempt to understand further the c-di-GMP signaling mechanisms and specificity, we applied microarray assay using Affermatrix genome array matrix.

Overview of microarray data

To gain insights into different gene

expression pattern between PA0861 mutant and wild type PAO1, microarray analysis was applied. Overnight culture was used as seed for 1:100 inoculation, and the cells were collected after 6 hours of growth, for RNA extraction, cDNA making and hybridization. We used Affetmatrix *P. aeruginosa* genome microarray that includes more than 5500 ORFs. Four biological replicates of each strain were analyzed and thus, eight readings were obtained for each ORF.

Fig. 1.A

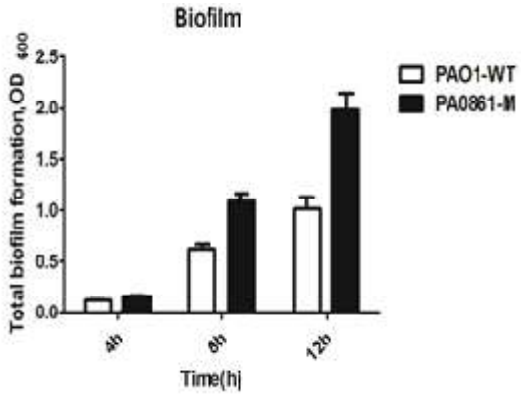
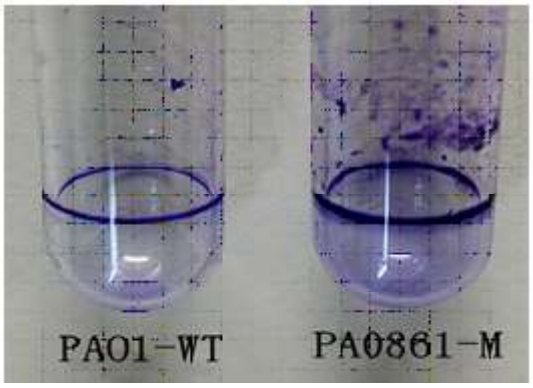


Fig. 1.B



1A Quantitative comparison of the biofilm formation by wild-type strain PAO1 and PA0861 mutants.
1B Visualization of bacterial biofilm formation on polystyrene tubes by crystal violet staining.
The data shown are means of triplicates, and the standard deviations are shown by error bars. *p-value* for 4h, 8h and 12h is 0.004, 0.001 and 0.001, respectively.

Fig. 1. Biofilm phenotypes of P. aeruginosa PAO1 an PA0861 (rbdA) transposon insertion mutant.

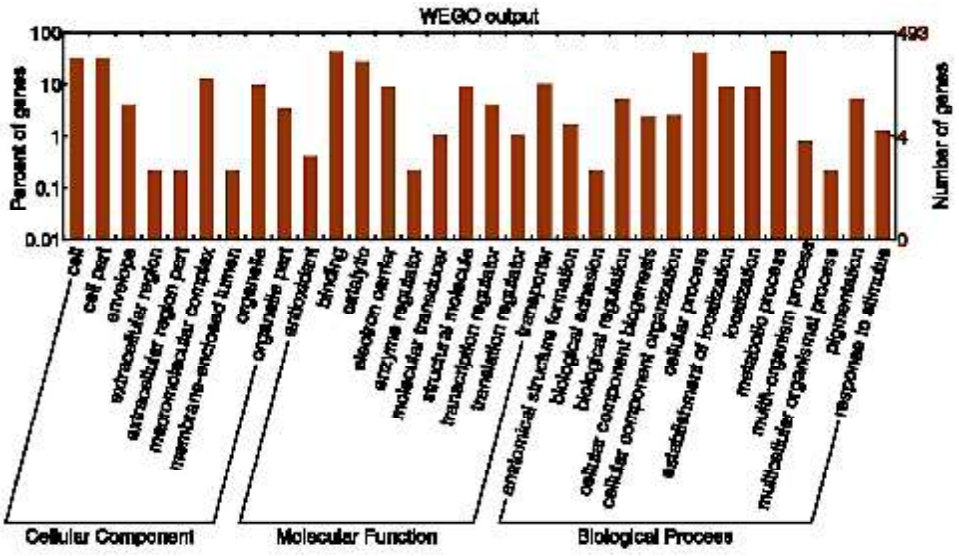
After the filtering process, a total of 2782 transcripts between wild type and mutant were different; of which, approximately 2400 transcripts upregulated and 382 downregulated for at least 2 folds. As the upregulated transcripts were much more, we adjusted the criteria to 6 fold increase or 2.5 fold decrease in our further analyses. Compared to wild type PAO1, PA0861 mutant showed 319 genes upregulated and 198 genes down-regulated significantly, with at least a 6 fold increase or 2.5 fold decrease in expression. The original data are available upon request.

To obtain an overall picture of the differentially expressed genes, GO analysis was applied. As shown in Table 1, the first 15 categories of differentially expressed genes in biological processes were presented, including translation (55 transcripts); generation of precursor metabolites and energy (35 transcripts); aerobic respiration (16 transcripts); acetyl-CoA catabolic process (12 transcripts); tricarboxylic acid cycle (12transcripts) coenzyme catabolic process (12 transcripts); acetyl-CoA metabolic process (12 transcripts); cofactor catabolic process (12 transcripts); cellular respiration (19 transcripts); energy derivation by oxidation of organic compounds (19 transcripts); and protein folding (11 transcripts). These genes account for forty-two percent (215/517) of the total differentially regulated genes.

Some of the biological processes, such as replication and DNA metabolism, signal transduction and nucleotide metabolism categories were not represented. The overall GO analysis result was summarized by WEGO (http://wego.genomics.org.cn/cgi-bin/wego/index.pl) and presented in Fig.2.

c-di-GMP likely to be involved in iron uptake

When examining the individual clusters, there emerged a category of "pigmentation" in the biological processes, which contains up to 26 transcripts (Fig.2), and was not very common in general microarray data. A variety of the colorful pigments, including pyocyanin (blue-green), pyoverdine (yellow-green and fluorescent), and pyorubin (red-brown), was produced in *P. aeruginosa* and involved in virulence. We therefore applied pathway analysis, to try to dig out more information related to PA0861 physiological function.



This analysis was carried out using the online resource WEGO (http://wego.genomics.org.cn/cgi-bin/wego/index.pl).

Fig. 2. Assignment of Gene Ontology (GO) categories of cluster I to cluster III.

The primers are listed below

Primer	Primer sequence	Tm	Fragment length
PA2385-F	TCCAGCCACTTCACCCTGTATC	57	1075bp
PA2385-R	GAATCCCGCTCTTCTCCACCT	57	
PA2392-F	GCCTGATTGGCTACCTGGATA	62	1021bp
PA2392-R	CCGTGGAACGACCAGAACA	62	
PA2394-F	GGAATACCGGCACAAGCG	62	643bp
PA2394-R	GAAGGTGAAGCCCGAAGACAG	62	
PA2395-F	CGGCACGGTGTTCAAGGAT	58	338bp
PA2395-R	ACAGCCAGGCGGCGTAGTTC	58	
GAPDH-F	CCTACACCAACGACCAGAACCT	57	316BP
GAPDH-R	TGATTGAAATCCACCGACACC	57	
PA2444-F	GCGGCAGGAAGACCACATC	61	582BP
PA2444-R	ATCGGGTTCGGGTAGAGGC		
PA2443-F	GCCACTGCCAGCATCAAGG	58	536BP
PA2443-R	TGCATCACCTGCCAGATACGC		
PA2418-F	CCTGGGAATCTACGGCAACG	60	404BP
PA2418-R	GCGGCGGGTTCATCTTGT		
PA2398-F	CAACGGAGCCAAGTGGAG	58	761BP
PA2398-R	CGAAGTAGGCGAGACTGGTATT		
PA2393-F	ACAAGGACGGACCGAACCA	58	409BP
PA2393-R	GGCAAGGGACGGGAAGAGT		
PA2403-F	CAGCAAGCCCTACGAAGATG	57	440bp
PA2403-R	CGCTCAGGCGATGGAAGT		
PA2388-F	CTGGCTGCAAGGCTGGATT	57	351bp
PA2388-R	GGAGTCAGGCTGCTGTCGTT		
PvdS-F	ATGTCGGAACAACTGTCTACCC	57	443bp
PvdS-R	TTGGCGATGTCCTTCTGTG		

Through the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://en.wikipedia.org/wiki/KEGG) and David (http://david.abcc.ncifcrf.gov/) pathway analysis, the first 10 significant KEGG pathways ($P<0.05$) were listed in Table 2. In addition to genes related to ribosome, citrate cycle (TCA cycle), and energy production, what prominent is that 5 genes related to siderophore biosynthesis were also activated in PA0861 mutant (Table 2), which seems to be in consistency with the GO analysis result (Fig. 2).

Siderophores are composed of a conserved fluorescent chromophore linked to a short peptide, which differs among species and strains. They are excreted and specifically chelate Fe^{3+} with a high affinity[23]. There are two major types of siderophores, pyoverdine and pyochelin, in *P aeruginosa*.

Siderophores are produced by nonribosomal peptide synthesis processes[24]. Through chemical and genetic analysis, the majority of synthetases and accessory enzymes related to pyoverdine and pyochelin production has been identified[25, 26, 27]. For pyoverdine biosynthesis, almost all genes cluster at a single (*pvd*) locus in the PAO1 genome[27], which span the region for approximately 120 kb, from PA2383 to PA2452[27]. There are totally about 70 genes, including *pvdS*, the major iron starvation sigma factor of *Pseudomonas aeruginosa*[28]. Compared to pyoverdin biosynthesis, the genes involved in pyochelin production is less abundant, which are mainly localized from PA4220 to PA4231.

The physiological functions of siderophores are closely related to iron uptake. Iron belongs to one of the most abundant elements on earth. However, iron restraint is encountered during bacteria growth: Fe^{3+} is almost insoluble; the soluble Fe^{2+} is limited and particularly sequestered by host proteins. Bacteria have

Table 1. Functional distribution of genes whose expression level changed significantly between wild type P. aeruginosa PAO1 and PA0861 mutant

Term	Count	P Value
GO:0006412~translation	55	1.00E-39
GO:0006091~generation of precursor metabolites and energy	35	1.43E-12
GO:0009060~aerobic respiration	16	3.09E-11
GO:0046356~acetyl-CoA catabolic process	12	3.32E-10
GO:0006099~tricarboxylic acid cycle	12	3.32E-10
GO:0009109~coenzyme catabolic process	12	2.49E-09
GO:0006084~acetyl-CoA metabolic process	12	1.31E-08
GO:0051187~cofactor catabolic process	12	3.81E-08
GO:0045333~cellular respiration	19	5.83E-08
GO:0015980~energy derivation by oxidation of organic compounds	19	1.64E-07
GO:0006457~protein folding	11	3.04E-05
GO:0022900~electron transport chain	16	1.19E-04
GO:0055085~transmembrane transport	10	6.63E-04
GO:0006732~coenzyme metabolic process	17	0.00108208
GO:0006221~pyrimidine nucleotide biosynthetic process	5	0.007481572

Table 2. Significant KEGG pathways in PA0861 mutant

Term	Count	P Value	FDR
pae03010:Ribosome	45	1.17E-45	1.49E-42
pau00020:Citrate cycle (TCA cycle)	14	7.50E-09	9.57E-06
pae00190:Oxidative phosphorylation	17	4.65E-08	5.94E-05
pap00020:Citrate cycle (TCA cycle)	12	3.88E-07	4.95E-04
pae01053:Biosynthesis of siderophore group nonribosomal peptides	5	1.75E-04	0.223501438
pae00240:Pyrimidine metabolism	11	2.07E-04	0.264247343
pae02010:ABC transporters	25	5.77E-04	0.734299992
pae00632:Benzoate degradation via CoA ligation	10	6.89E-04	0.875515625
pag00240:Pyrimidine metabolism	10	8.26E-04	1.049578694
pae00910:Nitrogen metabolism	10	0.002926664	3.672607057

developed the powerful iron acquisition systems to overcome this limitation: pyoverdines and pyochelin from nonproteinogenic precursors could solubilize ferric iron and accelerate iron transport, and thus enhance bacteria growth[25].

Moreover, siderophores functions are not limited to nutrition. In *P. aeruginosa*, pyoverdine not only regulates its own production but also controls production of two other virulence factors, exotoxin A and PrpL protease[29]. Pyoverdine is therefore acted as a signaling molecule.

Table 3. List of q-value and fold change of genes selected for RT-PCR analysis.

Gene ID	q-value(%)	Fold Change
PA2385	0.034722757	3.8983
PA2388	2.513383988	1.3129
PA2392	0.204340455	2.2493
PA2394	0	4.5287
PA2395	0.081778576	3.3334
PA2398 (fpvA)	0.129979704	3.273
PA2403	0.129979704	2.7319
PA2418	0	7.6558
PA2443 (sdaA)	2.513383988	1.028
PA2444 (glyA2)	8.816976733	0.9899
pvdS	0.058761589	3.4272

Furthermore, both pyoverdines and pyochelin siderophores are indicated to play important roles in the processes of infection, which has been reviewed by Pierre Cornelis recently[26]. At the time of acute infections, *P aeruginosa* produces the high-affinity pyoverdine for iron uptake, which also functions as a signal molecule for acute virulence factors production[26]. Additionally, RNA analysis of cystic fibrosis (CF) samples implies that both pyoverdine and pyochelin are involved in cystic fibrosis chronic infection[30]. Recently, it has been reported[31], inhibition of expression of the iron-starvation ó-factor *PvdS*, and thereby repressing the production of pyoverdine, PrpL protease, and exotoxin A, will lead to suppressed *P. aeruginosa* pathogenicity.

It was also reported that iron is intimately linked to biofilm formation in *P. aeruginosa*[32, 33, 34]. The connections of quorum sensing, motility, biofilm formation and iron availability seems to be intertwined and complicated, however, it likely to be demonstrated that, in *P. aeruginosa*, iron acquisition supports normal biofilm formation by using the endogenous pyoverdine (at relatively low external iron concentrations) or pyochelin (at higher iron levels)[32].

Table S1 and S2 Microarray data of fold change of pyoverdine and pyochelin biosynthetic genes

Table S1

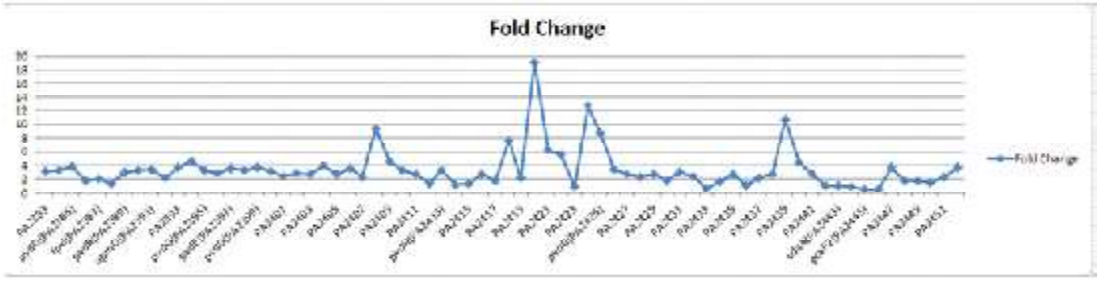
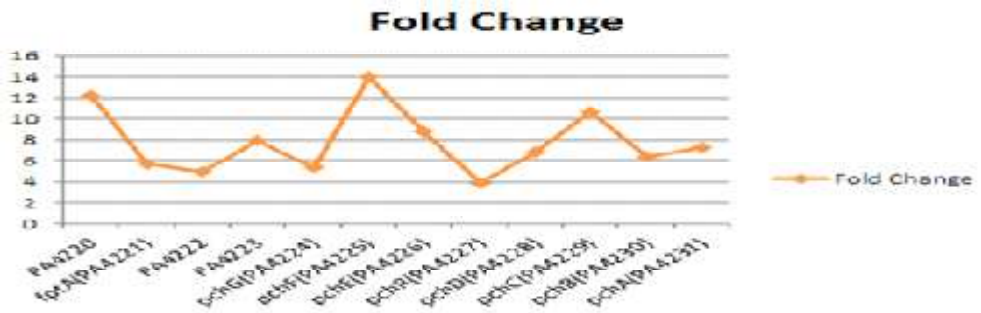
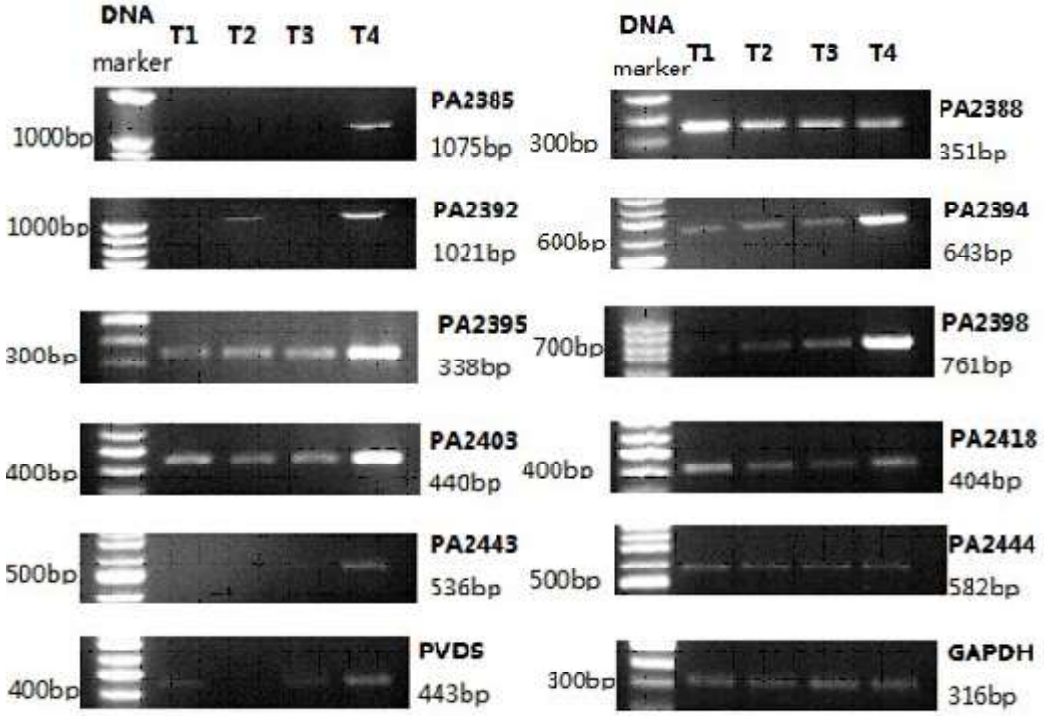


Table S2

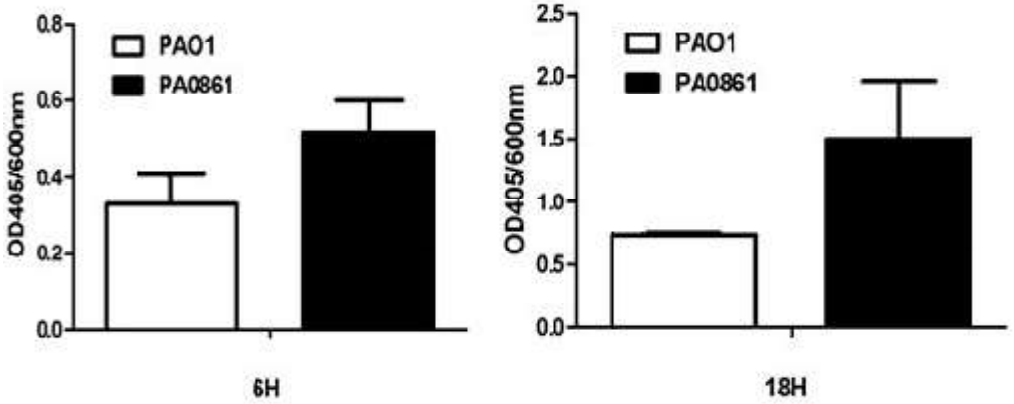


We firstly examined the genes known to be involved in siderophores synthesis (Table S1and S2). For the 70 genes related to pyoverdine biosynthesis (S1), a total of 51 genes showed increased expression levels, ranging from 2 to 19 folds in PA0861 mutant. For that of pyochelin synthetic genes (Table S2), all 11 genes (PA4220-PA4231, including pchABCDEFG, *pchR* and *fptA*)



Agarose gel electrophoresis of RT-PCR products of wild type PAO1 (Test 1 and 3) and mutant PA0861 (Tests 2 and 4) for 6 hour (T1 and T2) and 18 hours (T3 and T4). cDNA templates were normalized using GAPDH

Fig. 3. Transcriptional comparison of pyoverdine and pyochelin biosynthetic genes between PA0861 mutant and wild typw PAO1



The data shown are means of triplicates, and the standard deviations are shown by error bars. *p-value* is 0.019 (6h) and 0.046 (18h).

Fig. 4. Comparison of pyverdine production between wild type PAO1 and PA0861 mutant

were upregulated, with *pchR* at the lowest fold change of 3.86 fold of upregulation, and *pchF* at the highest of 14 fold. This expression comparison profile indicated that the pyoverdine and pyochelin synthetic genes are increased in PA0861 mutant.

There were 19 genes related to pyoverdine biosynthesis not to be affected by PA0861 (Table S1). For some of the 19 genes, the standard deviation was rather large (Table S1 and data not shown). Another possibility is that the bacteria were cultured for 6 hours in our microarray analysis, which may not be long enough for some of the genes to be upregulated. Further investigations will be needed.

To corroborate the microarray data, 11 genes were selected randomly for the semi-quantitative RT-PCR analysis (Fig. 3). Of the 11 genes, PA2385, PA2392, PA2394, PA2395, PA2398, PA2403, PA 2443 and *pvdS* were demonstrated to be upregulated, while PA2388 and PA2418 showed reduced expression at both 6 and 18 hours, and gene PA2444 seemed to be of no difference between wild type PAO1 and PA0861 mutant (Fig.3). There is only one exception of PA2418, which was shown to be downregulated in RT-PCR and highly upregulated in our array data (Fig.3 and Table 3).

Although the two methods are different in displaying the results, 10 out of 11 genes in RT-PCR analysis presented the same expression trend in both analysis (Fig. 3 and Table 3). These results, in general, confirmed that the differential gene expression pattern based on our microarray data are reliable. We thus obtained clues that the siderophores synthetic genes are affected by the c-di-GMP metabolizing gene PA0861.

Comparison of pyoverdine production between wild type PAO1 and PA0861 mutant

We further compared the products of pyoverdine in both wild type PAO1 and PA0861 mutant. The total concentrations of pyoverdine in PAO1 and in PA0861 mutant (grown in King B medium as the iron-depleted medium) were estimated spectrophotometrically[35]. As shown in Fig. 4, the PA0861 mutant had an increased ability to produce pyoverdine.

Pyoverdine (and also pyochelin) are related to iron uptake, which has a positive effect on biofilm formation. Upon current data, we deduced that in PA0861 mutant, it might be the upregulated pyoverdine (and maybe pyochelin) expression that contributes to increased iron uptake, and furthermore, to the increased biofilm formation. PA0861 (or *rbdA*) was shown to be linked to positive regulation of bacterial motility and production of rhamnolipids, and also negative regulation of production of exopolysaccharides[21]. Nevertheless, our transcriptional profiling comparison between PA0861 mutant and the wild type PAO1 indicates the increased c-di-GMP with increased iron uptake, and finally, increased biofilm formation. The current study might therefore shed light for further understanding of the physiological functions of PA0861.

DISCUSSION

In a recent study, it was reported that overproduction of WspR, a well-known DGC, which leads to an artificial increase of c-di-GMP, will lead to an increase of pyoverdine production in the *P. aeriginosa* PAO1 wild-type and mutant strains tested[36]. On the contrary, overexpression of RocR, a well-known PDE, displays the opposite function[36]. This observation is consistent to what we record in current research.

PA0861 or RbdA, which was reported to display the catalytic activity of PDE, once inactivated, the cellular concentration of c-di-GMP should be increased; with increased cellular c-di-GMP, the expression of pyoverdine and pyochelin would be upregulated; and consequently, biofilm formation in the mutant strain will be increased. Our observation thus links c-di-GMP, the well-established secondary messenger, with iron uptake in *P. aeruginosa,* though further investigations will be needed to disclose the signaling mechanisms.

ACKNOWLEDGEMENTS

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