An investigation of the phenotypic and genotypic changes in *Pseudomonas putida* CP1 following substrate-dependent autoaggregation

A thesis submitted to Dublin City University in fulfilment of the requirements for the award of the degree of Doctor of Philosophy

by

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I bow my head with great reverence to Him for giving me strength, inspiration and the cause behind every effort.

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Abstract

An investigation of the phenotypic and genotypic changes in *Pseudomonas putida* CP1 following substrate-dependent autoaggregation

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Pseudomonas putida CP1 was isolated from soil. The organism is capable of the degradation of a wide range of substrates and can grow on all three mono-chlorophenols. Substrate induced autoaggregation was observed when the bacterium was grown on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol and 200 ppm 4-chlorophenol and also when the organism was grown on 0.5% (w/v) fructose. Autoaggregation which is the aggregation of a single bacterial species to form clumps or clusters of cells, was attributed to substrate stress. Phenotyic and genotypic changes in the organism accompanying autoaggregation had not previously been studied. Aggregation was accompanied by the production of extracellular polymeric substances (EPS). A biochemical analysis of the EPS showed that the main constituents were carbohydrate (40% w/v) and protein (50% w/v) together with lower levels of DNA (<10% w/v). The extent of EPS production and the size of the aggregates increased with increasing stress as did the aggregation index and the hydrophobicity of the cells. A comparative study of *P. putida* CP1 and *P. putida* KT2440 using the API20NE system showed a close similarity between the two organisms. However, microarray gene expression profiling using a *P. putida* KT2440 Genome Oligonucleotide Array (Progenika, Spain) showed that 316 genes involved in metabolism and adaptation were differentially expressed in *P. putida* CP1. Global transcriptomic profiling studies showed that *P. putida* CP1 growing on mono-chlorophenols resulted in the induction of genes encoding for proteins involved in the outer membrane, the cytoplasmic membrane, efflux systems and general stress responses. Growth of the bacterium on fructose (0.5%, w/v) was 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. Two novel genes, associated with autoaggregation, were identified using transcriptomic analysis. They were PP1961 which encodes for TetR, a transcriptional regulator and PP4936 which encodes for an O-antigen polymerase.

List of Abbreviations

ABC	ATP binding cassette
AI	Aggregation index
BATH	Bacterial adherence to hydrocarbon
c-di-GMP	cyclic-di-guanosine monophosphate
cDNA	Complementary DNA
cfu	colony forming unit
DAPI	4', 6'-diamidino-2-phenylindole
DAVID	Database for Annotation, Visualization and Integrated Discovery
DNS	3, 5-dinitrosalicylic acid
EDP	Entner-Doudoroff pathway
EDTA	Ethylenediaminetetraacetic acid
EMP	Embden-Meyerhoff-Parnas pathway
EPA	Environmental protection agency
EPS	Extracellular Polymeric Substances
ETBR	Ethidium bromide
FDR	False discovery rate
FITC	Fluorescein isothiocyanate
KEGG	Kyoto Encyclopedia of Genes and Genomes
LPS	Lipopolysaccharide
MOPS	3-(N-morpholine)-propanesulfonic acid
OD	Optical desity
ppm	part per million
RNase	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate

TCS	Two-component system
TEMED	N, N, N, N-Tetramethyl-ethylenediamine
Tris	Tris-(hydroxyl-methyl)-methylamine (2-amino hydroxymethyl) propane-1, 3-diol
TRITC	Tetramethyl Rhodamine Isothiocyanate
x g	Times gravity

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1.0 INTRODUCTION

1 Introduction

1.1 Pseudomonads

Metabolically versatile bacteria such as members of the genus *Pseudomonas* play a key role in eliminating pollutants from the environment. They are therefore exploited in biotechnological processes to clean up contaminated soils and wastewaters (Hallsworth *et al.,* 2003). The genus *Pseudomonas* belongs to the bacterial family *Pseudomonadaceae* which also includes the genera *Azomonas, Azotobacter, Cellvibrio, Mesophilobacter, Rhizobacter* and *Rugamonas*. These bacteria are widespread in soil and water. They are strictly aerobic Gram-negative and non-sporulating bacteria. Based on biochemical characteristics, they are oxidase and catalase positive, non-acid fast rods, which are generally straight, but maybe slightly curved, 0.5 – 1.0 μm in diameter and 1.5 – 5 μm in length. They are motile by one or several polar flagellae and rarely nonmotile. In some species lateral flagellae of short wavelength may also be formed. They do not ferment carbohydrates, do not fix nitrogen and are not photosynthetic. Most species are inhibited when grown in acidic conditions (pH 4.5 or lower) and do not require organic growth factors. The optimum growth temperature for most strains is 28^{o}C, but many are capable of growth in between 4-45°C. Most of the species do not accumulate granules of poly-β-hydroxybutyrate, but accululation of poly-hydroxyalkanoates of monomer length higher than C_4 may occur when growing on alkanes or gluconate (Bergey's Manual of Systematic Bacteriology, 2005).

Pseudomonas species are subdivided on the basis of rRNA homology into five similarity groups. There are about forty species. Group I is the largest group and includes fluorescent strains such as *Pseudomonas aeruginosa, Pseudomonas fluorescens* and *Pseudomonas putida* and the plant pathogens *Pseudomonas syringae* and *Pseudomonas cichorii*. It also includes many important non-fluorescent species such as *P. stutzeri* and *P. mendocina.* The members of groups II, III, IV and V were moved into new or previously existing genera such as *Burkholderia*, *Xanthomonas* and *Comamonas* based on 16S rRNA gene analysis. The closely related bacteria to the genus *Pseudomonas* include species of the

aerobic, free-living, nitrogen fixing *Azotobacter-Azomonas* complex and cellulolytic species of the genus *Cellvibrio* (Moore *et al.,* 2006).

Pseudomonads cover the most diverse and ecologically significant groups of bacteria in a wide range of environmental niches including those connected with plants, animals and human pathogens. The ability to metabolize a variety of diverse nutrients and to form biofilms enables these bacteria to survive in a variety of habitats (Moore *et al.,* 2006). The genus *Pseudomonas* represents a diverse group of medically, environmentally and biotecnologically important bacteria. *Pseudomonas aeruginosa* is an opportunistic pathogen for humans and other mammals and is associated with infections in immunocompromised patients. The survival success of this species is due to its tolerance to many antimicrobial drugs (Oberhardt *et al.,* 2008). Some *Pseudomonas* species isolated from the environment affect plant growth through their inhibition of fungal plant pathogens or by their effects on the roots of plants. They are also found to be involved in food industry related to the spoilage of meats, poultry and fish and can be present in tap water and in hospital saline solutions. The ability of *Pseudomonas* to develop biofilms in certain conditions has led to a major problem in particular pipeline systems (Moore *et al.,* 2006).

Pseudomonads have been used in bioremediation and biotransformation, as biocontrol agents for plant growth promotion and in the production of low-molecular weight compounds, polymers and recombinant proteins (Rehm 2008). In the genus *Pseudomonas*, the following species have demonstrated ability to metabolise chaotropic environmental pollutants and have been used for their degradation;

- *P. alcaligenes* – polycyclic aromatic hydrocarbons (PAH) (O'Mahony *et al*., 2006)
- *P. mendocina* – toluene (Tao *et al.,* 2004)
- *P. resinovorons* – carbazole (Widada *et al.,* 2002)
- *P. putida* –organic solvents (Farrell and Quilty, 2002a)
- *P. stutzeri* – chlorinated hydrocarbons (Ryoo *et al.,* 2000)

Many enzymes isolated from Pseudomonads and whole *Pseudomonas* cells have been used as biocatalysts in industrial biotransformation. Enzymes isolated from *Pseudomonas* that has been applied in biotransformation reactions include aminopeptidase, benzaldehyde dehydrogenase, benzoate dioxygenase, dehalogenase, lipase and oxidase (Rehm, 2008). The use of whole cells in biotransformation has emerged as a key tool in the industrial synthesis of bulk chemicals, pharmaceuticals and agrochemical intermediates including the conversion of ferulic acid to vanillin (Walton and Narbad, 2000).

Pseudomonas species are among the most competent rhizosphere colonizers of soil (Moore *et al.,* 2006). The rhizosphere is defined as a zone around the plant root that contains high amounts of microbial activity due to the secretion of organic and amino acids by the plants. A few strains of *P. fluorescens* and *P. putida* have been described as biocontrol agents (Haas and Défago, 2005). *Pseudomonas* species can inhibit the colonization of plants by other microorganisms and are seen as important agents for biocontrol of plant diseases (Moore *et al.,* 2006). Pseudomonads are capable of synthesizing a variety of secondary metabolites and biopolymers that are related to biotechnology. Figure 1.1 illustrates the biosynthesis of various compounds by *Pseudomonas* species.

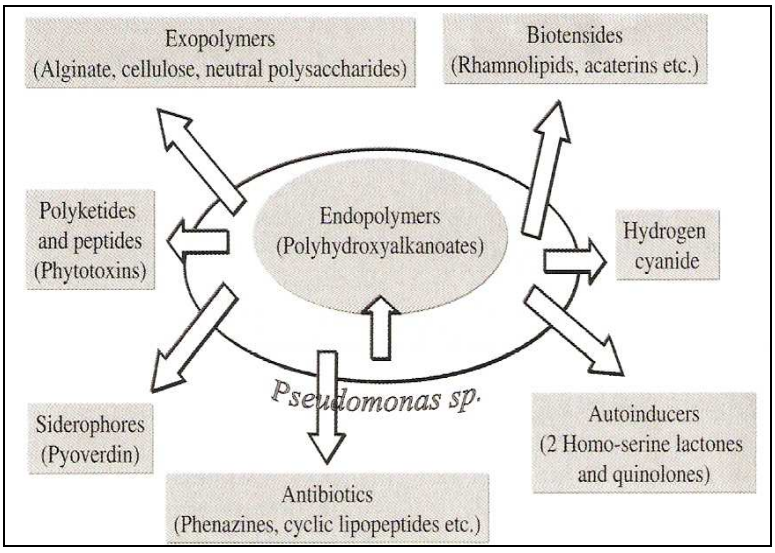


Figure 1.1 Biosynthesis capacity of Pseudomonads (Rehm, 2008).

1.1.1 *Pseudomonas putida*

Pseudomonas putida is a non-pathogen that belongs to the *Gammaproteobacteria* and is frequently isolated from polluted soil, the rhizosphere and water. It 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).

Pseudomonas putida CP1 is the subject of this study. The organism was isolated from soil and has been investigated in our laboratory because of its ability to degrade a wide range of pollutants including all three mono-chlorophenols. The use of chlorophenols in agriculture or industrial processes can lead to their accumulation in the environment. The complete degradation of all three mono-chlorophenol isomers by a single degradation system in bacteria is difficult and has rarely been reported. Farrell and Quilty (2002a) showed that *P. putida* CP1 was capable of the complete degradation of up to 200 ppm 2- and 3-chlorophenol and, 300 ppm 4-chlorophenol using a modified *ortho*-cleavage pathway.The bacterium is interesting not just because of its degradative capabilities, but also because it autoaggregates when grown on certain concentrations of substrates. Formation of aggregates was observed in the culture medium during growth of *P. putida* CP1 on higher concentrations of the mono-chlorophenols, ≥200ppm 4-chlorophenol and ≥100ppm 2- and 3-chlorophenol (Farrell and Quilty, 2002a). The ability to autoaggregate on mono-chlorophenol was observed to be in the order of 3CP> 2CP> 4CP which was inversely related to the degradative ability of the bacterium 4CP> 2CP> 3CP. Autoaggregation was described by the authors as a stress response to the toxicity of the substrate.

Formation of aggregates was also observed when *P. putida* CP1 was grown on fructose although not on glucose. This might be explained by the higher chaotropicity value for fructose compared to glucose (Cray *et al.,* 2013). The aggregative ability of the bacterium has been shown to enhance the use of this organism commercially and in particular its use in bioaugmentation. Augmentation of a commercial mixed culture with *P. putida* CP1 resulted in the complete degradation of 2-chlorophenol via an *ortho*-cleavage pathway (Farrell and Quilty, 2002b) and successful bioaugmentation of activated sludge was reported by McLaughlin *et al.,* (2006). Success of the bioaugmentation was attributed to the survival of the added organism by becoming integrated in the sludge floc.

P. putida KT2440 is the best characterized strain of the species and has become a model bacterium worldwide. This strain is a plasmid-free derivative of a toluene-degrading organism initially known as *P. arvilla* mt-2 and subsequently reclassified as *P. putida* mt-2 (Palleroni, 2010). It is the first Gram-negative soil bacterium to be certified as a biosafety host for the expression of foreign genes. The organism is widely used in both laboratory studies and the development of biotechnological applications including bioremediation of contaminated sites, biocatalysis for the production of useful chemicals and the production of plant growth promoters as a plant rhizosphere protective agent (Yuste *et al.,* 2005).

Pseudomonas putida KT2440 has a wide range of nutrient acquisition systems including mono- and di-oxygenases, oxidoreductases, ferredoxins and cytochromes, dehydrogenases, sulfur metabolism proteins, efflux pumps and glutathione-S transfereases. The organism also has an extensive array of extracytoplasmatic function sigma factors, regulators and stress response systems which constitute the genomic basis for its nutritional versatility and opportunism. The metabolic diversity of the organism was exploited for the experimental design of novel catabolic pathways as well as for its application in the production of high value compounds (dos Santos *et al.,* 2004). These include the hyperproduction of polymers such as polyhydroxyalkanoates, industrially relevant enzymes, epoxides, substituted catechols and heterocyclic compounds.

Pseudomonas putida KT2440 was the first *P. putida* sequenced. Genomic sequencing and the analysis of the complete genome sequence of *P. putida* KT2440 has been published in 2002 (Nelson *et al.*, 2002). The genome is a single circular chromosome of 6.18 Mb in length with an average G + C content of 61.6%. A total of 5420 open reading frames (ORFs) with an average length of 998 bp were identified. Putative role assignments were identified for 3571 ORFs, with another 600 (11.1%) being unique to *P. putida* and 1037 (19.1%) of ORFs encoding conserved hypothetical proteins. The putative genes include the LysR transcriptional regulator family with 110 members, the response regulator receiver domain with 94 members and the ABC transporter family with 93 members. 27 families represented by more than 20 members are related to proteins involved in transcription regulation (LysR, AraC, TetR, Sigma-54, etc.), transport (ABC, major facilitator family MFS, binding-protein-dependent transport system and porins), environmental signaling (response regulator receiver domains, histidine kinases and TonB receptors) and catabolism (dehydrogenases, hydrolases, transferases and oxygenases). Table 1.1 lists the identified coding sequences to The Institute for Genomic Research (TIGR) system of metabolic categories in *P. putida* KT2440. The majority of these categories include metabolic pathways associated with a stress response.

Table 1.1 Metabolic categories of annotated genes in *P. putida* KT2440 (Rehm, 2008).

Metabolic category	**Number of annotated genes**
*Amino acid biosynthesis	126
Biosynthesis of cofactors, prostheric groups and carriers	149
*Fatty acid and phospholipid metabolism	112
Central intermediary metabolism	79
*Energy metabolism	459
*Purines, pyrimidines, nucleosides and nucleotides	65
DNA metabolism	118
*Transcription	66
*Protein synthesis	132
Protein fate	180

*Cellular processes	361
*Regulatory functions	535
*Signal transduction	140
*Transport and binding proteins	656
*Cell envelope	327
Related to mobile elements	183
Unknown function	504
Conserved hypothetical proteins	1039
Hypothetical proteins	600

*Metabolic pathways associated with a stress response

Pseudomonas putida KT2440 shows a close similarity and relationship with the pathogenic species *P. aeruginosa*. Comparative genome analysis between both microbial genomes (http://www.tigr.org) revealed 85% of the *P. putida* KT2440 genome has homologues in the *P. aeruginosa* PAO1 genome. The genome of *P. putida* contains 1231 ORFs that are absent from that of *P. aeruginosa* including 226 conserved hypothetical proteins, 575 hypothetical proteins, a cellulose biosynthesis operon, a novel polysaccharide biosynthesis operon, 36 putative transposable elements, three phage, nine group II introns, two toluene resistance proteins, 75 proteins involved in energy metabolism and type IV pilus biosynthesis genes. The key virulence factors present in *P. aeruginosa* but absent in the *P. putida* KT2440 genome include genes for exotoxin A, elastase, exolipase, phospholipase C, alkaline protease, a type III secretion pathway, two N ramp manganese/ iron transporters and an operon for the synthesis of rhamnolipids (Nelson *et al.,* 2002). The genome of *P. putida* KT2440 contains 894 paralogous gene families which is considerably higher than that of the genome of *P. aeruginosa* strain PAO1, which has 809 putative paralogs. This large number of paralogous gene families showed the high degree of functional versatility of *P. putida*. It also contains 105 distinguishable genomic islands that provide increased metabolic proficiency as well as defense against several kinds of biotic and abiotic stresses (Yuste *et al.,* 2005).

The *Pseudomonas putida* KT2440 genome contains 36 Insertion sequence (*IS*) elements with the majority of the *IS* elements being present in multicopies. The high number of IS elements may be related to the versatile catabolic ability of *P. putida* KT2440 which also requires an increased level of genome plasticity for adaptation to various environments. Insertion sequence (*IS*) elements are associated with genes and operons encoding accessory functions (i.e., non-housekeeping), catabolic enzymes and pathways, pathogenicity factors and protective functions against noxious agents. Moreover, *IS* elements mediate gene rearrangements and facilitate the dissemination by horizontal transfer of gene clusters among bacterial populations (Weinel *et al.,* 2002).

Other atypical regions containing mobile elements and determinants of catabolic pathways have been identified in the *P. putida* KT2440 genome. Codon composition analysis revealed 39 regions of the genome greater than 10 kb in length that have atypical composition. The majority of them have a G + C content that is different from that of the rest of the genome (Nelson *et al.,* 2002). Many of these regions encode bacteriophage genes, transposable elements or determinants for proteins involved in the metabolism of aromatic compounds. In total, 90 transposon and 92 prophage determinants have been detected.

The *Pseudomonas putida* KT2440 genome reveals that a 35-bp sequence having the structure of an imperfect palindrome which is repeated more than 800 times throughout the genome. More than 80% of these sequences are extragenic and designated as Repetitive Extragenic Palindromic (REP) sequences. Of these, 82% are situated within 100 bp (and many within 30 bp) of the end of a neighboring gene. Given the positions and distribution of REP sequences in the *P. putida* genome, it was suggested that they may serve to allow DNA gyrase to bind and relax DNA when excessive supercoiling is generated, thereby playing an important role in the regulation of gene expression (Aranda-Olmedo *et al.,* 2002).

Similar to the *P. putida* F1, *P. putida* GB-1 and *P. putida* W619 genomes, *P. putida* KT2440 displayes a higher percentage of genes putatively coding for signal transduction mechanisms pointing to the presence of sophisticated regulatory networks to control gene expression in the function of a highly variable environment, inorganic ion transport and metabolism. However the *P. putida* genomes were less dense in genes putatively involved in carbohydrate transport and metabolism and replication, recombination and repair (Wu *et al.,* 2011).

Strains of *P. putida* are reported to metabolise a variety of substrates including toxic compounds like chemically stable aromatic xenobiotic compounds in the environment. Consistent with the extensive metabolic versatility for the degradation of aromatics, *P. putida* KT2440 encodes more putative transporters for aromatic substrates than any currently sequenced microbial genome including multiple homologues of the *Acinetobacter calcoaceticus* benzoate transporter BenK and of the *P. putida* 4-hydroxybenzoate transporter PcaK. In addition, KT2440 has 23 members of the BenF/PhaK/OprD family of porins that includes outer membrane channels implicated in the uptake of aromatic substrates (Nelson *et al.,* 2002). *P. putida* KT2440 lacks catabolic plasmids but is known to have a chromosomal pathway for the degradation of benzoate via catechol and 3-oxoadipate. The organism has revealed genetic determinants for putative enzymes able to transform a variety of other aromatic compounds including ferulate, coniferyl- and coumaryl alcohols, aldehydes and acids, vanillate, *p*-coumarate, *p*-hydroxybenzoate and protocatechuate, related to lignin, which is generated during decomposition of plant materials and abundant in the rhizosphere. These compounds constitute a carbon pool for rhizosphere associated microorganisms (Nelson *et al.,* 2002).

1.1.2 Gram-negative bacterial cell structure

The primary site of action for defence against environmental threats is the cell membrane. The cytoplasmic membrane of bacterial cells, a phospholipid bilayer, is a matrix in which various enzymes and transport proteins are embedded. It plays a vital role in solute transport, maintaining the energy status of the cell,

regulation of the intracellular environment, turgor pressure, signal transduction and energy transducing processes (Sardessai and Bhosle, 2002).

The envelope of Gram-negative bacteria (Figure 1.2) contains two distinct membranes, an inner and an outer membrane separated by the periplasmic space, a hydrophilic compartment that includes a layer of peptidoglycan (Ayala *et al.*, 2012).

- The inner membrane (also known as cytoplasmic or cell membrane) is a symmetric phospholipid bilayer in which proteins are embedded. The integral transmembrane proteins span the inner membrane with α-helical transmembrane domains, whereas lipoproteins are anchored to the membrane via an N-terminal lipid modification and face toward the periplasm. Peripheral inner membrane proteins are also associated at either cytoplasmic or periplasmic side of the inner membrane.
- The outer membrane is an asymmetric lipid bilayer with phospholipids forming the inner leaflet and lipopolysaccharides (LPS) forming the outer leaflet. Proteins and lipoproteins are also inserted in the outer membrane lipid bilayer. Typically, outer membrane proteins are transmembrane β-barrel structures and function as porins, channels, receptors and other transport machineries that mediate the communication with the extracellular environment.

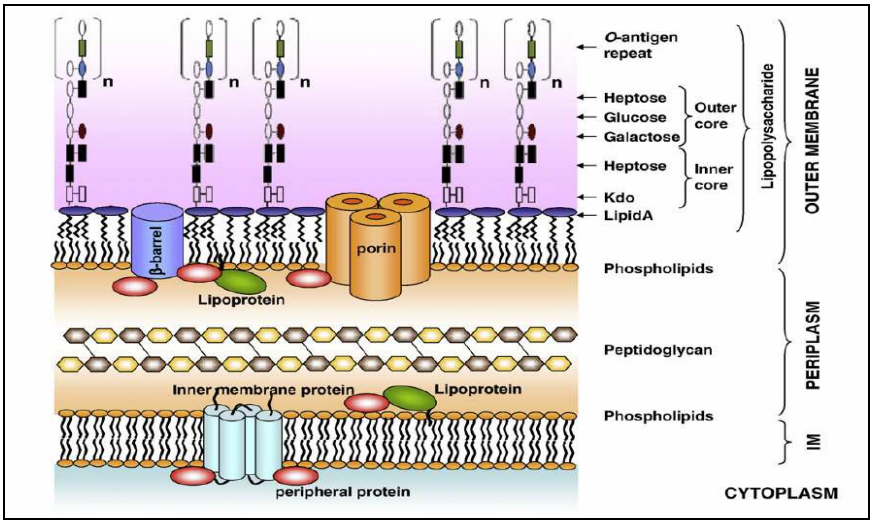


Figure 1.2 Structure of cell envelope in Gram-negative bacteria (Sperandeo *et al.*, 2009).

The bacterial cell wall differs from that of all other organisms by the presence of peptidoglycan. Peptidoglycan is responsible for the rigidity of the bacterial cell wall and for the determination of cell shape. Peptidoglycan is a polymer that consists of long glycan chains that are cross-linked via flexible peptide bridges to form a strong but elastic structure. In Gram-negative bacteria, the peptidoglycan is covalently attached to the outer membrane via lipoprotein. Gram-positive bacteria which lack an outer membrane, have a thick cell wall which contains covalently linked, charged polymers, such as teichoic acid and proteins that are anchored covalently or noncovalently to the cell wall (Scheffers and Pinho, 2005).

Lipopolysaccharides (LPS) are the major components of the outer surface of Gram-negative bacteria (Caroff and Karibian, 2003). LPS is essential for the viability of most Gram-negative bacteria and the Kdo2-lipid A moiety represents the minimal structure that is essential for growth (Sperandeo *et al.,* 2009). The hydrophobic lipid A component of LPS secures these molecules in the outer membrane and the core oligosaccharide links the lipid A region to the O-antigen or O-polysaccharide. The location of the LPS in the outer leaflet of the outer membrane allows interaction with the environment;

- Lipid A is composed of a phosphorylated diglucosamine moiety substituted with fatty acids and responsible for the biological activities of LPS.
- The core oligosaccharide region is attached to lipid A which can be divided into an inner and outer core. The inner core contains L-*glycero*-D-*manno*-heptose and 3-deoxy-D-*manno*-octulosonic acid (KDO) and the outer core are composed of hexose sugars such as D-glucose (D-Glc).
- O-antigen is known as A band (homopolymer) and B band (heteropolymer) (Sabra *et al.,* 2003). The A-band O-polysaccharide region is composed of D-rhamnose (D-Rha) residues arranged as trisaccharide repeating units. The A-band D-rhamnan polysaccharide is composed of approximately 70 D-Rha residues, which is equivalent to 23 repeating units. This is shorter than the B-band O antigen which is composed of more than 50 repeating units.

The terms smooth and rough are often used to describe the LPS phenotype. Attachment of the O-antigen to core-lipid A results in a smooth LPS phenotype, while core-lipid A lacking O-antigen is referred to as rough LPS (Rocchetta *et al.*, 1999). Some Gram negative bacteria express rough LPS (including *N. gonorrhoeae*) that do not express an O-antigen. They do however have a core region including KDO, also termed lipooligosaccaharide (LOS). Other Gram negative bacteria including *E. coli* and *Salmonella* express smooth LPS that normally have an O-antigen (repeating unit of sugars).

1.1.3 Metabolism in *Pseudomonas putida*

Pseudomonas putida KT2440 has an incomplete glycolytic pathway due to a lack of 6-phosphofructokinase like other Pseudomonads and rhizosymbionts. It uses a complete Entner-Doudoroff (ED) route for utilization of glucose and other hexoses. However it has genes coding for a fructose-1, 6-biphosphate and for glucose-6-phosphate isomerase which are involved in the early stages of gluconeogenesis. Similar to *P. aeruginosa* PAO1, *P. putida* KT2440 lacks the aldose-1-epimerase and glucose-1-phosphatase. All genes for the pentose phosphate pathway, the TCA cycle, the glyoxylate shunt, as well those for the oxidative and electron transport chain are present in the genomes of the *P. putida* KT2440 (Moore *et al.*, 2006). Three energy-dependent sugar- uptake mechanisms have been characterized in bacteria;

- the uptake of galactose, xylose and lactose in *E. coli* are conducted by the major facilitator superfamily transporters and operate by proton symport,
- the phosphoenolpyruvate dependent phosphotransferase system (PTS) for transporting glucose, fructose, mannose and sucrose which mainly operate in Gram-negative bacteria,
- the periplasmic binding protein dependent ATP-binding cassette (ABC) transporters, which are ubiquitous membrane protein complexes that use the energy generated from ATP hydrolysis for uptake or export of various solutes across biological membranes (Wei *et al.*, 2012).

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);

- glucose can be transported through a dedicated ABC uptake system (involving PP1015 to PP1018) into the cytoplasm. It is phosphorylated to glucose-6-phosphate and further to 6-phosphogluconate. Glucose can also diffuse into the periplasm and be converted there to gluconate and then to 2-ketogluconate. Both of these intermediates are then transported into the cytoplasm to be transformed into 6-phosphogluconate and to 2-keto-6-phosphogluconate. All upstream intermediates from glucose converge towards 6-phosphogluconate, which can then enter either the Entner-Doudoroff (ED) pathway or the pentose phosphate pathway (Lessie and Phibbs Jr, 1984),
- fructose metabolism occurs via a PTS system. Fructose is imported through a typical PTS permease (FruA) consisting of a fusion of EIIBFru and EIICFru domains. Fructose is converted first to fructose-1-phosphate (F1P) and then to fructose-1, 6-bisphosphate and next enter the ED pathway and/or a standard glycolytic Embden-Meyerhof-Parnas (EMP) route (Nogales *et al.*, 2008).

The scheme in Figure 1.3 summarizes the network of reactions in cells growing on either sugar as the sole carbon source. The upper metabolic domain includes the Entner-Doudoroff (ED) pathway, the Embden-Meyerhof-Parnas (EMP) glycolytic route and the pentose phosphate pathway (PPP). The lower metabolic domain comprises the phosphoenolpyruvate-pyruvate-oxaloacetate (PEPPyr-OAA) node, including the pyruvate shunt and the TCA cycle.

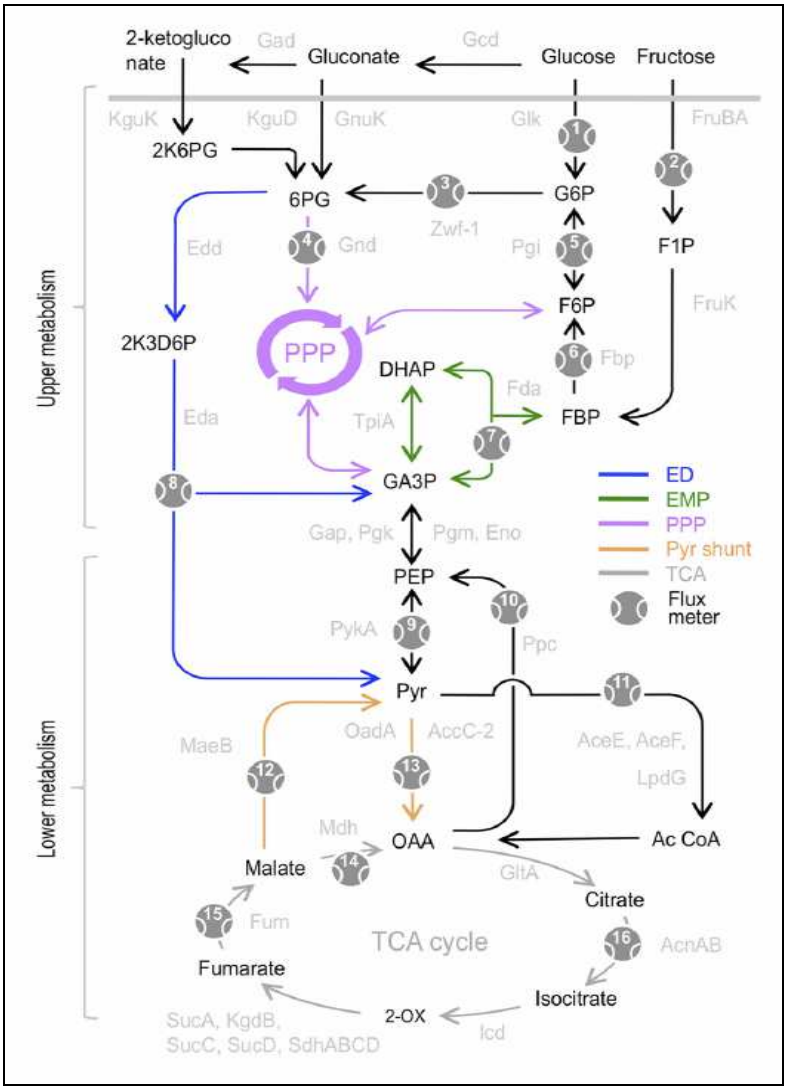


Figure 1.3 Glucose and fructose metabolism in *P. putida* (Chavarría *et al.*, 2012).

The catabolic potential including predicted pathways for energy production and metabolism of organic compounds of *P. putida* KT2440 is highlighted in Figure 1.4. Predicted transporters are grouped by substrate specificity, inorganic cations inorganic anions, carbohydrates, amino acids, peptides, amines, purines, pyrimidines and other nitrogenous compounds, carboxylates, aromatic compounds and other carbon sources, water, drug efflux and other. The energy-coupling mechanisms of the transporters are also illustrated including the solutes transported by channel proteins, secondary transporters, ATP-driven transporters and transporters with an unknown energy-coupling mechanism. The P-type ATPases indicate that they include both uptake and efflux systems. The central metabolism is involved at the outer and inner membrane, periplasmic space and the cytosol.

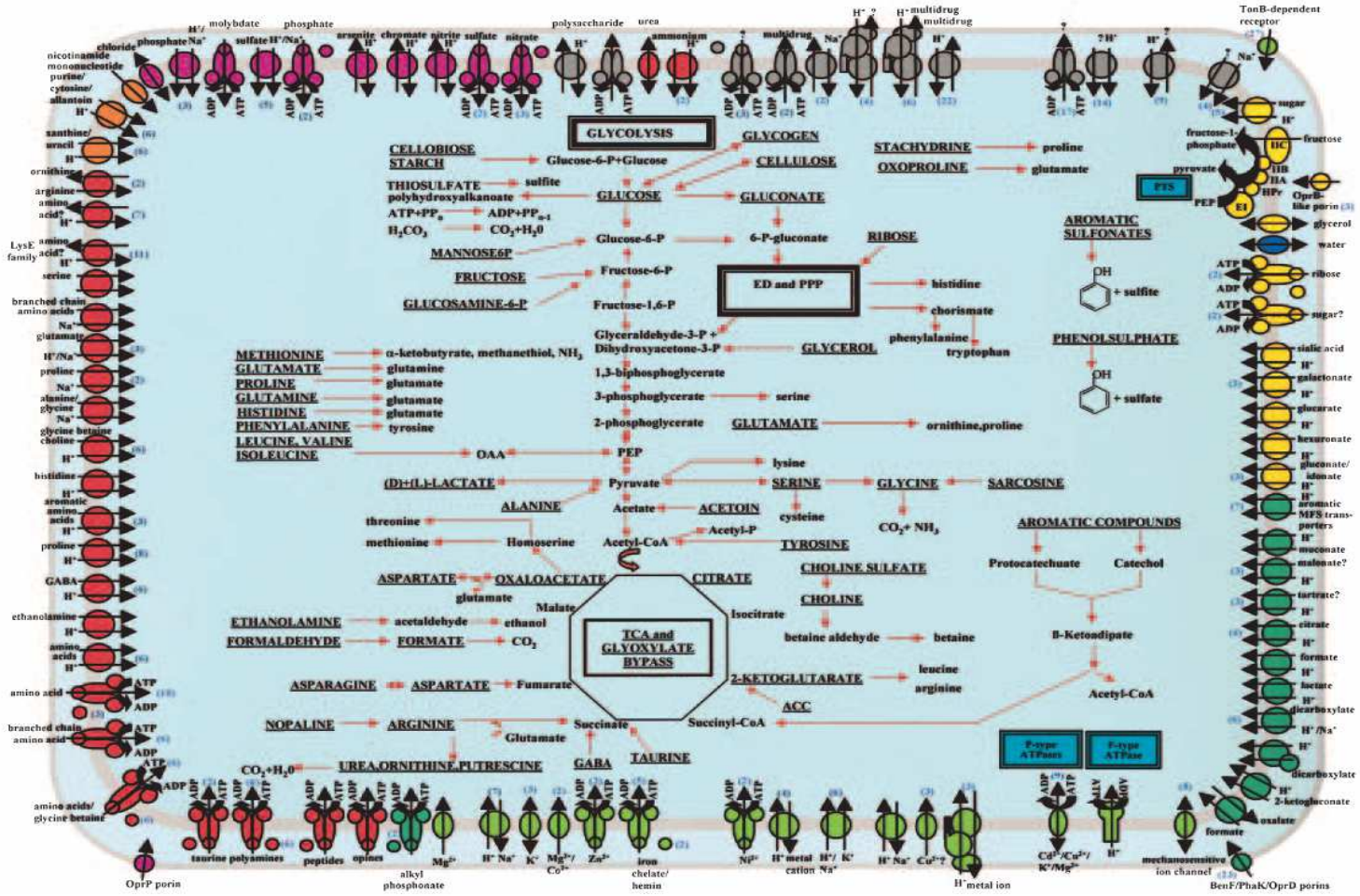


Figure 1.4 Overview of metabolism and transport in *P. putida* (Nelson *et al.*, 2002).

Substrate:

- inorganic cations (light green)
- inorganic anions (pink)
- carbohydrates (yellow)
- amino acids, peptides, amines, purines, pyrimidines and nitrogenous compounds (red)
- carboxylates, aromatic compounds and
- other carbon sources (dark green)
- water (blue)
- drug efflux and other (dark grey).

Solutes transport by:

- channel proteins (double-headed arrow)
- secondary transporters (two arrowed lines indicating both the solute
- and the coupling ion)
- ATP-driven transporters (ATP hydrolysis reaction)
- unknown mechanism (single arrow)
- The P-type ATPases (double-headed arrow)

1.1.4 Chlorophenol degradation

Pseudomonads are able to degrade various recalcitrant compounds like chlorinated aromatic hydrocarbons including chlorophenols that are present as pollutants in the environment (Jame *et al.,* 2010). Chlorophenols are chlorinated aromatic compound structures which are a major group of pollutants of environmental concern due to their toxicity and widespread use. They are known to be highly toxic, mutagenic and carcinogenic. Chlorophenols include pentachlorophenol (PCP), tetrachlorophenol (TeCP), trichlorophenol (TCP), dichlorophenol (DCP), and chlorophenol (CP). Each group has been used in industry for specific purposes (Solyanikova and Golovleva, 2004). Chlorinated phenolic compounds constitute an important class of pollutants because of their wide-spread use in industry e.g., pulp and paper, pesticides, herbicides, biocides and dyes. Monochlorophenols can be formed during wastewater chlorination and as a result of the breakdown of pesticides and chlorinated aromatic compounds. Chlorophenols can easily migrate within different aqueous environments and contaminate groundwaters since their solubility in water is relatively high (Armenante *et al.,* 1999). They are persistent in the environment, toxic and health risk related, thus they have been characterized as first priority pollutants by both the EU (European Union) and US EPA (United State Environmental Protection Agency) (Ye and Shen, 2004).

In natural environments, halogenated phenols usually adapt to different extents to microbial attack. For example, *para*-substituted halophenols are degraded more readily than *meta*- and especially, *ortho*-substituted ones; polyhalogenated phenols are less susceptible to microbial attack than monohalogenated phenols. Compared to chlorophenols, fluorophenols are reported to be attacked more easily. The position and nature of the halogen atom affects not only the rate of decomposition but also its pathway, resulting in the formation of various intermediates that are sometimes difficult to predict and can be more toxic and stable than the original halophenols (Finkel'shtein *et al.,* 2000). Generally chlorinated phenols are transformed via oxidative dechlorination in aerobic biodegradation (Steinle *et al.,* 1998) while in anaerobic conditions via reductive dechlorination. The recalcitrance of chlorophenols increases with increasing

number of chlorine substituents on the phenolic ring. The position of the chlorine substituents on the ring may also affect the biodegradability of such substances. The differences in the ability of microbes to degrade chlorophenols is influenced by the environment under which they act and the positions of the substituents (Annachhatre and Gheewala, 1996).

Metabolism of mono-chlorophenols

Chlorinated aromatics can be degraded by aerobic and anaerobic microorganisms by developing mechanisms to detoxify chlorophenols (Haggblom and Young, 1990). The use of microbial metabolic potential for eliminating environmental pollutants provides a safe and economic alternative to their disposal in waste dump sites compared to physico-chemical strategies. Biological methods are preferable to treat phenolic compounds because they are more economic and produce fewer byproducts. The application of physical and chemical methods includes adsorption, air stripping, solvent extraction and chemical oxidation to treat wastewaters containing phenolic and chlorophenolic compounds are expensive to operate (Ye and Shen, 2004; Monsalvo *et al.,* 2009).

A complete degradation of chlorinated aromatic compounds involves two steps including cleavage of the aromatic ring and the removal of the chlorine atom (Haggblom and Young, 1990). The initial step in the aerobic degradation of mono-chlorophenols is the transformation to chlorocatechols. 2- and 3-chlorophenol metabolism results in the formation of 3-chlorocatechol, while 4-chlorophenol is transformed to 4-chlorocatechol (Figure 1.5). Chlorocatechols are central metabolites in the aerobic degradation of a wide range of chlorinated aromatic compounds (Moiseeva *et al.,* 2002). These hydroxylated intermediates are then channeled into one or two possible pathways, either a *meta*-cleavage or *ortho*-cleavage pathway. Both types of pathways lead to intermediates of central metabolic routes, such as the tricarboxylic acid cycle (TCA cycle).

Aromatic compounds found naturally in the environment like phenol, benzene and methyl-substituted are typically broken down via the *meta*- cleavage pathway. Generally *meta*-cleavage of 3-chlorocatechol results in the inactivation of catechol

2, 3-dioxygenase either by 3-chlorocatechol itself, acting as a chelating compound (Klecka and Gibson, 1981), or by the production of a highly reactive acylchloride, the product of the cleavage of 3-chlorocatechol which binds irreversibly to the *meta*-cleavage enzyme. The *meta*-cleavage of 4-chlorocatechol results in the production of a chlorinated aliphatic compound 5-chloro-2-hydroxymuconic semialdehyde reported as being a dead-end metabolite (Wieser *et al.,* 1994).

The majority of known strains use a modified *ortho*-cleavage pathway for chlorophenol degradation. Normal 1,2-dioxygenases involved in catechol metabolism exhibit low activities for halogenated substrates. Enzymes with broad substrate specificities that have a high affinity for chloroaromatic substrates have been identified (Dorn and Knackmuss, 1978). In *Pseudomonas* sp. B13, catechol and chlorocatechol were assimilated via two separate *ortho*-cleavage pathways;

- *ortho* type-I pathway, involved in the degradation of unchlorinated aromatics, via catechol which catalyzed by the enzyme catechol 1,2-dioxygenase (pyrocatachase Type I)
- modified *ortho*- type II pathway, specific to the degradation of chlorinated aromatics via chlorocatechol (Dorn and Knackmuss, 1978)
- *ortho*- cleavage (Type II) of chlorinated aromatic compounds, catalyzed by the enzyme chlorocatechol 1,2-dioxygenase (pyrocatachase Type II) is generally necessary for complete degradation with a resulting release of the chloride atom.

Pseudomonas putida CP1 is able to degrade up to 200 ppm 2- and 3-chlorophenol and 300ppm 4-chlorophenol using an *ortho*-cleavage pathway. *Pseudomonas putida* CP1 possesses two different *ortho*-cleavage enzymes, a catechol 1,2-dioxygenase capable of metabolising catechol (*ortho*-I activity) and a chlorocatechol 1,2-dioxygenase with low substrate specificity capable of metabolising both catechol and chlorocatechols (*ortho*- II activity) (Farrell and Quilty, 2002a). *Pseudomonas putida* CP1 is capable of degrading 3- and 4-chlorocatechol which are known intermediates of chlorophenol degradation. Degradation of 4-chlorophenol, which occured via 4-chlorocatechol, was at a higher rate than the degradation of 2- or 3-chlorophenol, which was degraded via

3-chlorocatechol. This suggests that the initial step in degradation of chlorophenols, hydroxylation of chlorophenols to chlorocatechols was the rate-limiting step in degradation of chlorophenols by *P. putida* CP1.

Many reports have been published reporting the ability of *Pseudomonas* strains to degrade a wide range of chlorinated aromatic compounds as sole carbon source via the *ortho*-cleavage pathways. *Pseudomonas* sp. B13 was isolated for the degradation of 3-chlorobenzoate and has been shown to be capable of degradation of 4-chlorophenol as sole carbon source and the cometabolism of 2- and 3-chlorophenol via the modified *ortho*-pathway (Knackmuss and Hellwig, 1978). *Pseudomonas testosteroni* showed complete degradation of 2-, 3- and 4-chlorophenol using the *ortho*-cleavage pathway (Chih-Jen *et al.*, 1996). The *ortho*-pathway degradation was used by *Alcaligenes* sp. A-7, resulting in the degradation of 2-, 3- and 4-chlorophenol (Schwien and Schmidt, 1982).

Transformation of chlorocatechols via the modified *ortho*-cleavage pathway by chlorocatechol 1,2-dioxygenase yields chloromuconates (Figure 1.5). The chlorocatechol is oxidized at both carbon atoms carrying hydroxyl substituents, yielding two carboxylic acid (-COOH) groups at the end of a chain. Ring cleavage occurs between the hydroxyl-substituted carbons. *Ortho*-cleavage of 3-chlorocatechol yields 2-chloro-cis,cis-muconate while cleavage of 4-chlorophenol results in the production of 3-chloro-cis,cis-muconate. Following ring cleavage via the modified *ortho*-pathway, the products produced undergo cycloisomerisation. The chloro-substituted cic,cis-muconates are transformed resulting in the production of dienelactones (4-carboxymethylenebut-2-en-4-olides). This is a critical step in the degradation of chlorophenols as it results in the elimination of the chloro-substituent. The enzyme involved, chloromuconate cycloisomerase (cycloisomerase Type II) is homologous to muconate cycloisomerase (cycloisomerase Type I) involved in the degradation of catechol via the *ortho*-pathway. Cycloisomerase II exhibits higher activities with substituted-muconates than with unsubstituted substrates. Cycloisomerase II converts 2-chloro-cis,cis-muconate to the intermediate 5-chloromuconolactone and then dehalogenates the metabolite to form *trans*-dienelactone, while 3-chloro-cis,cis-muconate is converted to *cis*-dienelactone (Vollmer *et al.*, 1998).

The final steps involve the conversion of dienelactones produced following cycloisomeration into metabolites of the normal metabolism of the bacterium. This is achieved by the conversion of *cis*- and *trans*-dienelactone, by the enzyme dienelactone hydrolase to produce maleylacetate which is readily converted to intermediates of the TCA cycle and therefore may be utilised for the production of biomass, energy, carbon dioxide and water, resulting in complete degradation. Figure 1.5 illustrates the degradative pathways of *P. putida* by modified *ortho*-cleavage pathway.

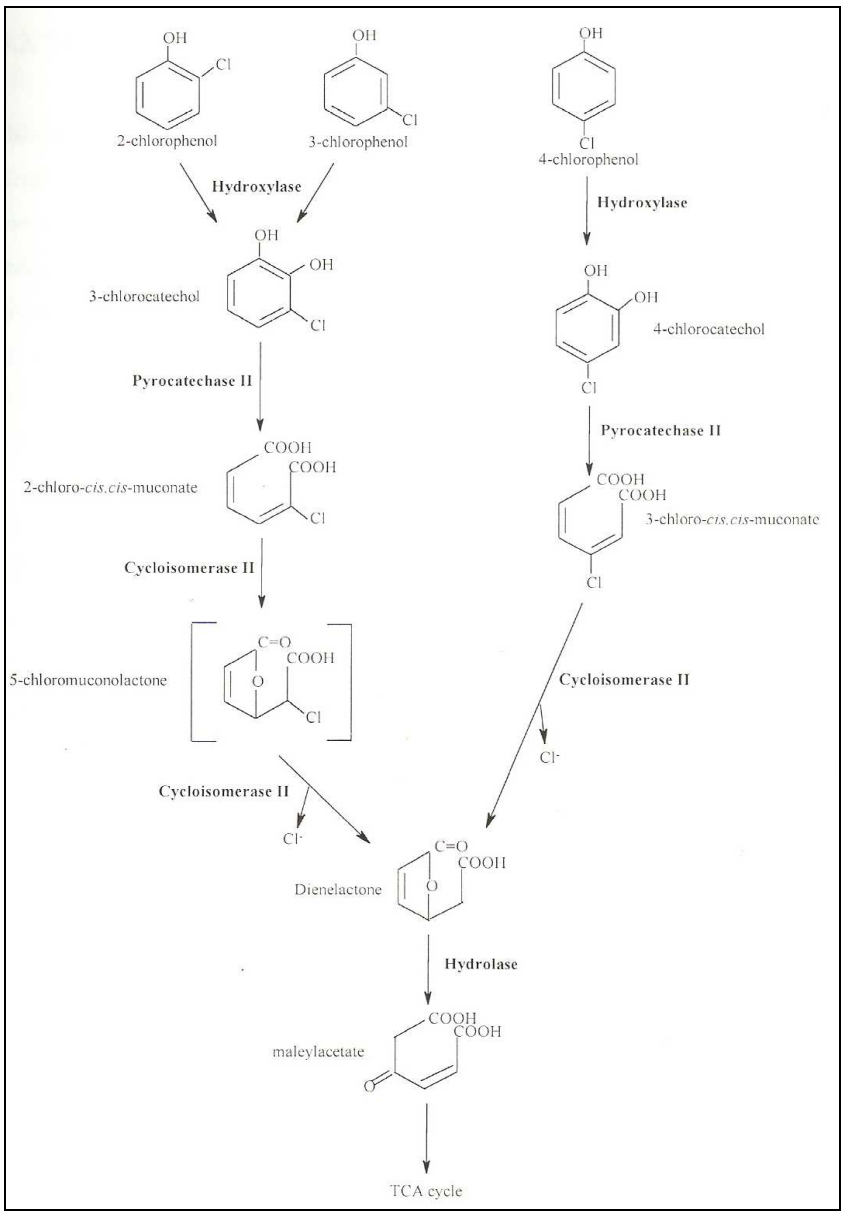


Figure 1.5 Degradative pathways of *P. putida* by the modified *ortho*-cleavage pathway (Knackmuss and Hellwig, 1978).

Microorganisms reported to degrade chlorophenols

The microorganisms used in biological treatment are usually aerobes, including *Pseudomonas* sp. (Farrell and Quilty, 2002a), *Alcaligenes* sp. (Elkarmi *et al.*, 2008; Jiang *et al.*, 2007) and *Rhodococcus* sp. (Martínková *et al.*, 2009; Goswami *et al.*, 2005). These aerobes are more efficient at degrading toxic compounds because they grow faster than anaerobes and usually transform organic compounds to inorganic compounds (Oh and Lee, 2002).

Gram-positive and Gram-negative bacteria have been identified which can utilize chlorophenols as a carbon and energy source under aerobic conditions (Table 1.2). Pure cultures are favoured for use in the majority of choroaromatic biodegradation studies. Among the strains used, the most common genus is *Pseudomonas*. *Pseudomonas* strains have a valuable potential to evolve entire catabolic sequences for specific degradative pathways. The organisms are present in large numbers in all major natural environments including terrestrial, freshwater and marine. Apart than that, they also have a remarkable nutritional versatility and are capable of utilizing a wide range of organic compounds as growth substrates.

Table 1.2 Examples of bacterial strains capable of growth on chlorinated phenols when supplied as a sole source of carbon and energy.

Bacterial strain	**Substrate**
Alcaligenes sp. A7-2 (Menke and Rehm 1992)	2CP, 4CP
Alcaligenes xylosoxidans JH1 (Hollender *et al.*, 2000)	2CP
Pseudomonas pickettii LD1 (Fava *et al.*, 1995)	2CP, 3CP, 4CP
Rhodococcus opacus 1G (Finkel'shtein *et al.*, 2000)	2CP, 3CP, 4CP
Pseudomonas putida CP1 (Farrell and Quilty 2002a)	2CP, 3CP, 4CP
Alcaligenes xylosoxidans JH1 (Hollender *et al.*, 2000)	3CP, 4CP
Pseudomonas sp. B-13 (Dorn and Knackmuss 1978)	4CP
Arthrobacter ureafaciencs CPR706 (Bae *et al.*, 1996)	4CP
Arthrobacter chlorophenolicus A6 (Nordin *et al.*, 2005)	4CP
Comamonas tertosteroni CPW 301 (Bae *et al.*, 1996)	4CP

The complete degradation of all three mono-chlorophenol isomers by a single degradation system is difficult and has rarely been reported. *Pseudomonas pickettii* LD1 degraded up to 0.57 mM 3-chlorophenol, 0.75 mM 4-chlorophenol and 1.51 mM 4-chlorophenol (Fava *et al.,* 1995). Zaitsev *et al.,* (1995) reported the ability of *Rhodococcus opacus* GM-14 to degrade up to 0.78 mM 3-chlorophenol and 1.95 mM 2- and 4-chlorophenol. In these studies no intermediates were detected in the culture medium during the degradation of chlorophenols, suggesting that any metabolites produced were degraded immediately. The complete degradation of 2-, 3- and 4-chlorophenol at concentration of 0.08 mM was conducted by immobilized cells of *Pseudomonas testosteroni* using the *ortho*-cleavage pathway (Chih-Jen *et al.,* 1996). Jame *et al.,* (2010) demonstrated the *Pseudomonas* sp. A4CP2 isolated from phenolic waste contaminated sites was able to degrade up to 800 ppm of phenol, 200 ppm of 2-chlorophenol and 350 ppm of 4-chlorophenol.

Pseudomonas putida CP1 is able to degrade a single concentration of 0.78 mM of 2-chlorophenol within 72 h, 3-chlorophenol within 80 h and 4-chlorophenol within 30 h in the order 4-chlorophenol> 2-chlorophnol> 3-chlorophenol (Farrell and Quilty, 2002a). The differences between the degradation of the mono-chlorophenols result from the differing positions of the chloride atom on the aromatic ring which, coupled with the electron - accelerating effect of the initial hydroxyl group, affects the introduction of a second hydroxyl group onto the aromatic ring. Due to the competing electron - accelerating / decelerating effects, the theoretical order of degradability should be 4-chlorophenol> 2- chlorophenol> 3–chlorophenol (Menke and Rehm, 1992) - the order that was observed during the degradation of the mono-chlorophenols by *P. putida* CP1. A strain identified as *Pseudomonas pickettii* LD1 isolated from an aerobic bacterial consortium was competent to totally degrade and dechlorinate 1.51 mM 2-chlorophenol, 0.57mM chlorophenol and 0.75mM 4-chlorophenol within 30, 30 and 40 hour of incubation. The rates of degradation followed the sequence of 2- CP > 4-CP > 3-CP (Fava *et al.,* 1995). These results are dissimilar from those reported for *Alcaligenes* sp. A7-2 strain, which was found to metabolize 4-chlorophenol faster than 2-chlorophenol, both in batch cultures containing free cells and in continuous cultures containing immobilized cells (Menke and Rehm, 1992).

Pseudomonads are not the only genus reported to perform the degradation of chlorophenolic compounds. *Comamonas testosteroni* JH5 used 4-chlorophenol as its sole source of energy and carbon up to a concentration of 1.8 mM and degraded it by the *meta*-cleavage pathway. Two key metabolites of the *meta*-cleavage pathway, 5-chloro-2-hydroxymuconic semialdehyde and 5-chloro-2-hydroxymuconic acid, were identified (Hollender *et al.,* 1997). *Arthrobacter ureafaciens* strain CPR706 reached the maximum degradation rate (about 0.054 mM h^{-1}) when the initial 4-chlorophenol concentration was between 0.9 and 1.6 mM, whilst was inhibited when the initial concentration increased to 2.0 mM (Bae *et al.,* 1996). *Rhodococcus opacus* 1CP was found to efficiently degrade 4-chlorophenol and 2, 4-dichlorophenol (Eulberg *et al.,* 1998). A Gram positive *Arthrobacter chlorophenolicus* A6 isolated from soil slurry degraded high concentrations of 4-chlorophenol up to 2.7 mM, together with other *p*-substituted phenols, such as 4-nitrophenol and 4-bromophenol (Nordin *et al.,* 2005). A bacterium from the *Bacillus* species has been identified with the ability to degrade 2-chlorophenol at initial concentration of 0.25-2.5mM. However cell growth was inhibited with the higher concentration of 2.5 mM (Al-Thani *et al.,* 2007).

1.2 Autoaggregation in bacteria

Bacteria exist in a form of aggregates or biofilms as an adaptation and protection strategy from environmental stress (O'Toole *et al.,* 2000). In the form of aggregates, bacteria are able to communicate and perform metabolic functions more efficiently than their planktonic forms (Eboigbodin *et al.,* 2005). This phenomenon exists in bacteria, yeasts, slime molds, filamentous fungi, algae and protozoa (Burdman *et al.,* 2000). A number of investigations have been carried out on bacterial autoaggregation including in several *Pseudomonas* strains (Farrell and Quilty, 2002a; Sanin *et al.,* 2003; Matz *et al.,* 2002; Panicker *et al.,* 2006; Klebensberger *et al.,* 2006).

Aggregation is the assembling of cells to form stable and contiguous structures with multicellular associations, occurring under certain physiological conditions. Cell aggregation may consist of similar units or dissimilar units. The aggregation of a single bacterial species is termed autoaggregation. The other term to replace

words for aggregation found in the literature include agglomeration, agglutination, association, clumping, coagulation, cohesion, and flocculation (Burdman *et al.*, 2000). Two principal elements that need to be present in any cell-aggregation system are physical movement and stable multicellular contacts to allow cells to come together. Biofilms and biological aggregates have many features in common (Costerton, 1999). Unlike the aggregation phenomenon, biofilms are defined as communities of microorganisms that are attached to a solid surface embedded in a polymer matrix (Madigan, Martinko and Parker, 2000). Biofilms can comprise a single microbial species or multiple microbial species and can form on a range of biotic and abiotic surfaces (O'Toole *et al.*, 2000).

From a general point of view, microbial aggregation systems can be classified into three main categories as they constitute: (1) a prelude to morphogenesis and differentiation, (2) a prelude to sexuality and parasexuality and (3) a mechanism for survival under adverse circumstances. Free-living nitrogen fixing bacteria such as *Azospirillum*, *Klebsiella*, and *Azotobacter*, which are known for their capacity to aggregate belong to the third category (Burdman *et al.*, 2000). All aggregates are accumulation of microorganisms, extracellular polymeric substances (EPS), multivalent cations, biogenic and inorganic particles as well as colloidal and dissolved compounds (Wingender and Flemming, 1999). Aggregated microbes being closely associated with one another allow better communication, structure, protection, stability and defence to harsh conditions including water shear force, predation and food limitation. Aggregated cells also display a higher degree of resistance to biocide compounds and higher metabolic activity than their planktonic counterparts (Bossier and Verstraete, 1996).

Microbial aggregation is of great significance in the scope of environmental research as well as biotechnological processes and medical studies. Application of microbial aggregation in aqueous solutions has been used for cell concentration, recovery and removal in various processes such as fermentation, portable water treatment and wastewater treatment. Microbial aggregation enhances efficiency of biological wastewater treatment processes through the selective separation of microbes and the collection of suspended solids. Efficient aggregation and proper settling of aggregates is important for the generation of good quality effluent in

the activated sludge process (Tse and Yu, 1997). However, aggregation can have a detrimental effect on environmental processes, such as bio-fouling of artificial surfaces and corrosion of metals. Aggregation and biofilm formation can also lead to the colonization of medical devices leading to infection (Eboigbodin *et al.*, 2007).

1.2.1 Extracellular polymeric substances (EPS)

Extracellular polymeric substances (EPS) are the key components in a number of models explaining the aggregation of microorganisms and the physicochemical properties of the extracellular matrix in aggregates and biofilms (Wingender and Flemming, 1999). EPS accelerates the formation of microbial aggregates by binding cells closely (Liu *et al.*, 2004). Wingender *et al.,* (1999a) defined EPS as representing different groups of macromolecules such as polysaccharides, proteins, nucleic acids, lipids and other polymeric compounds presented in the interior of various microbial aggregates. While Jahn *et al.,* (1999) suggested EPS were all polymers outside the cell wall which are not directly embedded in the outer membrane. EPS are mainly the high-molecular weight secretions from microorganisms and the products of cellular lysis and hydrolysis of macromolecules.

The forms of EPS that exist outside of cells can be subdivided into bound EPS (sheaths, capsular polymers, condensed gels, loosely bound polymers and attached organic materials) and free or soluble EPS (soluble macromolecules and slimes) (Jahn *et al.*, 1999; Laspidou and Rittmann, 2002). Bound EPS are closely bound with cells, while free EPS are weakly bound with cells or dissolved into the solution. Although the interaction between soluble EPS and cells is very weak, Sheng and Yu (2007) showed that soluble EPS also have a crucial effect on the microbial activity and surface characteristics of activated sludge. The structure of bound EPS is generally described by a two layer model (Figure 1.6).

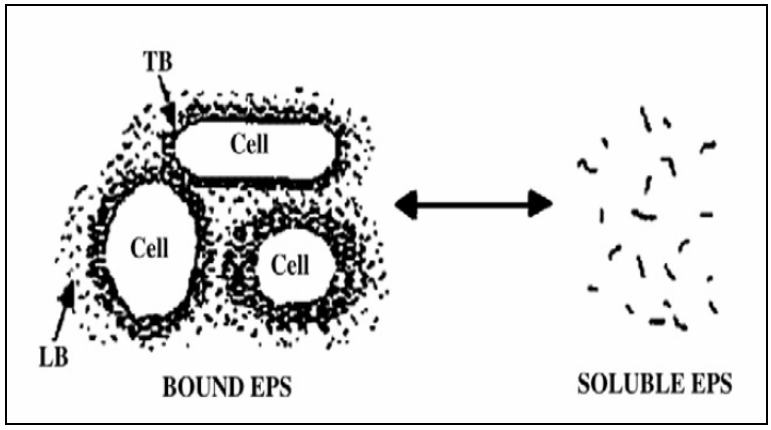


Figure 1.6 Sketch of EPS structure including soluble EPS and bound EPS composed of tight bound (TB) and light bound (LB) (Sheng *et al.*, 2010).

Proteins and carbohydrates are usually the predominant constituents, but other components such as multivalent cations, DNA, RNA and lipids have also been related to specific characteristics of EPS. Chemically, EPS are rich in high molecular weight polysaccharides (10 to 30 kDa) and generally have heteropolymeric composition. Bacterial EPS are generally rich in hexoses like glucose and galactose and the presence of sugars like arabinose helps in cell aggregation in bacteria. The chemical composition and physical properties of the polysaccharides in biofilm matrices can vary greatly due to the type of monomer units, the kind of glycosidic linkages (e.g. β-1,4, β-1,3 or α-1,6), and the occurrence of different organic and inorganic substitutions.

EPS also contain amounts of proteins, non-sugar moieties like uranic acid, pyruvates hexosamines, acetates, sulphate esters and small amounts of lipids and nucleic acid. These non-sugar components are attached to the sugar residues (Flemming and Wingender, 2002). The EPS molecules of most bacterial species are negatively charged under neutral pH conditions due to the presence of predominantly carboxylic and phosphoryl functional groups (Ueshima *et al.*, 2008). Besides protein and polysaccharides, other components found in aggregate EPS are nucleic acids. Although the percentage of nucleic acids is normally quite low relative to the protein and polysaccharide fractions, their role in aggregation may be more important (Frølund *et al.*, 1996; Liao *et al.*, 2001). The presence of extracellular DNA may play a role as a cell-to-cell interconnecting component in

many different biofilms. It was suggested that bacteria release DNA to exchange genetic material and to form and stabilize biofilms (Hendrickx *et al.*, 2003).

The biosynthesis of EPS serve many functions concerning promotion of cell adhesion, formation of microbial biofilms, aggregates, sludges and biogranules and protect cells from hostile environments (Pal and Paul, 2008; Ren *et al.*, 2009). In addition, EPS serve as biosorbing agents by accumulating nutrients from the surrounding environment and also play a crucial role in biosorption of heavy metals (Pal and Paul, 2008). EPS binds with cells through complex interactions to form a matrix structure with plenty of water that protects cells against dewatering (Wingender *et al.*, 1999a) and the harm of toxic substances (Sutherland, 2001). In conditions of nutrient shortage, EPS can serve as energy and carbon sources for bacteria (Zhang and Bishop, 2003). A list of EPS functions are summarized in Table 1.3.

Table 1.3 Functions of extracellular polymeric substances (EPS) (Wingender *et al.*, 1999b).

Function	**Relevance**
Adhesion to surfaces	Initial step in colonization, accumulation of bacteria on nutrient-rich surfaces in oligotrophic environments
Aggregation of bacterial cells, formation of aggregates and biofilm	Bridging between cells and inorganic particles trapped from the environment, immobilization of mixed bacterial populations, basis for development of high cell densities, generation of a medium for communication processes, cause for biofouling and biocorrosion events
Cell-cell recognition	Quorum sensing
Structural elements of biofilms	Mediation of mechanical stability of biofilms (frequently in conjunction with multivalent cations), dermination of the shape of EPS structure (capsule, slime, sheath)
Protective barrier	Resistance to nonspecific and specific host defenses
Retention of water	Prevention of desiccation under water-deficient conditions
Sorption of exogenous organic compounds	Scavenging and accumulation of nutrients from the environment, sorption of xenobiotics (detoxification) and volatile organic compounds.
Sorption of inorganic ions	Accumulation of toxic metal ions (detoxification),

	promotion of polysaccharide gel- formation, mineral formation
Enzymatic activities	Digestion of exogenous macromolecules for nutrient acquisition, release of biofilm cells by degradation of structural EPS of the biofilm

According to Sheng *et al.,* (2010), the production of EPS can be influenced by various factors including substrate type, nutrient content, growth phase and external conditions. Bacteria would excrete more EPS under unfavourable conditions. Nutrient levels have a significant effect on EPS production and composition (Pal and Paul, 2008). Jia *et al.,* (1996) found during the exponential growth phase, the EPS content increased with cultivation time, but during the stationary phase it decreased with increasing cultivation time. Metal concentration may influence the EPS contents because the EPS bound with cells via ion bridging with multivalent metals. Higgins and Novak (1997) mentioned that the protein content in the sludge EPS increased at higher Ca^{2+} or Mg^{2+} concentrations, while higher Na^{+} concentrations resulted to a lower protein content.

1.2.2 Interactions involved in the formation of aggregates

Several mechanisms have been suggested as being involved in aggregate formation highlighting the importance of surface properties in the interactions. The main interactions are:

- the polymer bridging model - the extracellular polymeric substances (EPS) which contain different negatively charged groups, are bound together by means of divalent and/or trivalent cations to form large polymeric networks in which the different aggregate constituents such as single bacteria are embedded. It has been suggested that the interactions between the aggregate components can be described by the DLVO theory (which is named after Derjaguin and Landau, Verwey and Overbeek) for colloidal stability where van der Waal's forces and the electrostatic repulsive forces caused by interpenetration of electrical double layers are added together to describe the interparticle interactions.

- other non-DLVO interactions such as hydrophobic interactions have been suggested as being important for the flocculation of activated sludge and other bacterial aggregates as well (Wilen *et al.,* 2003).

Three types of noncovalent interactions have to be considered as cohesive forces between the components within the EPS matrix that contribute to the stability of aggregates (Wingender and Flemming, 1999).

- Dispersion forces - exhibited by nonpolar molecules because of movements of the electrons in interacting molecules. They are present between all chemical groups and usually represent the main part of the total interaction force in condensed matter. They are generally weaker than ionic bonds and hydrogen bonds.
- Hydrogen bonds – form mainly between hydrogen atoms of hydroxyl groups and electronegative oxygen or nitrogen atoms that are abundant in polysaccharides and proteins.
- Electrostatic interactions – occur in between charged molecules (ions) and between permanent or induced dipoles. Divalent cations such as Ca^{2+} act as bridges contributing overall binding forces. Positively charged groups of amino sugars in polysaccharides or amino acids in proteins can interact with negatively charged groups providing cohesion forces. Electrostatic interactions are the major importance for the stability of EPS matrix.

The polymer cation bridging theory is one of the mechanisms describing aggregate formation as illustrated in Figure 1.7. Cells are held together through negatively charged EPS, which bridges with cations. EPS act as glue binding the different aggregate constituents together by electrostatic forces described by the DLVO theory. Bridging is by multivalent cations such as Mg^{2+}, Ca^{2+} and Fe^{3+}, entanglement of EPS molecules and hydrophobic interactions (Wilén *et al.,* 2008).

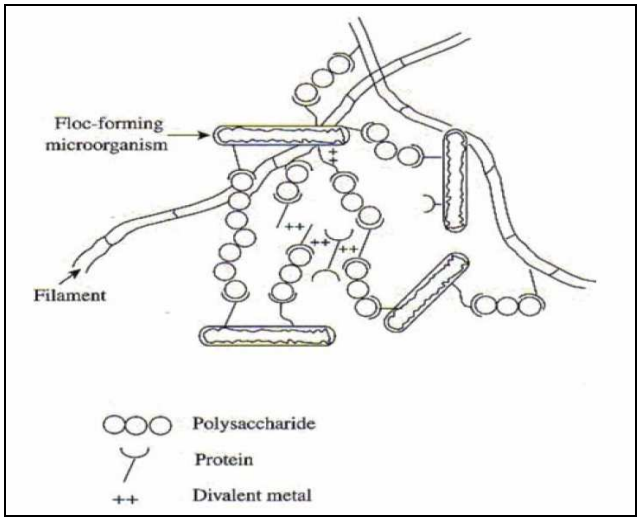


Figure 1.7 Schematic structure of aggregates (Nguyen *et al.*, 2007).

Surface hydrophobicity is an important property of bacteria which is correlated to the aggregation or adhesion of cells. Hydrophobic interactions define the relations between water and low water-soluble molecules (hydrophobes). Hydrophobes are nonpolar molecules and usually have a long chain of carbons that do not interact with water molecules. In biological systems hydrophobic interactions are the strongest long-range non-covalent interaction. The environmental factors influencing the surface characteristics and adhesion properties of bacteria include the source of nutrients, growth rate, growth phase, growth conditions, temperature and pH (Sanin *et al.*, 2003).

Microbial aggregation is generally attributed to exopolymeric bridging of bacterial cells. Bridging of aggregate components involves ionic interactions between charged functional groups of biopolymers and divalent cations and may also be mediated through hydrophobic moieties of exopolymers and specific protein–polysaccharide interactions (Chaignon *et al.*, 2002). The results from microbial surface hydrophobicity tests are used to understand microbial adhesion from physical–chemical perspective since microbial adhesion is an interplay of hydrophobicity, Van der Waals forces and electrostatic interactions.

Hydrophobicity measurement methods are universal and some are still debated. Several methods have been developed including:

- partitioning in aqueous two phase systems

- hydrophobic interaction chromatography
- salt aggregation
- polystyrene microsphere attachment
- contact angle measurements
- microbial adhesion to hydrocarbons

Bacterial strains with different cell surface hydrophobicity/hydrophilicity properties have been isolated from activated sludge (Jorand *et al.*,, 1994; Zita and Hermansson, 1997). Some strains were shown to modify their cell surface hydrophobicity characteristics depending on the growth phase. Strains with hydrophobic surfaces flocculate better than strains with hydrophilic surfaces (Jorand *et al.*, 1994) and adhesion to pre-existing flocs is enhanced by hydrophobic cell surfaces (Zita and Hermansson, 1997; Olofsson *et al.*, 1998). Environmental factors such as oxygen availability and the presence of toxic substances can also impact cell surface hydrophobicity. Oxygen limitation seems to lower cell surface hydrophobicity and this effect is most pronounced during the stationary phase (Palmgren *et al.*, 1998).

The fact that bacterial cells can adapt their surface characteristics according to growth phase, toxicity and oxygen and nutrient availability, indicates that physiology may impact aggregate structure. The EPS in microbial aggregates containes charged groups (e.g., carboxyl, phosphoric, phenolic and hydroxyl groups) and polar groups (e.g., aromatics, aliphatics in proteins and hydrophobic regions in carbohydrates) (Flemming and Leis, 2002). The presence of hydrophilic and hydrophobic groups in EPS molecules indicates that EPS are amphoteric. The formation of hydrophobic areas in EPS advantages for organic pollutant adsorption (Späth *et al.*, 1998).

1.2.3 Genotypic changes in autoaggregation

Information on genotypic changes in autoaggregation are still lacking especially in *Pseudomonas 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. Table 1.4 lists the microorganisms exhibiting autoaggregation and the genetic factors involved.

Table 1.4 List of microorganisms which exhibit autoaggregation and the genes involved.

Organisms	**Aggregation Factor**	**Genes Involved**
Pseudomonas solanacearum (Chapman and Kao, 1998)	EPS production	Structural gene clusters: opsI, opsII, rgnII, and epsI
Pseudomonas. putida (Espinosa-Urgel *et al.*, 2000)	Attachment to plastics, glass and corn seeds	lapA
Escherichia coli (Schembri *et al.*, 2001)	Type 1 fimbriae	fimH
Pseudomonas fluorescens (Hinsa *et al.*, 2003)	Outer-membrane protein LapE association with LapA	lapE and lapA
Pseudomonas aeruginosa PAO1(Jackson *et al.*, 2004)	EPS production	psl
Pseudomonas. aeruginosa PAK (Vasseur *et al.*, 2005)	EPS production	pel
Shewanella oneidensis MR-1 (Windt *et al.*, 2006)	Medium	aggA
Pseudomonas aeruginosa (Klebensberger *et al.*, 2007; Klebensberger *et al.*, 2009)	Response to SDS	siaA and siaD
Sinorhizobium meliloti (Sorroche *et al.*, 2012)	EPS prouction	expR

A study on *Shewanella oneidensis* conducted by 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. Cucarella *et al.*, (2001) found the locus *bap* (for biofilm associated protein) encodes a novel cell wall associated protein of 2, 276 amino acids (Bap) in *Staphylococcus aureus.* The 2276-aa *bap* products displayed an organizational similarity with an outer membrane protein of *P. putida* involved in adhesion to seeds which is highly correlated with the ability of this bacterium to produce a biofilm on abiotic surfaces. Primary attachment, intercellular aggregation and biofilm formation studies showed that Bap promotes primary attachment as well as the second step of biofilm formation.

Enteroaggregative *Escherichia coli* (EAggEC 17-2) is defined by its characteristic aggregative adherence (AA) ability. AA in EAggEC 17-2 is associated with the presence of plasmid encoded, bundle-forming fimbriae with diameters of 2 to 3 nm. AggR was found to promote expression of the *aggA* gene under a variety of conditions of temperature, osmolarity, oxygen tension, and medium. At acid pH, *aggA* expression was maximal and was regulated by both AggR-dependent and AggR-independent mechanisms (Nataro *et al.,* 1994).

A number of surface factors are also known to be implicated in autoaggregation of *E. coli*, such as Antigen 43 (Ag43), the product of the flu gene, the AAF/I and AAF/II aggregative adherence fimbriae (Schembri *et al.,* 2001). Due to its excellent cell-to-cell aggregation characteristics, Ag43 expression confers clumping and promotes biofilm formation. Ag43 is found in most *E. coli* strains and can actually be expressed by a broad spectrum of Gram-negative bacteria. Ag43-induced bacterial clumping can also be a highly efficient defense mechanism (Schembri *et al.,* 2003).

Nielsen *et al.,* (2011) discussed the importance of two putative exopolysaccharide gene clusters named bacterial cellulose (bcs) and putida exopolysaccharide A (pea) in *P. putida* KT2440 biofilm formation and stability. A novel EPS locus known as putida exopolysaccharide A (*pea*) is important for cell surface interactions and for maintaining cell–cell interactions post attachment on abiotic

surfaces. Pea contains β-1, 3 and β-1, 4 linkages based on the cellulose sensitivity test. Pea is central to maintaining cell-cell interactions after attachment, possibly by maintaining matrix stability.

Pseudomonas aeruginosa is capable of producing at least three exopolysaccharides (alginate, cellulose and neutral exopolysaccharide) and one intracellular storage polymer (PHA). Alginates are non-repeating copolymers of β–D-mannuronic acid and α–L-guluronic acid, linked by 1-4 linkages. The alginates in *Pseudomonas* were contributed to the formation of differentiated biofilms. The entire alginate biosynthesis gene cluster of *P. aeruginosa* is transcribed under control of the algD promoter, which represent the major target for transcriptional regulation (Rehm, 2008).

Four flocculating genes Lg-*FLO1*, *FLO1*, *FLO5*, and *FLO9* and the protein Lg-Flo1p were identified as flocculation factors in *Saccharomyces cerevisiae*. FLO8 encodes a transcription factor required for flocculation; whereas FLO11 encodes a cell surface glycoprotein responsible for the formation of pseudohyphae, sliding motility and adhesion to agar and plastic surfaces (Heine *et al.*, 2009).

Surface molecules from *Bifidobacterium bifidum* play a role in mucin adhesion capability and the aggregation phenotype of this bacterial species. The involvement of surface-exposed macromolecules in the aggregation phenomenon was determined in *B. bifidum* LMG13195, DSM20456, DSM20239 and A8. A mucin binding assay of *B. bifidum* A8 surface proteins showed a high adhesive capability for its transaldolase (Tal). The gene encoding Tal from *B. bifidum* A8 was expressed in *Lactococcus lactis*, and the protein was purified to homogeneity. The pure protein was able to restore the autoaggregation phenotype of proteinase K-treated *B. bifidum* A8 cells. These findings suggest that Tal, when exposed on the cell surface of *B. bifidum*, could act as an important colonization factor favoring its establishment in the gut (González-Rodríguez *et al*., 2012).

1.3 Microbial stress responses

Stress is a factor that promotes aggregation (Klebensberger *et al*., 2006). Microbes will grow at a maximum growth rate when supplied with sufficient nutrients and optimal growth temperature, pH, oxygen levels and solute concentrations. The existence of variations in any of these parameters can affect the maximum growth rate and thus can represent an environmental stress for the microbe. The ability of microbes to sense and respond to impromptu alterations in the environments is crucial to their survival. In reality, conditions that allow for maximal growth rates outside the laboratory are few and as a result most bacteria live in a constant state of stress. Bacteria have the ability to sense and respond to stress stimuli through coordinated changes in gene expression. Mechanisms for responding to environmental changes are universally present and changes usually lead to the synthesis of specific molecules that respond to the adverse environmental conditions (Boor, 2006). Table 1.5 describes common stress responses in the environment and the mechanisms involved in bacterial systems to cope with the stresses.

Microbial cells synthesize and accumulate low molecular-weight hydrophilic compounds named compatible solutes such as glycerol, mannitol, proline, betaine and trehalose that can protect their macromolecular apparatus under stressful conditions such as low water activity or extremes of temperature. Compatible solutes such as glycerol and trehalose may sometimes be implicated in turgor control but frequently act as protectants of macromolecular structures independently of osmotic processes. Microbial cells may even release compatible solutes into the extracellular in order to protect both sides of the plasma membrane. Compatible solutes protect diverse microbial species from stress induced by hydrophobic stressor which can up-regulate the synthesis of intracellular compatible solutes (Bhaganna *et al.,* 2010).

Table 1.5 General stress responses in bacteria.

Type of stress	**Response**
Chaotropic solutes (Hallsworth et al., 2003).	• Up-regulating proteins for protein stabilization, lipid metabolism, membrane structure, protein synthesis and energy metabolism. • Accumulation of compatible solutes.
Osmotic stress (Moat et al., 2002)	High Osmolality • Increase in k+ ion influx i.e uptake systems: trk, kdp and kup. • Decrease in intracellular putrescine levels due to increased excretion. • Synthesis of glutamate by two enzymes: glutamate dehydrogenase (gdh) and glutamate synthase (gs). • Accumulate the disaccharide trehalose Low Osmolality • Stretching of the cell envelope and activate stretch-activated channels. • Increase the permeability of the membrane • Synthesis of complex sugars, known as membrane-derived oligosaccharides (mdos).
Nutrient stress (Moat et al., 2002)	• Inducing the expression of protein involved in starvation-stress response (SSR). • Accumulation of at least two cellular nucleotides: cyclic 3,5-adenosine monophosphate (cAMP) and guanosine 3, 5-bis(diphosphate). • Two alternative sigma factors and σ^E encoded by the rpoS and rpoE genes respectively are key SSR regulators. • Expression of new or higher-affinity nutrient utilization systems; the degradation of cellular RNA, proteins and fatty acids, the reduction in the number of ribosomes, altering of the amounts and types of lipid components in the cytoplasmic membrane, an increase in the relative amounts of LPS in the outer membrane of Gram-negative bacteria and the condensation of chromosomal DNA in order to protect it from damage.

Temperature stress (Phadtare, 2004)	High temperature • Increase the rate of synthesis of a set of proteins called heat shock proteins (hsps). • Protein dnaJ and dnaK, the RNA polymerase σ^{70} subunit (rpod), groES, groEL, protease and lysU induced. • Heat shock increases expression of the σ^{H} regulon in σ^{H} levels, which in turn increases expression of the σh target genes. Lower temperature • The extracytoplasmic response pathways involve two partially overlapping signal transduction cascades: the σ^{E} and cpx systems. • Increased stability of RNA and DNA secondary structures • Reduced efficiency of replication, transcription and translation
pH and acid stress (Saito and Kobayashi, 2003)	• Induce the acid tolerance response (ATR). • Full induction of the ATR results in the increased expression of synthesized or existing proteins called acid shock proteins • Mg^{+2}-dependent proton translocating ATPase system important to acid tolerance in some organisms • Uses an arginine deiminase (ADI) pathway to generate ATP during acid stress. • Produces a powerful urease, a nickel-containing metalloenzyme that converts urea to carbon dioxide and ammonia.
Oxidative stress (Moat et al., 2002)	• Controlled by two major transcriptional regulators OxyR and SoxRS (Cabiscol et al., 2010). The OxyR regulon induced by H2O2 and the SoxRs induced by superoxid • *E. coli* produces a cytoplasmic Mn-SOD (SodA) and Fe-SOD (SodB) that protect DNA and proteins

	from oxidation. • A periplasmic Cu/Zn-SOD (SodC) protects the periplasmic and membrane constituents from exogenous superoxide. • NADH oxidase catalyzes the direct four-electron reduction of oxygen to water • A superoxide reductase system eliminating superoxide without the formation of molecular oxygen.

The autoaggregation phenomenon observed in *P. putida* CP1 can be related to the presence of a cellular stressor. Two major categories of cellular stressor are present in environmental pollutants. The first one is solutes that are ubiquitous and pervasive in the cellular environment. They are partitioned in the aqueous phase of all macromolecular systems and induce cellular water stress via their chaotropic properties. Second are the pollutants that partition into the hydrophobic domains of macromolecules and lipid bilayer. They disorder the macromolecules and lipid bilayer structures via chaotropic actions (Bhaganna *et al.,* 2010).

Chaotropic solutes weaken electrostatic interactions in biological macromolecules and influence water availability without having a major impact on cell turgor. Chaotropic compounds perturb macromolecule–water interactions, destabilize cellular macromolecules and inhibit growth. In *Pseudomonas putida*, they are potent mediators of water stress. The examples of chaotropic solutes are LiCl, ethanol, urea, ethylene glycol, phenol, guanidine hydrochloride and benzyl alcohol (Hallsworth *et al.,* 2003). Examples of hydrophobic substances are hydrocarbons, pesticides and diverse industrial chemicals. They introduce stress to biological systems and thereby affect their ability to mediate element and energy cycling. Such chemically diverse compounds may have distinct toxic activities for cellular systems and they may also share a common mechanism of stress induction mediated by their hydrophobicity (Bhaganna *et al.,* 2010).

The sustainability of the environment is threatened by pollution by persistent and non-persistent hydrophobic substances (Bhaganna *et al.,* 2010). Hydrophobic substances are well known for both their toxicity and their interference with the structural interactions of cellular macromolecules and lipid bilayers (Sikkema *et al.,* 1995). The degree of hydrophobicity of a compound is traditionally expressed as its partition coefficient in an octanol: water mixture, log $P_{\text{octanol–water}}$. Environmentally persistent compounds with a log P>3, and non-persistent molecules such as benzene (log $P \approx 2–3$), can bioaccumulate along natural food chains and have adverse effects on animal and human health and reproduction (Kelly *et al.,* 2007).

For substances with a log $P \leq 1.95$, there was a direct relationship between chaotropicity and hydrophobicity. For substances with a log P higher than 1.95, no chaotropic activity was detected. There is accordingly an inverse correlation between the polarity and chaotropicity of a compound. However, at log P values >1.95, hydrocarbons are insufficiently soluble to exert detectable chaotropic activity from within the aqueous phase of macromolecular systems (Bhaganna *et al.,* 2010). It has been proved that it is not the chemical structure of the solvent, but the concentration to which it accumulates in the cell membrane that plays a crucial role in determining toxicity. The greater the degree of the solvent accumulates in the membrane, the higher its toxicity (Sardessai and Bhosle, 2002).

In this study, *P. putida* CP1 was exposed to mono-chlorophenol. The concentrations that induced the formation of aggregates were between 0.5- 2 mM. This is the range of magnitude at which a compound with a log P of 2-2.5 would be expected to inhibit growth of *P. putida,* if its mode of action was as a hydrophobic stressor that ultimately induces a (chaotropicity-mediated) water stress (Bhaganna *et al*., 2010). Aggregation of the cells could be interpreted as a stress response that enabled the organism to withstand the environmental toxic shock.

1.3.1 Mechanisms of gene regulation under stress

Bacteria sense their environment through signalling proteins involved in two-component systems (TCS) which constitute the major signal trasduction system in bacteria (Alm *et al.,* 2006). 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 (Galperin, 2005).

Two-component signalling systems

Two-component systems are widely used by bacteria to regulate gene expression in response to environmental stimuli. Two-component systems respond to a variety of environmental signals and regulate numerous functions, including cell division, sporulation, motility, metabolism, communication, virulence and stress adaptation (Kivistik *et al.,* 2006). Prokaryotes generally use a simple phosphotransfer scheme, whereas phosphorelays and hybrid kinases dominate two-component signalling in eukaryotes (West and Stock, 2001). Phosphotransfer-mediated signaling pathways allow cells to sense and respond to environmental stimuli. The typical prokaryotic two-component system constitutes a membrane- bound histidine kinase and a response regulator (Figure 1.8). Histidine kinase contains a variable sensing domain and a conserved kinase domain. When sensing a stimulus, the sensor histidine kinase is activated and autophosphorylates at a conserved histidine (His) and influences gene expression by phosphorylating its cognate response regulator at a conserved aspartate (Asp). The response regulator is usually a DNA- binding transcription factor, which undergoes conformational changes upon phosphorylation that controls the expression of target genes (Quaranta *et al.,* 2009).

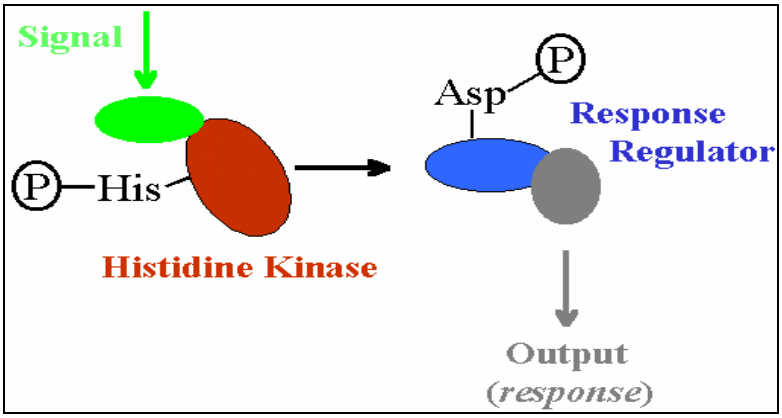


Figure 1.8 Two- component phosphotransfer scheme (West and Stock, 2001).

Sigma factors

In bacteria, alterations in gene expression are often controlled at the transcriptional level through changes in associations between the catalytic core of RNA polymerase and the different sigma factors present in a bacterial cell. Sigma factors are dissociable subunits of prokaryotic RNA polymerase. They control several iron- uptake pathways, tolerance to several stresses, alginate biosynthesis, expression of virulence factors and expression of outer-membrane porins.

Sigma factors are classified into two main protein families, σ^{54} and σ^{70} families (Figure 1.9) based on literature regarding *P. aeruginosa* (Boor 2006; Potvin *et al.*, 2007). Subunits comprising the σ^{54} family are often commonly referred to as σ^{N}. In addition to regulating nitrogen metabolism in a number of organisms, σ^{N} dependent genes also contribute to a diverse array of metabolic processes. Both *P. putida* KT2440 and *P. aeruginosa* specify 22 σ^{54}dependent transcriptional regulators. Many of the σ^{54} dependent regulators in KT2440 appear to belong to two-component systems and posses a domain that can be phosphorylated by a sensor-kinase protein in the N-terminal section.

The sigma 70 family has two subcategories: i) the primary sigma factor RpoD (σ^{70}) which is involved in the transcription of housekeeping genes and direct transcription of genes important for bacterial growth and metabolism ii) the alternative sigma factors which play important roles in transcription of stress

related genes are divided into four groups based on conservation of their primary sequences and structures;

- RpoS (σ^{32}) activates expression of numerous genes required to maintain cell viability as the cell leaves exponential growth conditions and moves into stationary phases,
- FliA (σ^{28}) controls flagellin synthesis in *P. aeruginosa*. The mechanism of *fliA* transcription is still unknown but is suggested to be constitutive (Dasgupta *et al.*, 2003),
- RpoH (σ^{32}) controls the heat shock regulation in *E. coli*. The role of RpoH in *P. putida* is not fully clarified,
- Extracytoplasmic function (ECF) are conserved across both Gram-positive and Gram negative species and consist of a large, phylogenetically distinct subfamily within the σ^{70} family. Members of the ECF subfamily are distinct from the rest of the σ^{70} family in that they regulate a wide range of functions involved in sensing and reacting to conditions in the membrane, periplasm, or extracellular environment. *Pseudomonas putida* KT2440 is reported to have 19 ECF sigma factors (Boor 2006; Martínez-Bueno *et al.*, 2002).

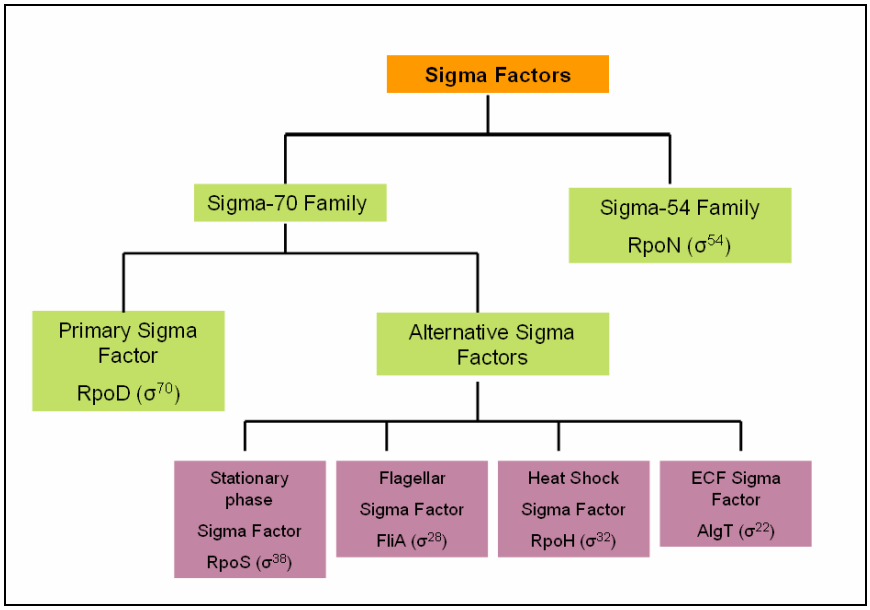


Figure 1.9 Sigma factors in *Pseudomonas aeruginosa* (Boor, 2006; Potvin *et al.*, 2007).

C-di-GMP

The formation of cell aggregates and biofilms in *P. aeruginosa* and other bacteria has been shown to involve cyclic-di-guanosine monophosphate (c-di-GMP) signalling. C-di-GMP-metabolizing proteins, di-guanylate cyclase and phosphodiesterase, which each possess one GGDEF and EAL domain as common domains. GGDEF domains are involved in synthesis and EAL domains involved in hydrolysis of c-di-GMP. The amino- acid sequences and protein structure in both domains share high similarity, even the proteins catalyze opposite biochemical reactions (Klebensberger *et al.,* 2007).

Levels of C-di-GMP are involved in a broad variety of cellular processes including the transition between the sessile and motile lifestyle in bacteria (Figure 1.10). The saturation of c-di-GMP directed processes was achieved by the expression of diguanylate cyclase leading to a high c-di-GMP production triggering the sessile lifestyle, favoring phenotypes such as extended biofilm formation that is associated with the adhesive matrix components, exopolysaccharides and fimbriae. While c-di-GMP depletion was achieved by the overexpression of a cytoplasmic phosphodiesterase led to the motility activity such as swimming, swarming and twitching motility. The overall architecture of GGDEF and/or EAL domain proteins (sensor output domain) is similar to that of sensor histidine kinases and methyl-carrier chemotaxis proteins due to the presence of an amino acid that can modulate the turnover of c-di-GMP in the same pattern as they modulate activities of histidine kinases (Römling *et al.*, 2005).

Cell aggregation is a stress response and serves as a survival strategy for *P. aeruginosa* strain PAO1 during growth on the toxic detergent Na-dodecylsulfate (SDS). This process involved the psl operon and is linked to c-di-GMP signalling. Transposon and site-directed mutagenesis revealed that siaD encoded a putative diguanylate cyclase involved in the biosynthesis of c-di-GMP and essential for SDS-induced aggregation (Klebensberger *et al.,* 2009).

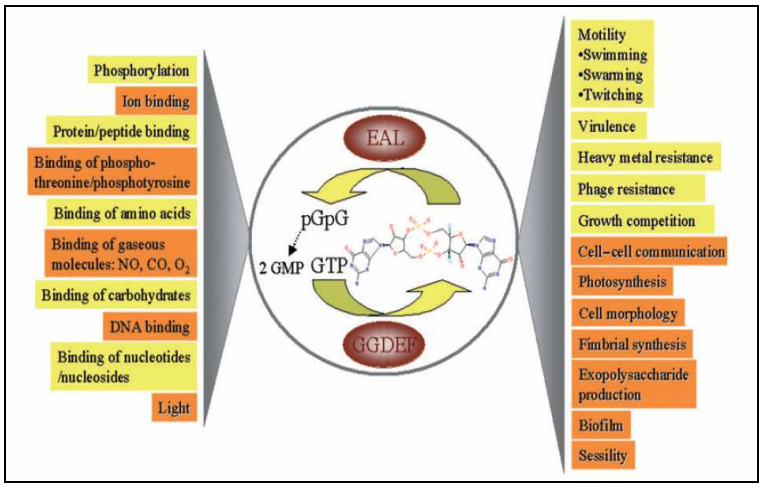


Figure 1.10 Known input signals and outputs of c-di-GMP metabolism (Römling *et al.*, 2005).

Chemotaxis and Flagellae

The first chemotaxis system of *P. aeruginosa* was described by (Moulton and Montie, 1979). Chemotaxis is a process whereby motile unicellular organisms direct their movement towards or away from gradients of specific substances either attractants or repellents. The process involves two separate systems including the chemo-receptors located in the bacterial cell membrane which are crucial for sensing the binding compounds and the transduction proteins which are involved in downstream signal transduction in response to the stimuli (Vladimirov and Sourjik, 2009). Molecular mechanisms of bacterial chemotaxis consist of methyl-accepting chemotaxis proteins (MCPs), cytoplasmic chemotaxis proteins (Che proteins) and flagellae (Figure 1.11).

Methyl-accepting chemotaxis proteins (MCPs) are transmembrane chemosensory proteins for environmental stimuli. They are reversibly methylated and function as homodimers. A cluster of chemotaxis genes has been located cheY, cheZ, cheA, cheB and cheJ. MCPs together with CheW modulate the autophosphorylation activity of CheA in response to temporal changes in stimulus intensity. MCPs are reversibly methylated at several glutamate residues by methyltransferase CheR and methylesterase CheB. CheB is another response regulator and receives the phosphoryl group from phosphorylated CheA. Phosphorylation of CheB increases

its methylesterase activity and the level of methylation of MCPs is controlled in response to environmental stimuli. This phenomenon called reversible methylation of MCPs, is important for chemical gradients sensing.

Changes in attractants or repellent concentrations are sensed by a protein complex consisting of transmembrane receptors (Tar, Tsr, Tap, Trg and Aer), an adaptor protein CheW and a histidine kinase CheA. Autophosphorylation activity of CheA is inhibited by attractant binding and enhanced by repellent binding to receptors. The phosphoryl group is rapidly transferred from CheA to the response regulator CheY. Phosphorylated CheY (CheYp) diffuses to the flagellar motors and changes the direction of motor rotation from counterclockwise to clockwise to promote tumbles. CheZ phosphatase, localized to sensory complexes through binding to CheA, ensures a rapid turnover of CheYp, which is essential to quickly readjust bacterial behavior. Receptor modification increases CheA activity and decreases sensitivity to attractants. Feedback is provided by CheB phosphorylation through CheA that increases CheB activity (Vladimirov and Sourjik, 2009).

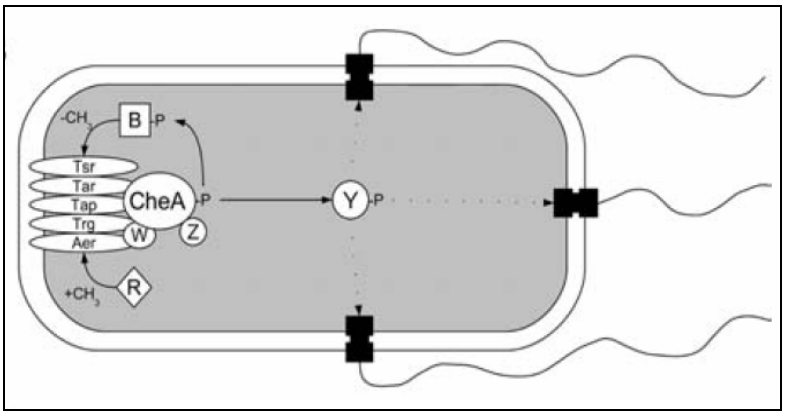


Figure 1.11 Chemotaxis pathway in *E. coli* (Vladimirov and Sourjik, 2009).

Flagellae are complex organelles generating motility. They are capable of propelling bacteria through liquids (swimming) and through highly viscous environments or along surfaces (swarming) (Linda, 2006). Six distinct types of bacterial surface motility are involved in bacteria including swimming, swarming, twitching, gliding, sliding and darting. Among these, swimming and swarming are flagella dependent (Harshey, 2003). Individual cells swim by flagellar rotation

and the number of flagella may differ depending on the species. For example *E. coli* and *S. typhimurium* can have up to 10 peritrichous flagellae (Harshey, 2003) while *P. aeruginosa* has a single polar flagellum (Dasgupta *et al.,* 2003) and an exception is *Burkholderia mallei* which is permanently immotile (Montie, 1998). *Pseudomonas putida* has been reported to have multiple polar flagellae and generally has between five and seven flagellae inserted at one end to form a tuft. The flagellar filaments have a conventional waveform and are usually 2 to 3 wavelengths long. Changes in direction are completed within 20 to 30 miliseconds (Harwood *et al.,* 1989).

Figure 1.12 shows the schematic representation of a typical flagellar assembly. The major parts of a flagellum include the basal body, motor, switch, hook, filament, caps, junctions and export apparatus. The basal body incorporated MS ring, P ring and L ring. The flagellar motor is around 50 nm and the filament is usually around 5-10 μm long and 20 nm in diameter (Harshey, 2003). The motor comprises a stator and rotor with MotA and MotB proteins form the stators. In Gram-negative bacteria MotA and MotB function together as a complex that generates the rotation of the filament (Dasgupta *et al.,* 2004). The flagellar motor of *P. aeruginosa* involves additional Mot proteins, e.g. MotC, MotD and MotY. MotAB together with MotY contribute mainly to motility in liquids, whereas MotC and MotD are less important for swimming (Doyle *et al.,* 2004). The rotor is made up of FliG proteins and generates the torque required for movement. The switch functions to change direction of the rotation. The hook is considered as a universal joint. The filament is the long cylindrical part which is turned by the motor.

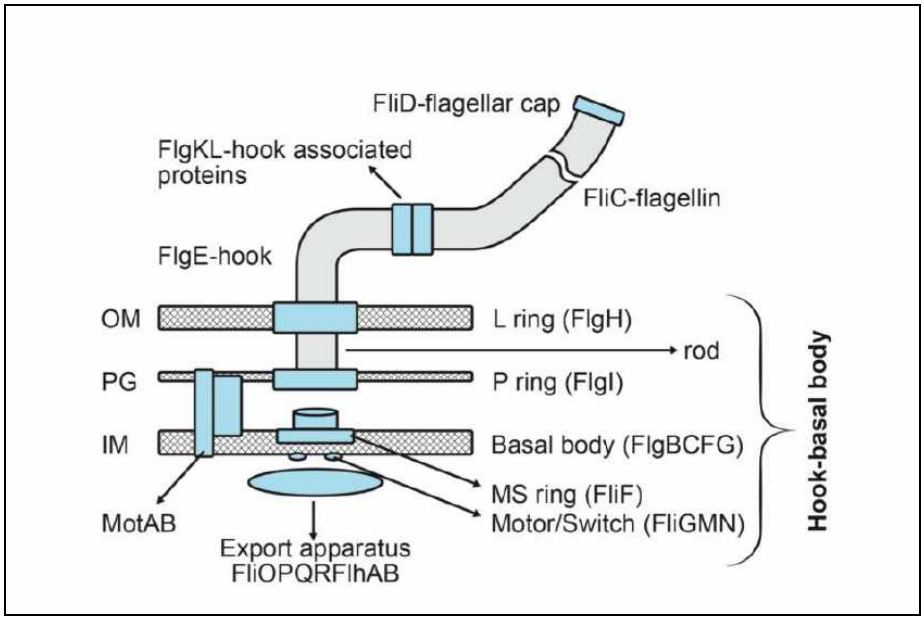


Figure 1.12 Flagellar assembly in *P. aeruginosa* (Jyot and Ramphal, 2008).

Flagellar rotations can be in a counter clockwise or clockwise direction. When flagellae rotate counter clockwise (CCW), the cell moves forward and the cell exhibits unidirectional swim called run. Meanwhile, when some of the flagellae rotate clockwise (CW) and others rotate CCW, cells start to tumble. Cells move in a way by alternating between run and tumble and the alternation is believed to be random. In a situation to exhibit chemotactic behavior, i.e., sensing the gradients of an attractant or repellent substrate, they change the frequency of tumble and run. When the cells sense increasing concentrations of attractants they tumble less frequently and swim longer times, whereas when they sense decreasing concentrations of attractants they tumble more and decrease run times (Berg, 2003). Contrary to swimming, swarming involves collective movement of the cells (Harshey, 2003). In order to swarm, cells become hyper-flagellated and elongated.

1.3.2 Molecular approaches used in understanding stress responses

Stress susceptibility and tolerance phenotypes could be assigned to specific genes but the links between gene and phenotype are often missing. Thus, it is important to use molecular studies to understand the fundamental concepts (Feder and Walser, 2005). Current advanced technologies have a great impact on molecular

biology. The available technologies known as transcriptomics, proteomics and metabolomics allow analysis of a large number of genes or proteins. Innovative high-throughput technologies allow measuring changes at various cellular levels (Figure 1.13).

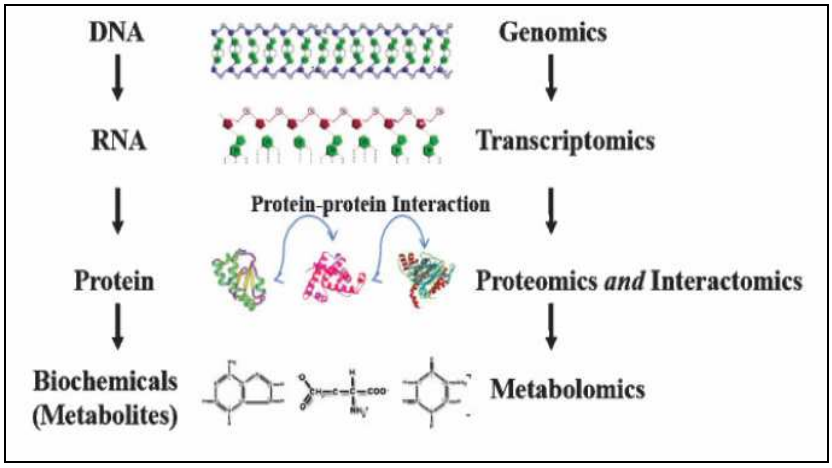


Figure 1.13 Schematic diagrams of various omics technologies targeting different types of cellular information (Zhang *et al.*, 2010).

Proteomics is used to profile all the proteins encoded by a given genome (Huan *et al.*, 2011). It can also be used to compare the protein profiles from different treatments and identify changes in protein expression levels using mass spectrometry and software databases (Lemos *et al.*, 2010). Two-dimensional electrophoresis (2-DE)-based proteomics remains useful in resolving protein extracts from organisms due to its high resolution and low expense (Choi *et al.*, 2012; Liu *et al.*, 2012). Renzone *et al.*, (2004) pointed out that proteomic technologies are powerful tools to study the physiological response of bacteria to various environmental stress conditions. However, neither the 2D-PAGE nor the LC-MS approach is close to saturating identifications in even small bacterial proteomes. Normally only 20–40% of the proteome can be detected and do not provide quantitative information (Zhang *et al.*, 2010).

Metabolomics can be used to characterize metabolic profiles in various biological systems (Lindon *et al.*, 1999; Daviss, 2005). Microbial metabolomics could be a powerful tool in understanding microbial metabolism and bridging the phenotype–genotype gap since it amplifies changes in the proteome and provides

a better representation of the phenotype of an organism (Bundy *et al.*, 2009). Similar to proteomics, comparisons on metabolomes can discover metabolic responses induced by stressors in organisms (Wu *et al.*, 2005; Pedras and Zheng, 2010). However, because metabolomics is a high-resolution analysis, it requires controllable experimental conditions, such as growth conditions and surrounding environment to validate methods. Therefore, checking method validation is one important application of microbial metabolomics, especially sample preparation and metabolite measurement (Putri *et al.*, 2013).

Transcriptomics allows quantitative measurements of the dynamic expression of mRNA molecules and their variation between different states at the genome scale, thus reflecting the genes that are being actively expressed at any given time. Several popular high-throughput transcriptomic strategies include;

- differential display or serial analysis of gene expression (SAGE),
- cDNA or oligonucleotide microarrays,
- next-generation sequencers for direct sequencing of the cDNA converted from whole transcriptomes and
- direct RNA sequencing (DRS) technology.

Transcriptomic technologies have been used in various microbial systems to explore genome-wide transcriptional activity and to define regulons, operon structure and perform comparative genotyping (Ye *et al.*, 2001). Understanding the composition of the expressed genome, information of what genes are expressed and the extent to which they are represented provides three different types of information;

- the set of genes expressed at a given time reflects cellular processes,
- help to elucidate the function of characterized genes and uncharacterized genes,
- the patterns and phenomena identified within the expression data can be used to address the complexities of the regulation of gene expression (Horak and Snyder, 2002).

Microarrays are among the common tools in transcriptomics used to understand the phenotypic changes in bacteria at a genetic level. It has become a widely used technology in molecular biology research to measure gene expression. Gene expression microarrays are used to screen for sets of genes involved in various biological processes. Genes identified on a DNA microarray become important for further study involving a larger sample set. Microarrays are required to validate that up- or down-regulation of the identified genes either is a direct consequence of the experimental conditions or if the regulation of that particular gene is instead related to some other cellular processes (Grimmond and Greenfield, 2001). It is a powerful tool for viewing the expression of thousands of genes simultaneously in a single experiment (Gao *et al.,* 2007).

DNA microarrays can be applied to investigate a single change in gene expression that might be the key to a given alteration in phenotype or to have an overview pattern of gene expression in order to understand the genetic regulatory system (Schulze and Downward, 2001). They allow understanding of various aspects of microbiology from the study of gene regulation and bacterial response to environmental changes to genome organization and evolution. They are useful in taxonomic and environmental studies because they permit the simultaneous monitoring of the expression of all genes in any bacterium (Ehrenreich, 2006). However, compared to classical expression measuring assays such as Northern blotting, analyzing gene expression data from microarrays is more complex due to the considerable amounts of data produced (Grimmond and Greenfield, 2001).

Three sources of error might occur at different stages of the experimental process including the microarray fabrication, the microarray experiment and the interpretation of data analysed. The validation strategies are important to alleviate the error. One of the widely used validation techniques is the quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). qRT-PCR is a technology used to quantify mRNA for gene expression profiling. The results obtained by real-time RT-PCR are enumerated by two strategies: the standard curve method (absolute quantification) and the comparative threshold method (relative quantification) (Jahn *et al.,* 2008). The qRT-PCR is a desirable method because

the assay can be completed in a short period. It is simple to prepare, highly sensitive and specific and is inexpensive (Filion, 2012).

Global analysis of mRNA abundance via genomic arrays such as transcriptional profiling is one approach to identify the genes involved in organisms experiencing environmental stress (Bar-Joseph *et al.,* 2012). Genomic cDNA or oligonucleotide arrays have considerable value in evolutionary studies of stress. Transcriptomics can be the method of choice if, for example, the objective is to identify those genes whose transcription might be linked or co-regulated under stress, or to deduce the nucleotide sequence responsible for high or low levels of gene expression. It may also guide researchers about already well-known genes that have been overlooked in designing candidate gene studies (Feder and Walser, 2005). Due to financial constraints and availability issues, very few studies employ more than one omics technology in any single investigation. However in the case of bacteria, the transcriptomic approach can be more powerful in the study of expression regulatory systems due to the smaller size of the genome (Zhang et al., 2010).

1.4 Aims and objectives of the project

Earlier studies in our laboratory had shown that *Pseudomonas putida* CP1 is a very effective organism for use in bioaugmentation products in the biological degradation of toxic chemicals (Farrell and Quilty, 2002) and also in the degradation of fats, oils and greases (Tzirita and Quilty, 2012). The fact that the organism autoaggregates when grown under certain conditions was shown by McLaughlin *et al.*, (2006) to offer a particular advantage to the organism when used in bioaugmentation products. However, autoaggregation by the organism had not previously been closely studied. This study therefore was designed to investigate phenotypic and genotypic changes in the organism associated with autoaggregation so that the use of the organism in the biological treatment of waste streams could be optimised.

The objectives of the study were to;

- characterize *P. putida* CP1 and compare it to *P. putida* KT2440,
- grow *P. putida* CP1 on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol, 200 ppm 4-chlorophenol and 0.5% (w/v) fructose to induce autoaggregation and study the kinetics of substrate removal,
- monitor phenotypic changes associated with autoaggregation including the aggregation index and the hydrophobicity of the cells, production of extracellular polymeric substances and changes to the lipopolysaccharide structure and
- identify changes in gene expression associated with the autoaggregation of *P. putida* CP1 by expression profiling studies at the mRNA level using a *P. putida* KT2440 Genome Oligonucleotide Array.

2.0 MATERIALS AND METHODS

2 Materials and methods

2.1 Bacterial cultures

Pseudomonas putida CP1 was isolated from soil. The organism was maintained at 4°C on nutrient agar and subcultured every two weeks. For the chlorophenol studies, the strain was maintained on chlorophenol agar. The agar was prepared by adding 1% bacteriological agar (Oxoid) to minimal medium (described below). Chlorophenol was added to the cooled agar to give 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol and 200 ppm and 50 ppm 4-chlorophenol as required (Farrell and Quilty, 2002). The cultures were maintained at 4°C and subcultured every month.

Pseudomonas putida KT2440 was obtained from the DSMZ-German Collection of Microorganisms and Cell Cultures GmBH, Braunschweig, Germany. The organism was maintained at 4°C on nutrient agar and subcultured every two weeks.

Escherichia coli 498, *Bacillus subtilis* DSMZ 10 and *Enterococcus faecalis* DSMZ 2570 were obtained from the DSMZ-German Collection of Microorganisms and Cell Cultures GmBH, Braunschweig, Germany and were used as control organisms for the cell characterization.

For long term preservation, bacteria were stored in glycerol. 800 µl of an overnight culture grown on nutrient broth was added to 200 µl of 80% (v/v) glycerol solution and mixed in an Eppendorf tube. The glycerol stocks were stored at -80°C immediately, for up to three years.

2.2 Chemicals

All chemicals used were of analytical grade and were obtained from Sigma Aldrich Chemical Company (UK) unless otherwise stated.

2.3 Buffers and solutions

Sodium Phosphate buffer

Sodium phosphate buffer (0.01 M) was prepared by dissolving 1.42 g/L Na_2HPO_4 and 1.2 g/L NaH_2PO_4 separately in distilled water. A total of 700 ml of dissolved Na_2HPO_4 was combined with 500 ml NaH_2PO_4 to give pH 7.0.

Potassium Phosphate buffer

Potassium phosphate buffer was prepared by dissolving K_2HPO_4 (104.5 g/L) and KH_2PO_4 (72.3 g/L) in distilled water. A total of 497 ml of dissolved K_2HPO_4 was combined with 503 ml KH_2PO_4 to give pH 6.8.

Tris-EDTA buffer

Tris-EDTA buffer was prepared by dissolving Tris (10 mM) and ethylenediaminetetra acetic acid (EDTA) (1 mM) in distilled water. The pH of the resulting solution was then adjusted to pH 8.0 with HCl (1M).

50X TAE DNA running buffer

50X Tris base, acetic acid and EDTA (TAE) DNA running buffer was prepared by dissolving Tris (2 M) and EDTA (0.05M) in distilled water and adjusting the pH to 8.0 with glacial acetic acid (1M). The 1X TAE DNA running buffer was prepared by combining 20 ml of 50X TAE buffer in 980 ml distilled water and was stored at room.

6X DNA Loading Dye

The loading dye was prepared by dissolving bromophenol blue (0.25 g) and sucrose (40 g) in 100 ml distilled water.

1X FA running buffer

1X FA gel running buffer was prepared by dissolving 100 ml 10X 3-(N-morpholine)-propanesulfonic acid (MOPS) and 20 ml formaldehyde (37% v/v) in 880 ml distilled water. 10X MOPS was prepared by dissolving 41.8 g 0.2M MOPS, 4.1 g 0.5 M NaOAc and 3.72 g 0.01 M EDTA in 1 L distilled water. The final solution was pH 7.

2X RNA loading buffer

2X RNA loading buffer was prepared by dissolving 0.72 ml formamide, 0.16 ml 10X MOPS, 0.26 ml formaldehyde, 0.1 ml glycerol, 0.08 ml saturated bromophenol blue and 0.18 ml distilled water. To make saturated bromophenol, solid bromophenol was added to distilled water with mixing. Bromophenol were added until no more will dissolve. Centrifuge the mixture to pellet the bromophenol and carefully pipette the saturated supernatant.

2X SDS-PAGE Sample loading buffer

Sample buffer was prepared by adding 2 ml 0.5 M Tris/HCl (pH 6.8), 1.6 ml sodium dodecyl sulfate (SDS) (20% w/v), 3.2 ml glycerol, 0.8 ml β-merkaptoethanol and 1.6 ml (1%) bromophenol blue to 6.8 ml of distilled water. All samples were diluted 1:4 and heated to 95°C for 5 minutes, prior to loading the SDS-PAGE gel.

SDS-PAGE Running (Towbin) buffer

This solution was prepared by dissolving 3.02 g Tris base, 14.4 g glycine and 1 g SDS in 900 ml distilled water.

Fixation buffer

Fixation buffer was prepared by adding 50 ml methanol, 10 ml acetic acid glacial and 50 µl formaldehyde to 40 ml distilled water.

Sensitizing buffer

Sensitizing buffer was prepared by combining 0.02 g $Na_2S_2O_3$ and 100 ml distilled water.

Silver nitrate staining solution

Silver nitrate staining solution was prepared by adding 0.2 g silver nitrate and 50 µl formalydehyde to 100 ml distilled water. This solution was prepared in a bottle wrapped with foil in order not to be exposed to light.

Developer solution

Developer solution was prepared by combining 6 g Na_2CO_3, 2 ml $Na_2S_2O_3$ and 50 µl formaldehyde in 100 ml distilled water.

Stop solution

Stop solution was prepared by combining 50 ml methanol, 10 ml acetic acid glacial and 40 ml of distilled water.

2.4 Media and gels

Pseudomonas Minimal Medium

Degradation studies were carried out in *Pseudomonas* minimal medium which was prepared as described by Goulding *et al.*, (1988). The trace salt solution was prepared separately in distilled water and stored in a dark bottle for 6-8 weeks.

K_2HPO_4	4.36 g
NaH_2PO_4	3.45 g
NH_4Cl	1.0 g
$MgSO_4.6H_2O$	0.912 g
Distilled water	1000 ml
Trace salts solution	1 ml

*The medium was adjusted to pH 7.0 with 0.2 M NaOH before autoclaving.

Trace Salts Solution

$CaCl_2.2H_2O$	4.77 g
$FeSO_4.7H_2O$	0.37 g
$CoCl_2.6H_2O$	0.37 g
$MnCl_2$	0.10 g
Na_2MoO_4	0.02 g
Distilled water	100 ml

Mono-chlorophenols were added to the *Pseudomonas* minimal medium following sterilisation. A range of mono-chlorophenol concentrations from 50 ppm to 200 ppm were used. The additional carbon sources, glucose 0.5% (w/v) and fructose 0.5% (w/v) were separately sterilized by filtration (Millex®GP, Millipore 0.22 µm filter) and added to the sterilized minimal media.

Chlorophenol agars were prepared by the addition of bacteriological agar, at a concentration of 1% (w/v) to the minimal medium. Following sterilisation, the agar was allowed to cool and the mono-chlorophenol was added to the agar to give the appropriate final concentration.

Preparation of gel agarose

Agarose gel, concentration 1% (w/v), was prepared by the addition of 0.3 g agarose (Sigma) to 30 ml of 1X TAE buffer. The mixture was heated up in microwave for 2 minute until the agarose was sufficiently dissolved. The solution was allowed to cool down and then 2 µl (10 mg/ml) ethidium bromide (ETBR) final concentration 0.4 µg/µl was added.

Formaldehyde agarose (FA) gel

Formaldehyde agarose (FA) gel was prepared for electrophoresis of RNA. The agar was prepared by adding 0.48 g agarose and 4 ml 10X MOPS in 36 ml distilled water. The mixture was heated up in microwave for 2 minute until the agarose was sufficiently dissolved. The solution was allowed to cool down and then 0.72 ml formaldehyde and 2 µl (10 mg/ml) ETBR (final concentration 0.4 µg/µl) was added then poured into the gel cast until fixed.

SDS-PAGE Resolving gel (15%)

The resolving gel used in SDS-PAGE was prepared by adding 2.5 ml 1.5M Tris HCl (pH 8.8), 100 µl SDS (10% w/v), 5 ml acrylamide bisacrylamide (30%/ 0.8% w/v) and 50 µl ammonium persulphate (APS) (10% w/v) to 2.3 ml distilled water. 10 µl of N, N, N, N-Tetramethyl-ethylenediamine (TEMED) was mixed with the

solution to catalyse the polymerization of acrylamide. The APS was freshly prepared on the day of use.

SDS-PAGE Stacking gel (4%)

The stacking gel used in SDS-PAGE was prepared by adding 1.26 ml 0.5M Tris-HCl (pH 6.8), 50 µl SDS (10% w/v), 0.66 ml acrylamide bisacrylamide (30%/ 0.8% w/v) and 50 µl APS (10% w/v) to 3 ml distilled water. TEMED (5 µl) was mixed with the solution to set the gel. The APS was freshly prepared on the day of use.

2.5 pH measurement

The pH was measured using an Orion Triode^{TM} pH electrode Model 91-57BN connected to an Orion bench top pH/ISE meter (model 920A).

2.6 Dry weight measurement

Aliquots of culture were filtered through 0.45 µm Pall filters. Filters were dried at 85°C for 2 h and then reweighed (Fakhruddin and Quilty, 2007).

2.7 Cell characterisation

Gram staining

The Gram stain was carried out on 24 hour cultures according to the method described by Harley and Prescott (1990). Gram positive cells appeared purple and Gram negative cells red.

Controls: Positive – *Bacillus subtilis* DSMZ 10
Negative – *Pseudomonas putida* CP1

Spore stain

A smear of a 48 hour culture was prepared and the spore stain was carried out as described by Harley and Prescott (1990). Endospores were detected microscopically using x100 oil immersion lens. The stained spore appeared green and the vegetative cell red.

Controls: Positive – *Bacillus subtilis* DSMZ 10
Negative – *Pseudomonas putida* CP1

Motility test

A 24 hour culture was examined microscopically in "hanging drop" preparations, using a x100 oil immersion. A "hanging drop" slide was prepared by placing a loopful of the bacterial suspension onto the centre of a coverslip. A depression slide onto which a ring of Vaseline had been spread around the concavity was lowered onto the coverslip, with the concavity facing down over the drop. When a seal had formed, the hanging drop slide was turned over and examined under the microscope.

Controls: Motile: *Pseudomonas putida* CP1
Non-motile: *Enterococcus faecalis* DSMZ 2570

Catalase activity

A loopful of 24 hour culture was transferred into a drop of 3% (v/v) hydrogen peroxide. Effervescence, caused by the liberation of free oxygen as gas bubbles, indicated a positive result.

Controls: Positive – *Bacillus subtilis* DSMZ 10
Negative – *Enterococcus faecalis* DSMZ 2570

Oxidase activity

Oxidase strips (Oxoid) impregnated with NNN'N' tetramethyl-p-phenylene-diamine dihydrochloride were used for the detection of bacterial cytochrome oxidase enzyme. The formation of a purple colour within 5-10 seconds indicated oxidase positive results.

Controls: Positive – *Pseudomonas putida* CP1
Negative – *Escherichia coli* 498

API 20NE system for Gram negative bacteria

The API identification system API 20NE (BioMerieux, Marcy-l' Etoile, France, http://www.biomerieux.com) for non-enteric Gram-negative rods was used according to the manufacturer's instructions. An overnight nutrient broth culture (10 ml) was harvested and washed twice with sterile phosphate buffer solution (4000 rpm for 15 minutes). The pellet was resuspended in 0.85% (w/v) NaCl (10 ml). The suspension was then used to inoculate a portion of the tests. For assimilation tests, 200 µl of this suspension was used to inoculate auxiliary medium supplied by the manufacturer and this was then used to inoculate the remaining tests. The strips were read and interpreted after incubation at 30°C for 24 hours. Identification was obtained using the Analytical Profile Index: the pattern of the reactions obtained was coded into a numerical profile. On a results sheet the test were separated into groups of three and a number 1, 2 or 4 was indicated for each. By adding the numbers corresponding to positive reactions within each group, a 7-digit number was obtained which constituted the numerical profile. Identification was then obtained using the identification software (API 20NE V6.0 database, apiweb.biomerieux.com) by manually entering the 7-digit numerical profile. The profile was listed along with the percentage of identification – an estimate of how closely the profile corresponded to the taxa relative to all the other taxon in the database and the T index – an estimate of how closely the profile corresponded to the most typical set of reactions for each taxon. The appearances of the positive and negative reactions are shown in Figure 2.1.

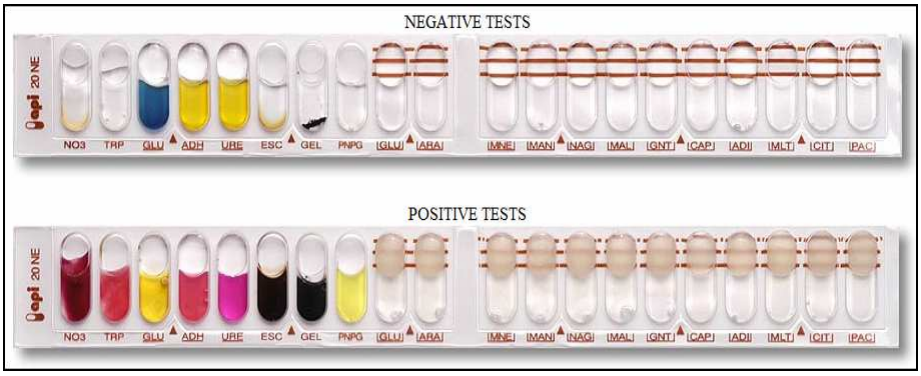


Figure 2.1 Negative and positive reactions on API 20NE.

2.8 Culture conditions and biodegradation studies

P. putida CP1 was grown up overnight in nutrient broth, centrifuged at 4000 rpm for 10 minutes and washed twice with 0.01 M sodium phosphate buffer, pH 7.0. The pellet was re-suspended to give an optical density at 660 nm of approximately 0.7. Biodegradation experiments were performed in 250 ml conical flasks containing (5% v/v) culture in 100 ml minimal medium following inoculation and addition of substrate. The cultures were incubated aerobically at 150 rpm and 30°C. Samples were aseptically removed at regular intervals and analysed for pH, chlorophenol concentration, reducing sugar and other parameters. Uninoculated control flasks were run in parallel.

Quantitation of chlorophenol concentrations

Chlorophenol concentrations were measured using the 4-aminoantipyrene colorimetric method based on the procedure detailed in Standard Methods for the Examination of Water and Wastewater (Clesceri *et al.,* 1998). Samples were centrifuged at 13,000 x g for 10 minutes and the resulting supernatants were diluted to bring the concentration into the range of the standard curve. The standards prepared were in the range 0-0.05 mg of chlorophenol in 10 ml distilled water. The samples and standards were treated by placing 10 ml in a test tube and adding 0.25 ml 0.5 N NH_4OH. The pH was then adjusted to 7.9 ± 0.1 with approximately 200 µl potassium phosphate buffer (pH 6.8). 100 µl of 2% (w/v) 4-aminoantipyrene solution was added and the tubes mixed well. The 100 µl of 8% (w/v) potassium ferricyanide was added and the tubes mixed well. The tubes were

allowed to stand for 15 minutes at room temperature. The absorbance was read at 500 nm and the concentrations were calculated from the standard curve (Figure 2.2).

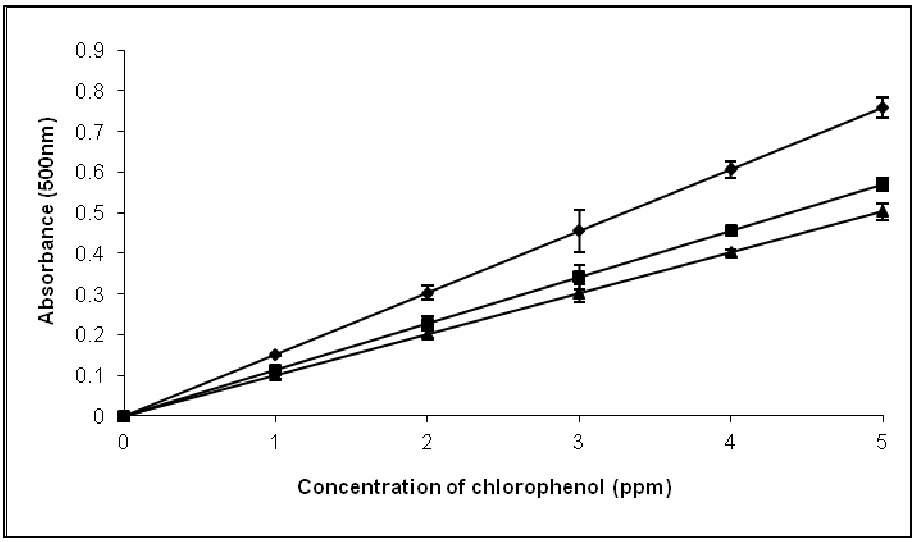


Figure 2.2 Standard curve for determination of 2-chlorophenol (♦), 3-chlorophenol (■) and 4-chlorophenol (▲) concentrations using the 4-aminoantipyrene colorimetric method.

Substrate removal rate were calculated in terms of substrate degraded per unit time from the end of the lag period (L) to the time of substrate disappearance (Td) and expressed as ppm/h. The lag period was defined as follows: in a plot of chlorophenol concentration versus time, the straight line was extrapolated to the initial chlorophenol concentration (S_0) and the intercept on the time axis was taken to be the length of the lag period. The time of degradation (T_d) was defined as the time at which no chlorophenol concentration was detected in the culture medium. The specific substrate removal rate was determined by the value of substrate removal rate divided by the biomass weight (g/L).

Reducing sugar estimation

Reducing sugars were determined by the dinitrosalicylate (DNS) colorimetric method (Miller, 1959). For the preparation of DNS reagent, 1 g 3, 5-dinitrosalicylic acid, 30 g potassium sodium tartarate, 50 ml distilled water and 16 ml 10% (w/v) sodium hydroxide were mixed together and warmed to dissolve. When cool, the volume was made up to 100 ml with distilled water. Reducing

sugar stock solutions were prepared by dissolving 2.0 g reducing sugar in 100 ml distilled water and stored at 4°C. Reducing sugar standard solutions were prepared by diluting 10 ml of stock reducing sugar solution to 100 ml volume. This gives a final concentration of 2 mg/ml. Bacterial cells were removed from samples by centrifugation (13,000 x g for 4 minutes) before assaying. One ml of standard and suitably diluted samples and 1 ml of water was placed in a test tube. 2 ml of DNS reagent was added. The tubes were capped and placed in a boiling water bath for 10 minutes. The tubes were then readily cooled and 10 ml of water added to each. Absorbance was read at 540 nm. The fructose concentrations were determined from the calibration curve of corresponding known standard concentrations (Figure 2.3).

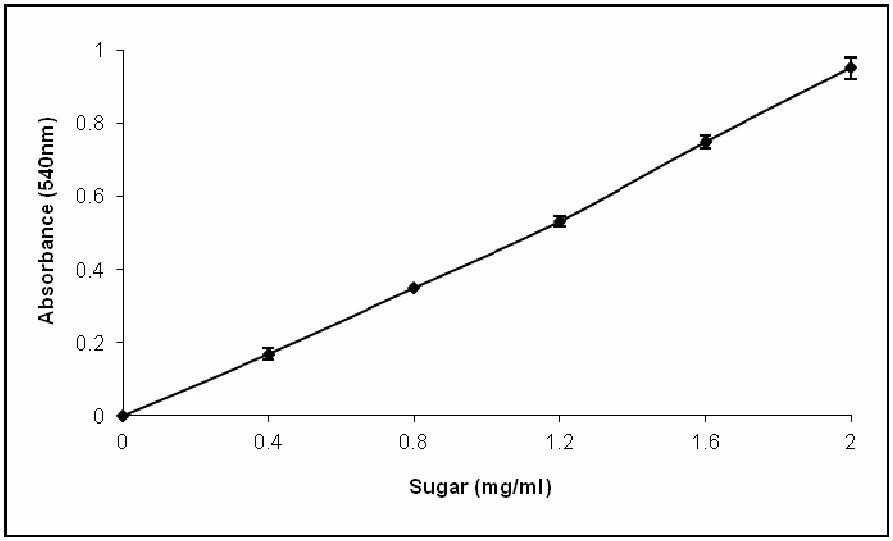


Figure 2.3 Standard curve for reducing sugar estimation using the dinitrosalicylate colorimetric method.

2.9 Microscopic studies

The stained planktonic cells were visualised on glass slides and aggregates were visualised using cavity glass slides. Aggregates for observation with the confocal laser scanning microscope (CLSM) were mounted in 1.2% (w/v) agarose gel. All preparations were sealed by using commercial nail varnish.

The Live/Dead Baclight Bacterial viability kit (Molecular Probes, Invitrogen) was used to determine the viability of bacterial cultures. An aliquot (1 ml) of culture

was added to 3 µl of the Live/Dead stain mixture SYTO 9 and propidium iodide (PI) (1:1). The tubes were gently tapped and incubated in the dark at the room temperature for 15 minutes. Stained samples (20 µl) were transferred to glass slides and covered with a cover slip (22 mm square) and observed using an epifluorescent microscope (Nikon T*i*-E) or a confocal laser scanning microscope (Leica DM IRE2 microscope connected to a Leica SP2 AOBS, Leica, Germany). The size of cells and aggregates was determined using phase contrast.

The epifluorescent microscope was fitted with three filter blocks with different excitation/emission wavelengths; FITC (490-525) TRITC (557-576) and DAPI (350-470). The fluorescence of SYTO 9 and PI was detected by excitation/emission at 480/500nm (FITC) for SYTO 9 and 490/635nm (TRITC) for PI. Live cells fluoresced green while dead cells fluoresced red. The individual images captured from FITC and TRITC filters were merged and the percentage of live/dead cells was calculated using the Nikon NIS-Elements software. Images were captured using the Nikon DS-U2 camera attached to the microscope.

For the visualization with CLSM, fluorescence of SYTO 9 and PI were detected by excitation/emission at 480/500nm (FITC) for SYTO 9 and 490/635nm (TRITC) for PI. The top, middle and bottom cross sections of each aggregate were captured with on average between 50-200 stacks and the images were processed with Leica Image Processing software.

Fluorochrome staining

The staining procedure was performed on aggregated bacterial cells to detect biochemical components as previously described by Chen *et al.,* (2007). In each test, aggregates were carefully collected from the sample suspension with a wide-mouthed pipette. The aggregates were kept fully hydrated during the staining procedure. FITC solution (10 g l^{-1}; 2.5 µl) was added first and left to incubate for 1 h. FITC stains all protein and amino-sugars of cells and EPS. This was followed by the addition of 10 µl of calcofluor white solution (Fluka) to stain the polysaccharides and incubated for 30 minutes. SYTO 63 (1.5 µl) was added to the aggregates and incubated for 15 minutes to stain the nucleic acid. The incubation

was carried out in the dark at room temperature. After each staining step, the aggregates were washed gently three times with PBS (Oxoid) for complete de-staining. Aggregates were transferred to cavity glass slides and covered with a cover slip (22 mm square) and observed with an epifluorescent microscope or Confocal laser scanning microscope (CLSM). Aggregates for observation under CLSM were mounted by embedding in agarose gel as described above. The fluorescence of calcofluor white was detected by excitation at 400 nm and from the emission width at 410–480 nm (blue). The FITC probe was detected by excitation at 488 nm and emission at 500–540 nm (green). The fluorescence of SYTO 63 was determined from excitation at 633 nm and emission at 650–700 nm (red). The individual images were captured from each filter and merged. The percentage of carbohydrate, protein and DNA was calculated using the Nikon NIS-Elements software.

2.10 Aggregate studies

Aggregate sizing

The epifluorescence microscopy (Nikon T*i*-E) was used with phase-contrast settings to determine the size of aggregates. The size was calculated using the object area measurement tool in Nikon NIS-Elements software.

Determination of aggregation index

The aggregation index (AI) of the cells was determined as the relative decrease of the optical density of a bacterial suspension at 660 nm after slow centrifugation at 4000 rpm for 2 minutes as described by Windt *et al.*, (2006). 10 ml of culture from each treatment was vigorously vortexed to disrupt the aggregates, and the OD_{total} of this suspension was determined. Another 10 ml of culture was centrifuged for 2 minutes at 4000 rpm without vortex treatment, providing $OD_{supernatant}$.

$$AI: [OD_{total}\text{-}OD_{supernatant}/OD_{total}].$$

Cell surface hydrophobicity assay (BATH)

The relative hydrophobicity was measured according to bacterial adherence to hydrocarbons (BATH) (Rosenberg *et al.*, 1980). 20 ml of culture was washed and suspended in 0.01 M sodium phosphate buffer pH 7.0. 0.3 ml of *n*-hexadecane (Sigma) were added to 3 ml of bacterial cell suspension (OD_{400}=0.6) in acid-washed test tubes (soak in 1% (v/v) sulfuric acid solution for overnight and rinse with deionised water). The suspension was vortex for 1 minute to ensure mixing and then left to stand for 15 minutes to allow separation of the two phases. The aqueous phase was carefully removed using a Pasteur pipette and the OD at 400 nm was read. The percentage of the cells bound to *n*-hexadecane was calculated using:

$$\textbf{BATH \%:}\ 100 \times \frac{\text{(OD 400 nm of washed cells - OD 400 nm of aqueous layer following extraction with } n\text{-hexadecane)}}{\text{OD 400 nm of washed cells}}$$

2.11 Extracellular polymeric substances (EPS) extraction

The extracellular polymeric substances (EPS) were extracted using the modification of the method described by Eboigbodin and Biggs (2008). Cells (10 ml) were harvested by centrifugation at 4000 rpm for 15 minutes at 4°C. The cell pellets were used for extraction of the bound EPS, and the supernatant was used for the free EPS extraction.

Bound EPS extraction

The cell pellets were washed twice with 0.9% NaCl to remove any traces of the media. The washed cells were resuspended in 1:1 volume of solution 0.9% NaCl and 2% EDTA then incubated for 60 minutes at 4°C. The supernatant was then harvested by centrifugation at 4000 rpm at 4°C for 30 minutes and then filtered through 0.45 µm membrane (Pall) as the EPS fraction for chemical analysis.

Free EPS extraction

After the initial harvesting by centrifugation, the collected supernatant was re-centrifuged at 4000 rpm for 30 minutes at 4°C to remove residual cells, and later the supernatant containing free E EPS was precipitated with 1:3 volume ethanol and stored at -20°C for 18 hours. Free EPS were then removed by centrifugation at 4000 rpm for 15 minutes at 4°C. The pellet was re-suspended in 10 ml ultra pure water and dialyzed against ultrapure water to remove the ethanol. Dialysis tubing of cellulose membrane (Sigma) was used for the dialysis process. The tubing was processed before every usage. In order to remove glycerin, tubing was placed under running water for 3 hours. Then the tubing was treated with 0.3% (w/v) sodium sulfide solution at 80°C for 1 minute to remove sulfur compounds. Later it was washed with hot water (60°C) for 2 minutes and followed by acidification with 0.2% (v/v) sulfuric acid and rinse with hot water to remove the acid.

Both bound and free EPS were stored at -20°C until needed for further analysis.

EPS biochemical characterization

Carbohydrate content was determined by the Dubois assay using glucose as the standard. Protein content was assayed using the Bradford method with bovine albumin serum (BSA) as the standard and nucleic acid was determined according to diphenylamine assay with calf thymus DNA as the standard. The sum of the amounts of total carbohydrate, proteins and DNA was considered to represent the total amount of EPS.

Dubois assay (Phenol/sulphuric acid method)

The test was conducted as described by Dubois *et al.,* (1956). 2 ml of sugar solution was added to a universal and 0.5 ml of 5% (w/v) phenol solution was added. Then 2.5 ml of concentrated sulphuric acid was added. The universals were allowed to stand for 10 minutes and later placed in a water bath at 30°C for about 20 minutes. The concentration of polysaccharides in the solution was

determined according to a calibration curve with glucose as the standard. The absorbance was measured at 490 nm. The calibration curve for Dubois assay is shown in Figure 2.4.

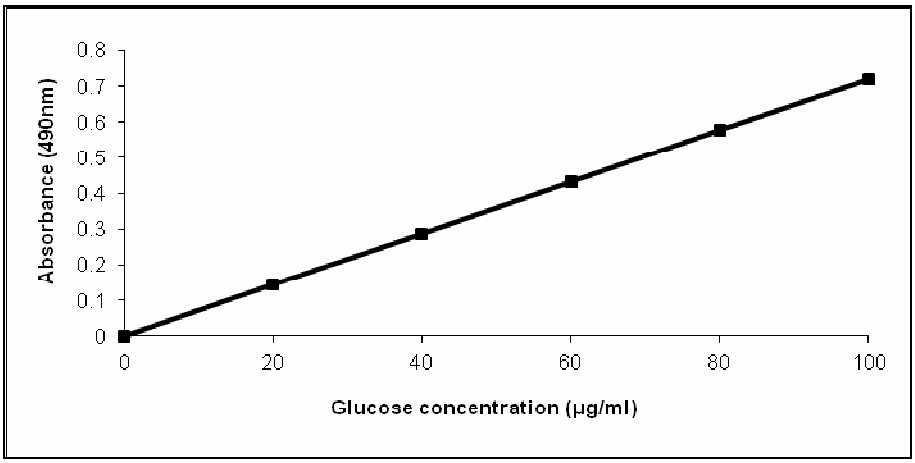


Figure 2.4 Calibration curve for the Dubois assay.

Bradford-Assay

The assay was performed as written according to Bradford (1976). 1 ml of Bradford reagent (Sigma) was added to 1 ml of sample and immediately vortexed. The mixture was left at room temperature for 5 minutes and the colour was measured at 595 nm. The concentration of the protein was determined by comparison to a standard curve with bovine albumin serum (BSA) from Sigma. The calibration curve for the Bradford assay is shown in Figure 2.5.

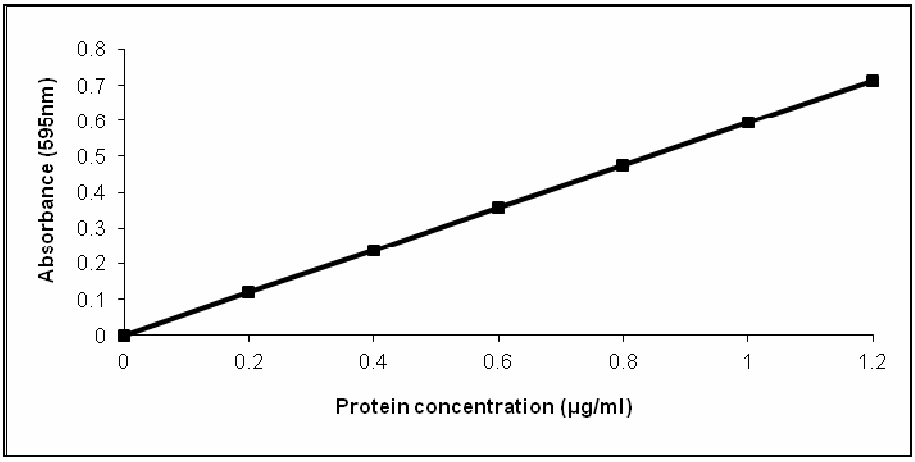


Figure 2.5 Calibration curve for the Bradford assay.

Test for DNA (Diphenylamine Assay)

The test to determine the DNA content of the sample was conducted according to Burton (1968). Diphenylamine reagent was prepared by adding 1.5 g diphenylamine to 100 ml glacial acetic acid and 1.5 ml sulphuric acid. On the day of usage, 100 µl acetaldehydes (1.6% v/v) were added to 20 ml of reagent. 2 ml of diphenylamine reagent was added to 1 ml of sample and left to incubate at room temperature overnight. The extinction was measured at 600 nm. The concentration was determined by comparison to a standard curve using highly polymerised calf thymus DNA (Sigma) as a standard. The calibration curve for the DNA assay is shown in Figure 2.6.

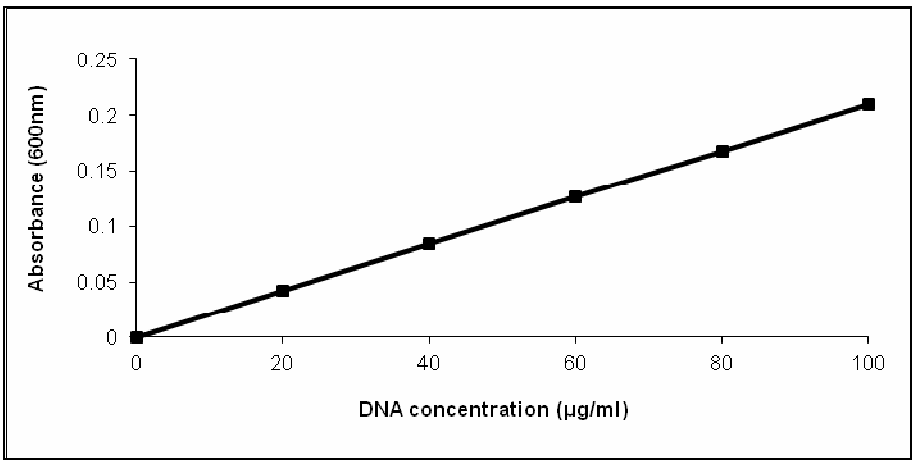


Figure 2.6 Calibration curve for DNA assay.

2.12 Lipopolysaccharide (LPS) extraction

LPS was extracted based on hot-phenol water method as described by Westphal and Jann (1965) with some modifications. 10 ml of bacterial suspension was centrifuged at 10000 x g for 5 minutes. The pellets were washed twice in PBS (pH 7.2) and re-suspended in 10 ml PBS and sonicated for 10 minutes on ice (70% power, ten times, with 1 minute interval at 4°C) with a Branson digital sonifier (Model 102c). An equal volume of 90% (w/v) phenol was added to the suspensions in a round bottom flask and was placed in a beaker containing water. The preparation was placed on a hot-plate and stirred at 65-70°C for 15 minutes. Suspensions were then cooled on ice and transferred to a centrifuge tube and

centrifuged at 8500 x g for 15 minutes. The aqueous layer was removed and the phenol phases were re-extracted with 2 ml of distilled water. Sodium acetate at 0.5 M final concentration (85 mg) and 10 volumes of 95% ethanol (20 ml) were added to the extracts and samples were stored at -20°C overnight to precipitate LPS. Tubes were centrifuged at 2000 x g at 4°C for 10 minutes and the pellets were resuspended in 1 ml distilled water. Dialysis against double distilled water at 4°C was carried out at the next step to remove the residual phenol in the aqueous phases. The membrane tubing was prepared as describe in Section 2.11. Final purified LPS were run on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and viewed with silver staining.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

LPS was resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970), which was performed using 15% (w/v) resolving gels overlaid with a 4% (w/v) stacking gel. The gel plates were cleaned prior to use with hot soapy water, rinsed with distilled water, then rinsed with ethanol and allowed to dry. Seals were placed between the gel plates and the plates were clamped together. The components for both the resolving gels and stacking gels were mixed. 5 ml of resolving gel was poured first and the gel was overlaid with 50% (v/v) isopropanol (to prevent air bubbles) and allowed to set for 30 minutes. The isopropanol was then removed and the stacking gel was poured above the resolving gel. A comb was then inserted into the stacking gel and the gel was allowed to set for 60 minutes.

The purified LPS was solubilized in sample buffer and boiled for 5 minutes. The ratio of sample: sample buffer was 1:4. 16 µl/ml from each sample was electrophoresed at 90 V for 1 hour and increased to 120 V for 1 hour using mini-PROTEAN electrophoresis instrument (Bio-Rad Laboratories, California, USA). PageRuler Plus prestained protein ladder (Fermentas) was used as a marker on all SDS-PAGE gels. Silver-staining was used to stain the SDS-PAGE gels. LPS from *Pseudomonas aeruginosa* 10 purified by phenol extraction (Sigma) was diluted to 10mg/ml and use as a control.

Silver staining

Polyacrylamide gel was placed in a polyethylene container and 100 ml fixing solution was added, agitated for 30 minutes at room temperature on an orbital shaker. Fixing solution was poured out and the gel was washed twice in 50% ethanol, 10 minutes for each wash. The gel was covered with sensitizing buffer and agitated gently 1 minute at room temperature then the gel was washed three times with distilled water, 30 seconds each wash. The gel was soaked in silver nitrate solution for 20 minutes and agitated gently on a shaker in the dark. The silver nitrate was poured out and the gel was washed three times with distilled water, 30 seconds each wash. The gel was soaked in 100 ml developer solution and agitated gently until the desired level of staining was achieved. The staining was stopped with stop solution then the solution was poured off and the gel was washed in water slowly for 10 minutes. The gel was photographed using the Imagemaster VDS image analysis system. The LPS band was quantified based on the marker. The appearance of the extracted LPS is shown in Figure 2.7.

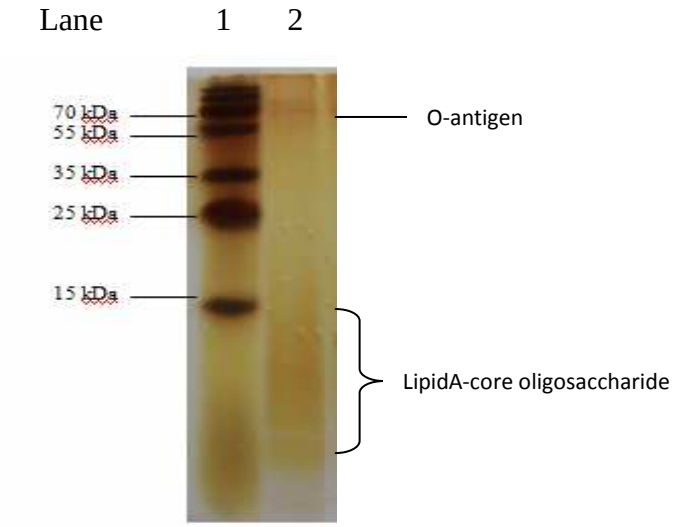


Figure 2.7 Appearance and quantification of DNA in gel agarose. Lane 1: Marker, Lane 2: LPS sample.

2.13 RNA preparation

RNA Extraction using the RNeasy Mini Prep Kit

Total RNA was extracted according to the protocol provided by Qiagen (RNeasy Mini Kit). Samples were harvested by centrifugation at 4000 rpm for 10 minutes at 4°C and pellets were immediately kept in RNA*later* (QIAGEN) solution and stored at 4°C before extraction. The mixture was centrifuged at 4000 rpm for 10 minutes at 4°C and the supernatant was decanted and if required the bacterial pellet could be stored at -20°C for up to 2 weeks or at -80°C for up to 4 weeks. For cell lysis, the cell pellets were resuspended in 100 µl Tris-EDTA buffer containing 1 $mg.ml^{-1}$ lysozyme. 350 µl of buffer RLT (supplemented with 10µl/ml of β-mercaptoethanol) was added and vortexed to loosen the pellets. The samples were homogenised by vortexing and centrifuged at 13,000 x g in a Biofuge 13 microfuge (Heraeus Instruments) for 2 minutes and supernatant was collected. One volume (250 µl) of absolute ethanol was added to the supernatant and mixed well by pipetting. This mixture was then loaded in 700 µl aliquots on to an RNeasy mini column, which was placed in a collection tube and centrifuged at 8,000 x g for 15 sec. Then 700 µl RW1 was loaded on to the column and centrifuged at 8,000 x g for 15 sec. This followed by 2 washes in buffer RPE by centrifuging at 8,000 x g for 15 sec. To completely dry the spin column, it was placed in a fresh collection tube and centrifuged at full speed for 1 minute to remove the carryover ethanol. Finally for the elution step, 30 µl of RNase free water was passed through the column by centrifugation at 8,000 rpm for 1 minute and followed with elution by 20 µl RNase free water.

RNA treatment with DNase

DNAse treatment was conducted to remove the genomic DNA contamination in the extracted RNA. Total of 2 µg of RNA was diluted in 8 µl of water. 1 µl of 10X Reaction Buffer and 1 µl of DNase I, Amplification Grade, 1 unit/µl (Sigma) were added in the reaction. The preparation were mixed gently and incubated for 15 minutes at room temperature. 1 µl of Stop Solution were added to bind calcium and magnesium ions and to inactivate the DNase I. The preparation was heated at

70°C for 10 minutes to denature both DNase I and RNA. RNA integrity and purity was checked by formaldehyde agarose gel electrophoresis and the Agilent 2100 Bioanalyzer and the NanoDrop ND-1000 was used to quantify the RNA concentration.

Formaldehyde (FA) gel agarose electrophoresis

The gel, prepared as described in 2.4 was poured into an electrophoresis apparatus and a comb was inserted to make the wells. When the gel was solidified, the comb was removed and the gel was placed in the device and immersed in 1X FA gel running buffer. One µl of a RNA sample were mixed with 1 µl 2X RNA loading buffer and the total 2 µl sample was transferred into the wells. Two microliters of marker, 10 kb smart ladder (MyBio Ltd., www.mybio.ie), were placed in the first well for comparison of molecular weights. The electrophoresis device, Bio-Rad system was configured to 90 V for 45 minutes. The bands on the gel became visible under UV light and the gel was photographed using the Imagemaster VDS image analysis system. Figure 2.8 illustrates the RNA before and after DNAse treatment. Sample 1 shows the 2 ribosomal RNA bands with genomic DNA contamination at high molecular weight. Sample 2 shows the 2 intact ribosomal RNA bands.

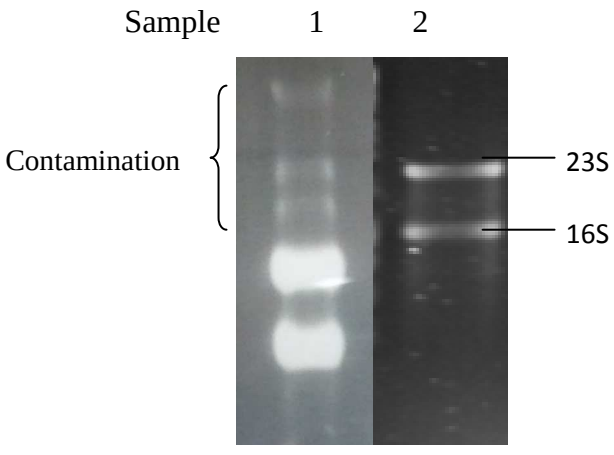


Figure 2.8 Appearance of RNA in two separate FA gels before and after DNAse treatment. Sample 1: before treatment and sample 2: DNAse treated RNA.

RNA quantification using the NanoDrop

The NanoDrop ND-1000 is a full-spectrum (220-750nm) spectrophotometer that measures 1 µl samples with high accuracy and reproducibility. The ND-1000 software automatically calculated the quantity of RNA in the samples using the OD_{260}. The software simultaneously measured the OD_{280} of the samples allowing the purity of the sample to be estimated. The desired value for purity OD_{260}/OD_{280} should be in the range of 1.8-2.0. A ratio of <1.6 indicates that the RNA may not be fully in solution. The protocol for RNA quantification using the Nanodrop illustrates in Figure 2.9.

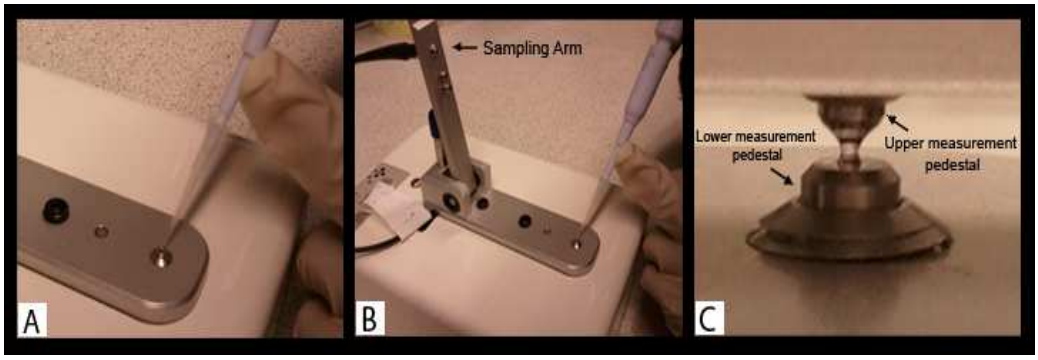


Figure 2.9 Measuring the quantity of RNA using the Nanodrop. Samples were quantified by loading 1µl onto the receiving fibre (A), the source fibre was connected to the sampling arm (B) and brought down into contact with the sample allowing a 1mm gap between the upper and lower pedestal (C), through which the light passed.

RNA quality assessment using the Bioanalyzer

A Total RNA 6000 LabChip kit (Agilent Technologies) was used with the Agilent 2100 Bioanalyzer to measure the integrity and purity of RNA samples. The gel was prepared by centrifuging 550 µl of the Nano gel Matrix through a spin filter at 1500 x g for 10 minutes. 65 µl aliquot of filtered gel was transfered into 0.5 ml RNase-free microfuge tubes. The gel-dye mixture was prepared by vortexing the RNA 6000 Nano dye for 10 seconds and spun down before 1 µl of dye added into 65 µl aliquot of filtered gel. The mixture was vortexed thoroughly and spun down at 13000 x g for 10 minutes at room temperature. The loading of the gel-dye mix was then carried out. A RNA Nano chip was placed onto the Chip Priming

Station. 9 µl of the Gel Dye mix was loaded into the well (third row, fourth column). The Chip Priming Station was closed and the plunger of the syringe was pressed down until held by the clip. After 30 seconds the plunger was released. After five seconds the plunger was slowly pulled up to the 1 ml position and the chip was removed from the Chip Priming Station. Finally, 9 µl of the Gel Dye Mix was pipetted into the bottom of each of the wells (first row, fourth column and second row, fourth column). 5 µl of the RNA 6000 Nano Marker was pipetted into the well marked ladder and into each of the 12 sample wells. 1 µl of each sample was pipetted into each of the 12 sample wells. RNA samples were heat denatured with a Stuart Block Heater SBH130D at 70°C for 2 minutes to minimize secondary structure formation before being loaded into the well. 1 µl of the Nano Marker was pipetted into the well without samples loaded. 1 µl of the ladder pipetted into the well marked ladder. The chip placed into the adapter of the IKA vortex mixer (2400 rpm) and vortexed for 1 minute. Finally, the RNA Nano Chip is placed into the receptacle of the Agilent 2100 Bioanalyzer and starts the software.

Figure 2.10 shows the differences between a degraded and intact RNA based on electropherogram profiles, (a) is an example of electropherogram of extreme degradation of RNA. The example lacks 16S and 23S peaks and consists solely of low molecular weight species. On the right side is the image of gel-like image of the degraded RNA sample with no specific band for 16S and 23S. (b) is an example of an electropherogram from high quality RNA with several characteristics. First, there are clear 16S and 23S peaks. Secondly, there should be low noise between the peaks and minimal low molecular weight contamination. On the right the software displays a gel-like image of the intact RNA.

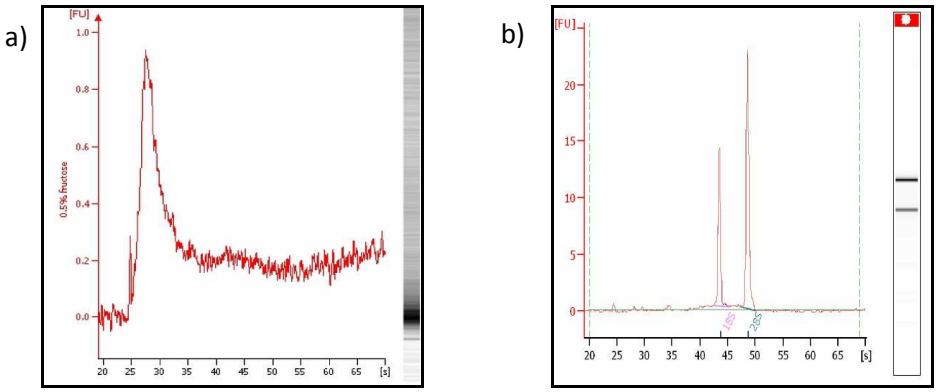


Figure 2.10 Electropherograms of RNA extracted from *P. putida* CP1 grown on 0.5% (w/v) fructose a) degraded RNA samples b) high quality RNA samples.

2.14 Microarray studies

P. putida KT2440 Genome Oligonucleotide Array

The microarray experiments carried out in this study used the *P. putida* KT2440 Genome Oligonucleotide Array (Progenika, Spain). The *P. putida* KT2440 DNA microarray contains 5539 oligonucleotides (50-mer) spotted in duplicate onto γ-aminosylane treated 25 x 75 mm microscope slides, covalently linked to the slide with UV light and heat. The spots, 180 mm in diameter, are separated from each other by 265 mm and arranged in 48 subarrays (16 columns and 15 rows each). The spotted area is ~17 x 54 mm. The oligonucleotides, designed by BioAlma (http://www.bioalma.com), are specific for each ORF in the array. These include the 5350 ORFs annotated in the *P. putida* KT2440 genome (obtained from http://www.ncbi.nlm.nih.gov, Refseq NC_002947; Gen-Bank Accession No. AE015451.1), the 140 ORFs defined for the TOL plasmid pWW0 (obtained from http://www. ncbi.nlm.nih.gov, Refseq NC_003350; GenBank Accession No. AJ344068.1) and other genes of diverse origins such as commonly used reporter genes (*lacZ, gfp*) or antibiotic resistance markers. Two oligonucleotides, coding for the *rpoD* and *rpoN* genes, respectively, are spotted at 20 different positions of the slide to serve as homogeneity controls. Negative controls (spotting buffer, 50% (v/v) dimethylsulfoxide) are spotted in duplicate at 203 positions evenly

distributed throughout the printed area. The array was printed by Progenika Biopharma (http://www.progenika.com).

Microarray experimental design

The array studies were conducted based on the experimental design shown in Figure 2.11 and included *P. putida* CP1 grown on nutrient broth OD_{660} 0.5, *P. putida* KT2440 grown on nutrient broth OD_{660} 0.5, *P. putida* CP1 grown on 100 ppm 2-chlorophenol, *P. putida* CP1 grown on 100 ppm 3-chlorophenol, *P. putida* CP1 grown on 200 ppm 4-chlorophenol, *P. putida* CP1 grown on 50 ppm 4-chlorophenol for 4 days and *P. putida* CP1 grown on 0.5% (w/v) fructose for 24 hours. Pure RNA was produced for each of the seven treatments. Three aliquots of pure RNA, each 20 µg was prepared for each treatment. Each aliquot was hybridized to the corresponding aliquot for each of 9 arrays as described in Figure 2.11. Therefore each array was carried out in triplicate.

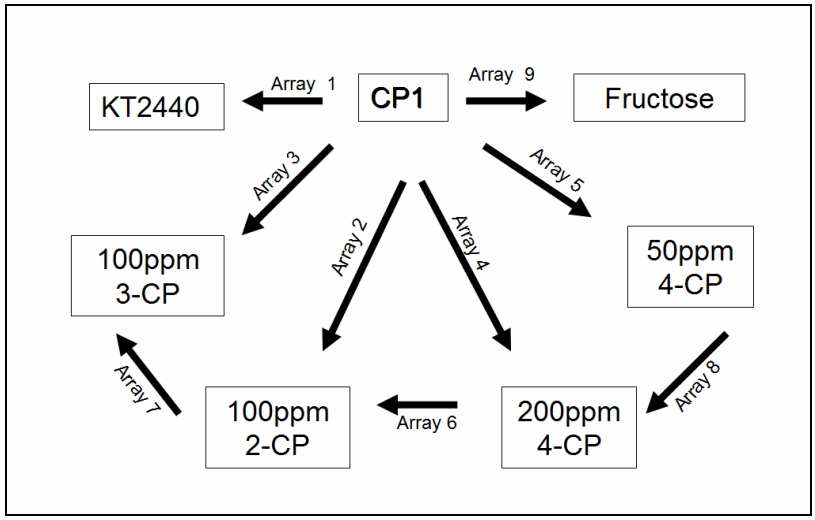


Figure 2.11 Microarray comparison design included a total of 9 arrays.
Array 1: *P. putida* CP1 grown on nutrient broth OD_{660} 0.5 compared to *P. putida* KT2440 grown on nutrient broth OD_{660} 0.5, Array 2: *P. putida* CP1 grown on nutrient broth OD_{660} 0.5 compared to *P. putida* CP1 grown on 100 ppm 2CP, Array 3: *P. putida* CP1 grown on nutrient broth OD_{660} 0.5 compared to *P. putida* CP1 grown on 100 ppm 3CP, Array 4: *P. putida* CP1 grown on nutrient broth OD_{660} 0.5 compared to *P. putida* CP1 grown on 200 ppm 4CP, Array 5: *P. putida* CP1 grown on nutrient broth OD_{660} 0.5 compared to *P. putida* CP1 grown on 50 ppm 2CP , Array 6: *P. putida* CP1 grown on 200 ppm 4CP compared to *P. putida* CP1 grown on 100 ppm 2CP, Array 7: *P. putida* CP1 grown on

100 ppm 2CP compared to *P. putida* CP1 grown on 100 ppm 3CP, Array 8: *P. putida* CP1 grown on 50 ppm 4CP compared to *P. putida* CP1 grown on 200 ppm 4CP and Array 9: *P. putida* CP1 grown on nutrient broth OD_{660} 0.5 compared to *P. putida* CP1 grown on 0.5% (w/v) fructose.

Hybridization and processing of microarrays

The hybridization and processing of the microarrays was conducted by Dr. Lisa Olohan from the School of Biological Sciences, University of Liverpool. This process included the cDNA synthesis and labeling with specific dyes, hybridization blocking, hybridization and post-hybridization including washing steps and array scanning.

cDNA synthesis

20 µg of total RNA was transformed to cDNA with Superscript III reverse transcriptase using random primers ($p(dN)_6$, Roche, Product no. 034731001).

Labelling

Fluorescently labelled cDNA for microarray hybridizations 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.

Hybridization blocking

Before the hybridization 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 hour at 42°C 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 minutes at room temperature in a microtitre plate centrifuge (Eppendorf 5804 with A-2-DWP rotor).

Hybridization

For the hybridization 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.

Hybridisation buffer

Hybridization buffer was prepared by combining 500 µl of 33.3% dextran sulphate and 500 µl of 20 × SSC and mixed. The mixture was filter-sterilised through a 0.2 µm membrane. 150 µl of the filtered dextran sulphate/SSC mixture added to 50 µl of 10% SDS, 250 µl of formamide (deionised, minimum 99.5% (GC); Sigma; F9037) and 50 µl of 20X Denhart's solution (Fluka; 30915). The hybridisation buffer was heated at 42°C for 30 minutes. 43 µl of hybridisation buffer added to the dried sample mixture and mixed by vortexing. The sample denatured by heating at 95°C for 5 minutes. The sample was centrifuged at 13000 rpm for 1 minute at room temperature and then placed at 37°C. The sample was loaded on to a pre-hybridised (blocked) array beneath a 22 × 60 mm lifterslip (Implen). The array was placed in pre-heated hybridisation chamber and the sealed chamber transferred to a 42°C oven for 18 h.

33.3% dextran sulphate was prepared by dissolving 0.5 g of dextran sulphate sodium salt (Sigma; D8906) to1.5 ml of nuclease-free water (Ambion; AM9937). The preparation were vortexed thoroughly and then heated at 55°C with rotation to mix, for approximately 10 minutes to ensure the dextran sulphate was completely dissolved. The remaining stock solution was stored at 4°C after use in the hybridisation buffer.

Post-hybridization

Post-hybridization was continued with 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 minutes. Array was washed in wash buffer 2 containing 0.5 × SSPE, 0.1% Tween 20 for 5 minutes followed by wash buffer 3 containing 0.5 × SSPE for 1 minute, wash buffer 4 containing 0.1 × SSPE for 15 s 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 1000 rpm for 5 minutes at room temperature in a microtitre plate centrifuge (Eppendorf 5804 with A-2-DWP rotor).

Array scanning

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

A gene list was generated and the statistical analysis of the microarray data set was completed by Dr. Yong Xiang Fang from the School of Biological Sciences, University of Liverpool. The statistical analysis included the data normalization and qualification. The results for each replica (median intensity for each channel) were normalized and statistically analysed using the LIMMA software package (Smyth, 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 normalized for each array through print-tip loess (Smyth and Speed, 2003) 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 and Hochberg (1995) to control the false discovery rate (FDR). The criterion for identifying differentially expression was based on 5% FDR.

Data filtration was done in the laboratory in DCU to generate a shorter list of differentially expressed genes. Only genes with average fold changes $\leq / \geq 1.2$ and adjusted *p*-values less than 0.05 (i.e., false discovery rate less than 5%) were identified as significantly differentially expressed genes. Datasets obtained from Progenika included the 5350 ORFs annotated in the *P. putida* KT2440 genome (obtained from www.ncbi.nlm.nih.gov, Refseq NC_002947; GenBank accession number AE015451.1). The information from the data obtained was then compiled with the public datasets downloaded from Pseudomonas Genome Database v2 (www.pseudomonas.com) for further analysis which contained a better annotation. Microsoft Access was used to generate the new annotation.

Genelists were arranged based on the LocusID, gene name, annotation, fold-change and *p*-value. A LocusID is a unique locus name that corresponds to a transcribed region in *P. putida* KT2440 genome. The format of the LocusID is, PPxxxx. The PP refers to *Pseudomonas putida*, the X correspond to the digit code, numbered from top to bottom of the chromosome. Annotation refers to the protein encoded by the gene which refers to the gene name. FC refers to the fold change which positive value means up-regulated and negative value means down-regulated.

Microsoft Access

MSAccess is a database-building package that was used to compare different gene lists. MSAccess allowed comparison of like genes across multiple lists. It allowed comparison of genes and *p*-values. It was also used as a repository and used for

database queries. It was also widely used for merging tables and adding annotations to genelists.

Venny

Venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html) is a Venn diagram-drawing software tool that was used to overlap and compare genelists for the comparative studies.

DAVID

The Database for Annotation, Visualization and Integrated Discovery DAVID (http://david.abcc.ncifcrf.gov/home.jsp) was used for the functional analysis for the genelists. The database provides a comprehensive set of functional annotation tools to understand the biological meaning behind large lists of genes including visualizing genes on KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway maps. An Entrez ID for each gene from the genelists is uploaded as the identifier for the analysis.

PSORTb version 3.0

PSORTb version 3.0 is protein sub-cellular localization predictor software for prokaryotes. The modules may act as a binary predictor, classifying a protein as either belonging or not belonging to a particular localization site, or they may be multi-category, able to assign a protein to one of several localization sites.

Genelist analysis strategy

Comparison 1: KT2440vsCP1

This genelist analysis compared *P. putida* CP1 with the reference organism (Array 1).

Comparison 2: CP1vs2CP, CP1vs3CP, CP1vs4CP$_{200}$ and CP1vs4CP$_{50}$

This genelist analysis compared *P. putida* CP1 grown on nutrient broth individually with those obtained when the organism was grown on 100 ppm 2-chlorophenol (Array 2), 100 ppm 3-chlorophenol (Array 3), 200 ppm 4-chlorophenol (Array 4) and 50 ppm 4-chlorophenol (Array 5). The four comparative studies (CP1vs2CP, CP1vs3CP, CP1vs4CP$_{200}$ and CP1vs4CP$_{50}$) were overlapped using Venny to identify similar responses in all mono-chlorophenols.

Comparison 3: 50 ppm4CP vs 200 ppm4CP

This genelist compared *P. putida* CP1 grown on higher concentration 200 ppm with lower concentration (50 ppm) 4-chlorophenol (Array 8).

Comparison 4: CP1-200 ppm4CP$_{200}$-100 ppm2CP-100 ppm3CP

This genelist analysis compared CP1 vs 200 ppm 4CP (Array 4), 200 ppm4CP vs 100 ppm 2CP (Array 6) and 100 ppm 2CP vs 100 ppm 3CP (Array 7). The differentially regulated genes from CP1 vs 200 ppm 4CP, 200 ppm4CPvs 100 ppm 2CP and from 100 ppm 2CP vs 100 ppm 3CP were overlapped using Venny. The overlapped genes were then analyzed using Microsoft Access to verify genes with consistent expression changes in fold change value.

Comparison 5: CP1-200 ppm4CP$_{200}$-100 ppm2CP-100 ppm3CP vs CP1-50 ppm 4CP

This genelist compared the genes with consistent expression changes in CP1-200 ppm4CP$_{200}$-100 ppm2CP-100 ppm3CP (Comparison 4) with CP1 vs 50 ppm 4CP (Array 5).

Comparison 6: CP1 vs. Fructose

This genelist compared *P. putida* CP1 grown on nutrient broth with *P. putida* CP1 grown on 0.5% (w/v) fructose (Array 9).

Comparison 7: The unique genes belong to only CP1-200 ppm4CP$_{200}$-100 ppm2CP-100 ppm3CP

Comparison 5 was overlapped with genes from Comparison 6 using Venny to identify common genes that are regulated during formation of aggregates in both conditions.

2.15 Quantitative real time RT-PCR (qRT-PCR)

The validation of genes identified from microarray study was conducted using qRT-PCR. Before running the assay, cDNA was synthesized by RT-PCR and specific primers were designed. The validation of primers was carried out using PCR. A High-Capacity cDNA Reverse Transcription Kit (Applied BioSystems, 4368814) was used for cDNA synthesis. Total 10 µl of RNA sample (200 ng) was mixed in 10 µl of RT reaction mix by pipetting up and down few times (Table 2.1). The tubes were then briefly centrifuged to spin down the contents and to eliminate any air bubbles and placed on ice until ready to be loaded in the thermal cycler which was programmed as described in Table 2.2. Positive and negative controls were prepared as described in Table 2.3.

Table 2.1 Reaction mixture for RT-PCR using High-Capacity cDNA Reverse Transcription Kit

Component	**Volume (µl)**
RNA	10
10X RT Buffer	2
25X dNTP Mix (100 mM) 0.8	0.8
MultiScribe Reverse Transcriptase	1
10X Random Primers	2
Nuclease-free H2O	4.2
Total	20

Table 2.2 Programme for thermal cycler for RT-PCR.

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 sec	∞

Table 2.3 Composition of the positive and negative controls for RT-PCR.

Component	**Positive control µl**	**Negative control µl**
RNA (2 µl (400ng) x 5 sample)	10	10
10X RT Buffer	2	2
25X dNTP Mix (100 mM) 0.8	0.8	0.8
MultiScribe Reverse Transcriptase	1	-
10X Random Primers	2	2
Nuclease-free H2O	4.2	5.2
Total	20	20

Primers for the amplification

Primer design was done using Primer Premier and the primers were ordered from Sigma. Six pairs of primers were designed. The details of the primer sequences are listed in Table 2.4. The primers were designed in the laboratory based on gene sequences available in Gene bank (http://www.ncbi.nlm.nih.gov). All primers were stock as 100 µM.

Table 2.4 List of primers.

Primer name	**Primer sequence**
fliE-F	CCATGTCGCTGCCCAAGGTAAC
fliE-R	ACGCCGCTCTTGCCGATTTC
PP132-F	AGCAAGGCGAGCAGCAATTACC
PP132-R	CCGAGTACACCACTGACCAGCA
PP4936 LPS-F	TCTGGTTCCTGGCGGCGATT
PP4936 LPS-R	GCCAACAGCAGCAGCACACT
PP1961-F	TGAGAACGCAAAGAAGCTGA
PP1961-R	GGGCTTTCGCTAGGCTAACT
RpoD-F	CCTGACCGTTGCCGAGATCAAG
RpoD-R	AGACCGATGTTGCCTTCCTGGA

PCR Amplification

In order to check the specific binding of the designed primer to the cDNA, PCR amplification was conducted using MyTaq Red mix Polymerase (MyBio). The cDNA concentration of all the samples was adjusted to 100 ng prior to the amplification step. The primers (Table 2.4) were used to amplify each gene. The amplification of the cDNA was conducted based on PCR reaction setup in Table 2.5. All the reactions were amplified in a Px2 Thermal Cycler (Thermo Electron Corporation, MA, USA) based on the PCR cycling condition in Table 2.6. The specificity of the primers was confirmed using agarose gel electrophoresis.

Table 2.5 Reaction mixture for PCR.

Component	**Volume (µl)**
Template (100ng)	1
Primers Forward (10µM)	1
Primers Reverse (10µM)	1
MyTaq Red Mix, 2x	25
ddH20	22
Total	50

Table 2.6 PCR cycling conditions.

Step	**Temperature (°C)**	**Time**	**Cycles**
Initial denaturation	95	1min	1
Denaturation	95	15s	35
Annealing	60	15s	
Extension	72	10s	
Final extension	72	7min	1

Agarose gel electrophoresis

The PCR products transferred to an agarose gel produced a band as described in Figure 2.12. The gel, prepared as described in Section 2.4, was poured into an electrophoresis apparatus and a comb was inserted to make the wells. When the gel was solidified, the comb was removed and the gel was placed in the device and immersed in 1X TAE gel running buffer. Five µl of a PCR product were mixed with 1 µl loading dye and the total 6 µl sample was transferred into the wells. Two microliters of marker, 100 bp DNA Ladder (New England Biolabs), were placed in the first well for the comparison of molecular weights. The electrophoresis device, Bio-Rad system, configured to 90 V for 45 minutes. The bands on the gel became visible under UV light and the gel was photographed using the Imagemaster VDS image analysis system. The PCR product yielded an amplicon sized around 100-200 bp.

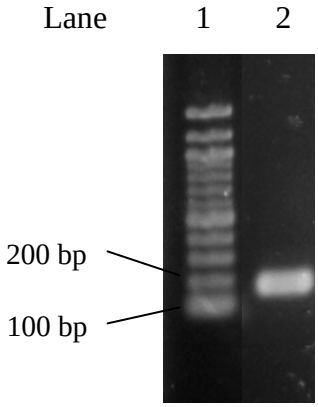


Figure 2.12 Appearance of PCR products on an agarose gel. Lane 1: Marker and Lane 2: PCR product.

2.16 Quantitative real time RT-PCR (qRT-PCR) assay

The Fast SYBR® Green quantitative Real-time PCR (qRT-PCR) analysis was performed using the Applied BioSystems 7500 Fast Real-time PCR System. In order to exclude any amplification product derived from genomic DNA or any other contaminant that could contaminate the RNA preparation, total RNA without reverse transcription was used as a negative control. Water on its own was

used as a negative control to detect the presence of any contaminating RNA or DNA.

i) Preparation of reaction-mix

The volume of each component needed for all the wells in each assay were calculated based on number of reactions. Three replicates of each reaction were prepared. The reaction mix tube were vortex briefly to mix the solutions and centrifuge briefly to spin down the contents and eliminate any air bubbles from the solutions. The qRT-PCR reaction setup was conducted based on Table 2.7. The cDNA templates were only combined into the aliquot of master mix in preparation of PCR reaction plate.

Table 2.7 Reaction mixture for qRT-PCR.

Component	**1 reaction (µl)**
Fast SYBR® Green Master Mix (2X)	10
Forward and Reverse Primers	1
cDNA template + H2O	2
H_20	7
Total	20

ii) Preparation of PCR reaction plate

18 µl of reaction master mix was added first to the MicoAmp fast optical 96-well reaction plate (Applied BioSystems, 4346906) followed by 2 µl of the cDNA. The plate was sealed before being placed in the thermal cycler (Applied BioSystems, 7500 Fast Real-time PCR System).

iii) PCR reaction conditions were based on Table 2.8.

Table 2.8 Thermal cycling conditions used for qRT-PCR.

Step	Temp (°C)	Duration	Cycles
Enzyme activation	95	20 sec	Hold
Denature	95	3 sec	40
Anneal/Extend	60	30 sec	

Cycle threshold (C_t)

The threshold is the level of detection or the point at which a reaction reaches a fluorescent intensity above background. The threshold line is set in the exponential phase of the amplification for the most accurate reading. The cycle at which the sample reaches this level is called the Cycle Threshold, Ct.

Relative Quantification (RT)

Relative quantification determines the change in expression of a target in a test sample relative to the calibrator sample (control). Changes in gene expression were calculated automatically in the Applied Biosystem 7500 System software using the $\Delta\Delta$ C_t method as follows:

ΔC_t (target) = C_t (target) – C_t (endogenous control)

ΔC_t (calibrator) = C_t (calibrator) – C_t (endogenous control)

$\Delta\Delta C_t = \Delta C_t$ (target) - ΔC_t (calibrator)

Fold changes = $2^{-\Delta\Delta Ct}$

rpoD gene was used as the endogenous control, and all samples included a no-RT control to assess genomic DNA contamination in the reactions.

2.17 Data analysis

All experiments were carried out in triplicate. All samples were analysed in triplicate. All data analysis was conducted using Microsoft Excel.

3.0 RESULTS

3 Results

Autoaggregation of *P. putida* CP1 had previously been observed in the laboratory but the phenomenon had not been studied in detail (Farrell and Quilty, 2002; Fakhruddin and Quilty, 2005). In this study it was of interest to identify phenotypic and genotypic changes in the cell following autoaggregation. *Pseudomonas putida* CP1 was grown on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol, 200 ppm 4-chlorophenol and 0.5% (w/v) fructose in order to produce aggregates. *Pseudomonas putida* KT2440, a non-aggregating organism was used as a control. The phenotypic responses observed included changes in cell surface hydrophobicity, the aggregation index of the cells, the biochemical composition of the extracellular polymeric substances (EPS) and the size of the aggregates. The genotypic response of the organism was determined using a Genome Oligonucleotide Array (Progenika, Spain). The array was prepared using *P. putida* KT2440. Studies of *P. putida* CP1 growing on mono-chlorophenols are described in Section 3.2 and on fructose in Section 3.3. The first section of results (Section 3.1) describes how *P. putida* CP1 compares with *P. putida* KT2440.

3.1 A comparative study of *P. putida* CP1 and *P. putida* KT2440

When *P. putida* CP1 and *P. putida* KT2440 were identified in the laboratory, they were both confirmed as Gram negative, non-spore forming and motile rods. The isolates were shown to be both oxidase and catalase positive (Table 3.1).

Table 3.1 Cell characteristics of *P. putida* CP1 and *P. putida* KT2440.

Characteristic	
Gram	-
Shape	Rod
Motility	+
Spore formation	-
Oxidase	+
Catalase	+

When the bacteria were tested using the API 20NE (BioMerieux) system, both isolates were identified as *Pseudomonas putida* with the identification percentage of 99.8% for *P. putida* CP1 and 99.7% for *P. putida* KT2440 (Table 3.2). The results showed the close similarity of the two bacteria. Both strains had the same reaction in 18 of the 20 tests on the API 20NE test strip. However, there were some exceptions. Arabinose assimilation was positive for *P. putida* CP1 and negative for *P. putida* KT2440 and mannose assimilation was negative for *P. putida* CP1 while positive for *P. putida* KT2440.

Table 3.2 API 20NE identification tests for *P. putida* CP1 and *P. putida* KT2440.

Test	***P. putida* CP1**	***P. putida* KT2440**
Reduction of nitrates	-	-
Indole production	-	-
Glucose acidification	-	-
Arginine dihydrolase	+	+
Urease	-	-
Esculin hydrolysis	-	-
Gelatin hydrolysis	-	-
B-galactosidase	-	-
Glucose assimilation	+	+
Arabinose assimilation	+	-
Mannose assimilation	-	+
Mannitol assimilation	-	-
N-acetyl-glucosamine assimilation	-	-
Maltose assimilation	-	-
Gluconate assimilation	+	+
Caprate assimilation	+	+
Adipate assimilation	-	-
Malate assimilation	+	+
Citrate assimilation	+	+
Phenyl-acetate assimilation	+	+
Cytochrome oxidase	+	+
Identification	*Pseudomonas putida* % ID: 99.8 T: 0.98	*Pseudomonas putida* %ID: 99.7 T: 0.98

Microarray gene expression profiling of *P. putida* CP1 and the reference organism, *P. putida* KT2440 was performed (Array 1). The array studies investigated 5539 genes of *P. putida* KT2440 in total. Results showed that only 316 genes were significantly differentially regulated in *P. putida* CP1 when compared to *P. putida* KT2440 and 85 of those were identified as unknown proteins. 230 genes were identified as significantly up-regulated ($P \leq 0.05$, Fold Change (FC) >1.2) and 86 genes were identified as significantly down-regulated ($P \leq 0.05$, FC< -1.2). Based on these limitations, the top 25 up- and 25 down-regulated genes in each study were selected.

Up-regulated gene transcripts

The 230 up-regulated genes were ranked by fold change and the top 25 genes are listed in Table 3.3. 56 of the 230 genes that were significantly up-regulated were identified with unknown function. Among the 25 top up-regulated genes, the top two are involved in a two-component system pathway. PP270 encodes for histidine kinase and fliC for flagelin FliC. A number of the top up-regulated genes, PP4065, PP4402, PP597, PP596, PP4403, PP4666 and PP4667, play a role in valine, leucine and isoleucione degradation. Other up-regulated genes included PP3533 involved in arginine and praline metabolism, fliD and PP4377 involved in flagella assembly, fadAx in fatty acid metabolism, PP1071 in ABC transporters, PP740 and PP2144 transcriptional regulators, hmgA involved in tyrosine metabolism and PP1776 which encoded for mannose-6-phosphate isomerase.

Two genes that are involved in genetic element transfer were identified among the top up-regulated genes. The gene PP2297 encodes for the integrative genetic element Ppu40 integrase and TnpA for Tn4652, transposase. Transposition is important in creating genetic diversity within species and adaptability to changing living conditions.

Table 3.3 Top 25 genes up-regulated in *P. putida* CP1 compared with *P. putida* KT2440.

Locus ID	**Gene Name**	**Annotation**	**Pathway**	**FC**	***p*-value**
PP270		integral membrane sensor signal transduction histidine kinase	Two-component system	6.276	0
PP4378	fliC	flagellin FliC	Two-component system; Flagellar assembly	5.874	0.00554
PP3533		ornithine cyclodeaminase	Arginine and proline metabolism	5.420	1.00E-05
PP2737		short chain dehydrogenase		4.658	0
PP4065		propionyl-CoA carboxylase	Valine, leucine and isoleucine degradation	4.246	0
PP4376	fliD	flagellar cap protein FliD	Flagellar assembly	4.134	1.00E-05
PP88		luciferase family protein		4.048	0
PP2297		integrative genetic element Ppu40, integrase		3.924	0
PP4621	hmgA	homogentisate 1,2-dioxygenase	Tyrosine metabolism	3.846	1.00E-05
PP740		MerR family transcriptional regulator	Transcription	3.778	6.00E-05
PP2215	fadAx	acetyl-CoA acetyltransferase	Fatty acid metabolism	3.640	0
PP1016		binding-protein-dependent transport systems inner membrane component		3.548	0.00116
PP4402	bkdA2	2-oxoisovalerate dehydrogenase, beta subunit	Valine, leucine and isoleucine degradation	3.486	1.00E-05
PP2964	tnpA	Tn4652, transposase		3.418	0.00042
PP597	mmsA-1	methylmalonate-semialdehyde dehydrogenase	Valine, leucine and isoleucine degradation	3.378	0.00021
PP2988		alcohol dehydrogenase		3.198	2.00E-05

PP4377		flagellin FlaG, putative	Flagellar assembly	3.176	1.00E-05
PP2311		TatD family hydrolase		3.166	0.00345
PP596		beta alanine--pyruvate transaminase	Valine, leucine and isoleucine degradation	3.148	0.00016
PP1071		amino acid ABC transporter	ABC transporters	3.104	0.00026
PP2144		TetR family transcriptional regulator	Transcription	3.098	0.0038
PP4403	bkdB	branched-chain α-keto acid dehydrogenase subunit E2	Valine, leucine and isoleucine degradation	3.074	6.00E-05
PP4666	mmsB	3-hydroxyisobutyrate dehydrogenase	Valine, leucine and isoleucine degradation	3.028	0.00044
PP4667	mmsA-2	methylmalonate-semialdehyde dehydrogenase	Valine, leucine and isoleucine degradation	3.010	3.00E-05
PP1776		mannose-6-phosphate isomerase	Fructose and mannose metabolism	2.914	0.00032

Down-regulated gene transcripts

86 genes were identified as significantly down-regulated and following ranking by fold change the top 25 genes are listed in Table 3.4. Twenty of the down-regulated genes were identified with unknown function. The top down-regulated 25 genes were involved in protein translation, pentose phosphate pathway, purine metabolism, bacterial chemotaxis, RNA degradation, arginine and praline metabolism, protein translation, pyrimidine metabolism and oxidative phosphorylation. The most down-regulated gene, PP1118, encoded for a recombinase-related protein with a fold change -7.1.

Table 3.4 Top 25 genes down-regulated in *P. putida* CP1 compared with *P. putida* KT2440.

Locus ID	**Gene Name**	**Annotation**	**Pathway**	**FC**	***p*-value**
PP1118		recombinase-related protein		-7.062	0
PP2468	rplT	50S ribosomal protein L20	Protein translation	-4.094	0
PP1360	groES	co-chaperonin GroES		-3.790	0.00039
PP5000	hslV	ATP-dependent protease peptidase subunit		-3.418	0.00209
PP722	prsA	ribose-phosphate pyrophosphokinase	Pentose phosphate pathway	-3.374	3.00E-05
PP5088	priA	primosome assembly protein PriA		-3.360	1.00E-05
PP1981		NifR3/Smm1 family protein		-3.296	0.0037
PP999	arcC	carbamate kinase	Purine metabolism	-3.144	2.00E-05
PP3414		methyl-accepting chemotaxis transducer	Bacterial chemotaxis	-2.974	0.04524
PP1361	groEL	chaperonin GroEL	RNA degradation	-2.840	0.00333
PP1000	argI	ornithine carbamoyltransferase	Arginine and proline metabolism	-2.742	0.00126
PP4709	rpsO	30S ribosomal protein S15	Protein translation	-2.564	7.00E-05
PP5337		LysR family transcriptional regulator		-2.530	0.00362
PP663		AsnC family transcriptional regulator		-2.50	0.03012
PP5393		heavy metal transport		-2.458	0.05144
PP4014	mnmA	tRNA-specific 2-thiouridylase MnmA		-2.418	0.00093
PP1771	cmk	cytidylate kinase	Pyrimidine metabolism	-2.380	0
PP4728	grpE	heat shock protein GrpE		-2.362	0.00515
PP5001	hslU	ATP-dependent protease ATP-binding subunit HslU		-2.340	0.00974
PP1522	cspA-1	cold shock protein CspA		-2.276	0.00928
PP77	betC	choline sulfatase		-2.222	0.00016

PP3218		NtaA/SnaA/SoxA family monooxygenase		-2.170	2.00E-05
PP626	ndh	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	Oxidative phosphorylation	-2.098	0.00134
PP1670		NLP/P60 protein		-1.988	0.00153
PP4409		phage integrase family site specific recombinase		-1.958	0.00639

Metabolic pathway analysis

Further analysis was carried out to determine the role of the 316 differentially regulated genes in pathways based on the biological information database KEGG. A total of 28 different metabolic pathways were identified in this analysis. Figure 3.1 summarizes these pathways and the number of genes and their regulation involved. In most of the pathways, more genes were induced than repressed. In the case of 10 pathways coding for ABC transporters, benzoate degradation via CoA ligation, beta-Alanine metabolism, tryptophan metabolism, butanoate metabolism, caprolactam degradation, geraniol degradation, styrene degradation, limonene and pinene degradation and pyruvate metabolism the genes were only induced. In the case of the oxidative phosphorylation pathway the genes involved were only repressed. The other pathways contained both up and down regulated genes.

Excluding genes encoding hypothetical proteins, the majority of induced genes belonged to the group involved in amino acid metabolism. Valine, leucine and isoleucine degradation pathways were associated with 17 induced genes. The second largest group were the genes involved with propanoate metabolism followed by ABC transporters, flagellar assembly, two-component system, fatty acid metabolism, butanoate metabolism, alanine, aspartate and glutamate metabolism and lysine degradation. The genes involved in these top 10 significant pathways are listed in Table 3.5. Some of the genes described are included in the list of the top 25 list of significantly up- and down-regulated genes and are highlighted in the Table 3.5.

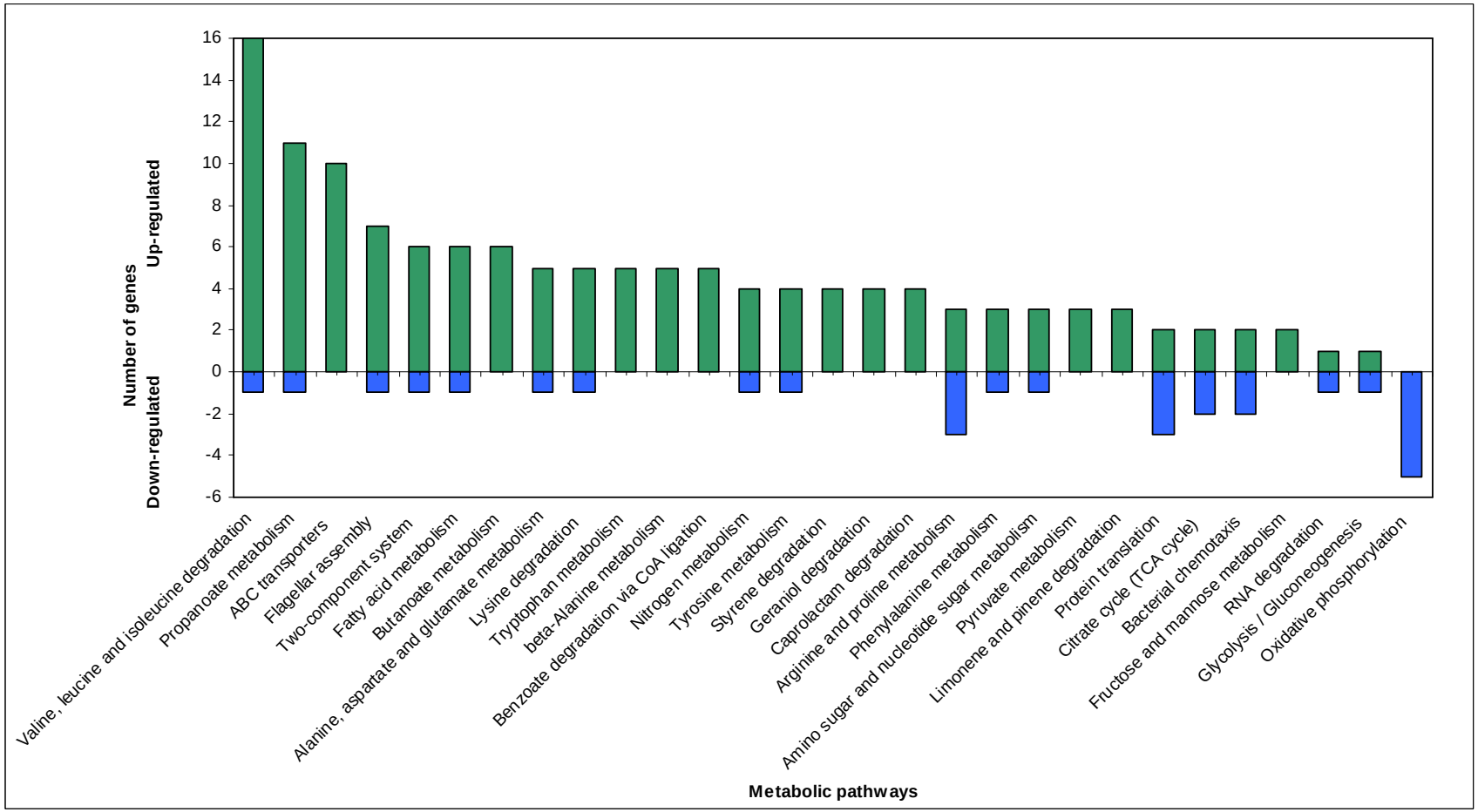


Figure 3.1 Metabolic pathways associated with the 316 differentially regulated genes in *P. putida* CP1 when compared with *P. putida* KT2440. Up-regulated genes (■) and down-regulated genes (■).

Table 3.5 The ten metabolic pathways associated with the most significantly differentially regulated genes in *P. putida* CP1 when compared to *P. putida* KT2440.

Pathway	**Locus ID**	**Gene name**	**Annotation**	**FC**	***p*-value**
Valine, leucine and isoleucine degradation	PP4065		propionyl-CoA carboxylase	4.246	0
	PP2215	fadAx	acetyl-CoA acetyltransferase	3.64	0
	PP4402	bkdA2	2-oxoisovalerate dehydrogenase, beta subunit	3.486	0.00001
	PP597	mmsA-1	methylmalonate-semialdehyde dehydrogenase	3.378	0.00021
	PP596		beta alanine--pyruvate transaminase	3.148	0.00016
	PP4403	bkdB	branched-chain alpha-keto acid dehydrogenase subunit E2	3.074	0.00006
	PP4666	mmsB	3-hydroxyisobutyrate dehydrogenase	3.028	0.00044
	PP4667	mmsA-2	methylmalonate-semialdehyde dehydrogenase	3.01	0.00003
	PP2214	fadB2x	short-chain dehydrogenase/reductase SDR	2.244	0.00136
	PP4066		gamma-carboxygeranoyl-CoA hydratase	2.234	0.00247
	PP4064	ivd	acyl-CoA dehydrogenase domain-containing protein	2.206	0.0002
	PP4404	lpdV	dihydrolipoamide dehydrogenase	1.874	0.00018
	PP4401	bkdA1	3-methyl-2-oxobutanoate dehydrogenase	1.79	0.00003
	PP4067		acetyl-CoA carboxylase, biotin carboxylase, putative	1.6	0.01097
	PP2217	fadB1x	enoyl-CoA hydratase/isomerase	1.302	0.0236
	PP4030		enoyl-CoA hydratase	1.224	0.0097
	PP4187	lpdG	dihydrolipoamide dehydrogenase	-1.63	0.05869
Propanoate metabolism	PP4065		propionyl-CoA carboxylase	4.246	0
	PP2215	fadAx	acetyl-CoA acetyltransferase	3.64	0

	PP597	mmsA-1	methylmalonate-semialdehyde dehydrogenase	3.378	0.00021
	PP596		beta alanine--pyruvate transaminase	3.148	0.00016
	PP4667	mmsA-2	methylmalonate-semialdehyde dehydrogenase	3.01	0.00003
	PP2335		methylcitrate synthase	2.548	0.00068
	PP4066		gamma-carboxygeranoyl-CoA hydratase	2.234	0.00247
	PP2334	prpB	2-methylisocitrate lyase	1.802	0.01555
	PP4487	acsA	acetyl-CoA synthetase	1.726	0.08591
	PP4067		acetyl-CoA carboxylase, biotin carboxylase, putative	1.6	0.01097
	PP2217	fadB1x	enoyl-CoA hydratase/isomerase	1.302	0.0236
	PP4030		enoyl-CoA hydratase	1.224	0.0097
	PP4186	sucC	succinyl-CoA synthetase subunit beta	-1.384	0.0294
ABC transporters	PP1071		amino acid ABC transporter	3.104	0.00026
	PP1139	livM	leucine/isoleucine/valine transporter permease subunit	2.318	0.00143
	PP1138	livG	leucine/isoleucine/valine transporter ATP-binding subunit	2.038	0.00209
	PP4485		polar amino acid ABC transporter inner membrane subunit	1.988	0.00008
	PP1140	braD	inner-membrane translocator	1.866	0.00761
	PP2456	rbsC	monosaccharide-transporting ATPase	1.728	0.01869
	PP1070		polar amino acid ABC transporter inner membrane subunit	1.602	0.00435
	PP885		dipeptide ABC transporter, periplasmic peptide-binding protein	1.578	0.01985
	PP1069		polar amino acid ABC transporter inner membrane subunit	1.258	0.00199

Flagellar assembly	PP4378	fliC	flagellin FliC	5.874	0.00554
	PP4376	fliD	flagellar cap protein FliD	4.134	0.00001
	PP4394	flgA	flagellar basal body P-ring biosynthesis protein FlgA	2.468	0.00026
	PP4396		FlgN family protein	2.298	0.0012
	PP4380	flgL	flagellar hook-associated protein FlgL	2.286	0.00113
	PP4388	flgE	flagellar hook protein FlgE	1.978	0.00204
	PP4375	fliS	flagellar protein FliS	1.872	0.00345
	PP4904	motB	flagellar motor protein MotB	-1.424	0.00041
Two-component system	PP270		integral membrane sensor signal transduction histidine kinase	6.276	0
	PP4378	fliC	flagellin FliC	5.874	0.006
	PP1186	phoP	winged helix family two component transcriptional regulator	2.046	0.031
	PP4503		winged helix family two component transcriptional regulator	1.33	0.006
	PP4489	phhR	transcriptional regulator TyrR	2.276	0.000
	PP5321	phoR	PAS/PAC sensor signal transduction histidine kinase	1.686	0.032
	PP4167	sixA	phosphohistidine phosphatise	-1.306	0.004
Fatty acid metabolism	PP2215	fadAx	acetyl-CoA acetyltransferase	3.64	0
	PP2214	fadB2x	short-chain dehydrogenase/reductase SDR	2.244	0.00136
	PP4066		gamma-carboxygeranoyl-CoA hydratase	2.234	0.00247
	PP4550	fadD2	long-chain-fatty-acid--CoA ligase	1.486	0.00243
	PP2217	fadB1x	enoyl-CoA hydratase/isomerase	1.302	0.0236
	PP4030		enoyl-CoA hydratase	1.224	0.0097
	PP3839	adhA	alcohol dehydrogenase	-1.448	0.02231

Butanoate metabolism	PP2215	fadAx	acetyl-CoA acetyltransferase	3.64	0.000
	PP3755	paaH	3-hydroxybutyryl-CoA dehydrogenase	2.746	0.000
	PP2214	fadB2x	short-chain dehydrogenase/reductase SDR	2.244	0.001
	PP4066		gamma-carboxygeranoyl-CoA hydratase	2.234	0.002
	PP2217	fadB1x	enoyl-CoA hydratase/isomerase	1.302	0.024
	PP4030		enoyl-CoA hydratase	1.224	0.010
Alanine, aspartate and glutamate metabolism	PP4065		propionyl-CoA carboxylase	4.246	0.000
	PP4038		dihydropyrimidine dehydrogenase	2.946	0.000
	PP2453	ansA	L-asparaginase, type II	2.58	0.002
	PP4037		putative oxidoreductase	1.632	0.001
	PP4030		enoyl-CoA hydratase	1.224	0.010
	PP2406	aroE	shikimate 5-dehydrogenase	-1.246	0.001
Lysine degradation	PP2215	fadAx	acetyl-CoA acetyltransferase	3.64	0.000
	PP2214	fadB2x	short-chain dehydrogenase/reductase SDR	2.244	0.001
	PP4066		gamma-carboxygeranoyl-CoA hydratase	2.234	0.002
	PP2217	fadB1x	enoyl-CoA hydratase/isomerase	1.302	0.024
	PP4030		enoyl-CoA hydratase	1.224	0.010
	PP4188	kgdB	dihydrolipoamide succinyltransferase	-1.74	0.043
Tryptophan metabolism	PP2215	fadAx	acetyl-CoA acetyltransferase	3.64	0.000
	PP2214	fadB2x	short-chain dehydrogenase/reductase SDR	2.244	0.001
	PP4066		gamma-carboxygeranoyl-CoA hydratase	2.234	0.002
	PP2217	fadB1x	enoyl-CoA hydratase/isomerase	1.302	0.024
	PP4030		enoyl-CoA hydratase	1.224	0.010

3.2 Phenotypic and genotypic responses of *P. putida* CP1 when grown on mono-chlorophenols

The phenotypic and genotypic responses of *P. putida* CP1 when grown on mono-chlorophenols were monitored. The ability of the organism to grow on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol, 200 and 50 ppm 4-chlorophenol was determined. Autoaggregation was monitored and the aggregates were characterised. Expression profiling analysis was carried out to identify genes associated with autoaggregation.

3.2.1 Phenotypic responses of *P. putida* CP1 when grown on mono-chlorophenols

The phenotypic responses of *P. putida* CP1 when grown on mono-chlorophenols were determined by monitored growth and substrate removal, aggregate formation and cell viability and aggregate characterization including EPS composition.

Growth of P. putida CP1 and P. putida KT2440 on mono-chlorophenols

P. putida CP1 and *P. putida* KT2440 were grown in liquid culture on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol, 200 and 50 ppm 4-chlorophenol. Previous studies had shown that *P. putida* CP1 aggregated when grown on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol and 200 ppm 4-chlorophenol although not on 50 ppm 4-chlorophenol (Farrell and Quilty, 2002). The chlorophenol was supplied as the sole carbon source. The pH of the medium in all cases was 6.9 and did not change in any system for the duration of the experiment. Substrate removal was monitored with time (Figure 3.2). *P. putida* KT2440 was unable to grow and remove any of the chlorophenols. In the case of *P. putida* CP1, 4-chlorophenol was removed more readily than 2-chlorophenol which was removed more readily than 3-chlorophenol (Figure 3.2). The lower concentration of 4-chlorophenol was removed fastest in 15 hours. Biomass was monitored throughout the experiment. Although *P. putida* CP1 could remove the substrate, there was little growth by the organism on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol and 200 ppm 4-chlorophenol. The only significant increase in

biomass was observed when *P. putida* CP1 was grown on 50 ppm 4-chlorophenol. The pattern of substrate removal by *P. putida* CP1 was a two stage process. A slow or lag period was followed by a rapid phase of removal. The lag period was shortest for 50 ppm 4-chlorophenol and longest for 100 ppm 2-chlorophenol and 100 ppm 3-chlorophenol (Table 3.6). When the rates of substrate removal and the specific rates of substrate removal were calculated for the two stages, the rates of substrate removal were in the order 4CP> 2CP> 3CP in the case of the higher concentrations of chlorophenols. The formation of aggregates of cells was observed after 72h growth on 100 ppm 2-chlorophenol and 200 ppm 4-chlorophenol and after 96h in the presence of 100 ppm 3-chlorophenol. No aggregate formation was observed during growth on the lower concentration of 50 ppm 4-chlorophenol.

The percentage viability of *P. putida* CP1, determined using the Live/Dead stain, decreased with time over the four day incubation (Table 3.7 and Figure 3.3). On day four, the percentage viability correlated with the ability of the organism to degrade the substrate – highest percent viability when the organism was grown on 4-chlorophenol and lowest viability when the organism was grown on 2-chlorophenol and 3-chlorophenol. Images of the organism (Figure 3.3) illustrate aggregation of the cells. The distribution of live and dead cells in the aggregate grown on 100 ppm 3-chlorophenol for 24 h was observed using confocal laser scanning microscopy (CLSM) at x60 magnification (Figure 3.4). The biomass was scattered evenly across the whole region. The biomass at the core was stained mainly by SYTO 9 indicating mainly live cells. Cells in the outer zone were stained by both propidium iodide and SYTO 9 randomly indicating a mixture of live and dead cells.

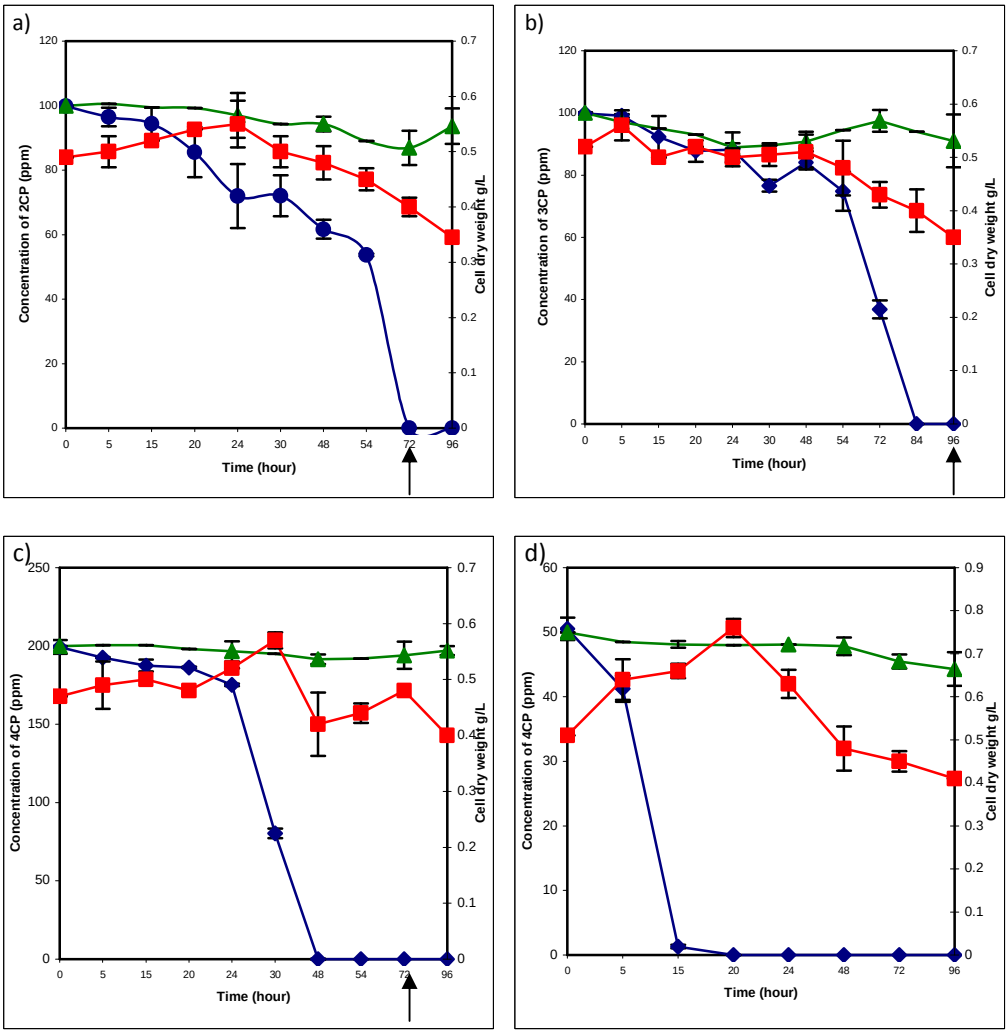


Figure 3.2 Degradation of a) 100 ppm 2-chlorophenol, b) 100 ppm 3-chlorophenol, c) 200 ppm 4-chlorophenol and d) 50 ppm 4-chlorophenol by *P. putida* CP1 and *P. putida* KT2440. (●) *P. putida* CP1 (ppm chlorophenol), (▲) *P. putida* KT2440 (ppm chlorophenol) and (■) Biomass of *P. putida* CP1 (g/L). Arrow indicates time of aggregate formation.

Table 3.6 Lag periods and substrate removal rates for *P. putida* CP1 grown on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol, 200 and 50 ppm 4-chlorophenol (Data from Figure 3.2).

Substrate and stage of removal	**Time (h)**	**Substrate removal rate ppm h^{-1}**	**Specific substrate removal rate ppm h^{-1} g^{-1} Biomass**
100 ppm 2CP			
Slow/lag phase	0-54	0.85 ± 0.01	1.53 ± 0.10
Rapid removal phase	54-72	2.98 ± 0.02	59.67 ± 0.46
100 ppm 3CP			
Slow/lag phase	5-54	0.5 ± 0.14	6.27 ± 0.47
Rapid removal phase	54-84	2.49 ± 0.21	31.2 ± 2.61
200 ppm 4CP			
Slow/lag phase	0-24	1.01 ± 0.22	20.2 ± 4.36
Rapid removal phase	24-48	7.28 ± 0.05	72.77 ± 0.52
50 ppm 4CP			
Slow/lag phase	0-5	1.76 ± 0.25	13.54 ± 1.92
Rapid removal phase	5-15	3.96 ± 0.09	39.62 ± 0.88

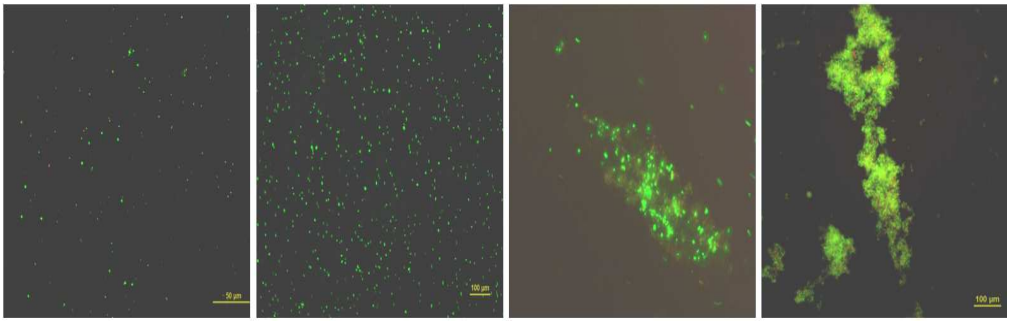
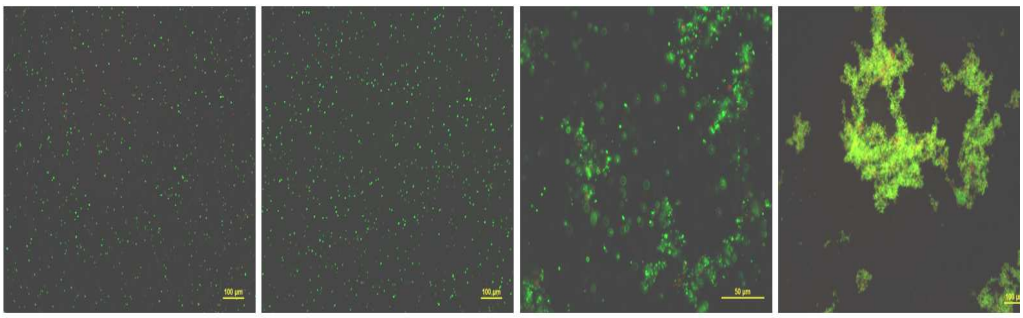
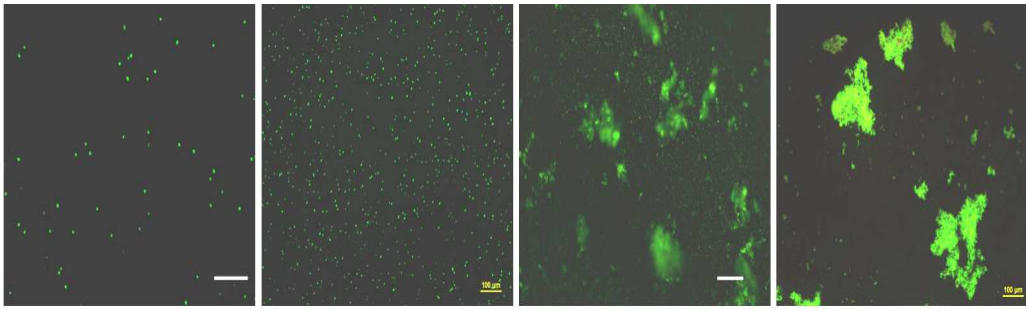
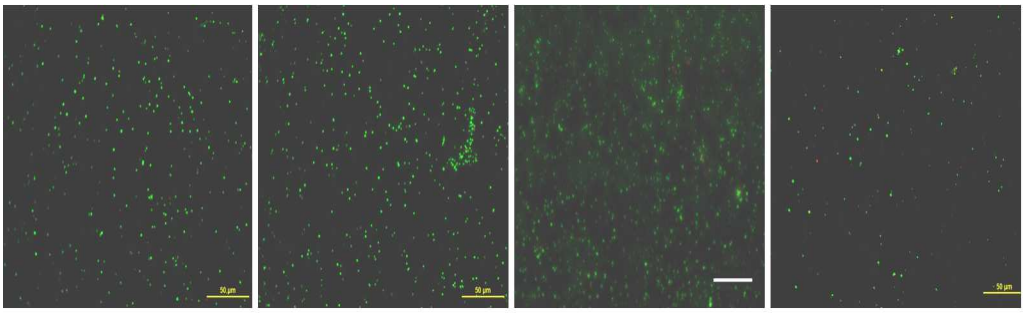
Day 1	Day 2	Day 3	Day 4
100 ppm 2-chlorophenol			
			
100 ppm 3-chlorophenol			
			
200 ppm 4-chlorophenol			
			
50 ppm 4-chlorophenol			
			

Figure 3.3 Microscopic images showing cell viability of *P. putida* CP1 stained with the Live/Dead stain following growth on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol, 200 and 50 ppm 4-chlorophenol over a four day period.

Table 3.7 The percentage viability of *P. putida* CP1 following growth on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol, 200 and 50 ppm 4-chlorophenol over a four day period (Data from Figure 3.3).

Treatment	**Day 1**	**Day 2**	**Day 3**	**Day 4**
100 ppm 2-chlorophenol	97 ± 6.3	80 ± 6.9	65 ± 5.7	64 ± 7.7
100 ppm 3-chlorophenol	81 ± 7	70 ± 7.5	64 ± 10	62 ± 5
200 ppm 4-chlorophenol	94 ± 7.4	83 ± 5.5	78 ± 5.4	76 ± 8
50 ppm 4-chlorophenol	97 ± 6	92 ± 6.8	81 ± 16.7	80 ± 9.4

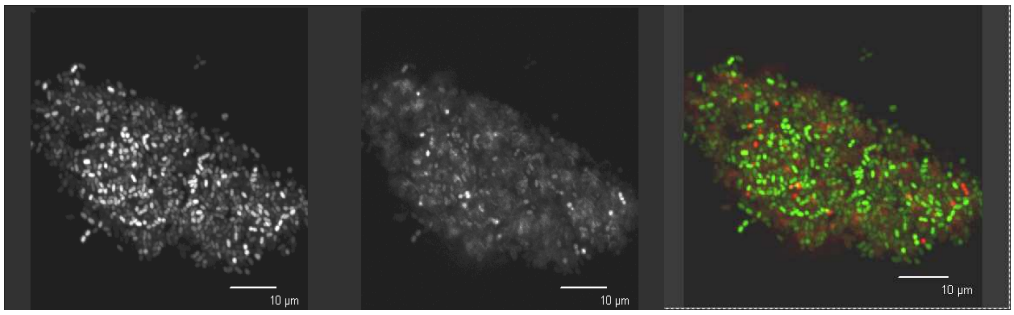


Figure 3.4 CLSM image of *P. putida* CP1 aggregates stained with the Live/Dead stain following growth on 100 ppm 3-chlorophenol for 4 days.

Analysis of the aggregates

The aggregation index, cell surface hydrophobicity and size of the aggregates were determined over a four day period as *P. putida* CP1 grew on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol, 200 and 50 ppm 4-chlorophenol. In the absence of aggregation, on day 1 and when the organism was grown on 50 ppm 4-chlorophenol, the aggregation index was in the order of 0.3 – 0.5. When cell aggregation was observed, the aggregation index increased with time of incubation. The aggregation index was highest when the organism was grown on 100 ppm 3-chlorophenol with a value of 0.94 ± 0.08 on day 4. This was followed by a value of 0.9 ± 0.02 on day 4 for 100 ppm 2-chlorophenol and 0.88 on day 4 when the organism was grown on 200 ppm 4-chlorophenol.

The BATH assay was used to determine the hydrophobicity of the aggregated cells. When the organism was grown on a lower concentration of 4-chlorophenol and did not form aggregates, the hydrophobicity of the cells was less than 7%. Hydrophobicity increased with time and there was a significant difference between the hydrophobicity of the aggregates from day 1 to day 4. In the presence of 100 ppm 3-chlorophenol, cell surface hydrophobicity was greatest at 52% on day 4, followed by 44% when the organism was grown on 100 ppm 2-chlorophenol and 32% when the organism was grown on 200 ppm 4-chlorophenol.

The aggregates increased in size from day 1 to day 4 of incubation. The aggregate size is only recorded in Table 3.8 where measurable aggregates were observed. Aggregates were observed on days 3 and 4 following growth on 100 ppm 2-chlorophenol and 200 ppm 4-chlorophenol, on day 4 following growth on 100 ppm 3-chlorophenol and no aggregates were formed when the organism was grown on 50 ppm 4-chlorophenol. On day 4, the size of the aggregates was largest at 22.47 ± 2.96 μm^2 (10^3) when the bacterium was grown on the most toxic substrate 3-chlorophenol. The average size of the aggregates was 19.15 ± 0.43 μm^2 (10^3) when the organism was grown on 100 ppm 2-chlorophenol and the smallest aggregate size was 9.35 ± 1.2 μm^2 (10^3) when the bacterium was grown on 200 ppm 4-chlorophenol (Table 3.8).

Table 3.8 Changes in aggregation index (AI), cell surface hydrophobicity (BATH %) and aggregate size when *P. putida* CP1 was grown on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol, 200 and 50 ppm 4-chlorophenol for four days.

Analysis	**Day**			
	1	**2**	**3**	**4**
100 ppm 2-CP				
AI	0.55 ± 0.05	0.67 ± 0.13	0.72 ± 0.04	0.9 ± 0.02
BATH %	17 ± 1.68	28 ± 1.06	29 ± 2.4	44 ± 1.01
Size μm^2 (10^3)	-	-	5.45	19.15 ± 0.43
100 ppm 3-CP				
AI	0.35 ± 0.19	0.48 ± 0.19	0.90 ± 0.02	0.94 ± 0.08
BATH %	10 ± 1.42	30 ± 1.25	40 ± 1.42	52 ± 1.7
Size μm^2 (10^3)	-	-	-	22.47±2.96
200 ppm 4-CP				
AI	0.32 ± 0.05	0.21 ± 0.1	0.6 ± 0.18	0.88
BATH %	10 ± 0.79	10 ± 1.07	14 ± 0.75	32 ± 1.64
Size μm^2 (10^3)	-	-	3.94 ± 1.2	9.35 ± 1.2
50 ppm 4-CP				
AI	0.3 ± 0.08	0.21 ± 0.1	0.25 ± 0.03	0.27 ± 0.01
BATH %	2 ± 0.38	6 ± 0.94	4 ± 1.02	6 ± 0.93

Extracellular polymeric substances (EPS)

The production of extracellular polymeric substances (EPS) by *P. putida* CP1 cultivated on mono-chlorophenols was investigated for the four day period. The levels of both bound and free EPS were quantified (Figure 3.5). In general, the levels of bound EPS were higher compared to the levels of free EPS in all treatments. In the absence of cell aggregation, the levels of EPS did not change significantly with time when the bacterium was grown on 50 ppm 4-chlorophenol. Where cell aggregation occurred, the levels of EPS increased steadily until day 4. The highest levels of EPS were detected when the organism was grown on 3-chlorophenol, then 2-chlorophenol and then 200 ppm 4-chlorophenol.

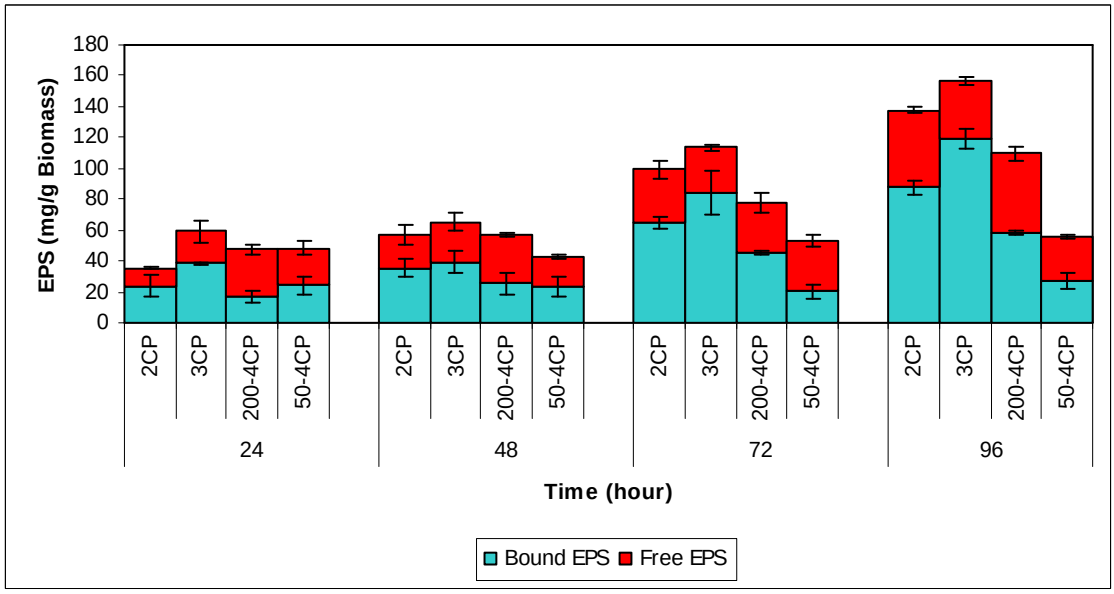


Figure 3.5 Distribution of bound and free EPS during growth of *P. putida* CP1 on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol, 200 and 50 ppm 4-chlorophenol.

Biochemical composition of EPS

A biochemical analysis of the EPS showed that the main components of the EPS were carbohydrate and protein in approximately equal proportions (Figure 3.6).

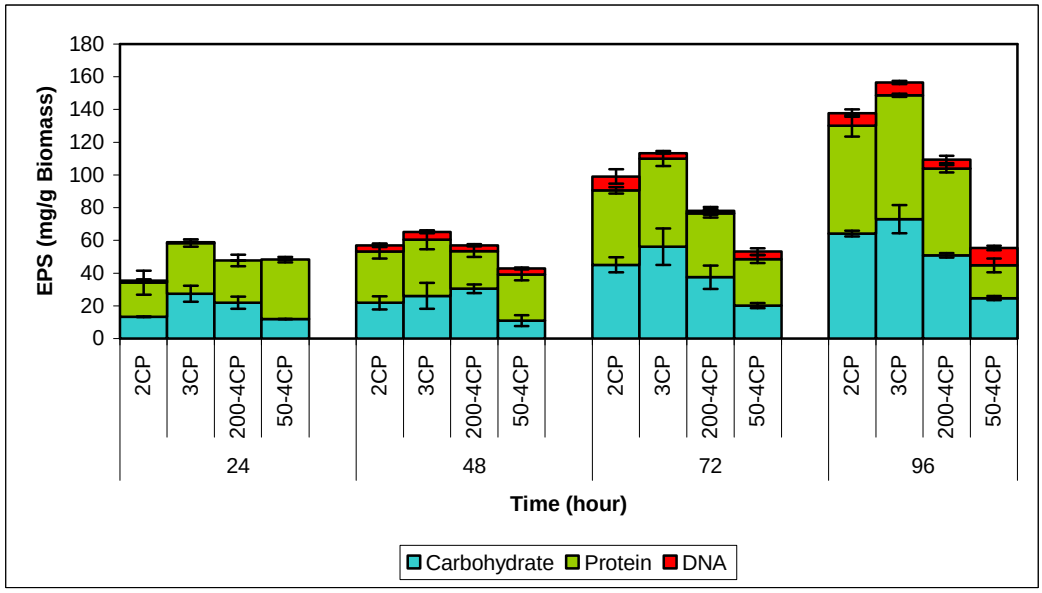


Figure 3.6 Biochemical composition of total EPS during growth of *P. putida* CP1 on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol, 200 and 50 ppm 4-chlorophenol.

When the biochemical composition of the bound and free EPS was individually analysed it was found that carbohydrate was the dominant component of the free EPS (Figure 3.7), while protein dominated the bound EPS (Figure 3.8). The third component of the EPS was DNA which formed less than 10% of the EPS. The levels of DNA were lowest on day 1 and increased with time suggesting that the DNA resulted from cell lysis.

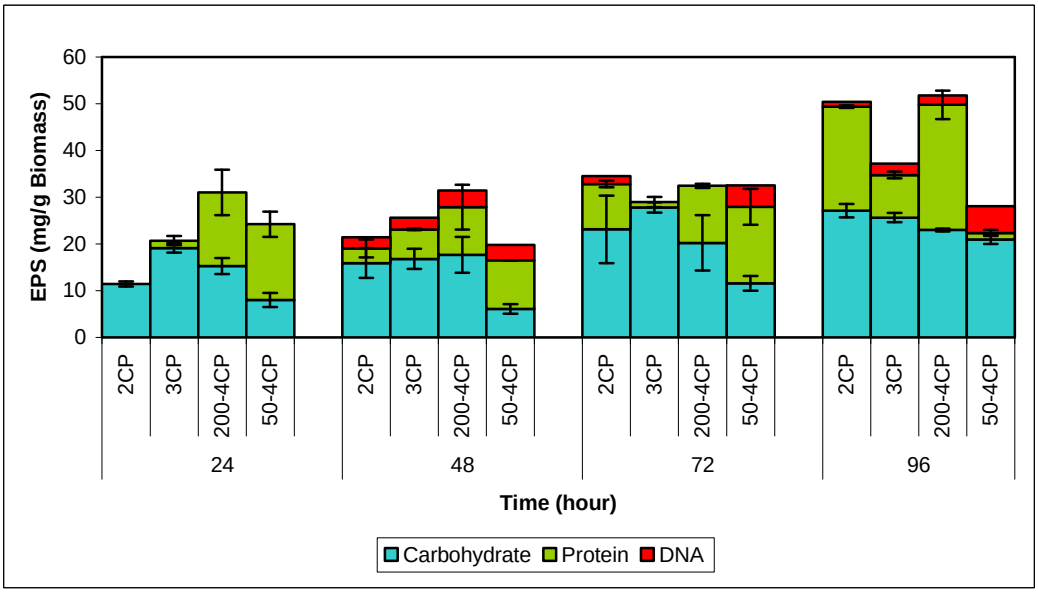


Figure 3.7 Biochemical composition of free EPS during growth of *P. putida* CP1 on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol, 200 and 50 ppm 4-chlorophenol.

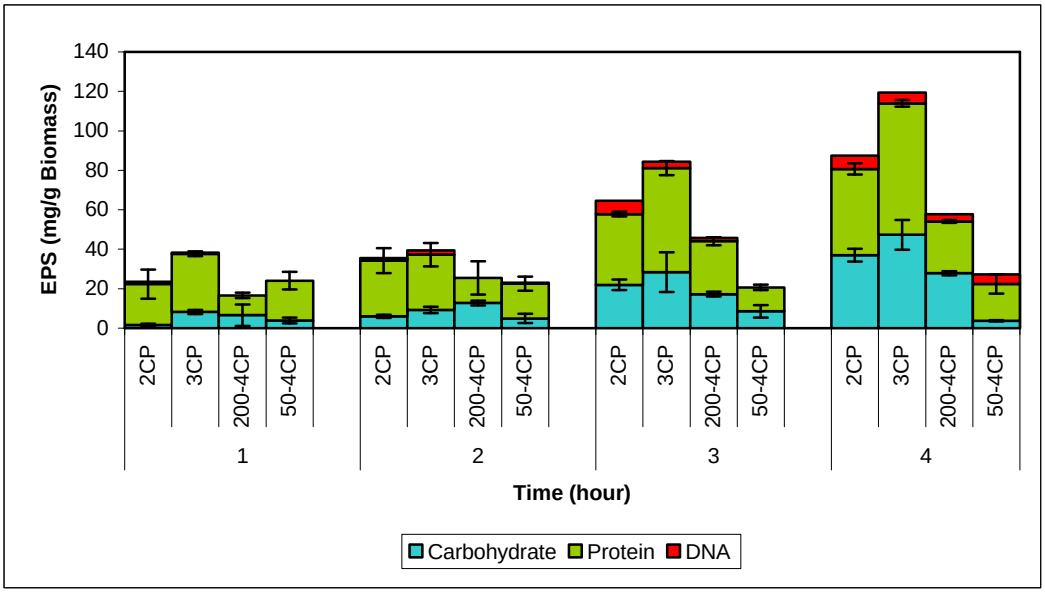


Figure 3.8 Biochemical composition of bound EPS during growth of *P. putida* CP1 on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol, 200 and 50 ppm 4-chlorophenol.

A microscopic examination of the EPS was carried out using specific fluorophores and epifluorescent microscopy. Calcofluor white was used to stain carbohydrate, FITC to stain protein and SYTO 63 to stain DNA. The results supported the biochemical analysis showing equal percentages of carbohydrate and protein and approximately 10% DNA (Figure 3.9). Confocal laser scanning microscopy (CLSM) was used to study the aggregates when the organism was grown on 100 ppm 3-chlorophenol. The distribution of the biochemical components of the EPS were seen to be randomly distributed (Figure 3.10).

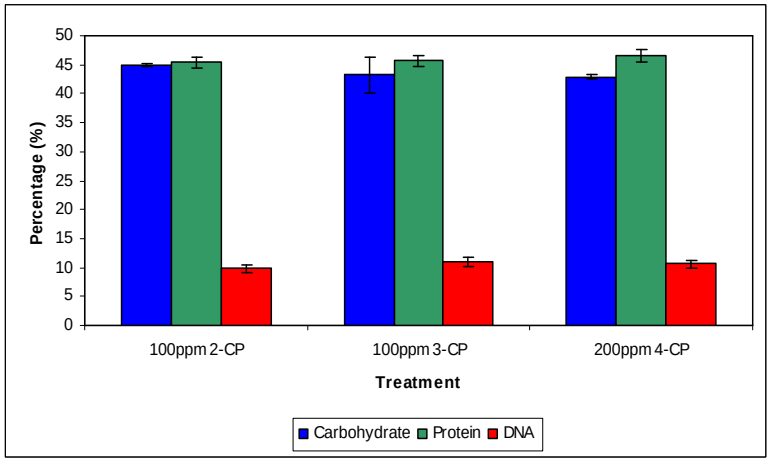


Figure 3.9 Percentage of carbohydrate, protein and DNA in EPS produced by *P. putida* CP1 following growth on 2-chlorophenol, 100 ppm 3-chlorophenol and 200 ppm 4-chlorophenol after four days based on microscopic observation.

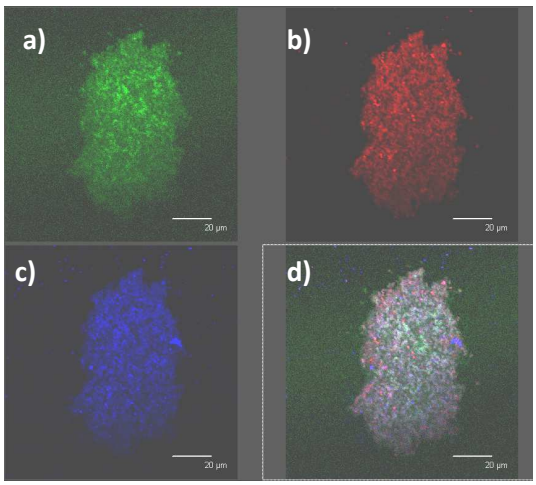


Figure 3.10 CLSM image of *P. putida* CP1 aggregated cells following growth on 100 ppm 3-chlorophenol for four days. a) FITC, b) SYTO 63 c) Calcofluor white and d) combined multi-channel image.

3.2.2 Expression profiling analysis

Expression profiling analysis was used to identify genetic factors governing autoaggregation when the organism was grown on mono-chlorophenols using a *P. putida* Genome Oligonucleotide Array (Progenika, Spain). Initially results obtained for *P. putida* CP1 grown on nutrient broth were compared individually with those obtained when the organism was grown on 100 ppm 2-chlorophenol (array 2), 100 ppm 3-chlorophenol (array 3), 200 ppm 4-chlorophenol (array 4) and 50 ppm 4-chlorophenol (array 5). Then a comparative study of 50 ppm4CP vs 200 ppm4CP (array 8) was carried out and finally a comparative study of CP1vs 200 ppm 4CP, 200 ppm4CPvs 100 ppm 2CP and 100 ppm 2CP vs 100 ppm 3CP was done. In each study the differentially regulated genes were identified, PSORTB v3 analysis was used to identify the cellular location of the proteins and metabolic pathway analysis was used to identify the main biological pathways.

Comparative study for CP1 vs 100 ppm 2-chlorophenol, CP1 vs 100 ppm 3-chlorophenol, CP1 vs 200 ppm 4-chlorophenol and CP1 vs 50 ppm 4-chlorophenol

The results obtained for *P. putida* CP1 grown on nutrient broth were compared individually with those obtained when the organism was grown on 100 ppm 2-chlorophenol (array 2), 100 ppm 3-chlorophenol (array 3), 200 ppm 4-chlorophenol (array 4) and 50 ppm 4-chlorophenol (array 5). The results are described in Figure 3.11 and show that a total of 982 genes were differentially regulated in the comparative study of CP1-100 ppm 2-chlorophenol, 556 genes were significantly up-regulated and 426 were identified as significantly down-regulated. A total of 1615 genes were differentially regulated when the comparative study of CP1-100 ppm 3-chlorophenol was carried out. 925 genes were significantly up-regulated and 690 genes were significantly down-regulated. The comparative study of CP1-200 ppm 4-chlorophenol showed 1015 genes were significantly up-regulated and 960 genes were down-regulated and a total of 1423 genes were differentially regulated when *P. putida* CP1 grown on nutrient broth was compared with *P. putida* grown on 50 ppm 4-chlorophenol. 857 genes were identified as significantly up-regulated and 566 genes were identified as

significantly down-regulated. All data generated were based on a *p*-value ≤ 0.05 and a fold change, FC> 1.2/ FC< -1.2.

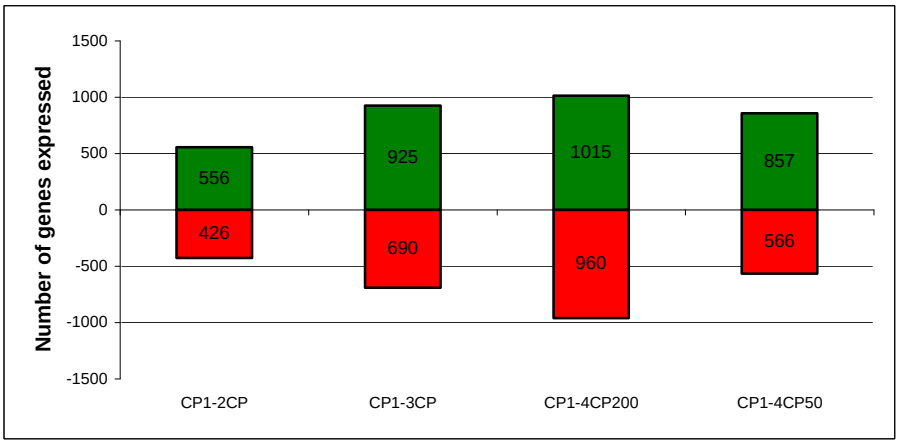


Figure 3.11 The number of differentially regulated genes in *P. putida* CP1 grown on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol, 200 and 50 ppm 4-chlorophenol when compared to *P. putida* CP1 grown on nutrient broth. Up- regulated genes (■) and down-regulated genes (■).

When the differentially regulated genes in the four comparative studies were overlapped using Venny, 484 genes were identified as common (Figure 3.12). 344 of these genes were identified as differentially up-regulated and 137 genes were down-regulated. Three of the overlapped genes were expressed in various patterns.

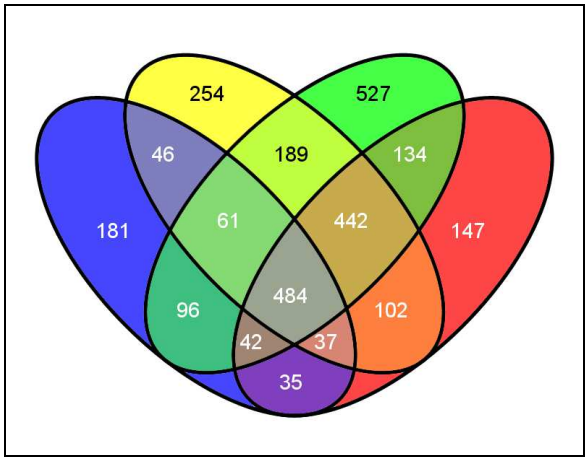


Figure 3.12 Venn diagram representing the genes common and uncommon to *P. putida* CP1 grown on 100 ppm 2-chlorophenol (■), 100 ppm 3-chlorophenol (■), 200 ppm 4-chlorophenol (■) and 50 ppm 4-chlorophenol (■) when compared to *P. putida* CP1 grown on nutrient broth.

Protein localization

It was of interest to determine the cellular location of proteins encoded by these 484 genes. PSORTB v3 analysis showed that 115 of the genes encoded proteins in an unknown location, 245 encoded proteins in the cytoplasm, 103 in the cytoplasmic membrane, nine in the periplasmic space, eight in the outer membrane, one for flagella and two proteins localized in an extracellular compartment (Table 3.9).

Table 3.9 Cell location of proteins encoded by genes differentially regulated in *P. putida* CP1 grown on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol, 200 and 50 ppm 4-chlorophenol when compared to *P. putida* CP1 grown on nutrient broth.

Protein location	**Gene number**		
	Induced	**Repressed**	**Total**
Cytoplasm	189	56	245
Cytoplasmic membrane	70	33	103
Periplasmic space	7	2	9
Outer membrane	5	3	8
Flagella	0	1	1
Extracellular	1	1	2
Unknown	70	45	115

Metabolic pathway analysis

A pathway analysis was conducted on the 484 overlapped genes based on the biological information database KEGG. A total of 14 different metabolic pathways were identified in this analysis (Figure 3.13). Nearly 10% of the genes played a role in protein translation. About 6% of the genes were involved in nucleotide metabolism, followed by 4.43% of the genes involved in oxidative phosphorylation and amino acid metabolism. Other pathways involving fewer genes included pathways for carbohydrate metabolism, two-component system, ABC transporters, bacterial secretion system, protein export, protein stabilization, LPS biosynthesis, fatty acid metabolism, transcription and bacterial chemotaxis.

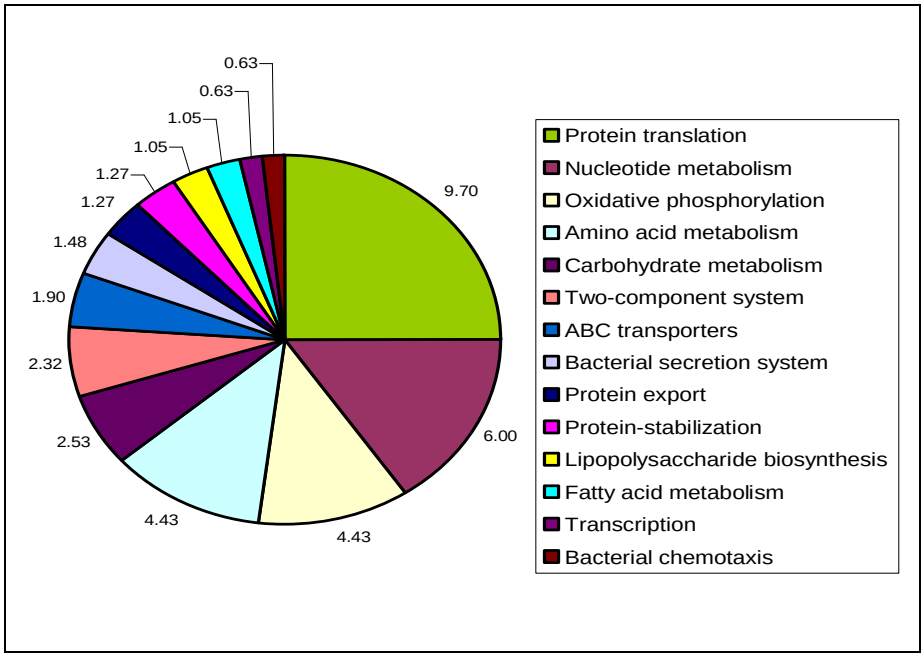


Figure 3.13 Percentage of metabolic pathways associated with differentially regulated genes in *P. putida* CP1 grown on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol, 200 and 50 ppm 4-chlorophenol when compared with *P. putida* CP1 grown on nutrient broth.

Comparative study 50 ppm4CP vs 200 ppm4CP

Aggregation was observed when *P. putida* CP1 was grown on the higher concentration of 4-chlorophenol but not on the lower concentration. A transcriptomic profile analysis was conducted by comparing the array results obtained for the two treatments. The analysis revealed a total of 626 genes differentially regulated. 264 genes were up-regulated (*p*-value $\leq$ 0.05, FC> 1.2). The top 25 up-regulated genes are listed in Table 3.10 and showed PP807 encoded for sigma 54 was the most induced gene. Other pathways induced included those for valine, leucine and isoleucine biosynthesis, carbohydrate metabolism, flagellar assembly, nucleotide metabolism, protein translation and ABC transporters.

Table 3.10 Top 25 genes up-regulated in *P. putida* CP1 grown on 200 ppm 4-chlorophenol when compared with *P. putida* grown on 50 ppm 4-chlorophenol.

Locus ID	**Gene Name**	**Annotation**	**Pathway**	**FC**	***p*-value**
PP807		sigma 54		8.338	0.00001
PP4680	ilvB	acetolactate synthase 3	Valine, leucine and	5.792	0.00016

		catalytic subunit	isoleucine biosynthesis		
PP4679	ilvH	acetolactate synthase 3 regulatory subunit	Valine, leucine and isoleucine biosynthesis	4.782	0.00079
PP4115		NolW domain protein		4.162	0.00018
PP1076	glpF	MIP family channel protein		4.15	0.00002
PP4487	acsA	acetyl-CoA synthetase	Glycolysis / Gluconeogenesis	3.63	0.00399
PP811	cyoups2	cyoups2 protein		3.62	0.00027
PP5347	accC-2	pyruvate carboxylase subunit A	Citrate cycle	3.576	0.01019
PP4390	flgC	flagellar basal body rod protein FlgC	Flagellar assembly	3.514	0.00002
PP1100	dcd	deoxycytidine triphosphate deaminase	Nucleotida metabolism	3.476	0.00007
PP47		DNA-binding heavy metal response regulator, putative		3.38	0.00343
PP1590	map	methionine aminopeptidase		3.264	0.00002
PP1692		Sel1 domain protein repeat-containing protein		3.242	0.00125
PP218		sensory box protein		3.202	0.01333
PP2848	ureF	urease accessory protein UreF		3.142	0.00043
PP5281	rpmG	50S ribosomal protein L33	Protein translation	3.134	0
PP1025	leuA	2-isopropylmalate synthase	Valine, leucine and isoleucine biosynthesis	3.054	0.02647
PP4385	flgG	flagellar basal body rod protein FlgG	Flagellar assembly	3.026	0
PP5282	rpmB	50S ribosomal protein L28	Protein translation	2.96	0.00008
PP4193	sdhC	succinate dehydrogenase, cytochrome b556 subunit	Citrate cycle	2.958	0.00534
PP4204		Cro/CI family transcriptional regulator		2.908	0.00055
PP8	rnpA	ribonuclease P		2.902	0.00006
PP4678	ilvC	ketol-acid	Valine, leucine and	2.896	0.00331

		reductoisomerase	isoleucine biosynthesis		
PP4194	gltA	type II citrate synthase	Citrate cycle	2.876	0.00157
PP1068		amino acid ABC transporter ATP-binding protein	ABC transporters	2.824	0.0001

362 genes were identified as significantly down-regulated and following ranking by fold change the top 25 genes are listed in Table 3.11. The top down-regulated 25 genes were involved in various pathways including two-component systems, carbohydrate metabolism, ABC transporters and amino acid metabolism. The most down-regulated gene, PP569, encoded for the MATE (Multi-drug and toxic compound extrusion) efflux family protein with a fold change -4.38.

Table 3.11 Top 25 genes down-regulated in *P. putida* CP1 grown on 200 ppm 4-chlorophenol when compared with *P. putida* grown on 50 ppm 4-chlorophenol.

Locus ID	**Gene Name**	**Annotation**	**Pathway**	**FC**	***p*-value**
PP569		MATE efflux family protein		-4.382	0.02925
PP2780		3-oxoacyl-(acyl-carrier-protein) synthase II		-3.484	0.00076
PP397		putative serine protein kinase, PrkA		-3.456	0.00003
PP2665	agmR	LuxR family two component transcriptional regulator	Two-component system	-3.268	0.00153
PP4902	orn	oligoribonuclease		-3.264	0.00003
PP589		Bcr/CflA family multidrug resistance transporter		-3.114	0.02183
PP334		ISPpu11, transposase		-3.112	0.00018
PP1585		antidote protein, putative		-3.088	0.00005
PP3249		aldo/keto reductase		-3.046	0.00565
PP4176		amidohydrolase 2		-3.022	0.0013
PP663		AsnC family transcriptional regulator		-2.932	0.02799
PP491		formate dehydrogenase, gamma subunit	Carbohydrate metabolism	-2.89	0.00237

PP3291		metallo-beta-lactamase family protein		-2.862	0.00727
PP4179	htpG	heat shock protein 90		-2.812	0.0022
PP3372	cpxR	winged helix family two component transcriptional regulator	Two-component system	-2.74	0.00632
PP3986		ISPpu13, transposase Orf1		-2.736	0.00076
PP4728	grpE	heat shock protein GrpE		-2.732	0.00509
PP3078		ABC transporter, periplasmic binding protein, putative	ABC transporters	-2.692	0.00396
PP967	hisC	histidinol-phosphate aminotransferase	Amino acid metabolism	-2.672	0.03147
PP1497		TetR family transcriptional regulator		-2.67	0.00041
PP3265		endonuclease/exonuclease/ phosphatase		-2.588	0.00182
PP2402		integral membrane sensor signal transduction histidine kinase	Two-component system	-2.578	0.0024
PP1227		cation efflux protein		-2.494	0.00213
PP3301		RND efflux membrane fusion protein		-2.488	0.00455
PP3086		RNA polymerase sigma-70 factor, putative		-2.47	0.00905

Protein localization

Analysis of the 626 differentially expressed genes, using PSORTB v3 showed the location in the cell of the encoded proteins (Table 3.12). 148 of the genes encoded proteins in an unknown location, 282 in the cytoplasm, 138 in the cytoplasmic membrane, 15 in the periplasmic space, 10 in the outer membrane, six for flagella and six proteins localized in an extracellular compartment.

Table 3.12 Cell location of proteins encoded by genes differentially regulated in *P. putida* CP1 grown on 200 ppm 4-chlorophenol when compared to *P. putida* CP1 grown on 50 ppm 4-chlorophenol.

Protein location	**Gene number**		
	Induced	**Repressed**	**Total**
Cytoplasm	127	155	282
Cytoplasmic membrane	45	93	138
Periplasmic space	5	10	15
Outer membrane	5	5	10
Flagella	6	0	6
Extracellular	2	4	6
Unknown	68	80	148

Metabolic pathway analysis

Following pathway analysis using the biological information database KEGG, significant metabolic pathways were identified based on *p*-value ($p \leq 0.05$) and the 14 most significant pathways are listed in Figure 3.14. Carbohydrate metabolism and amino acid metabolism pathways were observed to be dominant with 12.4% and 5.45% genes involved in each pathway respectively. Other pathways included those found to play a role in two-component systems, ABC transporters, oxidative phosphorylation, metabolism of cofactors and vitamins, flagellar assembly, nucleotide metabolism, protein translation, xenobiotic biodegradation and metabolism, bacterial chemotaxis, replication and repair, fatty acid metabolism and lipopolysaccharide (LPS) biosynthesis.

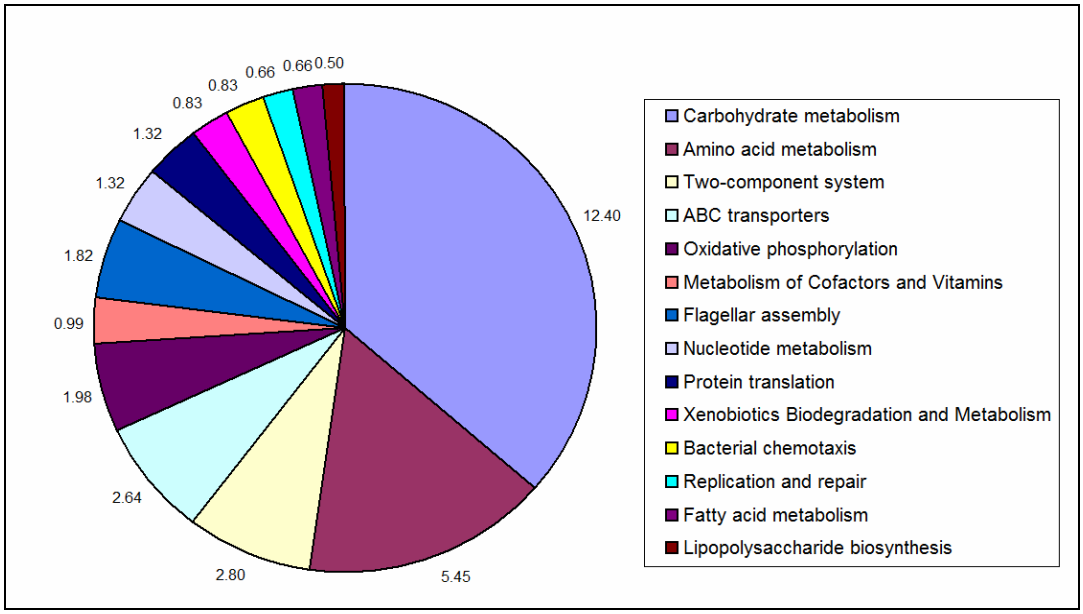


Figure 3.14 Percentage of metabolic pathways encoded by differentially regulated genes in *P. putida* CP1 grown on 200 ppm 4-chlorophenol compared to *P. putida* CP1 grown on 50 ppm 4-chlorophenol.

Comparative analysis of CP1 vs 200 ppm 4CP-100 ppm 2CP-100 ppm 3CP

The degree of aggregation was inversely related to the ability of *P. putida* CP1 to degrade the three mono-chlorophenols i.e. the aggregate size was greatest for 3-chlorophenol and smallest for 4-chlorophenol (Figure 3.15). To further investigate the genes involved with aggregation, the array data generated by Array 4: CP1 vs 200 ppm 4CP; Array 6: 200 ppm4CP vs 100 ppm 2CP and Array 7: 100 ppm 2CP vs 100 ppm 3CP were compared. In investigating the data, genelists were generated from the data and the data were compared based on consistent changes. A total of 1975 differentially regulated genes from CP1 vs 200 ppm 4CP, 1286 genes from 200 ppm4CPvs 100 ppm 2CP and 973 from 100 ppm 2CP vs 100 ppm 3CP were overlapped using Venny (Figure 3.16). The 937 overlapped genes were then analyzed using Microsoft Access to verify genes with consistent changes. The analysis revealed that only 51 genes were expressed in a consistent order.

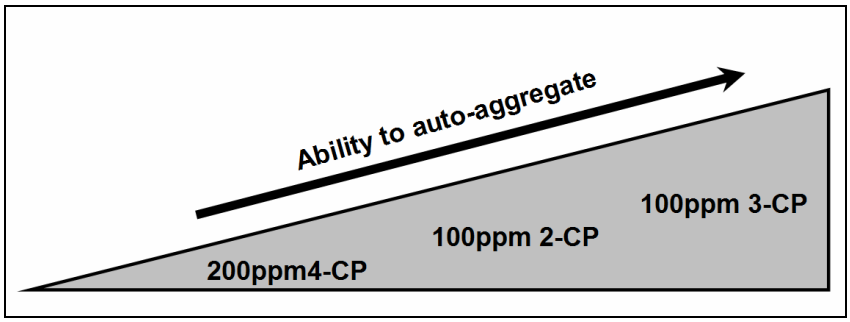


Figure 3.15 The autoaggregation phenomenon in *P. putida* CP1. Aggregates produced following growth on 100 ppm 3-chlorophenol> 100 ppm 2-chlorophenol> 200 ppm 4-chlorophenol.

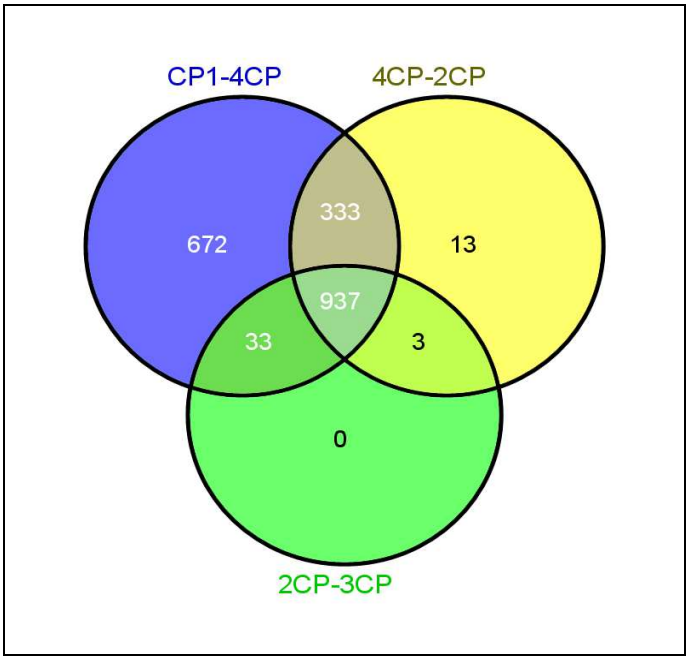


Figure 3.16 Venn diagram showing the numbers of genes for *P. putida* CP1 grown on 200 ppm 4-chlorophenol compared with *P. putida* CP1 grown on nutrient broth (■), *P. putida* CP1 grown on 100 ppm 2-chlorophenol compared with *P. putida* CP1 grown on 200 ppm 4-chlorophenol (■) and *P. putida* CP1 grown on 100 ppm 3-chlorophenol compared with *P. putida* CP1 grown on 100 ppm 2-chlorophenol (■).

33 genes of these genes were significantly up-regulated and 18 genes were identified as significantly down-regulated (p-value $\leq$ 0.05, FC>1.2/ < -1.2). To eliminate the genes common to *P. putida* CP1 when growing on non-aggregating and aggregating concentrations of chlorophenol, the genelists generated by *P. putida* CP1 vs. 200 ppm 4CP-100 ppm 2CP-100 ppm 3CP were overlapped with the results obtained with *P. putida* CP1 vs. 50 ppm 4CP (Figure 3.11). Of the

1423 genes identified in the *P. putida* CP1 vs. 50 ppm 4CP treatment, 44 were common to the list obtained for *P. putida* CP1 vs. 200 ppm 4CP-100 ppm 2CP-100 ppm 3CP (Figure 3.17). All of the 44 genes were expressed in the same direction. A total of 1379 genes were unique to *P. putida* CP1 vs. 50 ppm and 7 genes were unique to CP1 vs. 200 ppm 4CP-100 ppm 2CP-100 ppm 3CP.

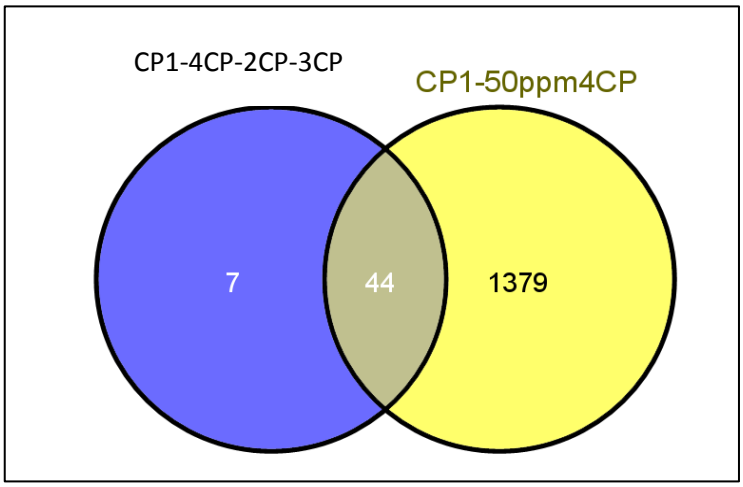


Figure 3.17 Venn diagram showing the numbers of genes common to two genelists; *P. putida* CP1 grown on nutrient broth compared to *P. putida* CP1 grown on 200 ppm 4-chlorophenol, 100 ppm 2-chlorophenol and 100 ppm 3-chlorophenol (■) and *P. putida* CP1 grown on 50 ppm 4-chlorophenol compared with *P. putida* CP1 grown on nutrient broth (■).

The 44 common genes were identified (Table 3.13). A total of 23 genes were up-regulated. The majority were involved in metabolic pathways. Other pathways included base excision repair which is a cellular mechanism that repairs damaged DNA, lipopolysaccharide biosynthesis, transcription, fatty acid metabolism, signal transduction and transport systems. Eight highlighted genes were down-regulated and included xcpZ involved in a secretion pathway, PP4888 involved in signal transduction, oprD encoding for a membrane protein, PP411 and PP145 involved in ABC transporters and 3 genes involved in metabolic pathways (PP180, gcl and bioB). Thirteen genes had an unknown function and included 6 induced genes and 7 repressed genes.

Table 3.13 Details of 44 genes and associated metabolic pathways common to two genelists; *P. putida* CP1 grown on nutrient broth compared to *P. putida* CP1 grown on 200 ppm 4-chlorophenol, 100 ppm 2-chlorophenol and 100 ppm 3-chlorophenol and *P. putida* CP1 grown on 50 ppm 4-chlorophenol compared to *P. putida* CP1 grown on nutrient broth (Data from Figure 3.17).

Locus ID	**Gene Name**	**Annotation**	**Pathway**	**FC CP1 vs. 200CP4**	**FC 200CP4 vs CP2**	**FC CP2 vs CP3**	**FC CP1 vs 50CP4**
PP5292	crc	exodeoxyribonuclease	Base excision repair	1.482	0.112	0.196	1.91
PP63		lipid A biosynthesis lauroyl acyltransferase putative	Lipopolysaccharide biosynthesis	1.222	0.126	1.228	0.238
PP1054	**xcpZ**	**type II secretion pathway protein XcpZ**	Secretion	**-1.694**	**-2.234**	**-0.556**	**-2.474**
PP1637		transcriptional regulator LysR family	Transcription	1.222	0.196	0.626	1.496
PP2232		transcriptional regulator Cro/CI family		1.946	0.498	0.89	2.418
NA		transcriptional		1.86	0.64	0.312	1.544
NA		transcriptional		1.58	0.058	0.1	1.68
PP5108	rpoH	RNA polymerase sigma-32 factor		1.632	0.17	1.444	1.482
PP124	engB	GTP-binding protein putative		2.302	0.776	0.266	3.35
PP4948		acyl-CoA dehydrogenase family protein	Fatty acid metabolism	1.826	0.608	0.15	3.27

PP3990		membrane protein putative	Signal transduction	2.38	1.08	0.478	2.486
PP2645	mgtB	magnesium-translocating P-type ATPase		2.622	0.25	0.566	5.038
PP3437		CBS domain protein		6.216	0.42	0.94	6.792
PP4888		**methyl-accepting chemotaxis transducer putative**		**-1.5**	**-0.226**	**-0.504**	**-2.582**
PP1206	**oprD**	**porin D**	Membrane protein	**-1.65**	**-0.422**	**-0.346**	**-2.324**
PP5355		sodium/proton antiporter putative	Transport system	2.42	0.366	0.344	1.806
PP2035	benE-1	benzoate transport protein		1.544	0.16	0.984	2.412
PP959	ttg2B	toluene tolerance ABC efflux transporter permease		1.212	0.124	0.638	1.62
PP145		**Na+/Pi cotransporter family protein**		**-1.788**	**-0.498**	**-0.936**	**-2.046**
PP411		**polyamine ABC transporter ATP-binding protein**		**-2.966**	**-1.956**	**-0.226**	**-3.906**
PP127		thiol:disulfide interchange protein DsbA family	Metabolic pathways	1.542	1.236	0.444	3.292
PP4823	purD	phosphoribosylamine--glycine ligase		3.002	0.198	0.398	3.922

PP626	ndh	NADH dehydrogenase		3.068	0.564	0.346	4.56
PP3338		ubiquinol oxidase subunit II-related protein		1.418	0.768	0.852	3.042
PP4999	pyrC	dihydroorotase multifunctional complex type		1.34	0.88	0.45	3.402
PP4252	ccoQ-1	cytochrome c oxidase cbb3-type CcoQ subunit		2.318	0.182	0.196	1.998
PP2160	queF	7-cyano-7-deazaguanine reductase		1.428	0.558	0.982	1.804
PP5092		NLP/P60 family protein		1.398	0.704	0.14	1.62
PP4297	**gcl**	**glyoxylate carboligase**		**-1.242**	**-0.024**	**-0.114**	**-1.478**
PP362	**bioB**	**biotin synthetase**		**-1.238**	**-1.094**	**-0.676**	**-4.632**
PP180		**cytochrome c family protein**		**-4.116**	**-0.034**	**-0.252**	**-4.09**

* Data in bold refer to down-regulated genes

Seven genes unique to *P. putida* CP1 vs. 200 ppm 4CP-100 ppm 2CP-100 ppm 3CP are described in Table 3.14. Four of these genes were up-regulated and included PP1961 encoding for TetR family transcriptional regulator, PP2934 encoding for dienelactone hydrolase which is the final enzyme involved in chlorophenol degradation, PP132 encoding for histidine kinase which is involved in signal transduction of a two-component system and PP4936 encoding for O-antigen polymerase. Three genes was repressed and included tig encoding for a trigger factor involved in protein folding, PP2073 encoding for an acetyltransferase and an unknown gene that encodes for ubiquinol.

Table 3.14 Details of seven genes with associated metabolic pathways that were differentially expressed when *P. putida* CP1 grown on nutrient broth was compared to *P. putida* CP1 grown on 200 ppm 4-chlorophenol, 100 ppm 2-chlorophenol and 100 ppm 3-chlorophenol.

Locus ID	**Gene Name**	**Annotation**	**Pathway/ function**	**FC CP1 vs 200 4CP**	**FC 200 4CP vs 2CP**	**FC CP2 vs 3CP**
PP1961		TetR family transcriptional regulator	Transcription	1.596	0.226	0.392
PP2934		dienelactone hydrolase	Chlorophenol degradation	1.434	0.084	0.09
PP132		multi-sensor signal transduction histidine kinase	Two-component system	1.402	0.022	0.312
PP4936		O-antigen polymerase	LPS biosynthesis	1.28	0.082	0.114
PP2299	tig	trigger factor	Protein translation	-1.208	-0.318	-0.062
PP2073		acetyltransferase	NA	-1.468	-0.144	-0.33
NA		ubiquinol	NA	-1.552	-0.588	-0.404

qRT-PCR

The fold change values described reflected the ability of the organism to aggregate which was in the order 3CP> 2CP> 4CP. Three genes PP132, PP1961 and PP4936 were selected for validation with qRT-PCR. The results obtained confirmed the array findings. In all three cases the trends of expression were 3CP> 2CP> 4CP (Figure 3.18) suggesting a role for all three genes in autoaggregation.

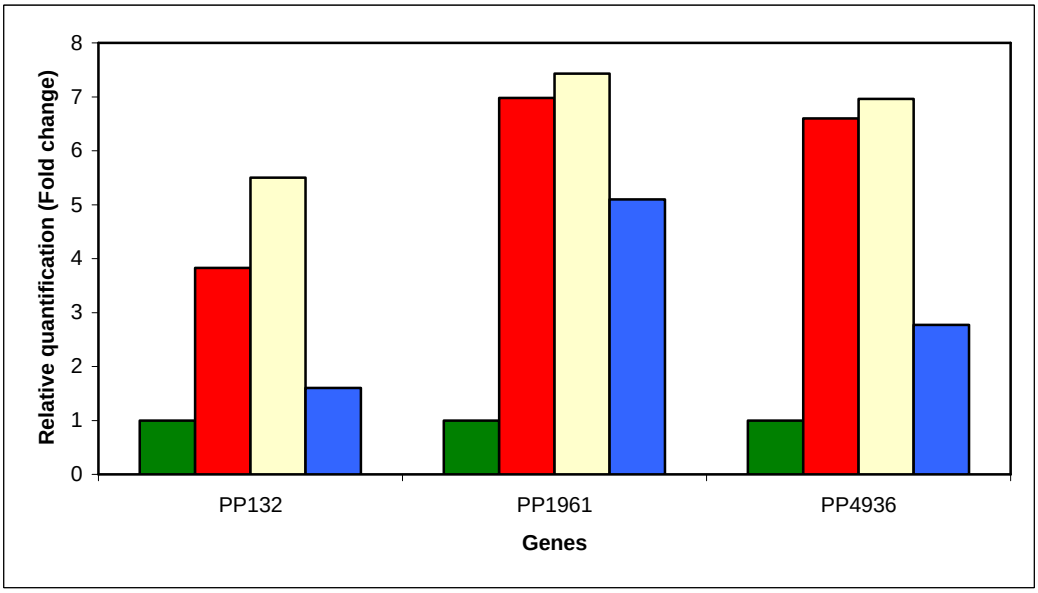


Figure 3.18 Relative quantification of gene expression of genes PP132, PP1961 and PP4936 in aggregated cells of *P. putida* CP1 grown on 100 ppm 2-chlorophenol (■), 100 ppm 3-chlorophenol (■) and 200 ppm 4-chlorophenol (■) compared to *P. putida* CP1 grown on nutrient broth (■).

3.3 Phenotypic and genotypic responses of *P. putida* CP1 when grown on 0.5% (w/v) fructose

Studies had shown that *P. putida* CP1 displayed autoaggregation when grown on 0.5% (w/v) fructose and so phenotypic and genotypic responses of *P. putida* CP1 when grown on 0.5% (w/v) fructose were monitored. The ability of the organism to grow on 0.5% (w/v) fructose and 0.5% (w/v) glucose was determined. Autoaggregation was monitored and the aggregates were characterised. Expression profiling analysis was carried out to identify genes associated with autoaggregation and to compare the findings with those obtained when the organism was grown on mono-chlorophenols.

3.3.1 Phenotypic responses of *P. putida* CP1 when grown on 0.5% (w/v) fructose

The phenotypic responses of *P. putida* CP1 when grown on 0.5% (w/v) fructose were determined by monitored growth and substrate removal, aggregate formation and cell viability and aggregate characterization including EPS composition.

Growth of P. putida CP1 and P. putida KT2440 on 0.5% (w/v) fructose and 0.5% (w/v) glucose

P. putida CP1 and *P. putida* KT2440 were grown in liquid culture on 0.5% (w/v) fructose and 0.5% (w/v) glucose. Growth was monitored until all the substrate was removed (Figure 3.19). Both sugars were degraded by the two bacteria. Sugar removal was preceded by a lag period. The lag was shortest for *P. putida* KT2440 where the lag was 2 hours for both substrates. The lag period for *P. putida* CP1 was twice that for glucose and four times longer for fructose. Fructose was completely removed by both organisms in 48h. However, glucose was utilised more readily than fructose in less than 20 hours. This was reflected in the rates of substrate removal (Table 3.15). The rate of glucose removal was double that of fructose and *P. putida* KT2440 removed both sugars more than twice as fast as *P. putida* CP1. The pH in all cases was initially 7.0 and fell to 6.2 – 6.4 in all systems with substrate utilization. The initial fall in pH was followed by a rise in

pH when maximum biomass values were achieved, except in case of *P. putida* CP1 grown on fructose. Biomass was monitored throughout the experiment. A significant increase in biomass was observed when *P. putida* CP1 and *P. putida* KT2440 were grown on both sugars. The removal of fructose by *P. putida* CP1 from the medium at 8 hours corresponded with aggregation of the bacterial cells. Aggregation continued with substrate removal and increased sharply when all the fructose was removed. The aggregation of the cells is illustrated in Figure 3.20. There was an increase in aggregate size with time and a colour change was also observed. No cell aggregation was observed when the organism was grown on glucose. Aggregation was absent when *P. putida* KT2440 was grown on either sugar.

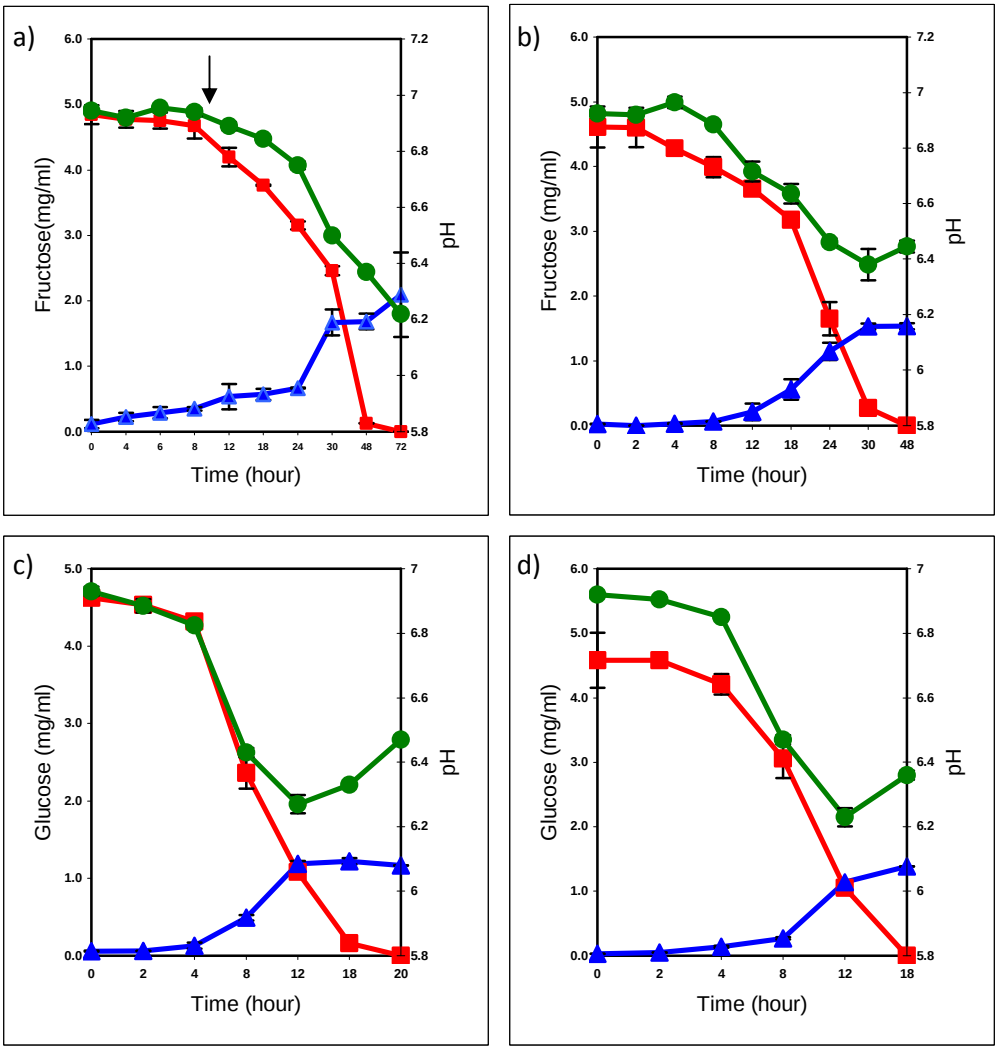


Figure 3.19 Growth of a) *P. putida* CP1 on 0.5% (w/v) fructose b) *P. putida* KT2440 on 0.5% (w/v) fructose c) *P. putida* CP1 on 0.5% (w/v) glucose and d) *P. putida* KT2440

on 0.5% (w/v) glucose. Sugar (■), cell biomass (▲) and pH (●). Arrow indicates time of formation of aggregates.

Table 3.15 Lag periods and substrate removal rates for *P. putida* CP1 and *P. putida* KT2440 grown on 0.5% (w/v) fructose and 0.5% (w/v) glucose (Data from Figure 3.19).

Treatment	**Lag period (h)**	**Removal rate ($mgml^{-1}h^{-1}$)**	**Specific substrate removal rate (h^{-1})**	**Presence of aggregates (h)**
CP1-Fructose	8	0.1143	5.25	8
KT2440-Fructose	2	0.1709	13.906	-
CP1-Glucose	4	0.2733	11.571	-
KT2440-Glucose	2	0.3054	26.813	-

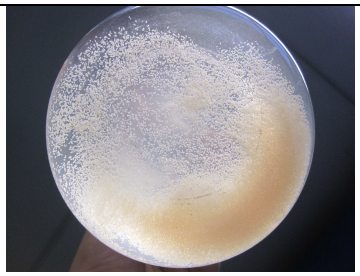
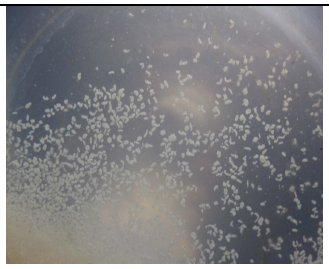
24 h	**48 h**	**72 h**
		
White aggregates with a clear medium	Yellow aggregates with a clear medium	Yellow aggregates with a cloudy medium

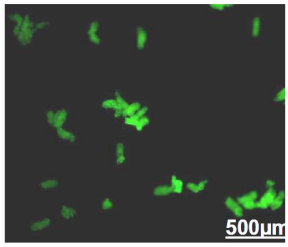
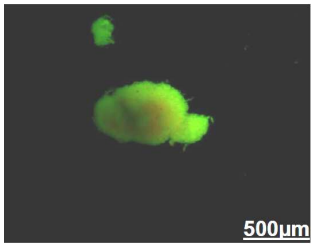
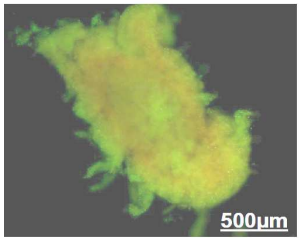
Figure 3.20 Appearance of aggregates when *P. putida* CP1 was grown on 0.5% (w/v) fructose after 24, 48 and 72 hours.

Analysis of the aggregates

The aggregation index, cell surface hydrophobicity and size and viability of the aggregates were determined over a three day period (Table 3.16). When cell aggregation was observed, the aggregation index increased with time of incubation. The aggregation index was 0.98 ± 0.01 after 24 h, 1.21 ± 0.22 on 48 h and 1.58 ± 0.08 on 72 h. Cell surface hydrophobicity increased with time and there was a significant difference between the hydrophobicity from day 1 to day 3.

The hydrophobicity as measured by adherence to *n*-hexadecane was 54% after 1 day, increasing to 66% on day 2 and 84 % on day 3. The aggregates increased in size as substrate decreased. The average size of the aggregates ranged from 25.79 μm^2 (10^{x3}) after 24 hours, 214.51 μm^2 (10^{x3}) after 48 hours and 1567.37 μm^2 (10^{x3}) after 72 hours. Microscopic observations showed that most of the cells in the aggregate were viable after 24 hours. However after 48 hours and 72 hours the core part of the aggregate was filled with dead cells.

Table 3.16 Changes in aggregation index (AI), cell surface hydrophobicity (BATH %), aggregate size and viability (%) during growth of *P. putida* CP1 on 0.5% (w/v) fructose following 24, 48 and 72 hours.

Time (h)	**24**	**48**	**72**
			
AI	0.98 ± 0.01	1.21 ± 0.22	1.58 ± 0.08
BATH (%)	54 ± 1.29	66 ± 2.11	84 ± 0.35
Size $\mu m^2(x10^3)$	25.79 ± 2.16	214.51 ± 13.92	1567.37 ± 10.9
Viability (%)	86 ± 3.6	68 ± 6.68	38 ± 6.25

The distribution of live and dead cells in the aggregate grown on 0.5% (w/v) fructose for 24 h was observed using confocal laser scanning microscopy (CLSM) at x60 magnification. The preliminary findings after interpretation of all sample slices revealed that the distribution of biomass in the aggregates followed a common pattern. The intensity of fluorescent signals under both channels was randomly distributed. Figure 3.21 shows the distribution of live and dead cells from the bottom, middle and top section of the aggregate. The biomass residing at the bottom was mostly stained by SYTO 9 in the peripheral zone, and the top and middle sections were stained by both propidium iodide and SYTO 9 randomly.

There were patches in the aggregates that were not stained by either dye suggesting the presence of extracellular material.

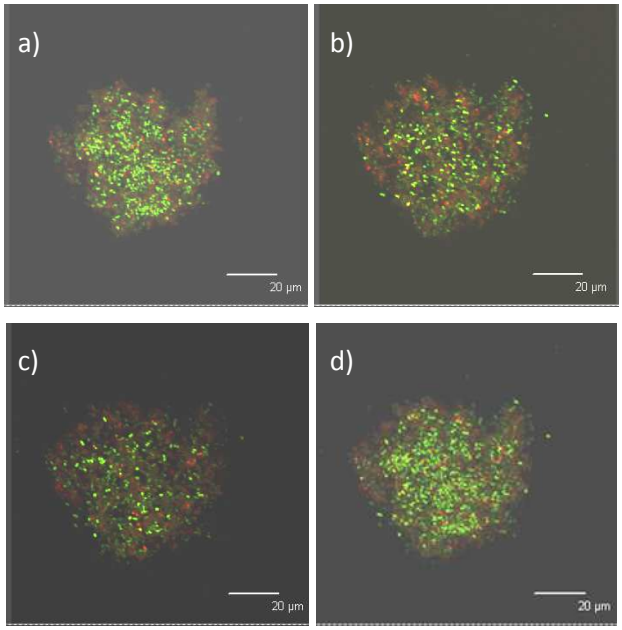


Figure 3.21 CLSM image of aggregated cells of *P. putida* CP1 grown on 0.5% (w/v) fructose following 24 hours stained with the Live/Dead stain. a) bottom b) middle c) top section and d) overall of the aggregate.

Extracellular polymeric substances (EPS)

The production of extracellular polymeric substances (EPS) by *P. putida* CP1 grown on 0.5% (w/v) fructose and 0.5% (w/v) glucose was investigated. The levels of both bound and free EPS were quantified biochemically (Figure 3.22). Low levels of EPS were detected following growth of *P. putida* CP1 on 0.5% (w/v) glucose for 24 hours. In contrast, high levels of EPS, twice the level detected when the organism was grown on glucose, was detected when the organism was grown on 0.5% (w/v) fructose for 24 hours. This production of EPS corresponded with the formation of aggregates by the organism. The production of EPS (µg/ml) was seen to increase as the size of the aggregates increased (Figure 3.22). When the EPS concentration was expressed in terms of the biomass the levels of EPS/unit biomass decreased with time (Figure 3.23).

The levels of bound EPS than free EPS were similar for the total EPS. When the biochemical composition of the EPS was investigated it was seen to comprise carbohydrate, protein and DNA as was the case when the organism was grown on mono-chlorophenols. The largest fraction of the EPS was protein which was largely bound EPS. A lower level of carbohydrate was detected and was found to be mainly free EPS. The levels of DNA were lowest and did not increase significantly with time.

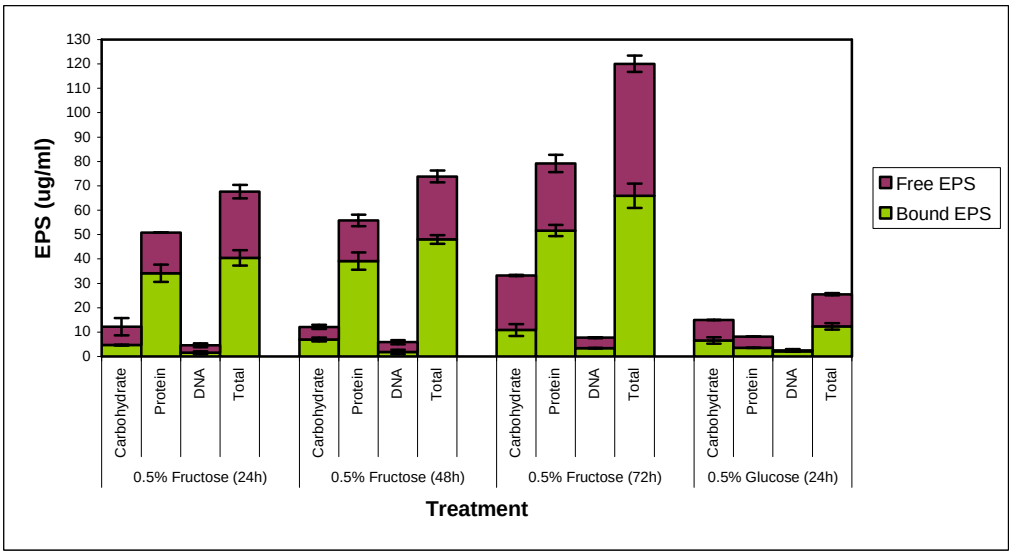


Figure 3.22 Composition of free and bound EPS (µg/ml) extracted from *P. putida* CP1 following growth on 0.5% (w/v) fructose up to 72 hours and 0.5% (w/v) glucose for 24 hours.

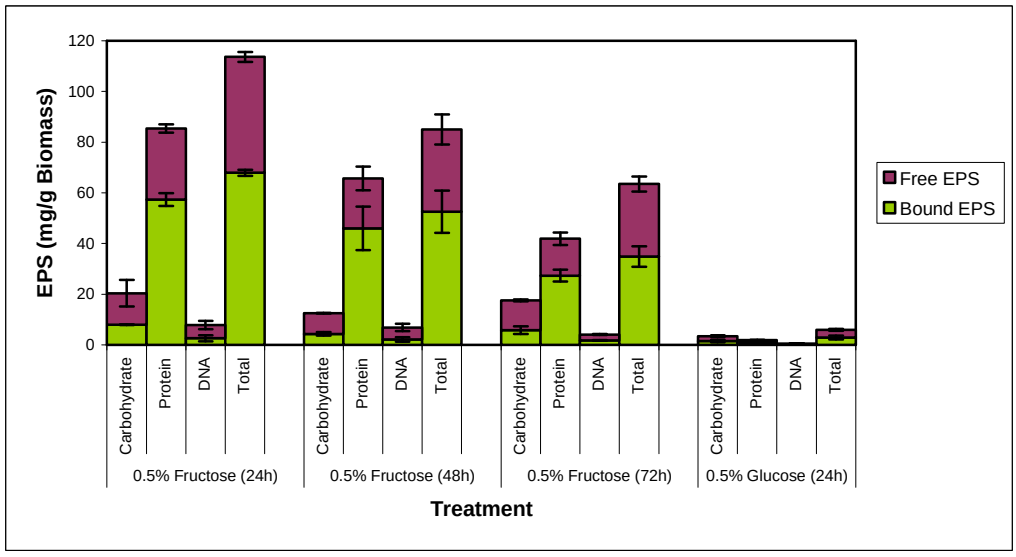


Figure 3.23 Composition of free and bound EPS (mg/g biomass) extracted from *P. putida* CP1 following growth on 0.5% (w/v) fructose up to 72 hours and glucose 0.5% (w/v) for 24 hours.

A microscopic examination of the EPS was carried out using specific fluorophores and epifluorescent microscopy. The results supported the biochemical analysis showing the EPS was composed mainly of protein and lower levels of carbohydrate and DNA. The levels of DNA however were seen to increase with time. Given that there was a decrease in cell viability this result suggests that cell lysis contributed to the levels of DNA (Figure 3.24). Confocal laser scanning microscopy (CLSM) was used to study the aggregates when the organism was grown on fructose after 24 hours. The distribution of the biochemical components of the EPS were seen to be randomly distributed (Figure 3.25).

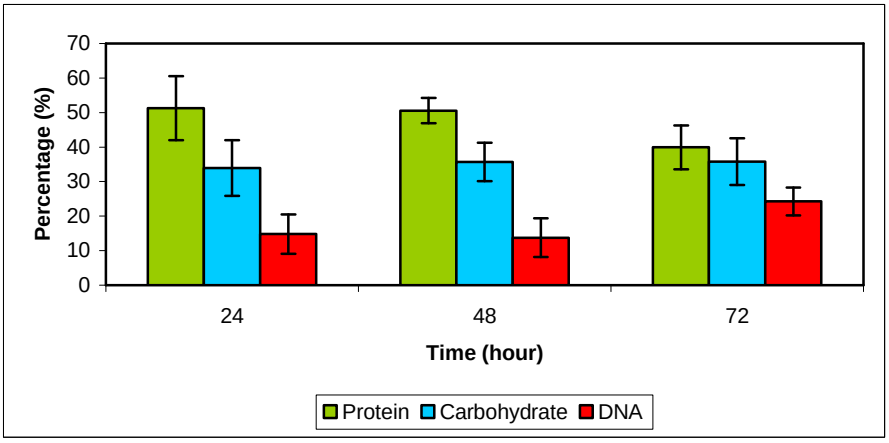


Figure 3.24 Percentage of protein, carbohydrate and DNA in extracellular polymeric substances (EPS) produced by *P. putida* CP1 following growth on 0.5% (w/v) fructose for up to 72 hours based on microscopic observation.

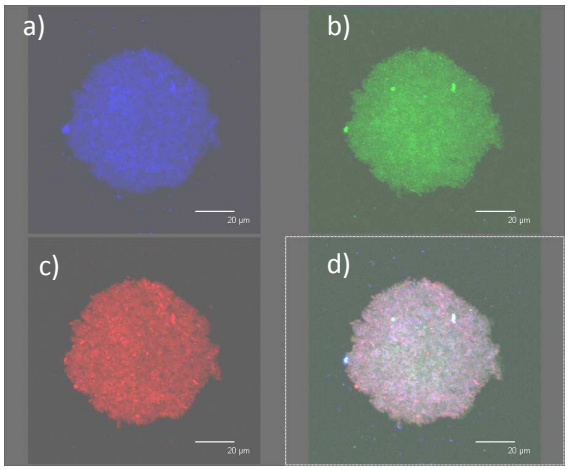


Figure 3.25 CLSM image of *P. putida* CP1 aggregated cells following growth on 0.5% (w/v) fructose for 24 hours. a) Calcofluor white, b) FITC and c) SYTO 63 and d) combined multi-channel image.

3.3.2 Expression profiling analysis

As was the case with the chlorophenols, expression profiling analysis was used to identify genetic factors governing autoaggregation when the organism was grown on fructose using a *P. putida* Genome Oligonucleotide Array (Progenika, Spain). Initially results obtained for *P. putida* CP1 grown on nutrient broth were compared with those obtained when the organism grown on fructose. Finally the result obtained following a study of CP1 vs. 200 ppm 4CP-100 ppm 2CP-100 ppm 3CP was compared with *P. putida* CP1 vs fructose. In each study the differentially regulated genes were identified, PSORTB v3 analysis was used to identify the cellular location of the proteins and pathway analysis was used to identify the main biological pathways.

Comparative study CP1vsFructose

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 (array 9), a total of 838 genes were significantly differentially regulated including 169 genes encoding for hypothetical proteins.

Up-regulated gene transcripts

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 3.17. 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.

Table 3.17 Top 25 genes up-regulated in *P. putida* CP1 following growth on 0.5% (w/v) fructose for 24 hours.

Locus ID	**Gene Name**	**Annotation**	**Pathway**	**FC**	***p*-value**
PP1185	oprH	outer membrane protein H1		6.872	0
PP814	cyoC	cytochrome o ubiquinol oxidase, subunit III	Oxidative phosphorylation	6.252	0
PP816	cyoE-2	protoheme IX farnesyltransferase	Oxidative phosphorylation	5.94	0.00001
PP5000		heat shock protein HslV		5.884	0.00005
PP813	cyoB	cytochrome o ubiquinol oxidase, subunit I	Oxidative phosphorylation	5.488	0.00001
PP1187	phoQ	integral membrane sensor signal transduction histidine kinase	Two-component system	5.04	0
PP1981		NifR3/Smm1 family protein		4.906	0.00049
PP5001		ATP-dependent protease ATP-binding subunit HslU		4.546	0.00015
PP459	rplV	50S ribosomal protein L22	Protein translation	4.286	0
PP449	rpsL	30S ribosomal protein S12	Protein translation	3.792	0.00081
PP5414	atpG	F0F1 ATP synthase subunit gamma	Oxidative phosphorylation	3.764	0.00005
PP480	rplQ	50S ribosomal protein L17	Protein translation	3.748	0.00002
PP5337		LysR family transcriptional regulator		3.738	0.0005
PP474	secY	preprotein translocase subunit SecY	Protein export; Bacterial secretion system	3.718	0.00379
PP470	rplR	50S ribosomal protein L18	Protein translation	3.632	0.00002
PP4895	miaA	tRNA delta(2)-isopentenylpyrophosphate transferase	Metabolic pathways	3.544	0.00012
PP4877	rpsF	30S ribosomal protein S6	Protein translation	3.464	0.00023

PP4728	grpE	heat shock protein GrpE		3.446	0.00088
PP6	yidC	putative inner membrane protein translocase component	Protein export; Bacterial secretion system	3.432	0.00005
PP4876	rpsR	30S ribosomal protein S18	Protein translation	3.382	0.00016
PP4874	rplI	50S ribosomal protein L9	Protein translation	3.284	0.0001
PP815	cyoD	cytochrome o ubiquinol oxidase	Oxidative phosphorylation	3.148	0.00583
PP5418	atpE	F0F1 ATP synthase subunit C	Oxidative phosphorylation	3.136	0.0001
PP4179	htpG	heat shock protein 90		3.126	0.00099
PP461	rplP	50S ribosomal protein L16	Protein translation	3.04	0.00098
PP4370	fliE	flagellar hook-basal body protein	Flagellar assembly	3.028	0.00044

Down-regulated gene transcripts

382 genes were identified as significantly down-regulated and following ranking by fold change the top 25 genes are listed in Table 3.18. 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 3.18 Top 25 genes down-regulated in *P. putida* CP1 grown on 0.5% (w/v) fructose for 24 hours.

Locus ID	**Gene Name**	**Annotation**	**Pathway**	**FC**	***p*-value**
PP5313	hupA	histone family protein DNA-binding protein		-9.31	0
PP1010	edd	phosphogluconate dehydratase	Pentose phosphate pathway	-8.47	0
PP1296	estB	carboxylesterase		-7.81	0
PP3443		glyceraldehyde-3-phosphate dehydrogenase, putative	Glycolysis / Gluconeogenesis	-5.69	0.00003
PP1012	gltR-2	winged helix family two component transcriptional regulator		-5.64	0.00001
PP4922	thiC	thiamine biosynthesis protein ThiC	Thiamine metabolism	-5.23	0
PP3745	glcD	glycolate oxidase subunit GlcD	Glyoxylate and dicarboxylate metabolism	-5.17	0.00007
PP1071		amino acid ABC transporter, periplasmic amino acid-binding protein	ABC transporters	-5.14	0
PP4659	ggt-2	gamma-glutamyltransferase	Taurine and hypotaurine metabolism	-4.72	0
PP2528	metY	O-acetylhomoserine aminocarboxypropyltransferase	Cysteine and methionine metabolism	-4.53	0.00001
PP794	fruK	1-phosphofructokinase	Fructose and mannose metabolism	-4.48	0.00032
PP3189	codA	N-isopropylammelide isopropylaminohydrolase	Pyrimidine metabolism	-4.40	0
PP1024	eda	keto-hydroxyglutarate-aldolase/keto-deoxy-phosphogluconate aldolase	Pentose phosphate pathway	-4.32	0.00012
PP545		aldehyde dehydrogenase family protein	Glycolysis / Gluconeogenesis	-4.17	0.00002
PP3514	hyuB	hydantoinase B/oxoprolinase	Arginine and proline metabolism	-4.12	0.00004
PP1009	gap-1	glyceraldehyde-3-	Glycolysis /	-4.09	0.00001

		phosphate dehydrogenase, type I	Gluconeogenesis		
PP5003	phaA	poly(3-hydroxyalkanoate) polymerase 1	Butanoate metabolism	-4.03	0.00017
PP1986	leuD	isopropylmalate isomerase small subunit	Valine, leucine and isoleucine biosynthesis	-3.90	0.00056
PP795	fruA	PTS system, fructose subfamily, IIC subunit	Fructose and mannose metabolism	-3.78	0.00002
PP362	bioB	biotin synthase	Biotin metabolism	-3.61	0.00042
PP793	fruB	phosphoenolpyruvate-protein phosphotransferase	Fructose and mannose metabolism	-3.59	0.00005
PP1139	livM	leucine/isoleucine/valine transporter permease subunit	ABC transporters	-3.46	0.00014
PP5007	phaF	poly(hydroxyalkanoate) granule-associated protein		-3.39	0.00001
PP1019	oprB-1	porin B		-3.12	0.00022
PP4256	ccoO-2	cbb3-type cytochrome c oxidase subunit II	Oxidative phosphorylation	-3.08	0.00003

Protein localization

PSORTB v3 analysis of the 838 differentially expressed genes showed the location in the cell of the encoded proteins (Table 3.19). The analysis showed 210 encoded proteins were localized in an unknown location, the majority of the proteins were localized in the cytoplasm (388), 182 in the cytoplasmic membrane, 31 in the periplasmic space, 22 in the outer membrane, 5 extracellular and 4 associated with flagellae.

Table 3.19 Cell location of proteins encoded by genes differentially regulated in *P. putida* CP1 grown on 0.5% (w/v) fructose for 24 hours.

Protein location	**Number of genes**		
	Induced	**Repressed**	**Total**
Cytoplasm	220	168	388
Cytoplasmic membrane	97	85	182
Periplasmic space	10	21	31

Outer membrane	7	15	22
Extracellular	2	3	5
Flagellar	4	-	4
Unknown	-	-	210

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 (Table 3.20). 5% of the genes were involved in protein translation, 4% in ABC transporters, 3% in oxidative phosphorylation and 2% in two-component systems. 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.

Table 3.20 Metabolic pathways (%) associated with differentially up-regulated and down-regulated genes when *P. putida* CP1 was grown on 0.5% (w/v) fructose for 24 hours.

Pathway	**Percentage**	**Up-regulated**	**Down-regulated**
Protein translation	4.65	39	0
ABC transporters	4.30	8	28
Oxidative phosphorylation	2.74	17	6
Two-component system	2.03	6	11
Arginine and proline metabolism	1.67	6	8
Purine metabolism	1.43	12	0
Butanoate metabolism	1.31	5	6
Propanoate metabolism	1.31	2	9

Pyrimidine metabolism	1.19	9	1
Alanine, aspartate and glutamate metabolism	1.19	8	2
Bacterial secretion system	1.07	6	3
Pyruvate metabolism	1.07	3	6
Valine, leucine and isoleucine degradation	1.07	3	6
Glycolysis / Gluconeogenesis	1.07	2	7
Lipopolysaccharide biosynthesis	1.07	6	3
Porphyrin and chlorophyll metabolism	0.95	5	2
Pentose phosphate pathway	0.95	2	6
Fatty acid biosynthesis and metabolism	0.84	8	8
Flagellar assembly	0.84	7	0
Bacterial chemotaxis	0.84	3	4
Histidine metabolism	0.72	3	3
Benzoate degradation via hydroxylation	0.72	3	3
Nitrogen metabolism	0.72	3	3
Tryptophan metabolism	0.72	2	4
Fructose and mannose metabolism	0.72	1	5
Citrate cycle (TCA cycle)	0.72	1	5
Glutathione metabolism	0.72	0	6
Terpenoid backbone biosynthesis	0.60	5	0
Protein export	0.60	5	0

Seven pathways involved with carbohydrate metabolism were identified among the significant pathways when the comparative study CP1vsFructose was carried out. These pathways are listed in Table 3.21. 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 theses genes was up-

regulated. The genes involved in each of the seven pathways are listed in Table 3.22.

Table 3.21 Numbers of pathways of carbohydrate metabolism associated with genes up-regulated and down-regulated when *P. putida* CP1 was grown on 0.5% (w/v) fructose for 24 hours.

Pathway	**Total**	**Up-regulated**	**Down-regulated**
Butanoate metabolism	11	5	6
Pyruvate metabolism	9	3	6
Glycolysis / Gluconeogenesis	9	2	7
Pentose phosphate pathway	8	2	6
Propanoate metabolism	11	2	9
Citrate cycle (TCA cycle)	6	1	5
Fructose and mannose metabolism	6	1	5

Table 3.22 Details of genes involved in carbohydrate metabolism pathways when *P. putida* CP1 was grown on 0.5% (w/v) fructose for 24 hours (Data from Table 3.21).

Pathway	**Locus ID**	**Gene name**	**Annotation**	**FC**	***p*-value**
Butanoate metabolism	PP4948		acyl-CoA dehydrogenase family protein	2.74	0.00224
	PP213	gabD	succinate-semialdehyde dehydrogenase	2.138	0.00006
	PP4192	sdhD	succinate dehydrogenase hydrophobic membrane anchor	1.6	0.0428
	PP214	gabT	4-aminobutyrate aminotransferase	1.518	0.00344
	PP3755	paaH	3-hydroxybutyryl-CoA dehydrogenase	1.378	0.00829
	PP4636		beta-ketothiolase	-1.2	0.04735
	PP2215	fadAx	3-ketoacyl-CoA thiolase	-1.474	0.03679
	PP2679		quinoprotein ethanol dehydrogenase putative	-2.856	0.00357
	PP2680		aldehyde dehydrogenase family protein	-2.902	0.00115
	PP5003	phaA	poly(3-hydroxyalkanoate)	-4.032	0.00017

			polymerase 1		
	PP545		aldehyde dehydrogenase family protein	-4.168	0.00002
Pyruvate metabolism	PP2929	nspC	carboxynorspermidine decarboxylase	2.26	0.03616
	PP4736	lldD	L-lactate dehydrogenase	1.98	0.00036
	PP1996	accD	acetyl-CoA carboxylase subunit beta	1.346	0.03157
	PP4636		acetyl-CoA acetyltransferase	-1.2	0.04735
	PP1607	accA	acetyl-CoA carboxylase carboxyltransferase subunit alpha	-1.456	0.02879
	PP2215	fadAx	acetyl-CoA acetyltransferase	-1.474	0.03679
	PP2680		aldehyde dehydrogenase family protein	-2.902	0.00115
	PP5366	lpd3	dihydrolipoamide dehydrogenase	-3.334	0.00034
	PP545		aldehyde dehydrogenase family protein	-4.168	0.00002
Glycolysis / Gluconeogenesis	PP4715	tpiA	triosephosphate isomerase	2.316	0.00168
	PP1616		D-isomer specific 2-hydroxyacid dehydrogenase family protein	2.156	0.00954
	PP2679		quinoprotein ethanol dehydrogenase, putative	-2.856	0.00357
	PP2680		aldehyde dehydrogenase family protein	-2.902	0.00115
	PP1011	glk	glucokinase	-2.904	0.00023
	PP5366	lpd3	dihydrolipoamide dehydrogenase	-3.334	0.00034
	PP1009	gap-1	glyceraldehyde-3-phosphate dehydrogenase, type I	-4.088	1.00E-05
	PP545		aldehyde dehydrogenase family protein	-4.168	2.00E-05
	PP3443		glyceraldehyde-3-phosphate dehydrogenase, putative	-5.694	3.00E-05

Pentose phosphate pathway	PP722	prsA	ribose-phosphate pyrophosphokinase	2.866	0.00084
	PP3416	gnuK	carbohydrate kinase, thermoresistant glucokinase family	1.94	0.01741
	PP4042	zwf-2	glucose-6-phosphate 1-dehydrogenase	-1.46	0.00652
	PP415	rpe	ribulose-phosphate 3-epimerase	-1.506	0.00666
	PP3378	kguK	PfkB domain protein	-1.746	0.03242
	PP1022	zwf-1	glucose-6-phosphate 1-dehydrogenase	-2.06	0.00031
	PP1024	eda	keto-hydroxyglutarate-aldolase/keto-deoxy-phosphogluconate aldolase	-4.316	0.00012
	PP1010	edd	phosphogluconate dehydratase	-8.47	0
Propanoate metabolism	PP214	gabT	4-aminobutyrate aminotransferase	1.518	0.00344
	PP1996	accD	acetyl-CoA carboxylase subunit beta	1.346	0.03157
	PP4636		acetyl-CoA acetyltransferase	-1.2	0.04735
	PP1607	accA	acetyl-CoA carboxylase carboxyltransferase subunit alpha	-1.456	0.02879
	PP2215	fadAx	3-ketoacyl-CoA thiolase	-1.474	0.03679
	PP2338	prpD	2-methylcitrate dehydratase	-1.674	0.02273
	PP4667	mmsA-2	methylmalonate-semialdehyde dehydrogenase	-1.68	0.00014
	PP4186	sucC	succinyl-CoA synthetase subunit beta	-1.704	0.02231
	PP2679		quinoprotein ethanol dehydrogenase, putative	-2.856	0.00357
	PP2680		aldehyde dehydrogenase family protein	-2.902	0.00965
	PP545		aldehyde dehydrogenase family protein	-4.168	0.00002
Citrate cycle (TCA cycle)	PP4192	sdhD	succinate dehydrogenase, hydrophobic membrane anchor protein	1.6	0.0428

	PP944	fumC-1	fumarate hydratase	-1.43	0.04476
	PP4186	sucC	succinyl-CoA synthetase subunit beta	-1.704	0.02231
	PP2336		aconitate hydratase	-1.832	0.03289
	PP4011	icd	isocitrate dehydrogenase, NADP-dependent	-2.284	1.00E-05
	PP5366	lpd3	dihydrolipoamide dehydrogenase	-3.334	0.00034
Fructose and mannose metabolism	PP4715	tpiA	triosephosphate isomerase	2.316	0.0016
	PP2037		class II aldolase/adducin domain protein	-1.5	0.01638
	PP1277	algA	mannose-1-phosphate guanylyltransferase/mannose-6-phosphate isomerase	-2.276	0.00085
	PP793	fruB	phosphoenolpyruvate-protein phosphotransferase	-3.582	0
	PP795	fruA	PTS system, fructose subfamily, IIC subunit	-3.782	0
	PP794	fruK	1-phosphofructokinase	-4.478	0.00032

Four pathways associated with autoaggregation according to Sauer and Camper (2001) was also identified with this study (Table 3.23). The genes involved in these 4 pathways are listed in Table 3.24. 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.

Table 3.23 Numbers of up-regulated and down-regulated genes involved in pathways related to autoaggregation when *P. putida* CP1was grown on 0.5% (w/v) fructose for 24 hours.

Pathway	**Total**	**Up-regulated**	**Down-regulated**
ABC transporters	36	8	28
Flagellar assembly	7	7	0
Two-component system	17	6	11
Lipopolysaccharide biosynthesis	9	6	3

Table 3.24 Details of genes involved in pathways related to autoaggregation when *P. putida* CP1 was grown on 0.5% (w/v) fructose for 24 hours.

Pathway	**Locus ID**	**Gene name**	**Annotation**	**FC**	***p*-value**
ABC transporters	PP237	ssuA	aliphatic sulfonate ABC transporter periplasmic ligand-binding protein	2.814	0.02764
	PP240	ssuB	aliphatic sulfonates transport ATP-binding subunit	2.032	0.00707
	PP164	lapG	Large adhesion protein	1.928	0.002
	PP3077		binding-protein-dependent transport systems inner membrane component	1.642	0.00013
	PP983		permease YjgP/YjgQ family protein	1.522	0.00434
	PP2767		branched-chain amino acid ABC transporter, ATP-binding protein, putative	1.444	0.00961
	PP2155	lolD	lipoprotein releasing system, ATP-binding protein	1.372	0.04227
	PP2240		ABC transporter	1.31	0.01107
	PP4843		branched-chain amino acid ABC transporter, permease protein, putative	-1.224	0.0211
	PP5178	potH	binding-protein-dependent transport systems inner membrane component	-1.258	0.00599
	PP619		branched-chain amino acid ABC transporter, periplasmic amino acid-binding protein	-1.268	0.03529
	PP3637		sulfonate ABC transporter, ATP-binding protein, putative	-1.282	0.01447
	PP884		dipeptide ABC transporter,	-1.292	0.02319

			periplasmic peptide-binding protein		
	PP2592		ferric siderophore ABC transporter, ATP-binding protein	-1.326	0.02062
	PP1141	braC	extracellular ligand-binding receptor	-1.33	0.00186
	PP5179	potG	spermidine/putrescine ABC transporter ATPase	-1.356	0.0018
	PP232	tauB	taurine transporter ATP-binding subunit	-1.372	0.04175
	PP3222		ABC transporter, permease protein	-1.424	0.01362
	PP5171	cysP	sulfate ABC transporter, periplasmic sulfate-binding protein	-1.478	0.02814
	PP5180	potF-1	putrescine ABC transporter, periplasmic putrescine-binding protein	-1.526	0.01685
	PP295		glycine betaine/L-proline ABC transporter, permease protein	-1.616	0.02005
	PP5177	potI	ornithine carbamoyltransferase	-1.658	0.00463
	PP1137	braG	branched chain amino acid ABC transporter ATP-binding protein	-1.75	0.00099
	PP4865		inner-membrane translocator	-1.818	0.00362
	PP3213		ABC transporter, periplasmic binding component-related protein	-2.024	0.01911
	PP1298	aapQ	polar amino acid ABC transporter inner membrane subunit	-2.124	0.00018
	PP1297	aapJ	general amino acid ABC transporter, periplasmic binding protein	-2.146	0.00045

	PP1138	livG	leucine/isoleucine/valine transporter ATP-binding subunit	-2.202	0.0037
	PP1299	aapM	polar amino acid ABC transporter inner membrane subunit	-2.54	0.00018
	PP1070		polar amino acid ABC transporter inner membrane subunit	-2.7	0.00018
	PP226		polar amino acid ABC transporter inner membrane subunit	-2.732	0
	PP227		cystine transporter subunit	-2.78	0.00005
	PP1140	braD	inner-membrane translocator	-2.968	0.00069
	PP1139	livM	leucine/isoleucine/valine transporter permease subunit	-3.46	0.00014
	PP1071		amino acid ABC transporter, periplasmic amino acid-binding protein	-5.138	0
	PP4867		extracellular ligand-binding receptor	-6.666	0.00001
Two-component system	PP1187	phoQ	integral membrane sensor signal transduction histidine kinase	5.04	0
	PP5046	glnA	glutamine synthetase, type I	2.678	0.00001
	PP4340	cheY	response regulator receiver protein	1.802	0.00561
	PP109		cytochrome oxidase assembly	1.618	0.01937
	PP5183		glutamine synthetase, putative	1.534	0.00676
	PP2100		two-component sensor protein	1.35	0.02904
	PP4636		acetyl-CoA	-1.2	0.04735

			acetyltransferase		
	PP4521		aerotaxis receptor, putative	-1.466	0.00095
	PP2215	fadAx	acetyl-CoA acetyltransferase	-1.474	0.03679
	PP4161	kdpA	potassium-transporting ATPase subunit A	-1.702	0.03055
	PP1401	dctD	two component, sigma54 specific, transcriptional regulator, Fis family	-1.772	0.00507
	PP3583		acriflavin resistance protein	-1.822	0.01014
	PP802	chev-1	chemotaxis protein CheV	-1.87	0.0068
	PP5320	phoB	winged helix family two component transcriptional regulator	-1.884	0.00027
	PP4647	luxR	LuxR family transcriptional regulator	-1.898	0.03228
	PP1181		winged helix family two component transcriptional regulator	-1.942	0.02548
	PP4333	CheW	CheW domain-containing protein	-3.554	0.00003
Flagellar assembly	PP4370	fliE	flagellar hook-basal body protein FliE	3.028	0.00044
	PP4385	flgG	flagellar basal body rod protein FlgG	2.514	0.00004
	PP4344	flhA	flagellar biosynthesis protein FlhA	2.03	0.03343
	PP4384	flgH	flagellar basal body L-ring protein	1.962	0.00337
	PP4383	flgI	flagellar basal body P-ring protein	1.646	0.02366
	PP4357	fliN	flagellar motor switch protein	1.558	0.00338
	PP4369	fliF	flagellar MS-ring protein	1.272	0.01441
Lipopolysaccharide biosynthesis	PP59		D,D-heptose 1,7-bisphosphate phosphatase	2.628	0.00426
	PP63		lipid A biosynthesis lauroyl acyltransferase	2.348	0.00017
	PP1604	lpxB	lipid-A-disaccharide	1.674	0.00245

			synthase		
	PP1323	gmhA	phosphoheptose isomerase	1.566	0.04328
	PP4936		O-antigen polymerase	1.516	0.00587
	PP1611	kdsA-1	2-dehydro-3-deoxyphosphooctonate aldolase	1.274	0.00988
	PP1603	lpxA	UDP-N-acetylglucosamine acyltransferase	-1.258	0.02614
	PP956		YrbI family phosphatase	-2.084	0.00931
	PP1807	kdsA-2	2-dehydro-3-deoxyphosphooctonate aldolase	-2.094	0.02054

Comparative study CP1 vs. 200 ppm 4CP-100 ppm 2CP-100 ppm 3CP and CP1vsFructose

To distinguish the genes involved in aggregation, the seven unique genes from the comparison of CP1 vs. 200 ppm 4CP-100 ppm 2CP-100 ppm 3CP (Table 3.14) were overlapped with 838 differentially regulated genes in *P. putida* CP1 vs. fructose using Venny (Figure 3.26). Four genes were unique to responses to mono-chlorophenols and 835 unique to responses to fructose. Three genes were common and are listed in Table 3.25. Two were up-regulated, PP1961 encoding for TetR family transcriptional regulator and PP4936 encoding for O-antigen polymerase protein. PP2073 encodes for acetyltransferase and was down-regulated. All three genes had also been associated with aggregation of the organism on chlorophenols (Section 3.2.2).

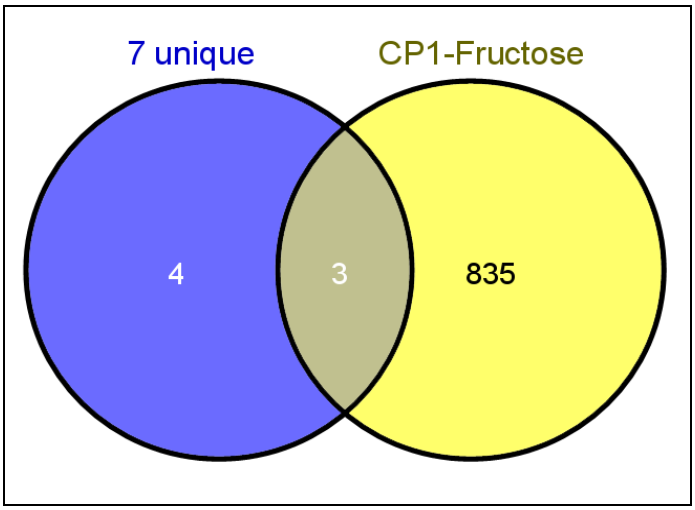


Figure 3.26 Venn diagram showing the numbers of genes common to two genelists. *P. putida* CP1 grown on nutrient broth compared to *P. putida* CP1 grown on 200 ppm 4-chlorophenol, 100 ppm 2-chlorophenol and 100 ppm 3-chlorophenol (■) and *P. putida* CP1 grown on 0.5% (w/v) fructose (■).

Table 3.25 Three common genes expressed in *P. putida* CP1 aggregated cells when the organism was grown on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol, 200 ppm 4-chlorophenol and 0.5% (w/v) fructose.

Gene name	**Annotations**	**FC CP1 vs. 200-4CP**	**FC 200-4CP vs 2CP**	**FC 2CP vs 3CP3**	**FC CP1 vs. fructose**
PP1961	TetR Family Transcriptional Regulator	1.596	0.226	0.392	1.716
PP4936	O-antigen polymerase	1.28	0.082	0.114	1.516
PP2073	Acetyltransferase	-1.468	-0.144	-0.33	-1.44

qRT-PCR

qRT-PCR was performed to verify the expression data. Three genes were selected for the validation and included PP1961, PP4936 and fliE. PP1961 and PP4936 had also been associated with aggregation in chlorophenol. FliE is involved in flagellar assembly. Seven genes are involved with this pathway all of which were

up-regulated when the organism was grown on fructose. FliE was examined because it was the most up-regulated of these genes. The results validated the findings from the array study. There was an 11.46 fold up-regulation recorded in PP1961, 4.56-fold change up-regulation for PP4936 and 8.856-fold change up-regulation for fliE (Figure 3.27). PP4936 encodes for an O-antigen polymerase. O-antigen being part of bacterial lipopolysaccharide (LPS), the production of LPS when the organism was grown on mono-chlorophenols and fructose was investigated.

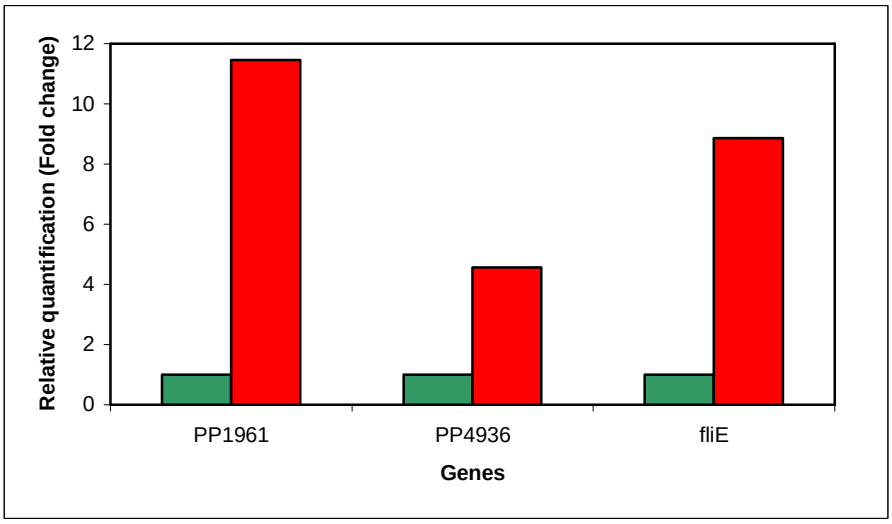


Figure 3.27 Relative quantification of gene expression of genes PP1961, PP4936 and fliE in aggregated cells of *P. putida* CP1 grown on 0.5% (w/v) fructose (■) compared to *P. putida* CP1 grown on nutrient broth (■).

Lipopolysaccharide production by P. putida CP1 during autoaggregation

The LPS profiles of *P. putida* CP1 and *P. putida* KT2440 grown on nutrient broth are illustrated in Figure 3.28. A commercial LPS sample from *P. aeruginosa* 10 (Sigma) was used as a positive control. The O-antigen located at 70 kDa is similar for all three organisms. The lipid A and core-polysaccharide components which migrate furthest due to their size of 10-25 kDa, are seen as a dark spot at the base of the gel. LPS was extracted from *P. putida* CP1 following growth on all three mono-chlorophenols on days 1 and 4 and following growth on fructose for 24 hours. Following growth on the mono-chlorophenols for 1 day, the O-antigen profile changed for all four treatments - 100 ppm 2-chlorophenol, 100 ppm 3-

chlorophenol, 200- and 50 ppm 4-chlorophenol, with a band visible with a molecular weight less than 70 kDa. Although cell aggregation had not been detected at this point, the result suggests a response to the presence of a toxic substrate. Following four days of growth on the mono-chlorophenols, aggregation of *P. putida* CP1 was observed when the organism was grown on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol, 200 ppm 4-chlorophenol but not 50 ppm 4-chlorophenol. Further changes in the O-antigen profile were observed where aggregation had occurred including when the organism was grown on 0.5% (w/v) fructose (Figure 3.28). An additional band was observed on the gel between 55-35 kDa.

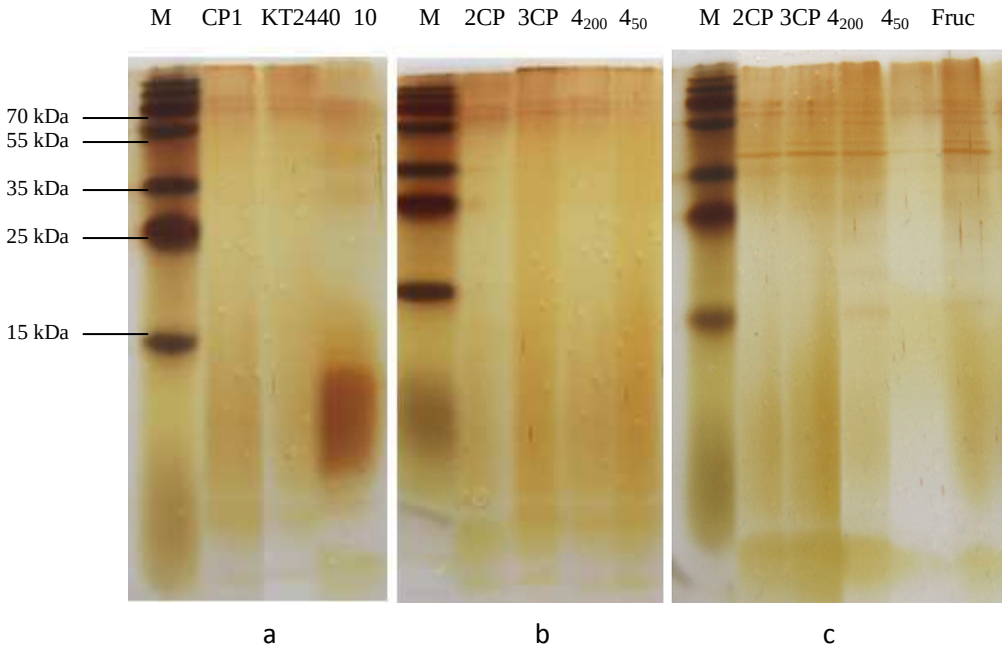


Figure 3.28 LPS profiles on SDS-PAGE gel. a) M: marker, CP1: *P. putida* CP1 grown on nutrient broth, KT2440: *P. putida* KT2440 grown on nutrient broth and 10: *P. aeruginosa* 10 grown on nutrient broth; b) M: marker, 2CP: *P. putida* CP1 grown on 100 ppm 2-chlorophenol, 3CP: 100 ppm 3-chlorophenol, $4CP_{200}$: 200 ppm 4-chlorophenol and $4CP_{50}$: 50 ppm 4-chlorophenol (day 1); c) M: marker, 2CP: *P. putida* CP1 grown on 100 ppm 2-chlorophenol, 3CP: 100 ppm 3-chlorophenol, $4CP_{200}$: 200 ppm 4-chlorophenol and $4CP_{50}$: 50 ppm 4-chlorophenol (day 4) and Fruc: 0.5% (w/v) fructose (24 hours).

4.0 DISCUSSION

4 Discussion

4.1 Comparison of *P. putida* CP1 and *P. putida* KT2440

Pseudomonas putida is an environmentally ubiquitous bacterium. Habitats used by *Pseudomonas* include dominant habitat types and also less frequently used habitats. These rarely used habitats can strongly influence ecological and evolutionary processes. Competition among *Pseudomonas* species has resulted in niche partitioning and is therefore a potentially important contributor to the observed variability in habitat use (Remold *et al.,* 2011). Screening of rhizobacteria with plant growth-promoting properties in potato fields in the Central Andean Highlands using 16S rRNA gene sequences identified the presence of *Pseudomonas* isolates (Ghyselinck *et al.,* 2013). Cray *et al.,* (2013) listed *Pseudomonas putida* among the dominant microbial groups which developed in open habitats and in high-nutrient concentrations. They reported that *Pseudomonas* species were the dominant microbes in Kunming, China on rocks polluted with crude oil. They also identified the presence of *Pseudomonas* species in hydrocarbon contaminated soil and in the rhizosphere of apple trees (Cray *et al.*, 2013).

Pseudomonas putida CP1 was the subject of this study. The organism was isolated from soil and has been studied for many years in our laboratory. The bacterium displays autoaggregation when grown on certain substrates and it was of interest to study phenotypic and genotypic responses associated with this phenomenon. Genotypic responses were studied using the *P. putida* KT2440 Oligonucleotide Array by Progenika and so the two bacteria were first compared. Based on cell characterisation, both organisms are motile Gram-negative rod shaped bacteria, positive for oxidase and catalase with no spore formation, characteristic of the genus *Pseudomonas* (Moore *et al.,* 2006). The characterization of species conducted by using classical phenotypic tests such as growth on specific media, enzymatic reactions and microscopy are time-consuming and unsuitable for large populations of closely related bacteria. Bacterial identification kits aim to make identification and characterization easier.

The most commonly used is the commercial API system, which is a rapid and reliable confirmatory identification approach. Following the use of the API 20NE system, it was observed that the identification value of *P. putida* CP1 was 99.8% and *P. putida* KT2440 was 99.7%. The bacteria differed only in their ability to assimilate arabinose and mannose. Many studies have approved the usage of API 20NE in bacterial identification. The accuracy of the API 20NE test was proven by a positive assimilation of arabinose by *Burkholderia pseudomallei* isolates in the API 20NE system identical to the results obtained from growth of the isolates on L-arabinose agar (Wuthiekanun *et al.,* 1996). Johnsen *et al.,* (1996) suggested that identification of members of the fluorescent *Pseudomonas* group using API 20NE was reproducible and a better system than the Biolog GN. He found that identification by API 20NE was in good agreement with classical test results for *P. putida*.

There are limitations in the application of commercially available identification systems. While the databases have a significant degree of accuracy for common species, there can be difficulties in identifying uncommon strains and infrequently isolated species. The technique has also shown that not all strains within a given species may exhibit a particular characteristic and the same strain may give different results upon repeated testing (Bosshard *et al.,* 2006). Adley and Saieb (2005) demonstrated the insufficient accuracy of the API 20NE system for *Ralstonia pickettii* identification. Two different commercial identification systems were used to identify 48 isolates of *R. pickettii*. Their findings showed that only 60% of the isolates were identified as *R. pickettii* with the API 20NE system compared to 90% with the RapID NF Plus system. The RapID NF Plus system is enzyme based with an ability to detect weakly oxidizing *R. pickettii* strains. All strains identified with RapID NF Plus were positive for glutmyl-β-naphthylamide, which is a key test for the identification of *R. pickettii* for this system. Uğur *et al.*, (2012) suggested that phenotypic classification techniques are not sufficient for identification of *Pseudomonas* species. He combined phenotypic and genotypic characterization of *Pseudomonas* species isolated from the marine environment using API 20NE and 16S rDNA sequencing. Among 20 isolates, 17 showed good agreement between both methods. The data suggested that the procedures for the identification of *Pseudomonas* sp. based on phenotypic characteristics should be

verified by molecular methods to obtain results that are meaningful as well as accurate. Bosshard *et al.,* (2006) compared phenotypic with molecular identification for non-fermenting Gram-negative bacilli (non-*Pseudomonas aeruginosa*) in the clinical laboratory using API 20NE and 16S rRNA sequencing. 16S rRNA gene sequence analysis showed 92% of the isolates were assigned to species level and 8% to the genus level and using API 20NE 54% was assigned to species and 7% to genus level. Thus he suggested that the molecular approach was more effective for the identification of nonfermenting Gram-negative bacilli.

Comparative transcriptomics-based approaches have played a fundamental role in investigations of complex cellular responses of *P. putida* strains. *P. putida* KT2440 is used worldwide as a workhorse for genetic and physiological studies and for the development of biotechnological applications (Nelson *et al.,* 2002). *P. putida* KT2440 is among the *P. putida* strains that have been fully sequenced. Thus, the genomic database of *P. putida* KT2440 serves as a standard reference for transcriptomic and proteomic studies in other strains. The consequence of the *P. putida* KT2440 full genome sequencing and annotation has led to a great interest among researchers in this strain (Ballerstedt *et al.,* 2007). Various studies have relied on the *P. putida* KT2440 genomic microarray in order to investigate transcriptomic profiling of this strain itself or another strain during various case studies including *P. putida* DOT-TIE (Duque *et al.,* 2007), *P. putida* S12 (Ballerstedt *et al.,* 2007) and *P. putida* KT2442 (Poblete-Castro *et al.,* 2012). As transcriptome profiling is based on the highly-sensitive detection of DNA–cDNA hybridization, DNA sequence similarity determines the validity of the analyses. The microarray platforms derived from strain KT2440 provide a valid framework for the study of nonsequenced *P. putida* strains. The solvent-tolerant, *P. putida* S12 and other *Pseudomonas* strains showed a value for biotechnology but the strains had not been sequenced. Thus, the metabolic potential of *P. putida* S12 was investigated by comparative proteomics and transcriptomics analyses based on the database information for *P. putida* KT2440 (Ballerstedt *et al.,* 2007). A similar approach has been conducted by Murray *et al.,* (2001) who used genome sequence information from the fully sequenced *Shewanella oneidensis* strain MR-1 to determine the connection between strains in the *Shewanella* genus of the γ-proteobacteria and MR-1.

Transcriptomics has informed our understanding of stress. The technique can be used to confirm changes in gene expression while also highlighting numerous unexpected genes for future study. Analyses of stress studies have shown mRNA abundance appears as a proxy for protein activity that may underlie variation in fitness (Feder and Walser, 2005). Fitness is important for an organism's response to abiotic stress and evolution and for their ability to survive and reproduce. At the biochemical level, fitness is primarily a result of the ability of proteins to function in their intra and extracellular surrounding, to integrate the diverse functions of the individual cells and organelles and to determine the levels of non-proteinaceous components. A study by Glanemann *et al*., (2003) showed mRNA concentrations of protein activity were closely linked to fitness. However, their study of the enzymes of amino acid biosynthesis in the bacterium *Corynebacterium* showed that protein activity sometimes exceeded mRNA abundance and vice versa. Thus, it is difficult to generally predict protein activity from quantitative transcriptome data. Feder and Walser (2005) pointed out that mRNA abundance provides little information on protein activity and fitness and thus cannot be substituted for detailed functional and ecological analyses of candidate genes.

DNA microarray studies were conducted by growing both organisms on nutrient broth at 30^{o}C. A comparison between *P. putida* CP1 and *P. putida* KT2440 showed a close similarity between both strains. Of the 5539 genes studied only 316 genes involved in 28 biological pathways were differentially regulated. The top 25 up- and down-regulated genes listed were based on the analysed genelists where genes were identified as significantly up-regulated ($P \leq 0.05$, Fold Change (FC) ≥ 1.2) and down-regulated ($P \leq 0.05$, Fold Change (FC) ≤ 1.2). The level of fold-changes in gene expression can be considered to be statistically, technically and biologically meaningful with the presence of a control to observe the background expression which should be much lower than that of the test samples. Researchers have combined the fold change and *p*-value to provide more meaningful results by setting cutoffs for both the fold change and *p*-value as in the volcano plot (Feng *et al.,* 2012). It has recently been suggested that differentially-expressed genes in a microarray experiment are best identified using fold-change, rather than a t-statistic because the former results in lists of differentially-

expressed genes that are more reproducible. This is particularly true for shorter gene lists due to the relatively unstable nature of the variance (noise) estimated in the *t*-statistic measurement. Furthermore, the impact of normalization methods on the reproducibility of gene lists becomes minimal when the fold change, instead of the *p*-value is used as the ranking criterion for gene selection (Shi *et al.,* 2006).

The genes that were differently expressed were mostly involved in core metabolic activity including pathways related to central energy and amino acid metabolism and adaptive response pathways. The metabolism and transport of amino acids in both strains were different at the expression level. Expression studies revealed an increase in the expression of genes involved in ABC transport systems in *P. putida* CP1. The genes of these systems governed the uptake of periplasmic amino acids (PP1071), leucine, isoleucine, and valine transporters (livM and livG), polar amino acids (PP4485, PP1070 and PP1069) monosaccharides (rbsC) and dipeptides (PP885). This increased expression was accompanied by expression of genes involved in the assimilation of valine, leucine and isoleucine (PP4065, PP4067, PP0596, bkdA1, bkdA2, bkdB, mmsA-1, mmsA-2 and mmsB), alanine, aspartate and glutamate (PP4547), lysine and tryptophan (fadAx, fadB2x, fadB1x, PP4066 nad PP4030), arginine and proline (PP3533 and PP5182), tyrosine (hpd, hmgA, PP4620 and PP4619) and phenylalanine metabolism (phhA and PP1791). These changes reflect adaptation of *P. putida* CP1 to the environment, allowing the bacterium to interconnect diverse pathways and to use this central metabolite as a universal substrate for various anabolic and catabolic processes.

In the pathway related to central energy metabolism, a gene involved in fructose and mannose metabolism, PP1776 was among the top 25 up-regulated genes in *P. putida* CP1 when compared to *P. putida* KT2440. Based on the KEGG pathway database, this gene shares a similar function to algA. Both genes encode for mannose-6-phosphate isomerise, which is involved in alginate polymerization. AlgA catalyses the conversion of fructose-6-phosphate to mannose-6-phosphate and converts mannose-1-phosphate to guanosine diphosphate (GDP)-mannose, before algD catalyzes the conversion of GDP-mannose to GDP-mannuronic acid (Ramsey and Wozniak, 2005). The induction of PP1776 involved in alginate biosynthesis has suggested an association of *P. putida* CP1 with cell attachment

and stress tolerance (Chang *et al.*, 2007). Variations in amino acid and central energy metabolism were also shown in the study conducted by Ballerstedt *et al.*, (2007). They carried out a transcriptomics analysis of solvent-tolerant *P. putida* S12 and found 12.7% of the genes involved in amino acid transport and 12% of the genes involved in carbohydrate transport and metabolism differed with that found in *P. putida* KT2440.

The adaptation mechanisms that differed in *P. putida* CP1 when compared to *P. putida* KT2440 were related to the pathways involved in a two-component system, flagellar assembly, lipid transport and metabolism and expression of mobile genetic elements. The most up-regulated genes were PP270 encoding for histidine kinase and fliC encoding for flagellin fliC. Both were involved in a two-component system (TCS) indicating a difference between the two strains in their response to environmental change. Tens of different two-component systems have been identified in members of the genus *Pseudomonas* (Putrinš *et al.,* 2010). TCS are composed of a membrane-located sensor with histidine kinase activity and a cytoplasmic response protein with a signal-accepting receiver domain. Two-component pathways enable cells to sense and respond to stimuli by inducing changes in transcription. Environmental signals sensed by membrane proteins are transduced to a response regulator by phosphorylation. PhoR-PhoP two-component protein was also found to be up-regulated in this study. In *Escherichia coli and Bacillus subtilis*, the two-component pathways PhoB/PhoR and PhoP/PhoR were shown to be involved in sensing phosphate (Pi) limitation (Prágai *et al.,* 2004). The PhoR/PhoP TCS of *Streptomyces lividans* was previously shown to allow the growth of the bacteria under conditions of Pi limitation. The system allowed bacteria to cope with limited availability of Pi by sensing Pi limitation in the growth medium. Strategies were then triggered aimed at scavenging and transporting trace amounts of Pi that is usually present in the growth medium as metal phosphates or by recycling phosphate present in phosphate-rich cellular constituents. They proposed that a Pi limitation correlated with a low adenylate charge thus triggering the activation of the central metabolic pathways and generating an internal oxidative stress. An oxidative stress being potentially deleterious for the cell, the bacteria have to precisely regulate the

balance between the necessary stimulation of its central metabolism and the production ATP (Ghorbel *et al.,* 2006).

Flagella are complex organelles involved in motility systems, regulated by many genes which allow bacteria to be propelled through liquids and on surfaces (Linda, 2006). Expression data showed that the transcription of genes encoding the flagellar proteins fliC, fliD, flgA, PP4396, flgL, flgE and fliS were induced suggesting a role for mobility in the bacterial recognition/interaction process of *P. putida* CP1. Variation in gene expression associated with flagellae among species of *P. putida* has been reported. The *P. putida* W619 genome contains a large cluster of genes involved in flagellar biosynthesis (PputW619_3655-3737) which are similarly organized in *P. putida* KT2440, *P. putida* GB-1 and *P. putida* FI, except that *P. putida* W619 contains within the flagellar biosynthesis cluster, a gene cluster involved in LPS biosynthesis and sporulation(Wu *et al.,* 2011).

The primary reaction of cells to environmental stresses takes place at the level of the cell envelope including changes in lipid transport and metabolism. Six of the genes induced in *P. putida* CP1 included fadAx (acetyl-CoA acetyltransferase), fadB2x (short-chain dehydrogenase), fadD2 (long-chain-fatty-acid-CoA ligase), fadB1 and PP4030 (enoyl-CoA hydratase) and PP4066 (gamma-carboxygeranoyl-CoA hydratase). These genes are involved in fatty acid metabolism and the alteration in gene expression in *P. putida* CP1 highlighted differences between the bacterium and *P. putida* KT2440 in fatty acid metabolism. Pseudomonads are able to metabolize a broad range of fatty acids (Poblete-Castro *et al.,* 2012).

Among the various putative genomic islands, many are suggested to be acquired via horizontal gene transfer due to the presence of integrases or mobile genetic elements (transposons, IS elements). Putative genomic islands have conferred *P. putida* species with many capabilities, including heavy metal resistance, aromatic compound degradation and stress responses which are relevant for the specific niche adaptation of this species (Wu *et al.,* 2011). Two genes that are involved in genetic element transfer were identified among the top up-regulated genes in *P. putida* CP1. These included PP2297 which encodes for the integrative genetic element Ppu40 integrase and TnpA which encodes for Tn4652 transposase.

Tn4652 transposase is a derivative of the toluene degradation transposon Tn4651 that belongs to the Tn3 family of transposons. Functional analysis of the tnpA promoter revealed that it is active in *P. putida* but silent in *E. coli*, indicating that some *P. putida*-specific factor is required for transcription from this promoter. The tnpA promoter activity was shown to be modulated by an integration host factor IHF. The presence of an IHF-binding site upstream of the tnpA promoter enhanced the promoter activity (Hõrak and Kivisaar, 1998). Among the transposable elements available in plasmids of *Pseudomonas* species only Tn4652 has the ability for the insertion of plasmid genes into the *P. putida* chromosome (Mae and Heinaru, 1994). Differences in bacterial adaptation to the environment were shown by Wu *et al.,* (2011). He carried out a comparative genomic study of four *P. putida* strains –KT2440, W619, F1 and GB-1. Differences were influenced by horizontal gene transfer and niche-specific functions encoded on genomic islands. The microevolution characteristics of an organismic lineage is the result of extrinsic (biotic and abiotic selection pressures) and intrinsic forces (the genetic prerequisites for horizontal gene transfer and the effective population size). Both forces determine the power of genetic drift (Selezska *et al.,* 2012).

4.2 Phenotypic responses of *P. putida* CP1 following growth on mono-chlorophenol and fructose

The ability of microorganisms to degrade all three mono-chlorophenol isomers is rarely reported. In this study the ability to use mono-chlorophenols as a sole carbon source was only observed with *P. putida* CP1 and not with the reference organism *P. putida* KT2440. *P. putida* KT2440 is a plasmid-free derivative of a toluene-degrading bacterium, derived from a well-known toluene-degrading organism *P. putida* mt-2 (Palleroni, 2010). Unlike *P. putida* KT2440, *P. putida* mt-2 harbours the conjugative plasmid pWW0, which confers oxidative catabolism of toluene/xylenes. Nilsson *et al.,* (2011) identified that the presence of the conjugative plasmid may also promote biofilm formation in *P. putida* mt-2. *P. putida* F1 isolated from a polluted creek is considered the best reference strain for the degradation and growth of *P. putida* on aromatic hydrocarbons including benzene, toluene, ethylbenzene and *p*-cymene. The bacterium has a broad

substrate toluene dioxygenase that can oxidize indole, nitrotoluenee, chlorobenzenes and including chlorophenols (Wu *et al.,* 2011).

The growth on higher concentrations of mono-chlorophenols affected the yield of biomass, where the only significant increase in biomass was observed when *P. putida* CP1 was grown on lower concentrations of 4-chlorophenol. A similar pattern was observed in *P. putida* S12 where the growth on various solvents like toluene, ethylbenzene, propylbenzene, xylene, hexane, and cyclohexane reduced the biomass yield. The effect was related to the concentration of solvent and not to the chemical structure (Isken *et al.,* 1999). The reduction in yield is due to the high energy requirement by the organism. The energy is required by the organism to turn on an adaptation mechanism which requires an active efflux system. The requirement of high energy was also observed in the active export of solvents and enhanced phospholipid biosynthesis rate in *P. putida* S12 and *P. putida* Idaho (Pinkart and White, 1997).

Pseudomonas putida plays a major ecological role in environments that contain an array of chemically diverse aromatics and hydrocarbons which induce chaotropicity mediated stress. This bacterium is not only renowned for its metabolic versatility, but is also known for its tolerance to specific solutes and solvents (Hallsworth *et al.,* 2003; Bhaganna *et al.,* 2010). Cray *et al.,* (2013) reported how the organism was able to tolerate all substances within each group of stressor including hydrophobic compounds, surfactants, aromatic solutes, chaotropic salts and polysaccharide kosmotropic salts. They pointed out that *P. putida* is able to avoid contact with or prevent accumulation of stressors by using solvent-efflux pumps and synthesizing EPS to form a protective barrier. They also showed that the bacterium utilizes highly efficient macromolecule protective systems that are upregulated in response to multiple environmental stresses including protein-stabilization proteins, oxidative stress responses, upregulation of energy generation and overall protein synthesis.

Toxic organic solvents are extremely harmful for microorganism because they partition in the cell membrane and disorganize the structure by removing lipids and protein and can cause cell death. The response of bacteria to solvents varies.

While some bacteria can grow and metabolise the solvent as a carbon source, others simply tolerate the solvent and are unable to metabolise it. Segura *et al.*, (1999) described a number of *Pseudomonas* strains tolerant to toluene which did not use the compounds as a carbon source. Solvent-tolerant bacteria include *P. putida* DOT-T1E, a highly solvent-tolerant microbe isolated from a wastewater treatment plant (Segura *et al.,* 2005).

Growth of *P. putida* CP1 on higher concentrations of the mono-chlorophenols including 200 ppm 4-chlorophenol, 100 ppm 2-chlorophenol and 100 ppm 3-chlorophenol resulted in autoaggregation. The formations of aggregates were only present in the higher concentrations of mono-chlorophenol while no aggregated cells were observed during growth on lower concentrations of 4-chlorophenol (50 ppm) and nutrient broth. Previously, Farrell and Quilty (2002) hypothesized the phenomenon as a survival response of the cells to toxicity of the added substrates. Stress induced by chaotropic compounds is one factor among the possible triggers for active bacterial aggregation that allowed a protection mechanism and a strategy to survive (Gilbert *et al.,* 2002). *P. aeruginosa* PAO1 exhibited cell aggregation as an energy-dependent response to detergent stress which might serve as a survival strategy during growth with detergent SDS (Klebensberger *et al.,* 2006). The cell aggregation behaviour of *Sphingobium chlorophenolicum* (Flavobacterium sp., ATCC 39723) degrading pentachlorophenol (PCP) has been reported (Chang and Su, 2003). The formation of aggregates during degradation of 200 ppm pentachlorophenol was also observed with *P. putida* CP1 but data is not shown in this study. *Pseudomonas* sp. ZA1 could degrade a synthetic monoazo dye, Acid Violet-7. The bacterium was aggregated with aluminium sulfate (alum) for enforced immobilization. The aggregation efficacy was consistent (83–85%) in a pH range from 4 to 10. As the solution pH increased from 4 to 6.5, more aluminum ions $Al(OH)^{2+}$ were available to form hydroxy-bridged polymers for the benefit of aggregate formation (Tse and Yu, 1997).

P. putida CP1 surface hydrophobicity increased with the degree of substrate toxicity 3CP> 2CP> 4CP. This observation suggested that surface properties function as an adaptive mechanism and response to stress in the presence of a chaotropic stressor. The solvent-tolerant organism, *P. putida* DOT-T1E exhibited

higher cell surface hydrophobicity when grown on the toxic solvent 1-decanol (Neumann *et al.,* 2006). Other, factors such as growth rate (Wang and Han, 2007), growth substrate (Farrell and Quilty, 2002), pH, temperature (Mai and Conner, 2007; Hori *et al.,* 2009) and starvation (Sanin, 2003) have also been shown to influence the hydrophobic properties of a cell surface.

Cell surface hydrophobicity is associated with the ability of bacteria to autoaggregate. The ability of *P. putida* CP1 to autoaggregate in mono-chlorophenols increased with the aggregation index (AI) and cell surface hydrophobicity from day 1 to day 4 when *P. putida* CP1 achieved the maximum level of aggregation. The AI represents the fraction of cells associated in the aggregates and the cell surface hydrophobicity is a measure of the surface characteristics of the cell and its ability to adhere to other surfaces. Theoretically, the physicochemical properties of cell surfaces have a fundamental consequence on the autoaggregation phenomenon. When bacteria become more hydrophobic cell-to-cell adhesion increases (Liu *et al.,* 2004). The hydrophobic effect attributed to cell wall proteins and lipids might be among the factors that trigger the ability to aggregate in most microbes (Gogra *et al.,* 2010). Two species, *Burkholderia cepacia* capable of degrading n-alkanes and *Burkholderia multivorans* capable of degrading naphthalene as sole substrate showed an ability to form biofilms. The ability to attach to a glass surface was evaluated by the increase in cell surface hydrophobicity (Chakraborty *et al.,* 2010). A study conducted by Gogra *et al.,* (2010) showed a positive correlation between cell surface hydrophobicity and autoaggregation ability in diethyl phthalate (DEP) degrading organisms *Escherichia coli*, *Staphylococcus aureus* and *Aspergillus niger*. Exposure to alcohols in *P. putida* has changed the cell surface hydrophobicity leading to autoaggregation as an adaptation response to the toxic substrate (Tsubata *et al.,* 1997). Bossier *et al.,* (2000) proved that the autoaggregating mutant *Ralstonia eutropha* - like strain A3 was more hydrophobic than its non-aggregating parent, *Ralstonia eutropha* - like strain AE815. Rahman *et al.,* (2008) proved that good aggregation ability and surface hydrophobicity influenced the aggregation capability of *Bifidobacterium longum*. An aggregate forming bacterium *Pseudomonas* sp. KEWA-1 was isolated from a water treatment plant. The

organism was hydrophobic and formed an extensive capsule when aggregated in a dilute medium (Singh and Vincent, 1987).

Hydrophobic interactions define the strong attraction between hydrophobic molecules and surfaces in water. In biological systems hydrophobic interactions are the strongest long-range non-covalent interactions (Sanin *et al*., 2003). According to Del Re *et al.,* (2000) hydrophobicity results from one method should be required to be compared with another one because cell surface hydrophobicity methods do not measure the intrinsic microbial cell surface hydrophobicity but rather the bacterial adhesion to certain hydrophobic substrates. The cell surface hydrophobicity for *P. putida* CP1 was measured using the BATH assay. This technique was originally developed by Rosenberg *et al.,* (1980) who found that various bacterial strains possessing hydrophobic surface characteristics adhered to liquid hydrocarbons, whereas hydrophilic strains did not. BATH has been proposed as a simple and general technique for studying cell surface hydrophobicity. In recent years many studies have used this method to measure the hydrophobicity of aggregated cells (Sanin, 2003; Rahman *et al.,* 2008; Del Re *et al.,* 2000; Jain *et al.,* 2007). Since electrostatic interactions can only be minimized but cannot be eliminated entirely in these tests, selection of the organic phase used in tests becomes important. Compared to *n*-octane, *n*-hexadecane was proven to give representative results and used for hydrophobicity evaluations of bacteria (Sanin, 2003). This was also found to be the case in this study.

When *P. putida* CP1 was grown on mono-chlorophenols, the viability of the cells decreased with time and correlated with the ability of the organism to degrade the substrate and the degree of substrate toxicity. Viability studies of the aggregates were conducted using the Live/Dead Baclight kit which uses Syto 9 and propidium iodide. These dyes are widely used and recommended for microscopic examination and have been shown to be very effective, with minimal non-specific binding in the staining of complex biofilm communities (Neu and Lawrence, 1997) and bacterial aggregates (Klebensberger *et al.,* 2006). The aggregated cells of *P. putida* CP1 grown on mono-chlorophenol were observed to be viable suggesting that the aggregate was affording a protective mechanism to the cell.

A study by Neumann *et al.,* (2005) revealed that cells of different bacterial species enlarged the cell size and decreased the relative surface of a cell and consequently reduced the attachable surface for toxic aromatic compounds. A study conducted by Panicker *et al.,* (2006) showed that aggregates of *Pseudomonas* sp. 30-3 contained viable and metabolically active cells when grown at low temperatures. The planktonic aggregates and polyester attached biofilms of *P. aeruginosa* PAO1 were shown to contain densely packed viable cells which decreased in viability during starvation periods (Schleheck *et al.,* 2009). The viability in *P. putida* CP1 aggregates grown on mono-chlorophenol is suggested to be related to the production of EPS that provided protection to the cells (Panicker *et al.,* 2002). The determinations of cell viability are important to this study. When RNA extraction of *P. putida* CP1 grown on mono-chlorophenols was conducted the profile of cell viability showed all samples were in a viable state.

Many researchers have reported the production of EPS during growth of certain *Pseudomonas* sp. under stress conditions and at the same time have noted the role of EPS in aggregation. The production of EPS by *P. putida* CP1 during growth on mono-chlorophenols correlated with the ability to autoaggregate and the size of the aggregates. A similar pattern was found in three different strains of *Ethanoligenes harbinense* which showed the composition of EPS was related to the capabilities of autoaggregation (Ren *et al.,* 2009). EPS production in *P. putida* CP1 related to the degree of growth rate inhibition where total EPS was highest in 3-chlorophenol, thus suggesting that EPS production was a stress protection mechanism induced by the toxic substrate. EPS aids in the formation of a matrix that keeps bacteria together and protects bacteria against harmful environmental conditions (Chapman and Kao 1998). Sheng *et al.,* (2005b) analysed the EPS formed by *R. acidophila* when growing in the presence of 2, 4-dichlorophenol (2, 4-DCP) at different concentrations. The EPS composition was not significantly influenced by the increase in 2, 4-DCP concentration from 0 to 80 mg l^{-1}. However, at 100 mg l^{-1} of 2, 4-DCP, the EPS content increased 2.4 times. This study suggested that the production of EPS was greatly promoted in the presence of toxic substances, forming a protective shield for the cells against the adverse influences from the external environment.

The ability of bacteria to produce EPS in response to various environment stresses is an opportunity for the cells to survive. The production of EPS by *Pseudomonas* sp. CM10 protected the cells in the presence of a predator, as the cell aggregates exceeded the size of the predator and became inedible (Matz *et al.*, 2002). Cray *et al.*, (2013) pointed out that autoaggregation may enhance stress tolerance and reduce losses through grazing. They showed that grazing and predation influenced growth dynamics and at the same time caused extensive losses in microbial numbers. *Pseudomonas aeruginosa* and *Microcystis aeruginosa* were reported to be capable of forming multicellular structures called microcolonies which grazers and predators find difficult to attack. Formation of the microcolonies was associated with the synthesis of EPS by *M. aeruginosa* (Yang and Kong, 2012) and the use of flagella by *P. aeruginosa* (Matz *et al.*, 2004).

Sanin (2003) reported that the aggregation of *Pseudomonas* sp. A and *Pseudomonas* sp. D during starvation was due to the production of exopolysaccharides. *Pseudomonas* sp. 30-3, a toluene degrading microorganism isolated from oil-contaminated Antarctic soils formed aggregated cells when exposed to low temperatures. This was speculated to be due to the secretion of EPS thus protecting the organism from cold or frost damage (Panicker *et al.*, 2006). The production of bound EPS was higher compared to the free EPS in *P. putida* CP1 grown on mono-chlorophenols. The primary role of bound EPS may not be in the initial cell-to-cell adhesion but in holding cells together after adhesion. These findings suggest that free EPS can induce cell aggregation and their ability to induce aggregation may be due to its protein content (Eboigbodin and Biggs, 2008).

Two different approaches were applied in the quantification of biochemical components in the EPS. They were chemical analysis and microscopy using specific fluorescent dyes. During growth of *P. putida* CP1 on mono-chlorophenols, carbohydrate and proteins constituted the dominant component of the EPS and less than 10% DNA. This suggested that in the presence of mono-chlorophenols, both components were highly induced in a response to the toxic medium. In some other studies, carbohydrates were identified as the main constituent of the EPS while some found proteins to dominate (Zhang and Fang,

2001; Liu and Fang, 2003). The EPS composition of aggregated cells differs in quantity, structure and nature depending on the microorganisms within the aggregates and the extraction methods. EPS of biofilms is highly heterogeneous even among the same bacterial species and therefore its amounts, composition and function within the biofilm will differ (O'Toole *et al.,* 2000). The EPS biochemical composition of *P. putida* CP1 during growth on mono-chlorophenol showed a similar pattern with the autoaggregating bacterium *Ethanoligenes harbinense* YUAN-3. This organism is a hydrogen-producing bacterium with a capability to autoaggregate during the exponential phase. The study showed that carbohydrate and protein played an important part in the autoaggregation of YUAN-3. Analyses of EPS showed protein (21±0.8mg/g biomass) and carbohydrate (16.9±0.8 mg/g biomass) were dominant, while DNA was 3.5±0.5 mg/biomass (Ren *et al.,* 2009).

Carbohydrate and protein are two dominant components of EPS of cells. Jorand *et al.,* (1998) suggested that carbohydrate plays the major role in aggregation. However, many publications reported that the level of EPS protein in activated sludge systems was higher than carbohydrate and plays an important role in aggregation as well (Higgins and Novak, 1997; Liao *et al.,* 2001). Carbohydrate fractions play a dominant role in sludge settling and were suggested to play a major role in aggregation due to their capacity to form bridges between their negatively charged groups and divalent cations (Flemming and Wingender, 2001). Carbohydrates are believed to protect bacterial cells from desiccation, heavy metals, organic compounds or other environmental stresses, including host immune responses and to produce biofilms, thus to increase the chances of the cells to colonize special ecological niches (Onbasli and Aslim, 2009). A *P. putida* isolate produced an exopolymer consisting of glucose, galactose and pyruvate in a ratio of 1:1:1 and *P. putida* is also known to produce an alginate like polysaccharide. *Pseudomonas aeruginosa* PA14 formed an extracellular matrix that contained a carbohydrate-rich material with glucosidic linkages for its structural integrity (Friedman and Kolter 2004). The chemical constituents of EPS of *Pseudomonas* sp. 30-3 aggregated cells showed the presence of exopolysaccharides (EPS) which were made up of N-acetyl-D-glucosamine and N-acetylneuraminic acid (Panicker *et al.,* 2006). The EPS production suggested

that it may form a microenvironment around the cells helping them to avoid direct contact with bulk liquid water conferring protection from cold and frost damage. The presence of hydrophilic polymers which are kosmotropic that have a substantial hydration shell will help to remove water from the liquid phase. This stabilizes membranes and other macromolecular systems (Cray *et al*., 2013).

The function of proteins in the formation of aggregated cells is known to be associated with the primary adhesion events during cell attachment and biofilm development. In addition, protein has a role as exoenzymes in the exopolymer matrix (Jahn *et al.,* 1999). The protein contribution to aggregate binding strength is explained by hydrophobic interactions and polyvalent cations bridging to increase the stability of the biopolymer network (Jorand *et al.,* 1998). The EPS molecules of most bacterial species are negatively charged under neutral pH conditions due to the presence of predominantly carboxylic and phosphoryl functional groups (Ueshima *et al.,* 2008). High concentrations of protein production were also reported from studies of *P. putida* cell aggregation (Jahn *et al.,* 1999; Kachlany *et al.,* 2002). Proteins were found to be the main component for sludge aggregate formation (Liao *et al.,* 2001). The composition of EPS will govern the surface characteristics and therefore the aggregation ability of aggregates. Proteins and amino acids are hydrophobic components of EPS. As hydrophobicity is increased, the attachment of bacteria increases (Sanin, 2003).

Less than 10% of EPS composition in *P. putida* CP1 was DNA. A low level of DNA present in the EPS has been suggested to indicate the presence of few dead or lysed cells (Sheng *et al.,* 2010). However, in this study, the levels of DNA did not increase while the numbers of dead cells in the aggregates did increase. Suzuki *et al.,* (2009) pointed out that DNA is not only the result of cell lysis but can also result from the active production of extracellular nucleic acid. Delivery of DNA outside the cell can occur via the formation of membrane vesicles or can originate from lysis or autolysis induced by stress (Klebensberger *et al.,* 2006). It is suggested that the source of DNA is not only cell lysis, but an active excretion mechanism through membrane vesicles that are released during growth of the bacteria. This phenomenon has been previously observed in two distinct *P. aeruginosa* strains, and it was established that the vesicles contained DNA

(Kadurugamuwa and Beveridge, 1995). The accumulation of DNA within the aggregate matrix is proposed to contribute to the structural stability of aggregates (Liao *et al.,* 2001). Sakka and Takashi (1982) observed that bacterial aggregation involved the interaction of DNA on cell surfaces and the accumulation of DNA in the growth medium that acted as a natural flocculant. DNA is a charged molecule that may accumulate in the EPS and influence the biofilms structure. The accumulation of DNA in biofilms is important in horizontal gene transfer (Jahn *et al.,* 1999).

The EPS of the macroscopic aggregates formed by strain *P. aeruginosa* PAO1 during growth with SDS contained acidic polysaccharides and DNA (Klebensberger *et al.,* 2006). By treatment with DNase, these aggregates were disintegrated and viable cells were released. A low level of DNA was detected in the EPS produced by *P. putida* CP1 and lower concentration of DNase did not disperse the aggregated cells (data not shown). Watanabe *et al.,* (1998) isolated a marine bacterium belonging to the genus *Rhodovulum,* which could aggregate under specific growth conditions and the EPS from this bacterium was found to be mainly composed or RNA and protein. Whitchurch *et al.,* (2002) found that DNA may also play a structural role in *P. aeruginosa* biofilm architecture. In addition, Palmgren and Nielsen (1996) reported that the amount of DNA present in the EPS matrix was much higher than estimates of total intracellular DNA based on cell numbers, which seems to suggest that lysis is not the only process contributing to DNA accumulation in the floc matrix of activated sludge.

An ideal EPS extraction method should minimise cell lysis and not disrupt the EPS structure (Frølund *et al.,* 1996). According to Jahn *et al.,* (1999) the efficiency of extraction is defined as the total amount of EPS extracted from the total organic matter. Various approaches for EPS extraction have been developed however it is still difficult to compare results due to the lack of standard protocols. The EPS components and proportion ratios may vary depending on the sample source and the extraction method chosen (Eboigbodin and Biggs, 2008). The event of cell lysis may occur during EPS extraction. Most studies show the content of nucleic acid in EPS is usually low, while a high level after extraction indicates severe cell lysis. The extraction of EPS using the EDTA method is well-

known as an established method which gives reproducible results, releasing low levels of nucleic acids with low levels of cell lysis compared with other methods (Sheng *et al.,* 2005b; Sheng *et al.,* 2005a). However, the residual EDTA can contaminate the EPS extraction, thus dialysis is always needed for this method to prevent contamination (Comte *et al.,* 2006).

The use of epifluorescent microscopy was suggested by Nosyk *et al.,* (2008) to be one of the most promising techniques which allow the determination of the structure and composition of EPS *in situ* by staining different components of the cells with specific fluorescent markers. This approach however has no consistent sample preparation or staining method available up until now. Multi-staining methods allow a complete staining of the aggregated cell components and maintain the original form and structure of aggregated cells. The epifluorescence microscopic observation revealed the binding of calcofluor white to the aggregated cells. This suggested the presence of cellulose-like polymers containing β 1-4 linkages in the EPS. Calcofluor itself has an extremely conjugated and planar structure which allows binding to cellulose type polymer β 1-4 linked polymers via hydrogen bonding, dipolar interactions and van der Waals forces. Calcofluor binding of bacterial colonies is often associated with the production of cellulose (Spiers and Rainey, 2005). The commercially available cellulose enzymes preparation used in this study contained a mixture of enzymatic activities including glucosidic activities against 1, 4- and 1, 6-linked glucose polymers (data not showed). Therefore, the carbohydrate-containing matrix component need not necessarily to be a 1, 4-linked cellulose-like polymer but may contain 1, 6-linkages. Spiers and Rainey (2005) showed that the *P. aeruginosa* WS-5 biofilm contained cellulose which stained with calcofluor. However they also showed that *P. aeruginosa* PA14 which did not contain cellulose also stained with calcofluor.

The distribution of EPS formed when *P. putida* CP1 was grown on 3-chlorophenol was scattered throughout the cells based on a cross-section of the aggregates. The advantage of using CLSM is to be able to observe the clear distribution of the biochemical composition of aggregates. Normally in fluorescence microscopy the entire field of view of the sample is completely

illuminated, making the whole region fluoresce at the same time. The CLSM has an advantage to reduce the amount of light emanating and has a setup to minimize how much of the specimen is illuminated (Semwogerere and Weeks, 2005).

The difference between glucose and fructose utilization by *P. putida* CP1 and *P. putida* KT2440 may be related to the transport mechanism of fructose into the cells which is different to glucose transport. In most Pseudomonads, fructose is transported into the cell via a phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS) producing intracellular fructose-1-phosphate (Allenza *et al.,* 1982). Extracellular glucose is brought directly into the cell by an active transport system (Whiting *et al.,* 1976). The genus *Pseudomonas* metabolizes glucose exclusively by the Entner-Doudoroff (ED) pathway, in which 6-phosphogluconate is the key intermediate (Temple *et al.,* 1998). Similar findings to this study were observed during the growth of *P. putida* MAD2 by Chavarría *et al.,* (2012). During the exponential phase bacteria grew on fructose 60% more slowly than on glucose. The growth rate on fructose was consistent with a slower uptake of the carbohydrate (~0.75 mmol sugar/g biomass/hour). In contrast, cells grown on glucose consumed the sugar 6-fold faster (~4.79 mmol glucose/g biomass/hour). These differences suggested that cells might not only channel each of these sugars through different pathways but also adapt to different overall metabolic function. The chaotropicity of the substrates may also have played a role. Fructose has a chaotropic activity of +4.56 kJ kg^{-1} mol^{-1} considerably higher than the value for glucose which is +1.19 kJ kg^{-1} mol^{-1} (Cray *et al.,* 2013).

P. putida CP1 exhibits autoaggregation in the presence of 0.5% (w/v) fructose but not in 0.5% (w/v) glucose. There was slight change in the pH in both systems, thus this was not considered to be linked with the aggregation. 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. Sadasivan and Neyra (1985) have identified the aggregation phenomenon in *Azospirillum brasilense* and *Azospirillum lipoferum* during growth on minimal medium containing fructose and the presence of nitrate increased the aggregate yield. *Azospirillum brasilense* was unable to form aggregates in glucose as a carbon source. The phenomenon was believed to be related with the production of exocellular polymers including cellulose and β-linked polysaccharides. The aggregates were resistant to desiccation and were viable with the formation of cysts containing poly-(3-hydroxybutyrate) granules composed of basic units of polysaccharides complex with divalent cations. The granules supplied a high degree of resistance to desiccation. *Azospirillum brasilense* was reported to aggregate during the growth in high C: N medium containing fructose and ammonium chloride (Burdman *et al.,* 2000; Burdman *et al.,* 1998). Tomás *et al.,* (2005) demonstrated the ability of *Lactobacillus johnsonii* CRL to aggregate in growth media of low initial pH. 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.

The aggregation index was measured for *P. putida* CP1 growing on 0.5% (w/v) fructose and was found to increase steadily corresponding with the increase in aggregation. The increase of the aggregation index was coupled with an increase of cell surface hydrophobicity and aggregate size from day 1 to day 3 observations. This pattern was similar to that observed with autoaggregation in mono-chlorophenols. Microscopic observations showed that most of the cells in the aggregate formed on fructose were viable after 24 hours. However after 48 hours and 72 hours the core part of the aggregate was filled with dead cells. The observation was similar as that observed in the aerobic granules from a sequencing batch reactor (Toh *et al.,* 2003). Accumulation of dead cells at core regions may be related to the oxygen limitation factor (Chiu *et al.,* 2007).

The production of total EPS of aggregates grown on fructose correlated with the aggregate size, which was a similar trend to that observed in mono-chlorophenols. However, when the total EPS was expressed in mg/g biomass, the total EPS value

decreased in comparison to the value expressed in µg/ml, thus suggesting the bigger aggregate contained less EPS. A characterization of different fractions of microbial aggregates from an anaerobic membrane bioreactor showed that smaller aggregates contained a higher level of EPS. They had a denser and less porous gel layer which caused a high specific resistance. Small aggregates have a strong tendency to deposit on membrane surfaces due to lower transport velocity compared to larger aggregates. The adhesion ability is enhanced by polymeric interactions when the EPS content is increased (Lin *et al.*, 2011).

The proportion of biochemical components in EPS of *P. putida* CP1 aggregates grown on fructose was similar to those on mono-chlorophenols. However, the ratio of proteins and carbohydrates detected by the microscopic analysis was different to ratios obtained by chemical analysis. The ratios can be expected to be due to the different systems applied in each methods as observed by Neu and Lawrence (1997).

4.3 Genotypic responses of *P. putida* CP1 following growth on mono-chlorophenols

Transcriptomic analysis has been used successfully to gain a comprehensive insight into complex metabolic networks including organic solvent stress (Segura *et al.*, 2005; Wierckx *et al.*, 2008; Reva *et al.*, 2006). Expression profiling was conducted on *P. putida* CP1 grown on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol and 50- and 200 ppm 4-chlorophenol and compared to *P. putida* CP1 grown on nutrient broth. A total of 484 common differentially regulated genes were identified in the four comparative studies. The information presents an overview of the response of *P. putida* CP1 when grown on mono-chlorophenol as the chaotropic stressor and the connection with potential adaptation mechanisms. Adaptation of *P. putida* CP1 to mono-chlorophenol showed alterations of the gene expression at the outer membrane, cytoplasmic membrane, efflux systems and general stress proteins.

As described by Hallsworth *et al.*, (2003) when *P. putida* responded to stress there was up-regulation of various genes including those involved in protein

stabilization, lipid metabolism, membrane structure, protein synthesis and energy metabolism. Other responses to stress included ncreases in the amounts of LPS in the outer membrane of Gram-negative bacteria (Moat et al., 2002), activation of a two-component system (Kivistik *et al.*, 20060) and increased synthesis of stress proteins including the chaperonins and heat-shock proteins and increases in the synthesis and transport of amino acids (Bhaganna et al., 2013).

The majority of isolated solvent-tolerant bacteria are Gram-negative. Their possession of an outer membrane could explain the tolerance toward organic solvents. Changes at the level of the outer membrane to increased solvent tolerance include the stabilization of the membrane by magnesium and calcium ions. Other changes in the outer membrane involve membrane-embedded porins and lipopolysaccharide (Spiers *et al.,* 2002). Kasai *et al.,* (2001) showed that aromatic compounds may enter the cell periplasm through porins in the outer membrane. Our results indicate that mono-chlorophenols may enter the cells of *P. putida* CP1 through the outer membrane porin due to induction of the outer membrane proteins OprH and OprG and the aromatic compound specific porin PP3656. Transport of xylene across the outer membrane proceed through the XylN porin which is encoded in the operon required for the catabolism of toluene and xylene (Kasai *et al.*, 2001). A study of the solvent-tolerant *P. putida* S12 suggested that phenol enters the cell via porin B1 (Wierckx *et al.*, 2005).

Two mechanisms of movement of hydrocarbon across membranes have been identified: the transient channel and the solubility-diffusion mechanism. Firstly, the molecular motion of bilayer molecules can lead to the formation of transient channels across the membrane, through which hydrocarbon molecules can move. Secondly, short-lived cavities that appear in the polar, hydrophilic region(s) can facilitate entry of hydrophobic molecules into the membrane. Some hydrocarbon-degrading microbes respond to hydrophobic compounds by modifying their plasma membrane structure that facilitates the spontaneous entry of substrate molecules into the cell (Gupta *et al.,* 2008). Meanwhile, hydrophobic compounds that enter the cell from the extracellular environment may have to navigate organic matrices composed of kosmotropic material such as extracellular polysaccharides (EPS), glycoprotein S-layers, or the LPS and peptidoglycans of

the microbial cell wall (Sikkema et al., 1995). Such extracellular material can facilitate attachment to hydrophobic surfaces and this demonstrates an ability to interact with hydrophobic substances. EPS and other polymeric matrices may stop the transit of hydrophobic molecules towards the microbial cell (McCammick *et al*., 2010).

The lipopolysaccharide at the outer membrane is essential for barrier function, as it restricts movement of both hydrophilic and lipophilic compounds. It has been shown by Santos *et al.,* (2004) that phenol induces expression of proteins related to cell envelope biosynthesis in *P. putida* involved in lipopolysaccharide and peptidoglycane biosynthesis. A similar finding was found in this study during growth of *P. putida* CP1 on mono-chlorophenols which induced the expression of genes involve in lipopolysaccharide biosynthesis kdsA (2-dehydro-3-deoxyphosphooctonate aldolase), lpxB (lipid-A-disaccharide synthase), PP3316 (LPS core biosynthesis protein) and PP0063 (lipid A biosynthesis lauroyl acyltransferase). KdsA and lpxB are localized at the cytoplasm and PP0063 at the cytoplasmic membrane. Gene PP1993 (peptidoglycan-binding LysM) involved in peptidoglycan biosynthesis was also induced. It suggests that adaptation to mono-chlorophenol involves higher requirement for synthesis of cell envelope components. PP0063 encoding for lipid A biosynthesis lauroyl acyltransferase was induced in response to all mono-chlorophenols. Expression of PP0063 was found in *P. putida* KT2440 to cope with cold, benzoate and acid stresses. This suggests that it is essential for adapting lipid A fatty acid composition to environmental stress conditions (Reva *et al.,* 2006). In the case of *P. putida* CP1, alteration in genes involved in LPS biosynthesis presumably caused the changes in the LPS profile with appearance of O-antigen band with a lower molecular weight less than 70 kDa. The result suggests a response to the presence of a toxic substrate. A similar phenomenon was observed in *P. putida* Idaho where LPS composition changed when grown in the presence of a toxic substrate. A higher-molecular-weight LPS band disappeared and was replaced by a lower-molecular-weight band (Pinkart *et al.,* 1996). Low temperatures promote non-covalent interactions and, thereby, rigidify cellular macromolecules and membranes (Chin *et al.,* 2010). In contrast, chaotropic solutes induce a strong cellular stress

response known to disorder macromolecular systems and to disrupt cellular interactions (Hallsworth *et al.*, 2003; Cray *et al.*, 2013).

The changes in protein production at the cytoplasmic membrane counterbalance the changes in membrane fluidity (Wierckx *et al.*, 2008). In an earlier study, it was reported that *P. putida* CP1 contained a mixture of *trans* and *cis* isomers of unsaturated fatty acids but increased in levels of *trans* isomer during exposure to mono-chlorophenol (Fakhruddin and Quilty, 2006). The isomerisation of the membrane lipids *cis* into *trans*-unsaturated fatty acid catalysed by *cis-trans* isomerase increases the membrane ordering and decreases membrane fluidity (Sardessai and Bhosle, 2002). Transcriptomic analysis revealed the expression of ppiB and slyD both encoding for peptidyl-prolyl *cis-trans* isomerase probably involved in this isomerisation. The isomerisation of *cis*-unsaturated fatty acid appears to be heme-catalyzed (Holtwick *et al.*, 1999). This mechanism involved a protein of the cytochrome c type. It was suggested that this protein was involved in the transport of heme over the cytoplasmic membrane. An observation in the mutant *P. putida* IH-2000 with disruption in the cytochrome c operon showed inability to isomerize *cis*-unsaturated fatty acids resulting in sensitivity to solvents (Hirayama *et al.*, 2006). Expression of membrane-stabilization proteins such as *cis-trans* isomerase for membrane stabilization enhances tolerance to stress. Proteins that were involved in maintaining bilayer rigidity were expressed in *P. putida* during exposure to toluene and other hydrophobic stressors (Bernal *et al.*, 2007). The induction of ccoO-1, ccoN-1, ccoP-1 and ccoQ-1 encoded for cytochrome c oxidase presumely are related to the isomerisation of *cis*-unsaturated fatty acid in *P. putida* CP1. The *cis-trans* isomerase (Cti) activity is constitutively present and is located in the periplasm; it requires neither ATP nor any other cofactor such as NADPH or glutathione (Heipieper *et al.*, 2003).

Stress contributes to a competitive ability and a vigorous growth phenotype and reinforces stress biology leading to increased levels of energy generation (Cray *et al.*, 2013). The modification of expression genes involved in oxidative phosphorylation pathways was observed in *P. putida* CP1 response to mono-chlorophenols with the majority of these genes localised in the cytoplasmic membrane. The genes were ndh (FAD-dependent pyridine nucleotide-disulphide

oxidoreductase), atpB/D/E/F/G/H (F0F1 ATP synthase), cyoB/cyoC/cyoD (cytochrome o ubiquinol oxidase), ccoO-1, ccoN-1, ccoP-1, ccoQ-1 (cytochrome c oxidase), nuoA/G/J/K/L (NADH dehydrogenase) and nuoCD (bifunctional NADH: ubiquinone oxidoreductase subunit C/D). The expression suggested that *P. putida* CP1 increased the production of energy as an adaptation mechanism to grow on mono-chlorophenol. A similar mechanism was shown by *P. fluorescens* to overcome the deleterious effects by an adaptation mechanism to produce ATP (Lemire *et al.,* 2010). The changes in expression of oxidative phoshorylation were observed in *P. putida* S12 during exposure to a stress induced substrate (Wierckx *et al.,* 2008). Cray *et al.,* (2013) suggested that species that are able to dominate microbial communities are exceptionally well equipped to resist the effect of multiple stress parameters and have high-efficiency energy-generation systems. Toxicants such as toluene cause cross-membrane proton gradients to break down and induce the up-regulation of genes involved in toluene degradation and/or the synthesis of solvent extrusion pumps and genes involved in increasing energy generation for these pumps to remove the compound from the cell (Bhaganna *et al.,* 2010). Furthermore, microbes that can achieve a greater net gain in energy than competing species will have an ecological advantage (Cray *et al.,* 2013a).

On the other hand the effects of all three mono-chlorophenols on the core step in aerobic metabolism (the Krebs cycle) showed that enzymes involved in metabolic feeding, glyceraldehyde-3-phosphate dehydrogenase gap-2 and triosephosphate isomerise, tpiA were induced. It is likely that the sensing of stress induced by mono-chlorophenols is translated into an increase in the synthesis of tricarboxylic acid cycle enzymes, which is compensated when the toxic compound can be used as a carbon source. Unlike when *P. putida* KT2440 was exposed to stress induced by chaotropic substrates, the genes involved in metabolic pathways were repressed (Dominguez-Cuevas *et al.,* 2006). Induction of genes involved in amino acid arginine and praline metabolism observed including argI (ornithine carbamoyltransferase), argJ (ornithine acetyltransferase) and arcA that encoded for arginine deiminase. All proteins encoded by these genes are located in the cytoplasm. The arginine deiminase pathway in *P. aeruginosa* is one of the major routes used by the cell to obtain energy under anaerobic conditions where it generates 1 mol of ATP from 1 mol of L-arginine. The induction of this pathway

in *P. putida* CP1 could be related to the activation of another source to generate the necessary energy to extrude mono-chlorophenols. The increase in amino acids in the response to mono-chlorophenols probably reflects the need for these amino acids in the synthesis of the new proteome found in cells exposed to mono-chlorophenols as suggested by Dominguez-Cuevas *et al.,* (2006). This hypothesis is supported by the induction of the elongation factors for protein (tsf, nusA and greA) in this study. Compatible-solute synthesis under stress induced by specific hydrocarbons is unique to the bacterium *P. putida.* The amino acid compatible solutes, betaine and other amino acids protect microbial enzymes against diverse hydrophobic stressors (e.g.benzene and hexane) (Bhaganna *et al.,* 2010). Evidence for increases in synthesis and transport of proline, glutamate and other amino acids under stress in *P. putida* has been published for toluene, xylene and di-chlorophenol (Segura *et al.,* 2005; Dominguez-Cuevas *et al.,* 2006). In addition, stress tends to enhance global protein synthesis due to the increased energy requirement. Growth of *P. putida* on phenol showed an increase in protein concentration involved in protein synthesis which is 30S ribosomal protein S1 (rpsA) and translation elongation factor G (fusA) (Hallsworth *et al.,* 2003).

Our finding showed an increased expression of fatty acid biosynthesis and metabolism genes by *P. putida* CP1 grown on mono-chlorophenols including fabD (malonyl CoA-acyl carrier protein transacylase) and PP4948 (acyl-CoA dehydrogenase domain-containing protein). Bacterial fatty acid biosynthesis required a three-carbon precursor, malonyl-coenzyme A (CoA), which is derived from acetyl-CoA by the action of acetyl-CoA carboxylase. The malonyl-CoA is transferred to the acyl carrier protein (ACP) by malonyl-CoA:ACP acyltransferase (FabD). Fab (fatty acid biosynthesis) pathway has been proposed to be the acyl donor for synthesis of acylated homoserine lactones (AHLs). The AHLs (or autoinducers) have received considerable attention in recent years since they are required for a regulatory phenomenon termed quorum sensing (Kutchma *et al.,* 1999). Lee *et al.,* (2012) described the insertion of fabD gene in *P. aeruginosa* with increased lipid content.

Genes associated with two-component systems are located in the cytoplasmic membrane. PhoQ encoding for integral membrane sensor signal transduction

histidine kinase was highly induced during growth on mono-chlorophenols. The involvements of two-component systems as a response to toxic substrate are rarely reported. Kivistik *et al.,* (2006) has demonstrated that ColRS two-component signal system regulates phenol tolerance of *P. putida* by adjusting permeability of the membrane to phenol. The importance of a two-component system highlighted the importance of a signalling mechanism in order to initiate the transcription of another gene response to mono-chlorophenols. It is an adaptive response to environmental changes and there is also evidence of participation of two-component systems in regulation of membrane permeability of bacteria.

Solvent-tolerant *P. putida* strains evolved several mechanisms to deal with toxic compounds including an active extrusion of a broad range of compounds through membrane-associated efflux pumps (Wierckx *et al.,* 2008). The efflux pump of resistance-nodulation-division (RND) family allows the flow in both cytoplasmic and outer membranes and is important for survival of the bacterium (Segura *et al.,* 1999). We observed the induction of PP0907 encoding for RND efflux membrane fusion proteins involved in the extrusion of toxic chemicals as a defense mechanism against the toxicity of mono-chlorophenols. The protein localization prediction showed the protein was located in the cytoplasmic membrane. An active efflux system extrudes the organic solvent from the membrane which will allow the survival of the bacterium. This mechanism allows the cell to slow down the rapid influx of solvents into the cytoplasmic membrane by reducing the accumulation rate of organic solvent in the membrane.

In *P. putida* CP1, seven genes were identified common to all mono-chlorophenols suggesting their involvement in the stress response (hslU, dnaK, htpG, grpE, groES, clpB and hfq). Some of the genes encoded for heatshock protein and functioned as chaperones which most probably increased the refolding of unfolded proteins. The analysis in this study reveals valuable information on the cellular responses to the presence of mono-chlorophenols and the presence of chaperones contribute to the stabilization of proteins affected by this organic solvent. Genes coding for production of chaperonins, heatshock proteins and other proteins involved in protein stabilization (e.g. DnaK, GroEL, GroES, GrpE, HtpG,

HtpX, IbpA and trigger protein) can be up-regulated by diverse hydrocarbons including benzene, toluene, xylene and hexane in both prokaryotic and eukaryotic species (Bhaganna *et al.,* 2010). Growth of *P. putida* on phenol showed an increase in protein concentration involve in protein stabilization heat shock protein htpG, dnaK, GroEL, lbpA and trigger protein (Hallsworth *et al.,* 2003). A similar finding was found when *P. putida* KT2440 was grown on aromatic compounds which induced a number of stress response genes including hslU, dnaK, htpG, grpE and groES (Dominguez-Cuevas *et al.,* 2006). A study conducted by Segura *et al.,* (2005) revealed GroES was among the stress response genes induced during exposure of *P. putida* DOT-T1E to toluene. In many microorganisms, groES prevents protein aggregation and assists in protein folding under different stress conditions. It has previously been reported that overexpression of groESL in *Clostridium acetobutylicum* results in an increase in butanol tolerance (Tomas *et al.,* 2003). In *P. aeruginosa* FRD1 proteins DnaK and GrpE associated with cellular stress responses, including chaperons and proteins involved in post-translational folding and stabilization showed increased abundance in biofilm grown cells (Patrauchan *et al.,* 2007). Overall in global transcriptomic analysis during growth of *P. putida* CP1, it is reasonable to conclude that the responses involved are mechanisms that may assist in regulation of biosynthesis of membrane components to maintain cell membrane intactness and membrane homeostasis during growth and division.

The exact role of flagellar genes in solvent-tolerant bacteria has not been widely studied, but it was shown that the flagellar genes in the mutant of *P. putida* S12 resulted in the decrease of membrane stability and solvent-sensitive phenotype. When *P. putida* cells are grown on toluene, xylene and 3-methylbenzene, they seem to use a strategy consisting of decreased motility and the inhibition of large portions of the basic metabolic machinery (Dominguez-Cuevas *et al.,* 2006). In another finding, exposure of *P. putida* to chaotropic stressors like LiCl, ethanol, urea, phenol, guanidine hydrochloride and benzyl alcohol showed repression of flagellin (FliC) which suggests that growth inhibition may reduce motility. Regulatory interactions between stress responses and flagellum biosynthesis have been established in several microbial species (Hallsworth *et al.,* 2003). We observed that stress caused by mono-chlorophenols led to the increase of motility

functions and the massive induction of pathways for the metabolism of unavailable carbon sources. The flagella assembly pathways are important for the cells motility. The assembly and functioning of molecular machines for motility consume large amounts of ATP. This strategy of expending energy in the pursuit of a better environment may reflect an autoaggregation response that forms part of mechanisms by which bacteria adapt to hostile environments. Our data suggested the relation of induction of energy metabolism through oxidative phosphorylation and flagella assembly pathways as a response to mono-chlorophenols. Both pathways are essential for survival and induction of energy metabolism could be a way to generate energy for stress endurance programs.

The induction of genes involved in flagellar assembly were only observed when *P. putida* CP1 autoaggregated on chlorophenols (100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol and 200 ppm 4-chlorophenol). However genes involved in flagellar assembly in each treatment were random including the degree of expression. The observation was probably due to the different motility responses showed by *P. putida* CP1 against various concentrations of the substrate.

The genetic response of *P. putida* CP1 following formation of aggregates on 200 ppm 4-chlorophenol were compared to planktonic cells grown on 50 ppm 4-chlorophenol. The formation of aggregates in 200 ppm 4-chlorophenol was strongly induced by a regulation sigma factor, a flagellar assembly pathway, two component systems and the LPS biosynthesis pathway.

The most highly induced gene in this comparative study was PP0807 encoding for sigma factor-54. Many of the sigma-54 dependent regulators in *P. putida* appear to belong to two-component systems and posses a domain that can be phosphorylated by a sensor-kinase protein in the N-terminal section. Sigma-54 has been described as a central regulator in many bacteria and has been linked to a multitude of cellular processes like nitrogen assimilation and important functional traits such as motility and biofilm formation. It has highly-represented and conserved genetic associations with genes that concern the transport and biosynthesis of the metabolic intermediates of exopolysaccharides, flagella, lipids, lipopolysaccharides, lipoproteins and peptidoglycan.

Comparative analysis on *P. putida* showed an association between sigma-54 to carbon metabolism and flagellar biosynthesis (Francke *et al.,* 2011). The comparative study showed that differentially regulated genes were mainly involved in carbohydrate and amino acid metabolism. Dominguez-Cuevas *et al.,* (2006) highlighted that activation of genes involved in metabolic processes is not the only effect of exposing *P. putida* cells to aromatic hydrocarbons, as these compounds also trigger a solvent tolerance response. Formation of aggregates influenced the expression of genes involved in carbohydrate metabolism mainly in the Krebs cycle and glycolysis. AcsA encodes for acetyl-coA synthetase. The *acsA* gene product expressed in *Escherichia coli* showed acyl-CoA synthetase activity toward butyric acid and CoA as substrates, with butyryl-CoA being synthesized (Hashimoto *et al.,* 2005). Proteins mainly involved in valine, leucine and isoleucine biosynthesis included ilvB, ilvH and leuA. The induction of this pathway suggested an increase in the production of propionyl-Coa leading to the production of succinate and pyruvate (Fonseca *et al.,* 2011).

The regulation of flagellar assembly genes was not observed in the growth of *P. putida* CP1 in 50 ppm 4-chlorophenol. This was further evidence for the role of flagellae in autoaggregation. Flagellar assembly pathway was activated during formation of aggregates in 200 ppm 4-chlorophenol including the flagellar basal body protein (flgA, flgB, flgC, flgG, flgH, flgI and flhA), flagellar biosynthesis protein fliQ, flagellar biosynthesis regulator flhF, flagellar MS-ring protein fliF and anti sigma28 factor flgM. Dasgupta *et al.,* (2003) explained a model of flagellar gene regulation in *P. aeruginosa. P. aeruginosa* and *P. putida* are 85% similar in their genomic sequences; it is likely that mechanisms are similar for both species (Nelson *et al.,* 2002). Flagellar synthesis is energetically costly and if the environmental conditions are not in favor of flagellar motility, suppression of flagella synthesis occurs. The importance of flagellae for cell aggregation and biofilm development has been well described (Pratt and Kolter, 1998; Klausen *et al.,* 2003). Flagellar gene expression is related to motility or flagella's ability to sense the environments or an attempt to migrate to favorable environments as a stress response. It is also possible that flagellar export apparatus is involved in exporting other proteins unrelated to flagellar assembly under environment stress. The mutations in the flagellar export machinery led to solvent sensitivity in *P.*

putida DOT-T1E (Segura *et al.,* 2004). This finding showed that the presence of intact flagellar machinery is critical for the solvent-tolerant response in this strain which was in agreement with the finding from *P. putida* S12 (Kieboom *et al.,* 2001).

Two-component systems were involved in the formation of 200 ppm 4-chlorophenol aggregates when compared to planktonic cells grown on 50 ppm 4-chlorophenol. Bacterial survival mechanisms for monitoring environmental parameters have evolved surface-exposed signal transduction systems, typically comprised of transmembrane proteins that channel the input from sensory modules to intracellular responses. These signaling systems include the chemotaxis receptors and histidine protein kinases together with their cognate response regulators (Mascher *et al.,* 2006). A gene, PP3663 encoded for GGDEF domain-containing protein have the diguanylate cyclase activity for the synthesis of second messenger bis-(2',5')-cyclic diguanylic acid (di-GMP). These domains were found to be involved in the regulation of biofilm formation (Ramos, 2004). Amador *et al*., (2010) has described the response regulator, CbrB (PP4696) as part of the CbrAB two-component system which affects chemotaxis, stress tolerance and biofilm development in *P. putida* KT2442. The transcriptomic profile of a *cbrB* mutant, lacking GGDEF domain has affected biofilm formation, exopolysaccharide biosynthesis or transport and genes implicated in flagellae. Another sigma54 specific, transcriptional regulator belongs to the Fis family and was expressed in the formation of aggregates. Fis regulates the flagellar movement of bacteria and investigations by Jakovleva *et al.,* (2012) indicated that this transcriptional regulator modulates *P. putida* biofilm formation and motility. In this study cheW associated with chemotaxis was up-regulated. It is part of signaling clusters which contain a receptor together with a histidine kinase CheA. The bacterial chemotaxis signal-transduction system functions to regulate the changes in the direction of rotation of the flagellar motors, which in turn affects the cellular swimming motility pattern (Vladimirov and Sourjik, 2009).

A gene encoding for lipopolysaccharide (LPS) biosynthesis lpxK tetraacyldisaccharide 4'-kinase was induced in *P. putida* CP1 during formation of aggregates on 200 ppm 4-chlorophenol. It has been described by Emptage *et al.,*

(2012) that the tetraacyldisaccharide 4′-kinase (lpxK) catalyzes the sixth step in the biosynthetic pathway of lipid A which play a role in 3-deoxy-D-manno-oct-2-ulosonic acid Kdo2-lipid A biosynthesis. In *E. coli*, LpxK has catalyzed the transfer of a phosphate group from ATP to the 4′-position of disaccharide-1-phosphate to form lipid IVA. LpxK is the only kinase of the pathway that acts upon a lipid substrate at the membrane. A study by Sampathkumar *et al.,* (2006) of *Campylobacter jejuni* biofilms indicated the upregulation of enzymes involved in modifications of the lipid, carbohydrate and protein components of the membrane including the upregulation of membrane-modification enzymes tetraacyldisaccharide 4'-kinase (lpxK).

In general, the aggregative response followed the order 3CP > 2CP> $4CP_{200}$ which may reflect the degree of toxicity of each of these chemicals. The comparative study showed seven genes unique to autoaggregation based on consistant expression changes. The genes included 4 up-regulated genes, PP1961 encoding for TetR Family transcriptional regulator, PP132 encoding for histidine kinase which is involved in signal transduction of a two-component system, PP4936 encoding for O-antigen polymerase and PP2934 encoding for dienelactone hydrolase which is the final enzyme involved in chlorophenol degradation.

An important link between the environment and the physiological state of bacteria is the regulation of the transcription of a large number of genes by global transcription factors (Martınez-Antonio and Collado-Vides 2003). TetR family members trigger a cell response to react to an external stimulus in the environment. In most cases, the adaptive responses are mediated by transcriptional regulators (Ramos *et al.,* 2005). The TetR family transcriptional regulators represent the third most frequently occurring transcriptional regulator family found in bacteria. TetR family proteins are also involved in various other important biological processes, such as biofilm formation, biosynthesis of antibiotics, catabolic pathways, multidrug resistance, nitrogen fixation, stress responses, and the pathogenicity of Gram-negative and Gram-positive bacteria. All the proteins belonging to the TetR family share a high degree of sequence similarity at the DNA-binding domain located at the N-terminal region (Yu *et al.,* 2010; Chattoraj *et al.,* 2011). Among the transcriptional regulators, the stress was

also sensed predominantly by members of the LysR family. Low temperature, urea, and benzoate had similar effects on the expression of LysR regulators that corresponded to positive correlations with the global transcriptional profile. Growth of *P. putida* at low pH, however, did not affect expression of the LysR regulators (Matilla *et al.,* 2011). One of the global regulators, Fis (factor for inversion stimulation), regulates positively the flagellar movement of *Pseudomonas putida* during colonization of plant roots (Jakovleva *et al.,* 2012). AlgR controls transcription of *algC*, encoding a dual-function enzyme necessary for both lipopolysaccharide (LPS) (Coyne Jr *et al.,* 1994) and alginate production (Yu *et al.,* 1997).

PP132 encoding for multi-sensor signal transduction histidine kinase was found to be up-regulated in the consistent changes of mono-chlorophenol to induce the formation of aggregates in *P. putida* CP1. Two-component pathways enable cells to sense and respond to stimuli by inducing changes in transcription. A previous study showed that a two-component regulatory system and the synthesis of the O-antigen lipopolysaccharides are important for *P. fluorescens* WCS365 for cell attachments (Hinsa *et al.,* 2003). Adaptive responses are controlled by two-component signal transduction systems enabling bacteria to respond to a large number of environmental signals (Kivistik *et al.,* 2006).

The third gene PP4936 encodes for O-antigen polymerase. This highlighted the importance of this gene to express the production of lipopolysaccharide for aggregate formation. Lipopolysaccharide (LPS) has been implicated in *P. aeruginosa* and *P. fluorescens* biofilm formation (Spiers and Rainey, 2005). However, Reva *et al.,* (2006) found that during the exposure of *P. putida* to urea, downregulation of genes for PP4936 suggests that there is an adaptive modification of the cell envelope fatty acids and lipopolysaccharides. Adhesiveness of *Pseudomonas* species has also been associated with the presence and composition of LPS (Williams and Fletcher, 1996).

The enzyme dienelactone-hydrolase is involved in the final steps in the degradation of chlorophenols following cycloisomerisation process. The conversion of cis- and trans-dienelactone by dienelactone hydrolase producing

maleylacetate which is readily converted to intermediates of the TCA cycle and therefore may be utilised for the production of biomass, energy, carbon dioxide and water, resulting in complete degradation (Schlömann *et al.,* 1993).

Tig encoding for trigger factor protein was repressed in this study. This gene involved in protein export and acts as a chaperone by maintaining the newly synthesized protein. Previous studies showed that many of the chaperons and post-translational modification proteins are induced during biofilm growth of *P. aeruginosa.* The biofilm growth in *P. aeruginosa* PAO1 and *P. aeruginosa* FRD1 have affected the abundance of chaperons and proteins involved in post-translational modification including tig gene (Patrauchan *et al.,* 2007).

4.4 Genotypic responses of *P. putida* CP1 following growth on fructose

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 of 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 recognizable 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 (Williams *et al.,* 2007). 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 transcriptomics studies 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).

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 localization of proteins encoded by the differentially regulated genes was mainly located in the cytoplasm and the cytoplasmic membrane.

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 hours. 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 involvement of two-component systems in formation of autoaggregation was a similar response observed in the case of mono-chlorophenols. The influence of outer membrane proteins has been identified as adhesins for bacteria cells which can be utilized 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 encoding for flagellar hook-basal body protein was among the top up-regulated genes in the study and has been validated with qRT-PCR. 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. Our microarray study also showed up-regulation of many flagellar genes with the formation of aggregates in the comparative study in 50ppm4CP vs 200ppm4CP. 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 colonization of potato and wheat roots and reduced cellular adhesion of *P. aeruginosa* to a polystyrene surface (Sauer and Camper, 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).

4.5 Common genes expressed in *P. putida* CP1 following growth on mono-chlorophenol and fructose

A common response was found during formation of aggregates on mono-chlorophenol and fructose. TetR family transcription regulator was found to be induced in both responses of *P. putida* CP1 grown on mono-chlorophenols and fructose as was PP4936 encoding for O-antigen polymerase. This similarity in regulation might propose that the TetR family transcription regulator controls the expression of PP4936 for the production of lipopolysaccharide for aggregate formation. The finding was supported with changes in the O-antigen profile from aggregated cells grown on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol, 200 ppm 4-chlorophenol and 0.5% (w/v) fructose. An additional band was observed on the gel between 55-35 kDa.

A role for the intact O-antigen of LPS in cell attachment in *P. putida* CP1 can be supported by another study. Dekkers *et al.,* (1998) have observed *P. fluorescens* WCS365 mutants lacking the O-antigen of LPS were defective for colonization and growth whereas one mutant (PCL1205) with a shorter O-antigen chain was defective in colonization ability. The O-specific region located on the very outer cell surface especially has an effect on surface properties. Wzy, an O-antigen polymerase located in the O-antigen biosynthetic locus *Francisella tularensis* has been characterised. The function is to catalyze the addition of newly synthesized O-antigen repeating units to a glycolipid consisting of lipid A, inner core polysaccharide, and one repeating unit of the O-polysaccharide (Kim *et al.,* 2010). Adhesiveness of *Pseudomonas* species is related to the presence and composition of LPS. Substantially reduced attachment to biotic and abiotic surfaces was observed in O-polysaccharide deficient *Pseudomonas* spp. and in *E. coli* strains with mutations in the LPS core biosynthesis genes *rfaG*, *rfaP*, and *galU* (Sauer and Camper, 2001).

The genes PP1961 (TetR family transcriptional regulator) and PP4936 (O-antigen polymerase) were validated by qRT-PCR. The transcription of the *rpoD* gene encoding the house keeping sigma70 factor was used as the housekeeping gene for qRT-PCR in this study. It has been identified as an ideal housekeeping gene for this task due to its stable expression levels in *P. putida* (Dominguez-Cuevas *et al.*, 2006) and *P. aeruginosa* (Savli *et al.*, 2003). The results confirmed those obtained with the microarrays, although differential expression values were in several cases higher than those delivered by the microarrays. The results obtained with the microarrays corresponded to the degree of expression change, mainly in the case of mono-chlorophenols; although the fold-change values were probably underestimated in some cases.

5.0 CONCLUSIONS AND FUTURE WORK

5 Conclusions and future work

Conclusions

When *Pseudomonas putida* CP1 was grown on 100 ppm 2-chlorophenol, 100 ppm 3-chlorophenol, 200 ppm 4-chlorophenol and 0.5% (w/v) fructose the organism formed clumps of cells or aggregates. The aggregation of cells of one species is also referred to as autoaggregation. Phenotypic and genotypic changes in the organism suggested that the aggregative response was a response to substrate stress. Phenotypic changes monitored during growth on these substrates showed changes in the cell surface together with the production of extracellular polymeric substances (EPS). EPS cements cells together and affords protection from adverse environmental conditions. When genotypic studies were carried out, aggregated cells showed the up-regulation of two genes in particular - PP1961 (TetR family transcriptional regulator) and PP4936 (O-antigen polymerase). Both of these genes are associated with stress responses and cell surface changes. These genes associated with autoaggregation in this environmental isolate may function as biomarkers for, or functional targets in, the maintenance of the phenotype. Given that the aggregated form of the bacterium has shown potential for use in bioaugmentation, an in-depth understanding of the phenomenon could enhance the use of this organism in biological wastewater treatment systems.

Future work

In this project, the growth of *P. putida* CP1 was investigated on chlorophenols and fructose when supplied as the sole source of carbon. The experiments were also carried out under a limited number of environmental conditions of temperature, pH and nutrients. Further work would investigate the growth of the bacterium under a wider range of environmental conditions and further analyses including the measurement of the chaotropicity of the substrate would be included.

P. putida CP1 was found to aggregate when grown on certain concentrations of fructose. The aggregative response was thought to be due to a deficiency in the metabolic capability of the organism. Future work will investigate the metabolic capability of the bacterium more closely using a metabolomics and/or proteomic approach.

The production of extracellular polymeric substances (EPS) is pivotal to cell aggregation. This study used biochemical methods to analyse the composition of the EPS. Such methods are lacking in accuracy and future work would investigate the composition of the EPS using more sophisticated approaches such as GC-MS analysis. It would also be useful to extend the microscopic analysis used in this study to quantify the EPS using confocal microscopy.

Surface changes in the cell are important in the study of aggregation. Changes in lipopolysaccharide (LPS) production were studied in this project using SDS-PAGE. A more in-depth analysis of the changes in the LPS using Western blotting would be useful.

The genes PP1961 and PP4936 were found to play a key role in aggregation. This would require further validation. Future work will investigate using knock-out gene expression at the mRNA level and a proteomic study using two-dimensional (2D) electrophoresis gel.

Genotypic studies in this project highlighted the role of flagellae in aggregation. Future work would further investigate this finding by focusing on the phenotype of the cell using scanning electron microscopy.

6.0 BIBLIOGRAPHY

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7.0 APPENDIX

7 Appendix

Research Products

Conference Proceedings:

- Aqma, W. S and Quilty, B, 2012, Fructose-Induced Cell Aggregation In *Pseudomonas putida* CP1. In Proceedings of the 5th International Conference on Sustainable Energy and Environmental Protection 5th to 8th June 2012 – Part II: Environment & Clean Technologies, pp.223-228. Edited by Abdul Ghani Olabi and Khaled Benyounis, Dublin City University, Ireland.

Oral presentations:

- Aqma, W. S and Quilty, B., 2010, "A study of the physiological response of *Pseudomonas putida* CP1 to toxic stress", International Conference on Environment (ICENV'10), Pulau Pinang, Malaysia.
- Aqma, W. S and Quilty, B, 2012, "Fructose-Induced Cell Aggregation In *Pseudomonas putida* CP1", International Conference on Sustainable Energy and Environmental Protection (SEEP2012), 5-8 June 2012, Dublin City University, Dublin, Ireland.

Poster presentations:

- Aqma, W.S and Quilty, B., 2009, ''Analysis of the aggregative behaviour of *Pseudomonas putida* CP1" School of Biotechnology Research Day, Dublin, Ireland, Dublin City University (DCU).
- Aqma, W.S and Quilty, B., 2010, ''Isolation of high quality RNA from aggregated cells of *Pseudomonas putida* CP1", School of Biotechnology Research Day, Dublin, Ireland, Dublin City University (DCU).
- Aqma, W.S and Quilty, B., 2010, "Isolation of high quality RNA from aggregated cells of *Pseudomonas putida* CP1", ENVIRON 2010 Conference, Limerick, Ireland, Limerick Institute of Technology.
- Aqma, W.S and Quilty, B., 2010, "Effect of mono-chlorophenols on surface hydrophobicity of *Pseudomonas putida* CP1", Irish Division of the

Society for General Microbiology Autumn: Insect-mediated microbial diseases of humans and animals; Current problems and future threats, Maynooth, Ireland, National University of Ireland (NUI).

- Aqma, W.S and Quilty, B., 2011, "The production of extracellular polymeric substances (EPS) By *Pseudomonas putida* CP1", School of Biotechnology Research Day, Dublin, Ireland, Dublin City University (DCU).
- Aqma, W.S and Quilty, B., 2012, "Growth of *Pseudomonas putida* on sugars", School of Biotechnology Research Day, Dublin, Ireland, Dublin City University (DCU).
- Aqma, W. S, Doolan, P. and Quilty, B., 2012, "Stress response of *Pseudomonas putida* CP1 to substrate stress", Microbial Stress: from Molecules to Systems, Belgirate (VB), Maggiore Lake, Italy.