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

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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

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(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 trasduction 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).

Nutrient 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 and 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) K_2HPO_4, 4.36; NaH_2PO_4, 3.45; $(NH_4)_2SO_4$, 1.26; $MgSO_4 \cdot 6H_2O$, 0.912; and 1 mL/L trace salts solution. The trace salts solution contained (g/100 mL): $CaCl_2 \cdot 2H_2O$, 4.77; $FeSO_4 \cdot 7H_2O$, 0.37; $CoCl_2 \cdot 6H_2O$, 0.37; $MnCl_2$, 0.10; and $Na_2MoO_4 \cdot 2H_2O$, 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 4×10^8 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 OD_{660} 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 by 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 0.1 × 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-

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Aldrich; P7949). Then the array was dried by centrifugation at 1000 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 bitt 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 (7145)9) 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 expression was based on 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 cluster 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/1iqtjknco4Ic1b1P93z5d4ebbwztNbFtO/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.

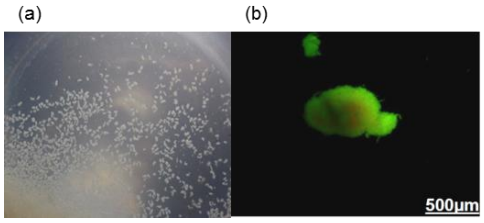

Figure 1: Appearance of aggregates when *P. putida* CP1 was grown on 0.5% (w/v) fructose after 24 h. a) aggregates in medium and b) microscopic observation at 600× magnification.

Up-regulated gene transcripts

A total of 456 genes were identified as significantly up-regulated ($p \leq 0.05$, 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, rplI, 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.

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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 HslV		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		NifR3/Smm1 family protein		4.906
PP5001		ATP-dependent protease ATP-binding subunit HslU		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	fliE	flagellar hook-basal body protein	Flagellar assembly	3.028

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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		glyceraldehyde-3-phosphate dehydrogenase, putative	Glycolysis / Gluconeogenesis	-5.69
PP1012	gltR-2	winged helix family two component transcriptional regulator		-5.64
PP4922	thiC	thiamine biosynthesis protein ThiC	Thiamine metabolism	-5.23
PP3745	glcD	glycolate oxidase subunit GlcD	Glyoxylate and dicarboxylate metabolism	-5.17
PP1071		amino acid ABC transporter, periplasmic amino acid-binding protein	ABC transporters	-5.14
PP4659	ggt-2	gamma-glutamyltransferase	Taurine and hypotaurine metabolism	-4.72
PP2528	metY	O-acetylhomoserine aminocarboxypropyltransferase	Cysteine and methionine metabolism	-4.53
PP794	fruK	1-phosphofructokinase	Fructose and mannose metabolism	-4.48
PP3189	codA	N-isopropylammelide isopropylaminohydrolase	Pyrimidine metabolism	-4.40
PP1024	eda	keto-hydroxyglutarate-aldolase/keto-deoxy- phosphogluconate aldolase	Pentose phosphate pathway	-4.32
PP545		aldehyde dehydrogenase family protein	Glycolysis / Gluconeogenesis	-4.17
PP3514	hyuB	hydantoinase B/oxoprolinase	Arginine and proline metabolism	-4.12
PP1009	gap-1	glyceraldehyde-3-phosphate dehydrogenase, type I	Glycolysis / Gluconeogenesis	-4.09
PP5003	phaA	poly(3-hydroxyalkanoate) polymerase 1	Butanoate metabolism	-4.03
PP1986	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	ABC transporters	-3.46
PP5007	phaF	poly(hydroxyalkanoate) granule-associated protein		-3.39
PP1019	oprB-1	porin B		-3.12
PP4256	ccoO-2	cbb3-type cytochrome c oxidase subunit II	Oxidative phosphorylation	-3.08

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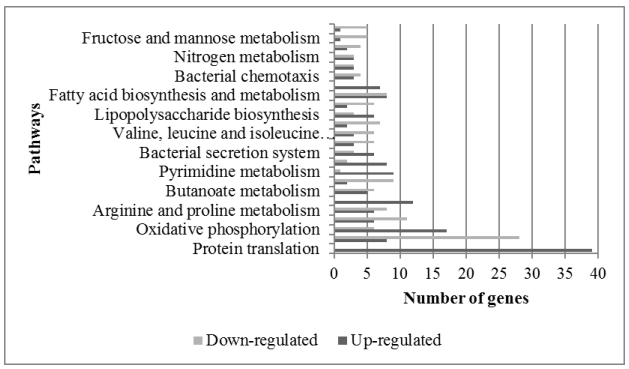

Figure 2: Breakdown of responses of *P. putida* CP1 grown on 0.5% (w/v) fructose for 24 h according to functional categories.

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 CP1vs Fructose 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 isomerise was detected to be up-regulated in fructose and mannose metabolism. TpiA played a role in the conversion glyceraldehyde-3-phosphate and 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 *nuoA* 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 transripts 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.

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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 sulfonate ABC transporter periplasmic ligand-binding protein	2.814
	PP240	ssuB	aliphatic sulfonates 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 YjgP/YjgQ family protein	1.522
	PP2767		branched-chain amino acid ABC transporter, ATP-binding protein, putative	1.444
	PP2155	lolD	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
	PP2100		two-component sensor protein	1.35
Flagellar assembly	PP4370	fliE	flagellar hook-basal body protein FliE	3.028
	PP4385	flgG	flagellar basal body rod protein FlgG	2.514
	PP4344	flhA	flagellar biosynthesis protein FlhA	2.03
	PP4384	flgH	flagellar basal body L-ring protein	1.962
	PP4383	flgI	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 biosynthesis	PP59		D,D-heptose 1,7-bisphosphate phosphatase	2.628
	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-deoxyphosphooctonate aldolase	1.274

OprH 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. Edrington *et al.* (2011) has suggested that the function of OprH is to provide increased stability to the outer membranes by interacting with lipopolysaccharide molecules. OprH is genetically linked to the PhoP-PhoQ two-component regulatory system that is up-regulated and over expressed in response to Mg^{2+}-limited growth conditions. It is a major component of the *P. aeruginosa* PAO1 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 flagellae to induce the production of aggregates during growth on fructose. FliE

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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 fliN, flagellar MS-ring protein fliF and flagellar biosynthesis protein flhA (Figure 3).

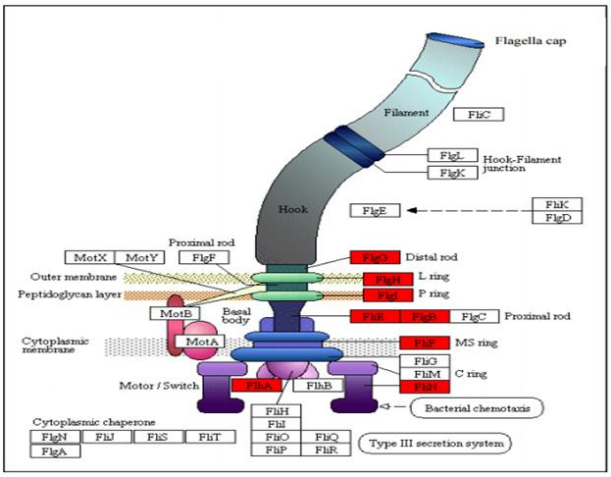

Figure 3: KEGG pathway map analyses of differentially expressed genes (> 1.2-fold) in flagella assembly.

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), *fliEFGHIJ* (class-2), *fliLMNOPQRflhB* (class-2), *flgBCDE* (class-3), *flgFGHIJKL* (class-3) and *flgMN* (class-2 & 4) as decribed 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. *flgM*, 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).

The microarray results showed that gene expression in LPS biosynthesis including gmhA, gmhB, 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.

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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). Genomotyping 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.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Belkys, C. S., Chungyu, C., Chenggang, W., Bryan, T. and Hung T. T. (2017). Electron transport chain is biochemically linked to pilus 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., Niubó, 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 chaotropicity and kosmotropicity. *Environmental Microbiology* **15(1), 287-296.**

Dasgupta, N., Wolfgang, M. C., Goodman, A. L., Arora, S. K., Jyot, J., 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.**

Fakhruddin, 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., Sternberg, C., Molin, S. and Tolker-Nielsen, T. (2005). 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), 4232-4238.**

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., Crauwels, 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., Klebensberger, J., Webb, J. S., McDougald, D., Rice, S. A. and Kjelleberg, S. (2009). *Pseudomonas aeruginosa* PAO1 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 Polissi, 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.**

Velázquez, F., di Bartolo, I. and de Lorenzo, V. (2004). Genetic evidence that catabolites of the Entner-Doudoroff pathway signal C source repression of the σ54 Pu promoter of *Pseudomonas putida*. *Journal of Bacteriology* **186(24), 8267-8275.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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.**

 ISSN (print): 1823-8262, ISSN (online): 2231-7538