Transcriptomic profiling of substrate-dependent autoaggregation in *Pseudomonas putida* CP1

Wan Syaidatul Aqma*, Padraig Doolan³, Brid Quilty²,³

₁School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia.

₂School of Biotechnology, Dublin City University, Dublin 9, Ireland.

³National Institute for Cellular Biotechnology (NICB), Dublin City University, Dublin 9, Ireland.

Email: syaidatul@ukm.edu.my

ABSTRACT

**Aims:** *Pseudomonas putida* CP1 is an interesting environmental isolate which exhibits substrate-dependent autoaggregation when the organism was grown on 0.5% (w/v) fructose. Autoaggregation is a process of a single bacterial species to develop clumps of cells during a substrate stress. This study was carried out to investigate the genetic changes in the bacterium during aggregate formation.

**Methodology and results:** *P. putida* CP1 was grown on 0.5% (w/v) fructose in batch culture at 30 °C and 150 rpm. The removal of fructose from the medium corresponded with aggregation of the cells which started after 8 h incubation. Microarray gene expression profiling using a *P. putida* KT2440 Genome Oligonucleotide Array (Progenika, Spain) showed that 838 genes involved in metabolism and adaptation were differentially expressed in *P. putida* CP1. Global transcriptomic profiling studies showed that *P. putida* CP1 growing on fructose resulted in the induction of genes encoding for proteins mainly involved in protein translation, ABC transporters, oxidative phosphorylation and two-component systems (TCS). Novel genes, associated with autoaggregation, were identified using transcriptomic analysis involved in ABC transporter, TCS, flagella assembly and lipopolysaccharide biosynthesis. It was also associated with the up-regulation of genes involved in the flagellar assembly including the fliE gene which encodes for the flagellar hook-basal body protein.

**Conclusion, significance and impact of study:** The identification of new genes involved in autoaggregation formation is important to understand the molecular basis of strain variation and the mechanisms implicated in cell-cell communication.

**Keywords:** *Pseudomonas putida* CP1, sugar metabolism, autoaggregation

INTRODUCTION

*Pseudomonas putida*, a non-pathogen that belongs to the Gammaproteobacteria is among the best studied species of the metabolically versatile and ubiquitous genus of the Pseudomonads. They are known as rapidly growing bacteria and are nutritional opportunists that are able to recycle organic wastes in aerobic and microaerophilic compartments of the environment for the maintenance of environmental quality (Wu et al., 2011). Members of the species are recognised for their metabolic versatility and tolerance to solutes and solvents and to chemically diverse aromatics and hydrocarbons that induce chaotropicity mediated stress (Cray et al., 2013).

Bacteria colonise every habitat on the earth facilitated by their ability to survive and adapt to environmental stress conditions. The formation of aggregates was one of the survival strategies by bacteria in a stressful condition (Marles-Wright and Lewis, 2007). An example of advantageous microbial aggregation is the enhanced efficiency of biological wastewater treatment processes through the selective separation of microbes and the suspended solids (Eboigbodin et al., 2007). Aggregates are stabilised by a matrix of extracellular polymeric substances (EPS) that consist of polysaccharides, proteins and DNA. EPS are a major component of bioaggregates for keeping the aggregates together in a three-dimensional matrix and provides energy and carbon when substrate is limited. They also protect the cells from any harsh external environment (Aqma and Quilty, 2015).

Various aggregation factors have been identified as Windt et al. (2006) discovered that the agglutination protein AggA was involved in the hyper-aggregating phenotype. AggA is a necessary factor for the increased surface and cell adhesion of the hyper-aggregating bacterium. Nielsen et al. (2011) discussed the importance of two putative exopolysaccharide gene clusters named bacterial cellulose (bcs) and putida exopolysaccharide A.

DOI: http://dx.doi.org/10.21161/mjm.503

*Corresponding author*
(pea) in *P. putida* KT2440 biofilm formation and stability. In addition, bacteria sense their environment through signalling proteins involved in two-component systems (TCS) which constitute the major signal transduction system in bacteria (Mattos-Granera et al., 2017).

Signal transduction systems function as intracellular information-processing pathways that link external stimuli to specific adaptive responses. Other proteins like sigma factors, cyclic-di-guanosine monophosphate (c-di-GMP) related proteins and methyl-accepting chemotaxis with flagella proteins are also involved in signal transduction (Valentini and Filloux, 2016).

NPK availability has been suggested to influence aggregation in *P. putida*. A previous study by Fakhruddin et al. observed that *P. putida* CP1 aggregated when grown on fructose but not when grown on glucose (Fakhruddin and Quilty, 2005). Pseudomonads conducted an incomplete glycolytic pathway for utilisation of glucose and other hexoses due to a lack of 6-phosphofructokinase. Glucose and fructose are similar hexoses with virtually identical energy values but they are predicted to follow different routes into the central metabolism of *P. putida* (Velázquez et al., 2004). Information on genotypic changes in autoaggregation are still lacking especially in *P. putida*. The identification of new genes involved in autoaggregation formation is important to understand the molecular basis of strain variation and the mechanisms implicated in cell–cell communication. The results of expression profiling on genes regulated in aggregative behaviour and the identification of key aggregation induced genes varies depending on the strains used. Not much work has been done to investigate the transcriptional response of *P. putida* to utilise fructose using microarray methodologies. To address this, we look at changes in the transcriptome of *P. putida* CP1 during growth in fructose to investigate the genetic changes in the bacterium during aggregate formation.

**MATERIALS AND METHODS**

**Microorganism and growth media**

*Pseudomonas putida* CP1 were grown overnight in 10 mL nutrient broth, washed twice with 0.01 M sodium phosphate buffer, pH 7.0, and resuspended until the optical density of the culture at 660 nm was 0.7. Inoculums were grown in 100 mL of minimal medium to which fructose and glucose were added individually. The minimal medium contained (g/L) K₂HPO₄, 4.36; NaH₂PO₄, 3.45; (NH₄)₂SO₄, 1.26; MgSO₄·6H₂O, 0.91; and 1 mL/L trace salts solution. The trace salts solution contained (g/100 mL): CaCl₂·2H₂O, 4.77; FeSO₄·7H₂O, 0.37; CoCl₂·6H₂O, 0.37; MnCl₂, 0.10; and Na₂MoO₄·2H₂O, 0.02. The pH of the medium was adjusted to 7.0 with 2 M NaOH and the medium was autoclaved at 121 °C for 15 min. A total of 0.5% (w/v) fructose was added to the flasks following sterilisation of the medium. The flasks were inoculated with a 5% (v/v) inoculum, which corresponded to approximately 4×10⁸ cells/mL, and were incubated at 150 rpm and 30 °C. Uninoculated control flasks were incubated in parallel.

**Design of genomic DNA microarray**

The microarray experiments carried out in this study used the *P. putida* KT2440 Genome Oligonucleotide Array (Progenika, Spain). The array was printed by Progenika Biopharma (http://www.progenika.com).

**Hybridisation and processing of microarrays**

Total RNA were purified from *P. putida* CP1 grown in nutrient broth with OD₆₀₀ of 0.5 and *P. putida* CP1 grown on 0.5% (w/v) fructose for 24 h according to the protocol provided by Qiagen (RNeasy Mini Kit). Three aliquots of pure RNA, each 20 µg was prepared for each treatment. 20 µg of total RNA was transformed to cDNA with Superscript III reverse transcriptase using random primers (p(dN)₆, Roche, Product no. 034731001). Fluorescently labelled cDNA for microarray hybridisations was obtained using the Superscript Plus Indirect cDNA Labelling System (Alexa 647 or Alexa 555 dyes) from Invitrogen according to the manufacturer’s instructions with some modifications. Labelling efficiency was assessed using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies) and the labeled cDNA was stored at −20 °C until hybridisations were carried out. Before the hybridisation process, the microarray was blocked by immersion in blocking solutions containing 250 mL of 20 × SSC (UltraPure; Invitrogen; 15557-044), 10 mL of 10% SDS (Invitrogen; 24730-020), 10 g of BSA (Fraction V; Sigma; A-3294), 740 mL of RO (reverse osmosis purified) water. The blocking solutions were filter-sterilised through a 0.2 µm filter then pre-heated at 42 °C. Slides were incubated in blocking solution for 1 h at 42 °C and then washed in RO water (room temperature) for 30 sec. The steps were repeated thrice, using fresh RO water each time followed by another immersion in isopropanol. The slides were dried by centrifugation at 1000 rpm for 5 min at room temperature in a microtitre plate centrifuge (Eppendorf 5804 with A-2-DWP rotor). For the hybridisation set up, initially the hybridisation chamber (Genetix) was preheated to 42 °C before use. The samples were combined to be co-hybridised and evaporated to complete dryness by centrifugation under vacuum. The hybridisation buffer was prepared freshly for each batch of arrays to be hybridised. Post-hybridisation was continued with the washing steps and the scanning process. An array/lifterslip sandwich was submerged in Wash buffer 1 containing 2 × SSPE, 0.1% Tween 20 (wash buffer 1 was pre-heated to 42 °C overnight to remove lifterslip). The array was then transferred to a rack in a container of fresh WB1 and washed for 5 min. Array was washed in wash buffer 2 containing 0.5 × SSPE, 0.1% Tween 20 for 5 min followed by wash buffer 3 containing 0.5 × SSPE for 1 min, wash buffer 4 containing 1 × SSPE for 15 sec and finally dipped in wash buffer 5 containing 0.05 × SSPE, UltraPure 20 × SSPE Buffer (Invitrogen; 15591-043) SigmaUltra Tween 20 (Sigma-
Aldrich; P7949). Then the array was dried by centrifugation at 8000 rpm for 5 min at room temperature in a microtitre plate centrifuge (Eppendorf 5804 with A-2-DWP rotor). Arrays were scanned using an Agilent DNA microarray scanner with SureScan High-Resolution Technology (Agilent Technologies, Stockport, Cheshire, UK) at a resolution of 10 μm and 100% PMTs for both the red and green channels to generate two 16 bit Tiffs for each array. Scanned images were flipped from upper left to lower right in Agilent Feature Extraction software and the data extracted using BlueFuse for Microarrays (version 3.6 (71459) software (BlueGnome, Great Shelford, Cambridge, UK).

Microarray data analysis

The statistical analysis included the data normalisation and qualification. The results for each replica (median intensity for each channel) were normalised and statistically analysed using the LIMMA software package (Smyth and Speed, 2004). Background subtraction was performed using a method implemented in LIMMA designed to yield positive corrected intensities (i.e. to avoid negative intensity values). Differential expression was calculated using linear models and empirical Bayes moderated t-statistics (Smyth and Speed, 2003; Smyth, 2004). The resulting log-ratios were normalised for each array through print-tip loess and expression values were scaled to achieve consistency among arrays. Each probe was tested for changes in expression over replicates by using moderated t-statistics (Smyth, 2004). The p-values were adjusted for multiple testing as described by Benjamini et al. (1995) to control the false discovery rate (FDR). The criterion for identifying differentially expressed genes was set to 0.5% FDR. Data filtration was done to generate a shorter list of differentially expressed genes. Only genes with average fold changes ≤ 1.2 and adjusted p-values less than 0.05 (i.e., false discovery rate less than 5%) were identified as significantly regulated genes. Only genes with average fold changes ≤ / ≥ 1.2 were used to generate a shorter list of differentially expressed genes. The resulting log-ratios were normalised for each array through print-tip loess and expression values were scaled to achieve consistency among arrays. Each probe was tested for changes in expression over replicates by using moderated t-statistics (Smyth, 2004). The p-values were adjusted for multiple testing as described by Benjamini et al. (1995) to control the false discovery rate (FDR). The criterion for identifying differentially expressed genes was set to 0.5% FDR. Data filtration was done to generate a shorter list of differentially expressed genes. Only genes with average fold changes ≤ 1.2 and adjusted p-values less than 0.05 (i.e., false discovery rate less than 5%) were identified as significantly differentially expressed genes based on Gene Ontology (GO) classification. Pathview tools (Weijun et al., 2017) were used to visually map clusters of genes involved in KEGG pathways and processes for pathway-specific and molecular overview purposes. Complete microarray data has been deposited at: https://drive.google.com/file/d/1qtlknco4le1b1P93z5d4e8bwztNbfIo/view?usp=sharing

RESULTS AND DISCUSSION

The aggregation of the cells is illustrated in Figure 1. P. putida CP1 exhibits autoaggregation in the presence of 0.5% (w/v) fructose. The observation suggested that the organism was under stress which could be attributed to the more complex mode of transport of this particular sugar into the cell (Temple et al., 1998). The ability to aggregate in fructose has been observed as well in a study conducted by Borrego et al. (2000). Mycobacterium sp. MB-3683 aggregated most in fructose followed by glycerol and glucose. The cells were found to have the highest hydrophobicity in the fructose medium and lowest in the glucose medium. Azospirillum brasilense was reported to aggregate during the growth in high C:N medium containing fructose and ammonium chloride (Burdman et al., 2000). The size of P. putida CP1 aggregates on fructose was reduced and showed dispersal when the substrate was fully utilised (data not shown). This is supported by Schleheck et al. (2009) who suggested that the dispersion of aggregates in P. aeruginosa PAO1 was related to the nutrient carbon and nitrogen starvation.

When changes in gene expression were compared for P. putida CP1 grown on nutrient broth with the organism grown on 0.5% (w/v) fructose, a total of 838 genes were significantly differentially regulated including 169 genes encoding for hypothetical proteins.

Up-regulated gene transcripts

A total of 456 genes were identified as significantly up-regulated (p ≤ 0.05, Fold Change (FC) >1.2). Genes were ranked by fold change and the top 25 genes are listed in Table 1. The most up-regulated gene was PP1185 which encodes for the outer membrane protein H1 with a fold change of 6.87. A number of the top up-regulated genes, rplV, rpsL, rplQ, rplR, rpsF, rpsR, rplL, rplP and rplD play a role in protein translation. Other up-regulated genes included cyoC, cyoE-2, cyoB, atpG, cyoD and atpE involved in oxidative phosphorylation, fliE involved in flagella assembly, phoQ in a two-component-system, secY, yidC and secE in protein export and bacterial secretion systems.

Down-regulated gene transcripts

A total of 382 genes were identified as significantly down-regulated and following ranking by fold change the top 25 genes are listed in Table 2. The top down-regulated 25 genes were involved in carbohydrate metabolism, amino acid metabolism and ABC transporters. The most down-regulated gene, hupA encoded for histone family protein DNA-binding protein with a fold change -9.31. Genes involved in fructose metabolism, fruK, fruA and fruB were also down-regulated in this comparative study. Two genes, phaA and phaF, associated with poly-3-hydroxyalkanoate (PHA) were also listed.
Table 1: Top 25 genes up-regulated in *P. putida* CP1 following growth on 0.5% (w/v) fructose for 24 h.

| Locus ID | Gene Name | Annotation                                                                 | Pathway                                | Fold-change |
|----------|-----------|----------------------------------------------------------------------------|----------------------------------------|-------------|
| PP1185   | oprH      | outer membrane protein H1                                                  |                                        | 6.872       |
| PP814    | cyoC      | cytochrome o ubiquinol oxidase, subunit III                               | Oxidative phosphorylation              | 6.252       |
| PP816    | cyoE-2    | protoheme IX farnesyltransferase                                          | Oxidative phosphorylation              | 5.94        |
| PP5000   |           | heat shock protein HsIV                                                   |                                        | 5.884       |
| PP813    | cyoB      | cytochrome o ubiquinol oxidase, subunit I                                 | Oxidative phosphorylation              | 5.488       |
| PP1187   | phoQ      | integral membrane sensor signal transduction histidine kinase             | Two-component system                   | 5.04        |
| PP1981   |           | NilR3/Smm1 family protein                                                  |                                        | 4.906       |
| PP5001   |           | ATP-dependent protease ATP-binding subunit HsIV                           |                                        | 4.546       |
| PP459    | rplV      | 50S ribosomal protein L22                                                  | Protein translation                    | 4.286       |
| PP449    | rpsL      | 30S ribosomal protein S12                                                  | Protein translation                    | 3.792       |
| PP5414   | atpG      | F0F1 ATP synthase subunit gamma                                            | Oxidative phosphorylation              | 3.764       |
| PP480    | rplQ      | 50S ribosomal protein L17                                                  | Protein translation                    | 3.748       |
| PP5337   |           | LysR family transcriptional regulator                                      |                                        | 3.738       |
| PP474    | secY      | preprotein translocase subunit SecY                                       | Protein export; Bacterial secretion system | 3.718       |
| PP470    | rplR      | 50S ribosomal protein L18                                                  | Protein translation                    | 3.632       |
| PP4895   | miaA      | tRNA delta(2)-isopentenylpyrophosphate transferase                        | Metabolic pathways                    | 3.544       |
| PP4877   | rpsF      | 30S ribosomal protein S6                                                   | Protein translation                    | 3.464       |
| PP4728   | grpE      | heat shock protein GrpE                                                   |                                        | 3.446       |
| PP6      | yidC      | putative inner membrane protein translocase component                     | Protein export; Bacterial secretion system | 3.432       |
| PP4876   | rpsR      | 30S ribosomal protein S18                                                  | Protein translation                    | 3.382       |
| PP4874   | rplI      | 50S ribosomal protein L9                                                   | Protein translation                    | 3.284       |
| PP815    | cyoD      | cytochrome o ubiquinol oxidase                                            | Oxidative phosphorylation              | 3.148       |
| PP5418   | atpE      | F0F1 ATP synthase subunit C                                               | Oxidative phosphorylation              | 3.136       |
| PP4179   | htpG      | heat shock protein 90                                                      |                                        | 3.126       |
| PP461    | rplP      | 50S ribosomal protein L16                                                  | Protein translation                    | 3.04        |
| PP4370   | flfE      | flagellar hook-basal body protein                                         | Flagellar assembly                     | 3.028       |

Malays. J. Microbiol. Vol 14(6) Special Issue 2018, pp. 503-512
DOI: http://dx.doi.org/10.21161/mjm.1461809

ISSN (print): 1823-8262, ISSN (online): 2231-7538
Table 2: Top 25 genes down-regulated in *P. putida* CP1 grown on 0.5% (w/v) fructose for 24 h.

| Locus ID | Gene Name | Annotation | Pathway                                      | Fold-change |
|----------|-----------|------------|----------------------------------------------|-------------|
| PP5313   | hupA      | histone family protein DNA-binding protein |                              | -9.31       |
| PP1010   | edd       | phosphogluconate dehydratase               | Pentose phosphate pathway    | -8.47       |
| PP1296   | estB      | carboxylesterase                           |                              | -7.81       |
| PP3443   | gntR-2    | glyceraldehyde-3-phosphate dehydrogenase, putative winged helix family two component transcriptional regulator | Glycolysis / Gluconeogenesis | -5.69       |
| PP1012   | thiC      | thiamine biosynthesis protein ThiC         | Thiamine metabolism         | -5.23       |
| PP4922   | glcD      | glycolate oxidase subunit GlcD             | Glyoxylate and dicarboxylate metabolism | -5.17       |
| PP1071   | metY      | O-acetylhomoserine aminocarboxypropyltransferase | ABC transporters          | -5.14       |
| PP4659   | ggt-2     | gamma-glutamyltransferase                  | Taurine and hypotaurine metabolism | -4.72       |
| PP2528   | fruK      | 1-phosphofructokinase                      | Cysteine and methionine metabolism | -4.53       |
| PP794    | codA      | N-isopropylmalide isopropylaminohydrolase | Pyrimidine metabolism      | -4.48       |
| PP1024   | eda       | keto-hydroxyglutarate-aldolase/keto-deoxy- phosphogluconate aldolase | Pentose phosphate pathway | -4.32       |
| PP545    | hyuB      | hydantoinase B/oxoprolinase                | Glycolysis / Gluconeogenesis | -4.17       |
| PP3514   | gap-1     | glyceraldehyde-3-phosphate dehydrogenase, type I | Arginine and proline metabolism | -4.12       |
| PP1009   | phaA      | poly(3-hydroxyalkanoate) polymerase 1      | Butanoate metabolism       | -4.03       |
| PP5003   | leuD      | isopropylmalate isomerase small subunit    | Valine, leucine and isoleucine biosynthesis | -3.90       |
| PP795    | fruA      | PTS system, fructose subfamily, IIC subunit | Fructose and mannose metabolism | -3.78       |
| PP362    | bioB      | biotin synthase                           | Biotin metabolism          | -3.61       |
| PP793    | fruB      | phosphoenolpyruvate-protein phosphotransferase | Fructose and mannose metabolism | -3.59       |
| PP1139   | livM      | leucine/isoleucine/valine transporter permease subunit poly(hydroxyalkanoate) granule-associated protein | ABC transporters         | -3.46       |
| PP5007   | phaF      | poly(3-hydroxyalkanoate) polymerase 1      | Butanoate metabolism       | -4.03       |
| PP1019   | oprB-1    | porin B                                  |                              | -3.12       |
| PP4256   | ccoO-2    | cbb3-type cytochrome c oxidase subunit II  | Oxidative phosphorylation   | -3.08       |
Metabolic pathway analysis

A pathway analysis was conducted for the 838 overlapped genes based on the biological information database KEGG. A total of 29 different metabolic pathways were identified in this analysis. 5% of the genes were involved in protein translation, 4% in ABC transporters, 3% in oxidative phosphorylation and 2% in two-component systems (Figure 2). A smaller number of genes were involved in pathways associated with carbohydrate metabolism including pentose phosphate pathway, glycolysis, fructose and mannose metabolism, amino sugar and nucleotide sugar metabolism, pyruvate metabolism and the TCA cycle and with amino acid metabolism pathways including arginine and proline metabolism, alanine, aspartate and glutamate metabolism, valine, leucine and isoleucine degradation, histidine metabolism, glutathione metabolism and tryptophan metabolism. A total of 0.84% genes were involved in cell motility including chemotaxis and flagellar assembly.

Seven pathways involved with carbohydrate metabolism were identified among the significant pathways when the comparative study CP1 vs P. putida was carried out. Butanoate metabolism and propanoate metabolism had the highest number of genes associated with the pathway. The pathway involved in fructose and mannose metabolism had the lowest number of associated genes (6) and only one of these genes was up-regulated.

Transcriptomic analysis showed tpiA encoding triosephosphate isomerase was detected to be up-regulated in fructose and mannose metabolism. TpiA played a role in the conversion of glyceroldehyde-3-phosphate to dihydroxyacetone. Unexpectedly, three genes encoding for phosphotransferase cluster, fruA, fruB and fruK were down-regulated. The genome of P. putida KT2440 encodes only five recognisable proteins belong to the phosphoenolpyruvate (PEP)-carbohydrate phosphotransferase system (PTS) including the FruA and FruB to form a complete system for fructose intake (Velazquez et al., 2007; Pfluger and De Lorenzo, 2008). The fruA- and fruB-encoded proteins appeared to form the only system in P. putida for the intake of sugars (fructose) through the phosphorylation-linked transport. A study showed the P. putida KT2440 with fruB mutant was unable to metabolise fructose. This proved that mechanisms of entry of fructose in P. putida can only be mediated by the multiphosphoryl transfer protein FruB along with FruA (Velazquez et al., 2007). This suggests that P. putida CP1 uses a different transport system for fructose which need to be investigated in the future. Modifications in the electron transport chain were also observed during growth on fructose. The expression of the nuaA genes, which encode the NADH dehydrogenase, increased. This enzyme has a key role in feeding electrons into the electron transport chain (Belkys et al., 2017). In addition, the expression of succinate dehydrogenase (sdhD), which besides its role in the TCA cycle also feeds electrons into the electron transport chain, also increased.

Genes involved in translation were identified at a high level in P. putida CP1 grown on fructose in contrast to other functions. This was a similar observation to that found in a study conducted by Ballerstedt et al. (2007) where the number of transcripts of different Pseudomonads were identified at a high frequency (>80%) related to the translation. The induction of these genes presumably to trigger the growth in the active system. The induction of genes RPL1, RPL25 and RPS33 related to ribosomal proteins was observed in the yeast Saccharomyces cerevisiae during growth on a nonfermentable carbon source with the addition of carbon sources glucose and fructose. Expression of all ribosomal protein genes occurs upon addition of a rapidly fermentable sugar to cells growing on a nonfermentable carbon source or upon addition of a nitrogen source to nitrogen-starved cells related to the stimulation of growth under these conditions (Pernambuco et al., 1996).

Pathway related to autoaggregation

Four pathways associated with autoaggregation according to Sauer et al. (2001) were also identified with this study. The genes involved in these 4 pathways are listed in Table 3. Most of the genes were linked with ABC transporters, 17 genes were involved in two-component systems, 9 genes were involved in LPS biosynthesis and 7 genes regulated in flagellar assembly.

Fructose-induced aggregation was associated with increased expression of genes involved in ABC transporters, two-component system, fatty acid biosynthesis, lipopolysaccharide biosynthesis and flagellar assembly pathway. The localisation of proteins encoded by the differentially regulated genes was mainly located in the cytoplasm and the cytoplasmic membrane.
OpR is encoding the outer membrane protein H1 appeared to be highly induced during aggregation of P. putida CP1 on 0.5% (w/v) fructose after 24 h. Edington et al. (2011) has suggested that the function of OpR is to provide increased stability to the outer membranes by interacting with lipopolysaccharide molecules. OpR is genetically linked to the PhoP-PhoQ two-component regulatory system that is up-regulated and over expressed in response to Mg²⁺-limited growth conditions. It is a major component of the P. aeruginosa PA01 and P. fluorescens 2P24 outer membrane (Yan et al., 2009). To support this argument, it was found among up-regulated genes involved in the two-component system was phoQ (integral membrane sensor signal transduction histidine kinase) together with cheY (response regulator receiver protein) and PP2100 (two component sensor protein). CheY is responsible for the transduction of the chemical signal to the flagellar switch. The influence of outer membrane proteins has been identified as adhesins for bacteria cells which can be utilised in a variety of environments (Hinsa et al., 2003).

ABC transport system consisted of lapG encoded for large adhesion protein. Gjermansen et al. (2005) found lapG and PP0165, encoding a putative periplasmic protein and a putative transmembrane protein containing GGDEF and EAL domains in P. putida. They identified the importance of these genes for formation and starvation-induced dispersion of P. putida biofilms. These two proteins were also found to regulate adhesion, probably via the adhesiveness of bacterial cells through c-di-GMP signaling in a phosphorelay-mediated signaling event.

All genes involved in flagellar assembly were up-regulated, suggesting the role of flagella to induce the production of aggregates during growth on fructose. FliE

Table 3: Details of genes involved in pathways related to autoaggregation when P. putida CP1 was grown on 0.5% (w/v) fructose for 24 h.

| Pathway                   | Locus ID | Gene name | Annotation                                               | Fold-change |
|---------------------------|----------|-----------|----------------------------------------------------------|-------------|
| ABC transporters          | PP237    | ssuA      | aliphatic sultonate ABC transporter periplasmic ligand-binding protein | 2.814       |
|                           | PP240    | ssuB      | aliphatic sultonates transport ATP-binding subunit        | 2.032       |
|                           | PP164    | lapG      | Large adhesion protein                                    | 1.928       |
|                           | PP3077   |          | binding-protein-dependent transport systems inner membrane component | 1.642       |
|                           | PP983    |          | permease YigP/YigQ family protein                         | 1.522       |
|                           | PP2767   |          | branched-chain amino acid ABC transporter, ATP-binding protein, putative | 1.444       |
|                           | PP2155   | loID      | lipoprotein releasing system, ATP-binding protein         | 1.372       |
|                           | PP2240   |          | ABC transporter                                          | 1.31        |
| Two-component system      | PP1187   | phoQ      | integral membrane sensor signal transduction histidine kinase | 5.04        |
|                           | PP5046   | glnA      | glutamine synthetase, type I                             | 2.678       |
|                           | PP4340   | cheY      | response regulator receiver protein                       | 1.802       |
|                           | PP109    |          | cytochrome oxidase assembly                               | 1.618       |
|                           | PP5183   |          | glutamine synthetase, putative                            | 1.534       |
|                           | PP22100  |          | two-component sensor protein                              | 1.35        |
| Flagellar assembly        | PP4370   | fliE      | flagellar hook basal body protein FliE                    | 3.028       |
|                           | PP4385   | fliG      | flagellar basal body rod protein FliG                     | 2.514       |
|                           | PP4344   | fliA      | flagellar biosynthesis protein FliA                       | 2.03        |
|                           | PP4384   | fliH      | flagellar basal body L-ring protein                       | 1.962       |
|                           | PP4383   | fliI      | flagellar basal body P-ring protein                       | 1.646       |
|                           | PP4357   | fliN      | flagellar motor switch protein                            | 1.558       |
|                           | PP4369   | fliF      | flagellar MS-ring protein                                 | 1.272       |
| Lipopolysaccharide        | PP59     |          | D,D-heptose 1,7-bisphosphate phosphatase                   | 2.628       |
| biosynthesis              | PP63     |          | lipid A biosynthesis lauroyl acyltransferase              | 2.348       |
|                           | PP1604   | lpxB      | lipid A-disaccharide synthase                             | 1.674       |
|                           | PP1323   | gmhA      | phosphoheptose isomerase                                  | 1.566       |
|                           | PP4936   |          | O-antigen polymerase                                      | 1.516       |
|                           | PP1611   | kdsA-1    | 2-dehydro-3-deoxyphosphocononate aldolase                 | 1.274       |
encoding for flagellar hook-basal body protein was among the top up-regulated genes in the study. Seven genes involved in flagellar assembly were found to be up-regulated in the microarray analysis of *P. putida* CP1 vs fructose including the flagellar basal body protein (FlIE, flgG, flgH, flgI), flagellar motor switch protein flIM, flagellar MS-ring protein flIF and flagellar biosynthesis protein flhA (Figure 3).

The microarray results showed that gene expression in LPS biosynthesis including gmhA, gmbB, lpxB, PP4936 and kdsA was altered when *P. putida* CP1 was grown on fructose. The relation between bacterial stress, LPS production and aggregate formation has been poorly explored. However, several reports have identified that LPS of *E. coli* K12, *Salmonella enterica*, *Vibrio cholera* and *Pseudomonas aeruginosa* are known to play an important role in biofilm formation (Yeom et al., 2012). Previously, transcriptome analysis of LPS deletion mutants revealed a significant degree of reduction in biofilm formation of *E. coli* K12 (Niba et al., 2007). The production of LPS in cell aggregates appeared to be a protection mechanism for *P. putida* CP1. LPS is a major component of the Gram-negative bacterial outer membrane and is important as a permeability barrier and for the resistance against complement-mediated cell lysis (Sperandeo et al., 2009). They also form a protective extracellular barrier against the penetration of potentially noxious molecules by divalent cation-mediated LPS-LPS interactions (Edrington et al., 2011). LPS layer of the outer membrane affects surface properties such as charge and hydrophobicity (Neumann et al., 2006).

**CONCLUSION**

The results showed one of the major contributions of the identification of new genes involved in carbohydrate metabolism and autoaggregation formation in *Pseudomonas putida* CP1 to understand the molecular basis of strain variation and the mechanisms implicated in cell-cell communication. The genes identified here provide insight into autoaggregation of environmental isolates and may function as biomarkers for, or functional targets in the maintenance of this phenotype in *P. putida* CP1 culture.

**ACKNOWLEDGEMENTS**

This research was supported by funding from Universiti Kebangsaan Malaysia (UKM), Ministry of Higher Education of Malaysia (MoHE) and Dublin City University, Ireland.

**REFERENCES**

Aqma, W. S. and Quilty, B. (2015). Influences of extracellular polymeric substances (EPS) for autoaggregation of *Pseudomonas putida* CP1 during growth on monochlorophenol. *Malaysian Journal of Microbiology* 11(3), 246-253.

Ballerstedt, H., Volkers, R. J. M., Mars, A. E., Hallsworth, J. E., Santos, V. A. M., Puchalka, J., van Duuren, J., Eggink, G., Timmis, K. N. and deBont, J. A. M. (2007). Genotypotyping of *Pseudomonas putida* strains using *P. putida* KT2440-based high-density DNA microarrays: Implications for transcriptomics studies. *Applied Microbiology and Biotechnology* 75(5), 1133-1142.

---

**Figure 3:** KEGG pathway map analyses of differentially expressed genes (> 1.2-fold) in flagella assembly.

All of the flagellar genes that we detected in both comparative studies as differentially expressed in aggregated cells belonged to genes in the 6 of the 17 flagellar operons known in *P. aeruginosa*: flgA (class-2), flIEFGHJ (class-2), flIIIMNOPQRTflhB (class-2), flgBCDE (class-3), flgFGHIJKL (class-3) and flgMN (class-2 & 4) as described by (Dasgupta et al., 2003). Many expressed genes encode proteins of the basal body: FlIE, FlgB and FlgC form the proximal rod, FlgF and FlgG form the distal rod and FlIM is a motor-switch component. flIM, on the other hand, is the negative regulator of flagellin synthesis (flIC). flgM is expressed as a class-2 gene and as an anti-sigma factor, prevents expression of the FlIA sigma factor-dependent class-4 genes such as flIC. No class-4 gene, other than flgM, which is considered both a class-2 and class-4 gene, was differentially expressed at any of the sampling times. With observation of only class 2- and class-3 genes but not class-4 strongly suggests that neither flagellation nor hyperflagellation of cells took place during the stress, indicating a different role for the expression of some flagella synthesis operons. The investigation of flagellae regulation in formation aggregates was not yet been highlighted but the primary function of flagellae in biofilm formation is assumed to be in transport and in initial cell-to surface interactions. The absence of flagellae impaired *P. fluorescens* and *P. putida* in colonisation of potato and wheat roots and reduced cellular adhesion of *P. aeruginosa* to a polystyrene surface (Sauer et al., 2001).
Belkys, C. S., Chungyu, C., Chenggang, W., Bryan, T. and Hung T. T. (2017). Electron transport chain is biochemically linked to plius assembly required for polymicrobial interactions and biofilm formation in the Gram positive Actinobacterium Actinomycesoris. mBio 8, 3e00399-17.

Benjamini, Y. and Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. Journal of the Royal Statistical Society: Series B (Statistical Methodology) 57(1), 289-300.

Borrego, S., Niubò, E., Ancheta, O. and Espinosa, M. E. (2000). Study of the microbial aggregation in Mycobacterium using image analysis and electron microscopy. Tissue and Cell 32(6), 494-500.

Burdman, S., Okon, Y. and Jurkevitch, E. (2000). Surface characteristics of Azospirillum brasilense in relation to cell aggregation and attachment to plant roots. Critical Reviews in Microbiology 26(2), 91-110.

Cray, J. A., Russell, J. T., Timson, D. J., Singhal, R. S. and Hallsworth, J. E. (2013). A universal measure of chaotropy and kosmotropy. Environmental Microbiology 15(1), 287-296.

Dasgupta, N., Wolfgang, M. C., Goodman, A. L., Arora, S. K., Jyot, L., Lory, S. and Ramphal, R. (2003). A four-tiered transcriptional regulatory circuit controls flagellar biogenesis in Pseudomonas aeruginosa. Molecular Microbiology 50(3), 809-824.

Eboigbodin, K. E., Ojeda, J. and Biggs, C. A. (2007). Investigating the surface properties of Escherichia coli under glucose controlled conditions and its effect on aggregation. Langmuir 23, 6691-6697.

Edrington, T. C., Kintz, E., Goldberg, J. B. and Tamm, L. K. (2011). Structural basis for the interaction of lipopolysaccharide with outer membrane protein H (OprH) from Pseudomonas aeruginosa. Journal of Biological Chemistry 286(45), 39211-39223.

Fakruddin, A. N. M. and Quilty, B. (2005). The influence of glucose and fructose on the degradation of 2-chlorophenol by Pseudomonas putida CP1. World Journal of Microbiology and Biotechnology 21, 1541-1548.

Gjermansen, M., Ragas, P., Berns, C., Molin, S. and Tolker-Nielsen, T. (2003). Characterization of starvation-induced dispersion in Pseudomonas putida biofilms. Environmental Microbiology 7(6), 894-904.

Hinsa, S. M., Espinosa-Urgel, M., Ramos, J. L. and O'Toole, G. A. (2003). Transition from reversible to irreversible attachment during biofilm formation by Pseudomonas fluorescens WCS365 requires an ABC transporter and a large secreted protein. Molecular Microbiology 49(4), 905-918.

Marles-Wright, J. and Lewis, R. J. (2007). Stress responses of bacteria. Current Opinion in Structural Biology 17, 755-60.

Mattos-Granera, R. O. and Duncan, M. J. (2017). Two-component signal transduction systems in oral bacteria. Journal of Oral Microbiology 9(1), 1400858.

Neumann, G., Cornelissen, S., van Breukelen, F., Hunger, S., Lippold, H., Loffhagen, N., Wick, L. Y. and Heipieper, H. J. (2006). Energetics and surface properties of Pseudomonas putida DOT-T1E in a two-phase fermentation system with 1-decanol as second phase. Applied and Environmental Microbiology 72(6), 4322-4328.

Niba, E. T. E., Naka, Y., Nagase, M., Mori, H. and Kitakawa, M. (2007). A genome-wide approach to identify the genes involved in biofilm formation in E. coli. DNA Research 14(6), 237-246.

Nielsen, L., Li, X. and Halverson, L. J. (2011). Cell-cell and cell-surface interactions mediated by cellulose and a novel exopolysaccharide contribute to Pseudomonas putida biofilm formation and fitness under water-limiting conditions. Environmental Microbiology 13(5), 1342-1356.

Pernambuco, M. B., Winderickx, J., Crouwe, M., Griffioen, G., Mager, W. H. and Thevelein, J. M. (1996). Glucose-triggered signalling in Saccharomyces cerevisiae: Different requirements for sugar phosphorylation between cells grown on glucose and those grown on non-fermentable carbon sources. Microbiology 142(7), 1775-1782.

Pfluger, K. and De Lorenzo, V. (2008). Evidence of in vivo cross talk between the nitrogen-related and fructose-related branches of the carbohydrate phosphotransferase system of Pseudomonas putida. Journal of Bacteriology 190(9), 3374-3380.

Sauer, K. and Camper, A. K. (2001). Characterization of phenotypic changes in Pseudomonas putida in response to surface-associated growth. Journal of Bacteriology 183(22), 6579-6589.

Schleheck, D., Barraud, N., Klebsensberger, J., Webb, J. S., McDougal, D., Rice, S. A. and Kjelleberg, S. (2009). Pseudomonas aeruginosa PA01 preferentially grows as aggregates in liquid batch cultures and disperses upon starvation. PLoS ONE 4(5), 5513.

Smyth, G. K. (2004). Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Statistical Applications in Genetic and Molecular Biology 3(1), 3.

Smyth, G. K. and Speed, T. (2003). Normalization of cDNA microarray data. Methods 31(4), 265-273.

Sperandeo, P., Dehò, G. and Poliassi, A. (2009). The lipopolysaccharide transport system of Gram-negative bacteria. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 1791(7), 594-602.

Temple, L. M., Sage, A. E., Schweizer, H. P. and Phibbs, J. P. V. (1998). Carbohydrate catabolism in Pseudomonas aeruginosa. Pseudomonas 10, 35.

Valentini, M. and Filloux, X. A. (2016). Biofilms and cyclic di-GMP (c-di-GMP) signaling: Lessons from Pseudomonas aeruginosa and other bacteria. The Journal of Biological Chemistry 291(24), 12547-12555.

Veiañquez, F., di Bartolo, I. and de Lorenzo, V. (2004). Genetic evidence that catabolite of the Entner-Doudoroff pathway signal C source repression of the γ54 Pu promoter of Pseudomonas putida. Journal of Bacteriology 186(24), 8267-8275.
Velázquez, F., Pfluger, K., Cases, I., De Eugenio, L. I. and De Lorenzo, V. (2007). The phosphotransferase system (PTS) formed by PtsP, PtsO, and PtsN proteins controls production of polyhydroxyalkanoates in Pseudomonas putida. Journal of Bacteriology 189(12), 4529-4533.

Weijun L., Pant, G., Bhavnasi, Y. K., Blanchard, S. G. and Brouwer, C. (2017). Pathview Web: User friendly pathway visualization and data integration. Nucleic Acids Research 45, 501–508.

Windt, W. D., Gao, H., Kromer, W., Damme, P. V., Dick, J., Mast, J., Boon, N., Zhou, J. and Verstraete, W. (2006). AggA is required for aggregation and increased biofilm formation of a hyper-aggregating mutant of Shewanella oneidensis MR-1. Microbiology 152, 721-729.

Wu, X., Monchy, S., Taghavi, S., Zhu, W., Ramos, J. and Van Der Lelie, D. (2011). Comparative genomics and functional analysis of niche-specific adaptation in Pseudomonas putida. Federation of European Microbiological Societies Microbiology Reviews 35(2), 299-323.

Yan, Q., Gao, W., Wu, X. G. and Zhang, L. Q. (2009). Regulation of the PcoI/PcoR quorum-sensing system in Pseudomonas fluorescens 2P24 by the PhoP/PhoQ two-component system. Microbiology 155(1), 124-133.

Yeom, J., Lee, Y. and Park, W. (2012). Effects of non-ionic solute stresses on biofilm formation and lipopolysaccharide production in Escherichia coli O157: H7. Research in Microbiology 163(4), 258-267.