An investigation of mono-chlorophenol degradation and the bioaugmentation of activated sludge by two *Pseudomonas putida* species.

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 hereby declare that this material, which I now submit for assessment on the program of study leading to the award of Ph D is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work

Signed Henry McLaughlin

I D No 97970441

Date 28-11-2006

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Abstract

The degradation of all three mono-chlorophenols by two *Pseudomonas putida* species, A(a) and CP1, was investigated *Pseudomonas putida* A(a) metabolised 4-chlorophenol to completion via the *meta*-cleavage pathway but the degradation of 2- and 3-chlorophenol was incomplete *Pseudomonas putida* CP1 degraded all three chlorophenol isomers to completion via the modified *ortho*-cleavage pathway

The genes for the key enzymes of the chlorophenol degradative pathways, catechol 2,3-dioxygenase of the *meta*-cleavage pathway from *P putida* A(a) and chlorocatechol 1,2-dioxygenase of the modified *ortho*-cleavage pathway from *P putida* CP1 were amplified by PCR and identified by nucleotide sequencing

Large plasmids were detected in both strains Southern hybridization to DIG-labelled probes confirmed the presence of the genes for chlorocatechol 2,3-dioxygenase on the 117 kb plasmid in A(a) and chlorocatechol 1,2-dioxygenase on the 110 kb plasmid in CP1

The bacteria were tagged by inserting the gene for green fluourescent protein (GFP), into the chromosomes of *P putida* A(a) and *P putida* CP1 using Tn*7* and Tn*5* based delivery vectors Tn*7* inserted specifically at a neutral chromosomal site in *P putida* A(a) and an unknown site in *P putida* CP1

Bioaugmentation of activated sludge resulted in the complete removal of 4-chlorophenol GFP was used as a visual marker to observe the location of the introduced strains, revealing that CP1 Tn*7*-*gfp* was located almost entirely within the sludge flocs while *P putida* A(a) Tn*7*-*gfp* was evenly distributed throughout the mixed liquor *P putida* CP1 Tn*7*-*gfp* survived in greater numbers than *P putida* A(a) Tn*7*-*gfp*

The addition of the bacteria had a protective effect on the indigenous baterial population however this effect was more pronounced with the addition of *P putida* CP1 Tn*7*-*gfp* The floc-forming ability of *P putida* CP1 Tn*7*-*gfp* may result in its enhanced survival leading to better degradation of 4-chlorophenol

Abbreviations

5-Cl-HMS	5-chloro-2-hydroxymuconic semialdehyde
ATP	Adenosine triphosphate
cfu	Colony forming unit
EDTA	Ethylenediaminetetraacetic acid
GFP	Green fluorescent protein
Gn	Gentamycin
MIC	Minimum inhibitory concentration
NADP	Nicotinamide adenine dinucleotide phosphate
PCR	Polymerase chain reaction
Tn	Transposon

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1.0 Introduction

1 1 Aromatic Pollutants in the environment.

Synthetic organic compounds are widely distributed in the environment due to their extensive production and use in almost all human activities Aromatic compounds are not foreign to nature-the benzene ring is a prevalent component of lignin in wood and is the second most common monomer in the environment However, modern industrial practices may result in the addition of various substitutions to the aromatic ring resulting in the creation of compounds which do not normally occur in the environment These compounds are termed xenobiotics Pollutant or xenobiotic compounds enter the natural environment either directly e g through the application of pesticides, chemical spillage or losses during production or indirectly e g through improper disposal techniques, contamination of aqueous run-off or by leachates (Hardman *et al*, 1993)

One group of man-made chemical pollutants are the chloroaromatics Chlorinated compounds are amongst the most extensively studied because of the highly publicised problems associated with 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (DDT), other pesticides and numerous industrial solvents Hence, chlorinated compounds serve as the basis for most of the information available on the biotransformation of synthetic compounds (Chaudhry and Chapalamadugu, 1991)

They persist in the environment due to their unusal substitution, their highly condensed aromatic ring and excessive molecular size (Atlas and Bartha, 1998) Chloroaromatics consist of a diverse range of chemicals such as chlorophenols, chlorobenzoic acids, chlorobenzenes, chlorobiphenyls, chloroanilines and chlorotoluenes Organohalogen compounds such as the chloroaromatics are placed on the "Black List" of the European Union Chemicals placed on this list tend to be highly toxic (Hardman *et al*, 1993)

Among the chloroaromatic compounds, the chlorophenols are one of the most extensively studied In industry, chlorophenols are obtained in large, industrial and commercial scales by chlorinating phenol or hydrolyzing chlorobenzenes (Czaplicka, 2004) Chlorophenols are common environmental contaminants originating from their use as wide-spectrum biocides in industry and agriculture Pulp bleaching and incineration of organic materials in the presence of chloride also give rise to chlorophenols This has resulted in soil and groundwater contamination by chlorophenols (Puhakka and Melin, 1996) Chlorophenols consist of a benzene ring, -OH group and atoms of chlorine Some of the properties of the main 19 chlorophenol compounds are shown in Table 1 In general, these compounds dissolve weakly in water and their water solubility decreases with increasing number of chlorine atoms in a molecule, thereby affecting their mobility and bioavailability The lipophilicity of chlorophenols increases with increasing chlorine substitutions, thereby increasing their tendency to bioaccumulate Increased water solubility and decreased lipophilicity makes the mono-chlorophenols particularly suitable for biodegradation studies

Information on the levels of hazardous waste quantities in Ireland is available in the National Waste Report for 2004 (EPA, 2005) and is shown in Table 2 As can be seen from this table, chloroaromatics which are found in chlorinated organic solvents, pesticides and PCB waste, constitute a large proportion of the chemical waste generated in Ireland The main sources of this organic waste are the chemical and pharmaceutical industries

1 2 Biodegradation.

Biodegradation involves the breakdown of organic compounds either through biotransformation into less complex metabolites or through mineralization into organic minerals, H_2O and CO_2 (aerobic) or CH_4 (anaerobic) (Singh and Ward, 2004) Complete mineralization of a compound is the most desirable of the processes as it generates carbon and energy for microbial growth and leads to the disappearance of the xenobiotic compound

Table 1 Selected Properties of chlorophenols (Czaplicka, 2004)

No	Compound	Formula	Molecular weight	Solubility g/l
1	2-Chlorophenol	C_6H_5ClO	128 56	28
2	3- Chlorophenol	C_6H_5ClO	128 56	26
3	4- Chlorophenol	C_6H_5ClO	128 56	27
4	2,3- Dichlorophenol	$C_6H_4Cl_2O$	163 00	Na
5	2,4-Dichlorophenol	$C_6H_4Cl_2O$	163 00	4 5
6	2,5-Dichlorophenol	$C_6H_4Cl_2O$	163 00	Na
7	2,6-Dichlorophenol	$C_6H_4Cl_2O$	163 00	Na
8	3,4-Dichlorophenol	$C_6H_4Cl_2O$	163 00	Na
9	3,5-Dichlorophenol	$C_6H_4Cl_2O$	163 00	Na
10	2,3,4-Trichlorophenol	$C_6H_3Cl_3O$	197 45	0 22
11	2,3,5-Trichlorophenol	$C_6H_3Cl_3O$	197 45	0 22
12	2,3,6-Trichlorophenol	$C_6H_3Cl_3O$	197 45	Na
13	2,4,5-Trichlorophenol	$C_6H_3Cl_3O$	197 45	0 948
14	2,4,6-Trichlorophenol	$C_6H_3Cl_3O$	197 45	0 434
15	3,4,5-Trichlorophenol	$C_6H_3Cl_3O$	197 45	Na
16	2,3,4,5-Tetrachlorophenol	$C_6H_2Cl_4O$	231 89	0 166
17	2,3,4,6-Tetra chlorophenol	$C_6H_2Cl_4O$	231 89	0 183
18	2,3,5,6-Tetrachlorophenol	$C_6H_2Cl_4O$	231 89	0 100
19	PCP	$C_6\ Cl_5OH$	266 4	0 014

Na = not available

Table 2 Hazardous waste generation in Ireland in 1995 by waste type (EPA, 2005)

Waste Type	**Total Quantity Generated** (tonnes)
Organic and chlorinated organic solvents	170,088
Oil waste	24,472
Paint/ink/varnish waste	7855
Pesticides and herbicides	600
PCB waste	19

When studies into the biodegradability of a particular compound are being carried out, consideration must be given not only to the concentration at which a particular compound is released into the environment, but to the chemical and toxicological properties of the chemical Since degradation of chemicals is generally microbially mediated, any structural feature of a chemical precluding or retarding its attack by microbes will lead to its accumulation in the environment, i e to recalcitrance of the particular compound (Leisinger, 1983) Recalcitrance is a term that is applied to any xenobiotic that is attacked slowly or not at all by microbial enzymes Studies carried out have shown that relatively small changes in chemical structure can appreciably alter a chemicals susceptibility to degradation The following molecular features generally increase the recalcitrance of a xenobiotic to biodegradation (Boethling, 1993)

1 Branching, especially tertiary (N) and quaternary (C)
2 Polymerisation
3 Presence of halogen, nitro, nitroso, aryl sulphonate, aryl ammo and azo substitutions (especially when there is a multiple substitution)
4 Aliphatic ether linkages
5 Polycyclic residues, especially with more than three fused rings
6 Heterocyclic residues

The ability of micro-organisms to degrade an organic chemical is governed not only by the chemical structure, but also by the environment in which it is found Many variables contribute to the ability of micro-organisms to degrade any particular compound The extent and rate of biodegradation depends on many factors including pH, temperature, oxygen, microbial population, degree of acclimation, accessibility of nutrients, chemical structure of the compound, cellular transport properties and chemical partitioning within the growth medium (Singh and Ward, 2004) Aspects of requirements for biodegradation are presented schematically in Figure 1

Biodegradation of recalcitrant compounds will only take place if the required environmental factors are favourable to the degradative bacteria Microorganisms will not grow sufficiently unless the required nutrients (macronutrients such as nitrogen and phosphorous and micronutrients such as essential trace elements and sometimes growth factors) are present at a suitable temperature/pH/salinity, in the presence of oxygen (for aerobic micro-organisms), in order for biodegradation to occur The concentration at which the chemical is found also can affect biodegradation Low substrate concentrations may result in the failure of the chemical to induce sufficient levels of enzyme activity with the result that degradation may occur more slowly than expected or not at all At high concentrations, biodegradation may also fail to take place due to the toxicity of the chemical to the microorganisms

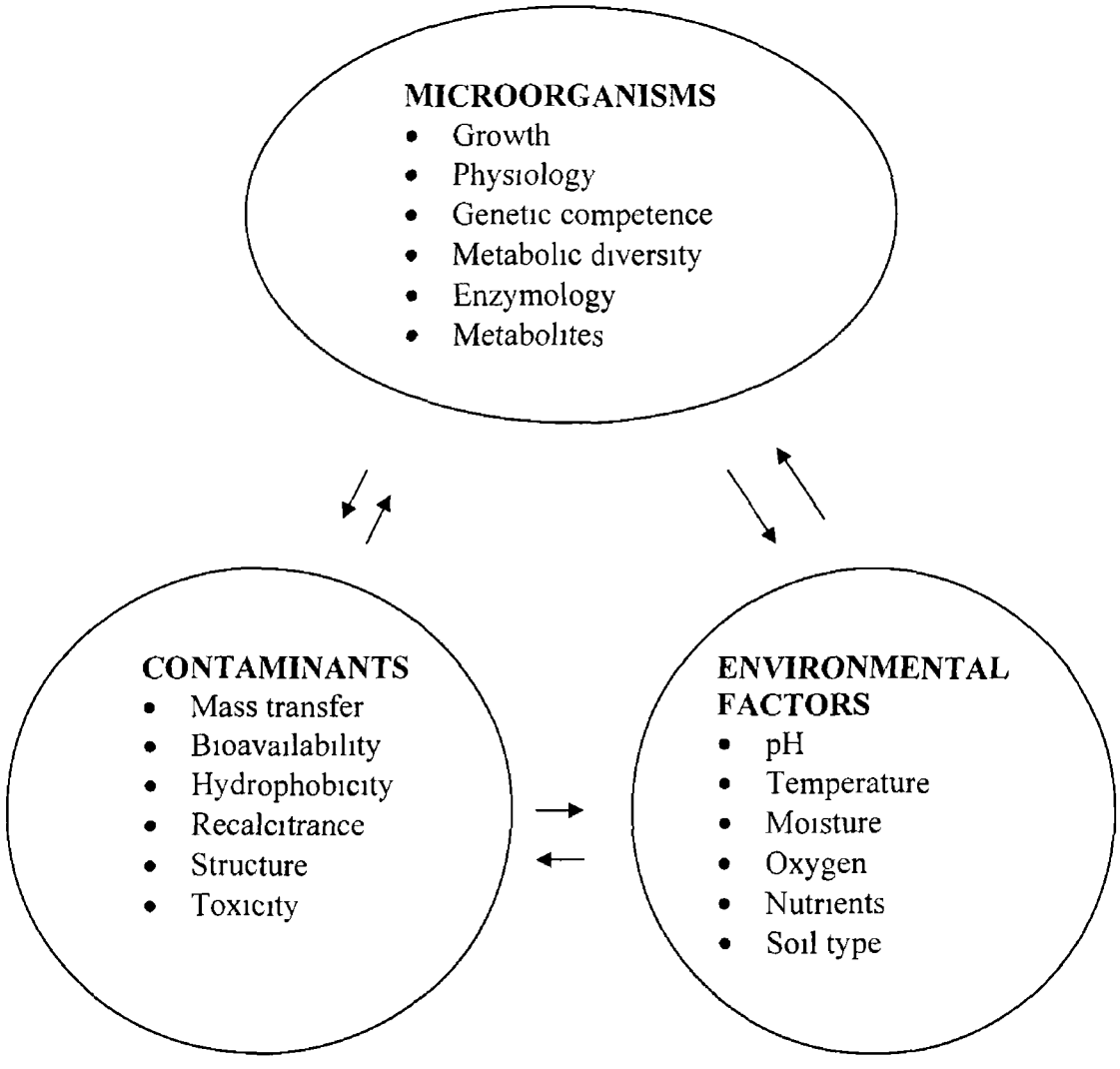

Figure 1 Variables contributing to the ability of micro-organisms to degrade xenobiotics (Singh and Ward, 2004)

1.3 Toxicity of chlorophenols to humans and microorganisms

All chlorinated phenols have been found to have possible endocrine-disrupting effects caused by interfering with the transport of thyroid hormones (van der Berg, 1990) Human exposure to chlorophenols is evident from the presence of chlorophenols in Canadian drinking water After PCP, 4-chlorophenol was among the chlorophenols most often identified in drinking water (Czaplicka, 2004)

Phenols are also toxic to individual cells and bacteria due to the fact that they uncouple the cells respiration (Escher *et al* , 1996) The basis of this effect is that phenols are fat soluble enabling them to traverse the cell membrane, coupled with the fact that they are weak acids, forming a phenolate ion when the hydrogen of the hydroxyl group dissociates from the parent compound The dissociation constant (pK_a) of the compound will depend on the type of substituents on the phenol For example, the pK_a of 4-chlorophenol is as high as 9 4, whereas that for 2, 4, 5-trichlorophenol is as low as 6 0 (Czaplicka, 2004) Thus a phenolate ion may take up a proton on the outside of the cell membrane, pass through the membrane and deposit the proton on the cytosolic side The phenolate ion may then diffuse out through the cell membrane to complete the cycle Phenols have also been found to form dimers which take part in the uncoupling cycle The effect of this uncoupling activity of phenols is that they become lethal to microorganisms over a certain concentration level with the level varying depending on the microorganism and phenol in question (Escher *et al* , 1996)

Another mechanism of chlorophenol toxicity is related to disruptions of the lipid bi-layer in microbial membranes Dissolution of chlorophenols in microbial cell membranes disturbs structural integrity and leads to permeabilization This causes inhibition of membrane-bound proteins such as Na^+/K^+-ATPase and the glucose transport system (Jensen, 1996)

1 4 Degradation of mono-chlorophenols.

Microorganisms use naturally occurring and synthetic chemicals for their growth For the acquisition of carbon and energy to sustain growth of bacteria and fungi, synthetic substrates that are extensively degraded are simply another substrate form which the population can obtain the necessary elements or energy for biosynthetic reactions Chloroaromatic compounds can be degraded under anaerobic and aerobic conditions Individual species rarely bring about complete mineralization of target compounds under anaerobic conditions, with the species carrying out the initial transformation coexisting with other anaerobes that carry out the later steps (Alexander, 1994) Microorganisms that mineralize aromatic compounds are widespread, while those that degrade chloroaromatics are rare However, aerobic degradation has been reported and many bacteria have been isolated that are capable of the complete degradation of chloroaromatic compounds (Hardman, 1991) The degradation process can be divided into three steps, formation of a ring cleavage substrate, subsequent cleavage of the aromatic ring and finally assimilation into central metabolism

1.4.1 Formation of a ring cleavage substrate.

Before the aromatic ring can be cleaved, microorganisms must invest energy in the form of reducing power to modify the ring Under aerobic conditions, this normally occurs by the formation of a ring cleavage substrate that contains at least two hydroxyl groups, usually situated *ortho* or *para* to each other (Pieper and Reineke, 2000) The initial step in the aerobic degradation of mono-chlorophenols is their transformation to chlorocatechols This step may be carried out by monohydroxylation or dihydroxylation Degradation of 2- and 3-chlorophenol generally results in the production of 3-chlorocatechol, while metabolism of 4-chlorophenol leads to the production of 4-chlorocatechol In general, compounds that already contain a hydroxyl group, such as phenols,salicylate and hydroxybenzoate, are monohydroxylated as the initial transformation(Haggblom, 1992, Mishra *et al* , 2001) The addition of a single hydroxyl group to an aromatic ring is typically performed by a monooxygenase enzyme, which

reduces two atoms of dioxygen to one hydroxyl group and one H_2O molecule by the concomitant oxidation of NAD(P)H (Häggblom 1992; Harayama *et al.*, 1992)

The hydroxyl groups can be viewed as a "handle" which enables enzymes to cleave the ring. By introducing these hydroxyl groups, a variety of starting compounds are transformed into a few common intermediates, such as chlorocatechol (Fig. 2) (Schlömann, 1994).

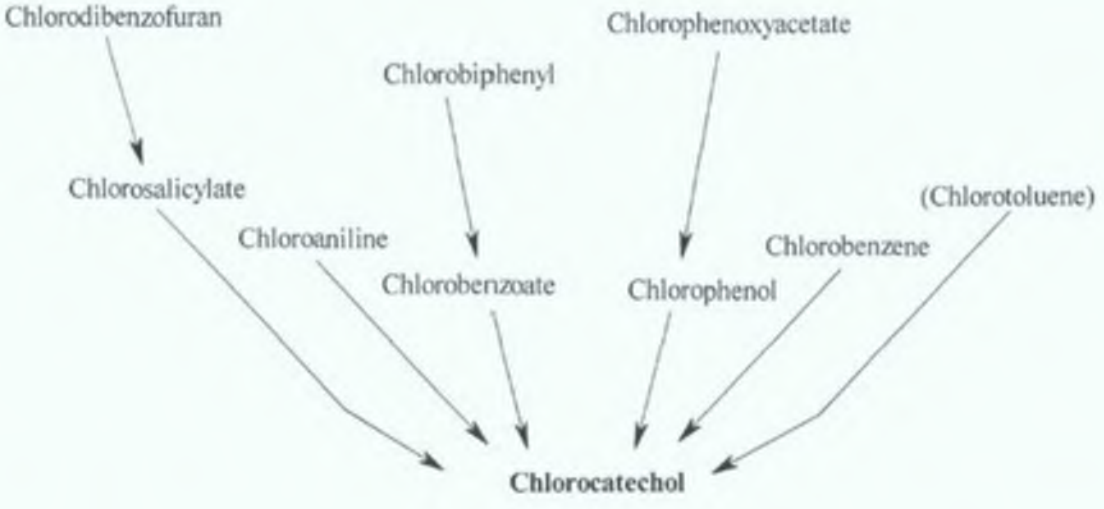

Figure 2: The central role of chlorocatechols in the oxidation of chlorinated aromatic compounds (Schlömann, 1994).

The next step in the degradation of these dihydroxylated intermediates is the breakage of the aromatic ring. This is performed by ring cleavage dioxygenases. There are two major mechanisms of ring cleavage and they are performed by enzymes that have evolved

independently of one another (Eltis and Bolin, 1996, Que and Ho, 1996) *Meta-* cleavage, or extradiol cleavage, occurs between two adjacent carbon atoms, one carrying a hydroxyl group and the other being unsubstituted, while *ortho-* cleavage (intradiol cleavage) occurs between adjacent carbon atoms substituted with hydroxyl groups Both types of pathways lead to intermediates of central metabolic routes, such as the tricarboxylic acid cycle (TCA cycle)

1 4 2 The *ortho-* cleavage pathway

Normal 1,2-dioxygenases involved in catechol metabolism exhibit low activities for halogenated substrates (Schmidt and Knackmuss, 1980) Compared with ordinary dioxygenases, enzymes with broad substrate specificities that have a high affinity for chloroaromatic substrates have been identified (Dorn and Knackmuss, 1978) Transformation of chlorocatechols via the "modified" *ortho-* cleavage pathway by chlorocatechol 1,2-dioxygenase yields chloromuconates (Fig 3) (Schlomann, 1994) The chlorocatechol is oxidised 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-chlorocatechol results in the production of 3-chloro-*cis,cis*-muconate

1 4.3 Cycloisomerisation.

Following ring cleavage via the modified *ortho-* pathway, the products produced undergo cycloisomeration as described by Schmidt and Knackmuss (1980) The chloro-substituted *cis,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 (Fig 3) 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

Both cycloisomerases catalyse similar reactions and differ with respect to their substrate specificities and product formation Cycloisomerase II has been shown to exhibit higher activities with substituted-muconates than with unsubstituted substrates (Schmidt and Knackmuss, 1980) Cycloisomerase I responsible for the conversion of *cis,cis*-muconate to muconolactone also converts 2-chloro-*cis,cis*-muconate to mixtures of 2- and 5-chloromuconolactone by carrying out both 1,4 and 3,6-cycloisomerisations of the substrate (Vollmer *et al*, 1994) and 3-chloro-*cis,cis*-muconate to form the bacteriotoxic protoanemonin (Blasco *et al*, 1995) Cycloisomerase I is unable to further metabolise 2- or 5-chloromuconolactone Cycloisomerase II however 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 step in the degradation of chlorophenols is 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

The genes for the modified *ortho*- pathway are generally located on catabolic plasmids (van der Meer *et al*, 1992) The chlorocatechol 1,2-dioxygenase and chlorocycloisomerase genes of these operons are significantly homologous to their counterparts in the ordinary *ortho*- cleavage pathway genes, while the relationship between dienelactone hydrolase and 3-oxoadipate enol-lactone hydrolase is more distant (Schlomann, 1994) Plasmids that contain the genes for chloroctaechol degradative pathways, the modified *ortho*- cleavage pathway, have been well investigated The structures of the corresponding operons are almost identical (Fig 4) The *clc* and *tcb* genes, for example, are organized identically while the *tfd* operon differs only in the absence of an open reading frame between *tfd*D and *tfd*E (Reineke, W, 1998) A comparison of the sequences shows a high identity between the corresponding enzymes

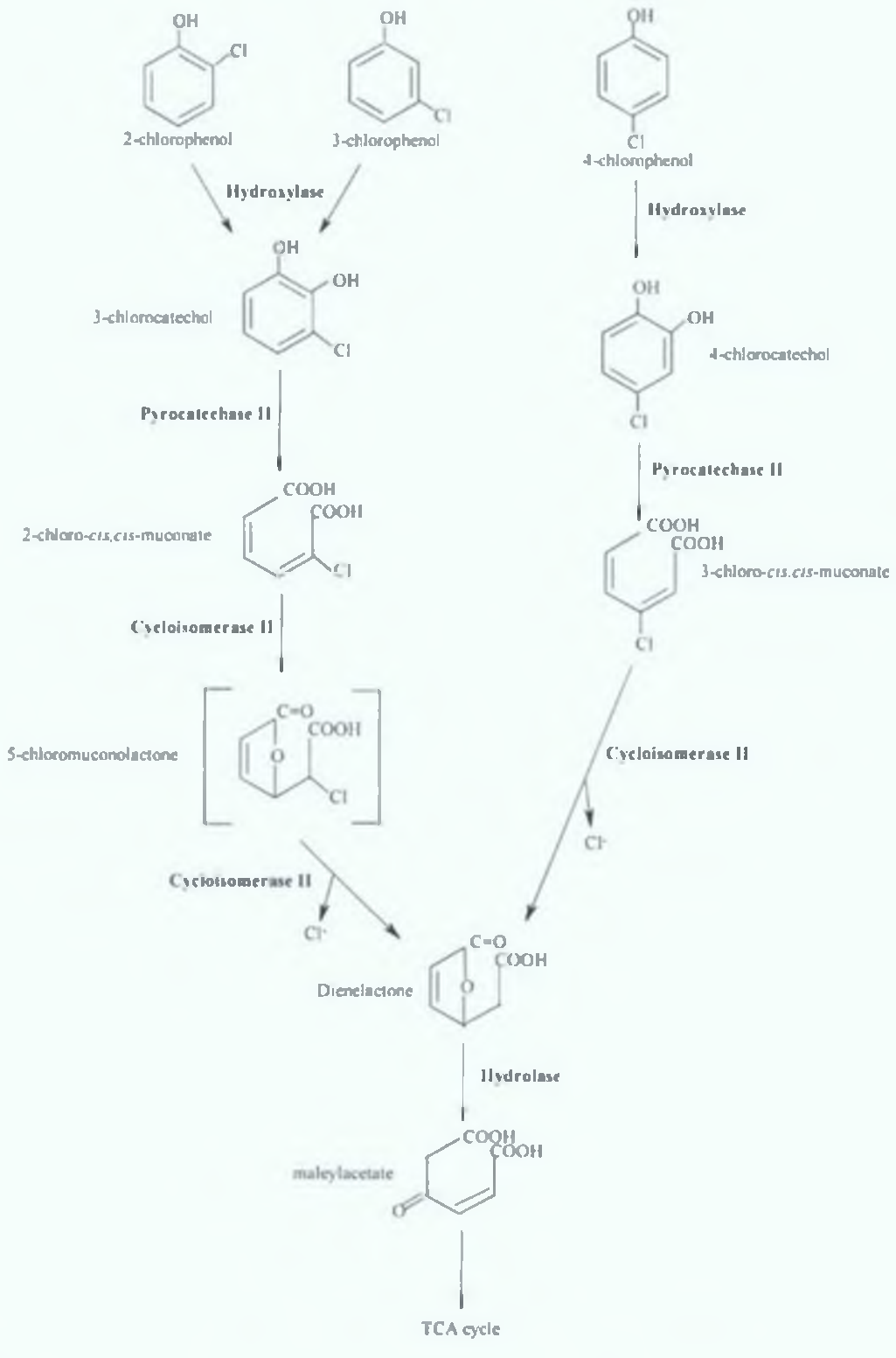

Figure 3: The degradation of mono-chlorophenols via the modified *ortho*- pathway (Knackmuss, 1981).

of the different bacteria. The gene products of the regulatory genes, TfdR, ClcR and TcbR are members of the LysR family and act as positive regulators.

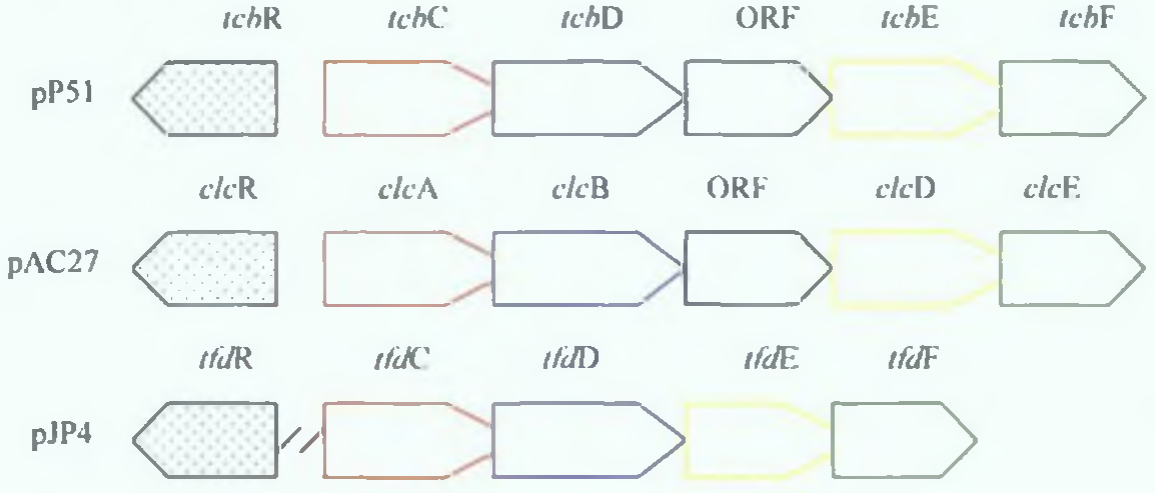

Figure 4: Operon structure of the modified *ortho*- pathway with adjacent regulatory genes (tcb, trichlorobenzene; clc, chlorocatechol; tfd, 2, 4-dichlorophenoxyacetic acid). Each arrow corresponds to a gene or open reading frame (ORF). Homologous genes are colored the same. The genes of the chlorocatechol 1,2-dioxygenases (*tcb*C, *clc*A, *tfd*C) are red, those of the chloromuconate cycloisomerases (*tcb*D, *clc*B, *tfd*D) are blue, those of the dienelactone hydrolases (*tcb*E, *clc*D, *tfd*E) are yellow, and those of the maleylacetate reductase (*tcb*F, *clc*E, *tfd*F) are colored green. The regulator genes *tcb*R, *clc*R, and *tfd*R are marked with dots. tfdR is located several kilobases upstream of *tfd*CDEF. Arrows without tips denote an overlap of the reading frames. (Reineke, 1998).

1 4 4 The *meta*- cleavage pathway

The alternative route to *ortho*- cleavage of chlorocatechols, is ring cleavage via the *meta*- cleavage pathway by the enzyme catechol 2,3-dioxygenase In general, *ortho*- cleavage is required to bring about complete mineralization of chlorophenols as *meta*- cleavage can result in dead-end pathways Dead-end pathways result in two ways, generation of a metabolite which may not be degraded further or generation of a metabolite which acts as a specific enzyme inhibitor The latter is called "lethal synthesis" and can result from competitive inhibition of an essential enzyme, removal of an essential metal co-factor from the enzyme's active site by a chelating agent, or from an irreversible form of inhibition (Bartels *et al* , 1984)

Generally *meta*- cleavage of 3-chlorocatechol, produced following hydroxylation of 2- and 3-chlorophenol, results in the inactivation of catechol 2,3-dioxygenase Two mechanisms have been proposed by which 3-chlorocatechol may interfere with the activity of catechol 2,3-dioxygenase The first mechanism is by 3-chlorocatechol itself, acting as a chelating compound (Klecka and Gibson, 1981) 3-chlorocatechol may chelate the iron co-factor required for the catabolic activity of catechol 2,3-dioxygenase causing a large build up of chlorocatechols Following chelation, enzyme activity may be reactivated by treatment with ferrous iron and a reducing agent Alternatively, *meta*- cleavage of 3-chlorocatechol can lead to the production of a highly reactive acyl chloride, 5-chloroformyl-2-hydroxypenta-2,4-dienoic acid, which has been proposed to act as a suicide compound, binding irreversibly to catechol 2,3-dioxygenase with a subsequent release of chloride, destruction of metabolic activity and an accumulation of chlorocatechols (Fig 5) (Bartels *et al*, 1984) In both cases the accumulated chlorocatechols then polymerise due to auto-oxidation resulting in the production of brown/black coloured chlorocatechol polymers

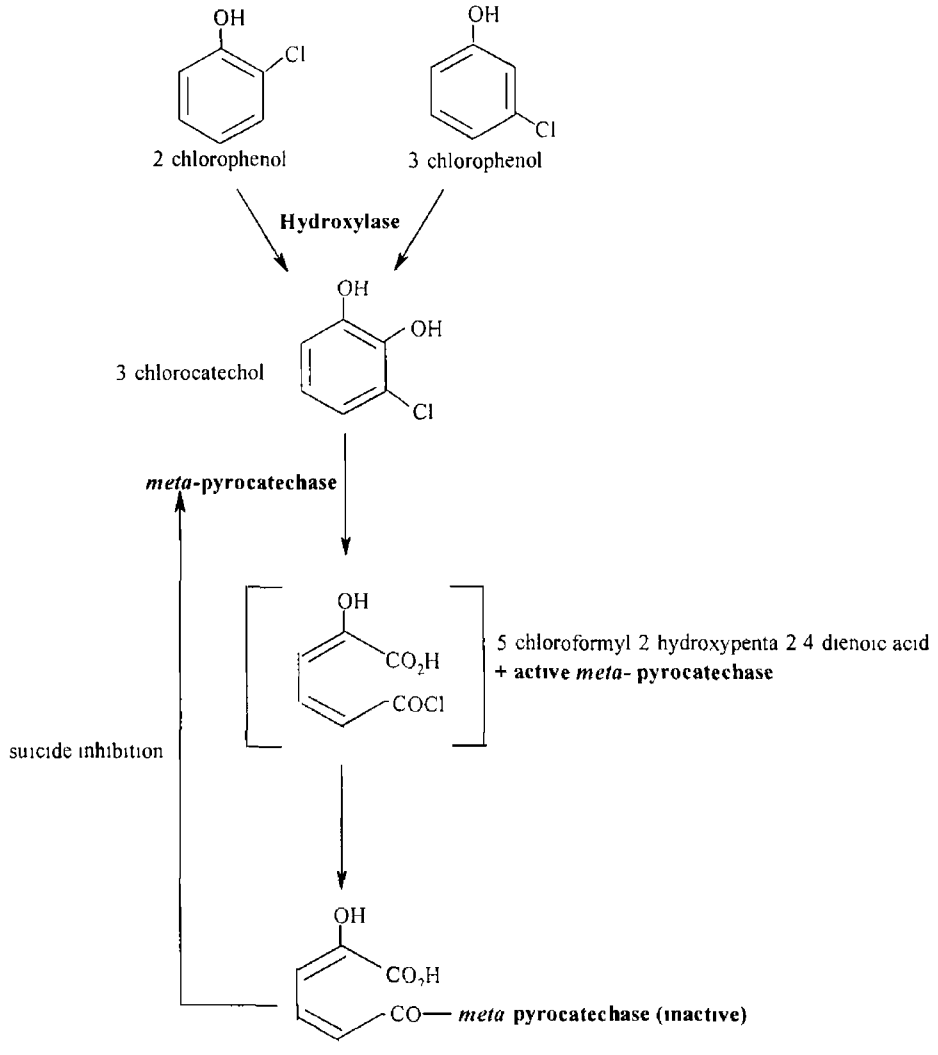

Figure 5 The *meta*- cleavage of 3-chlorocatechol (Bartels *et al* , 1984)

The complete degradation of chloro-aromatics via 3-chlorocatechol by a *meta*- cleavage pathway has been demonstrated in the organism *P putida* GJ31 (Mars *et al* , 1997) but is extremely rare The proposed pathway by which *P putida* GJ31 degrades 3-chlorocatechol is shown in Figure 6 *P putida* GJ31 appears to possess a catechol 2,3-dioxygenase which is able to convert 3-chlorocatechol to 2-hydroxy-*cis,cis*-muconic acid, an intermediate of the *meta*- cleavage of catechol, avoiding the rapid inactivation of the enzyme by the highly reactive acyl chloride usually associated with the *meta*- cleavage of catechol 2,3-dioxygenase 2-hydroxy-*cis,cis*-mucomc acid can then be degraded by the normal *meta*- cleavage pathway involved in the degradation of catechol

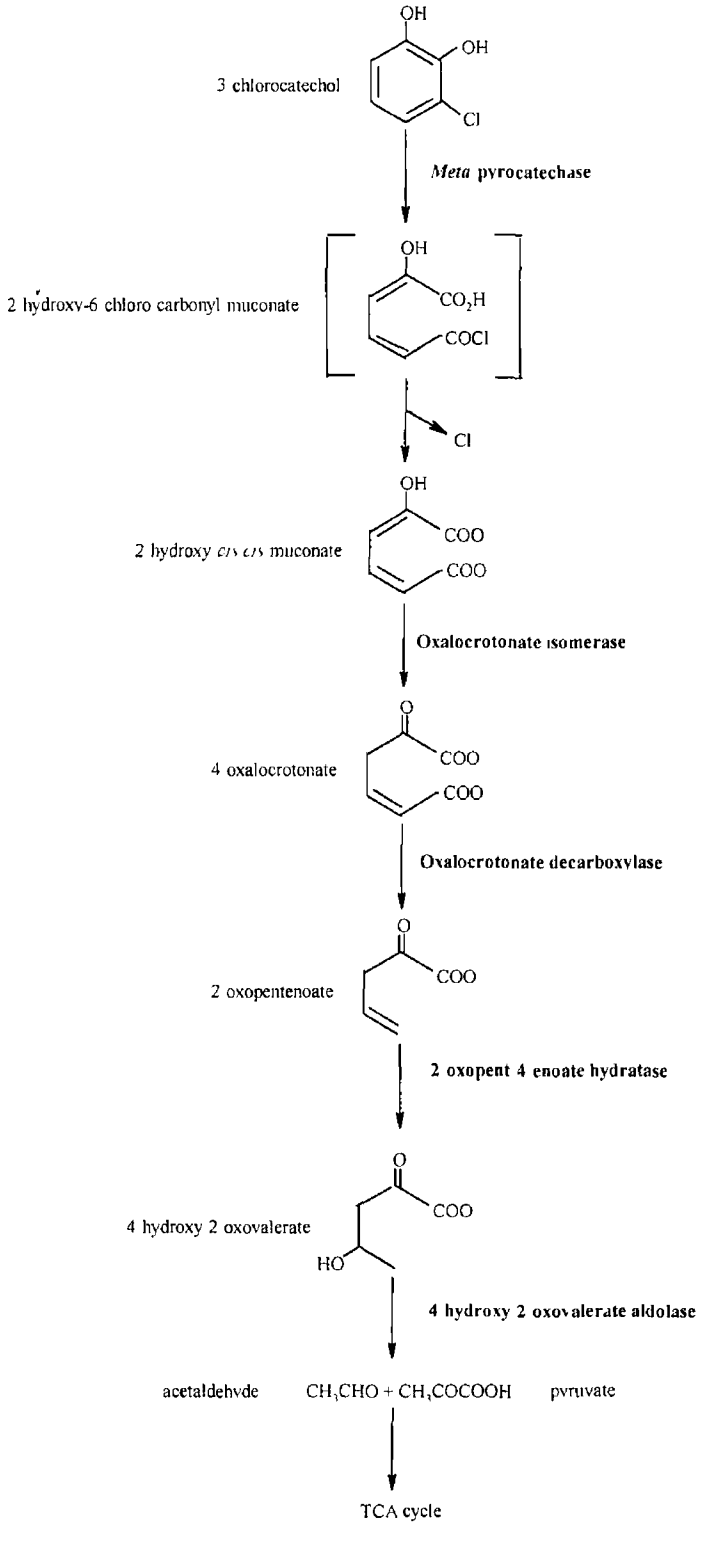

Figure 6 The *meta*- cleavage of 3-chlorocatechol by *Pseudomonas putida* GJ31 (Mars *et al* , 1997)

The *meta-* cleavage of 4-chlorocatechol, produced following metabolism of 4-chlorophenol, results in the production of a chlorinated aliphatic compound, 5-chloro-2-hydroxy-muconic semialdehyde (Weiser *et al*, 1994) which has been widely reported as being a dead-end metabolite (Reineke *et al*, 1982, Westmeier and Rehm, 1987) Accumulation of this compound, resulting from the fact that this product ordinarily cannot be further metabolised, causes an intense lime/yellow coloration of the culture medium (Knackmuss, 1981)

However recent reports have shown that further metabolism of 5-chloro-2-hydroxymuconic semialdehyde may occur, resulting in complete degradation of 4-chlorophenol via a *meta-* cleavage pathway (Sung Bae *et al*, 1996, Hollender *et al*, 1997) Although the exact mechanism by which 4-chlorophenol is degraded via 4-chlorocatechol by a *meta-* cleavage pathway is unclear, it is likely to be similar to a proposed mechanism as described by Arnesdorf and Focht (1995) for the degradation of 4-chlorobenzoate (Fig 7)

5-chloro-2-hydroxymuconic semialdehyde is transformed to 5-chloro-2-hydroxymuconic acid and 5-chloro-2-hydroxy-pent-2,4-dienoic acid (enol) by the enzymes 2-hydroxymuconic semialdehyde hydrolase, 4-oxalocrotonate isomerase and 4-oxalocrotonate decarboxylase 5-chloro-2-hydroxy-pent-2,4-dienoic acid is then transformed to 5-chloro-4-hydroxy-2-oxopentanoic acid which is further transformed to chloroacetic acid and pyruvic acid Chloroacetic acid may then be dehalogenated to form glycolate, which may be utilised along with pyruvic acid in the TCA cycle (McCullar *et al*, 1994) leading to the production of biomass, energy, carbon dioxide and water

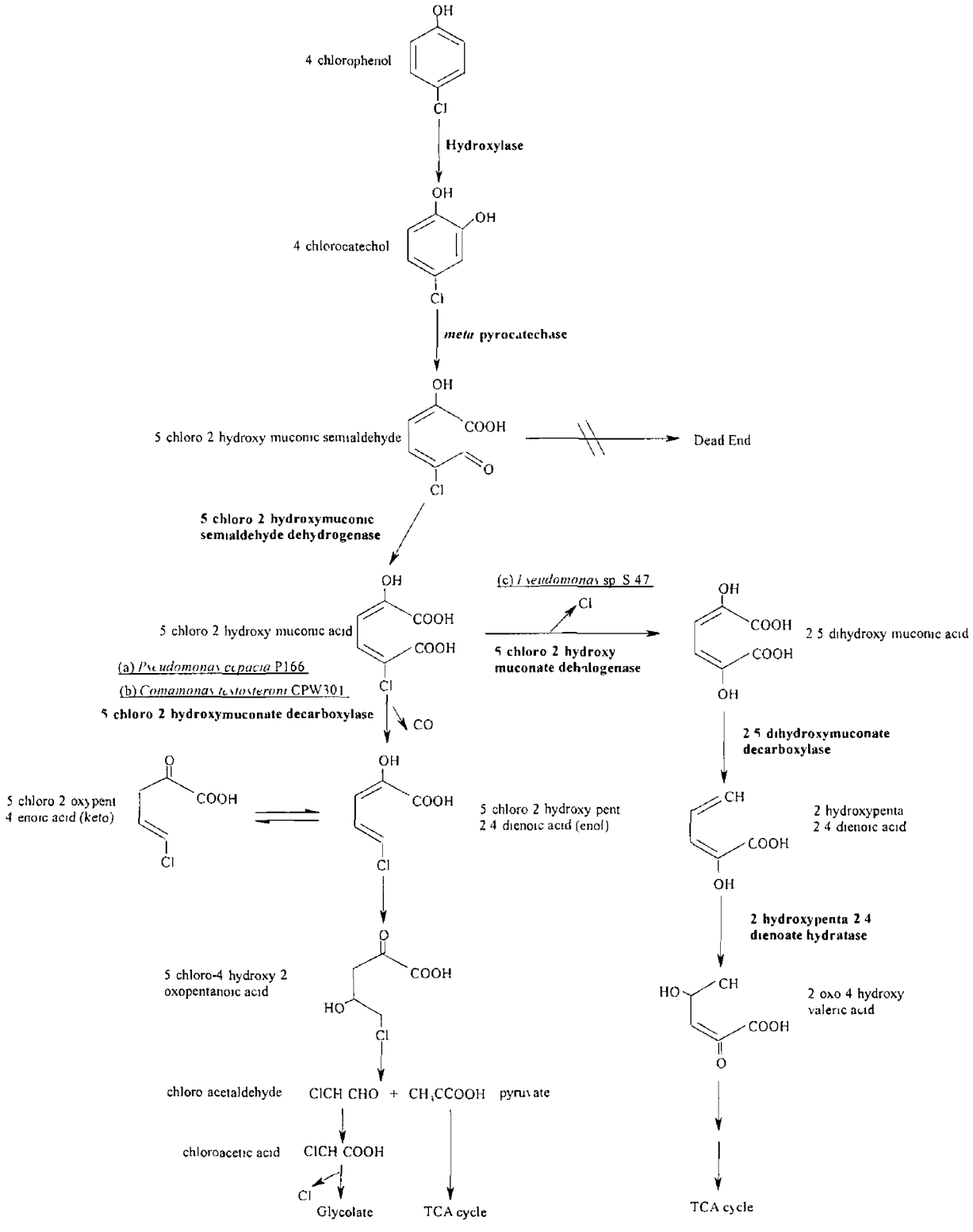

Figure 7 The *meta*- cleavage of 4-chlorophenol (a) Arnesdorf and Focht, 1995, (b) Hollender *et al* , 1997, (c) Seo *et al* 1998)

Similarly, Seo *et al* (1997 & 1998) reported the complete degradation of 4-chlorobenzoate, via 4-chlorocatechol, using a *meta-* cleavage pathway by a *Pseudomonad*, sp S-47 (Fig 7) They described how 4-chlorocatechol was initially transformed to 5-chloro-2-hydroxymuconic semialdehyde and then 5-chloro-2-hydroxymuconic acid *Pseudomonas* sp S-47 appeared to dehalogenate the substrate at this stage, converting 5-chloro-2-hydroxymuconic acid to 2-hydroxypenta-2,4-dienoic acid via 2,5-dihydroxy-muconic acid 2-hydroxypenta-2,4-dienoic acid is a normal metabolite following the degradation of catechol via the *meta-* pathway and was easily metabolised to form pyruvate and acetaldehyde which may be utilised by the TCA cycle (Seo *et al*, 1998)

Despite these examples, the *meta-* cleavage of chloroaromatics usually results in dead-end pathways, resulting in large accumulations of polychlorinated catechol polymers The destruction of *meta-* cleavage activity by the chlorocatechols and their inefficient oxidation by unmodified *ortho-* cleavage enzymes explains the instability and dark coloration of activated sludges suddenly loaded with chloroaromatics (Bartels *et al*, 1984) Consequently the degradation of chloroaromatics in wastewaters generally requires the action of the modified *ortho-* cleavage pathway for their efficient removal from wastewaters

Among the genes for *meta-* cleavage of aromatic compounds, the *xyl* genes, harbored by the TOL plasmid pWW0 of *P putida* strain PaW1, are the best characterized These genes form two functional units, the upper and the *meta-* operon A simplified model is shown in Figure 8 The upper operon,*xylCMABN* encodes three enzymes that oxidize toluene and xylene to benzoate and toluate, respectively The function of the protein encoded by *xylN* is unknown The promoter of the upper pathway genes, Pu, is regulated positively by the regulatory gene *xylR* This gene contains the information for a protein, which enhances transcription after the binding of an inducing molecule (toluene, m-xylene, and the respective benzoates) XylR belongs to the NtrC family of σ^{54} – dependent transcriptional regulators The lower-pathway or *meta* operon is composed of

13 genes that encode enzymes for the conversion of methylbenzoates to the central intermediates pyruvate, acetaldehyde and acetate via (methyl)catechols (Reineke, 1998)

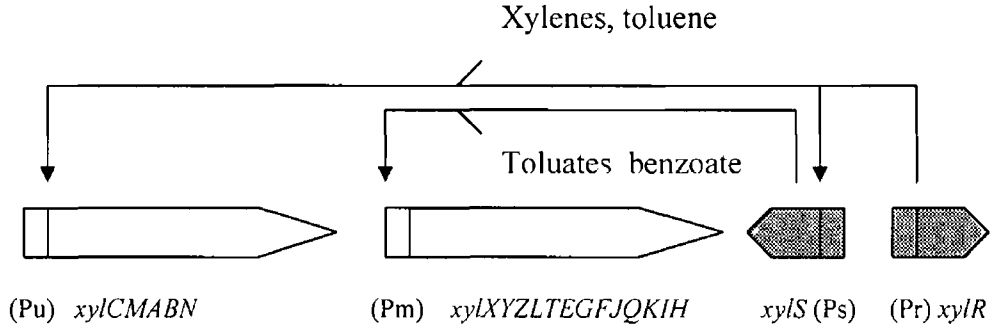

Figure 8 Simplified model of the regulation of *xyl* gene operons (*xyl*, xylene) The upper operon (*xyl*CMABN) codes xylene monooxygenase, benzylalcohol, and benzaldehyde dehydrogenase, while 13 enzymes are encoded by the *meta-* operon (*xyl*XYZLTEGFJQKIH), including toluate 1,2-dioxygenase (xylXYZ), toluate dihydrodiol dehydrogenase (XylL), and catechol 2,3-dioxygenase (XylE) The regulator genes are shown in gray The promoter regions are marked by small boxes The arrows indicate the induction by XylR and XylS regulatory proteins in concert with the respective aromatic effectors (Reineke, 1998)

1 5 Monitoring of bacterial inoculants during the bioremediation process

When a microbial inoculant is introduced into an environment it can be desirable to track its survival and dispersal There are techniques in existence that allow for such information to be gained The bioaugmentation strategy can then be changed based on this data to optimize the removal of the desired pollutant The fate of the introduced strains can be determined using biomarkers A marker gene is a DNA sequence which

has been introduced into an organism which confers a distinctive genotype or phenotype to enable monitoring in any given environment (MAREP, 1998. Janson et al. 1999). Desirable properties in a marker gene are that they should be easy to detect, specific, stable, suitable for in situ detection, non-toxic and able to confer the ability to monitor single cells to allow quantification. Additional benefits that may be present are the lack of a need to use exogenous substrate and the ability to give information about the metabolic state of the cell. No marker gene meets all of these criteria so the choice of biomarker depends on the system studied and the question to be addressed (Jansson, 1995). Another possibility is to combine different marker systems within a cell so that more information can be gained.

1.5.1 Tagging with marker genes.

The marker gene may be introduced into the target strains chromosome or on a plasmid. The introduction of the marker gene on a plasmid has the advantage that the sensitivity of detection of the target cells may be greater if the protein expression is higher due to a high copy number of the plasmid bearing the marker gene. However it is preferable to introduce the marker gene into the chromosome of the target strain. This eliminates any questions over plasmid stability under environmental conditions, which do not provide any selective pressure (e.g. with antibiotics) for the tagged strain to maintain the plasmid. Chromosomal marking also reduces the risk of transfer of the marker gene to other microorganisms in the environment.

Homologous recombination or transposition may be used to introduce a marker gene into the chromosome. Homologous recombination involves the use of a vector where the marker gene is flanked by sequences from a non-coding or non-essential region of the chromosome of the recipient. The delivery vector, e.g. a plasmid unable to replicate in the absence of the π protein (λ *pir*), and therefore in the target organism, once inside the cell facilitates the integration of the marker gene into the chromosome by homologous recombination. The marker gene is more commonly introduced into the target organism on a transposon. Typically a minitransposon is used. Minitransposons were developed by

de Lorenzo et al (1990) and Herrero et al (1990) to overcome the problems associated with natural transposons Non-essential sequences were eliminated leading to a substantial reduction in transposon size and the resulting recombinant minitransposons were much simpler to handle than natural transposons, inherited in a stable fashion and did not provoke DNA rearrangements In minitransposons, the transposase is not included in the part of the transposon that is integrated into the chromosome of the target organism Instead the transposase is located outside the transferred part and the entire construct is located on a plasmid that is not able to replicate in the recipient cell The marker gene must be transposed into the genome of the recipient when the plasmid is introduced or it is lost Since the transposase enzyme is not transferred, there is no risk of subsequent transposition events occurring after the marker gene has been introduced

1.5 1 1 Antibiotic resistance marker genes.

Antibiotic resistance markers are easy to detect and enumerate by plating onto selective media containing the antibiotic of choice and counting the colonies One of the most commonly used antibiotic resistance markers is the nptII gene coding for neomycin or kanamycin resistance Other antibiotic resistance markers used include chloramphenicol, tetracycline, streptomycin and ampicillin among others (Jernberg and Jansson, 2002, Jansson, 2003) The main advantage of using antibiotic resistance genes as biomarkers lies in the ease with which tagged bacteria can be identified and selected for on antibiotic-containing media Environmental samples can be plated on selective medium containing antibiotic so that only cells with the specific antibiotic resistance genes will grow (Jansson and de Bruijn, 1999) Before an antibiotic resistance marker is used, background expression from the indigenous microbial population in a particular environment should be taken into account to ensure specific detection of the resistance marker Strains that have been marked with an antibiotic resistance marker should not be released into the field since there are serious concerns about the spread of antibiotic resistance among pathogenic bacteria

1.5 1 2 Heavy Metal Resistance Markers

An alternative to antibiotic resistance as a selective biomarker is heavy metal resistance Heavy metal resistance genes exist for mercury, copper and cadmium amongst others (Daunert *et al*, 2000) The main disadvantage of using heavy metal resistance genes as markers is that the metals used are often toxic to humans Therefore care is required when preparing selective media and strict regulations must be adhered to when disposing of media An attractive option is the use of tellurite resistance Unlike many of the other heavy metals, tellurite is non-toxic at the concentrations used for selection, while remaining highly selective against the indigenous microflora Colonies expressing tellurite resistance are also easily visualised as black colonies on selective medium containing tellurite (Jansson and de Bruijn, 1999)

1.5 1 3Metabolic/chromogenic markers

Chromogenic marker genes possess the ability to cleave a specific substrate to produce a coloured product The most commonly used chromogenic marker genes are the *lac*ZY and *xyl*E genes (Atlas and Bartha, 1998) The *lacZ* gene encodes the β-galactosidase enzyme This enzyme cleaves the disaccharide lactose into glucose and galactose When cells expressing β-galactosidase are grown on medium containing the synthetic compound X-gal (5-bromo-4-chloro-3-indolyl- β-D-galactoside), the β-galactosidase cleaves the X-gal and produces an insoluble blue pigment and the colonies turn blue A disadvantage with the use of *lacZY* as a marker gene is that there can be high background from environmental microorganisms (Jansson, 2003) Another metabolic/chromogenic marker that has been used for monitoring bacteria is the *xyl*E gene which encodes the enzyme for catechol 2, 3-dioxygenase Catechol 2, 3-dioxygenase produces 2-hydroxymuconic semialdehyde from catechol Colonies expressing catechol 2, 3-dioxygenase turn yellow when sprayed with catechol (Jansson and de Bruijn, 1999)

1.5.1.4 Bioluminescent Markers

Bioluminescent luciferase markers are either eukaryotic firefly luciferase (*luc*) or prokaryotic luciferase (*lux*) in origin. Bacteria tagged with bioluminescent markers can be easily identified on the basis of light production by a variety of methods such as detection of light-emitting colonies or CCD camera detection (Jansson and de Bruijn, 1999). The firefly luciferase gene has been used as a marker for detection of specific bacteria from different environmental samples including freshwater, sediment and soil (Moller and Jansson, 1998). The *luc*-tagged cells do not produce light unless luciferin is added to the sample and therefore expression of luciferase does not interfere with cell growth. Both bacterial and eukaryotic luciferase enzymes are dependent on the energy reserves of the cell for light output: $FMNH_2$ for bacterial luciferase and ATP for eukaryotic luciferase. Luciferase activity therefore reflects the metabolic status of the tagged cells and the light output may vary with the growth stage of the cells and presence of growth substrate (Jansson, 2003).

1.5.1.5 Green fluorescent Protein (GFP)

The gene encoding green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* was originally cloned and expressed in *E. coli* and a range of other cell types (Chalfie et al., 1994). The wild-type GFP protein autofluoresces by emitting green light with a wavelength of 509 nm upon excitation with UV light in the 395-nm range. Organisms expressing that have expressed GFP include plants, mammalian cell lines, insects fungi and yeast (March et al., 2003). GFP has been used to study the dynamics and/or distribution of *gfp*-labelled bacteria in soils, water systems, rhizospheres, activated sludges, biofilms, and root nodules. The use of GFP as a marker has allowed researchers to detect added cells and determine more precise data about cell counts, survival and spatial location than has previously been possible (Errampalli *et al.*, 1999). A unique aspect of GFP fluorescence is that no cofactors or exogenous substrates are required for

fluorescence, with the exception of oxygen which is briefly necessary for autooxidation of the GFP protein to facilitate autofluorescence (Zimmer, 2002) The GFP is stable under a number of extreme conditions It persists at high temperatures (up to 65°C) and pH values (6-12) and is stable in the presence of many denaturants and proteases and can withstand paraformaldehyde treatment, thereby allowing detection of green fluorescence in fixed samples (Errampalli *et al* , 1999) The GFP protein, encoded by the *gfp* gene, has been shown to have a barrel-like structure consisting of 11 beta-sheets, with a central helix enclosing the chromophore (Ormo *et al* , 1996, Yang *et al* , 1996) Various mutant derivatives of GFP have been created with shifted excitation or emission spectra for enhanced fluorescence properties (Table 3) Furthermore, GFP is available in many different colors such as red, cyan, yellow, and magenta For example, one of the mutants, P4, is a GFP variant with the same excitation maximum as wild-type GFP, but with a blue shifted emission maximum, resulting in blue fluorescent protein (BFP) (Jansson and de Bruijn, 1999) In particular, many of the "red-shifted" GFP variants, having the excitation wavelength shifted toward a higher wavelength, have been found useful owing to the increased solubility of GFP protein and enhanced fluorescence intensity in bacteria In addition, the longer wavelength of fluorescence is more compatible with common filters used for fluorescence microscopy (Jansson and de Bruijn, 1999) and the effect of photobleaching at these longer wavelengths is lower than at 395 nm (Errampalli *et al* , 1999) Single cell detection of bacteria labelled chromosomally with one copy of red shifted GFP is possible using epifluorescence microscopy (Cormack *et al*, 1996) Unstable variants have also been created to enable study of transient gene expression (Andersen *et al* , 1998)

Because of the stability of GFP once it is formed, and the fact that no co-factors are needed for expression of the marker phenotype once fluorescence is established, the protein is ideal for microbial ecology applications as even starved cells may be detected (Backman *et al* , 2004, Unge *et al* , 1999)

Table 3 A selection of GFP mutants used as markers or reporters for bacteria (Tombolini and Jansson, 1998, Andersen *et al*, 1998, Crameri *et al*, (1996))

GFP mutant designation	Mutation	Excitation maximum, nm	Emission maximum, nm
Wild –type		395 (475)[a]	508
P11	Ile-167-Thr	471 (396)[a]	502 (507)
P4	Tyr-66-His	382	448
S65T	Ser-65-Thr	489	511
RSG4	Phe-64-Met	490	505
	Ser-65-Gly		
	Gln-69-Leu		
Mut 1	Phe-64-Leu	488	507
	Ser-65-Thr		
Mut 2	Ser-65-Thr	481	507
	Val-68-Leu		
Mut 3	Ser-65-Gly	501	511
	Ser-72-Ala		
Mut 3*	Ser-2-Arg	501	511
	Ser-65-Gly		
	Ser-72-Ala		
GFP uv	Phe-99-Ser	360-400	508
	Met-153-Thr		
	Val-163-Ala		
GFP5	Val-163-Ala	395, 473[b]	509
	Iso-167-Thr		
	Ser175-Gly		

[a] The value in parentheses is a minor peak

[b] The excitation intensity is similar at both wavelengths

However, GFP does have some potential drawbacks as a marker The chromophore forms slowly after translation, the protein cannot be used in completely anaerobic environments due to the oxygen requirement for chromophore formation and it can be difficult to distinguish GFP fluorescence from background fluorescence (Zimmer, 2002)

GFP fluorescence may be detected by a variety of methods Colonies expressing the phenotype may be distinguished directly with a lamp emitting light in the near-UV range Microcolonies of bacteria expressing GFP, for example colonizing a seed surface, may be studied using a stereomicroscope equipped with a UV-lamp (Tombolini *et al*, 1999, Unge and Jansson, 2001) Single cells may be detected by epifluorescence microscopy or confocal scanning laser microscopy, the latter technique gives very high-resolution images (Olofsson *et al*, 1998) Another technique for detection of single *gfp*-tagged cells is by flow cytometry (Backman *et al*, 2004, Unge *et al*, 1999) In this approach, the cells are passed between a laser beam and a detector, one by one, and a variety of parameters may be measured such as fluorescence intensity, cell size and cell shape Thousands of cells can be analyzed per second, and thus the method is very efficient Real-time PCR has also been used for quantification of GFP-tagged cells (Wang et al , 2004)

1 6 Activated Sludge

Municipal sewage treatment plants are an important component of wastewater infrastructure In addition to domestic sewage, effluents from industrial facilities are sometimes also discharged into municipal sewage treatment plants for (further) treatment before being released into the environment, though many industrial facilities are equipped with their own wastewater treatment processes Wastewater treatment may be divided into three main stages or phases Primary treatment refers to the removal of insoluble matters such as grit, grease or scum from water by screening and/or sedimentation Secondary treatment is employed to remove soluble organic matters that are oxygen demanding, usually by the action of microorganisms, especially bacteria Tertiary treatment is designed to further remove suspended solids and dissolved organic and/or inorganic materials from the effluent from secondary treatment Secondary treatment of

wastewater may take several forms such as the trickling filter However the activated sludge process is the most widely used The activated sludge contains a consortium of biological organisms, which usually include bacteria, protozoa, and rotifers, of which bacteria form the major community (Ren, 2004)

The activated sludge process consists of an aerobic treatment that oxidises organic matter to CO_2, H_2O, NH_4, and new cell biomass The microbial cells form flocs, allowing a straightforward separation of the newly formed microbial biomass and treated effluent in a clarification tank A conventional activated sludge process is shown in Figure 9

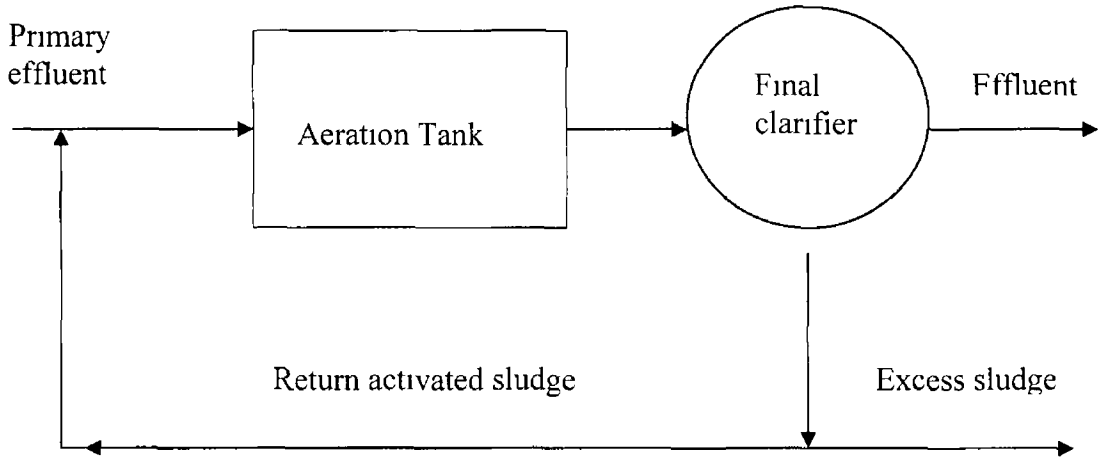

Figure 9 Conventional activated sludge system

Aerobic oxidation of organic matter is carried out in the aeration tank Primary effluent is introduced and mixed with the return activated sludge to form the mixed liquor Air is provided using diffused or mechanical aeration One of the most important characteristics of the activated sludge process is the recycling of a large proportion of the biomass, making the mean cell residence time (i e sludge age) much greater than the hydraulic retention time This helps maintain a large number of microorganisms that effectively

oxidise organic compounds in a relatively short time The sedimentation tank is used for the sedimentation of microbial flocs produced during the oxidation phase in the aeration tank A portion of the sludge in the clarifier is recycled back into the aeration tank and the remainder is "wasted" to maintain a proper food-to-microorganism ratio A low food-to-microorganism ratio means that the microorganisms in the aeration tank are starved, leading to a more efficient wastewater treatment (Bitton, 1999a)

When encountered during the biological treatment of industrial and domestic wastewaters, toxic inhibition by xenobiotics may be a major problem There are 2 concerns over the fate of organic toxicants in wastewater treatment plants

1 Biodegradation of organic toxicants in wastewater treatment plants It is desireable that xenobiotics be mineralized to CO_2 or, at least, to less toxic metabolites
2 Toxicity of xenobiotics to wastewater treatment organisms There can be subsequent reduction in the removal of biogenic organic compounds (i e , lower BOD reduction) or inhibition of nitrification and methane production (Bitton, 1999b)

1 7 Flocculation and its importance during bioaugmentation.

Aerobic wastewater treatment relies heavily on the ability of microorganisms to aggregate, allowing a straight forward separation of the formed microbial biomass and the effluent in the final settling tank The activated sludge process is operated as a continuous bioreactor with feedback of the biocatalyst, which ensures rapid oxidation of pollutants present in the influent and also stabilises the system against variations in in influent composition Process conditions are regulated and cell growth is minimised in order to obtain flocculation and a highly clarified effluent (Van Limbergen *et al* , 1999) The formation of well settling activated sludge flocs is based on the ability of the microbial community to aggregate Bacteria, particularly the Gram-negative bacteria, constitute the major component of sludge flocs They are responsible for the oxidation of

organic matter and nutrient transformations and produce polysaccharides and other polymeric materials that aid in the flocculation of microbial biomass (Bitton, 1999) For bioaugmentation of activated sludge, the inoculated strains should integrate into the flocs to avoid washout from the reactor (Van Limbergen *et al* , 1999) Furthermore, the flocs may form a protective area for the bacteria against predatory protozoa, which feed mostly on free suspended microorganisms and cells which can integrate into the flocs may benefit from enhanced survival (McClure et al , 1989, Bossier and Verstraete 1996) Protozoa are not able to reach the bacteria which are located in the inner part of a floc Therefore, the selective pressure exerted on activated sludge micro-organisms by predatory protozoa makes floc formation more likely It is likely that flocculation occurs as a result of predation and not due to bacteria sensing the presence of protozoa (Bossier and Verstraete, 1996)

Another theory proposed for the flocculation of micro-organisms is aggregation in response to physical stress such as unfavourable environmental growth conditions or a response to chemical stress such as toxic shock loading of chemicals Micro-organisms growing in the middle of aggregates may be sheltered from such stressful conditions (Bossier and Verstraete, 1996)

Flocculation of activated sludge occurs in a three stage process (Verstraete and van Vaerenbergh, 1986) Firstly ionic bridges formed by polyvalent cations such as Fe^{3+} and Ca^{2+} bind individual bacterial cells Next, gluey substances, consisting of polysaccharides and DNA/RNA from dead cells, make up a matrix in which cells are embedded and by which they stick to one another Finally, the matrix of cells attach themselves to filamentous micro-organisms (sometimes fungi, but most often bacteria) forming the flocs found in activated sludge

For inoculation, selection of strains that flocculate or integrate into the sludge flocs stand a better chance of survival and persistence during bioaugmentation of activated sludge

1 8 Chloroaromatic-degrading microorganisms.

Degradation of chloroaromatic compounds has been demonstrated in a wide variety of microorganisms Degradation of mono-chlorophenols has been shown to occur using both pure and mixed cultures The biodegradation of chloroaromatics has been mostly studied using pure strains This is due to the tendency of microbiologists to work with isolated pure strains and the ease with which information may be obtained on biochemical, physiological, genetic and molecular mechanisms in such pure strains

Many *Pseudomonas* strains have been shown to degrade chloroaromatic compounds as their sole source of carbon via the *meta-* and *ortho*-cleavage pathways The best known chloroaromatic-degrading strain is *Pseudomonas* sp B13 It was initially isolated for the degradation of 3-chlorobenzoate and has been shown to be capable of the degradation of 4-chlorophenol as sole carbon source and the cometabolism of 2- and 3-chlorophenol (Knackmuss and Hellwig, 1978) Degradation takes place via the modified *ortho-* pathway which is generally required for complete degradation This *ortho-* cleavage ability was transferred to a strain of *Alcaligenes*, sp A-7 possessing a broad spectrum phenol hydroxylase, resulting in a transconjugant strain of *Alcaligenes*, sp A 7-2, which was capable of degrading 2- and 4-chlorophenol and the cometabolism of 3-chlorophenol (Schwein and Schmidt, 1982)

A strain of *P putida*, CLB250, has been shown to be capable of the degradation of 2-chlorobenzoate as sole carbon source, with stoichiometric releases of chloride using the *ortho-* cleavage pathway (Engesser and Schulte, 1989) *Pseudomonas pickettii* LD1 had the ability to completely degrade 2-, 3- and 4-chlorophenol as sole carbon source Degradation of the chlorophenol isomers was accompanied by a stoichiometric release of chloride and the absence of any chlorocatechol accumulations in the culture medium suggesting that metabolism was via the *ortho-* cleavage pathway (Fava *et al* , 1995)

Pseudomonas strains capable of the degradation of chloroaromatics via the *meta-* cleavage pathway have also been isolated Spain and Gibson (1988) showed the

transformation of mono-chlorophenols to their corresponding chlorocatechols by *P putida* F1 However, there was an accumulation of chlorocatechols during ring cleavage via the *meta*- cleavage pathway *P putida* GJ31 has been shown to be capable of growth on toluene and chlorobenzene simultaneously and possesses a *meta*- cleavage pathway capable of the complete degradation of 3-chlorobenzoate via the intermediate 3-chlorocatechol (Mars *et al*, 1997) *Pseudomonas cepacia* P166 was capable of the degradation of mono-chlorobiphenyls via the *meta*- cleavage pathway (Arnesdorf and Focht 1994) Seo *et al* (1997) demonstrated the degradation of 4-chlorobenzoate via a *meta*- cleavage pathway by *Pseudomonas* sp S-47 Degradation of 2- and 3-chlorobiphenyl resulted in a dead-end pathway, while complete degradation of 4-chlorobiphenyl was reported When a strain of *Pseudomonas testosteroni* was immobilized, it was able to degrade low concentrations of 3- and 4-chlorophenol to completion (Lu *et al*, 1996)

Degradation of chloroaromatics has been reported in other genus apart from *Pseudomonas Arthrobacter chlorophenolicus* A6 could degrade 4-chlorophenol and other *p*-substituted phenols such as 4-nitrophenol and 4-bromophenol via hydroxyquinol (Westerberg *et al*, 2000) The complete degradation of 4-chlorophenol via a *meta*-cleavage pathway by *Comamonas testosteroni* CPW301 was demonstrated by Sung Bae *et al* (1996) and by *Comamonas testosteroni* JH5 (Hollender *et al*, 1997) A strain of *Rhodococcus opacus*, GM-14, was capable of the complete degradation of all three mono-chlorophenols, with a stoichiometric release of chloride (Zaitsev *et al*, 1995)
Degradation of mono-chlorophenols has also been demonstrated by the yeast *Rhodotorula glutinis* (Katayama-Hirayama *et al*, 1994) Degradation occurred via the *ortho*- cleavage pathway, and led to the complete degradation of 3- and 4-chlorophenol The non-specific ligninolytic activities of some fungi, in particularly white rot fungi, allows them to degrade complex mixtures of pollutants, including substituted aromatics *Phanerochaete chrysosporium* has been shown to be capable of degrading 2-chlorophenol (Lewandowski *et al*, 1990, Wang and Ruckenstein, 1994) Rubio Perez *et al* (1997) reported the degradation of 2-, 3- and 4-chlorophenol by *Phanerochaete*

chrysosporium Hofrichter *et al*, (1994) reported partial degradation of 4-chlorophenol by the soil fungus *Penicillium frequentans* with a 35% stoichiometric release of chloride

1.9 *Pseudomonads.*

The term *Pseudomonad* is used to describe strictly aerobic, Gram-negative, non-sporulating bacteria They are oxidase positive, non-acid fast rods, which are generally straight but maybe slightly curved, 0 5 - 1 μm in diameter and 1 5 - 5 μm in length They are generally motile, with polar flagellae The energy-yielding metabolism is respiratory, never fermentative and therefore they generally do not ferment carbohydrates They do not fix nitrogen and are not photosynthetic The optimum growth temperature for most strains is 28° C but many are capable of growth in the range of 4 - 43° C The incapacity to hydrolize gelatine is the characteristic that has classically defined *P putida* and separate it from *P aeruginosa* and *P fluorescens* (Bergey's Manual of Systematic Bacteriology, 2005) The majority of the strains have been assigned to biovar A (biotype A of Stanier et al , 1966), which is considered typical

Pseudomonas spp are ubiquitous in nature and are found in most temperate, aerobic and semi-arobic soil and water habitats They have simple nutritional requirements and grow rapidly on standadard laboratory media and therefore often overgrow other microorganisms that may be present in the sample (Timmis, 2002) The predominant feature of the Pseudomonads is their biochemical diversity (Stanier *et al*, 1966) Strains of *Pseudomonas* spp are nutritionally omnivorous, capable of growth on over 100 different compounds and therefore play an important role in decomposition, biodegradation and the carbon and nitrogen cycles *Pseudomonas* spp have been reported as as being capable of growing on alkanes, mono- and polycyclic hydrocarbons, salycilate, heterocyclics, phenolics, and aliphatic and aromatic halogenated compounds (Timmis, 2002) Of these compounds, the aromatic compounds are particularly interesting due to the biochemical intricacies of the various pathways by which they are metabolised (Palleroni, 1986) The ability of *Pseudomonads* to utilise such a wide range of compounds makes them a vital component of any biodegradative system and, in

particular, those involved in the treatment of wastewaters derived from the chemical industry Total aerobic counts in activated sludge are in the order of 10^8 CFU/mg of sludge (Bitton, 1999, McClure 1990) The majority of these have been identified as *Commomonas-Pseudomonas* (Table 4) In soil, Pseudomonads are widespread and constitute up to 87% of soil isolates (Ridgeway et al , 1990)

Table 4 Distribution of Aerobic Heterotrophic Bacteria in Standard Activated sludge (Bitton, 1999, Hiraiashi *et al* , 1989)

Genus or group	Percentage of total isolates
Commomonas-Pseudomonas	50 0
Alcaligenes	5 8
Pseudomonas (fluorescent group)	1 9
Paracoccus	11 5
Unidentified (Gram-negative rods)	1 9
Aeromonas	1 9
Flavobacterium	13 5
Bacillus	1 9
Micrococcus	1 9

1 10 Bioaugmentation

Many industrial wastes can contain man-made chemicals which due to their unfamiliarity to microorganisms may be resistant to degradation by indigenous microbial populations This can cause great difficulties in the biological treatment of wastes, as accumulation of such xenobiotics can cause a complete breakdown of the treatment process (Erb *et al*, 1997) When the intrinsic microorganisms at a polluted site are not sufficient to degrade a pollutant, inoculation with an exogenous strain or strains, in a process known as bioaugmentation may be a useful approach Bioaugmentation is the application of indigenous or allochthonous wild-type or genetically-modified organisms to polluted

hazardous waste sites or bioreactors in order to accelerate the removal of undesired compounds (Van Limbergen *et al* , 1998)

Addition of certain organisms can increase the biological diversity and metabolic activity of an indigenous population Such an increase in the biological diversity broadens the gene pool available to the population in times of environmental stress Horizontal gene transfer has played a large role in microbial adaptation to xenobiotics in the environment (Tsuda, 1999) Horizontal gene transfer may occur through (1) uptake of naked DNA (transformation), (2) mediation by bacteriophage (transduction) or (3), physical contact and the exchange of genetic material such as plasmids or conjugative transposons between microorganisms (conjugation) Such processes may be viewed as gene bioaugmentation The potential advantages of such a system, where the degradative genes are in a mobile form such as a plasmid, as opposed to the traditional cell bioaugmentation approach are (1) introduction of degradative genes into indigenous microorganisms that are already adapted to survive and proliferate in the environment, and (2) no requirement for long term survival of the host strain (Gentry et al , 2004)

Properly applied, bioaugmentation has demonstrated the ability to

1 Reduce process instability caused by fluctuations in organic loadings
2 Improve the degradation of target substrates
3 Improve removal efficiencies for biological oxygen demand (BOD) and chemical oxygen demand (COD)
4 Speed recovery from plant upsets or prevent upsets
5 Reduce the inhibitory effects of toxic substrates
6 Improve mixed liquor flocculation
7 Reduce sludge and scum from aerobic and anaerobic digesters and lagoon deposits
8 Induce or stabilise nitrification and recover nitrates (Huban and Plowman, 1997)

All of the above result in a more efficient treatment process thus lowering operational and energy costs

Nußlein *et al* (1992) successfully used two genetically modified micro-organisms, *Pseudomonas* sp B13FR1 (pFRC20P), designated FR120 and *Pseudomonas putida* KT2440 (pWWO-EB62), designated EB62 to augment activated sludge Strain FR120 contained an assembled *ortho*-cleavage route which allowed for the degradation of 3-chlorobenzoate and 4-methylbenzoate, while strain EB62 harboured pWWO-EB62, a derivative of the TOL plasmid which allowed for the degradation of 4-ethyl benzoate via a modified toluate degradation pathway Augmentation of the activated sludge for the degradation of 3-chlorobenzoate and 4-methylbenzoate with FR120 allowed for faster and more complete degradation than was observed without augmentation FR120 also displayed a protective effect on the indigenous population against the toxicity of 3-chlorobenzoate and 4-methylbenzoate at higher concentrations Augmentation of the activated sludge with KT2440 for the degradation of 4-ethylbenzoate was less successful with degradation rates only slightly increased over the control activated sludge system

When the phenol-degrading strain, *Pseudomonas putida* ATCC1172 was inoculated into a sequencing batch activated sludge reactor, phenol removal was maintained at 95-100% for at least 41 days In the unaugmented control reactor phenol removal declined from 100% to 40% over the same period (Selvaratnam *et al*, 1997)

An activated sludge isolate, *Commomonas testosteronii* I2 was capable of the degradation of 3-chloroaniline When the strain was inoculated into a lab-scale semi continuous activated sludge system, complete degradation was achieved, while in the unaugmented system, no degradation at all occurred (Boon *et al*, 2000)

Bioaugmentation using bacteria with degradative capabilities does not always lead to successful degradation of the target compound A 3-chlorobenzoate degrading strain, *Pseudomonas putida* UWC1 (pD10) was inoculated into a laboratory scale activated sludge unit Despite the survival of *P putida* UWC1 within the activated sludge unit and the stable maintenance of the degradative plasmid pD10, no breakdown of 3-chlorobenzoate was observed (McClure *et al*, 1991b) Two of the indigenous bacteria which were isolated, strain AS2 and *Pseudomonas putida* 2 8, which had taken up the

plasmid from *Pseudomonas putida* UWC1 (pD10) in plate-filter matings, were reinoculated into the activated sludge unit and survived at higher numbers than was observed for *P putida* UWC1 Strain AS2 and *P putida* 2 8 also enhanced the degradation of 3-chlorobenzoate (McClure *et al*, 1991a) These results indicate that the use of strains well adapted to their environmental conditions can be an important consideration for a successful bioaugmantation strategy These studies demonstrate that simple addition of pure cultures possessing metabolic capabilities to activated sludge does not guarantee enhanced degradation The ability to metabolise a chemical is a necessary but not a sufficient condition for the organism to effect the transformation of xenobiotics in a natural environment Reasons suggested for the failure of introduced micro-organisms to degrade pollutants include

1 The concentration of the compound in nature may be too low to support the growth of the inoculated species
2 The natural environment may contain substances that are toxic or inhibitory to the growth and activity of the added organisms
3 The growth rate of the organism may be slower than the rate of predation for example by protozoa, so that the rate of activity of the predators reduces the cell density of the inoculated species
4 The introduced species are exposed to intense competition with indigenous micro-organisms better adapted to the prevailing environmental conditions
5 Wastewaters normally contain a complex spectrum of more easily assimilated carbon sources than xenobiotics and so genes for xenobiotic degradation are not expressed
6 Inoculated strains, serving as a "gene pool" survive, but others are not competent for gene transfer
7 The organisms may fail to flocculate and can be washed out of the system resulting in increased turbidity of the mixed liquor Organisms that fail to flocculate also more susceptible to grazing by protozoa

(Goldstein *et al*, 1985 and Gallert and Winter, 1999)

1 10.1 Plasmids and their role in biodegradation and bioaugmentation

Most of the genes involved in the degradation of xenobiotics are part of operons encoded on broad host range, conjugative or mobilisible plasmids TOL plasmids are self transmissible and contain operons which encode the enzymes required for the degradation of methylbenzenes, such as toluene, and xylenes via the *meta*-cleavage pathway The best characterized plasmids involved in the degradation of methylaromatics are the TOL plasmid (pWWO), in which the *xyl* genes encode the enzymes for the degradation of methylbenzenes via methylbenzoates, and the NAH7 plamsid, in which the *nah* and *sal* genes encode the enzymes for the degradation of naphthalene via salicylate Another *meta*-cleavage pathway is encoded by the *dmp* genes on pVII50 of *Pseudomonas putida* CF600, and is involved in the degradation of phenols, cresols and 3,4-dimethylphenol The catabolic genes of plasmid pNAH7 and TOL are clustered in two operons while the *dmp* genes from plasmid pVII50 form a single operon The genes of the *ortho*-cleavage pathway are located on the chromosome and are involved in the degradation of catechol and protocatechuate The characterization of chlorinated aromatic degrading strains demonstrated another set of genes for the modified *ortho*-cleavage pathway enzymes The enzymes, involved in the degradation of chlorocatechols, have wider substrate specificity than the ordinary *ortho*-cleavage pathway enzymes Therefore, the chlorocatechol degradative pathway is also called the modified *ortho*-cleavage pathway (van der Meer et al , 1992)

The three most extensively studied modified *ortho*-cleavage pathway genes are (1) the *clc*ABD operon from *Pseudomonas putida* (pAC27), (2) the *tfd*CDEF genes from *Ralstonia eutropha* JMP134(pJP4) and (3) the *tcb*CDEF operon from *Pseudomonas* sp strain P51(pP51) In these three organisms the chlorocatechol 1,2-dioxygenase appears to be linked to the rest of the genes in a single operon (van der Meer et al , 1992)

Most plasmids up to now are large plamids of more than 50 kbp and, if classified, belong to known incompatibility groups (incompatibility grouping determined using fluorescent pseudomonads as hosts) Degradation of compounds that are mostly man-made, such as

chloroaromatics, are often encoded by promiscuous, broad host range IncP-1 plasmids (Top et al , 2002)

Catabolic plasmids allow the horizontal spread of degradative genes among microbial communities, therefore the deliberate dissemination of a catabolic plasmid in an ecosystem could possibly be exploited as a bioremediation tool During bioaugmentation, maintaining high levels of active inoculants can be a challenge as introduced strains may have to compete with indigenous microorganisms for resources and space and face predation from protozoa as well as being exposed to unfavourable conditions To avoid the need for the inoculum to survive and remain functional for long periods, strains with the relevant genetic information on transferable catabolic plasmids may be used The long term survival of the introduced strain is no longer needed following horizontal transfer to one or various well established and competitive indigenous bacteria in an ecosystem (Top et al , 2002)

1.11 Aims of the project

1 To investigate the biochemical pathway involved in the degradation of 4-chlorophenol by *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1

2 To characterise the key gene for 4-chlorophenol degradation in two strains, *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1 and to determine if these genes were located in large plasmids harboured by these strains

3 To tag *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1 with GFP using a transposon

4 To study the degradation of 4-chlorophenol in activated sludge augmented with GFP-tagged derivatives of *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1

2. Materials and Methods

2 1 Materials

2 1.1 Organisms

-*Pseudomonas* and *Ralstonia* strains

-*P putida* CP1 was obtained from Prof Fabio Fava, University of Bologna, Italy, who isolated it from nature and was separately maintained on 2-chlorophenol (1 56 mM),3-chlorophenol (0 78 mM) or 4-chlorophenol (1 56 mM)

-*P putida* A(a) was isolated from a commercially produced bioaugmentation product, Biolyte HAB and was obtained from International Biochemicals Ltd , Dublin This bioaugmentation product was specially formulated to degrade a variety of substituted aromatic compounds and it contained at least eleven microorganisms comprising bacteria belonging to the genera *Pseudomonas* and *Actinomycetes*, together with a species of fungus, *Trichoderma harazanium* The strain was maintained on Tryptone Soy Agar and 4-chlorophenol agar

-*P putida* mt-2 pWW0 (TOL plasmid) and the cured derivative *P putida* KT2440 were obtained from the National Collection of Industrial and Marine Bacteria (NCIMB) and the National Collection of Food Bacteria, Aberdeen, Scotland (http //www ncimb co uk) The strains were maintained on Tryptone Soy Agar (TSA)

-*R eutropha* JMP134 pJP4 and the cured derivative *R eutropha* JMP222 were obtained from DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures,

http //www dsmz de) These strains were maintained on 2, 4-dichlorophenoxyacetic acid and TSA respectively

Recombinant *E. coli* strains

- *E coli* S17 1 λ*pir* pTGN-mini-Tn*5gfp*-Km was provided by Professor X Tang, National University of Singapore and was maintained on Luria Bertani (LB) agar with Kanamycin (10μg/ml)

- *E coli* XL1 Blue pBK-miniTn7-*gfp*2 and *E coli* SM10/λ*pir* pUX-BF13 were provided by Professor Birgit Koch, Royal Veterinary and Agricultural University, Frederiksberg, Denmark and were maintained on LB agar containing Kanamycin (25 μg/ml) and ampicillin (100 μg/ml) respectively

- *E coli S17-1* was obtained from Dr Paul Clarke, School of Biotechnology, Dublin City University and was maintained on Luria Bertani (LB) agar with Streptomycin (10μg/ml)

Maintenance of organisms

-Organisms were maintained on plates and slants at $4^{O}C$ Glycerol stocks were prepared by adding 20% (v/v) glycerol to an overnight liquid culture and freezing at $-70^{O}C$ Stocks were also maintained using Protect beads stored at $-70^{O}C$

-Activated sludge

Activated sludge was obtained from an industrial wastewater treatment plant

2.1 2 Media

All media unless otherwise stated were obtained from Oxoid and were sterilised by autoclaving at 121^{O}C for 15 minutes

Pseudomonas minimal medium

The medium of Goulding *et al* (1988) was prepared by combining the ingredients in distilled water and adjusting the pH to 7 0 with 2M NaOH The trace salts solution was prepared separately in distilled water and was stored in a dark bottle for 6-8 weeks

Pseudomonas minimal medium

K_2HPO_4	4 36g
NaH_2PO_4	3 45g
NH_4Cl	1 0g
$MgSO_4$ $6H_2O$	0 912g
Distilled water	100ml
Trace salts solution*	1ml

pH 7 0

Trace salts solution*	**g100ml^{1}**
$CaCl_2$ $2H_2O$	4 77
$FeSO_4$ $7H_2O$	0 37
$CoCl_2$ $6H_2O$	0 37
$MnCl_2$	0 10
$NaMoO_4$	0 02

Carbon Sources

1 56mM 4-chlorophenol was added to the *Pseudomonas* minimal medium following sterilisation

Filter sterilised 2, 4-dichlorophenoxyacetic acid (Na salt) was added to the minimal medium following sterilisation at a concentaration of 0 05% from a 20 mgml^{-1} stock solution

Agar was 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 carbon source was added to the agar to give the appropriate final concentration

Luria Bertani Broth

Luria Bertani (LB) broth was prepared according to the method of Maniatis *et al* (1982) The ingredients were combined in distilled water

LB Broth

	g/L
Tryptone	10 0
Yeast Extract	5 0
NaCl	10 0

Agar was prepared by the addition of bacteriological agar, at a concentration of 1% (w/v) to the LB Broth

2.1.3 Buffers and solutions

20 x SSC

A stock solution of 20X SSC comprised sodium chloride (3M) and sodium citrate (0 3M) in distilled water The pH was adjusted to 7 0 with sodium hydroxide This solution was stored at $4^{O}C$ and diluted as required

50X TAE

Tris-acetate buffer was prepared by dissolving Tris (2M) and ethylenediaminetetra acetic acid (0 05M) in distilled water and adjusting the pH to 8 0 with glacial acetic acid The buffer was stored at room temperature and diluted as required

TE Buffer

Tris EDTA buffer was prepared by dissolving Tris (10mM) and EDTA (1mM) in distilled water and adjusting to pH 8 0 with 2M HCl

Potassium phosphate buffer

Potassium phosphate buffer was prepared as outlined by Clerescı *et al*, (1998) by dissolving K_2HPO_4 (104 5g/L) and KH_2PO_4 (72 3g/L) in distilled water The pH of the resulting solution should be 6 8

TRIS-HCL Buffer

Tris-HCl buffer (0 033M) was prepared by dissolving Tris (0 033M) in distilled water and adjusting the pH to 7 6 using 2 M HCl

Sodium Phosphate Buffer

Sodium phosphate buffer (0 01M) was prepared by dissolving Na_2HPO_4 (0 01M) and NaH_2PO_4 in distilled water The pH of the resulting solution was then adjusted to pH 7 0

Antibiotic stock solutions

Gentamicin and kanamycin for use in LB medium for the maintenance of recombinant *E coli* strains and for addition to *Pseudomonas* minimal medium for the selection of antibiotic resistant strains were prepared as 20mg/ml stock solutions in deionised water The solutions were then filter sterilized and aliquots were stored at $4^{O}C$ (gentamicin) and $-20^{O}C$ (kanamycin) Cycloheximide stock solutions for addition to plate count agar and *Pseudomonas* minimal medium agar for the inhibition of growth of yeast and moulds were prepared by dissolving 50 mg/ml cycloheximide in ethanol and stored at $-20^{O}C$

$FeSO_4$ Stock solution

$FeSO_4$ stock solution (100mM) was prepared by adding $FesO_4$ to distilled water and filter sterilizing Stocks were maintained at $4^{O}C$

Prewashing Solution

Prewashing solution was prepared by dissolving maleic acid (100 mM), NaCl (150 mM) and Tween 20 (0 3%(v/v)) in distilled water

Hybridization Solution

Hybridization solution was prepared by dissolving N-lauryolsarcosine (0 1%(w/v)), SDS (0 02%(w/v)), blocking reagent (1% from a 10% blocking reagent stock solution) in 5X SSC

Buffer 1 (Washing Buffer)

Washing buffer was prepared by dissolving Tween 20 (0 3% (v/v)) in 0 1 M maleic acid, 0 15 M NaCl adjusted to pH 7 5 with solid NaOH

Buffer 2 (Blocking Stock Solution)

Blocking stock solution was prepared by dissolving blocking reagent (10% (w/v)) in buffer 1 with constant stirring on a heating block (65 °C) Buffer 2 was autoclaved at 121 °C for 15 minutes and stored at 4 °C

2 1.4 Source of Chemicals

Chemicals were obtained from a number of sources including Reidel-de-Haen, BDH, and Sigma Aldrich

2 1 5 Source of Molecular Biology Reagents

Restriction enzymes were supplied by Sigma, Missouri, USA

Wizard Genomic DNA Purification Kit was supplied by Promega Corp , Madison, USA

DIG DNA labelling and detection system was supplied by Roche Molecular Diagnostics

GenElute Plasmid Miniprep Kits were supplied by Promega Corp , Madison, USA

High Pure PCR product purification kit was supplied by Boehringer Mannheim, Germany

2 2 Methods

2.2 1 Inocula preparation of *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1

Pseudomonas putida A(a) and *Pseudomonas putida* CP1, taken from chlorophenol agar plates, were grown up overnight in nutrient broth, washed twice with 0 01 M sodium phosphate buffer, pH 7 0, resuspended to give an optical density at 660nm of approximately 0 7 for CP1 or 0 5 for A(a) and used to inoculate chlorophenol or activated sludge flasks The approximate inocula sizes (v/v) were 5 0% = 1 2 x 10^7 cell/ml, 1 0% = 2 4 x 10^6 cell/ml and 0 5% = 1 2 x 10^6 cell/ml

2 2 2 Biodegradation studies

Biodegradation experiments on pure cultures were performed in 1L conical flasks containing a final culture volume of 250ml following inoculation and addition of 1 56 mM chlorophenol Flasks were inoculated with *Pseudomonas putida* A(a) or *Pseudomonas putida* CP1 at a concentration of 0 5 %, 1 0% or 5% and incubated aerobically at 150 rpm and 30 °C Samples were aseptically removed at regular intervals and analysed for pH, cell density, chlorophenol concentration and free chloride concentration Uninoculated control flasks were incubated in parallel

For the activated sludge experiments, duplicate systems were set up in 1L flasks with a final working volume of 200 ml The solids were allowed to settle and the supernatant was replaced with a synthetic effluent consisting of *Pseudomonas* minimal medium containing 1 56 mM 4-chlorophenol Flasks were inoculated with *P putida* A(a) gfp or *P putida* CP1 gfp (0 5% v/v, 1 0%v/v and 5 0% v/v) The systems were operated under aerobic batch culture conditions with shaking at 150 rpm and 30°C Samples were aseptically removed at regular intervals for analysis

2.2.3 Determination of cell numbers

The plate count technique was used to determine cell numbers Prior to carrying out cell counts on agar, sludge flocs were dispersed by purging samples through a sterile needle (21G x 1 5 in, Sigma-Aldrich) 5 times (Boon et al , 2000) Total cell counts in activated sludge were determined by performing cell counts on Oxoid plate count agar The numbers of chlorophenol degrading organisms were determined by plating onto *Pseudomonas* minimal medium chlorophenol agar The numbers of *P putida* A(a) gfp and *P putida* CP1 gfp, following their addition to the activated sludge, were enumerated by plating onto *Pseudomonas* minimal agar containing gentamicin (20 μg/ml) and cycloheximide (100 μg/ml) All plates were incubated at 30^0 C for 72 hours

2 2.4 Identification and metabolic profiles

Biolog Identification

Identification and metabolic profiles were carried out using the Biolog microlog system (BIOLOG Inc , Hayward, Calif , USA) according to the manufacturers' instructions GN microplates, for Gram negative organisms were used An isolated colony was transferred to Biolog inoculating fluid using sterile swabs to give the correct cell density required by the system Cell densities were compared against GN-NENT (Gram negative Non-enteric) turbidity standards supplied by the company 150μl of the resulting inoculum was added to each of the 96 test wells in the microtitre plate The plates were incubated for 16 - 24 hours at 30 °C before results were interpreted using Biolog automated microlog software Identification is obtained by comparison of the results obtained with those of database patterns supplied in the software Similarity values must be > 0 5 after 16 – 24 hours, while the distance value must be < 5 0 for a valid identification to occur

API Test

The API 20NE, for non-enteric Gram negative rods, was also used to identify and obtain metabolic profiles from both tagged and untagged *P putida* strains The identification system was carried out according to the manufacturers' instructions (bioMerieux sa, Marcy-l'Etoile, France) The inocula were prepared as follows an overnight nutrient broth culture (10ml) was harvested in a Labofuge 6000 bench-top centrifuge (5,000 rpm for 10 minutes) and washed once with sterile 0 01 M sodium phosphate buffer (Section 2 1 3) The pellet was resuspended in 0 85% (w/v) NaCl (10ml) and used to inoculate a portion of the tests For assimilation tests, 200µl of this suspension was used to inoculate auxiliary medium supplied by the manufacturer and this was then used to inoculate the remaining tests

2 2 5 Measurement of chlorophenol concentrations

Chlorophenol concentrations were measured using a 4-aminoantipyrene colorimetric method based on the procedure detailed by Clerescı *et al*, (1998) Samples were centrifuged at 5,000 rpm for 10 min 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 10ml distilled water The samples and standards were treated by placing 10ml in a test tube and adding 0 25ml 0 5N NH_4OH The pH was then adjusted to 7 9 ± 0 1 with approximately 200µl potassium phosphate buffer (pH 6 8) 100µl of 2% (w/v) 4-aminoantipyrene solution was added and the tubes mixed well The 100µl of 8% (w/v) potassium ferricyanide was added and the tubes mixed well The tubes were allowed to stand for 15 min at room temperature The absorbance was read at 500nm and the concentrations were calculated from the standard curve

Sample standard curves for the mono-chlorophenols are presented in Figure 10

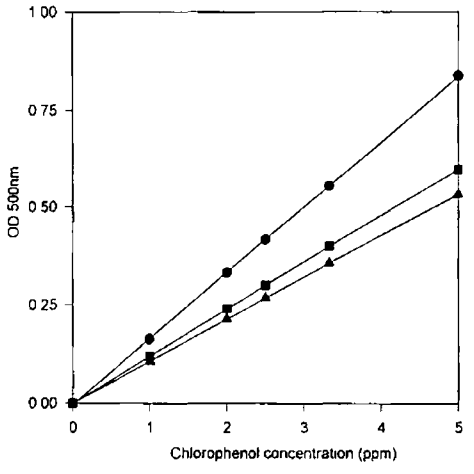

Figure 10 Standard curve for mono-chlorophenols using colorimetric assay Symbols ● 2-chlorophenol, ■ 3-chlorophenol and ▲ 4-chlorophenol

2 2 6 Chloride assay

Chloride release was followed with an Orion chloride specific electrode (model 9417) The electrode was calibrated with reference to NaCl standards and chloride concentrations were calculated using the direct readout capability of an Orion benchtop pH/ISE meter (model 920A) Samples and standards were diluted with 2% 5M $NaNO_3$ ionic strength adjusting solution

2 2 7 Measurement of pH

Following the aseptic removal of a sample from a flask, the pH was measured using an Orion Triode^{TM} pH electrode Model 91-57BN connected to an Orion benchtop pH/ISE meter (model 920A)

2 2.8 Enzyme Assays

Preparation of cell-free extracts

Overnight cultures of cells grown on minimal medium containing 2-, 3-, or 4-chlorophenol (100 ml) were harvested by centrifugation at 5000 rpm for 10 mins and washed twice in 0 033 M tris-HCl buffer (pH 7 6) The cells were broken by sonication for 3 mm (30 sec on, 30 sec off) at 16 amplitude microns using a Labsonic U (B Braun) The cell suspension was kept on ice throughout sonication Whole cells and debris were removed by centrifugation at 20,000 rpm for 20 mins and 0-4°C The cell free extract was kept on ice and assayed as soon as possible for catechol dioxygenase activity

Catechol 1,2-dioxygenase activity

Catechol dioxygenase activity was measured by following the formation of *cis, cis*-muconic acid, the ortho-cleavage product of catechol using the method of Dorn and Knackmuss (1978) The 3-ml reaction mixture contained the following reagents made up in 1 mM EDTA 2 ml 50 mM Tris-HCl buffer (pH 8 0), 0 7ml distilled water, 0 1 ml 100 mM 2-mercaptoethanol and 0 1 ml cell-free extract The contents of the cuvette were mixed by inversion and 0 1 ml catechol (1mM) was added and the contents mixed again *Cis, cis*- muconic acid formation was followed by an increase in the absorbance at 260nm over a period of 5 min

Chlorocatechol 1,2-dioxygenase activity

Chlorocatechol 1,2-dioxygenase activity was measured by following the formation of 2-chloromuconic acid, the *ortho*-cleavage product of 3-chlorocatechol The procedure used was as for Type 1 activity with 3-chlorocatechol (1mM) or 4-chlorocatechol used in place of catechol (1mM)

Catechol 2,3-dioxygenase activity

Catechol 2,3-dioxygenase activity was measured by following the formation of 2-hydroxymuconic semialdehyde, the *meta*-cleavage product of catechol, using the method of Feist and Hegeman (1969) The 3-ml reaction mixture contained the following reagents made up in 1 mM EDTA 2ml 50 mM Tris-HCl buffer (pH7 5), 0 6ml distilled water, 0 2ml cellfree extract The contents were mixed by inversion and 0 2ml catechol (100mM), 3-chlorocatechol (1mM) or 4-chlorocatechol (1mM) were added and the contents mixed again The production of 2-hydroxymuconic semialdehyde was followed by an increase in absorbance at 375nm over 5 min

Calculation of enzyme activity

Activities were calculated using the following extinction coefficients for each reaction product catechol at 260nm = 16,800 l/mol/cm, 3-chlorocatechol at 260nm = 17,100 l/mol/cm, 4-chlorocatechol at 260nm = 12,400 l/mol/cm, 4-chlorocatechol at 379nm = 40,000 l/mol/cm and catechol at 375nm = 36,000 l/mol/cm (Sung Bae *et al* ,1996) One unit of enzyme activity was defined as the amount of enzyme that catalysed the formation of 1 µmol of product formed per minute in 1 ml reaction mixture and was calculated using the following formula (John R A , 1995)

$$\text{Activity (U/min)} = [(\Delta\ OD/\ \Delta T) / \varepsilon] \times V \times 1000$$

where $\Delta\ OD/\ \Delta T$ = increase in optical density per minute

ε = molar extinction coefficient (1 mol ^{1}cm ^{1})

V = reaction volume (3 ml)

Protein determination

The protein concentration in the cell-free extract was determined by the method of Lowry *et al*, (1951) Reagent A (2 5ml) was added to 0 5ml of suitably diluted sample or standard The tubes were left for 10 minutes in the dark at room temperature Reagent B (0 125ml) was then added and the tubes were left for 25 minutes at room temperature in the dark The tubes were then mixed by vortexing and left for a further 5 minutes The absorbance was read at 600nm Standards were prepared with bovine serum albumin (0 - 1 mg ml^{-1})

Reagent A 50ml 0 2% Na_2CO_3 in 0 1M NaOH
0 5ml 1% $CuSO_4$
0 5ml 2% NaK tartarate

Reagent B 50% (v/v) Folins - Ciocalteau in water

2.2 9 Measurement of intermediates of 4-chlorophenol metabolism

The production of the *meta-* cleavage product of 4-chlorocatechol, 5-chloro-2-hydroxymuconic semialdehyde (5-Cl-HMS) was followed by measurement of the optical density at 380nm following removal of cells by centrifugation

2 2.10 DNA purification

Preparation of template DNA for PCR

Template DNA was prepared by resuspending 100ul of an overnight culture in 200ul of sterile distilled water Cells were lysed by boiling for 10 min followed by freezing for 10min Cell debris was sedimented by centrifugation at 14, 000 rpm and 4° C for 15 mm For the template, 2 µl of the supernatant was used for a 50ul PCR reaction

Purification of plasmid DNA from recombinant *E coli* cultures

Plasmid DNA was purified from recombinant *E coli* strains using the Genelute Plasmid Miniprep Kit (Sigma, Saint Louis, Missouri) according to the instructions of the manufacturer

Isolation of chromosomal DNA from GFP-tagged *P putida* CP1

Chromosomal DNA was isolated using a Wizard Genomic DNA isolation kit (Sigma) according to the instructions of the manufacturer

Isolation of plasmid DNA from *Pseudomonas* and *Ralstonia* spp

(Modified from the method of Hansen and Olsen (1983))

A 250 ml culture was harvested at 7, 000 rpm for 10 minutes in a Sorvall RC-5B high speed centrifuge The resulting pellet was resuspended in 6 mls of 25% (^{W}/v) sucrose in 0 05 M Tris (pH 8 0) From this point on, all further additions were mixed by gentle inversion of the tube Firstly, 1 ml of lysozyme (5 mg/ml in 0 025 M Tris, pH 8 0, freshly prepared) was added and the tubes left on ice for 5 minutes Next, 2 5 mls of EDTA (0 25 M, pH 8 0) was added and the tubes were left on ice for a further 5 minutes The additin of 2 5 mls SDS (20% w/v in TE buffer) followed by a1-2 minute incubation at 55^{O}C completed cell lysis 0 75 mls of a freshly prepared 3 M solution of NaOH was added and the tubes mixed by inversion for three minutes 6 0 ml of TRIS (2M, pH7 0) was added and the tubes mixed thoroughly Next, 3ml of SDS (20%w/v in TE) was added, immediately followed by 6 ml of ice cold 5M NaCl Tubes were then left on ice for 6hr or overnight at 4^{O}C The precipitated chromosome membrane complex was then removed by centrifugation at 20,000 rpm for 30 min at 4^{O}C The supernatant was removed to a fresh centrifuge tube and 1/3 volume of PEG 6000 (42% w/v in 0 01M sodium phosphate buffer pH7 0) was added The tubes were mixed by inversion and left overnight at 4^{O}C before centrifugation at 7,000 rpm for 6 min and 4^{O}C The resulting

DNA pellet was resuspended in TE Plasmid DNA was visualized by gel electrophoresis on a 0 7% agarose gel for 1 hr at 100mV

Caesium chloride ethidium bromide density centrifugation

The method of Sambrook et al (1982) was used The crude plasmid solution was placed in a 10 ml glass cylinder and the volume adjusted to 6 5 mls with TE buffer 6 5 g of solid caesium chloride was added and the solution mixed gently until all of the salt had dissolved Then, 0 52 mls of ethidium bromide solution (10 mg/ml in distilled water) was added to the caesium chloride solution The caesium chloride solution was then transferred to a heat-seal tube The remainder of the heat seal tube was filled with paraffin oil and balanced in pairs to within 0 005 g of each other (including lids), using a balancing stock solution of TE buffer (6 5 mls), caesium chloride (6 5 g) and ethidium bromide (0 52 mls) The tubes were sealed and the density gradient was formed by centrifugation at 50,000 rpm for 24 hours at $18^{O}C$ in an ultracentrifuge

After centrifugation, two bands of DNA were located in each tube The lower band containing covalently closed circular DNA was extracted with a syringe in the presence of UV light as follows a hypodermic needle was inserted into the top of the tube to allow air to enter In order to minimise the possibility of contamination, the upper band containing nicked circular and linear DNA was first removed by inserting a hypodermic needle, bevel side up, just below the band, and withdrawing it into the syringe The lower band was then withdrawn in a similar manner and transferred to a microtube

Removal of ethidium bromide and caesium chloride from the purified DNA

The ethidium bromide was removed as follows an equal volume of 1-butanol saturated with water was added to the DNA solution and the two phases mixed by gentle inversion The solution was then centrifuged at 13, 000 rpm for 3 minutes to separate the phases

The upper layer was removed and the extraction repeated until all of the pink colour was removed from the aqueous solution

The cesium chloride was removed from the DNA solution by dialysis Dialysis tubing was boiled in 10 mM Na_2EDTA for 10 minutes and then boiled in distilled water for a further 10 minutes The plasmid solution was dialysed against TE twice, including once overnight The resulting purified plasmid DNA was stored at $4^{O}C$

Restriction of purified DNA

When necessary, restriction of purified genomic or plasmid DNA was carried out according to the instructions of the manufacturer The resulting restriction profiles were visualized following overnight eletrophoresis on a 0 7% agarose gel at 35 mV

2.2.11 Polymerase Chain Reaction

PCR amplification reactions

The 50µl reaction mix contained 1X Taq polymerase buffer, 3mM MgCl2, 200µM each of dATP, dCTP, dGTP, dTTP (Promega), 0 5µM each of primers (synthesized by MWG BIOTECH) (Table 5) and 1 25 U of Taq polymerase The reaction mixes were overlaid with 50µl mineral oil All ingredients were added from a mastermix and template DNA was added last to avoid cross contamination PCR was performed on a Hybaid Omni Gene thermal cycler using Taq polymerase (Promega) PCR products were visualized by electrophoresis on a 1 0 % agarose gel for 1 hr at 100mV

Amplification of the gene for chlorocatechol 1,2-dioxygenase

The PCR protocol for the amplification of chlorocatechol 1, 2-dioxygenases was as follows initial denaturation for 3 min at 94°C, 30 cycles denaturing for 45 sec at 94°C,

annealing for 30 sec at 62°C, extension for 2 min at 72°C and a final extension step for 6 min at 72°C

Amplification of the gene for catechol 2,3-dioxygenase

The PCR protocol for amplification of catechol 2, 3-dioxygenases was as follows initial denaturation for 3 min at 94°C, 30 cycles denaturing for 1 min at 94°C, annealing for 1 min at 58°C, extension for 2 min at 72°C and a final extension step for 6 min at 72°C

Amplification of the gene for GFP

The PCR protocol for amplification of *gfp* was as follows initial denaturation for 3 min at 94°C, 25 cycles denaturing for 1 min at 94°C, annealing for 1 min at 55°C, extension for 1 min at 72°C and a final extension step for 7 min at 72°C

Amplification of intergenic region between the glmS gene and the cat gene

The PCR reaction mixtures for the amplification of the intergenic region between the *glm*S gene and the *cat* gene were subjected to 94°C for 3 min, followed by 30 cycles of 52°C for 1 min, 72°C for 1 min, and 94°C for 1 min, and one cycle of 52°C for 1 min and 5 minutes at 72°C

Sequencing and analysis of PCR products

PCR products were purified using the Highpure PCR product purification kit (Roche Molecular Diagnostics) according to the instructions of the manufacturer and sequenced by MWG Biotech (Germany) The DNA sequences obtained were compared with known sequences using Genbank and amino acid sequences were obtained using the EMBL

eubacterial protein translation program Sequence alignments were carried out using the Clustal W multiple sequence alignment program

Table 5 Oligonucleotide primers used for PCR

Gene	Sequence	Reference
Chlorocatechol 1,2-dioxygenase (F)	5'-TGGCA(C/T)TC(G/C)ACGCC(C/T)GAT-3'	Kleinsteuber *et al*, (1998)
Chlorocatechol 1,2-dioxygenase (R)	5'-TTC(A/G)AAGTATTGCGTGGT-3'	
catechol 2, 3-dioxygenase (F)	5'-CGACCTGATC(AT)(CG)CATGACCGA-3'	Mesarch et al, (2000)
catechol 2, 3-dioxygenase (R)	5'-T(CT)AGGTCA(GT)(AC)ACGGTCA-3'	
GFP (F)	5'-ATGAGTAAAGGAGAAGAACTTTTCACTGG-3'	Tresse, 2001
GFP (R)	5'-CTATTTGTATAGTTCATCCATGCCATGTGT-3'	
GlmS	5'-TTT TTC GTC TCA GCC AAT CCC-3'	Koch *et al*, 2002
Cat	5'-GTA ATC TGG CGA AGT CGG TG-3'	

2 2 12 Phylogenetic tree

Sequences were downloaded from the NCBI website into the DNAstar package Editseq They were then imported and aligned in the DNAstar package Megalign Clustal X gives an accurate tree from aligned sequences of the exact same length therefore from the aligned sequences, portions of each sequence were highlighted to give a sequence comparisom of the exzact same length This alignment was then opened in Clustal X,

which aligns, bootstraps and provides a statistical relationship of the compared sequences (Thompson et al , 1997) The resultant tree was then viewed by Treview (Page, 1996)

2.2.13 Southern Hybridization

Labelling of dioxygenase probes and GFP probes

Random primer labelling of linearized probe DNA with digoxigenin was carried out using the DIG DNA labelling kit in accordance with the manufacturers instructions (Roche Molecular Diagnostics)

First, 1 μg of the appropriate PCR product was diluted to a total volume of 15 μl in H_2O and denatured by boiling for 10 minutes followed by chilling on ice The kit reagents were then added to the denatured DNA in the following order

2 μl hexanucleotide mixture
2 μl dNTP labelling mixture
1 μl Klenow enzyme

The tube was centrifuged briefly and was incubated at 37°C for 20 hours The reaction was stopped by adding 2 μl of EDTA (0 2 M, pH 8 0) The labelled DNA was then used directly for hybridization or stored at -20°C for up to 6 months The stored probe had to be denatured prior to use This was done by denaturing the DIG-labeled DNA probe by boiling for 5 minutes and rapidly cooling on ice

Southern hybridization

Southern transfer and hybridization (Southern, 1975) were performed as detailed in the method of Maniatis *et al* (1982)

Transfer of DNA from agarose gel to nitrocellulose paper

Following electrophoresis one corner corner of the gel was cut away in order to mark its orientation on the nitrocellulose The gel was soaked twice for 15 minutes in 0 25 M HCl at room temperature and then washed very well with water (4-5 minutes) The DNA was denatured by soaking the gel in several volumes of 1 5 M NaCl and 0 5 M NaOH for 1 hour at room temperature with constant shaking The gel was neutralized by soaking in several volumes of 1 M Tris HCl (pH 8 0) and 1 5 M NaCl for one hour at room temperature with constant shaking At this stage the pH of the ball was below 8 5 The transfer took place overnight

For the Southern blot, a piece of nitrocellulose (Sigma) was cut about 1-2 mm larger than the gel in both dimensions Gloves were worn at all times when handling the nitrocellulose The nitrocellulose was floated on the surface of 2 X SSC until completely wet from beneath and then submerged completely for 5 minutes This ensured even wetting of the nitrocellulose, eliminating air bubbles The gel was placed on a 3MM Whatman filter paper wick, the ends of which were in contact with 20 X SSC The wet nitrocellulose was laid carefully on the gel and any air bubbles removed by rolling a glass teat tube over the surface Two pieces of Whatman filter paper soaked in 2 X SSC were placed on top of the nitrocellulose and the stack built up with paper towels A weight was placed on the top to aid the transfer Parafilm was placed around the edges of the gel in order to prevent the towels coming into contact with the gel or 20 X SSC, thereby preventing the liquid bypassing the nitrocellulose

Transfer was allowed to proceed overnight The towels and filter paper were then removed and the positions of the wells were marked on the nitrocellulose with a pencil or ball point pen They were then washed in 6 X SSC for 5 minutes to remove any fragments of agarose and air dried at room temperature The dried filter was then placed between two pieces of 3MM paper and baked at $80^{O}C$ for two hours The filter could then be stored at room temperature or used for hybridization

Hybridization to digoxigenin-labelled probe

Baked filters were floated on the surface of a solution of 2 X SSC until wet from beneath and were then submerged for 5 minutes The filters were then washed in several volumes of prewashing solution (section 2 1 3) at 50^{O}C for 30 minutes with occasional agitation

The filters were prehybridized in heat sealable plastic bags with at least 20 ml of hybridization solution per 100 cm^2 of filter

They were incubated at 42 ^{O}C for at least one hour The solution was distributed from time to time The solution was then replaced with 2 5 ml of hybridization solution per 100 cm^2 of filter, containing freshly denatured labelled DNA The filters were incubated at 42 ^{O}C overnight and the solution was redistributed occasionally

Following incubation, the filters were removed from the plastic bags and washed twice in 2 X SSC and 0 1 % (w/v) SDS for 5 minutes at room temperature (50 ml/100cm^2 filter) The filters were twice washed for 15 minutes at 68^{O}C in 0 1 X SSC and 0 1% (w/v) SDS (50 ml/100 cm^2 filter) The filters were then used directly for detection or stored air dried for later detection

All the following incubations were performed at room temperature All the reactions were carried out with agitation except the colour development The volumes were calculated for a 100 cm^2 filter size

The membranes were washed briefly (1 minute) in several volumes of buffer 1 and then incubated for 30 minutes in 100 ml of buffer 2 (section 2 1 4) The membranes were then incubated for 30 minutes in 20 ml of diluted antibody conjugate solution (150 mU/ml, 1/5000 in buffer 2) Unbound antibody was removed by washing twice fo 15 minutes in buffer 1 The membranes were equilibrated for 2 minutes with 20 ml of buffer 3 and then incubated in 10 ml of colour substrate solution (4 5 µl/ml in buffer 3) in the dark The colour was allowed to develop overnight without shaking The reaction was stopped by

washing the filters for 5 minutes with 50 ml of TE buffer. The results were documented by scanning the filter.

2.2.14 Tagging of Pseudomonas species with GFP

Preparation of competent *E. coli* S17-1

E. coli S17-1 was streaked ont an LB plate and grown overnight at 37°C. A single colony was then looped into 1 ml and grown overnight with shaking at 37°C. The 1 ml was diluted 1 in 100 in an Erlenmeyer flask and incubated at 37°C with vigorous shaking for 3-4 hours until OD 600 nm (1 cm) = 0.6 (2 x10^8 cells/ml). The culture was centrifuged at 3,000 rpm for 10 min. and 4°C in a Sorvall RC-5B centrifuge. The pellet was resuspended in a 1/2 volume ice cold 50 mM $CaCl_2$ and left to stand for 15 mins on ice (1 hour if the cells were to be frozen). The tube was centrifuged at 3,000 rpm for 10 min. and 4°C in a Sorvall RC-5B. The resulting pellet was resuspended in 1/10th volume of ice cold 50 mM $CaCl_2$ for immediate use. For long term storage of competent cells at –70°C, the pellet was resuspended in 20% glycerol, 50 mM $CaCl_2$. The cells were quickly aliquoted into 200 µl amounts in pre-chilled eppendorfs and frozen at –70°C.

Transformation of competent *E. coli* S17-1

Purified plasmid pBK-miniTn7-*gfp*2 (10 ng) was placed in a sterile microfuge tube. To this was added 100 µl of the competent cells that had been briefly thawed between the fingers. The tube was swirled gently and placed on ice for 30 minutes. The cells were then heat-shocked by placing the tube in a waterbath at 42°C for 90 s after which they were returned to ice for 2 minutes. 1 ml of sterile Luria-Bertani medium was added to the tube and the cells incubated at 37°C for 1 hour with gentle agitation (200 rpm). The cells were centrifuged for 1 min at 6,000 rpm and the supernatant removed. The pellet was gently resuspended in 100 µl of sodium phosphate buffer and plated out on LB agar plus 40ug/ml gentamicin. The plates were incubated overnight at 37°C. Two plates were used for each transformation, 90% of the transformation (i.e. 90 µl) was spread on the

first one, while 10% (i e 10 µl) was spread on the second to ensure single colonies were obtained

Plate matings

For the plate mating, 100µl each of an overnight culture of the recipient organism and the donor organisms, *E coli* S17-1 pBK-miniTn7-*gfp*2 in conjunction with the helper strain *E coli* SM10λ *pir* pUX-BF13, or *E coli* S17 1 λ*pir* pTGN were washed twice in sodium phosphate buffer to remove any antibiotics 20 µl of each was added to a sterile microfuge tube and centrifuged at 4000 rpm for 5 minutes The resulting pellet was resuspended in 10 ul of sodium phosphate buffer and spotted on to an LB plate The plates were incubated at 30^{O}C overnight

Selction of transformants

A portion of the overnight plate mating was removed with a sterile inoculating loop and resuspended in 1 ml of sterile sodium phosphate buffer Serial dilutions of this suspension were plated out on selective medium (*Pseudomonas* minimal medium containing 1 56mM 4-chlorophenol or LB agar with gentamicin or kanamycin at a concentration of 20 µg per ml) to obtain single colonies Antibiotic-resistant colonies appearing after several days were purified by restreaking onto fresh agar plates Cells transformed with pBK-mmiTn7-*gfp*2 were screened for the expression of GFP using epifluorescence microscopy Cells transformed with pTGN were screened for the expression of GFP under a long wavelength UV lamp

Minimum inhibitory concentration

GFP-tagged cells were grown overnight in nutrient broth Cells were washed in sodium phosphate buffer and 100 µl were spread on LB agar containing a range of Gentamicin

concentrations from 0 to 70μg/ml and the plates were incubated for 24 hours The minimum inhibitory concentration was then recorded

2 2 15 Image analysis microscopy

Microscopic images of the bacterial samples were recorded using a Hamamatsu C5810 colour chilled 3CCD camera (Hamamtsu Photonics K K , Hamamatsu City, Japan) attached to a Zeiss Axioskop microscope at 400X magnification equipped with Zeiss Filter set 09 (excitation at 450-490, beamsplitter at 510 and emission at 515) using Optimas 6 5® acquisition software (Media Cybernetics, Bothell, WA)

2.2.16 Statistical Analysis

All data analysis was carried out using Sigma Plot Version 1 2 Scientific Graph System (Jandel Corporation) The values shown in all graphs are the means ± S D for duplicate samples Linear regression analysis was used to estimate the rate of decrease in log number of *P putida* numbers per hour The slopes of the regression lines were calculated to give the log decrease in *P putida* numbers / ml / hour For all regression lines, R^2 was >0 9

3 Results

3 1 Degradation of mono-chlorophenol isomers by *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1.

The aerobic degradation of of 1 56mM 2-, 3-, and 4-chlorophenol by *P putida* A(a) and *P putida* CP1 was investigated in batch cultures Parameters measured during the degradation of the mono-chlorophenols included the removal of substrate, the production of metabolites, the activity of the key enzymes involved in the degradation of mono-chlorophenols and the morphology of the strains during degradation studies

3.1.1 Degradation of 1 56mM 2-, 3- and 4-chlorophenol isomers by *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1

2-chlorophenol

The degradation of 1 56 mM 2- chlorophenol by *P putida* A(a) is shown in Figure 11 2-chlorophenol degradation stopped after 71 hours Degradation of the chlorophenols was incomplete as indicated by the non-stoichiometric release of chloride The pattern of chloride release for 2- chlorophenol mirrored the pH drop observed The initial chloride release was accompanied by a corresponding drop in pH of approximately 0 2 pH units which reached a plateau as the chloride release also reached completion 1 56 mM 2-chlorophenol was degraded by *P putida* CP1 within 94 hours (Fig 11), with a stoichiometric release of chloride indicating complete degradation Chloride release was accompanied by a decrease in the pH of the culture medium of approximately 0 16 pH units

3-chlorophenol

The degradation of 3-chlorophenol by *P putida* A(a) was accompanied by a non-stoichiometric release of chloride, which indicated that chlorophenol was not metabolised to completion (Fig 11) Metabolism of 3-chlorophenol by *P putida* A(a) came to a stop after 192 hours The pattern of chloride release for 3-chlorophenol mirrored the drop in pH The initial chloride release was accompanied by a corresponding drop in pH of approximately 0 2 pH units which reached a plateau as the chloride release also reached completion 3-chlorophenol was less efficiently degraded by *P putida* CP1 than 2-chlorophenol *P putida* CP1 was capable of the complete degradation of 1 56 mM 3-chlorophenol within 240 hours Degradation resulted in a stoichiometric release of chloride which was concurrent with chlorophenol removal and resulted in a pH drop of 0 16 units

4-chlorophenol

Of the mono-chlorophenol isomers, 4-chlorophenol was the only one to be degraded to completion by *Pseudomonas putida* A(a) 1 56 mM 4-chlorophenol disappeared from the medium within 50 hours and there was a stoichiometric release of chloride within 115 hours The pH dropped by approximately 0 25 pH units after 80 hours and remained constant thereafter 4-chlorophenol degradation by *P putida* CP1 was the most efficient *P putida* CP1 completely degraded 1 56 mM 4-chlorophenol within 33 hours, faster than 2-chlorophenol (94 hours) and 3-chlorophenol (240 hours) Chlorophenol removal was accompanied by stoichiometric releases of chloride indicating complete degradation of 1 56 mM 4-chlorophenol Degradation of 1 56 mM resulted in a pH drop of 0 13 units

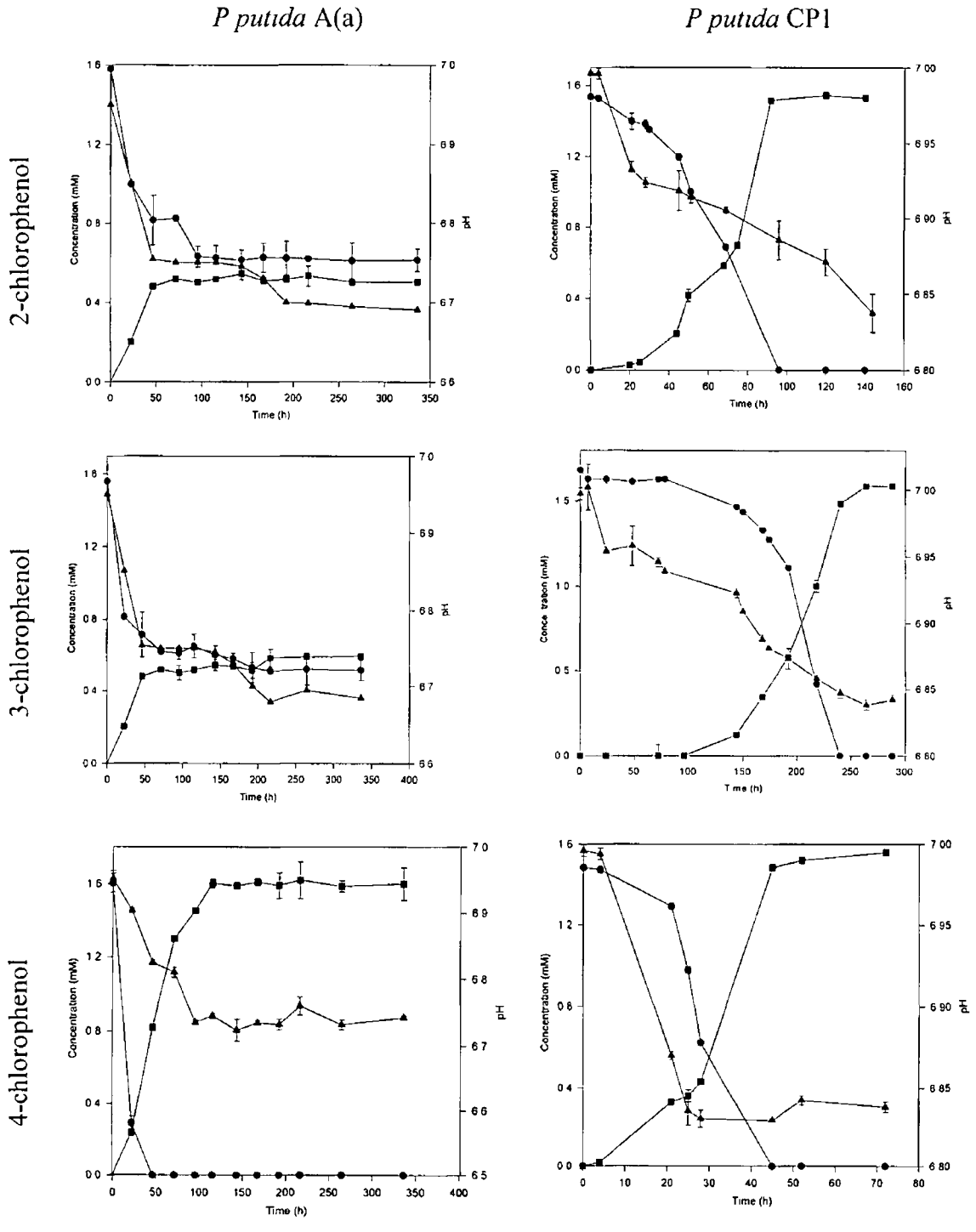

Figure 11 The degradation of 2, 3 and 4-chlorophenol (1 56 mM) by *P putida A(a)* and *P putida* CP1 Symbols ● 4-chlorophenol, ■chloride release and ▲ pH

3 1 2 Ring cleavage enzyme activities, colour production and cell morphology during the degradation of 2-, 3- and 4- chlorophenol by *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1

3-chlorocatechol is a known intermediate of the degradation of 2- and 3-chlorophenol and 4-chlorocatechol is a known intermediate in the degradation of 4-chlorophenol The key enzymes involved in catalysing ring fission of 3-chlorocatechol and 4-chlorocatechol (chlorocatechol 1,2-dioxygenase and catechol 2,3-dioxygenase) were assayed (Table 6) The presence of 1,2-dioxygenase indicates the presence of *ortho-* cleavage activity and the presence of 2,3-dioxygenase indicates the presence of *meta-* cleavage activity Colour production and morphology was also monitored during degradation of the mono-chlorophenols in pure culture by both organisms The *meta-* cleavage pathway of monochlorophenols is known to give rise to coloured intermediates, while the modified *ortho-* cleavage pathway is not known to result in coloured metabolites

P putida CP1 did not display *meta*-cleavage activity towards any of the substrates assayed when grown on 2-, 3-, and 4-chlorophenol, indicating the absence of any meta-cleavage activity in the organism There was *ortho*-cleavage activity against catechol when grown on all 3 monochlorophenols When grown on 2- and 3-chlorophenol, there was modified *ortho*-cleavage activity against 3-chlorocatechol and when grown on 4-chlorophenol there was modified *ortho*-cleavage activity against 4-chlorocatechol, indicating that degradation of monochlorophenols was via a modified *ortho*-cleavage pathway by *P putida* CP1

The growth of *P putida* CP1 on each of the mono-chlorophenol isomers resulted in the formation of large flocs of cells in the culture medium (Fig 12) Growth of *P putida* CP1 on 4-chlorophenol resulted in numerous smaller clumps throughout the culture medium, while growth on 2- and 3-chlorophenol resulted in fewer but larger flocs Focculation occurred during the lag period of chlorophenol degradation and chlorophenol degradation did not proceed until *P putida* CP1 formed clumps in culture medium There was no production or accumulation of coloured intermediates in the growth medium

during the degradation of mono-chlorophenols by *P putida* CP1 (Fig 12) which is typical of the modified *ortho*- cleavage pathway

P putida A(a) did not display *ortho*-cleavage activity towards any of the substrates assayed when grown on 2-, 3-, and 4-chlorophenol, indicating the absence of any *ortho*-cleavage activity in the organism (Table 6) There was *meta*-cleavage activity against catechol when grown on all 3 monochlorophenols No catechol 2, 3-dioxygenase activity was detected during 2- and 3-chlorophenol degradation by *P putida* A(a) as activity is irreversibly inactivated by 3-chlorocatechol There was catechol 2, 3-dioxygenase activity against 4-chlorocatechol when grown on 4-chlorophenol, indicating that degradation of 4-chlorophenol proceeded via a *meta*-cleavage pathway in this organism

Experiments carried out into the degradation of 2- and 3-chlorophenol by *P putida* A(a) had shown the accumulation of brown/black coloured intermediates in the culture medium (Fig 12) Metabolism of 2- and 3-chlorophenol caused an accumulation of this degradative intermediate in the culture medium typical of the coloured polymers produced as a result of autooxidation of 3-chlorocatechol, an intermediate in the degradation of 2- and 3-chlorophenol Together with the non-stoichiometric release of chloride during the degradation of 2- and 3-chlorophenol, these phenomena were typical of the ring cleavage of 3-chlorocatechol by the *meta*- cleavage pathway (Bartels *et al*, 1984) Enzyme assays had already confirmed that degradation of 4-chlorophenol by *P putida* A(a) proceeded via a *meta*- cleavage pathway During the degradation of 4-chlorophenol, a lime/yellow colour accumulated in the medium (Fig 2) The lime/yellow colour with an absorption maximum of 379nm at pH 7 0 and 334nm at pH 2 0 (Fig 13) disappeared reversibly upon acidification and was typical of 5-chloro-2-hydroxymucomc semialdehyde, known to be the *meta*- cleavage product of 4-chlorocatechol, an intermediate in the degradation of 4-chlorophenol

However, unlike the brown/black colour produced during 2- and 3-chlorophenol degradation, this lime/yellow colour did not remain in the medium During the

degradation of 4-chlorophenol, the lime/yellow colour became visible within 2 hours, developed to form a straw yellow colour and finally disappeared after 340 hours, suggesting further metabolism of the yellow intermediate.

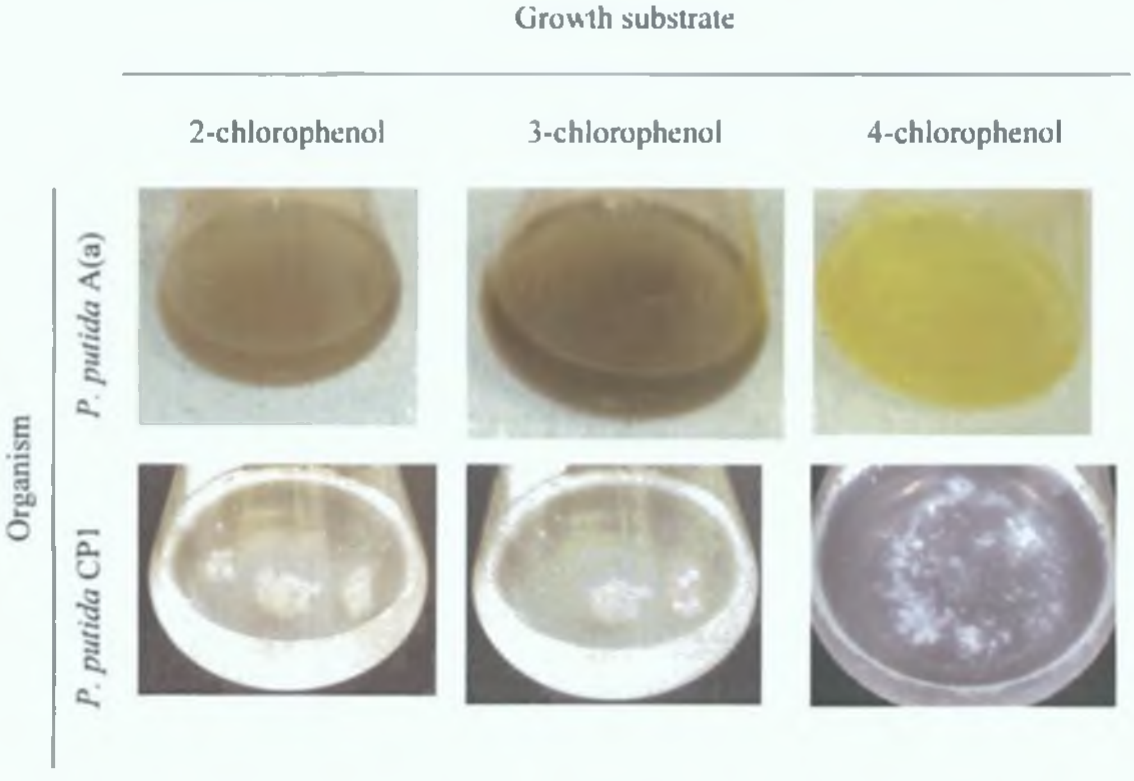

Figure 12: Morphology and colour production following 48 hours growth of *P. putida* A(a) and *P. putida* CP1 on 1.56 mM mono-chlorophenol.

Table 6 Dioxygenase activites in *P putida* A(a) and CP1 following growth on 1 56 mM monochlorophenol isomers at 30°C for 24 hrs

		Specific Activity (U/mg) Substrate					
		catechol		4-chlorocatechol		3-chlorocatechol	
Growth Substrate (24 hr)	Enzyme	*P putida* A(a)	*P putida* CP1	*P putida* A(a)	*P putida* CP1	*P putida* A(a)	*P putida* CP1
2-chlorophenol	Chlorocatechol 2, 3-dioxygenase	9 75	<0 01	<0 01	<0 01	<0 01	<0 01
	Chlorocatechol 1, 2-dioxygenase	<0 01	47 63	<0 01	n/d	<0 01	16 41
3-chlorophenol	Chlorocatechol 2, 3-dioxygenase	11 24	<0 01	<0 01	<0 01	<0 01	<0 01
	Chlorocatecol 1, 2-dioxygenase	<0 01	51 3	<0 01	n/d	<0 01	14 25
4-chlorophenol	Chlorocatechol 2 3-dioxygenase	10 185	<0 01	17 57	<0 01	<0 01	<0 01
	Chlorocatecol 1, 2-dioxygenase	<0 01	53 84	<0 01	18 52	<0 01	n/d

n/d = not determined

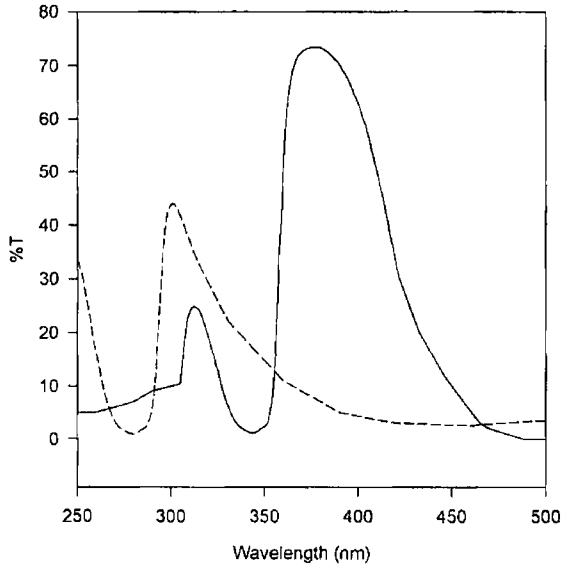

Figure 13 UV spectrum, at pH 7 00 —— and pH2------ resulting from the development of yellow coloration of the culture medium during degradation of 4-chlorophenol by *Pseudomonas putida* A(a)

The development of 5-chloro-2-hydroxymuconic semialdehyde following the degradation of 4-chlorophenol by *P putida* A(a) was monitored by measurement of the optical density at 380nm and is shown in Figure 14 Optical density measurements rose sharply in the initial 24 hours, reached a peak and began to drop corresponding to the development and disappearance of the yellow colour and the release of chloride into the culture medium The further metabolism of 5-chloro-2-hydroxymuconic semialdehyde was demonstrated by the fall in OD 380nm measurements, the disappearance of the lime/yellow colour and the stoichiometric releases of chloride in the presence of *P putida*

A(a) When 5-chloro-2-hydroxymuconic semialdehyde was incubated following the removal of *P putida* A(a) cells by filtration, its disappearance from the culture fluid was significantly lower (Fig 14), while the yellow colour remained in the culture medium indicating that removal in the presence of *P putida* A(a) was not due to spontaneous decomposition Unlike *P putida* CP1, *P putida* A(a), did not form flocs in the presence of monochlorophenols

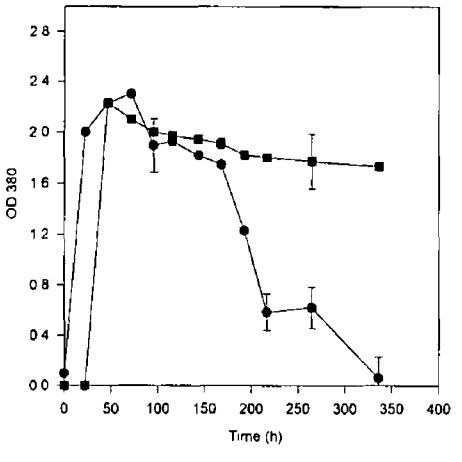

Figure 14 Development and disappearance of 5-chloro-2-hydroxymuconic semialdehyde during the degradation of 4-chlorophenol by *P putida* A(a) ● and its disappearance in the absence of any microbial inoculum ■

3 2 Genetic characterisation of *P putida* A(a) and *P putida* CP1

It was of interest to confirm the presence of the genes for catechol 2, 3-dioxygenase, the key enzyme of the *meta*-cleavage pathway in *P putida* A(a) and catechol 1,2-dioxygenase, the key enzyme of the modified *ortho*-cleavage

pathway in *P putida* CP1 It was also of interest to characterise the genes and to investigate the location of the genes

3 2 1 Investigation of the presence of dioxygenase genes

The presence of the genes for catechol 2,3-dioxygenase and catechol 1,2-dioxygenase was investigated using PCR followed by sequence analysis

PCR amplification

The primers used recognised evolutionarily conserved DNA sequences within their respective dioxygenase genes which allowed for their use in the detection of a wide range of diverse dioxygenase genes

PCR products are shown in Figure 15 *P putida* mt-2, with the catechol 2,3-dioxygenase gene *xyl*E, was used as a positive control for the PCR amplification of catechol 2,3- dioxygenase In the case of *P putida* mt-2 a PCR product of the expected size, 238 bp, was obtained (Lane 4) A PCR product of similar size was obtained for *P putida* A(a) (Lane 5) suggesting the presence of a catechol 2,3-dioxygenase gene Chlorocatechol 1,2-dioxygenase was not amplified from A(a) (lane 6), indicating the lack of the gene in A(a)

A PCR product of 261bp was obtained from *Ralstonia eutropha* JMP134 as expected (Lane 2) *Ralstonia eutropha* JMP134 posseses the chlorocatechol 1,2-dioxygenase gene, *tfd*C A similar size product was obtained from *P putida* CP1 (Lane 3) suggesting the presence of a chlorocatechol 1,2-dioxygenase gene in *P putida* CP1 Chlorocatechol 1,2-dioxygenase was not amplified from A(a) (lane 7), indicating the lack of the gene in CP1

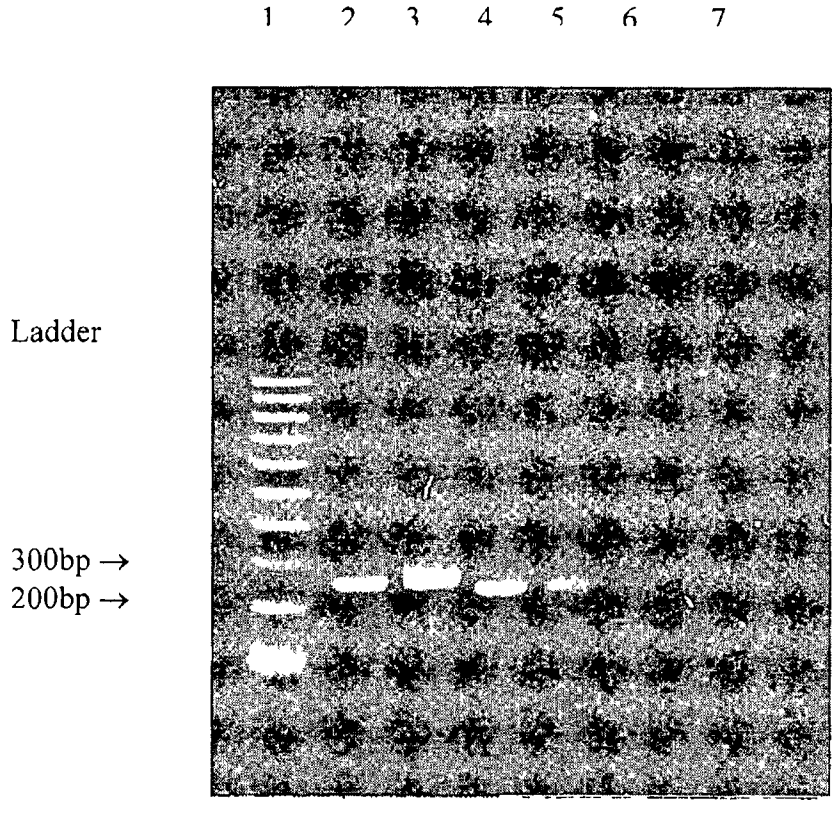

Fig 15 Agarose gel electrophoresis of PCR products

Lane 1	100-bp DNA ladder
Lane 2	chlorocatechol 1,2-dioxygenase PCR products amplified from *R eutropha* JMP134 pJP4
Lane 3	chlorocatechol 1,2-dioxygenase PCR products amplified from *P putida* CP1
Lane 4	catechol 2,3-dioxygenase PCR products amplified from *P putida* mt-2
Lane 5	catechol 2,3-dioxygenase PCR products amplified from *P putida* A(a)

Sequence analysis of PCR products .

To confirm that the PCR products obtained from *P. putida* A(a) and *P. putida* CP1 were indeed amplified from the catechol 2,3-dioxygenase gene in *P. putida* A(a) and the catechol 1,2-dioxygenase gene in *P. putida* CP1, the PCR products were sequenced. Comparison of the sequences obtained from *P. Putida* A(a) and *P. putida* CP1 with dioxygenase genes of known sequence confirmed the identity of the PCR products .The partial nucleotide sequence of catechol 2,3-dioxygenase from *P. putida* A(a) exhibited 95%, 86% and 84% homology with analogous enzymes encoded by *dmp*B from the *P. putida* CF600 plasmid pVI150, nahH from the *P. putida* plasmid pNAH7 and *xyl*E from the *P. putida* mt-2 plasmid pWWO respectively (Fig. 16A). Homology at the amino acid level with the same organisms was 100%, 89% and 89% respectively (Fig 16B).

The partial nucleotide sequence of chlorocatechol 1,2-dioxygenase from *P. putida* CP1 exhibited 99% and 68% homology with analogous enzymes encoded by *clc*A from the *P. putida* AC866 plasmid pAC27 and *tfd*C from the *R. eutropha* JMP134 plasmid pJP4 respectively (Fig. 16C). Homology at the amino acid level, with the same organisms was 100% and 67% respectively (Fig. 16D).

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Aa          1 TCGACATAGGCCCGACCCGCCACGGCCTGACCCATGGCAAGACCATTTACTTCTTCGACC
dmpB      756 TCGACATAGGCCCGACCCGCCACGGCCTGACTCACGGCAAGACCATCTACTTCTTCGACC
nahH      728 TCGATATCGGCCCGACCAGGCACGGCCTGACTCAGGGCAAGACCATTTATTTCTTCGACC
xylE      764 TCGATATCGGCCCAACCCGCCACGGCCTCACTCACGGCAAGACCATCTACTTCTTCGACC
              **** ** ***** *** * ******** ** ** ********** ** **********

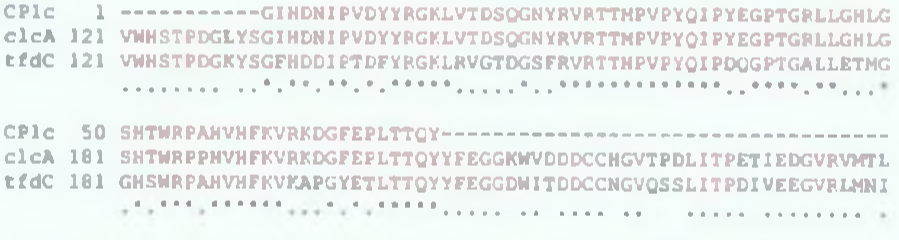
Aa         61 CCTCGGGCAACCGCAACGAGGTGTTCTGCGGTGGCGATTACAACTACCAGGATCACAAGC
dmpB      816 CTTCGGGCAACCGCAACGAGGTGTTCTGTGGCGGCGATTACAACTACCAGGACCACAAGC
nahH      788 CGTCCGGCAACCGCTGCGAGGTGTTCTGCGGCGGGAATTACAACTATCCGGATCATAAGC
xylE      824 CGTCCGGTAACCGCAACGAAGTGTTCTGCGGGGGAGATTACAACTACCCGGACCACAAAC
              * ** ** ******  *** ******** ** **  ********** * *** ** ** *

Aa        121 CCGTGACCTGGCTGGCCAAGGACCTGGGCAAGGCCATTTTTTACCACGACCGGGTGCTCA
dmpB      876 CCGTGACCTGGCTGGCCAAGGACCTGGGCAAGGCCATTTTTTACCACGACCGGGTGCTCA
nahH      848 CGGTGACTTGGTTGGCCAAGGATGTGGGCAAGGCGATCTTCTATCACGACCGGGTGCTCA
xylE      884 CGGTGACCTGGACCACCGACCAGCTGGGCAAGGCGATCTTTTACCACGACCGCATTCTCA
              * ***** ***    ** *  *  ********** ** ** ** ********  * ****
```

A DNA sequence alignment of the PCR product amplified from *P. putida* A(a).

B Amino acid sequence alignment of the deduced amino acid sequence of the PCR product amplified from *P. putida* A(a).

C DNA sequence alignment of the PCR product amplified from *P. putida* CP1.



D Amino acid sequence alignment of the deduced amino acid sequence of the PCR product amplified from *P. putida* CP1.

Fig 16 (A) DNA sequence alignment of catechol 2,3-dioxygenases from *P putida* A(a), dmpB from the *P putida* CF600 plasmid pVI150, nahH from the *P putida* plasmid pNAH7 and xylE from the *P putida* mt-2 plasmid pWWO **(B)** The corresponding amino acid sequence alignment The nucleotide and ammo acid sequences which are conserved among catechol 2,3-dioxygenases are indicated by asterisks **(C)** DNA sequence alignment of chlorocatechol 1,2-dioxygenases from *P putida* CP1, *clcA* from the *P putida* AC866 plasmid pAC27 and *tfdC* from the *R eutropha* JMP134 plasmid pJP4 **(D)** The corresponding amino acid sequence alignment The nucleotide and amino acid sequences which are conserved among catechol 1,2-dioxygenases are indicated by asterisks

Phylogenetic tree

The complete nucleotide sequence of the chlorocatechol 1,2-dioxygenase from *Pseudomonas putida* CP1 has been obtained and this was used to create a phylogenetic tree to classify the dioxygenase enzyme from CP1 in relation to other known dioxygenase enzymes The phylogenetic tree obtained is shown in Figure 17

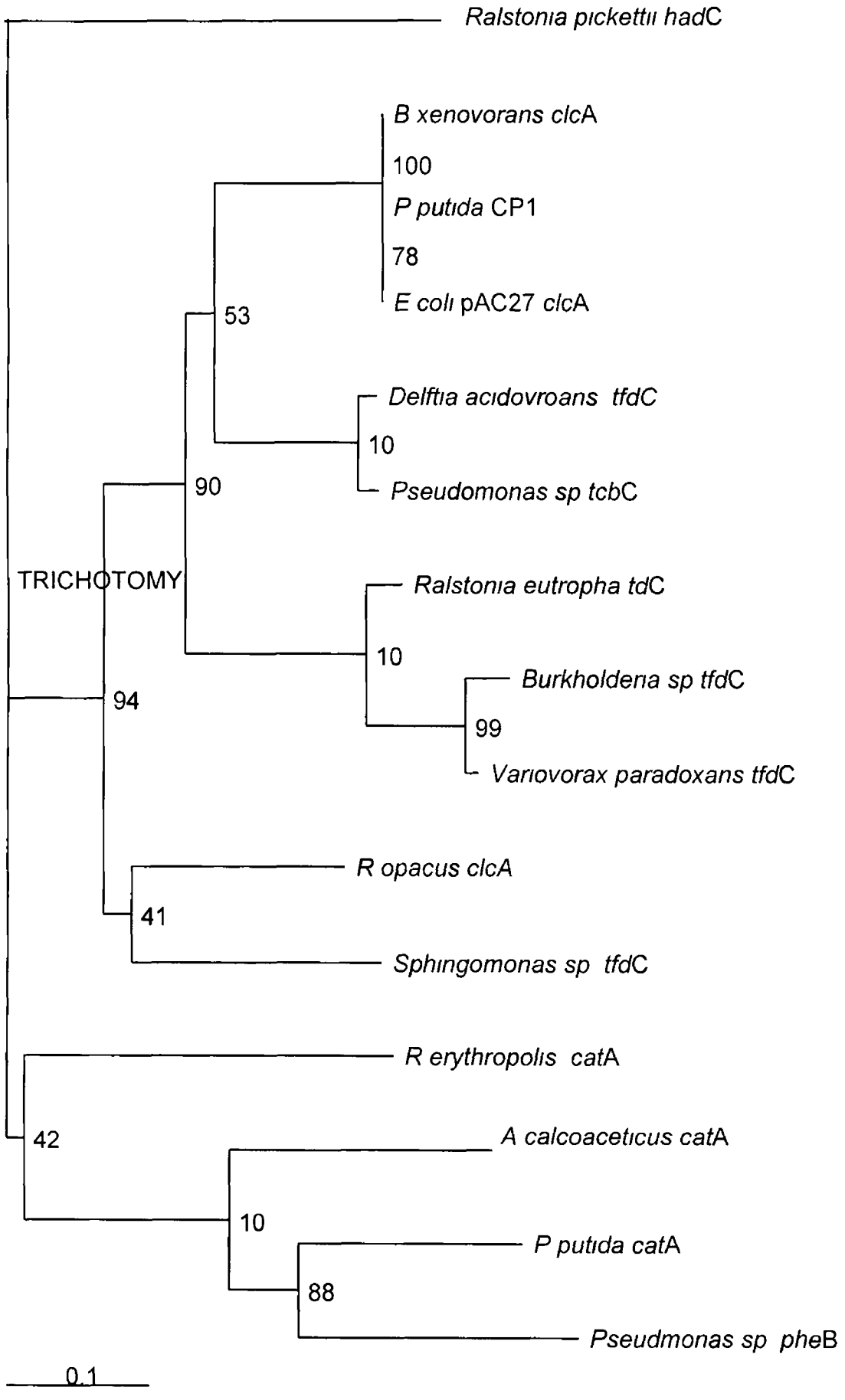


Figure 17 The phylogenetic tree shows an alignment comparing the 1,2-dioxygenase gene from *P putida* CP1 with the *clc* A from *R opacus* 1CP (X99622), *E coli* pAC27 (M16964), *Burkholderia xenovorans* (YP_559868) *tfd*C from *Pseudomonas sp* pEST4011 (U32188), *Ralstonia eutropha* (M35097), *Sphingomonas* sp (AF068242), *Variovorax paradoxans* (AF068237), *Burkholderia* sp (AF068238), *Delftia acidovorans* (AAM76776), *cat*A of *A calcoaceticus* (Z36909), *P putida* (U12557), *phe*B of *Pseudomonas* sp EST1001 (M57500) and the *had*A from *Ralstonia picketti*

3 2 2 Determination of the location of the genes

Since chlorocatechol 1,2-dioxygenase and catechol 2,3-dioxygenase genes are often found on large degradative plasmids, the presence of such plasmids was investigated Restriction analysis followed by DNA probing was used to identify the location of the genes on the plasmid

The plasmid profiles of the organisms

Plasmid DNA was isolated and separated by agarose gel electrophoresis (Fig 18) Marker strains were used to establish the approximate size of the plasmids The marker strains used were *R eutropha* JMP134 (lane 1) plasmid pJP4 (78 kb) and *P putida* mt-2 (lane 4) plasmid pWWO (117 kb) A cured strain of *R eutropha* JMP134 was used as a chromosomal marker (lane 1) Examination of the plasmid profiles obtained from both *P putida* A(a) (lane 5) and *P putida* CP1 (lane 3) confirmed the presence of a single large plasmid band in both strains The size of the plasmid in *P putida* A(a) was estimated to be under 117kb while that in *P putida* CP1 was over 117kb, indicating that both plasmids were different in regard to molecular weight

Restriction Analysis

The plasmid DNA from each organism was purified by cesium chloride density centrifugation and subjected to restriction analysis Single digests were carried out using *Eco*R I, *Bam*H I, *Hin*d III and *Xho* I Double digests were carried out using combinations of *Bam*H I, *Hin*d III, and *Xho* I with *Eco*R I

*Eco*R I digests of pWW0 containing the *xyl*E gene from the bacterium *P putida* mt-2 was used as a control for catechol 2,3-dioxygenase and from pJP4 containing the *tfd*C gene for chlorocatecolcatechol 1, 2-dioxygenase from the bacterium *Ralstonia eutropha* JMP134

1 2 3 4 5

Plasmid bands →

Chromosomal DNA→

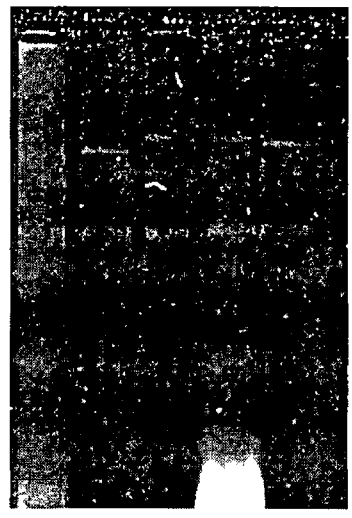


Fig 18 Profiles of plasmid DNA isolated from CP1 and A(a)

Lane 1	*R eutropha* cured strain	
Lane 2	*R eutropha* JMP134 pJP4	(78kb)
Lane 3	*P putida* CP1	(>117kb)
Lane 4	*P putida* mt-2 pWWO	(117kb)
Lane 5	*P putida* A(a)	(<117kb)

Uncut plasmid DNA from each organism together with the restricted DNA was visualised on a 0 7 % agarose gel with lambda size markers in the range 2kb to 33 5kb The restriction profile of *P putida* A(a) is shown in Figure 19 and the restriction profile of *P putida* CP1 is shown in Figure 20

The large plasmids from both organisms were successfully cut with all the enzymes used and the DNA restriction fragments generated ranged in size from approximately 33kb to less than 2kb

The restriction fragment patterns were complex with large numbers of fragments being produced and as anticipated, the number of fragments varied depending on

the restriction enzymes used The absence of homology between restriction fragment patterns from the purified plasmid DNA from both strains A(a) and CP1 was further evidence that the plasmids were not the same

Hybridisation Studies

A chlorocatechol 2,3-dioxygenase probe comprising the 238 bp PCR product amplified from the gene for chlorocatechol 2,3-dioxygenase in *P putida* A(a) and a chlorocatechol 1,2-dioxygenase probe comprising the 261 bp PCR product amplified from the gene for chlorocatechol 1,2-dioxygenase in *P putida* CP1 were prepared

The probe for chlorocatechol 2,3-dioxygenase hybridized with the large plasmid band from uncut plasmid DNA from *P putida* A(a) (lane 2, Figure 19B) and to a number of restriction fragments (lanes 3 to 9) The probe also hybridized to the plasmid DNA from the TOL plasmid, pWW0 (lane 10) From these results it could be concluded that the large plasmid present in *P putida* A(a) encoded the gene for chlorocatechol 2,3-dioxygenase

The probe for chlorocatechol 1,2-dioxygenase hybridized with the large plasmid band from uncut plasmid DNA from *P putida* CP1 (lane 2, Figure 20B) and to a number of restriction fragments (lanes 3 to 9) The probe also hybridized to the plasmid DNA from the plasmid pJP4 (positive control containing the chlorocatechol 1,2-dioxygenase gene *tfdC*) (lane 10) From these results it could be concluded that the large plasmid present in *P putida* CP1 encoded the gene for chlorocatechol 1,2-dioxygenase

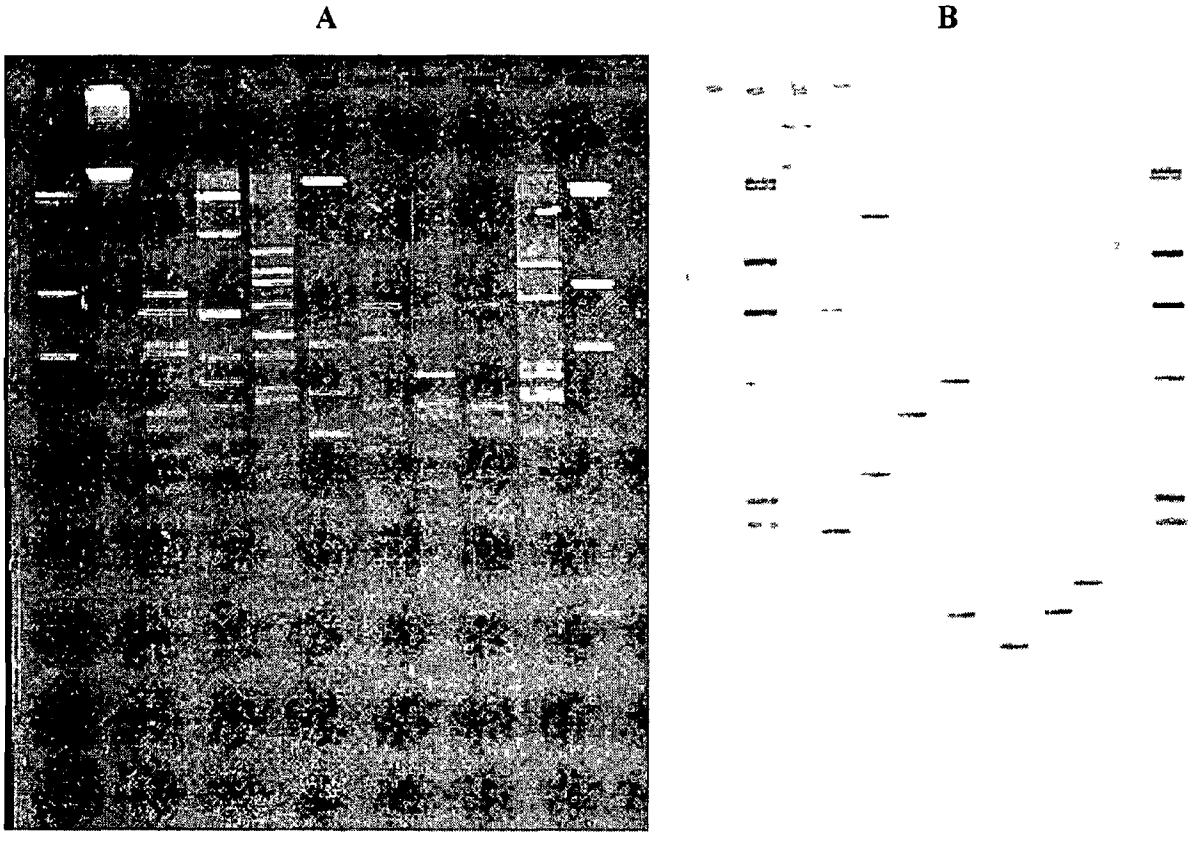


Figure 19 Restriction analysis of purified plasmid DNA from *Pseudomonas putida* A(a) **(A)**, and **(B)** Hybridyzation of purified plasmid DNA from *Pseudomonas putida* A(a) with a probe for catechol 2,3-dioxygenase

Lane 1 DNA molecular weight marker

Lane 2 uncut A(a) plasmid

Lane 3 A(a) plasmid + *Eco*R I

Lane 4 A(a) plasmid + *Bam*H I

Lane 5 A(a) plasmid + *Hind* III

Lane 6 A(a) plasmid + *Xho* I

Lane 7 A(a) plasmid + *Eco*R I + *Bam*H1

Lane 8 A(a) plasmid + *Eco*R I + *Hind* III

Lane 9 A(a) plasmid + *Eco*R I + *Xho* I

Lane 10 pWWO + *Eco*R1

Lane 11 DNA molecular weight marke

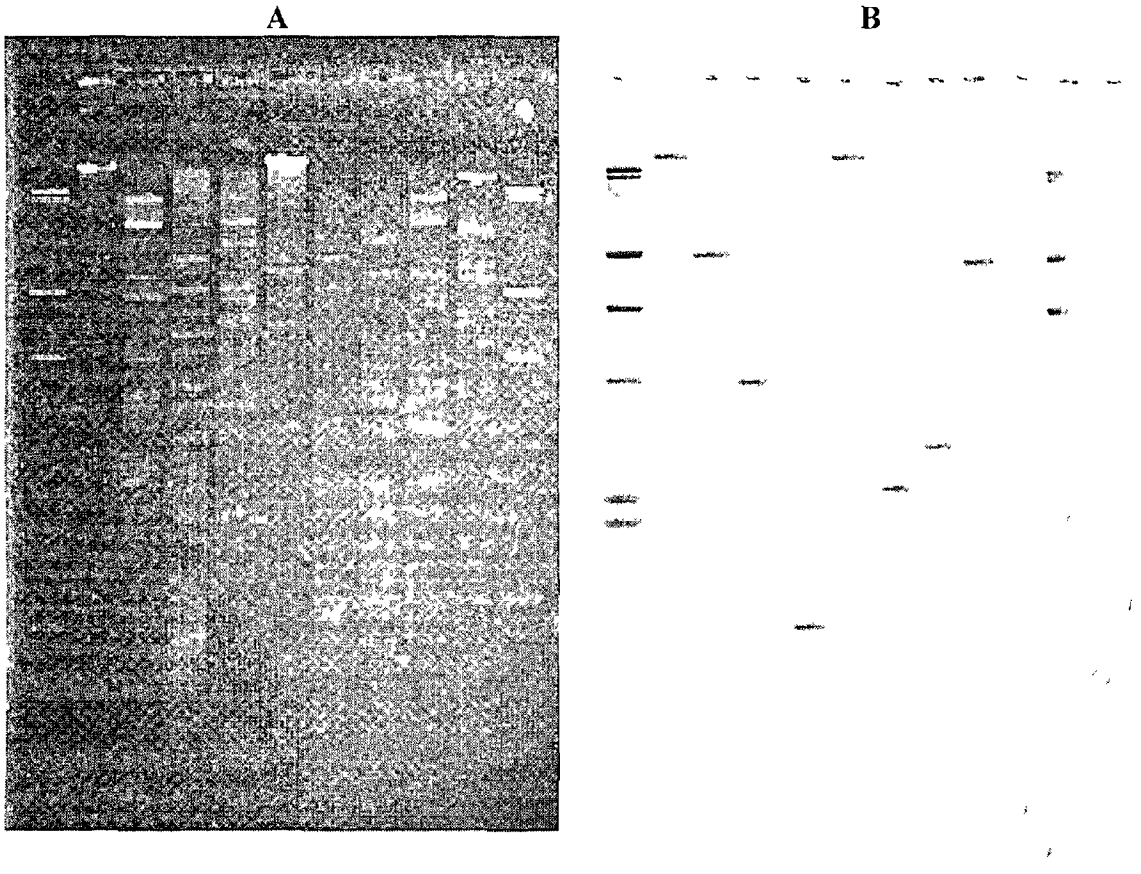


Figure 20 Restriction analysis of purified plasmid DNA from *Pseudomonas putida* CP1 **(A)**, and **(B)**, Hybridyzation of purified plasmid DNA from *Pseudomonas putida* CP1 with a probe for chlorocatechol 1,2-dioxygenase

Lane 1	DNA molecular weight marker
Lane 2	uncut CP1 plasmid
Lane 3	CP1 plasmid + *Eco*R I
Lane 4	CP1 plasmid + *Bam*H I
Lane 5	CP1 plasmid + *Hind* III
Lane 6	CP1 plasmid + *Xho* I
Lane 7	CP1 plasmid + *Eco*R I + *Bam*H I
Lane 8	CP1 plasmid + *Eco*R I + *Hind*III
Lane 9	CP1 plasmid + *Ec* R I + *Xho* I
Lane 10	pJP4 + *Eco*R I
Lane 11	DNA molecular weight marker

3 3 Tagging of *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1 with Green Fluorescent Protein

To facilitate tracking of these strains following addition to activated sludge, green fluorescent protein (GFP) was chosen as a marker Once inserted into the bacterial chromosome, the gene for GFP can be used as a marker to visually distinguish introduced GFP-labelled bacteria from indigenous bacteria in any given environment

3 3 1 Tagging of *P putida* A(a) and *P putida* CP1 with *gfp* UV using a Tn*5*-based delivery vector

Chromosomal insertion of the gene for GFP UV was studied using a Tn*5* based delivery vector, pTGN *gfp* UV was a mutated GFP gene that was optimised for fluorescence under a long wave handheld UV light *E coli* S17 11 λ *pir* was the host for plasmid pTGN that contained the transposon Tn5 Transposon Tn*5* contained promoterless genes for GFP UV, kanamycin and gentamicin resistance Transfer of plasmid pTGN to the recipient strains, *P putida* A(a) or *P putida* CP1 was carried out by conjugation with *E coli* S17 11 λ *pir* plasmid pTGN

3 3 1 1 Transformation of *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1 with Transposon Tn*5*-*gfp* UV by conjugation

Suitable dilutions of the plate mating between the donor, *E coli* S17 11 λ *pir* plasmid pTGN and the recipient, *P putida* A(a) or *P putida* CP1 gave rise to exconjugants after 4 days on selective medium (mineral medium with 20 μg/ml kanamycin and 1 56 mM 4-chlorophenol as carbon sourceor LB agar with 20 μg/ml kanamycin and 1 5mM $FeSO_4$) After incubation for a further 2 or 3 days, single colonies expressing GFP under illumination with a long wave handheld UV light were visible Typically 200 exconjugants per plate were obtained, but of these, approximately 1 in 400 expressed *gfp* to the extent that the colony was fluorescent to the naked eye Several intensely fluorescent colonies were isolated and purified and the most intensely fluorescent was

selected for further use. The two most intensely fluorescent strains are shown growing on tryptone soy agar (TSA) supplemented with 0.45mM $FeSO_4.7H_2O$ (Fig. 21 A and B). Biofluorescent colonies that had been transformed with *gfp* UV did not fluoresce when excited at 450-490 nm under an epifluorescence microscope.

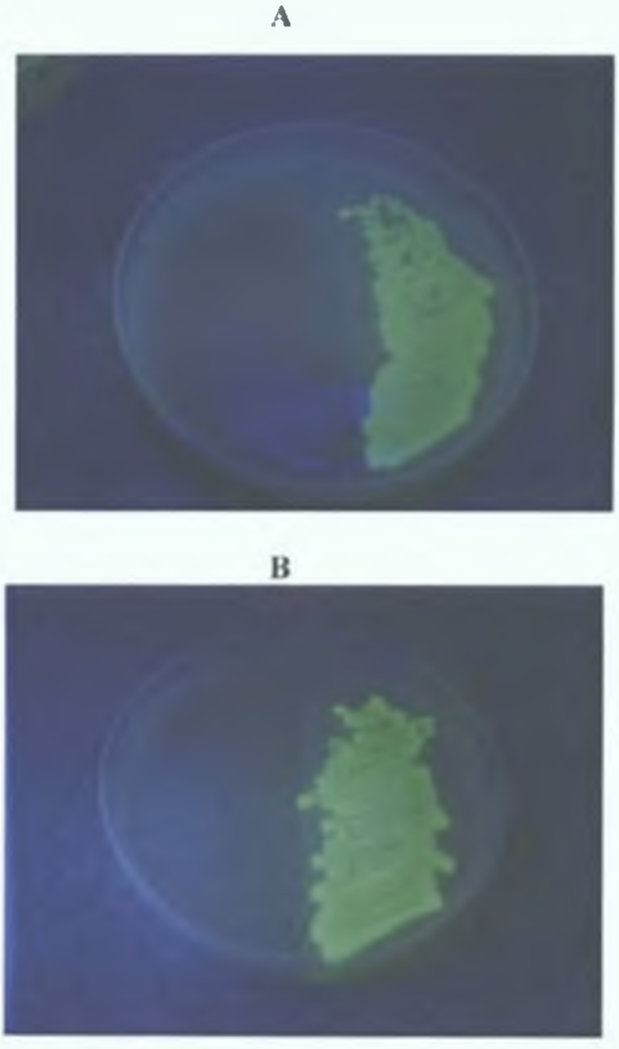


Figure 21: Parental and *gfp*-UV-transformed colonies. (A) *Pseudomonas putida* A(a) and (B) *Pseudomonas putida* CP1 cultured overnight on TSA containing 0.45mM $FeSO_4.7H_2O$ and excited under a long wave UV light (365 nm).

3 3 1 2 Confirmation of chromosomal integration of Tn*5-gfp* in *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1

Integration of Tn*5-gfp* into the genome of *P putida* A(a) and *P putida* CP1 was confirmed by PCR using two *gfp* specific primers Amplification products were analyzed using agarose gel electrophoresis and a band at approximately 720 bp was considered positive for the *gfp* fragment

Along with a 100 bp marker DNA ladder (Lanes 1and 6), PCR products were amplified from the intensely fluorescent Tn*5-gfp* UV transformed strains (Figure 22) Amplification products of 720 bp were detected in both transformed strains (Lanes 3 and 5), confirming the presence of the *gfp* fragment in these strains No amplification product was detected in the parental strain (Lanes 2 and 4), confirming the absence of *gfp* in wild type *P putida* A(a) and *P putida* CP1

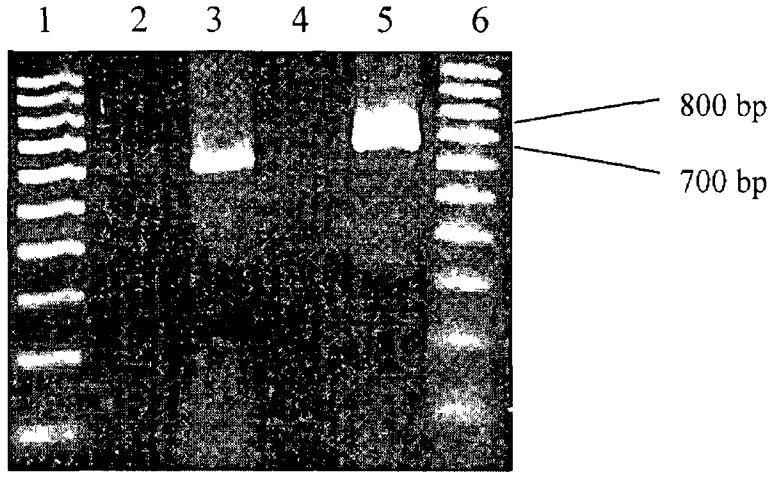


Figure 22 Gel electrophoresis of PCR amplification fragments from *P putida* A(a) and *P putida* CP1 using primers specific for the amplification of the gene for GFP

Lanes 1 and 6	100 bp marker
Lane 2	P putida A(a) wild type
Lane 3	P putida A(a) transformed with Tn5-gfp UV
Lane 4	P putida CP1 wild type
Lane 5	P putida CP1 transformed with Tn5-gfp UV

3 3 2 Tagging of *Pseudomonas putida* CP1 and *Pseudomonas putida* A(a) with Green Fluorescent Protein mut3* using a Tn*7*-based vector

In order to obtain GFP-tagged strains expressing the GFP mut3* gene, a Tn*7*-based vector was used The gene for GFP mut3* (Table 3) was optimised for fluorescence under an epifluorescence microscope

E coli XL1-Blue was the host strain for plasmid pBK-miniTn*7*-*gfp*2 that contained the mini Tn*7* transposon miniTn*7*-*gfp*2 The transposon also contained marker genes for gentamicin and chloramphenicol resistance Plasmid pBK-miniTn*7*-*gfp*2 was purified from *E coli* XL1-Blue and used to transform competent *E coli* S17-1 *E coli* S17-1 was

chosen as the donor to allow conjugative transfer of plasmid pBK-miniTn*7*-*gfp*2 into *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1 The tagging system also required the use of the helper strain *E coli* SM10 λ *pir* plasmid pUX-BF13 Plasmid pUX-BF13 provided the Tn*7* transposition functions in *trans* Following conjugation between the two donor strains, *E coli* S17-1 plasmid pBK-miniTn*7*-*gfp*2 and *E coli* SM10 λ *pir* plasmid pUX-BF13, and the recipient, either *P putida* A(a) or *P putida* CP1, putative GFP-tagged colonies of the recipient strain were isolated on plates using selective media

3 3 2 1 Transformation of *E coli* S17-1 with pBK-miniTn*7*-*gfp*2

E coli XL1-Blue was the host strain for plasmid pBK-miniTn*7*-*gfp*2 that contained the mini Tn*7* transposon miniTn*7*-*gfp*2 Plasmid DNA from this strain was isolated using plasmid DNA minipreps and used to transform chemically competent *E coli* S17-1 by heat shock Transformants were detected on selective medium containing gentamicin (20 μg/ml) A long wave UV lamp was used to detect transformants, which appeared as colonies after 2–3 days incubation and fluoresced intensely due to the high copy number of the plasmid in transformed cells One fluorescent colony of *E coli* S17-1 was isolated and further tested to confirm successful transformation with plasmid pBK-miniTn*7*-*gfp*2 Transformation of *E coli* S17-1 with plasmid pBK-miniTn*7*-*gfp*2 was confirmed by gel electrophoresis of plasmid DNA isolated from *E coli* S17-1 alongside pBK-miniTn*7*-*gfp*2 from *E coli* XL1-Blue (Fig 23) Examination of the plasmid bands confirmed that following transformation with pBK-miniTn*7*-*gfp*2 from *E coli* XL1-Blue (lane 2), *E coli* S17-1, which previously contained no plasmid DNA (lane 4), now contained a plasmid of identical size as *E coli* XL1-Blue plasmid pBK-miniTn*7*-*gfp*2 (lane 3) These results confirmed that *E coli* S17-1 was successfully transformed with plasmid pBK-miniTn*7*-*gfp*2

1 2 3 4

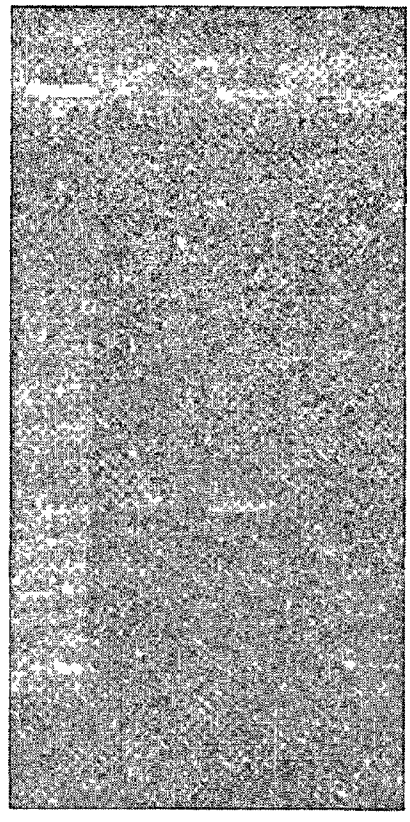


Figure 23: Plasmid profiles of pBK-miniTn*7*-*gfp*2 isolated from *E coli* XL1 Blue and transformed *E coli* S17-1

Lane 1	Marker
Lane 2	pBK-miniTn*7*-*gfp*2 isolated from *E coli* XL1 Blue
Lane 3	Transformed *E coli* S17-1
Lane 4	*E coli* S17-1

3 3 2 2 Transformation of *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1 with transposon Tn*7-gfp* by conjugation.

E coli S17-1 pBK-miniTn*7-gfp*2 was used as the donor in a triparental mating with the helper strain, *E coli* pUX-BF13 and *P putida* A(a) or *P putida* CP1 as the recipient organism to introduce the gene for GFP into the chromosome of the recipient strain via Tn7 transposition Plasmid pUX-BF13 provided the Tn*7* transposition functions in *trans*

Triparental matings were carried out on peptone yeast extract agar overnight at 30 ^{O}C Suitable dilutions of the mating mixture spread on selective mineral medium containing 4-chlorophenol as carbon source (1 56mM) and gentamicin (20ug/ml) gave rise to single colonies after 4 days A portion of a single colony was tested for fluorescence under a fluorescence microscope to confirm expression of GFP mut3* Expression of *gfp* mut3* resulted in intensely fluorescent cells that were easily visible at the single cell level (Fig 24) Following confirmation of fluorescence, colonies were streaked on to fresh selective mineral medium for further analysis Green fluorescence was not observed in the *P putida* A(a) or *P putida* CP1 parental strains These results indicated that *P putida* A(a) and *P putida* CP1 were successfully transformed with Tn*7-gfp* following conjugation When excited by a UV light (365 nm), the *gfp*mut3*-transformed colonies did not fluoresce

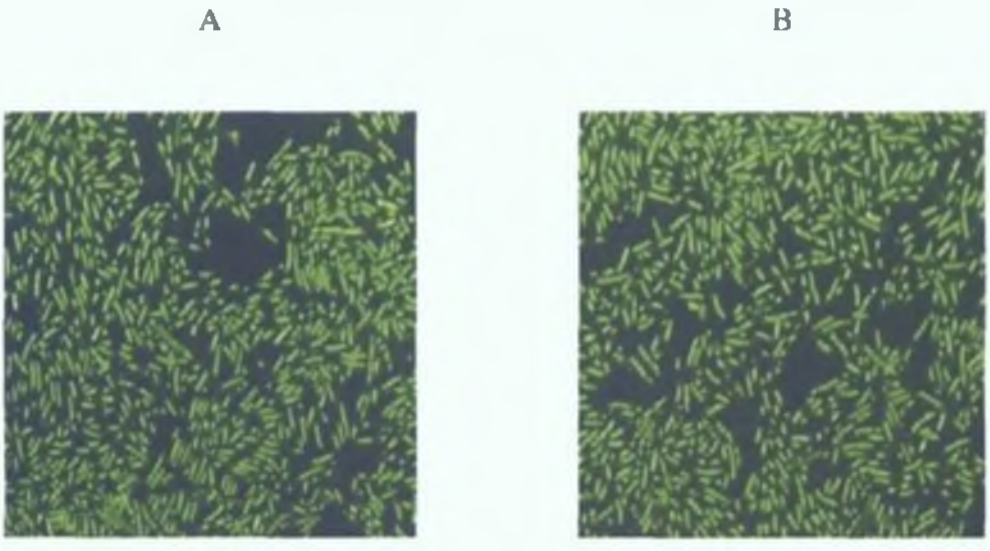


Figure 24: Epifluorescence micrographs of GFP-tagged cells. (A) *P. putida* A(a) and (B) *P.putida* CP1.

3.2.2.3 Confirmation of chromosomal integration of Tn7*gfp* in *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1.

It was important to confirm that Tn7*gfp* inserted in the chromosome of *P. putida* A(a) and *P. putida* CP1 and not on the large degradative plasmids harboured by these strains since integration into the plasmid could result in transmission of the gene for GFP via conjugation in the presence of other bacteria. It was also important to verify that *gfp* expression was a result of integration of the gene for GFP and not a result of uptake of the Tn7-*gfp* vector plasmid.

Integration of Tn7-*gfp* into the genome of *P. putida* A(a) was confirmed by PCR using two *gfp* specific primers. Amplification products were analyzed using agarose gel electrophoresis and a band at approximately 720 bp was considered positive for the *gfp*

fragment In many Gram-negative bacteria, Tn7 transposes into a specific target site on the chromosome located in the intergenic region downstream of the *glm*S gene Primers corresponding to the conserved region of *glm*S and a primer targeting the *cat* gene originating from Tn7-*gfp* were used to amplify the intergenic region between these two genes by PCR

PCR products amplified from ten independently isolated GFP expressing strains and from the parental *P putida* A(a) were separated using agarose gel electrophoresis The resulting gel is shown in Figure 5 Amplification products of 720 bp were detected in all ten GFP expressing strains (Lanes 2-11, Fig 25A), confirming the presence of the *gfp* fragment in these strains No amplification product was detected in the parental strain (Lane 12), confirming the absence of *gfp* in wild type *P putida* A(a) In *P putida* CP1, no PCR product was obtained from the parental strain while an approximately 720-bp PCR product was obtained from 10 of the corresponding *gfp*-tagged strains (Fig 25B)

3.3 2 4 Identification of the Tn7 insertion site in *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1.

Primers corresponding to the conserved region of *glm*S and a primer targeting the *cat* gene originating from Tn7-*gfp* were used to amplify the intergenic region between these two genes by PCR In *P putida* A(a), an approximately 668-bp PCR product was obtained from 10 of the *gfp*-tagged strains and no PCR product was obtained from the parental strain (Fig 26) No PCR products were obtained from *P putida* CP1 parental strains or *gfp*-tagged strains indicating that the Tn7 transposon did not integrate at the same neutral site in the chromosome as in A(a) This indicated a site specific insertion of Tn7 near the *glm*S gene in *P putida* A(a) but not in *P putida* CP1

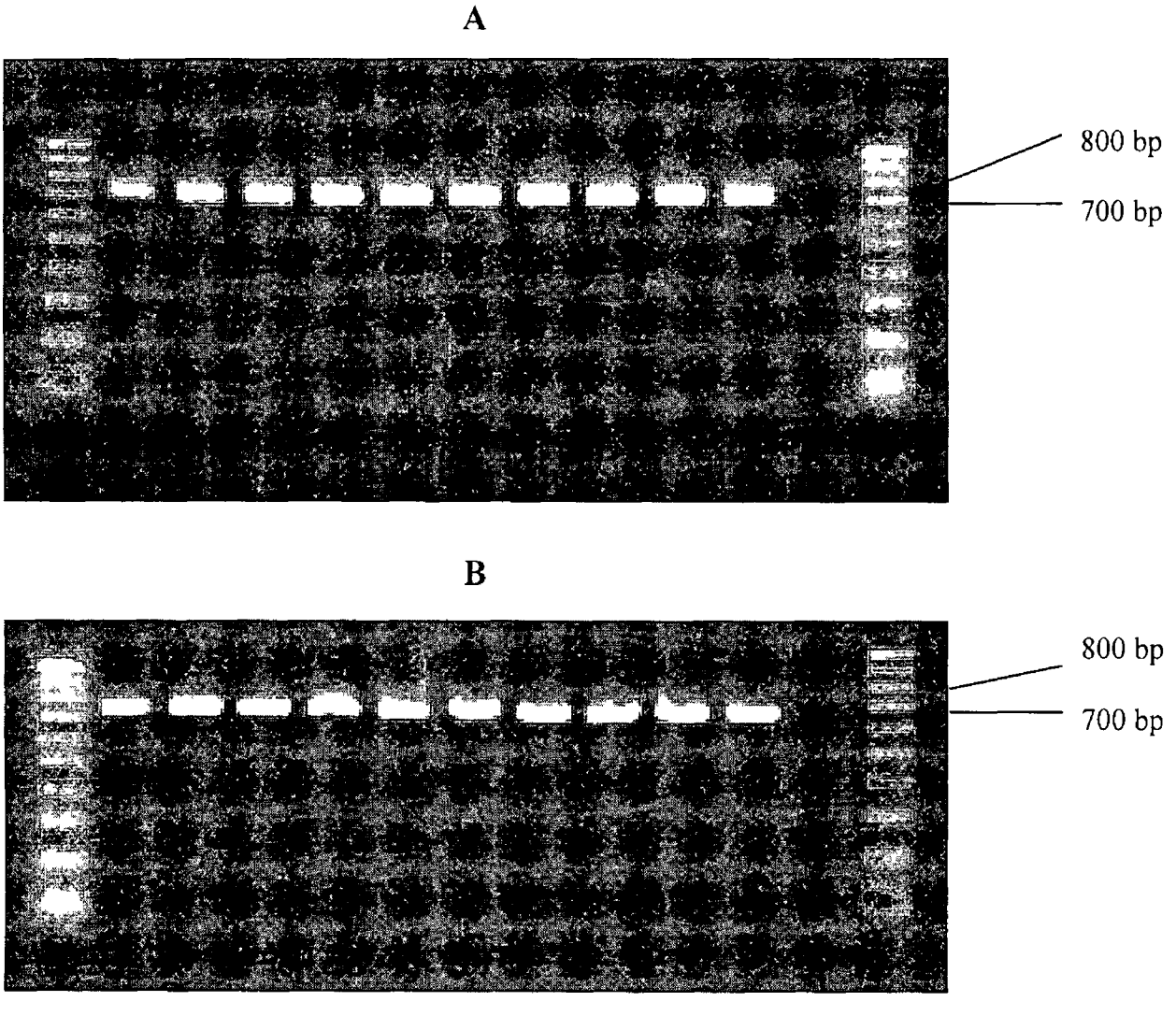


Figure 25 Agarose gel electrophoresis of PCR products amplified using *gfp*-specific primers from 10 independently obtained GFP-tagged strains of (**A**), A(a) and (**B**), CP1

Lanes 1 and 13	DNA molecular weight marker (1000 bp-100 bp)
Lanes 2-10	GFP-tagged strains 1 to10
Lane 11	parental strain/wild type

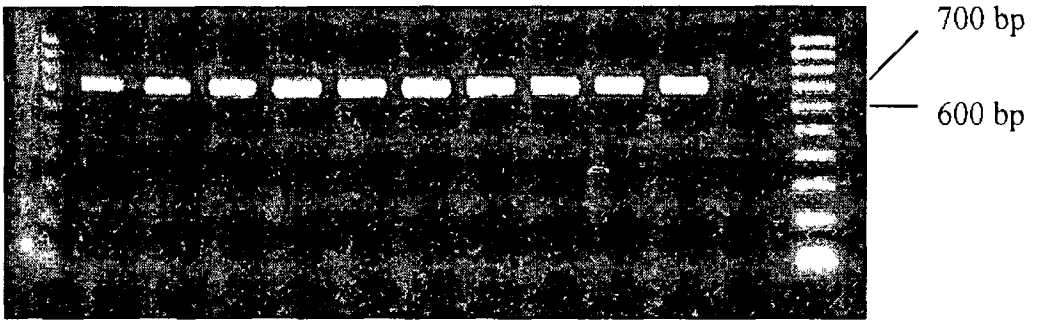


Figure 26 Agarose gel electrophoresis of 668bp PCR products amplified from *P putida* A(a) using a primer recognising *glmS* in combination with a PCR primer recognising the *cat* gene encoded by the Tn*7*-*gfp* construct

Lanes 1 and 13, DNA molecular weight marker

Lanes 1-10, A(a) gfp starins 1 to10

Lane 11, A(a) parental strain/wild type

3 3 2.5 Investigation into the specificity of insertion of Tn7-gfp in *P putida* CP1

Although site specific insertion of Tn*7* near the *glm*S gene in *P putida* A(a) had been demonstrated, this was not the case for *P putida* CP1 In order to determine whether Tn*7* inserted at a specific site or at a random site in the chromosome, restricted genomic DNA from the ten separately isolated *gfp*-transformed strains (Fig 27) was subjected to Southern hybridization using a probe for *gfp* (Fig 8) Results showed that insertion of Tn*7*-*gfp* occurred at the same site on the chromosome in all ten isolates This indicated site-specific insertion of Tn7-*gfp* in *P putida* CP1

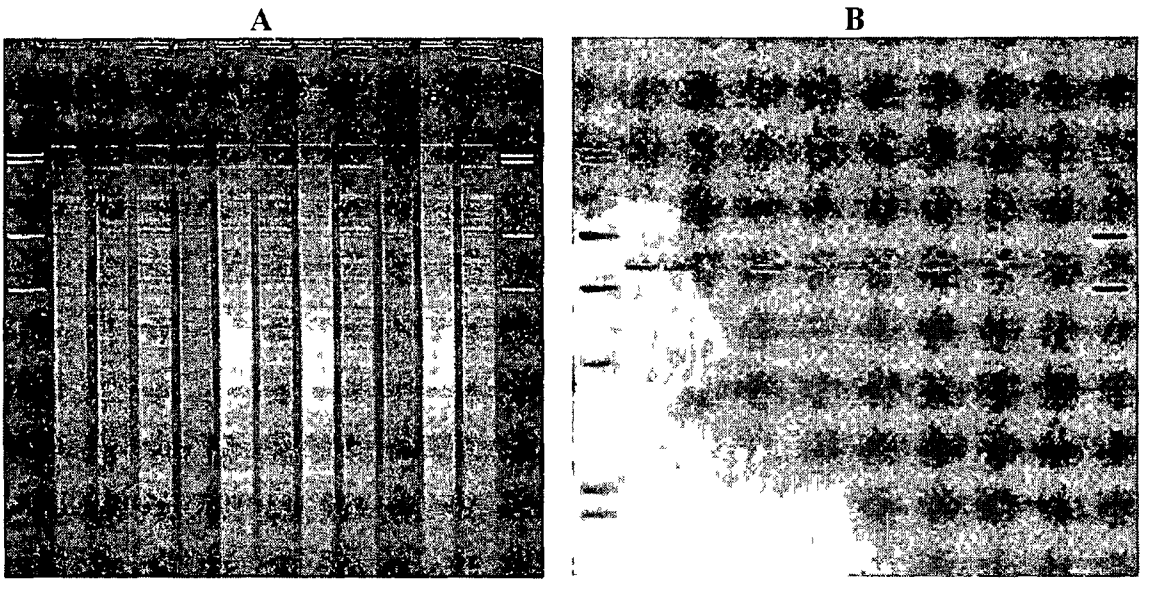


Figure 27 Genomic DNA from *P putida* CP1 cut with Sal1 (A) and (B), hybridization with a probe for *gfp*

Lanes 1 and 13	marker
Lanes 2 to 11	GFP-tagged *P putida* strains 1-10
Lane 12	*P putida* (parental strain)

3 3 2 6 Southern blot of plasmid DNA from *P putida* CP1 with a *gfp* probe

It had been established that Tn7-gfp inserted at a specific but unknown site in the genome of *P putida* CP1 It was important to verify that Tn7-*gfp* inserted into the chromosome and not into the large degradative plasmid harboured by this strain since integration into the plasmid could result in transfer of the gene for GFP via conjugation in the presence of other bacteria Plasmid DNA from *P putida* CP1 was cut with Eco R1 and Eco R1 + Xho1 and the DNA was separated by agarose gel electrophoresis (Fig 28A, lanes 2 and 3) alongside a molecular weight marker (Lane 1), uncut plasmid DNA from *P putida* CP1 (Lane 2) and the Tn7-gfp vector, plasmid pBK-miniTn7-gfp2 (Lane 5) The gel was transferred on to a nylon membrane by Southern blotting and hybridized with a *gfp*-

specific probe (Fig 9B) The probe for *gfp* hybridized with the *gfp* vector (Lane 5) but not with plasmid DNA from *P putida* CP1 (Lanes 2,3 and 4) These results indicated that the transposition of Tn7-*gfp* had occurred on to the chromosome and not the large plasmid

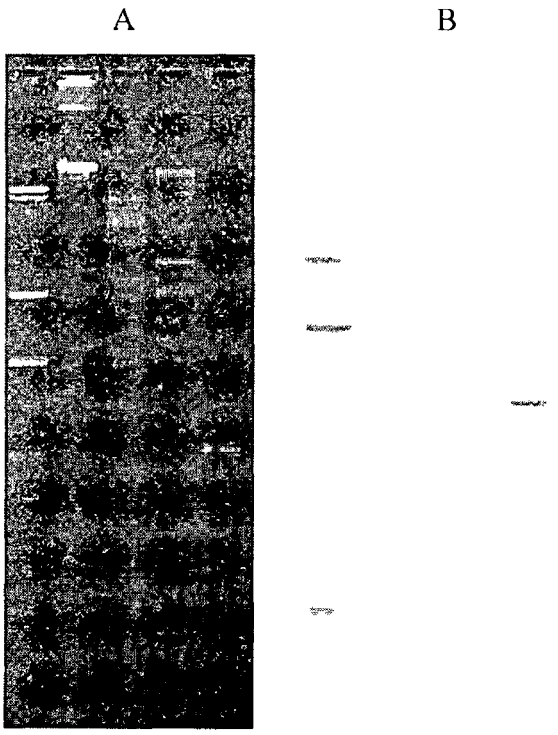


Figure 28 Agarose gel electrophoresis of plasmid DNA from *P putida* CP1 and pBK-miniTn7-*gfp2* (**A**) and Sothern blot with *gfp* probe (**B**)

Lane 1 Marker
Lane 2 uncut plasmid DNA from CP1
Lane 3 plasmid from CP1 + EcoR1
Lane 4 plasmid from CP1 + EcoR1 + XhoI
Lane plasmid pBK-mimTn7-gfp2

3 3 3 Metabolic profiles of parental and GFP-transformed A(a) and CP1

In addition to visual selection and verification of chromosomal insertion of the GFP gene, it was important to verify that the *gfp* inserts did not impair or alter the metabolic activity of the organisms The metabolic profile of each organism was tested using the Biolog GN and API 20 NE systems

3.3.3 1 Metabolism of carbon sources using the Biolog system

P putida A(a), *P putida* CP1 and the corresponding strains transformed with GFP UV and GFPmut3* were compared for their ability to utilize various carbon sources using the Biolog system The results are shown in Tables 7 and 8 The metabolic profiles obtained revealed no differences in the oxidation of 95 substrates between the GFP-tagged strains and the corresponding parental strains These results indicated that the *gfp* insert did not alter the ability of any of the strains to utilize any of the 95 carbon substrates tested

Identification using the Biolog GN system identified A(a) as being a *Pseudomonas putida* strain (biovarA) similarity value – 0 62, distance – 5 93, and probability -100% (Table 7) and CP1 as being a *Pseudomonas putida* strain, similarity value – 0 633, distance – 4 15, and probability -100% (Table 8)

3 3.3 2 Metabolic profiles using the API 20 NE system

There was no difference between the GFP-tagged and parental strains of A(a) (Table 9) and CP1 (Table 10) obtained from the API 20 NE system The two wild-type strains differed only in their ability to assimilate mannitol

Identification using API 20NE tests gave a 97 6% i d , t = 0 75, good identification for *Pseudomonas putida* A(a) and 99 9% i d , t = 0 98, very good identification for *Pseudomonas putida* CP1 strain

Table 7 Results of Biolog GN identification for wild type and GFP-tagged strains of *Pseudomonas putida* A(a)

Assimilation Test	*P. putida* A(a) wild type	*P. putida* A(a) Tn*7-gfp*	*P. putida* A(a) Tn*5-gfp*UV	Assimilation Test	*P. putida* A(a) wild type	*P. putida* A(a) Tn*7-gfp*	*P. putida* A(a) Tn*5-gfp*UV
Water	-	-	-	i-erythritol	-	-	-
α-cyclodextrin	-	-	-	D-fructose	+	+	+
Dextrin	+	+	+	L-fucose	-	-	-
Glycogen	+	+	+	D-galactose	-	-	-
Tween 40	+	+	+	Gentiobiose	-	-	-
Tween 80	+	+	+	α-D-glucose	+	+	+
N-acetyl-D-galactosamine	-	-	-	m-inositol	-	-	-
N-acetyl-D-glucosamine	-	-	-	α-D-lactose	-	-	-
Adonitiol	-	-	-	Lactulose	-	-	-
L-arabinose	-	-	-	Maltose	-	-	-
D-arabitol	-	-	-	D-mannitol	-	-	-
Cellobiose	-	-	-	D-mannose	+	+	+
D-melibiose	-	-	-	Acetic acid	+	+	+
ß-methyl D-glucoside	-	-	-	*cis*-aconitic acid	+	+	+
D-psicose	-	-	-	Citric acid	+	+	+
D-raffinose	-	-	-	Formic acid	+	+	+
L-rhamnose	-	-	-	D-galactomc acid lactone	-	-	-
D-sorbitol	-	-	-	D-galacturonic acid	+	+	+
Sucrose	-	-	-	D-gluconic acid	+	+	+
D-trehalose	-	-	-	D-glucosaminic acid	-	-	-

Turanose	-	-	-	D-glucuronic acid	+	+	+
Xylitol	-	-	-	α-hydroxybutyric acid	+	+	+
Methyl pyruvate	+	+	+	ß-hydroxybutyric acid	+	+	+
Mono-methyl succinate	+	+	+	γ-hydroxybutric acid	+	+	+
p-hydroxyphenylacetic acid	+	+	+	Bromo succinic acid	+	+	+
Itaconic acid	-	-	-	Succinamic acid	+	+	+
α-keto butyric acid	+	+	+	Glucuronamide	+	+	+
α-keto glutaric acid	+	+	+	Alaninamide	+	+	+
α-keto valeric acid	+	+	+	D-alanine	+	+	+
D,L-lactic acid	+	+	+	L-alanine	+	+	+
Malonic acid	+	+	+	L-alanyl-glycine	+	+	+
Propionic acid	+	+	+	L-asparagine	+	+	+
Quinic acid	+	+	+	L-aspartic acid	+	+	+
D-saccharic acid	+	+	+	L-glutamic acid	+	+	+
Sebacic acid	-	-	-	Glycyl-L-aspartic acid	-	-	-
Succinic acid	+	+	+	Glycyl-L-glutamic acid	+	+	+
L-histidine	+	+	+	Urocanic acid	-	-	-
Hydroxyl L-proline	+	+	+	Inosine	+	+	+
L-leucine	+	+	+	Uridine	-	-	-
L-ornithine	+	+	+	Thymidine	-	-	-
L-phenylalanine	-	-	-	Phenyl ethylamine	+	+	+
L-proline	+	+	+	Putrescine	+	+	+
L-pyroglutamic acid	+	+	+	2-amino ethanol	+	+	+
D-serine	+	+	+	2,3-butanediol	+	+	+
L-serine	+	+	+	Glycerol	+	+	+
L-threonine	+	+	+	D,L-α-glycerol phosphate	-	-	-
D,L-carnitine	+	+	+	glucose-1-phosphate	-	-	-
γ-amino butyric acid	+	+	+	glucose-6-phosphate	-	-	-

Table 8 Results of Biolog GN identification for wild type and GFP-tagged strains of *Pseudomonas putida* CP1

Assimilation Test	CP1 wild type	CP1 Tn7-gfp	CP1 Tn5-*gfp* UV	Assimilation Test	CP1 wild type	CP1 Tn7-*gfp*	CP1 Tn5-*gfp* UV
Water	-	-	-	i-erythritol	-	-	-
α-cyclodextrin	-	-	-	D-fructose	+	+	+
Dextrin	+	+	+	L-fucose	-	-	-
Glycogen	+	+	+	D-galactose	-	-	-
Tween 40	+	+	+	Gentiobiose	-	-	-
Tween 80	+	+	+	α-D-glucose	+	+	+
N-acetyl-D-galactosamine	-	-	-	m-inositol	-	-	-
N-acetyl-D-glucosamine	-	-	-	α-D-lactose	-	-	-
Adonitiol	-	-	-	Lactulose	-	-	-
L-arabinose	+	+	+	Maltose	-	-	-
D-arabitol	-	-	-	D-mannitol	-	-	-
Cellobiose	-	-	-	D-mannose	+	+	+
D-melibiose	-	-	-	Acetic acid	+	+	+
ß-methyl D-glucoside	-	-	-	*cis*-aconitic acid	+	+	+
D-psicose	+	+	+	Citric acid	+	+	+
D-raffinose	-	-	-	Formic acid	+	+	+
L-rhamnose	-	-	-	D-galactonic acid lactone	+	+	+
D-sorbitol	-	-	-	D-galacturonic acid	+	+	+
Sucrose	-	-	-	D-gluconic acid	+	+	+
D-trehalose	-	-	-	D-glucosammic acid	-	-	-
Turanose	-	-	-	D-glucuronic acid	+	+	+
Xylitol	-	-	-	α-hydroxybutyric acid	+	+	+
	+	+	+		+	+	+

Methyl pyruvate Mono-methyl succinate	+	+	+	ß-hydroxybutyric acid γ-hydroxybutric acid	-	-	-
p-hydroxyphenylacetic acid	-	-	-	Bromo succinic acid	+	+	+
Itaconic acid	-	-	-	Succinamic acid	+	+	+
α-keto butyric acid	+	+	+	Glucuronamide	+	+	+
α-keto glutaric acid	+	+	+	Alanmamide	+	+	+
α-keto valeric acid	+	+	+	D-alanine	+	+	+
D,L-lactic acid	+	+	+	L-alanine	+	+	+
Malonic acid	+	+	+	L-alanyl-glycine	+	+	+
Propionic acid	-	-	-	L-asparagine	+	+	+
Quinic acid	+	+	+	L-aspartic acid	+	+	+
D-saccharic acid	+	+	+	L-glutamic acid	+	+	+
Sebacic acid	-	-	-	Glycyl-L-aspartic acid	-	-	-
Succinic acid	+	+	+	Glycyl-L-glutamic acid	+	+	+
L-histidine	+	+	+	Urocanic acid	+	+	+
Hydroxyl L-proline	+	+	+	Inosine	+	+	+
L-leucine	+	+	+	Uridine	+	+	+
L-ornithine	+	+	+	Thymidine	-	-	-
L-phenylalanine	+	+	+	Phenyl ethylamine	+	+	+
L-proline	+	+	+	Putrescine	+	+	+
L-pyroglutamic acid	+	+	+	2-ammo ethanol	+	+	+
D-serine	+	+	+	2,3-butanediol	+	+	+
L-serine	+	+	+	Glycerol	+	+	+
L-threonine	+	+	+	D,L-α-glycerol phosphate	-	-	-
D,L-carnitine	+	+	+	glucose-1-phosphate	-	-	-
γ-amino butyric acid	+	+	+	glucose-6-phosphate	-	-	-

Table 9 Results of API 20NE identification for strains A(a)

Test	*P putida* A(a)	*P putida* A(a) Tn7-*gfp*	*P putida* A(a) Tn5 -GFPuv
Reduction of nitrates	-	-	-
Indole production	-	-	-
Glucose acidification	-	-	-
Arginine Dihydrolase	+	+	+
Urease	-	-	-
Esculin Hydrolysis	-	-	-
Gelatine hydrolysis	-	-	-
ß-galactosidase	-	-	-
Glucose assimilation	+	+	+
Arabinose assimilation	-	-	-
Mannose assimilation	-	-	-
Manmtol assimilation	+	+	+
N-Acetyl-Glucosamine assimilation	-	-	-
Maltose assimilation	-	-	-
Gluconate assimilation	+	+	+
Caprate assimilation	+	+	+
Adipate assimilation	-	-	-
Malate assimilation	+	+	+
Citrate assimilation	+	+	+
Phenyl-acetate assimilation	+	+	+
Cyctochrome oxidase	+	+	+

Table 10 Results of API 20NE identification for strains CP1

Test	*P putida* CP1	*P putida* CP1 Tn*7*-*gfp*	*P putida* CP1 Tn*5*-GFPuv
Reduction of nitrates	-	-	-
Indole production	-	-	-
Glucose acidification	-	-	-
Arginine Dihydrolase	+	+	+
Urease	-	-	-
Esculin Hydrolysis	-	-	-
Gelatine hydrolysis	-	-	-
ß-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	+	+	+
Cyctochrome oxidase	+	+	+

3 3 4 Minimum inhibitory concentration of gentamicin and kanamycin in *gfp* transformed cells

The transposons Tn*7*-*gfp* and Tn*5*-*gfp* contained marker genes for gentamicin and kanamycin respectively It was important to determine the minimum inhibitory concentration of each antibiotic if these resistance markers were to be used in selective plate counts of *gfp* transformed strains

The minimum inhibitory concentration (MIC) of gentamicin and kanamycin was determined for the parental strains of *P putida* A(a) and *P putida* CP1 This was compared with the minimum inhibitory concentration of gentamicin in Tn*7*-*gfp* transformed cells which contained a marker gene for gentamicin resistance The minimum inhibitory concentration of kanamycin was also determined for the strains that had received the Tn*5*-*gfp* insert which contained a marker gene for kanamycin resistance The MIC was determined by spread plating the cultures on plates containing increasing amounts of antibiotic and determining the concentration at which the antibiotic had an inhibitory effect on colony forming units

The results showed that for the wild type strains, the minimum inhibitory concentration for gentamicin in *P putida* A(a) and *P putida* CP1 was between 2 to 4 ug/ml (Table 11) This indicated that both the wild type strains of *P putida* A(a) and *P putida* CP1 were not gentamicin resistant Following transformation with Tn*7*-*gfp*, which contained a marker gene for gentamicin resistance, both strains became resistant to gentamicin For the transformed cells, the MIC for gentamicin was between 50 and 60 ug/ml

The minimum inhibitory concentration for kanamycin was between 2 and 4 ug/ml for the wild type strains and was >70 ug/ml for the *P putida* A(a) and *P putida* CP1 strains that had been transformed with Tn5-gfp containing a kanamycin resistance marker gene (Tables 12) These results indictaed that the transformed cells were resistant to kanamycin

Table 11 Minimum inhibitory concentration of gentamicin in wild type and Tn7-*gfp* transformed *P putida* A(a) and *P putida* CP1

Strain	MIC gentamicin (μg/ml)
P putida A(a)	2-4
P putida A(a) Tn7-*gfp*	50-60
P putida CP1	2-4
P putida CP1 Tn7-*gfp*	>70

Table 12 Minimum inhibitory concentration of kanamycin in wild type and Tn5-*gfp* transformed *P putida* A(a) and *P putida* CP1

Strain	MIC kanamycin (μg/ml)
P putida A(a)	2-4
P putida A(a) Tn5-*gfp*UV	>70
P putida CP1	2-4
P putida CP1 Tn5-*gfp*UV	>70

3 4 The augmentation of activated sludge with two GFP-tagged strains of *Pseudomonas putida*, A(a) and CP1

The bioaugmentation of activated sludge with *P putida* A(a) and CP1 was studied To facilitate their tracking in the mixed microbial community GFP-tagged strains were used Prior to their use in bioaugmentation experiments, it was desirable to determine whether insertion and expression of the gene for GFP had any effect on the degradative

capabilities of the GFP-tagged strains Since both organisms were capable of the degradation of 4-chlorophenol, this was chosen as the target compound for the bioaugmentation studies The effect of inoculum size on the degradation of 4-chlorophenol was also investigated Results from this section have been published (Mc Laughlin *et al*, 2006)

3 4 1 Degradation of 4-chlorophenol by *Pseudomonas putida* A(a) and CP1 and the corresponding GFP-tagged strains

The mutant *P putida* A(a) and *P putida* CP1 strains marked with Tn*5*-*gfp*UV and Tn*7*-*gfp*mut3* were compared with the wild type strains for their ability to degrade 4-CP All of the GFP-tagged strains were able to remove 4-chlorophenol from the medium, and release chloride (Fig 29) at similar rates to the parent strain (Section 3 1 1, Fig 11) Therefore the ability of *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1 to degrade 1 56 mM 4-CP was not hampered by chromosomal insertion of either marker gene

The strains marked with Tn*7*-*gfp* were chosen for the bioaugmentation studies since the mut3* version of GFP was intensely fluorescent under an epifluorescence microscope and the tagged strains could be visualised at the single cell level It had also been confirmed that the gene for GFP integrated at a non-coding neutral chromosomal site in *P putida* A(a) and at an unknown but specific site in the chromosome of CP1 thereby preventing possible plasmid-mediated transfer to indigenous members of the activated sludge community

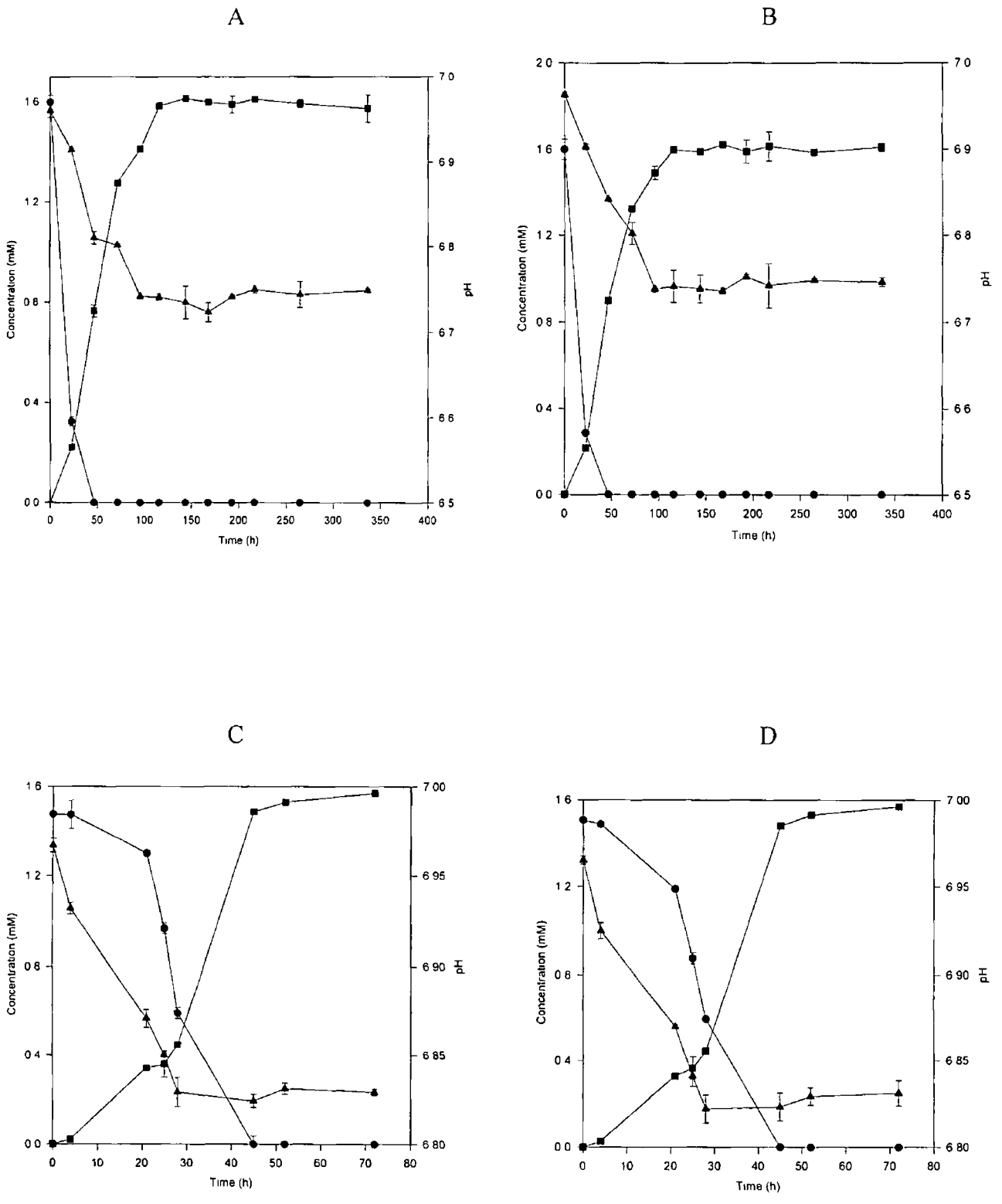


Figure 29 The degradation of 4-chlorophenol (1 56 mM) by (A) *P putida* A(a) Tn7-*gfp*, and (B) *P putida* A(a) Tn5-*gfp*, (C) *P putida* CP1 Tn7-*gfp* and (D) *P putida* CP1 Tn5-*gfp* Symbols ● 4-chlorophenol, ■ chloride release and ▲ pH

3 4 2 Degradation of 1 56mM 4-chlorophenol by Activated Sludge augmented with 0 1, 0 5 and 5 0% *Pseudomonas putida* A(a) Tn7-gfp and *Pseudomonas putida* CP1 Tn7-gfp and their monitoring during augmentation of activated sludge

P putida A(a) was capable of degrading 4-chlorophenol to completion using a *meta*-cleavage pathway while *P putida* CP1 was capable of degrading it using an *ortho*-cleavage pathway Both strains had antibiotic resistance and visual markers inserted into their chromosomes It was therefore of interest to add these xenobiotic degrading organisms to activated sludge to evaluate their effect on the degradative ability of the sludge and to follow their survival and location within the sludge Degradation of 4-chlorophenol by activated sludge augmented with 0 5% (v/v), 1 0% (v/v) and 5 0% (v/v) inoculum of *P putida* A(a) Tn*7-gfp* and *P putida* Tn*7-gfp* was examined to determine the optimum inoculum size

No degradation of 4-chlorophenol by the activated sludge was observed, indicating the absence of any organisms in the sludge with the ability to degrade 4-chlorophenol (Fig 30A) Addition of 5 0 %, 1 0% and 0 5% inocula sizes of *P putida* CP1 Tn*7-gfp* to activated sludge resulted in the complete removal of 4-chlorophenol within 48 hours (Fig 30C) Addition of 5 0 % inoculum of *P putida* A(a) Tn*7-gfp* to activated sludge resulted in the removal of 4-chlorophenol within 48 hours (Fig 30B) Degradation with the 0 5% and 1 0% inoculum was less successful as 4-chlorophenol removal was accompanied by a dark brown colour which developed in the medium after 48 hours indicating incomplete degradation The production of colored metabolites during degradation of 4-chlorophenol by augmented activated sludge was investigated The production of colored metabolites is indicative of the metabolic pathway utilized by the system to degrade 4-chlorophenol

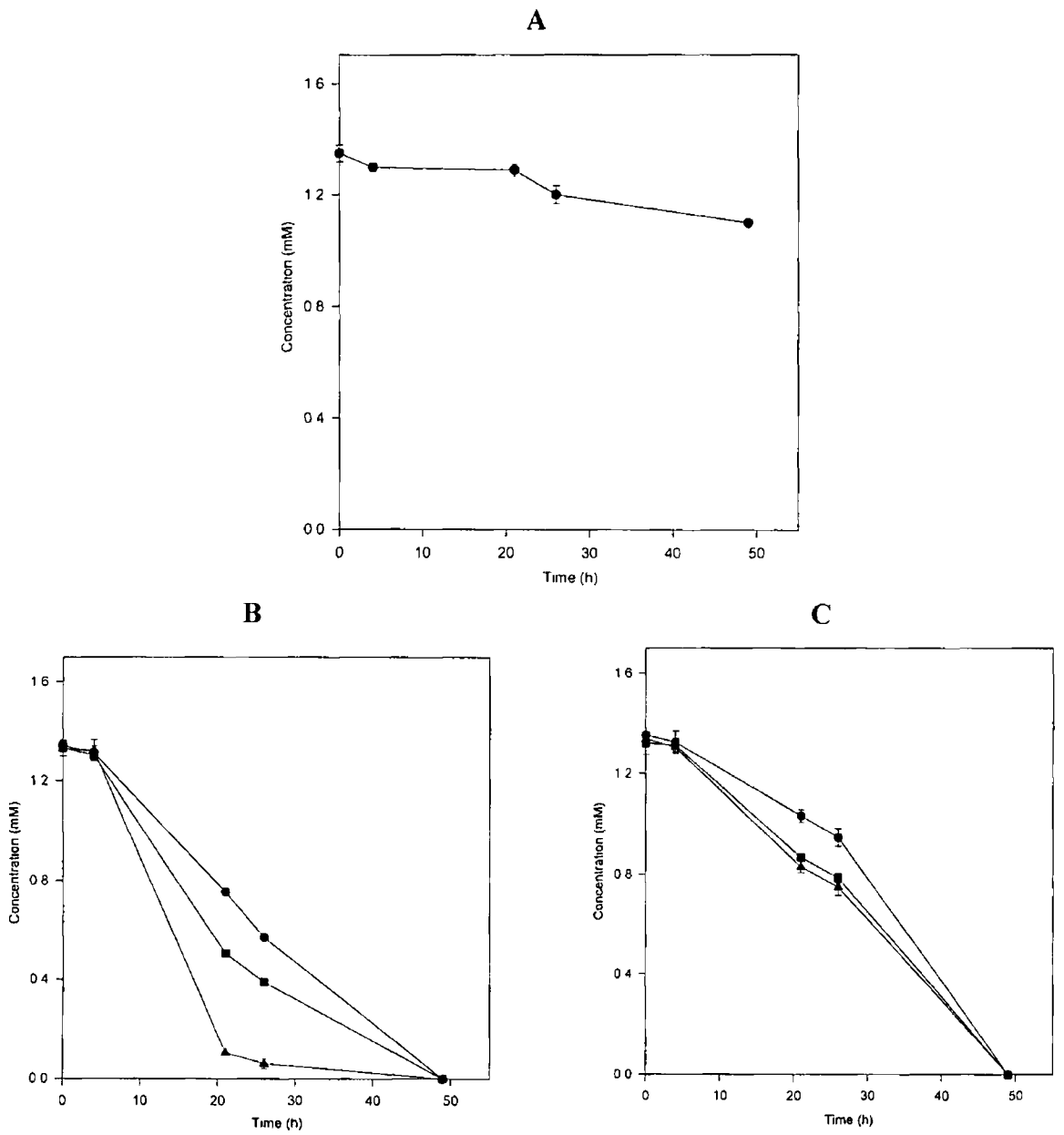


Figure 30 Degradation of 4-chlorophenol by activated sludge (**A**), augmented with (**B**) *P putida* A(a) and (**C**) *P putida* CP1 Symbols● 0 5% inoculum, ■ 1 0% inoculum and ▲ 5 0% inoculum

Production of 5-Cl-HMS during the degradation of 4-chlorophenol by activated sludge augmented with *Pseudomonas putida* A(a)

5-chloro-2-hydroxymuconic semialdehyde accumulation was not observed in activated sludge with 4-chlorophenol (Fig 31A) but was observed during the degradation of 4-chlorophenol by activated sludge augmented with A(a) Tn*7-gfp* (Fig 31B) Maximum accumulation was observed following 24 hours incubation Metabolite production was considerably higher in activated sludge augmented with 5 0% *P putida* A(a) Tn*7-gfp* than in sludge augmented with 0 5% and 1 0% *P putida* A(a) Tn*7-gfp* The yellow colour disappeared corresponding to a decrease in the OD 380nm in the presence of 5% A(a) Tn*7-gfp* within 75 hours However in the activated sludge augmented with 0 5% and 1 0% *P putida* A(a), a dark brown colour developed in the culture medium after 48 hours incubation

Production of 5-Cl-HMS during the degradation of 4-chlorophenol by activated sludge augmented with *Pseudomonas putida* CP1

5-chloro-2-hydroxymuconic semialdehyde accumulation, as indicated by an increase in the OD380 of the activated sludge augmented with *P putida* CP1, was observed after 24 hours incubation (Fig 31C) However the increase in OD380 was insignificant compared to that observed in the sludge augmented with *P putida* A(a) (Fig 31B) No accumulation of 5-chloro-2-hydroxymuconic semialdehyde was observed during the incubation of activated sludge amended with 4-chlorophenol

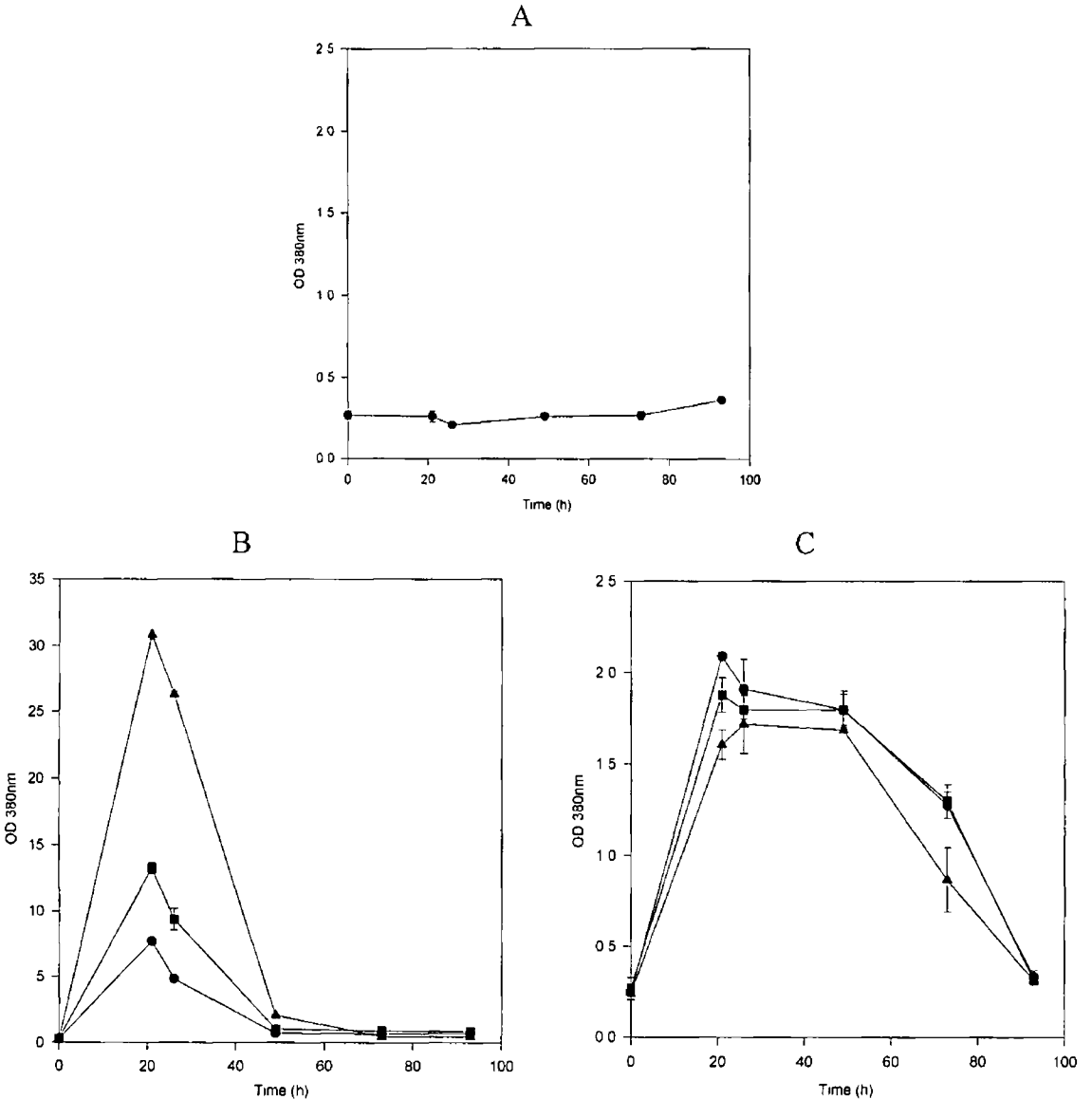


Figure 31 Accumulation of 5-chloro-2-hydroxymuconic semialdehyde during the degradation of 4-chlorophenol by (A) Activated Sludge, (B) Activated Sludge augmented with *P putida* A(a) Tn7-gfp and (C) Activated Sludge augmented with *P putida* CP1 Tn7-gfp Symbols ● 0 5% inoculum,■ 1 0% inoculum and ▲ 5 0% inoculum

3.4.3 Microbiological analysis of the activated sludge

The addition of *P putida* A(a) and *Pseudomonas putida* CP1 to activated sludge resulted in the degradation of 4-chlorophenol It was of interest to know whether the introduced strains responsible for the degradation survived in the sludge and whether the relative populations of the sludge microbial community changed during chlorophenol degradation During the degradation of the mono-chlorophenols by the activated sludge, microbiological analysis was carried out to determine the total chlorophenol degrading numbers, survival of the introduced strains, and the total bacterial numbers

4-chlorophenol degrading microorganisms in the un-augmented activated sludge

The addition of 4-chlorophenol to the activated sludge resulted in a more rapid decline in overall cell numbers (Fig 32B) than in activated sludge without 4-chlorophenol (Fig 32A), possibly due to the toxicity of 4-chlorophenol to the sludge microbial community There were no gentamicin resistant chlorophenol degraders detected in the activated sludge (Fig 32), indicating that these selection criteria were suitable for use with the gentamicin resistant GFP-tagged strains that were to be added to the sludge

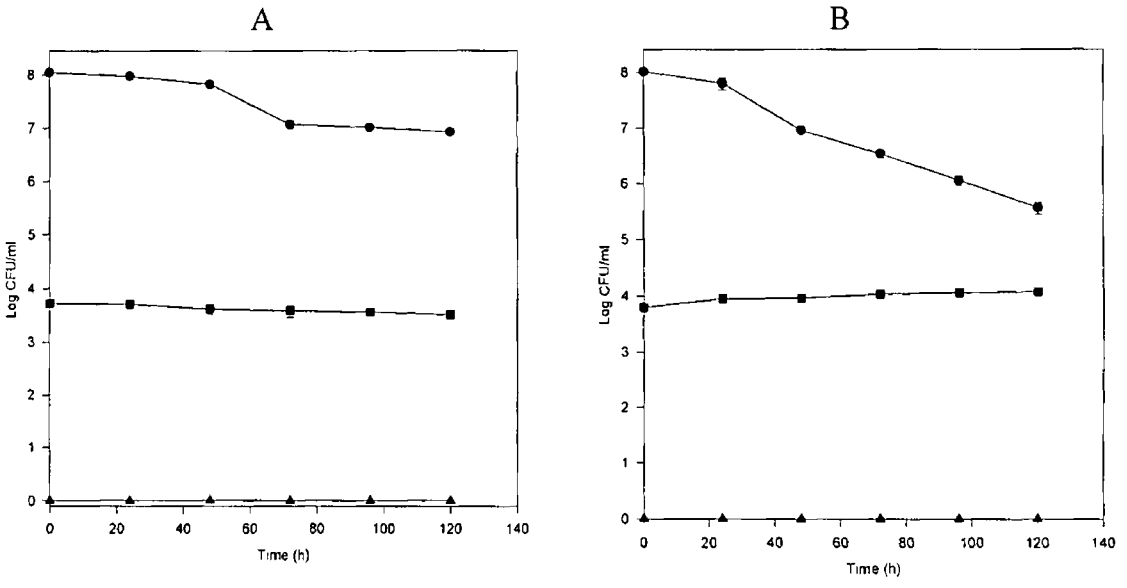


Figure 32 Cell counts in activated sludge (**A**) and activated sludge amended with 4-CP (**B**) Symbols ● cell counts on plate count agar and ■ cell counts on 4-CP agar and ▲ 4-CP+ Gn

Survival of GFP-tagged *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1 in activated sludge

During the degradation of the mono-chlorophenols by the activated sludge augmented with *P putida* A(a) Tn*7-gfp* and *P putida* CP1 Tn*7-gfp*, microbiological analysis was carried out to determine the total bacterial numbers, the total chlorophenol degrading numbers and the numbers of GFP-tagged *P putida* A(a) in the mixed culture The survival of *P putida* A(a) in the activated sludge is shown in Figure 33 and the survival of *P putida* CP1 in the activated sludge is shown in Figure 34

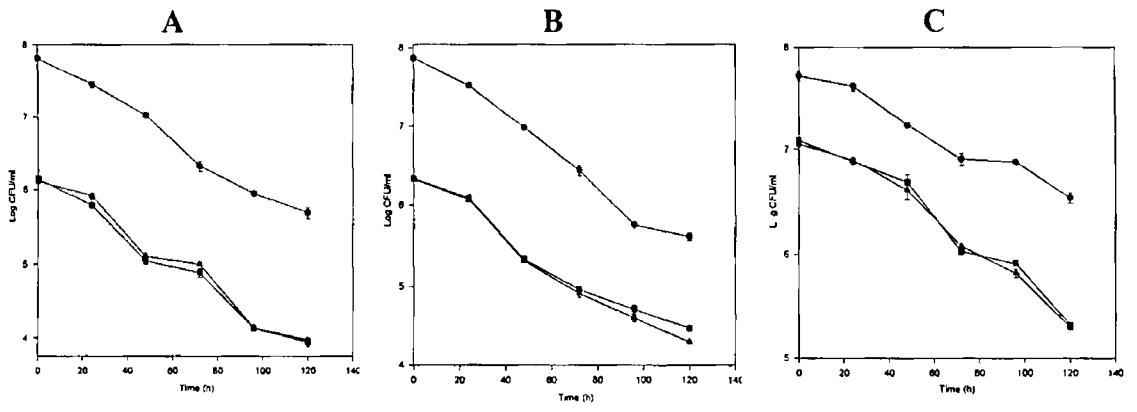


Figure 33 Cell counts during degradation of 4-chlorophenol in activated sludge augmented with (**A**) 0 5%, (**B**) 1 0% and (**C**) 5 0% *P putida* A(a) Tn*7-gfp* ● Total cell counts on PCA, ■ Total cell counts on 4-CP, ▲ cell counts on 4-CP+gentamicin

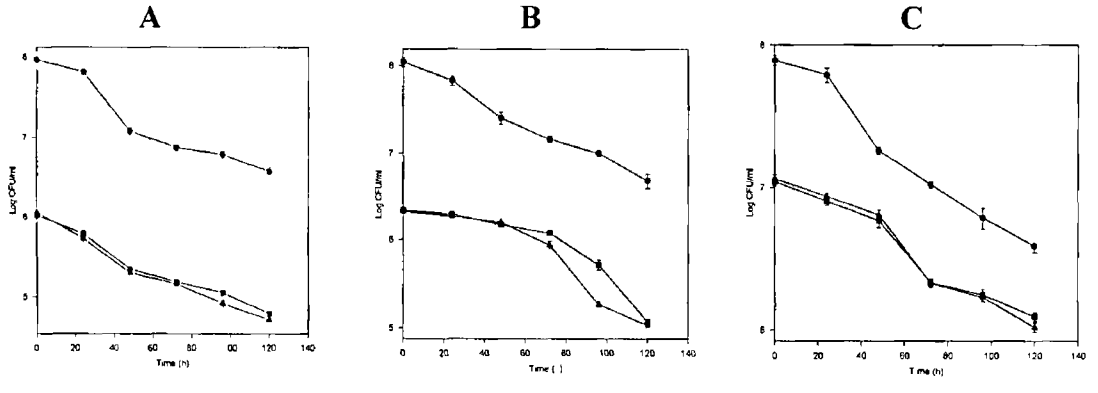


Figure 34 Cell counts during degradation of 4-chlorophenol in activated sludge augmented with (**A**) 0 5%, (**B**) 1 0% and (**C**) 5 0% *P putida* CP1 Tn*7-gfp* ● Total cell counts on PCA, ■ Total cell counts on 4-CP, ▲cell counts on 4-CP+gentamicin

P putida A(a) Tn*7-gfp* and CP1 Tn*7-gfp* numbers fell following addition of either strain to activated sludge at all inocula sizes (Figures 33 and 34) Linear regression analysis was used to estimate the rate of decrease in log number of GFP-tagged *P putida* numbers per

hour The slopes of the regression lines were calculated to give the log decrease in *P putida* numbers / ml / hour and are shown in Table 13 Decreases in *P putida* cell numbers were greater at the lower inoculum sizes (0 5% and 1 0%) for both organisms Furthermore, the decreases in *Pseudomonas putida* A(a) Tn*7-gfp* numbers were greater than the decreases in *Pseudomonas putida* CP1 numbers at all inocula sizes

Table 13 Decrease in log *Pseudomonas putida* numbers / ml / day following their addition to activated sludge

GFP-tagged Inoculum	Decrease in log number of cfu / ml / day
AS + 0 5% *P putida* CP1	0 2640
AS + 1 0% *P putida* CP1	0 2530
AS + 5 0% *P putida* CP1	0 2240
AS + 0 5% *P putida* A(a)	0 4613
AS + 1 0% *P putida* A(a)	0 4259
AS + 5 0% *P putida* A(a)	0 3574

Effect of the addition of *Pseudomonas putida* strains on activated sludge numbers

As observed with the drop in GFP-tagged *P putida* numbers, total viable numbers in the activated sludge decreased over time (Figures 33 and 34) Total cell numbers in activated sludge to which 4-chlorophenol had been added decreased at a rate above that observed in activated sludge without any 4-chlorophenol (Table 14) Addition of GFP-tagged *P putida* strains to the activated sludge resulted in smaller decreases in cell numbers than those observed in unaugmented activated sludge to which chlorophenol had been added Addition of *P putida* CP1 at the different inocula sizes resulted in drops in cell numbers similar to those observed in activated sludge to which no chlorophenol had been added Drops in cell numbers in activated sludge augmented with *P putida* A(a) were greater than those observed following addition of CP1 but less than those observed in the unaugmented sludge

Table 14 Decrease in log total numbers / ml / day following during degradation of 4-chlorophenol

GFP-tagged inoculum	Decrease in log number of cfu / ml / day
Activated Sludge	0 2654
Activated Sludge + 4cp	0 5116
AS + 0 5% *P putida* CP1	0 2928
AS + 1 0% *P putida* CP1	0 2876
AS + 5 0% *P putida* CP1	0 2789
AS + 0 5% *P putida* A(a)	0 4536
AS + 1 0% *P putida* A(a)	0 4251
AS + 5 0% *P putida* A(a)	0 3598

In situ visualisation of *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1 during the degradation of 4-chlorophenol in augmented sludge

The insertion of *gfp* into both *P putida* strains allowed for their distribution in the activated sludge to be investigated using epifluorescence microscopy Fluorescent cells were easily visualised at the single cell level in the activated sludge *P putida* CP1 Tn7-*gfp* cells were found to be almost entirely within the sludge flocs after 24 hours incubation, while *P putida* A(a) Tn7-*gfp* cells remained randomly distributed throughout the sludge mixed liquor (Figure 35)

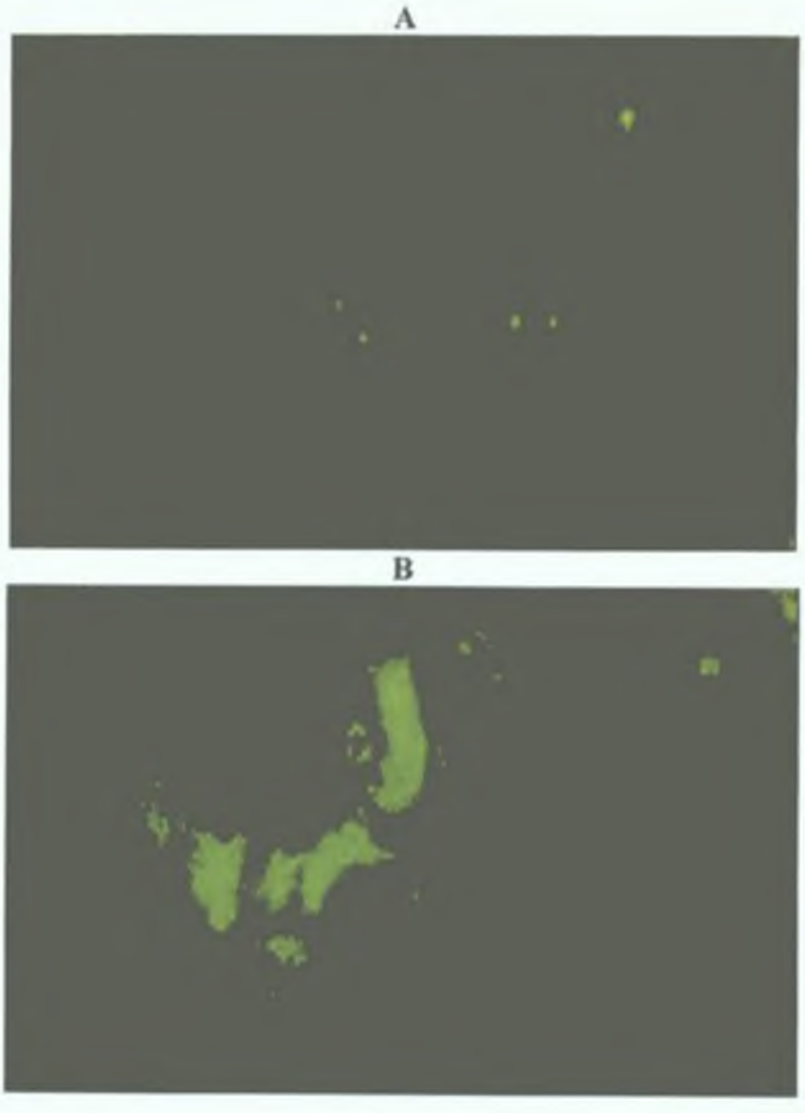


Figure 35: Location and survival of **(A)** *Pseudomonas putida*A(a)::Tn*7-gfp* and **(B)** *Pseudomonas putida* CP1::Tn*7-gfp* following their addition to activated sludge.

4. Discussion

There is concern over chlorophenols in the environment due to their persistence and resistance to microbial degradation, The degradation of 2-, 3- and 4-chlorophenol was investigated in two organisms, *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1

Pseudomonas putida A(a) was isolated by O' Sullivan, 1998, and was the only organism capable of growth on 4-chlorophenol isolated from Biolyte HAB (Haloaromatic Biodegradation) , a commercial bioaugmentation product Produced by International Biochemicals Ltd , Ireland, it was designed to provide a selection of organisms capable of degrading a range of substituted haoaromatic compounds The product was designed for use in conventional wastewater treatment plants and for the bioremediation of contaminated soils The formulation consisted of a mixed culture consisting of a minimum of eleven microbial strains including *Pseudomonads*, *Actinomycetes* and a fungus These microorganisms were said to have the ability to degrade chlorosubstituted benzenes, polychlorinated biphenyls and structurally related compounds They were grown in pure culture, harvested by centrifugation and preserved by freeze-drying before being blended to form the final formulation (manufacturers' literature)

Pseudomonas picketti LD1 was shown to degrade all 3 mono-chlorophenol isomers (Fava *et al*, 1995) *Pseudomonas putida* CP1, the second chlorophenol degrading isolate used in this study was purified from the same mixed culture as *Pseudomonas picketti* LD1 and was obtained from Professor Favio Fava, University of Bologna, Italy

The degradation of-mono-chlorophenol isomers by strains A(a) and CP1 was studied in the basal salts medium of Goulding et al (1998), which provided the nitrogen, phosphate and trace salts essential for the growth of microorganisms The 5% inoculum (v/v) used was approximately 1 2 x 10^7 cells/ml

In the pure culture inoculated with *Pseudomonas putida* A(a) degradation of 1 56 mM 2- and 3-chlorophenol was incomplete as indicated by the non-stoichiometric release of

chloride Incomplete degradation of 2- and 3-chlorophenol was accompanied by the accumulation of brown/black coloured intermediates in the culture medium The accumulation of this brown/black colour has been attributed to the polymerization of 3-chlorocatechol, an intermediate in the degradation of 2- and 3-chlorophenol by the *meta*-cleavage pathway (Haller and Finn, 1979, Kim and Hao, 1999) Haller and Finn (1979) reported the appearance of a melanin-like pigment during the degradation of 3-chlorobenzoate/benzoate mixtures by sludge The brown pigment appeared to result from the polymerization of 3-chlorocatechol The production of a brownish/gray colour during the metabolism of phenol/3-chlorophenol in batch studies by an *Acinetobacter* species was attributed to the accumulation and polymerization of chlorocatechols (Kim and Hao, 1999)

Studies by Fava *et al* , (1993b) proposed that simple autooxidation of chlorocatechol was not the only mechanism by which pigments were formed They suggested that polymerization may be the result of biological processes A peroxidase enzyme and a polyphenol oxidase were shown to actively oxidize 4-chlorocatechol to form 4-chloro-1,2-benzoquinone, a highly reactive substrate for pigment formation, during the co-metabolism of 3-chlorobenzoic acid by *Pseudomonas fluorescens* CP30 (Fava and Marchetti, 1991) Despite the activity of these enzymes, simple autooxidation of chlorocatechols still contributed significantly to polymerization and it has been shown that catechols oxidize and turn brown readily in aqueous solutions (Windholz et al , 1983)

The degradation of 2- and 3- chlorophenol results in the intermediate formation of 3-chlorocatechols and most meta-cleavage enzymes, catechol 2,3-dioxygenases, are inactivated very rapidly during the conversion of 3-chlorinated catechols resulting in an accumulation of 3-chlorocatechol There are two mechanisms whereby 3-chlorocatechol may have adverse effects on catechol 2,3-dioxygenase, one reversible and the other irreversible Reversible inactivation occurs when 3-chloroctechol acts as a chelating agent and binds to the catalytically active ferrous ion of catechol 2,3-dioxygenase (Klecka and Gibson, 1981) Irreversible inactivation, or suicide inhibition occurs due to

the production of a highly reactive acylchloride, 5-chloroformyl-2-hydroxy-penta-2,4-dienoic acid (Bartels et al , 1984) This has been proposed to act as a suicide compound, binding irreversibly to catechol 2,3-dioxygenase with a subsequent release of chloride and destruction of metabolic activity Enzyme assays were carried out for catechol 2,3-dioxygenase activity These enzymes catalyze the *meta*-cleavage of aromatic rings to form hydroxymuconic semialdehyde Results showed that *Pseudomonas putida* A(a) posessed *meta*-cleavage activity against 4-chlorocatechol, the ring cleavage substrate for 4-chlorophenol As metabolism of 2- and 3- chlorophenol by *Pseudomonas putida* A(a) led to some chloride release with a corresponding drop in pH, it was assumed that suicide inhibition of catechol 2,3-dioxygenase led to the accumulation of the dark brown colour observed in the medium This may also explain why no *meta*-cleavage enzyme activity was detected in cells grown on 2- and 3-chlorophenol prior to the assay

Degradation of 4-chlorophenol by *Pseudomonas putida* A(a) also took place using the *meta*-cleavage pathway Enzyme assays on cells grown on 4-chlorophenol revealed *meta*-cleavage activity towards 4-chlorocatechol, the meta-cleavage product of 4-chlorophenol However, cells grown on 2- and 3-chlorophenol did not show any activity towards 4-chlorocatechol, presumably as a result of suicide inactivation of catechol 2,3-dioxygenase by 3-chlorocatechol Metabolism of 4-chlorophenol by *Pseudomonas putida* A(a) resulted in a lime/yellow colouration of the culture medium, however, unlike the brown colour that resulted from the metabolism of 2- and 3-chlorophenol, this yellow colour did not remain in the culture medium The degradation of 4-chlorophenol by the *meta*-cleavage pathway has been known to give rise to the production of 5-chloro-2-hydroxymuconic semialdehyde, which can accumulate to give a yellow colouration of the culture medium (Knackmuss, 1981) In the presence of 4-chlorophenol, the culture medium turned yellow within 4 hours of incubation The spectrophotometric characteristics of this accumulated intermediate ($^{\lambda}$ max = 378 nm at pH 7 and $^{\lambda}$ max = 330 nm at pH 2 accompanied by reversible disappearance of the yellow colour upon acidification) corresponded to those of 5-chloro-2-hydroxymuconic semialdehyde, the *meta*-cleavage product of 4-chlorocatechol (Wieser *et al*, 1994, Farrell and Quilty, 1999)

This meta-cleavage product, 5-chloro-2-hydroxymuconic semialdehyde has been widely reported to toxic metabolites, preventing further degradation (Balfanz and Rehm, 1991, Weiser et al , 1994, Farrell and Quilty 1999) However, the complete degradation of 4-chlorophenols via the *meta*-cleavage pathway has occasionally been reported *Pseudomonas cepacia* P166 was capable of the complete degradation of 4-chlorobenzoate via a *meta*-cleavage pathway (Arnesdorf and Focht, 1994) One of the metabolites of degradation identified was 5-chloro-2-hydroxymuconic semialdehyde, which was shown to be further degraded, eventually resulting in complete removal (Arnesdorf and Focht, 1995) *Comamonas testestoroni* CPW301 produced 5-chloro-2-hydroxymuconic semialdehyde following the degradation of 4-chlorophenol via the meta-cleavage pathway and the yellow colour disappeared following further metabolism (Sung Bae *et al*, 1996) *Comamonas testestoroni* JH5 degraded 4-chlorophenol to completion via a meta-cleavage pathway and 5-chloro-2-hydroxymuconic semialdehyde was identified as an intermediate of metabolism (Hollender *et al* , 1997) This was further metabolised and accompanied by a stoichiometric release of chloride, indicating complete degradation The most recent example of the complete degradation of a para-substituted haloaromatic compound accompanied by the production of 5-chloro-2-hydroxymucomc semialdehyde and the stoichiometric release of chloride was reported by Seo et al (1998) in *Pseudomonas* sp S-47

Following the degradation of 4-chlorophenol by *Pseudomonas putida* A(a), the accumulated yellow colour dissapeared corresponding with a decrease in the optical density at 380nm Following removal of the cell biomass by filtration, 5-chloro-2-hydroxymuconic semialdehyde disappeared at a much slower rate than when in the presence of *Pseudomonas putida* A(a) Thus the removal of 5-chloro-2-hydroxymuconic semialdehyde by *Pseudomonas putida* A(a) was shown to be microbially mediated and not abiotic Spontaneous decomposition of 5-chloro-2-hydroxymuconic semialdehyde has been reported by Weiser et al (1994) During the degradation of 4-chlorophenol by Azotobacter sp GP1, 5-chloro-2-hydroxymuconic semialdehyde levels reached a peak after 10 hours, remained constant for 1 day and thereafter a slow decrease was observed

This slow decrease was consistent with the rate of spontaneous decomposition of 5-chloro-2-hydroxymuconic semialdehyde, indicating that further metabolism of the metabolite was not microbially mediated During degradation, 4-chlorophenol removal was complete after 24 hours and there was a stoichiometric release of chloride after 100 hours 5-chloro-2-hydroxymuconic semialdehyde appeared within 4 hours and reached a peak at 75 hours, after which it slowly degraded The presence of 5-chloro-2-hydroxymuconic semialdehyde, as determined by the optical density at 380nm, despite the stoichiometric release of chloride indicated that the degradation pathway of 4-chlorophenol by *Pseudomonas putida* A(a) followed the same metabolic pathway as that of *Pseudomonas* sp S-47 During this pathway, chloride is released before the hydroxymuconic acid is converted to a dieonoic acid by a decarboxylase enzyme 5-chloro-2-hydroxymuconic semialdehyde is converted to 2-5-dihydroxymuconic semialdehyde by the enzyme 5-chloro-2-hydroxymuconic dehalogenase accompanied by the release of chloride This indicates that the yellow colour present after the stoichiometric release of chloride may have been 2-5-dihydroxymuconic semialdehyde These metabolites are very similar, 2-hydroxymuconic semialdehyde has a peak absorption of 375nm and has a similar absorption spectrum in acidic and alkali environments as 5-chloro-2-hydroxymuconic semialdehyde (Loh and Chua, 2002)

The degradation of chlorophenols by *Pseudomonas putida* CP1 did not result in the formation of any coloured metabolites, which is indicative of the modified *ortho*-cleavage pathway Chlorocarechol 1,2-dioxygenase incorporates molecular oxygen into catechol as it cleaves between its hydroxylated carbons to produce *cis,cis*-muconic acid which is metabolised via the ß-ketoadipate pathway Most common in nature is catechol 1,2-dioxygenase, which exhibits little or no activity towards chlorocatechol Chlorocatechol 1,2-dioxygenase acts on chlorocatechols to produce the corresponding muconic acid derivatives and represents about 16% of the soluble protein in fully induced cells Ka-Leung *et al*, 1990) Modified *ortho*-cleavageactivity was confirmed in CP1 when enzyme assays were carried out The levels of enzyme avtivity were higher than those previously reported from the laboratory (Farrell, 2002) This resulted from a modification of the previously used method and the presence of EDTA in the enzyme

assay reaction mixture Muconate and chloromuconates absorb strongly at 260 nm, a wavelength at which the absorbance of catechol or chlorocatechol is slight Therefore the activity of the oxygenases can be determined by measuring the increment in absorbance at 260 nm in the presence of catechol Muconate cyclisomerase, the enzyme that acts on muconate and converts it to dienelactone, requires Mn^{2+} and, unlike the catechol oxygenases, is inhibited by EDTA Therefore quantitative muconate accumulation from catechol can be assured by adding EDTA to the assay mixture (Ka-Leung *et al*, 1990)

It was noted that during the degradation of mono-chlorophenols *Pseudomonas putida* A(a) did not flocculate or form clumps in the growth medium whereas *Pseudomonas putida* CP1 formed clumps within hours of inoculation The clumps resulting from incubation in 4-chlorophenol were smaller and more numerous than the clumps resulting from incubation in 2- and 3-chloeophenol which resulted in larger and less numerous clumps Microbes capable of flocculation have advantages over non-flocculating strains as they may remain within the sludge unit for longer periods of time through biomass recycling and flocs can confer protection from protozoa, which feed mainly on free-swimming microorganisms (van Limbergen et al, 1998, Eberl et al, 1997) When *Pseudomonas putida* CP1 was grown on phenol or low concentrations of mono-chlorophenol, there was an increase in biomass but no cell clumping Cells grown on higher concentrations of mono-chlorophenols formed large aggregates of cells in the culture medium and were more hydrophobic than those grown on the lower concentrations of substrate, suggesting that increased hydrophobicity and autoaggregation of *Pseudomonas putida* CP1 was a response to toxixity of added substrates (Farrell and Quilty, 2002)

Aerobic degradation of aromatic compounds occurs through the use of dioxygenase enzymes which cleave the aromatic ring It was therefore of interest to target the corresponding genes for amplification by PCR to confirm the presence of these genes in the *P putida* A(a) and *P putida* CP1 The dioxygenases involved most often cleave

catechol or chlorocatechol, the central intermediates of aromatic hydrocarbon degradation, by *meta-* or *ortho-* cleavage *Meta*-cleavage dioxygenases, or catechol 2, 3-dioxygenases are usually associated with the degradation of methylaromatic compounds such as benzene, toluene, xylene and naphthalene Modified *ortho*-cleavage dioxygenases, or chlorocatechol 1, 2-dioxygenases are usually associated with the degradation of chlorinated aromatic compounds such as chlorophenols and chlorobenzenes

Chromosomal DNA from *P putida* A(a) and the positive control, mt-2 (TOL plasmid), was purified and subjected to PCR amplification with the primers described DEG-F and DEG-R were able to amplify the expected 238-bp fragment from both strains but no fragment was amplified when the primers specific for chlorocatechol 1,-2-dioxygenase were used in A(a) Enzyme assays carried out on *P putida* A(a) had demonstrated *meta*-cleavage activity towards 4-chlorocatechol but a complete absence of any *ortho*-cleavage activity This was consistent with the results obtained from the PCR amplification of the dioxygenase genes in A(a) Sequencing of the PCR fragment amplified from A(a) confirmed the identity of the fragment, thus demonstrating that PCR using the primer pair DEG-F-DEG-R was a valid method for detection of the catechol 2, 3-dioxygenase gene in A(a) Sequence analysis of the partial nucleotide sequence indicated that it was unique at the nucleotide level with the highest homology shown to any other catechol 2, 3-dioxygenase belonging to *dmpB* from the *P putida* CF600 plasmid pVI150 Sequence alignments with the well characterized *meta*-dioxygenase genes *nahH* from the *P putida* plasmid pNAH7 and *xylE* from the *P putida* mt-2 plasmid pWWO (TOL) revealed 86% and 84% homology respectively Despite the fact that the nucleotide sequence of the 2, 3-dioxygenase from A(a) was unique, the amino acid sequence showed 100% homology with *dmpB*, the 2, 3-dioxygenase from *P putida* CF600 plasmid pVI150

The primers DEG-F and DEG-R were capable of detecting dioxygenase genes from the I 2 A subfamily of catechol 2, 3 dioxygenases This subfamily consists of two-domain iron containing enzymes that show a preference for monocyclic substrates and they are involved in the biodegradation of benzene, toluene, xylene and naphthalene (Eltis and

Bolin, 1996). Mesarch *et al.* (2000) successfully used these primers in a competitive PCR procedure to accurately and reproducibly quantify 2, 3-dioxygenase genes in complex environments such as petroleum-contaminated soil.

Purified chromosomal DNA from *P. putida* CP1 subjected to PCR with the chlorocatechol 1, 2-dioxygenase-specific primers described yielded PCR products of the expected size (261 bp). As was the case with A(a), these results were consistent with the enzyme assay results which showed activity for chlorocatechol 1, 2-dioxgenase but a total absence of catechol 2, 3-dioxygenase activity in CP1. The sequence of the PCR fragment identified it as coming from a chlorocatechol 1, 2-dioxygenase gene and confirmed the usefulness of these primers for detecting and identifying the gene for chlorocatechol 1, 2-dioxygenase in *P. putida* CP1. The partial nucleotide sequence of chlorocatechol 1, 2-dioxygenase from *P. putida* CP1 exhibited the highest homology, 99%, and 68% homology with analogous enzymes encoded by *clcA* from the *P. putida* AC866 plasmid pAC27 and *tfdC* from the *R. eutropha* JMP134 plasmid pJP4 respectively. Homology at the amino acid level, with the same organisms was 100% and 67% respectively.

These primers were originally developed to detect *tfd*C-homologous genes (Kleinsteuber *et al.*, 1998). The chlorocatechol 1, 2-dioxygenase gene, *tfd*C, is from one of the best characterised chloroaromatic degrading pathways, that of the 2, 4-D pathway encoded by the archetypal 2, 4-D plasmid pJP4. The primers were derived from conserved sequence motifs based on the known nucleotide sequence from known chlorocatechol 1, 2-dioxygenase genes and the primers were successful for the detection of diverse chlorocatechol 1, 2-dioxygenase genes in Gram-negative strains.

Phylogenetic analysis is a useful tool to approach and understand the origin and evolution of catabolic pathways involved in the degradation of xenobiotic compounds. From the phylogenetic tree, it can be seen that the chlorocatechol dioxygenase genes involved in chloroaromatic catabolic pathways are diverse and significantly divergent which suggests an ancient origin. Chlorocatechol degradative pathways are diverged from a common

ancestral pathway already adapted for chlorocatechol catabolism and chlorocatechol 1,2-dioxygenase genes are more similar to each other than to their counterparts in catechol metabolism (Schlomann, 1994) The tree confirms that the chlorocatechol 1,2-dioxygenase enzyme in *P putida* CP1 belongs to the same branch as chlorocatechol 1,2-dioxygenases from other organisms that are adapted for chlorocatechol metabolism and is related to other chlorocatechol 1,2-dioxygenase with different substrate specificities

The chlorocatechol catabolic pathway of *Rhodococcus opacus* 1CP developed independently from the corresponding pathways of proteobacteria (Eulberg *et al*, 1998) Hence, the chlorocatechol 1,2-dioxygenase gene, *Clc*A, from this strain, together with that of the *Sphingomonas* species form another, separate, branch on the tree While the genus *Sphingomonas* belong to a subgroup of the proteobacteria, a comparison of the genes encoding the degradation of 2,4-dichlorophenoxyacetic acid or biphenyl suggested that usually only a low degree of sequence similarity is found between sphingomonads and other *Proteobacteria* This indicated that in the evolution of degradative pathways, some kind of barriers must have existed between sphingomonads and other *Proteobacteria* (Basta *et al*, 2004)

The catechol 1,2-dioxygenas genes, *Cat*A, form another branch on the tree These genes are involved in the degradation of non-chlorinated aromatic substrates and the widely held view is that catechol metabolism evolved first and then gave rise to chlorocatechol catabolism This conclusion is consistent with aromatic compounds, which give rise to catechol during their degradation, being more common in nature than chloroaromatic compounds that are degraded via chlorocatechols (Eulberg *et al*, 1998) For the outgroup, *Had*C, the hydroxyquinol 1,2-dioxygenase from *Ralstonia picketti* was used The hydroxyquinol 1,2-dioxygenase used by this strain degrades 4-chlorophenol via hydroxyquinol and not 4-chloroctaechol, the more usual route employed by the modified *ortho*-cleavage pathway (Nordin et al , 2005)

The presence of large plasmids were demonstrated in *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1 The genes for the *meta*- and modified *ortho*-cleavage

degradative pathways are often found on such large plasmids Most of the catabolic plasmids that have been reported are large plasmids of more than 50 kbp (Top et al , 2002) Hybridization of the restricted plasmid DNA with labeled gene probes for catechol 2,3-dioxygenase and chlorocatechol 1,2-dioxygenase confirmed that the genes for *meta*- and *ortho*-cleavage in *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1 were located on the large plasmids Such an approach has been used widely to localize catabolic genes on plasmids Basta et al , (2004) demonstrated the presence of large plasmids in xenobiotic-degrading *Sphingomonas* strains and hybridization with labeled gene probes suggested the large plasmids were involved in the *meta*-cleavage pathway degradation of dibenzo-p-dioxin, dibenzofuran and naphthalenesulfonates Labeled probes for chlorocatechol 1,2-dioxygenase of the modified *ortho*-cleavage pathway were also used to localize the chlorocatechol catabolism genes on large plasmids in the 2-chlorobenzoate degrading strain *Achromobacter xylosoxidans* A8 (Jencova, 2004)

The biodegradative capabilities of *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1 made them interesting candidates for bioaugmentation It was therefore of interest to label the organisms with green fluorescent protein (GFP) to facilitate their monitoring in mixed microbial systems GFP-tagged strains have been used as a visual marker to track xenobiotic degrading microorganisms in many environments including activated sludge (Boon *et al* , 2000) and soil (Jernberg et al , 2002)

The gene for GFP has been assembled into mini-transposons for an easy delivery into the strains of interest For this study, the transposons used for chromosomal insertion of *gfp* were a Tn*5*- and a Tn*7* based delivery system Tn5 inserts at random into the chromosome whereas Tn7 inserts at a specific site named *att*Tn*7*

In this study, *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1 were tagged with GFP using a Tn*5* transposon The transposon, mini-Tn*5gfp-Km,* (Fig 36), contained a promoter-less artificial operon encoding both GFP and neomycin phosphotransferase II (Npt II) (Tang *et al*, 1999) When this transposon was used to mutagenize *A*

tumefaciens, a wide range of fluorescence intensities was detected among the Km-resistant colonies, suggesting that the *gfp-nptII* operon was an effective reporter gene In addition, the colonies of the lowest fluorescence were resistant to high concentrations of kanamycin, indicating that the *gfp-nptII* operon was also an effective selection marker Mini- Tn*5gfp-Km* was therefore bi-functional in that it could both select for operon fusions and report the expression levels The GFP variant used in this selection-reporter transposon, GFPuv, was optimized for optimal fluorescence when excited by a UV light (360-400nm) and compared with wild-type GFP, is more soluble and less toxic and fluoresces 18 times more brightly (Crameri et al , 1996)

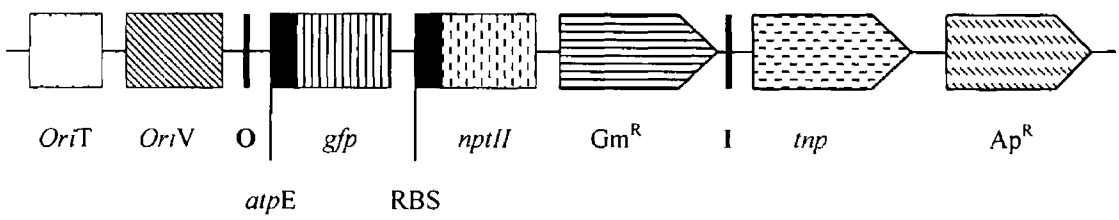


Figure 36 Selection-reporter transposon system The *gfp-nptII* operon is promoter-less The GFP ORF contained its own start codon and the *atpE* translational signal The NptII ORF included its own ribosome-binding site (RBS) and start codon Gm^R represents the gene encoding gentamicin resistance, *atpE*, the efficient translation region of the *E coli atpE* gene, **I** and **O**, inverted repeats of IS*50*, *tnp*, the gene encoding Tn5 transposase, *oriT*, RP4 transfer origin, *oriV*, R6K origin of replication (Tang *et al*, 1999)

Plate matings between *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1 with *E coli* S17 1 λ*pir* pTGN-mini-Tn*5gfp-Km* resulted in typically 200 mutant colonies on selective agar plates containing kanamycin and 4-chlorophenol of which approximately 1 in 400 expressed GFP to the extent that the colony was fluorescent to the naked eye under

illumination from a long-wavelength UV light This figure was low compared to the amount of transformants obtained on selective LB plates and the 15% reported by Tang *et al*, (1999) when the transposon was used to mutagenize *A tumefaciens* and may have been due to insertional inactivation of genes essential for growth on 4-chlorophenol Elvang *et al*, (2001), tagged the 4-chlorophenol degrading strain *A chlorophenolicus* A6 with GFP using Tn*5* minitransposons and found that the amount of transformants was higher on selective LB agar than on 4-chlorophenol agar However it was necessary to select onto 4-chlorophenol agar since the degradative ability of resultant mutants was lower without 4-chlorophenol selection

Since there was no growth of *Pseudomonas putida* A(a) or *Pseudomonas putida* CP1on control plates containing kanamycin, it was concluded that all kanamycin resistant colonies were transformants and that the transposon inserted downstream of weak promoters resulting in low expression of GFP in the majority of cases Tang *et al*, (1999) found that a wide range of fluorescence intensities was detected among the kanamycin resistant colonies and that even the colonies of lowest fluorescence were resistant to up to 400 µg ml of kanamycin

When screening for tansformants, 0 45 mM $FeSO_4$ $7H_2O$ was used to supplement selective LB agar as recommended by Timmis-Wilson and Bailey (2001) When viewed on standard bacterial agar, the fluorescence of siderophore producing *Pseudomonas* spp can mask normal levels of GFP fluorescence The additional iron suppresses siderophore biosynthesis to optimise fluorescence by GFP This may be useful where GFP is intended as a reporter or promoter probe to record variation in the levels of gene expression When grown on minimum medium containing 4-chlorophenol, siderophore biosynthesis was not observed However on the rich LB selective agar, the colonies were larger and fluoresced under UV light, which could easily be mistaken for GFP fluorescence, a problem that was eliminated by addition of iron to the media

Transformants of *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1 that were intensely fluorescent to the naked eye under UV illumination were isolated from 4-

chlorophenol agars for further analysis, since the main objective of this study was not promoter probing, but to obtain GFP-tagged strains capable of growth on 4-chlorophenol

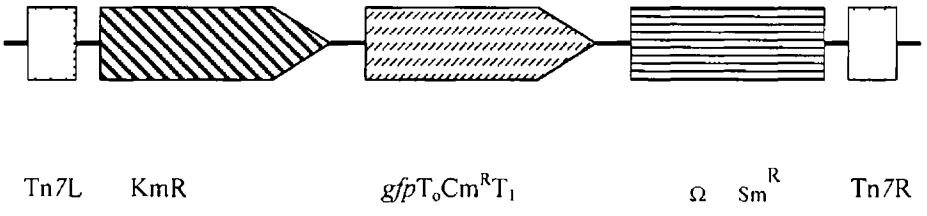


Figure 37 The Tn*7* element of the plasmid pBK-miniTn*7-gfp2* (Koch *et al*, 2001)

Although *E coli* S17 1 λ*pir* pTGN-mini-Tn*5gfp-Km* was used to successfully transform *Pseudomonas putida* A(a) and CP1 , another vector, pBK-mmiTn*7-gfp*2 was also used (Fig 37) This was a Tn*7*-based system for site specific insertion of GFP for tagging of target microorganisms (Koch et al , 2001) The *gfp* delivery plasmids contained a Tn*7* element with the intensely fluorescent mutant gfp gene, *gfp*mut3* (Cormack et al , 1996, Andersen et al , 1998) controlled by a modified *lac* promoter, $P_{A1/04/03}$ (Andersen et al , 1998) Strong fluorescence from *gfp*mut3* under the control of $P_{A1/04/03}$ has previously been observed in *Pseudomonas putida*, where the fluorescent cells could be observed after incubation under starvation conditions in soil even after two weeks (Ramos et al , 2000) The *gfp*mut3* mutant gene is more soluble than and is 21 times more fluorescent than the wild-type gene (Andersen et al , 1998) With an excitation maximum at 501 nm, it is more suited to the normal fluorescence filters found in standard fluorescent microscopes This Tn-7 based system also consisted of a helper plasmid pUX-BF13, which provided the Tn7 transposition functions in *trans*

Originally, plasmid pBK-miniTn*7*-*gfp*2 DNA and helper plasmid pUX-BF13 DNA was isolated and used for the transformation of *Pseudomonas* strains by Koch et al , (2001) as previously described by Højberg *et al*, (1999) using electroporation However, for this study pBK-miniTn*7*-*gfp*2 was used to transform the mobilizing strain, *E coli* S17-1 and a triparental mating procedure was used in conjunction with the helper strain, *E coli* SM10λ *pir* to transform the recipient organisms, *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1 by conjugation *E coli* S17-1 carried the transfer genes of the broad host range IncP-type plasmid RP4 integrated into their chromosomes and could utilize any Gram-negative bacterium as a recipient for conjugative DNA transfer (Simon *et al*, 1983) Following transformation of chemically competent *E coli* S17-1, transformed cells were easily visualized by the naked eye as green colonies, presumably due to the high plasmid copy number and expression of GFP from the constitutive promoter $P_{A1/04/03}$ As with plate matings with *E coli* S17 1 λ*pir* pTGN-mini-Tn*5gfp-Km*, miniTn*7*-*gfp*2 transformed cells were selected on 4-chlorophenol minimal medium containing gentamicin to ensure that the 4-chlorophenol degrading phenotype of transformants had not been lost due to insertion of the TN*7* element All of the resulting colonies were fluorescent under a fluorescence microscope

Chromosomal DNA from *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1 that had been transformed with the gene for GFP were subjected to PCR with primers specific for the gene for GFP and PCR products of the expected size were successfully amplified This confirmatory PCR test indicated that colonies that exhibited green fluorescence contained the *gfp* gene and that green fluorescence could be used as a visual marker (Errampalı et al , 1999) Amplification of the gene for gfp from *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1 also demonstrated the potential usefulness of PCR for detection of tagged strains in environmental samples Wang et al , (2004) used real time PCR (RTm-PCR) to detect *Pseudomonas putida* GN2 during 2-chlorobenzoate degradation in soil The strain contained the plasmid pGN2 which encoded the genes for GFP The RTm-PCR estimations of *Pseudomonas putida* GN2 numbers strongly correlated with those obtained from plate count methods during active 2-chlorobenzoated degradation However, after 2-chlorobenzoate degradation ceased, RTm-PCR estimates

were generally one order of magnitude lower than those from plate counts This underestimation was attributed to loss of the GFP-containing plasmid This problem could have been overcome if the gene for GFP had been inserted into the chromosome During the bioaugmentation of activated sludge, PCR was used to monitor the survival of GFP-tagged *Comamonas testosteroni* I2gfp (Boon et al , 2000) The detection limit was ca 5×10^5 CFU/ml, however, which was higher than that for the plating method

The tagged *Pseudomonas putida* strains were designated the names *Pseudomonas putida* A(a) GFPuv and Tn*7*-*gfp*2 and *Pseudomonas putida* CP1 GFPuv and Tn*7*-*gfp*2 In addition to the gene for GFP, both organisms had genes for antibiotic resistance inserted along with the transpson This resulted in resistance to gentamicin in the case of Tn*7* and kanamycin in the case of Tn*5* While the parental strains were inhibited by low concentrations of antibiotic, the tagged strains were resistant to greater than 70 μg/ml kanamycin and between 50 and 60 μg/ml gentamicin Insertion of the gene for GFP had no significant effect on the 4-chlorophenol degradation capabilities of the tagged strains This was particularly important to establish for the strains tagged using the Tn*5*-based vector, which inserts randomly into the chromosome, and may thereby cause insertional inactivation of host genes and positional effects affecting the expression of inserted genes (Sousa et al , 1997) GFP-tagged strains that show no difference from the wild type strains with regard to degradation of xenobiotic substrates have been widely reported (Elvang *et al* , 2001, Tresse *et al* , 1998, Errampalli et al , 1998) Results from API20NE and Biolog identification systems also indicated that the insertion of the GFP vectors caused no change in the substrate utilization patterns of the tagged strains No difference was found between the transformant and the wild type Therefore, insertion of the GFP gene did not alter the ability of the tagged strains to utilize the carbon sources tested The Biolog GN bacterial identification system has been used to show that chromosomal insertion of *gfp* did not affect the ability of the phenanthrene-degrading GFP-tagged strain, *Pseudomonas* sp UG14Gr to utilize the different carbon sources (Erramapali, 1998)

Colonies tagged with the GFPuv variant had the advantage of fluorescing under simple illumination with a UV lamp However, fluorescence under an epifluorescence microscope was not detectable, despite the fact that Suarez *et al*, (1997) stated that individual bacterial cells with a single copy of the gene could be readily observed under a fluorescence microscope For further studies, the strain tagged with miniTn*7*-*gfp*2 was selected The first reason was that tagged cells were visible at the single cell level under epifluorescence microscopy Secondly, the Tn*7* transpson used has been shown to insert at high frequency in one orientation as a single copy into a specific site named *att*Tn*7* Insertion into this site is directed by recognized sequences in the 3' end of glmS and because of this, Tn*7* insertion is believed to occur at a neutral site on the chromosome and should not affect its host Insertion of Tn*7* into this site is not deleterious to the host and provides a mechanism for the coexistence of Tn*7* and its host, and promotes the transmission of Tn*7* to daughter cells (Peters and Craig, 2001) The *glm*S gene encodes glucosamine synthetase, involved in N-acetyl glucosamine synthesis, required for cell wall biosynthesis and therefore present in most bacteria (Lambertsen *et al*, 2004, Choi *et al*, 2005) PCR analysis using a primer set that recognized th *glm*S gene and the *cat* gene encoding chloramphenicol acetyltransferase present on the vector showed that in *Pseudomonas putida* A(a), miniTn*7*-*gfp*2 inserted at this neutral site However in *Pseudomonas putida* CP1, miniTn*7*-*gfp*2 inserted at a specific but unknown site In many Gram-negative bacteria Tn*7* transposes into the specific target site located downstream of the *glm*S gene, but in *Pseudomonads* the situation is more uncertain (Koch *et al*, 2001) Site specific insertion of Tn*7* has been demonstrated in strains of *P fluorescens, P putida, P aeruginosa, P solanacearum and P syringae* (Barry, 1986, Hojberg *et al*, 1999, Staley *et al*, 1997, Boucher *et al*, 1985, Caruso and Shapiro, 1982, Shen *et al*, 1992) However the insertion site has not been identified in these strains While *glm*S genes are highly conserved, it may be possible that *Pseudomonas putida* CP1 has an unusual *glm*S gene that was not recognised by the primer It was necessary to demonstrate that the Tn*7* had not transposed onto the large plasmid present in *Pseudomonas putida* CP1 as this could lead to transfer of the GFP gene to other bacteria by conjugation This was accomplished when a probe for *gfp* failed to hybridize with plasmid DNA from *Pseudomonas putida* CP1

Also used for the tagging of *P putida* A(a) and CP1 were Tn*5* and Tn*7*-based transposons The plasmid for delivery of the Tn*5* transposon was pUTmini-Tn*5gfp* (Matthysse *et al*, 1996), and for the Tn*10* transpson, pLOFKm (Stretton *et al*, 1998) Following conjugation between donors and recipients, exconjugants grew on selective medium, indicating that the recipient cells were successfully transformed However, GFP fluorescence could not be detected under epifluorescence microscopy Both transpsons encoded the intensely fluorescent *gfp*(mut2) variant of GFP (Cormack et al, 1996), which had similar characteristics to *gfp*(mut3*), the gene for GFP that was encoded by the Tn*7*-based tagging system used in this study Unlike the Tn*7*-based system, these genes were promoterless in pUTmini-Tn*5gfp* and pLOFKm and insertion of Tn*5* and Tn*10* is random Despite the screening of numerous exconjugants, GFP fluorescence could not be detected This may have been due to the failure of Tn*5* or Tn*10* to insert near a strong promoter in either of the recipient strains, resulting in weak or undetectable levels of expression of *gfp*

API 20 NE tests and the Biolog identification system were used to obtain metabolic profiles of the parental *Pseudomonas putida* A(a) and CP1 and their GFP-tagged derivatives While CP1 had been identified previously in the lab using these identification systems (Farrell, 2000), the metabolic profiles obtained in this study served to identify A(a) for the first time using these tests API 20NE identification comprises 21 tests which are performed in cupules on a plastic strip where desiccated contents are reconstituted with a suspension of the test organism Some tests have to be overlayed with mineral oil to obtain the correct gaseous conditions Results are available in 24 - 48 hours and are represented as a seven digit profile number which may be read from the Analytical Profile Index API 20NE provides a quick and simple identification system which is capable of correctly identifying the majority of *Pseudomonas* species Studies carried out by Costas *et al* (1992) showed the correct identification of 90 4% of 146 *Pseudomonas* strains used 5 5% were not identified, while 4 1% were incorrectly identified

The Biolog identification system is based on tests for the oxidation of 95 substrates in a 96-well microtitre plate Each well contains a carbon source along with tetrazolium violet

which determines colorimetrically the increased respiration that occurs when cells oxidise the carbon source Reactions are read after 4 and 24 hours, thus providing a rapid identification Studies carried out by Costas *et al* (1992) showed the correct identification of between 74% and 79% of 114 *Pseudomonas* or *Pseudomonas*-like species depending on whether results were read using an automated plate reader or read manually The application of API 20NE and Biolog identification systems provided a reliable identification of A(a) as being a *Pseudomonas putida* belonging to the biovar A The majority of *Pseudomonas putida* strains have been consigned to biovar A, which is considered typical Biovar B differs from biovar A only in a few phenotypic characteristics-all biovar B strains utilise L-tryptophan, kynurenine, and anthranilate, and most use d-galactose as carbon source None of the strains of biovar B uses nicotinate (Bergey's Manual of Systematic Bacteriology, 2005)

Previous studies in our laboratory have shown an inability of activated sludge to efficiently degrade mono-chlorophenols to completion and using autoclaved sludge, the slight decrease in 4-chlorophenol concentration was attributed to abiotic adsorption to the sludge flocs In such cases, the indigenous population may be augmented by specialised bacteria, selected for the degradation of specific pollutants, in a process known as bioaugmentation As *Pseudomonas putida* A(a) metabolised 4-chlorophenol to completion via the *meta*-cleavage pathway and *Pseudomonas putida* CP1 degraded 4-chlorophenol to completion via the modified *ortho*-cleavage pathway, it was of interest to augment activated sludge with these strains It was hoped that addition of either strain to the activated sludge would bring about the degradation of 4-chlorophenol

No degradation of 4-chlorophenol by activated sludge was observed *Pseudomonas putida* A(a) Tn*7*-*gfp*2 and *Pseudomonas putida* CP1 Tn*7*-*gfp*2 were added to the activated sludge at three different inocula sizes, 0 5%, 1 0% and 5 0% (v/v)

Addition of *Pseudomonas putida* CP1 Tn*7*-*gfp*2 to the activated sludge, at all 3 inocula sizes, resulted in the removal of 4-chlorophenol within 48 hours However, this removal was accompanied by the production of yellow colored metabolites The production of 5-

Cl-HMS is usually associated with degradation via the *meta*-cleavage pathway (Knackmuss, 1981) The minor amounts of 5-Cl-HMS in the activated sludge augmented with *P putida* CP1 Tn*7-gfp* disappeared within 72 hours, and compared with that produced in the activated sludge augmented inoculated with *P putida* A(a) Tn*7-gfp*, indicated that degradation occurred predominantly using an *ortho-* cleavage pathway Attempts to carry out enzyme assays on the sludge were unsuccessful Sonication of the mixed liquor resulted in a black viscous liquid and even after dilution no enzyme activity could be detected The presence of 5-Cl-HMS has been found during biodegradation studies despite the fact that no *meta-* activity could be detected Radianingtyas et al (2003) reported the presence of a greenish yellow coloration of the culture medium during degradation of 4-chloroamline by a bacterial consortium capable of the degradation of 4-chloroaniline via an *ortho-* cleavage pathway despite the fact that no *meta-* cleavage activity could be detected They concluded that 2, 3-dioxygenase was not the main enzyme in the degradation of 4-chloroaniline One possible explanation for the appearance of 5-Cl-HMS could be that members of the indigenous sludge population possessed *meta*-cleavage activity against 4-chlorocatechol, an intermediate of 4-chlorophenol catabolism by *Pseudomonas putida* CP1, and were capable of further catabolism of the compound No 5-Cl-HMS production was observed in activated sludge alone when incubated with 4-chlorophenol, indicating that if such an indigenous population existed, it lacked the phenol hydroxylase activity necessary to oxidize 4-chlorophenol to 4-chlorocatechol

Addition of *P putida* A(a) Tn*7-gfp* to the activated sludge resulted in removal of 1 56mM 4-chlorophenol within 48 hours accompanied by the production of 5-chloro-2-hydroxymucomc semialdehyde, which was indicative of the *meta*-cleavage pathway Maximum accumulation was observed following 24 hours incubation Metabolite production was considerably higher in activated sludge augmented with 5 0% (v/v) *P putida* A(a) Tn*7-gfp* than that augmented with 0 5% and 1 0% (v/v) In the activated sludge, the yellow color disappeared in the presence of 5 0% (v/v) A(a) Tn*7-gfp* after 72 hours indicating complete degradation of 4-chlorophenol This was faster than in pure culture, where the disappearance of the yellow colour took 340 hours Therefore,

members of the activated sludge community may have played a part in the degradation of 5-chloro-2-hydroxymuconic semialdehyde This would be consistent with the possible explanation for the transient appearance of the yellow color in the activated sludge augmented with *Pseudomonas putida* CP1 Tn*7*-*gfp*2, i e members of the indigenous sludge population possessed *meta*-cleavage activity against 4-chlorocatechol

The addition of degradative bacteria to activated sludge does not always lead to improved degradation as was noted by McClure et al (1991) who reported the introduction of *P putida* UWC1 to activated sludge for the degradation of 3-chlorobenzoate Successful bioaugmentation relies on the ability of the introduced strain to survive at sufficient numbers in the new environment, in addition to its catabolic activities (Goldstein et al , 1985) In the activated sludge augmented with 0 5% and 1 0% (v/v) *P putida* A(a) Tn*7*-*gfp*, a dark brown colour developed in the culture medium after 48 hours incubation The accumulation of toxic halocatechols and their black auto-oxidation products is frequently observed in activated sludge in waste treatment plants subjected to shock loads of haloaromatics (Leisinger, 1986) The production of dark colours during the degradation of chloroaromatics is reported to be due to the abiotic polymerisation of accumulated metabolites (Bartels et al , 1984) This would indicate incomplete degradation of 4-chlorophenol by activated sludge augmented with 0 5% and 1 0% (v/v) *P putida* A(a) Tn*7*-*gfp* The success of the 5 0% inoculum size and the failure of the smaller inoculum sizes would indicate that inoculum size was a factor in the successful augmentation of the sludge with *P putida* A(a) Tn*7*-*gfp*

The survival of the *P putida* strains following their addition to activated sludge at different inoculum sizes was investigated *P putida* numbers fell following addition of either strain to activated sludge at all inocula sizes When the GFP-tagged strain *Pseudomonas putida* GREEN31 was inoculated into activated sludge, cell numbers decreased by 2-3 orders of magnitude within the first 3 days after addition to activated sludge (Eberl et al , 1997) This decrease was mainly the result of predation by protozoa so it is proposed that this was also the main reason for the decrease in introduced *Pseudomonas putida* strains in this study When the rates of decrease of GFP tagged

Pseudomonas putida strains in the sludge was compared, it was shown that *P putida* CP1 Tn*7*-*gfp* survived in higher numbers than *P putida* A(a) Tn*7*-*gfp* Survival of the strains at the different inocula sizes was in the order of 5 0% > 1 0% > 0 5% Inocula sizes of 0 5 and 1 0% were sufficient to bring about the complete degradation of 4-chlorophenol by *P putida* CP1 Tn*7*-*gfp* It is proposed that in this study, the 0 5 and 1 0 % size inocula of *P putida* A(a) Tn*7*-*gfp* did not survive in high enough numbers to bring about complete degradation of 4-chlorophenol Initially, the smaller inocula sizes were present in sufficient numbers to bring about the hydroxylation of 4-chlorophenol to 4-chlorocatechol, but the subsequent decrease in cell numbers were such that there were not enough cells left to further metabolise 4-chlorocatechol, which then polymerized to form the dark brown coloration which was seen in the sludge supernatant The ability of any organism to degrade a compound is dictated by several environmental and physiological factors (Goldstein et al , 1985) Among these factors, one possible reason for the failure of inoculation of contaminated sites with degradative bacteria is the inoculum size Ramadan et al (1990) used the *p*-nitrophenol degrading strain *Pseudomonas cepacia* for bioaugmentation of lake water The inoculum was capable of degrading *p*-nitrophenol at relatively high cell densities but not at low ones The failure of the small inoculum to mineralize *p*-nitrophenol was attributed to its failure to survive Because more cells were added with the large inoculum, a marked population decline might not result in the failure to degrade *p*-nitrophenol

Conjugal transfer of plasmids in activated sludge has been reported Geisenberger *et al*, (1999) reported the transfer of a GFP-tagged broad host range plasmid, RP4, from the *Pseudomonas putida* donor strain to indigenous bacteria in activated sludge Goris *et al*, (2003) reported the transfer of a GFP-tagged plasmid from *Pseudomonas putida* UWC to a diverse range of indigenous sludge bacteria Bathe *et al*, (2005) reported the horizontal transfer of pNB2 from *Pseudomonas putida* SM1443 *gfp*2x (unable to degrade 3-chloroaniline) correlated with the onset of 3-chloroaniline degradation

The presence of large degradative plasmids in *P putida* A(a) and *P putida* CP1 had been demonstrated These plasmids are often transferable and have a wide host range Because

of their high cell density and the presence of biodegradable organic matter, sludge flocs, which contain the majority of bacteria present in activated sludge, may form the ideal site for conjugation If this is true, successful integration of donors and subsequent plasmid transfer in activated sludge could be enhanced by using donor cells that easily form aggregates (Top et al , 2002) However, in this study there was no significant difference between the cell numbers capable of growth on 4-chlorophenol and the numbers of introduced *Pseudomonas putida* strains as determined by plating on selective media and therefore no evidence of plasmid transfer from either A(a) or CP1 to members of the indigenous community When performing colony counts on minimal medium containing 4-chlorophenol, no difference in colony morphology was observed, indicating that there were no transconjugants with a different morphology to A(a) or CP1 It is likely that the duration of this experiment was not sufficient for a detectable indigenous population of transconjugants to appear For evidence of plasmid transfer, continuous culture of the augmented sludge over a longer period of time may yield better results

The increased survival of *P putida* CP1 Tn*7-gfp* in activated sludge over *P putida* A(a) Tn*7-gfp* may have been due to its ability to flocculate in unfavourable conditions such as the presence of mono-chlorophenols (Farrell and Quilty, 2002) A common feature of a number of bacteria used successfully to augment activated sludges is their ability to flocculate McClure *et al* (1991a) described how the characteristic flocculation of strain AS2 may have been an important factor in the maintenance of a stable population following its introduction into an activated sludge unit facilitating 3-chlorobenzoate degradation Flocculation was also proposed by Watanabe et al (1996) as a reason for the successful augmentation of activated sludge by *Alcaligenes* sp E2 for the degradation of phenol Boon *et al* (2000) described how a phenotypic shift of *C testosteroni* I2 *gfp* to non-mucoid colony forming cells under unfavourable conditions may have been an advantage in the augmentation of activated sludge for the degradation of 3-chloroaniline During the bioaugmentation of activated sludge, *C testosteroni* I2 *gfp* was not randomly distributed, but clusterd within the sludge flocs Flocculation or incorporation of introduced bacteria into sludge flocs provides a protective environment against predation from protozoa that feed mainly on free suspended micro-organisms

(Eberl et al , 1997) For bioaugmentation of activated sludge, the inoculated strains should integrate into the flocs to avoid washout from the reactor (Van Limbergen *et al* , 1999) Using *gfp* labelled *P putida* CP1 and A(a) strains, CP1 was shown to be located in the activated sludge flocs, while A(a) was found to be evenly distributed throughout the activated sludge This ability to flocculate may have been important in the increased survival of *P putida* CP1 Tn*7*-*gfp*

In the activated sludge without 4-chlorophenol, there was a decrease in total cell numbers over time This may have been attributable to "natural causes" such as predation by protozoa and starvation The decrease in total cell numbers in activated sludge to which 4-chlorophenol had been added was greater than that observed in activated sludge with no 4-chlorophenol reflecting the toxicity of the substrate to the mixed microbial population The toxicity of 4-chlorophenol to microorganisms is well known (Hale *et al* , 1996, Boyd *et al* , 2001) When 0 5 and 1 0 % *P putida* A(a) Tn*7*-*gfp* were added to activated sludge with 4-chlorophenol, the decrease in cell numbers was similar to that observed in unaugmented activated sludge with 4-chlorophenol indicating a failure of augmentation at the lower inoculum size corresponding with the accumulation of polymerised chlorocatechols When 0 5, 1 0 and 5 0 % *P putida* CP1 Tn*7*-*gfp* and 5 0 % *P putida* A(a) Tn*7*-*gfp* were added to activated sludge with 4-chlorophenol, the decrease in overall cell numbers more resembled that of the unaugmented sludge without 4-chlorophenol than that to which 4-chlorophenol had been added This would indicate a protective effect of bioaugmentation on the indigenous population of the activated sludge by these inoculants

Boon *et al* , (2003) investigated the effects of a chloroaniline pulse on the composition of microbial communities in activated sludge augmented with the 3-chloroaniline strain *Commamonas testosteroni* I2 *gfp* In the bioaugmented reactor, the ammonia-oxidizing microbial community recovered in structure, activity and abundance, while in the nonaugmented reactor, ammonia oxidizers decreased drastically and the community composition changed and did not recover Erb *et al* , (1997), used the genetically engineered microorganism *Pseudomonas* sp B13 SN45RE, capable of the degradation of

mixtures of methyl- and chloroaromatics, to augment a laboratory scale sewage plant *Pseudomonas* sp B13 SN45RE significantly increased the degradation of phenol mixtures in the model system In the absence of the strain, shock loads of the phenol mixtures reduced the number of indigenous bacteria by three orders of magnitude, whereas in the augmented system, the indigenous community was protected and the continued functioning of the sewage system was assured In another experiment, *Pseudomonas* sp B13 SN45RE and its parent strain, *Pseudomonas* sp B13 were also used for the bioaugmentation of activated sludge In response to a shock load of phenols, inoculation with *Pseudomonas* sp B13 SN45RE protected the microbial community while in the system inoculated with the parent strain, the microbial community was severely disturbed The catabolic trait present in the genetically engineered *Pseudomonas* sp B13 SN45RE allowed for bioprotection of the activated sludge community from breakdown caused by toxic shock loading (Eichner et al , 1999)

Pseudomonas putida A(a) Tn*7-gfp*2 required a high inoculum size to degrade 4-chlorophenol and did not survive well or degrade 4-chlorophenol at low inoculum sizes The ability of *Pseudomonas putida* CP1 Tn*7-gfp*2 to survive and integrate into the activated sludge flocs coupled with its ability to degrade 4-chlorophenol at low inoculum sizes showed the potential of this strain for use in the treatment of xenobiotic containing activated sludge

5 0 Conclusions

- In pure culture, *Pseudomonas putida* A(a) degraded 4-chlorophenol to completion via a *meta*-cleavage pathway with the production of 5-chloro-2-hydroxymuconic semialdehyde while degradation of 2- and 3-chlorophenol was dead end *Pseudomonas putida* CP1 degraded all three mono-chlorophenol isomers via an *ortho*-cleavage pathway that did not give rise to any coloured metabolites in pure culture

- *Pseudomonas putida* A(a) and *Pseudomonas putida* CP1 harbored large plasmids of approximately 90 kb and >110 kb respectively These large plasmids gave restriction profiles that were different when subjected to single and double digests PCR and sequence analysis confirmed the presence of dioxygenase genes in both organisms The large plasmids were the location of these genes for the key enzymes for 4-chlorocatechol metabolism, chlorocatechol 2, 3-dioxygenase in A(a) and chlorocatechol 1, 2-dioxygenase in CP1

- *Pseudomonas putida* A(a) and CP1 were both labelled with the gene for GFP Insertion of the gene into the chromosome by a Tn7 transposon based delivery vector resulted in cells that were intensely fluorescent at the single cell level when viewed under an epifluorescence microscope In *P putida* A(a), Tn7 inserted at a non-coding neutral site in the chromosome In *P putida* CP1, Tn7 inserted at a specific but unknown site Insertion of the transposon did not have any detectable affect on the abilty of either organism to degrade 4-chlorophenol nor could any phenotypic effects be detected

- Augmentation of activated sludge by a 5 0% inoculum of *P putida* A(a) Tn*7-gfp* resulted in the complete removal of 4-chlorophenol while the 0 5 and 1 0% inocula sizes were unsuccessful Augmentation with CP1 Tn*7-gfp* resulted in complete 4-chlorophenol removal with all inocula sizes CP1 Tn*7-gfp* survived

in greater numbers than A(a) Tn*7-gfp* and this was attributed to its location within the sludge flocs

Further Study

The use of a continuous culture activated sludge unit could provide better data about the long term survival and activity of the inoculated strains

Molecular techniques could be used to analyse microbial communities without the need for isolation of individual strains Such methods could provide data about shifts in the composition of the indigenous microbial community in response to shock loads of xenobiotics and bioaugmentation with introduced strains

Further investigations of the large plasmids harboured by CP1 and A(a) could reveal if they are transferable and if transfer occurs during bioaugmentation of activated sludge One approach could be to label the plasmid with GFP to monitor transfer to the indigenous microbial community

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