THE CHARACTERIZATION OF *PSEUDOMONAS* SPECIES FROM A COMMERCIAL BIOAUGMENTATION PRODUCT.

A thesis presented for the
degree of PhD
by

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under the supervision of
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May 1993.

I hereby certify that this material, which I now submit for asssessment on the programme of study leading to the award of PhD is entirely my own work and has not been taken from the work of others save to the extent that such work has been cited and acknowledged within the text of my work.

Signed : Geraldine Mulcahy Date: 11-09-'93

THE WATER-BEARER

A flash of silver in the sun,
The salmon leaps cascading falls,
To reach at last the spawning place
Of clear and shining water.

I climbed Mount Halla in the sun,
A journey knowing sweat and pain,
To reach the summit in the clouds
A mirror of bright water.

My thumb singed on fair speckled flesh,
A sip from the crater of the gods;
And I repay their gifts to me
With pure, life-giving water.

Caren M. Mulcahy,
Dec. 1991.

This thesis is dedicated to
my parents for all their love
and encouragement.

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ABSTRACT

THE CHARACTERIZATION OF *PSEUDOMONAS* SPECIES FROM A COMMERCIAL BIOAUGMENTATION PRODUCT

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The *Pseudomonas* spp. from a commercial bioaugmentation product were characterized so that they might be monitored in a waste treatment system. The product CX 80 was chosen because of its use in the biodegradation of aromatic compounds. Four *Pseudomonas* spp. were selected - three *P. putida* spp., IGA 7.16, IGA 7.24 and IGA 0.92, and one *P. fluorescens* sp., IGA 13.42. The organisms were morphologically identical and were not readily distinguishable using biochemical tests. They had similar growth properties when grown on benzoate, naphthalene, phenol and m-toluate. IGA 7.16 alone was capable of growth on toluene and p-chlorophenol. A large plasmid band (85-120kb) was detected in IGA 7.16, IGA 7.24 and IGA 0.92. Southern hybridization to a ^{32}P-labelled probe and dot blot hybridization to a non-radioactively labelled probe confirmed the presence of a NAH plasmid in IGA 7.24 and a TOL plasmid in IGA 7.16.

P. putida IGA 7.24 was added to an activated sludge system in order to note the effect of bioaugmentation and to monitor the survival of the organism in the system. The response of the activated sludge to naphthalene with and without the addition of IGA 7.24 was similar. The systems adapted rapidly to naphthalene. Colony hybridization using a non-radioactively labelled NAH probe was more sensitive than vapour plates in the early detection of the emerging naphthalene degrading population.

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1 INTRODUCTION

1:1 PSEUDOMONADS

The ubiquity of pseudomonads in natural habitats and their remarkable nutritional versatility has led to their being the subjects of extensive research. The biodegradation of various organic compounds including aromatics has been studied at the biochemical and genetic level with a view to understanding and improving the degradation of these compounds in the environment. The application of selected and adapted *Pseudomonas* species to polluted soils or water may improve the removal of pollutants from the environment and their addition to waste treatment systems may improve the efficacy of treatment before effluents are discharged to the environment.

The term pseudomonad is commonly used to describe rod-shaped, Gram-negative, non-sporulated, polarly flagellated bacteria. Pseudomonads form a vast and heterogeneous group of organisms. The genus *Pseudomonas*, which was created by Migula in 1894, is the most important of the many genera of bacteria possessing the above characteristics (Palleroni, 1986). Members of the genus *Pseudomonas* occupy a dominant position in the biosphere in terms of variety of habitat and the number of species in a given habitat. *Pseudomonas* species are found in large numbers in all major natural environments, terrestrial, freshwater and marine, and in many different associations with plants and animals (Clarke, and Slater, 1986).

Members of this genus are described in Bergey's Manual of Systematic Bacteriology (1984) as being straight or slightly curved rods, 0.5 - 1μm in diameter and 1.5 - 5μm in length. Species are

motile by polar flagella, rarely non-motile although non-motile strains of various species are occasionally isolated from nature. *Pseudomonas* species are strictly aerobic and chemoorganotrophic. The optimum growth temperature for most strains is 28°C but many are capable of growth at temperatures in the range of 4 - 43°C.

Pigments of various chemical types are produced by many *Pseudomonas* species. They can be soluble in water and freely diffusible into culture media or they can remain associated with the cells. The best known of the soluble pigments are the fluorescent pigments of some members of RNA group 1. The fluorescent pseudomonads include *P. aeruginosa, P. fluorescens* and *P. putida*. These pigments act as siderophores, strong iron chelators, and allow growth in media having a low iron content. Another important soluble pigment is pyocyanin, a phenazine derivative characteristic of *P. aeruginosa*. Among the water-insoluble pigments that remain closely associated with cellular structures or diffuse only to a limited extent, several carotenoids and phenazine compounds have been identified.

Pseudomonas species are subdivided on the basis of RNA homology. There are five RNA groups. The best known species of the genus are included in group 1. *P. aeruginosa* is by far the best known especially in relation to medical microbiology. *P. fluorescens* and *P. putida* are other well known members of RNA group 1. Both are complex species and can be subdivided into a number of biovars or biotypes (Palleroni, 1986).

One of the most striking properties of members of

this genus is their remarkable nutritional versatility. Organic compounds readily used by *Pseudomonas* species include alcohols, aliphatic acids, amides, amines, amino acids, aromatic compounds, carbohydrates and hydrocarbons. In contrast to many other bacterial groups, many pseudomonads do not excel in the utilization of sugars. This ability to utilize a wide range of compounds makes *Pseudomonas* species an important component of activated sludge particularly that treating wastewater derived from the chemical industry. Of the various substrates used as sole carbon and energy source aromatic compounds are particularly interesting due to the biochemical intricacies of the various pathways by which they are metabolized (Palleroni, 1986).

Pseudomonads are readily isolated that can use aromatic compounds as their major sources of energy for growth since these compounds are widely distributed in natural environments. Many of the aromatic compounds found in the environment are derived from oil and coal, and the combustion of these and other organic materials (Dagley, 1986).

1:2 AROMATIC HYDROCARBONS IN THE ENVIRONMENT

1:2:1 REMOVAL OF AROMATIC COMPOUNDS FROM THE ENVIRONMENT

Organic compounds can be removed from the environment by a variety of mechanisms including biodegradation, auto-oxidation, adsorption, sedimentation, hydrolysis and photo-degradation. Biological processes are generally accepted to play the major role in nature (Painter, and King, 1985). The large scale production and use of organic compounds necessitates the treatment and safe

disposal of the hazardous wastes arising.

Little data are available on the aromatic wastes produced in Ireland and their disposal. Figures for the waste arising from the production of some hazardous organic compounds in Ireland for the years 1984 and 1985 were collated by An Foras Forbatha (Dec. 1986). These data are summarised in Table 1. The hazardous waste categories are defined by the EC (Toxic and Dangerous Waste) Regulations, 1982. As the chemical and pharmaceutical industry has grown considerably in the last decade these figures must be considered an underestimate of the situation today. The fate of much of this waste is unknown but traditionally there has been a reliance on landfill and incineration.

Table 1. Toxic and dangerous waste arising in Ireland (1984/85)

Waste Category	Quantities (tonnes/annum)
Organic Solvents	26,940
Biocides and phyto-pharmaceutical substances	7,680
Chlorinated Solvents	6,368
Phenols	600
Organic-halogen compounds (excluding other substances referred to in this listing)	17.4

There is considerable concern among the general public and environmentalists about traditional methods used to dispose of toxic wastes such as

incineration and landfill. As a result there is growing interest in the biological treatment of hazardous wastes. Many producers have biological treatment plants for the treatment of their less hazardous waste. The microorganisms in these systems can be inhibited or poisoned by the presence of more toxic compounds in the influent. Particularly attractive to these producers, is the possibility of bioaugmentation, adding microbes with the ability to degrade the toxic components of the waste. The development of improved biological systems for the treatment of hazardous waste requires detailed knowledge of the biochemical and genetic processes involved in microbial degradation.

1:2:2 BIODEGRADATION

Biodegradation can be defined as the breakdown of a compound by a living organism, for example bacteria or fungi. Ultimate biodegradation is the complete breakdown of a compound to fully oxidized simple molecules eg. CO_2, H_2O, NO_3^-, NH_4^+ with the formation of new cells (Painter and King, 1985). The extent of biodegradation of a compound in the environment can vary. The susceptibility of a compound to biodegradation is a function of a number of parameters. These include the chemical structure, the potential of the compound to associate with other organic and inorganic material in the environment and many physico-chemical factors such as pH, temperature, oxygen tension and salinity (Neilson *et al*, 1985).

A wide range of microorganisms have been found to possess biochemical pathways for the partial or complete degradation of a number of synthetic and

naturally-occurring aromatic hydrocarbons. Among the most widely studied catabolic pathways are those found in the genus *Pseudomonas*. Pseudomonads appear to have a remarkable potential to evolve entire catabolic sequences for xenobiotic compounds. This evolutionary flexibility may reflect the diversity of enzymes and pathways that have evolved to degrade naturally occurring organic compounds and may also involve genetic rearrangements within and between strains (Clarke and Slater, 1986).

1:2:3 CATABOLIC PLASMIDS

Many of the degradative pathways studied have been located on plasmids. Catabolic plasmids have been found in pseudomonads and a range of other bacteria. Degradative plasmids tend to be large, up to approximately 200kb (kilobase pairs) for the CAM plasmid (encoding a camphor degradative pathway). The degradative genes constitute only a small portion of the plasmid, for example a 40kb region of the TOL (toluene degradation) plasmid encodes the entire degradative pathway. The genes controlling transfer have also been located on some of these large plasmids but little is known about the function of the rest of the plasmid.

Catabolic plasmids can be either transmissible or non-transmissible. Plasmids found to be transmissible among *Pseudomonas* spp. have not been shown to be transferred to other genera. Most catabolic plasmids seem to be compatible with each other, that is they belong to different incompatibility groups, with the exceptions of CAM and OCT (n-alkane degradation), and NAH/SAL (naphthalene/salicylate degradation) and TOL. This

compatibility means that a single bacterial strain can carry a number of catabolic plasmids thus giving it a broader substrate range (Chakrabarty, 1976).

Many catabolic plasmids have been shown to share homologous regions. Most of these regions encode the degradation of catechol via the *meta*-cleavage pathway (section 1:3:2).

In a number of studies, a high degree of homology has been demonstrated between the *meta*-cleavage operons of the TOL, NAH and SAL plasmids (Lehrbach *et al*, 1983; Cane and Williams, 1986; Bayley *et al*, 1979). Further studies strongly indicated the same gene order on the *meta* operons of the TOL and NAH plasmids (Assinder and Williams, 1988).

A large body of evidence supports the theory that many different catabolic plasmids share a common ancestry. The organization of catabolic genes in distinct units (operons) may facilitate the assembly of new degradative plasmids and pathways. Genetic instability may promote rearrangement of DNA to form new pathways which are preserved by selective pressures (Yen and Serdar, 1988). Very many pathways have been described in *Pseudomonas* species for the degradation of a variety of aromatic compounds. The critical step in these pathways is the cleavage of the aromatic ring. The initial catabolic steps involve the insertion of two hydroxyl groups on the aromatic nucleus. Subsequent ring fission proceeds via *ortho* or *meta* cleavage of the hydroxylated aromatic.

1:3 BIODEGRADATIVE PATHWAYS IN *PSEUDOMONAS* SPECIES

Many different biodegradative pathways converge on a common intermediate - catechol. Catechol undergoes subsequent degradation by one of two pathways : the *ortho*-cleavage pathway or the *meta*-cleavage pathway. The *meta* pathway predominates in nature as the majority of aromatic compounds, especially methyl-substituted aromatics are degraded via the *meta* pathway while benzoate and chlorinated aromatics are metabolized via the *ortho* pathway (Williams and Murray, 1974). Due to differences in regulation, the *ortho* pathway is not normally expressed in cells containing the plasmid-encoded *meta* pathway (Williams and Worsey, 1976).

The initial product of *ortho*-fission of catechol (*cis, cis*-muconate) induces the enzymes of the *ortho* pathway. However, the enzymes of the *meta* pathway are induced by the primary substrates such as toluene or phenol. The activity of the *meta* enzymes prevents the accumulation of catechol and therefore the formation of *cis, cis*-muconate (Feist and Hegeman, 1969).

1:3:1 *ORTHO*-CLEAVAGE PATHWAY

The *ortho*-cleavage or β-ketoadipate pathway degrades catechol via β-ketoadipate to succinate and acetyl Co A (Figure 1). The structural genes for the enzymes involved are designated *cat* ABC and *pca* D and are shown in Figure 1. Initial cleavage of the aromatic ring of catechol is catalyzed by catechol 1,2-dioxygenase. This reaction is induced by the product, *cis, cis*-muconate and is also quite sensitive to catabolite repression (Wu *et al.* 1972). Catechol 1,2-dioxygenase consists of two

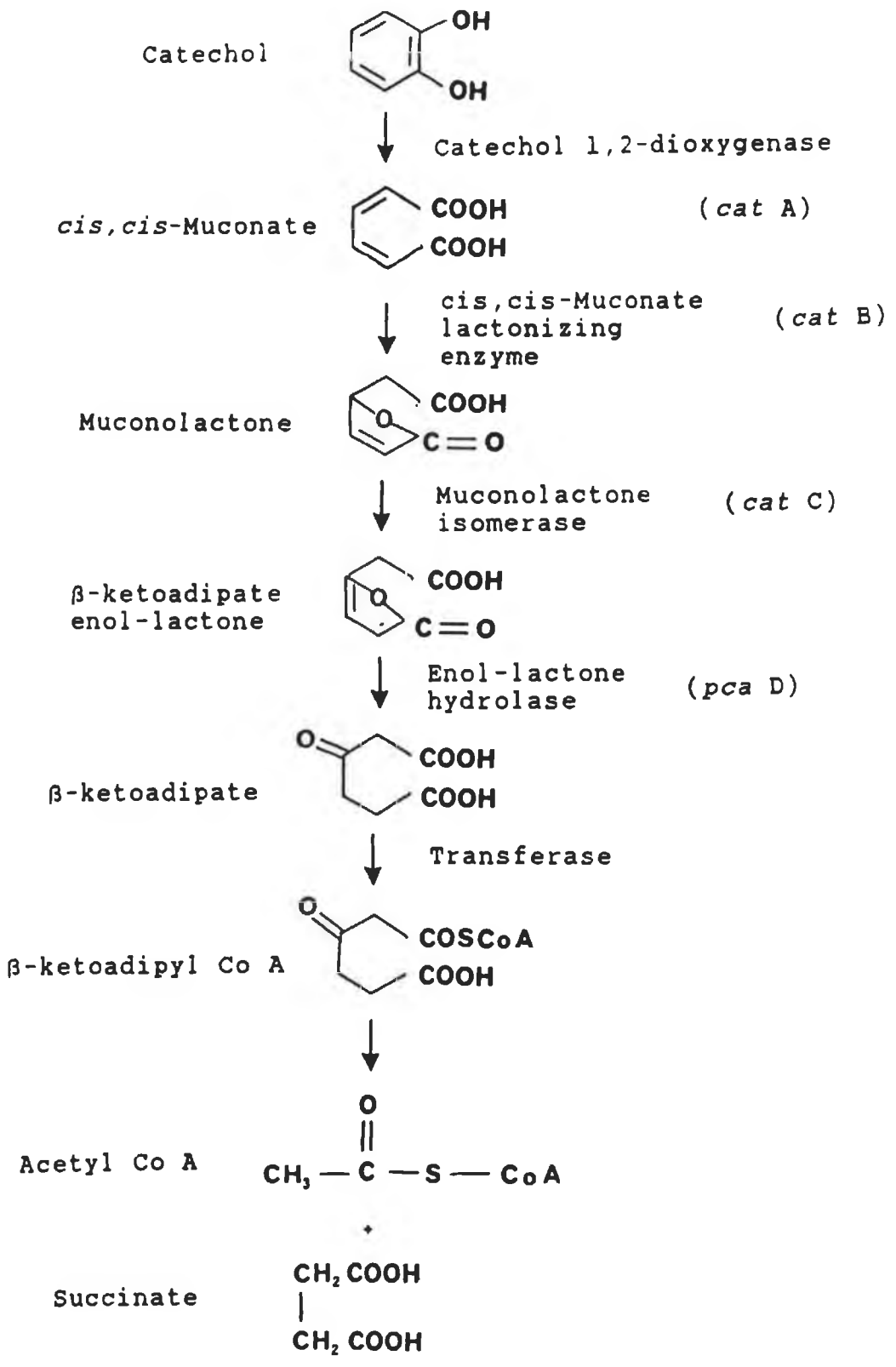


Figure 1. The *ortho*-cleavage pathway in pseudomonads (Wu *et al.*, 1972; Wheelis and Ornston, 1972)

non-identical subunits with a single active site. The ferric form of iron is the sole co-factor (Nozaki, 1979).

cis, cis-Muconate lactonizing enzyme (4-carboxy methyl-4-hydroxyisocrotonolactone lyase) is a decyclizing enzyme catalyzing the conversion of *cis, cis*-muconate to muconolactone and is induced by *cis, cis*-muconate (Hegeman, 1966). Muconolactone isomerase, also induced by *cis, cis*-muconate, converts muconolactone to enol-lactone which is further metabolized to β-ketoadipate by enol-lactone hydrolase. A transferase enzyme catalyzes the conversion of β-ketoadipate to β-ketoadipyl Co A which is then converted to succinate and acetyl Co A which feed directly into the TCA cycle. Enol-lactone hydrolase and transferase are induced by β-ketoadipate or β-ketoadipyl Co A (Wheelis and Ornston, 1972).

The *ortho*-cleavage pathway is chromosomally encoded. The structural genes for muconate-lactonizing enzyme (*cat* B) and muconolactone isomerase (*cat* C) have been shown to lie in close proximity on the chromosome. These enzymes share a common inducer (*cis, cis*-muconate) and their synthesis is co-ordinately controlled. Catechol 1,2-dioxygenase, though also induced by *cis, cis*-muconate, and its structural gene (*cat* A) are subject to independent control (Wu *et al*. 1972).

A regulatory gene, *cat* R has been located to the left of *cat* B on the *Pseudomonas putida* chromosome. Mutation studies of *cat* R suggest that it exerts positive control on the expression of *cat* B and C. Mutations in *cat* R do not affect the induction of catechol 1,2-dioxygenase. As well as being induced

in the presence of *cis, cis*-muconate, *cat* A is also subject to independent regulation by catabolite repression. This may be due to the role catechol 1,2-dioxygenase plays in the formation of *cis, cis*-muconate which acts as inducer for the subsequent enzyme reactions (Wu *et al*. 1972).

1:3:2 *META*-CLEAVAGE PATHWAY

Catechol is metabolized via the *meta*-cleavage pathway to pyruvate and acetaldehyde (Figure 2). The genes encoding the enzymes of the *meta* pathway are designated *xyl* E-K and are shown in Figure 2. As with the *ortho* pathway the initial reaction involves fission of the aromatic nucleus of catechol. This is achieved by the action of catechol 2,3-dioxygenase to produce 2-hydroxy-muconic semialdehyde. The active form of catechol 2,3-dioxygenase contains ferrous iron as a prosthetic group (Harayama and Rekik, 1990). 2-Hydroxymuconic semialdehyde can be metabolized via either the hydrolytic or dehydrogenative pathway, depending on the substrates.

2-Hydroxymuconic semialdehyde is degraded by the NAD^+-dependent dehydrogenase to 4-oxalocrotonate. The ring fission product of 4-methylcatechol, 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate (Figure 3) is also metabolized via the dehydrogenative pathway.

The product of 3-methylcatechol cleavage, 2-hydroxy-6-oxohepta-2,4-dienoate (Figure 4), is metabolized by the hydrolase to 2-oxopent-4-enoate and formate.

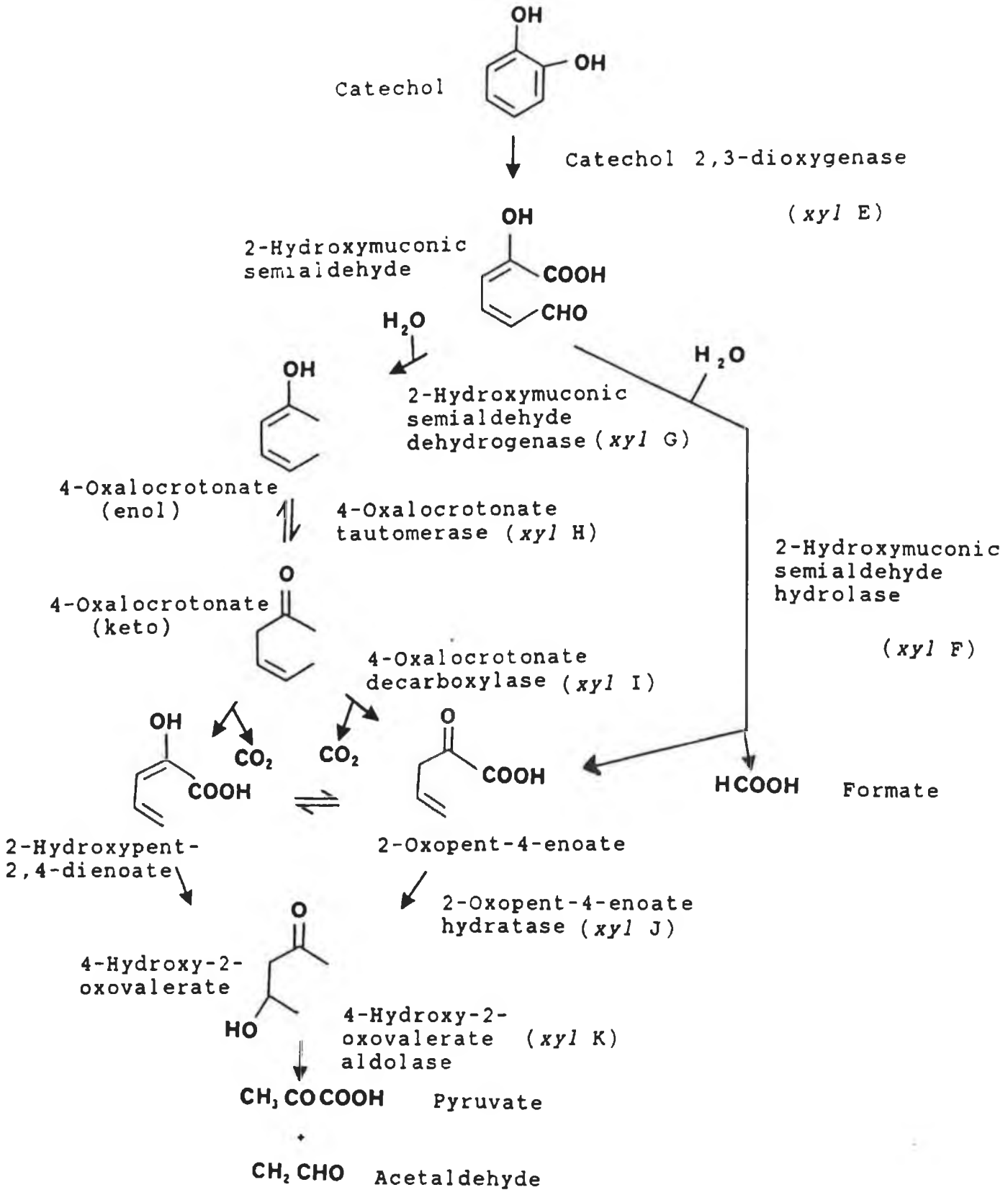


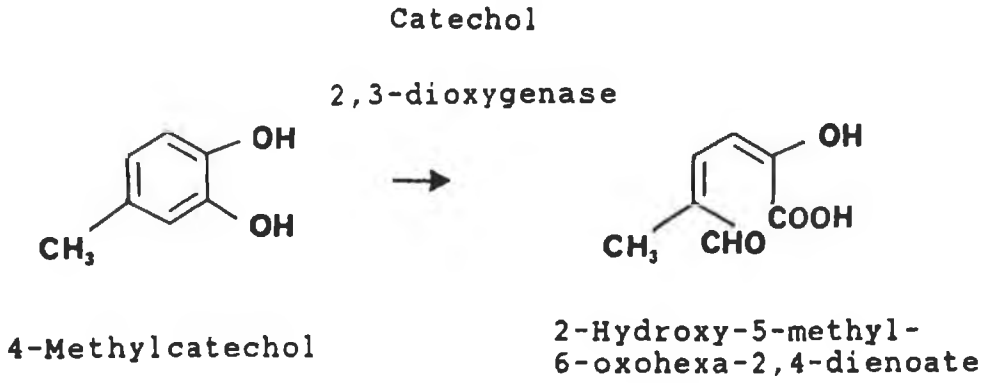
Figure 2. The *meta* cleavage pathway in pseudomonads (Bayly and Barbour, 1984 ; Wigmore *et al*, 1974 ; Harayama *et al*, 1989)

The dehydrogenase is inactive against 2-hydroxy-6-oxohepta-2,4-dienoate as it lacks an oxidizable aldehyde group (Harayama *et al.*, 1989).

The product of the 2-hydroxymuconic semialdehyde dehydrogenase reaction is the enol form of 4-oxalocrotonate (2-hydroxyhexa-2,4-diene-1,6-dionate). This is converted to the keto form (2-oxohex-4-ene-1,6-dienoate) by 4-oxalocrotonate tautomerase. The keto form is the substrate for 4-oxalocrotonate decarboxylase resulting in the formation of 2-oxopent-4 enoate with the release of CO_2. Mg^{2+} is required for decarboxylase activity (Harayama and Rekik, 1990 ; Bayly and Barbour, 1984).

At this point the hydrolytic and dehydrogenative pathways reconverge. 2-oxopent-4-enoate hydratase acts on 2-oxopent-4-enoate to form 4-hydroxy-2-oxovalerate. Harayama *et al.* (1989) found that the product of the decarboxylase was the enol compound 2-hydroxypent-2,4-dienoate and that this was spontaneously and reversibly transformed into 2-oxopent-4-enoate, the keto form. They found the enol form to be the substrate for the hydratase.

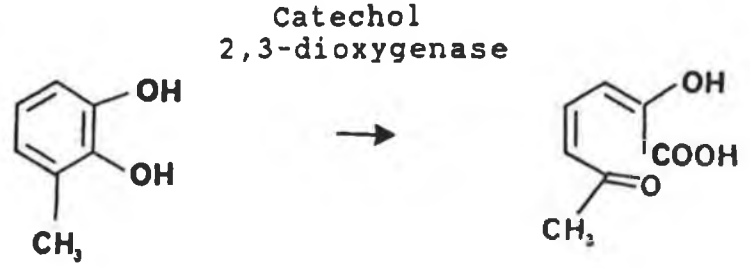
Their work also suggested that the hydratase and decarboxylase form a physical complex *in vivo* which may ensure the efficient transformation of the unstable intermediate 2-hydroxypent-2,4-dienoate. Finally, 4-hydroxy-2-oxovalerate aldolase catalyzes the conversion of 4-hydroxy-2-oxovalerate to the TCA intermediates, pyruvate and acetaldehyde.



4-Methylcatechol

2-Hydroxy-5-methyl-
6-oxohexa-2,4-dienoate

Figure 3. The cleavage of 4-methylcatechol by catechol 2,3-dioxygenase



3-Methylcatechol

2-Hydroxy-6-oxohepta-
2,4-dienoate

Figure 4. The cleavage of 3-methylcatechol by catechol 2,3-dioxygenase

The genes for the *meta*-cleavage pathway are organized into an operon, carried on a number of catabolic plasmids. As outlined previously there is a high degree of homology between the *meta*-cleavage operons of different plasmids. One of the best studied is that carried on the TOL plasmid, pWWO. The *meta* operon of pWWO comprises thirteen genes : *xyl* E,F,G,H,I,J,K which code for the structural genes of the enzymes responsible for the degradation of catechol to pyruvate and acetaldehyde as detailed in Figure 2 ; *xyl* X,Y,Z which encodes the 3 subunits of toluate 1,2-dioxygenase and *xyl* L which encodes 1,2-dihydroxy-3,5-cyclohexadiene-1-carboxylate dehydrogenase (these two enzymes catalyze the conversion of toluate or benzoate to catechol); also contained on the pWWO *meta* operon are two genes, *xyl* T and *xyl* Q, the functions of which are as yet unknown. The order of these genes within the operon is as follows: *xyl* X,Y,Z, *xyl* L, *xyl* T, *xyl* E, *xyl* G, *xyl* F, *xyl* J, *xyl* Q, *xyl* K, *xyl* I, *xyl* M (Harayama and Rekik, 1990).

A promoter is located upstream of *xyl* X and the regulatory gene *xyl* S is located downstream of *xyl* H. As with many degradative pathways studied, the *meta* cleavage pathway is under positive regulation. Toluate and benzoate act as inducers of the pathway in the presence of the *xyl* S gene product. The *meta* pathway can also be induced by the substrates for the TOL upper pathway eg. toluene, benzyl alcohol, when the products of both *xyl* S and *xyl* R (regulatory gene for upper pathway) are present (Worsey *et al.*, 1978).

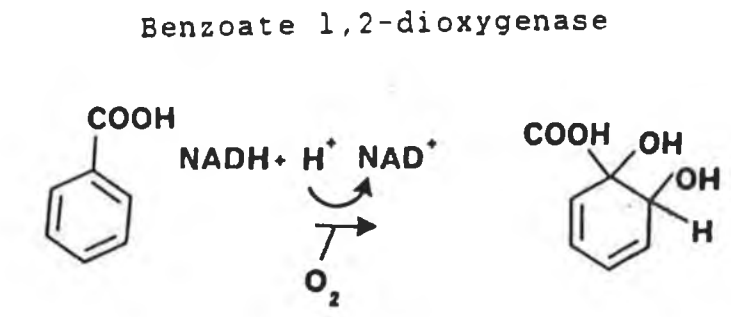
Overproduction of the *xyl* S gene product leads to constitutive expression of the *meta* pathway in the

absence of inducers, indicating the existence of both an active and inactive form of the *xyl* S protein, with activation occurring in the presence of the inducing compounds. Mutation studies involving *xyl* S have revealed the possible existence of a third regulatory gene. A mutation in *xyl* S prevents induction of the *meta* pathway by m-toluate but not by unsubstituted compounds such as benzoate. Benzoate interacts with the OP2, operator-promoter region of pWWO to induce the *meta*-pathway in the absence of known plasmid regulatory genes. Benzoate-induction of OP2 has only been demonstrated in *Pseudomonas spp.* suggesting the involvement of a chromosomal gene, possibly a regulatory gene for the chromosomally-encoded benzoate dioxygenase (Cuskey and Sprenkle, 1988).

The entire meta cleavage operon of pWWO is 10kb in size, making it one of the largest operons known in bacteria. Its size could pose a problem with regard to both transcription and stability of the mRNA for the operon. It has been suggested that the *meta* operon was formed by the fusion of two DNA modules each possessing its own promoter. The first comprising the genes for the conversion of toluate/benzoate to catechol, *xyl* X,Y,Z,L,(T) and the second encoding the degradation of catechol, *xyl* (T),E,G,F,J,Q,K,I,H (Harayama and Rekik, 1990).

1:4 BENZOATE DEGRADATION BY *PSEUDOMONAS* SPECIES

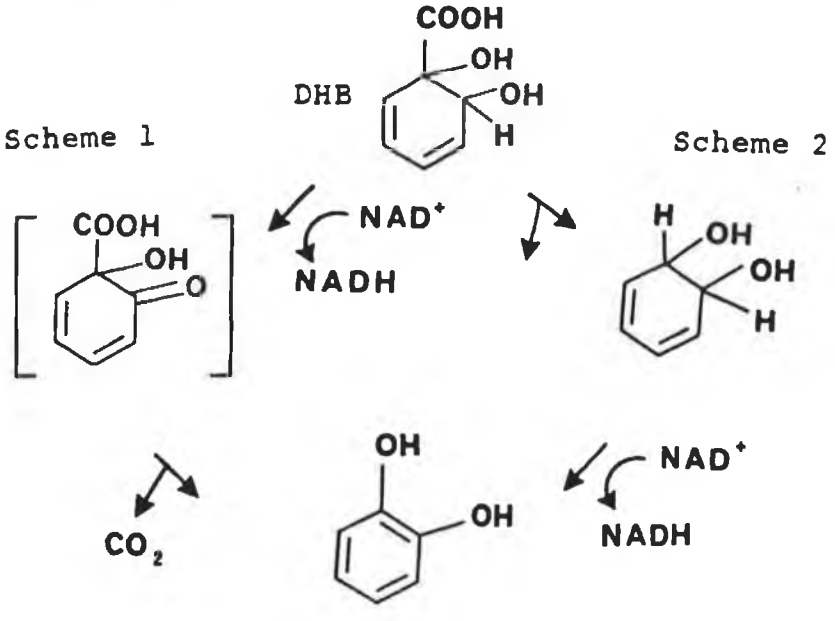
In most pseudomonads benzoate is degraded via the chromosomally-encoded *ortho* cleavage pathway. Benzoate is initially acted upon by benzoate-1,2-dioxygenase to form 2-hydro-1,2-dihydroxybenzoic acid (Figure 5).



Benzoate

1,2-Dihydro-1,2-dihyroxybenzoic acid (DHB)

Figure 5. Benzoate degradation by benzoate-1,2-dioxygenase



Catechol

Figure 6. The conversion of 1,2-dihydro-1,2-dihydroxybenzoic acid (DHB) by DHB dehydrogenase

Benzoate dioxygenase is a multicomponent enzyme consisting of an NADH-cytochrome c reductase component and a terminal oxygenase component. The reductase is an iron-sulphur flavoprotein containing one FAD (Flavin Adenine Dinucleotide) and one iron-sulphur centre of plant ferredoxin type with two iron and two labile sulphur atoms (Yamaguchi and Fujisawa, 1978). The oxygenase component is also an iron-sulphur protein. It contains two subunits, the larger polypeptide containing the iron-sulphur cluster. Benzoate dioxygenase also seems to contain three iron atoms as prosthetic groups in addition to the three iron-sulphur clusters (Yamaguchi and Fujisawa, 1982).

1,2-Dihydro-1,2-dihydroxybenzoic acid (3,5-cyclohexadiene-1,2-diol-1-carboxylic acid) is metabolized to catechol by the single component enzyme, DHB dehydrogenase (3,5-cyclohexadiene-1,2-diol-1-1carboxylic acid oxidoreductase). This enzyme requires NAD^+ for its action which involves a dehydrogenation and decarboxylation reaction. Two alternative schemes for the formation of catechol from dihydrodihydroxybenzoic acid are shown in Figure 6.

In scheme 1 dehydrogenation occurs first, yielding an unstable α-keto acid intermediate which undergoes spontaneous decarboxylation to catechol.

Decarboxylation of DHB occurs first in scheme 2 yielding 3,5-cyclohexa-diene-1,2-diol, followed by dehydrogenation to catechol. Experimental results obtained by Reiner (1972) favour the first route. Catechol is metabolized to acetyl Co A and succinate via the *ortho*-cleavage pathway.

The enzymes for benzoate degradation are chromosomally encoded and are ubiquitous in *Pseudomonas* spp. Benzoate can also be attacked by the TOL plasmid-encoded enzymes, toluate 1,2-dioxygenase and 1,2-dihydroxy-3,5-cyclohexadiene-1-carboxylate dehydrogenase. Toluate dioxygenase has a broad-substrate specificity while benzoate dioxygenase has a much narrower substrate range. However, both enzymes have a similar structure with an NADH-cytochrome *c* reductase component and an oxygenase component composed of two polypeptides. The three sub-unit proteins have similar molecular weights in both enzymes suggesting they are closely related (Harayama and Rekik, 1990).

Benzoate has been found to induce the OP2 operator-promoter of the TOL plasmid pWWO and a chromosomally-encoded regulatory gene for benzoate-dioxygenase is thought to be involved (Cuskey and Sprenkle, 1988). Little is known of the regulation of benzoate dioxygenase and DHB dehydrogenase. DHB dehydrogenase appears only to be induced during growth on benzoate. DHB cannot support the growth of many strains most likely due to the polarity of DHB which would make transport across the cell membrane difficult (Renier, 1971).

1:5 PHENOL DEGRADATION BY *PSEUDOMONAS* SPECIES

The plasmid-encoded, multicomponent enzyme, phenol hydroxylase catalyzes the formation of catechol by the hydroxylation of the aromatic nucleus of phenol as shown in Figure 7 (Shingler *et al.*, 1989). Catechol is further metabolized via the *meta*-cleavage pathway as described previously. The synthesis of phenol hydroxylase and the enzymes of

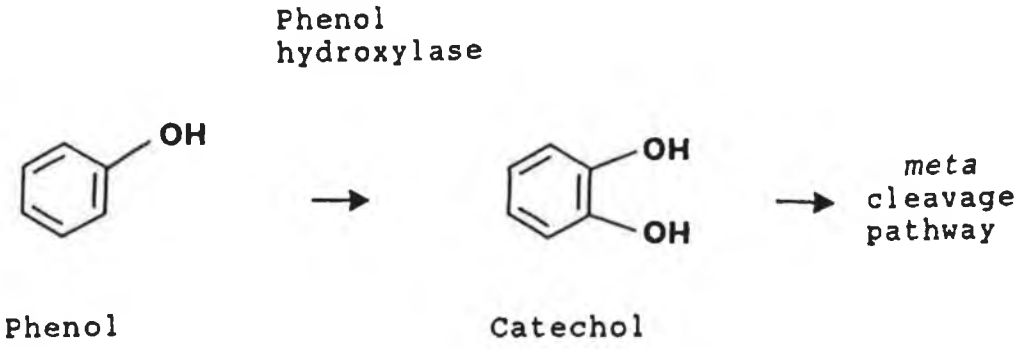


Figure 7. The hydroxylation of phenol in pseudomonads

the *meta* pathway are induced in the presence of phenol (Feist and Hegeman, 1969).

Meta-cleavage of catechol results in the formation of 2-hydroxymuconic semialdehyde which can be metabolized by either the NAD^+-dependent dehydrogenase or an hydrolase. In the case of phenol degradation the preferred route seems to be via the dehydrogenase activity. However, when 2-hydroxymuconic semialdehyde accumulates in the cells this seems to "drive" it down the hydrolytic pathway (Wigmore *et al.*, 1974).

Some pseudomonads with the ability to metabolize phenol carry TOL, NAH, SAL or related plasmids (Wong *et al*, 1978 ; Cane and Williams, 1982). A

natural multiplasmid system encoding phenol, m-toluate and salicylate metabolism was isolated from *P. putida* EST1001 (Kivisaar *et al.*, 1989).

1:6 CHLOROPHENOL DEGRADATION BY *PSEUDOMONAS* SPECIES

Most halogenated organics found in the environment are man-made. Chlorinated hydrocarbons are widely used as industrial solvents but their chief use is probably as herbicides and insecticides. The first weed-killers produced in 1945 were chlorophenol derivatives. Many chlorinated hydrocarbons are not readily degraded in the environment and can therefore accumulate. The danger to man and animals is that many chlorinated and non-chlorinated hydrocarbons are soluble in fat and can accumulate in body tissues until they eventually produce toxic effects (Caglioti, 1983).

Chlorophenols are toxic to a number of aerobic microorganisms because of their ability to uncouple oxidative phosphorylation. Many organisms have therefore developed mechanisms to detoxify such compounds, usually by simple chemical alterations in the structure eg. alkylation, esterification or nucleophilic displacement of the halogen substituent. However complete biodegradation of aromatic compounds is hindered by the presence of chlorine or other halogen substituents on the aromatic ring. Halogen atoms deactivate the benzene nucleus to electrophilic attack by dioxygen by withdrawing electrons from the ring. This deactivating effect increases with the number of halogen substituents (Neilson *et al.*, 1985).

The crucial step in the biodegradation of

chlorophenol and other haloaromatics is the removal of the halogen substituent. This can occur early in the degradative pathway with reductive, hydrolytic or oxygenolytic elimination of the substituent (Reineke and Knackmuss, 1988). Dehalogenation of haloaromatics by hydroxylation before ring cleavage has been demonstrated for several different substrates. Dehalogenating hydroxylases have been proposed to account for metabolism by pseudomonads of *para*-substituted haloaromatics via unsubstituted substrates for ring-cleavage enzymes (Weightman, *et al.*, 1984). Initial dehalogenation is, however, quite rare and the majority of haloaromatics are degraded via halocatechols. The elimination of the halide ion is a spontaneous reaction following cycloisomerization or linked to lactone formation during *ortho*-cleavage of the halocatechols (Knackmuss, 1981).

Fortuitous metabolism of haloaromatics requires initial catabolic enzymes, especially oxygenases, which can cope with both the steric and negative inductive effects of substituents. Phenol hydroxylase is induced in *Pseudomonas sp.* B13 during growth on 4-chlorophenol (Knackmuss and Hellwig, 1978). The toluene dioxygenase system exhibits very broad substrate and inducer specificities and can catalyze the conversion of a number of chlorophenols to their corresponding chlorocatechols (Spain *et al.*, 1989). The upper pathway enzymes of the TOL plasmid pWWO have also been shown to have relaxed substrate specificities. Toluene monooxygenase, benzyl alcohol dehydrogenase, benzaldehyde dehydrogenase and toluate dioxygenase can all act on chlorinated substrates (Abril *et al.*, 1989 ; Harayama *et al.*,

1986).

The action of the above enzymes on chlorinated aromatics results in the formation of chlorocatechols. Chlorocatechols interfere with oxygen-binding of catechol 1,2-dioxygenase due to the steric and inductive effects of the chloride atom resulting in the accumulation of toxic chlorocatechols and their black autooxidation products in culture supernatants (Bartels *et al.*, 1984). The *meta*-cleavage enzyme, catechol 2,3-dioxygenase readily cleaves 4-chlorocatechol yielding 5-chloro-2-hydroxymuconic semialdehyde, a dead-end product which accumulates in the culture medium resulting in an intense yellow colour (Figure 8) (Knackmuss, 1981).

3-chlorocatechol, formed from the oxidation of both 2- and 3-chlorophenol, is a suicide substrate for catechol 2,3-dioxygenase resulting in the generation of an acylchloride, 5-chloroformyl-2-hydroxypenta-2,3-dienoic acid (Figure 9). This product is an acylating agent which irreversibly inactivates catechol 2,3-dioxygenase, again resulting in the accumulation of chlorocatechol and its autooxidation products. Inactivation might also partly result from chelating the iron from the active centre of the enzyme. The destruction of *meta*-cleavage activity by chlorocatechols and their inefficient oxidation by *ortho*-cleavage resulting in the accumulation of black autooxidation products is a common phenomenon and explains the instability and dark colour of activated sludge suddenly loaded with chloroaromatics (Bartels *et al.*, 1984).

Ring cleavage, therefore, is a crucial reaction in chlorophenol degradation. Strains which

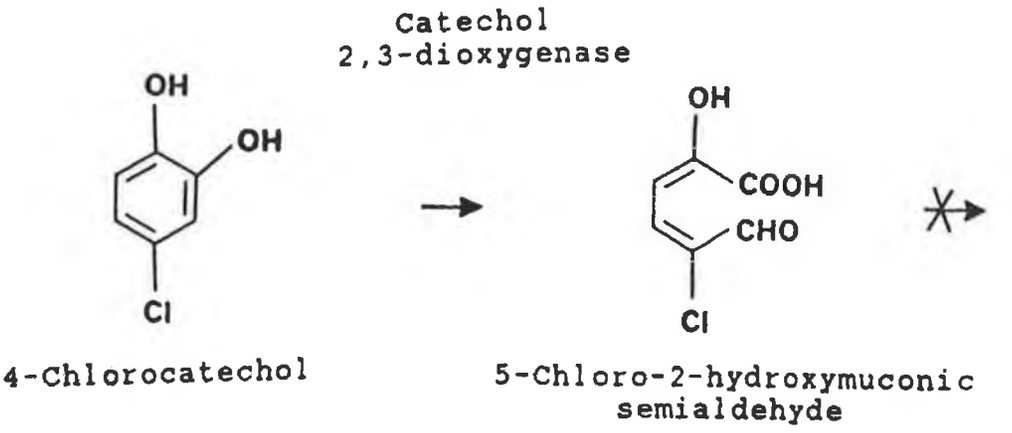


Figure 8. The *meta*-cleavage of 4-chlorocatechol

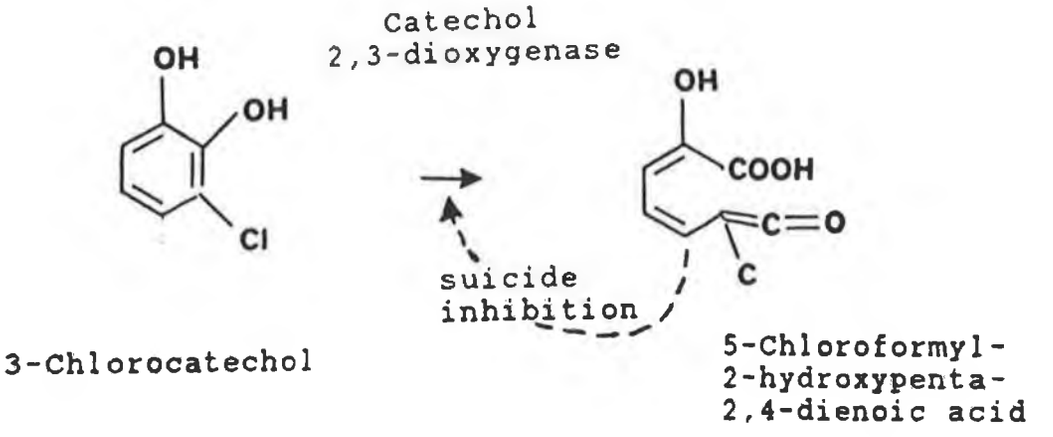


Figure 9. The *meta*-cleavage of 3-chlorocatechol

successfully utilize chloroaromatics as growth substrates have developed a modified *ortho*-cleavage mechanism (Figure 10). The enzymes of the modified pathway have relaxed substrate specificities and high activities for chloro-substituted substrates. Pyrocatechase II and cycloisomerase II are isoenzymes of the corresponding unmodified enzymes which exhibit some activity with chlorinated substrates suggesting that they are the ancestors of the modified enzymes selected during enrichment on chlorinated substrates. Hydrolase I and II are not true isoenzymes as there is not cross-reactivity between their two functions. Hydrolase II is highly specific for 4-carboxymethylene-but-2-en-4-olide but exhibits no activity for the ordinary 4-carboxymethyl-but-3-enolide of the unmodified pathway (Knackmuss, 1981).

Initial hydroxylation of chlorophenol to chlorocatechol can be catalyzed by a mono- or dioxygenase. The reaction requires molecular oxygen and usually NADH or NADPH. Ring cleavage of 3- or 4-chlorocatechol, catalyzed by pyrocatechase II (chlorocatechol 1,2-dioxygenase), is achieved by the insertion of oxygen between carbons one and two of the benzene nucleus. Lactonization of the resulting chloromuconic acid to unstable intermediates by cycloisomerase II leads to the spontaneous loss of a chloride ion and the formation of buterolides. Hydrolase II catalyzes lactone cleavage with the formation of maleylacetate which is further metabolized via the TCA cycle (Steiert and Crawford, 1985).

Complete degradation of chlorophenol and many other haloaromatics, therefore, requires the presence of broad substrate specific enzymes, inhibition of

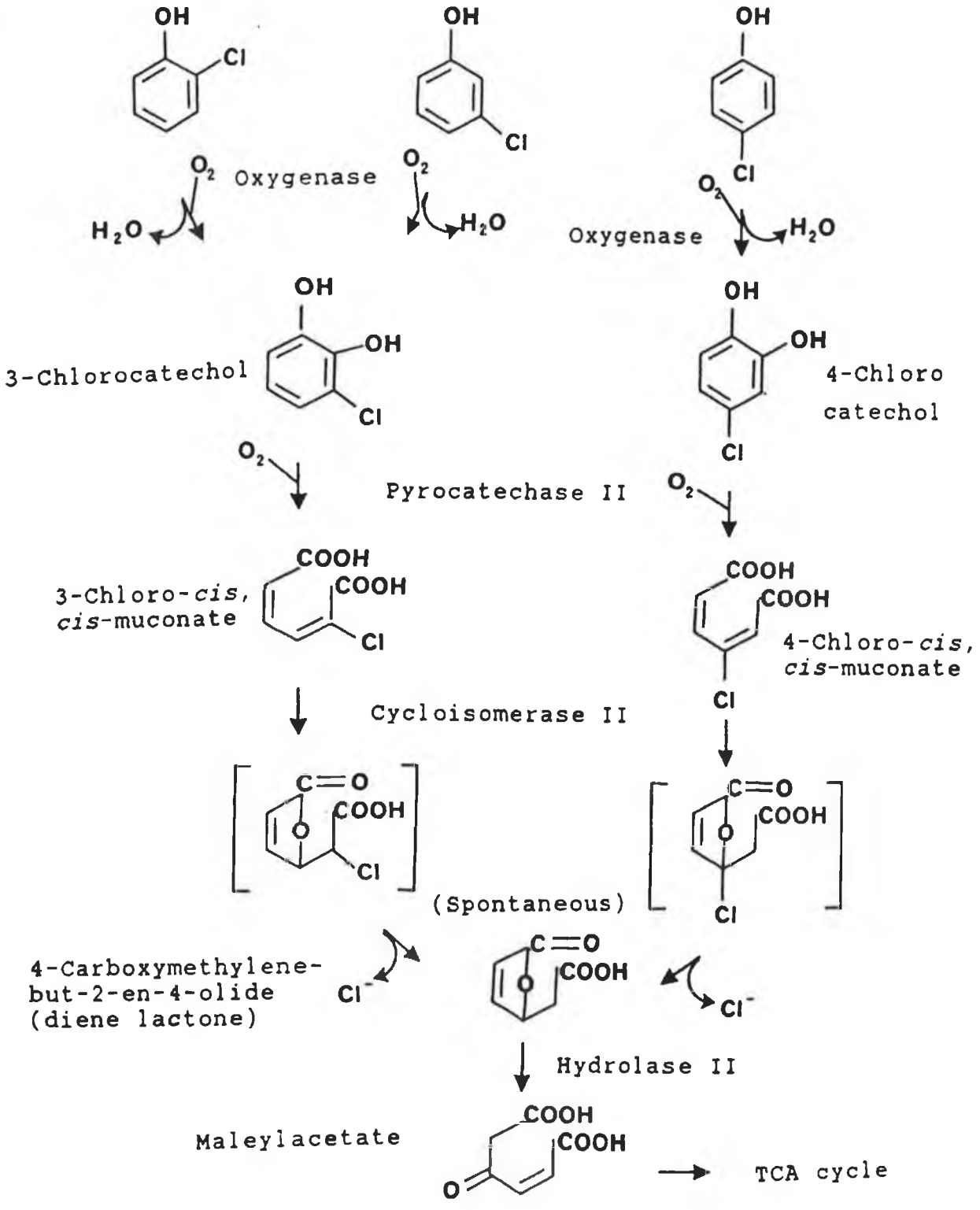


Figure 10. The degradation of chlorophenol in pseudomonads (Knackmuss, 1981)

unproductive *meta* pathway enzymes and the development of a modified *ortho*-cleavage pathway.

Although plasmids such as the TOL have been implicated in the initial metabolism of chloroaromatics, few plasmids are known which allow complete degradation of such substrates. Examples include the plasmids pAC25 and pAC31 which encode a pathway for the complete degradation of chlorobenzoates and pJP2 and pJP4 which encode for the degradation of 2,4-dichlorophenoxyacetic acid and 3-chlorobenzoate. In nature several plasmids and their gene products may interact to allow complete degradation of complex haloaromatic compounds. Chromosomally encoded enzymes such as those for the modified *ortho*-cleavage pathway also play a vital role (Karns *et al.*, 1984).

1:7 TOLUENE DEGRADATION BY PSEUDOMONAS SPECIES

Toluene is one of the many by-products formed during oil refining and coke production from coal. It is also used in plastics manufacture (Caglioti, 1983) and in the chemical industry. A number of pseudomonads possess the ability to utilize toluene and related compounds as their sole source of carbon and energy. In many pseudomonads this ability is conferred by the presence of a TOL plasmid. In some pseudomonads a chromosomally encoded pathway is responsible for toluene metabolism. These pathways are outlined in Figure 11 and Figure 12.

A third pathway for toluene degradation has been described in *Pseudomonas mendocina*, shown in Figure 14.

1:7:1 DEGRADATIVE PATHWAY ENCODED BY THE TOL PLASMID

The toluene degradative pathway encoded by the TOL plasmid pWWO is presented in Figure 11.

The structural genes for the enzymes involved are designated *xyl* A, B, C, D, L. Toluene undergoes initial oxidative attack at the methyl substituent to form benzyl alcohol. The toluene mono-oxygenase system requires NADH and FAD for this reaction (Gibson and Subramanian, 1984). Benzyl alcohol is further oxidized to yield benzaldehyde, benzoate and finally catechol, which then undergoes *meta*-fission.

Benzyl alcohol dehydrogenase catalyzes the reversible oxidation of benzyl alcohol to benzaldehyde with the concomitant reduction of NAD^+. Benzaldehyde dehydrogenase then catalyzes the irreversible oxidation of benzaldehyde to benzoate with a corresponding NAD^+ reduction. Both these enzymes are dimers of identical subunits and neither contain a prosthetic group (Shaw and Harayama, 1990).

Toluene oxygenase has quite a broad substrate specificity, oxidizing toluene, m- and p-xylene, m-ethyltoluene and 1,3,4-trimethylbenzene. It can also oxidize m- and p-chlorotoluene at a low rate. Benzyl alcohol dehydrogenase and benzaldehyde have a broader specificity, oxidizing m- and p-methyl-, ethyl- and chloro-substituted benzyl alcohols and benzaldehydes, as well as the unsubstituted compounds.

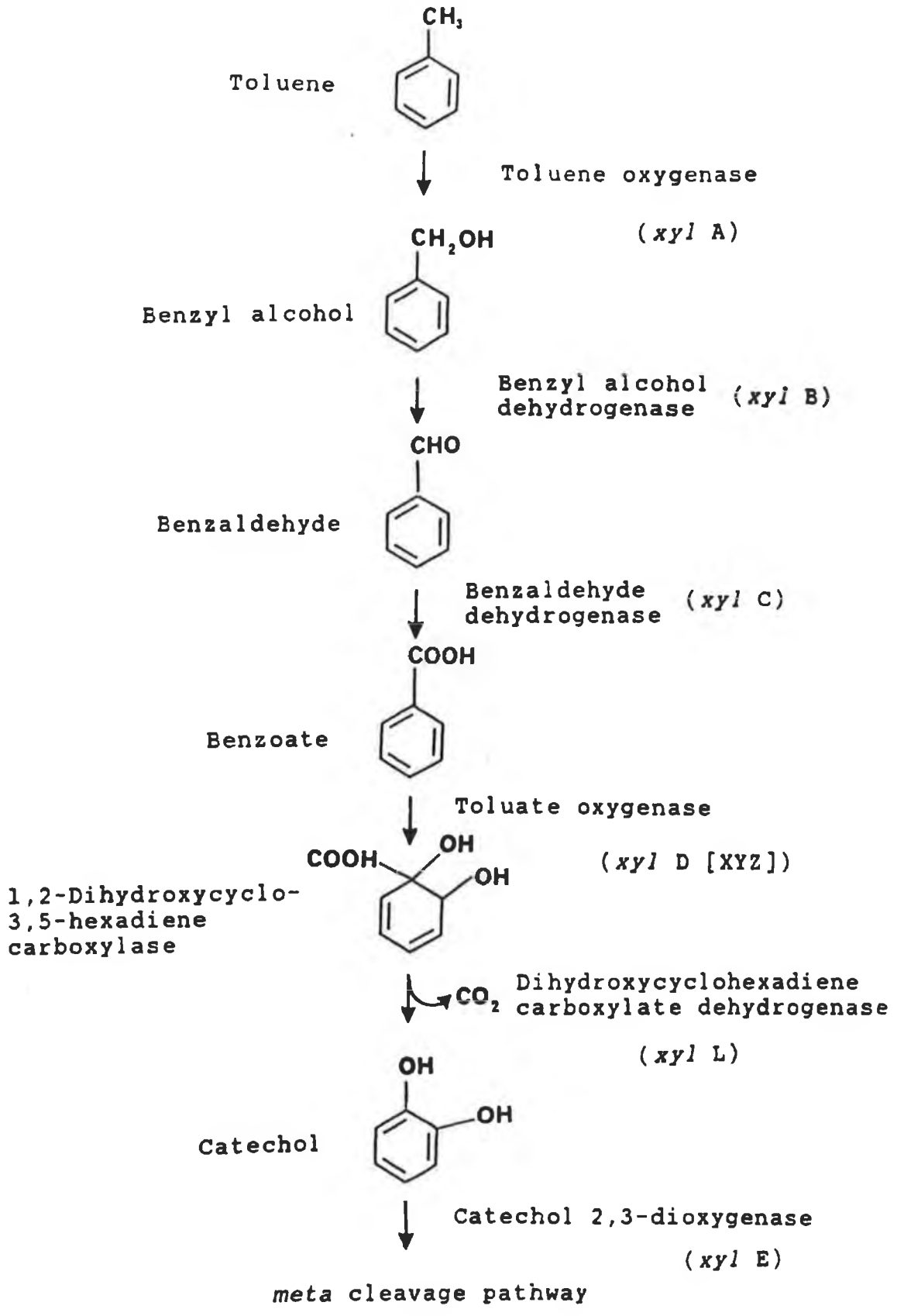


Figure 11. Toluene degradative pathway encoded by the TOL plasmid (Burlage, Hooper and Sayler, 1989)

Toluene oxygenase, therefore is the limiting step in the oxidation of chloro-substituted toluenes, preventing the accumulation of toxic dead-end products produced by *meta*-cleavage of chlorocatechols (Abril *et al.*, 1989).

pWWO, the archetypal TOL plasmid, was first described in *Pseudomonas arvilla* mt-2, renamed *Pseudomonas putida* mt-2 (Williams and Murray, 1974). Many other TOL plasmids have since been described, most of which show strong DNA homology to pWWO and encode similar biochemical pathways. pWWO is a large (117kb), self-transmissible plasmid belonging to the P-9 incompatibility group. The catabolic and regulatory genes are carried on approximately 40kb of the plasmid. The catabolic genes are organized into two operons, one encoding enzymes for the upper pathway (7kb) and the other encoding the *meta* pathway enzymes (10kb). The two operons are separated by a segment approximately 10kb in length (Harayama *et al.*, 1986). The genes for replication and conjugal transfer have been located only roughly. The structural genes for the upper pathway enzymes are given in Figure 11. Those of the *meta* pathway enzymes are shown in Figure 2.

Although control of the upper and lower (*meta*) TOL pathways is not fully understood, two regulatory genes have been located on the TOL plasmid, *xyl* R and *xyl* S. Substrates for the upper pathway enzymes eg. toluene, benzyl alcohol are inducers of the pathway when the *xyl* R product is present. The product of *xyl* S combines with the carboxylic acids, benzoate or m-toluate to induce the *meta* pathway. The substrates for the upper pathway can induce both lower and upper pathways when the

products of both *xyl* R and *xyl* S are present (Worsey *et al.*, 1978). This prevents the build-up of toxic intermediates and prevents the escape of metabolite pools that might act as a chemoattractant for competing organisms (Burlage, *et al*, 1978). Research indicates that the TOL degradative genes are under positive control at the level of transcription initiation (Harayama *et al.*, 1986.

The TOL plasmid has been shown to be stably maintained in the host, even during growth on non-selective media. Under carbon and energy-limiting conditions, the presence of the TOL plasmid places a strain on the host resulting in loss or deletion of the plasmid (Euetz and van Andel, 1991). The TOL plasmid can be lost or undergo large, plasmid-specific deletions when grown on benzoate. Cells carrying the TOL plasmid metabolize benzoate via the *meta*-cleavage pathway. The *ortho*-cleavage pathway is not normally expressed in such cells due to differences in the regulation of the two pathways. The chromosomally-encoded *ortho* pathway appears to permit faster growth on benzoate. Therefore, a positive selection exists for cells that have lost the plasmid (Williams and Worsey, 1976). Benzoate is also thought to selectively inhibit the growth of cells containing wild-type or deleted TOL plasmids (Stephens and Dalton, 1988).

The 40kb fragment which encodes the entire degradative pathway on the TOL plasmid has been found on the chromosome of some strains that have lost the TOL plasmid and these strains are still capable of toluene and xylene degradation (Broda *et al.*, 1988). In studies of continuous cultures by Keshavarz *et al.*(1985) approximately 1% of the

total population retained the TOL plasmid after 600h growth under benzoate-limiting conditions. However, the TOL^+ population recovered to 100% when the medium was returned to m-toluate. Plasmid stability was greater in TOL^+ cells isolated from benzoate cultures. The plasmid loss may be due to a failure in the control of partitioning during cell division. This failure is not total and a residual low level of plasmid-containing bacteria persist in the population, which proliferate when toluene is reintroduced to the culture.

1:7:2 CHROMOSOMAL TOLUENE DEGRADATIVE PATHWAY

An alternative chromosomally-encoded pathway for toluene degradation has been described in *Pseudomonas putida* F1 (Zylstra and Gibson, 1989). Toluene is metabolized to 3-methylcatechol via *cis*-toluene dihydrodiol. 3-methylcatechol is further metabolized via the *meta*-cleavage pathway (section 1:3:2). Toluene degradation by *P. putida* F1 is outlined in Figure 12 (Zylstra *et al.*, 1988). The structural genes for this pathway are designated *tod* A-F.

Toluene dioxygenase catalyzes the initial reaction by incorporating both atoms of molecular oxygen into the aromatic nucleus to form *cis*-toluene dihydrodiol ((+)-*cis*-1,2-dihydroxy-3-methylcyclohexa-3,5-diene). Toluene dioxygenase is a multicomponent, membrane-associated enzyme system comprising ferredoxin $_{TOL}$ reductase, ferredoxin $_{TOL}$ and ISP $_{TOL}$. Ferredoxin $_{TOL}$ reductase is a flavoprotein which accepts electrons from NADH and transfers them to a small iron-sulphur protein, ferredoxin $_{TOL}$. Ferredoxin $_{TOL}$ then reduces ISP $_{TOL}$,

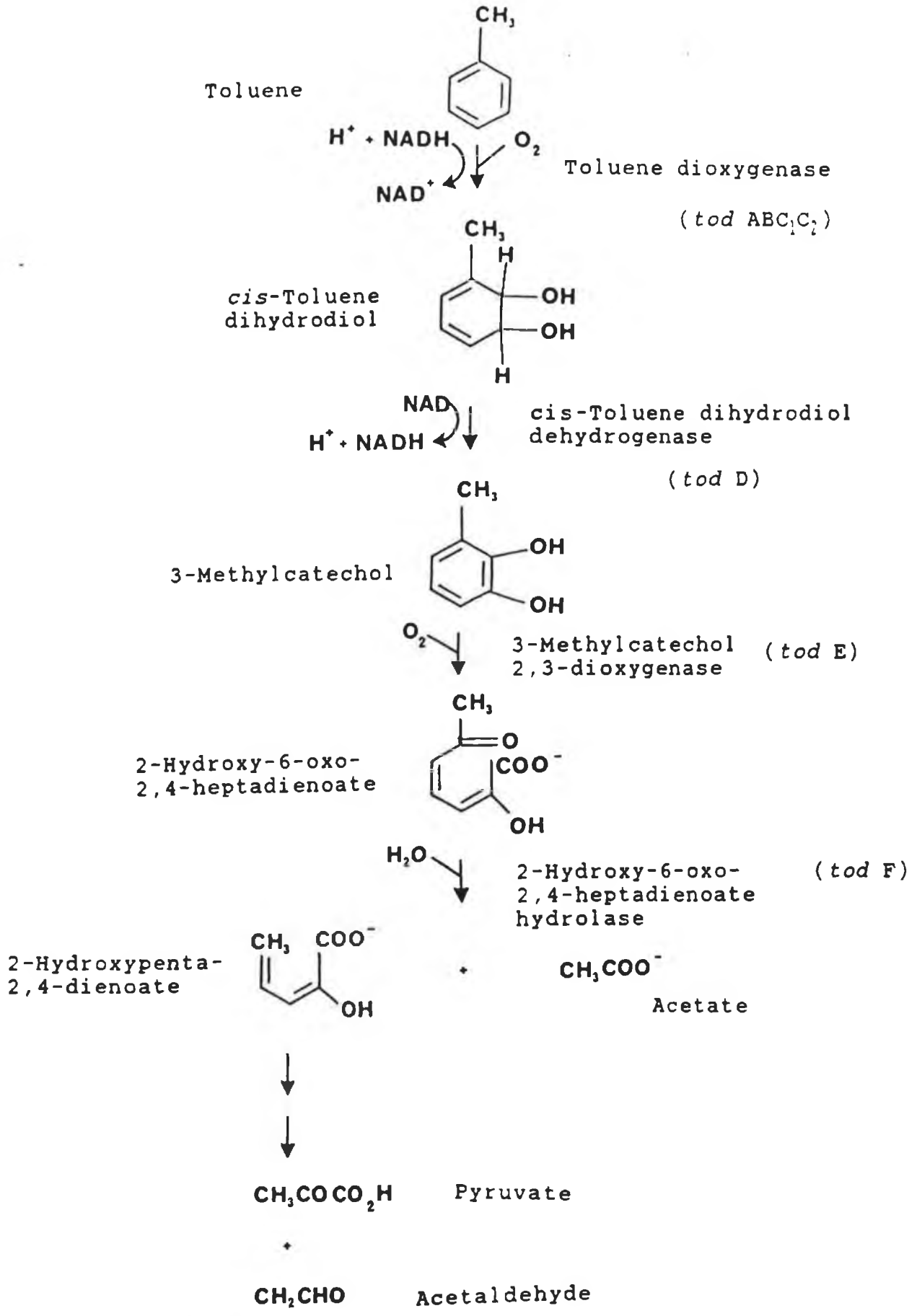


Figure 12. The degradation of toluene by the chromosomal pathway of *P. putida* F1

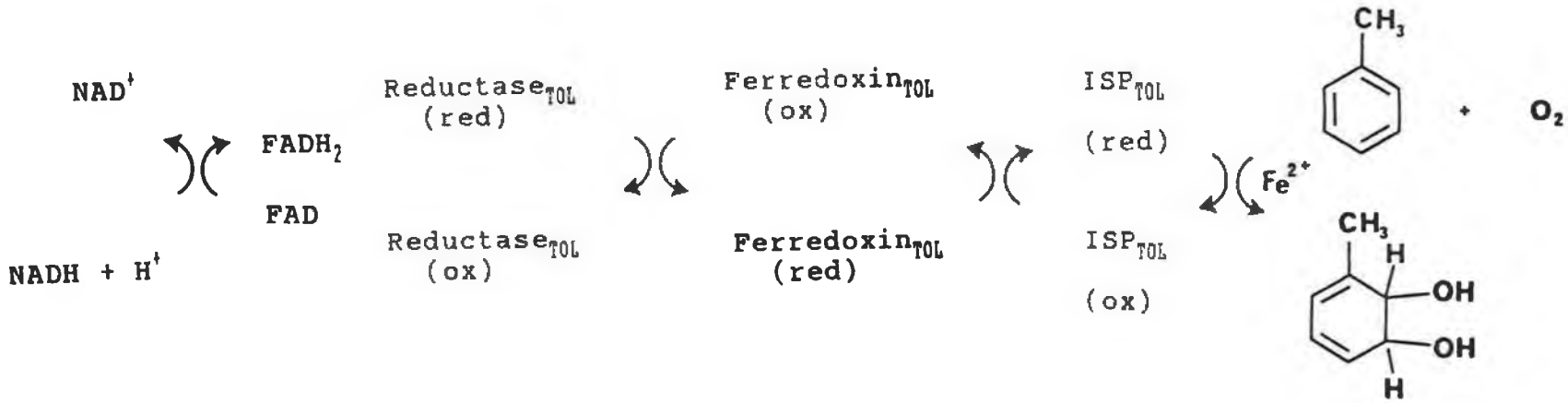


Figure 13. Proposed electron transport scheme for toluene dioxygenase

NAD - Nicotinamide adenine dinucleotide
FAD - Flavin adenine dinucleotide
red - reduced
ox - oxidized

the terminal oxygenase component, which is a large iron-sulphur protein. The reduced oxygenase catalyzes the oxidation of toluene to *cis*-toluene dihydrodiol. The action of toluene dioxygenase is summarized in Figure 13 (Gibson and Subramanian, 1984).

cis-Toluene dihydrodiol is further oxidized to 3-methylcatechol by an NAD^+-dependent dehydrogenase (Figure 12) (Rogers and Gibson, 1977). *Meta*-cleavage of 3-methylcatechol by the dioxygenase yields 2-hydroxy-6-oxo-2,4-hepta-dienoate, which is further metabolized to 2-hydroxypenta-2,4-dienoate and acetate by a hydrolase. A detailed description of the *meta*-cleavage of 3-methylcatechol to the TCA intermediates, pyruvate and acetaldehyde, is described in section 1:3:2.

The structural genes encoding the aforementioned enzymes have been designated *tod* A, B, C_1, C_2, D, E, F as shown in Figure 12. These genes form part of the *tod* operon for toluene degradation and are carried on the chromosome of *Pseudomonas putida* F1. A series of mutations and complementation analyses performed by Zylstra *et al.* (1988) revealed the order of transcription of the genes in the *tod* operon to be : *tod* F, *tod* C_1, *tod* C_2, *tod* B, *tod* A, *tod* D, *tod* E. Further studies indicated that the *tod* genes are under positive control.

1:7:3 TOLUENE DEGRADATIVE PATHWAY IN *PSEUDOMONAS MENDOCINA*

A route for toluene degradation specific to *P. mendocina* has been described and is summarized in Figure 14.

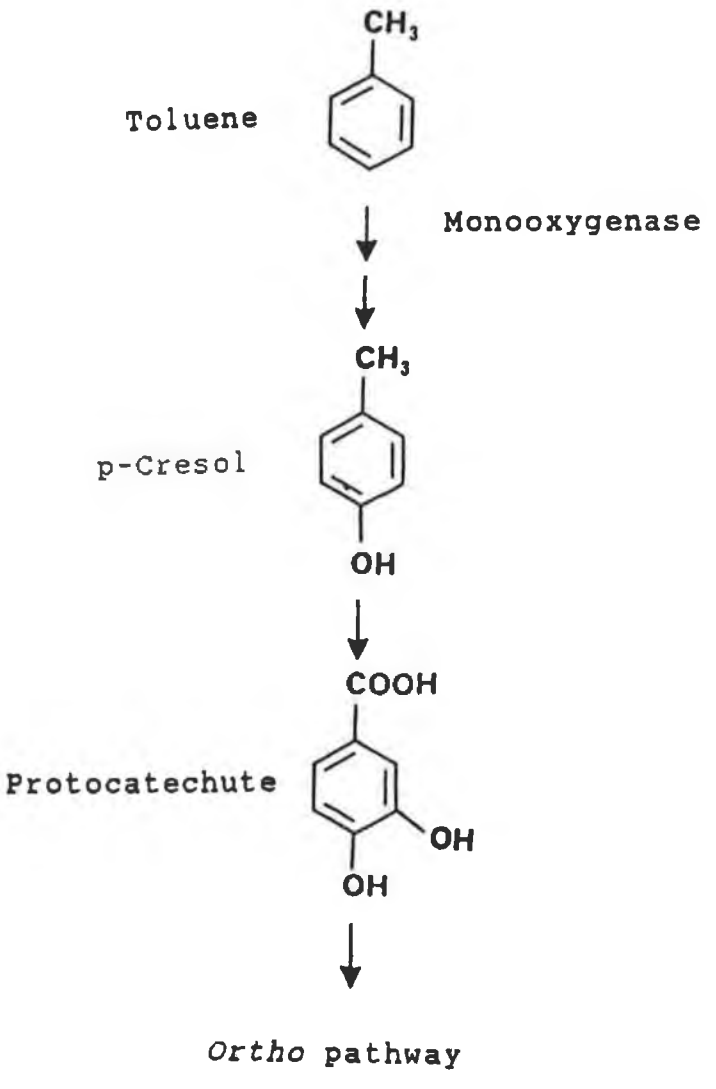


Figure 14. Toluene degradation in *P. mendocina*

Toluene is oxidized to p-cresol by a plasmid encoded monooxygenase with toluene-3,4-oxide as an intermediate. The monooxygenase is a multicomponent enzyme system with flavin and haem as prosthetic groups (Gibson, 1987). p-Cresol is further oxidized to protocatechute which serves as a substrate for *ortho*-ring fission (Finette *et al.*, 1984).

1:8 NAPHTHALENE DEGRADATION BY *PSEUDOMONAS* SPECIES

Naphthalene is formed during the production of coke from coal and is a by-product of oil refining procedures (Caglioti, 1983).

The biochemistry of naphthalene degradation by bacteria was first studied in 1943 (Davies and Evans, 1964). The following pathway for naphthalene degradation has been demonstrated in pseudomonads (Figure 15) (Yen and Serdar, 1988). The capital letters prefixed by *nah* represent the genes encoding the corresponding enzymes.

The initial reaction of the pathway is catalyzed by naphthalene dioxygenase, a multi-component, membrane-associated enzyme system of similar composition to toluene dioxygenase, described previously. It consists of a terminal iron-sulphur containing oxygenase (ISP $_{NAP}$); reductase $_{NAP}$, an iron-sulphur flavoprotein which is the initial electron acceptor, shuttling electrons from NADH to the terminal oxygenase and ferredoxin $_{NAP}$ also an iron-sulphur protein, which acts as an intermediate electron carrier. The action of these components of naphthalene dioxygenase is shown in Figure 16 (Haigler and Gibson, 1990).

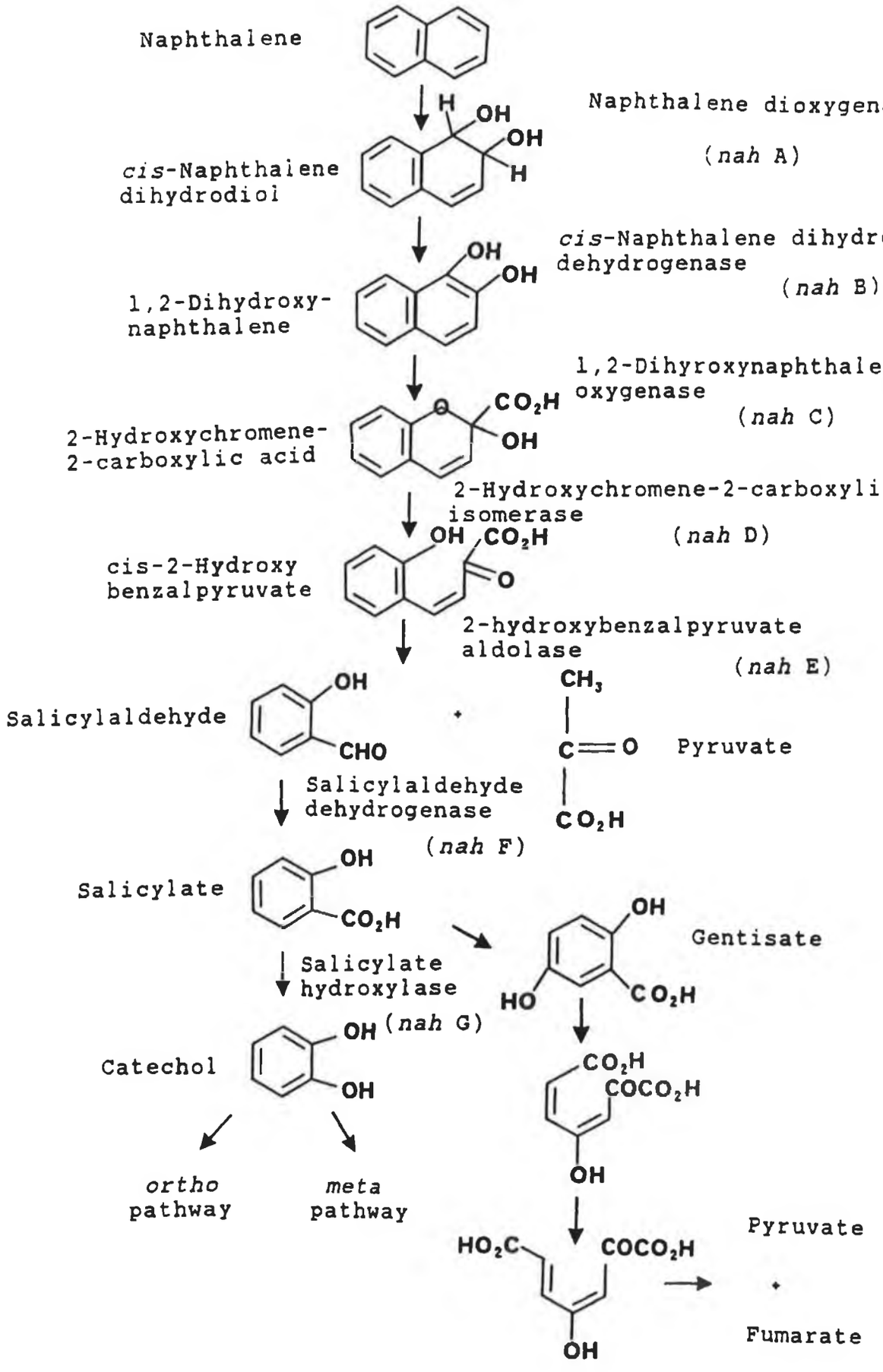


Figure 15. Naphthalene and salicylate catabolic pathways in pseudomonads.

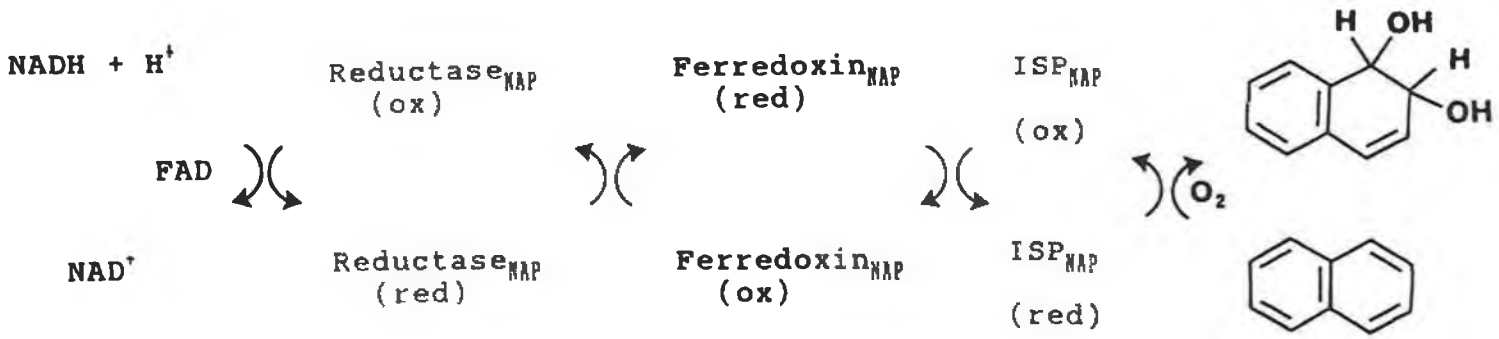


Figure 16. Proposed electron transport scheme for naphthalene dioxygenase

NAD - Nicotinamide adenine dinucleotide
FAD - Flavin adenine dinucleotide
red - reduced
ox - oxidized

The second step in the metabolism of naphthalene is the oxidation of *cis*-naphthalene dihydrodiol (*cis*-1,2-dihydroxy-1,2-dihydronapthalene) to 1,2-dihydroxynaphthalene by *cis*-naphthalene dihydrodiol dehydrogenase. This enzyme requires NAD as an electron acceptor (Yen and Serdar, 1988). 1,2-dihydroxynaphthalene is cleaved by a dioxygenase to yield 2-hydroxychromene-2-carboxylic acid, which is subsequently converted to *cis*-2-hydroxybenzalpyruvate by an isomerase. *cis*-2-Hydroxybenzalpyruvate is converted to salicylaldehyde and pyruvate in a reaction catalyzed by an aldolase. Salicylaldehyde is further oxidized to salicylate by an NAD^+-dependent dehydrogenase.

In most case salicylate is converted to catechol by salicylate hydroxylase which is further metabolized via the *ortho*- or *meta*-cleavage pathways. Salicylate hydroxylase (monooxygenase) requires NADH or NADPH as electron donors and FAD as a co-factor (Nozaki, 1979). The formation of gentisate from salicylate has been proposed during naphthalene metabolism by *Pseudomonas fluorescens* and *Pseudomonas alcaligenes* (Yen and Serdar, 1988).

A number of plasmids carrying naphthalene catabolic genes have been demonstrated in *Pseudomonas* spp. All naphthalene catabolic plasmids belong to the incompatibility groups P7 or P9. All are self-transmissible. To date all naphthalene plasmids studied encode the same upper pathway (naphthalene through salicylate) and most carry the same lower pathway for salicylate degradation indicating a close relationship between all naphthalene degradative plasmids (Yen and Serdar, 1988).

The best-studied naphthalene catabolic plasmid is NAH 7, first identified in *Pseudomonas putida* strain G1. NAH 7 is 83kb in size and belongs to the P9 incompatibility group. All the characterized catabolic genes are contained within a 30kb region of NAH7. These genes are arranged into two *nah* operons, separated by a 7kb fragment (Lehrbach and Timmis, 1983). The first operon encodes degradation of naphthalene to salicylate (upper pathway). This comprises the genes *nah* ABCDEF as shown in Figure 15. The second operon comprises *nah* G, coding for salicylate hydroxylase, and the genes *nah* HINLJK, coding the lower (*meta*-cleavage) pathway enzymes which convert catechol to acetaldehyde and pyruvate. Mutation studies revealed that these two operons are transcribed from *nah* A to *nah* F and from *nah* G to *nah* K, respectively.

A regulatory gene, *nah* R, is located between the two operons and its product is required for the expression of both operons. *Nah* R appears to be transcribed constitutively as the levels of *nah* R-mRNA are not altered significantly by the presence or absence of an inducer. The *nah* R protein may be present in active and inactive forms, with a shift towards the production of active protein in the presence of an inducer. Naphthalene and its oxidation products, naphthalene dihydrodiol, dihydroxynaphthalene and hydroxychromene carboxylic acid do not induce the catabolic pathway encoded by the NAH 7 plasmid. A low-level constitutive production of the upper pathway enzymes probably converts naphthalene to salicylate, which then induces both upper and lower pathways. Increased synthesis of mRNA for the two *nah* operons which occurs in the presence of salicylate indicates that

induction is at the transcriptional level (Yen and Serdar, 1988).

As indicated in Figure 15 catechol can also be metabolized via the chromosomally-encoded *ortho*-cleavage pathway. This can occur when mutations in the *meta*-cleavage pathway allow catechol to accumulate thus inducing the *ortho* pathway (section 1:3:1). In some strains all or portions of the naphthalene catabolic pathway are encoded on the chromosome and therefore catechol is metabolized via the *ortho* pathway (Frantz and Chakrabarty, 1986). In *P. putida* NP the upper pathway enzymes are specified by chromosomal genes that can integrate into a large plasmid, pDTG11 found in this strain (Yen and Serdar, 1988).

1:9 ACTIVATED SLUDGE

The activated sludge process was originally developed by Ardern and Lockett in 1914 and since then has been improved through a number of engineering and operation developments. Activated sludge processes are continuous flow, dispersed, flocculated growth processes in which the bacterial flocs, described as activated sludge, are separated from the treated effluent by sedimentation in a clarifier and recycled to the aeration tank where they are recontacted with the wastewater undergoing treatment (Hamer, 1985). The activated sludge process is commonly used for the treatment of both domestic sewage and industrial wastewater. In Ireland most chemical plants treat their effluent in biological treatment systems (O'Flaherty, 1989). Successful operation is dependent on the capability of the biomass (sludge) to assimilate the waste and

convert it into cells and suspended solids, and to form a floc particle that will settle (Chambers, 1982).

Biomass composition is determined by both the waste characteristics and process operation parameters. The biological treatment of most waste streams depends on the 'ubiquity principle' which states that bacteria are ubiquitous and the most effective species of microorganisms for a given environment will eventually establish themselves in that environment (Zachopoulis and Hung, 1990). The bacterial population which arises in a bioreactor as a result of physiological and genetic adaptation is unique from the point of species diversity (Lange *et al*, 1987). The predominant microbes in activated sludge processes are chemoheterotrophic bacteria. The microbes best suited are those with the ability to grow as either microbial aggregates or those able to become directly associated with such aggregates so as to be recycled. As far as effective pollutant biodegradation is concerned the predominant microbes are those of the family Pseudomonadaceae especially the genera *Pseudomonas* and *Zooglea* and also members of the genera *Flavobacterium* and *Alcaligenes* (Hamer, 1985).

1:9:1 ACCLIMATION

Acclimation is a period of physiological, morphological and genetic adaptation of the biomass to a new environment. Adaptation can be either phenotypic or genotypic. In phenotypic adaptation the genetic information of the cell is unchanged but the degree of expression of certain genes is altered. This may be common during short term fluctuations in the environment. Genotypic

adaptation involves changes in the genetic information of the cell and these changes are transmitted to daughter cells. There are two basic methods of genetic change : mutation and recombination. Mutation is a change in the nucleotide sequence of the DNA occurring within an individual cell. Recombination is any process whereby genes from two separate cells are brought together into the one cell, for example, transformation, conjugation, transduction and genetic engineering. Genotypic adaptation may be common during chronic changes in the environment (Senthilnathan and Ganczarzyk, 1989).

The length of time required for acclimation to various pollutants can range from a few days to several weeks. As there is little or no detectable mineralization during acclimation it is important to minimize the time required. The length of the acclimation period can be affected by the presence of a secondary carbon source or the rarity of the microbes that can degrade certain chemicals. The presence of inhibitory compounds or a high enough concentration of a chemical to inhibit those microbes capable of metabolizing it will also affect the time needed for acclimation (Wiggins and Alexander, 1988). When acclimated organisms are subjected to a different environment they tend to adapt to the new environment and as a result may lose the ability to grow at the expense of the original substrate. Phenotypic adaptations are temporary and therefore more rapidly lost than genotypic adaptations. This process of deadaptation of acclimated biomass may influence the biodegradability of organic pollutants which are discharged intermittently (Senthilnathan and Ganzarczyk, 1989). For such industries which

produce a variety of products on staggered schedules it has been suggested that a background level of the pollutant in the influent would help to maintain the bacterial population responsible for its mineralization (Eckenfelder, 1989).

1:9:2 PROBLEMS ENCOUNTERED

Although a naturally developed microflora can provide adequate treatment for many waste streams, there are still some waste streams which present specific problems. The shift towards marketing a more diversified product range with shorter production cycles has affected the pattern and quality of effluent being generated by chemical and pharmaceutical companies. One of the problems frequently encountered in treatment systems is specific pollutant removal (O'Flaherty, 1989).

Recent changes in legislation in Europe and in the U.S. have meant that more consideration must be given to levels of residual toxic or inhibitory compounds rather than just overall BOD or COD removal. There are a number of factors affecting the biodegradation and achievable effluent concentrations of toxic organics in the activated sludge process. These include acclimation, influent concentration of the pollutant, wastewater characteristics and sludge retention time.

The nature and concentration of various wastewater constituents can affect the removal of a specific pollutant. In a multisubstrate system such effects as catabolite repression, non-competitive, uncompetitive and competitive inhibition can occur between the various compounds which are potential substrates for the biomass. The accumulation of

oxidation by-products in the activated sludge unit can also cause major problems as these may be toxic or inhibitory both to sludge biomass and the aquatic environment if they pass through the system. One of the major problems at present is that most of these by-products are unidentifiable (Eckenfelder, 1989). Other wastewater characteristics which can influence pollutant biodegradation include pH, temperature, dissolved oxygen and the availability of inorganic nutrients and enzyme co-factors.

Despite the inherent ability of the bacteria within a wastewater treatment plant to adjust to various selective pressures in their environment, many treatment plants can not respond rapidly enough to prevent them from failing to produce the required standard of effluent. The relationship between sludge retention time and the residual pollutant concentration for a given pollutant is dependent on the particular treatment plant and the biomass (Stevens, 1989).

In systems treating toxic or inhibitory waste, bacterial diversity is reduced by the increased selective pressure on the population due to the presence of inhibitory compounds. Reducing the types of bacteria present can diminish the genetic pool of the population and consequently its ability to adapt to changes in the environment or waste composition. As a result hazardous waste treatment processes are often plagued by upsets and are unable to degrade new compounds entering the waste stream (Lange *et al.*, 1987).

These failures may manifest themselves as poor

BOD/COD or pollutant removal, turbidity in the final effluent, poor sludge settlement, foaming, odour problems, loss of nitrification or other effects. In such cases there are four possible measures open to plant operators:- to modify influent characteristics, to modify mode of operation (eg. sludge return or wastage rates, aeration, etc.), to change plant design by building extra capacity or reorganizing treatment units, or to introduce new components to the biomass community by adding sludge from another works or through bioaugmentation (Stevens, 1989).

1:10 BIOAUGMENTATION OF ACTIVATED SLUDGE

Bioaugmentation or biomass enhancement is the addition of selected organisms to a treatment system in order to improve its efficiency. The abilities of the selected organisms can be improved by mutation or genetic engineering. The addition of genetically engineered microorganisms to a treatment plant constitutes a release of such organisms to the environment as most plants are open systems with final effluents being discharged into local waterways and bioreactors that are open to the atmosphere. There is considerable public and legislative opposition to the release of engineered organisms, therefore most companies producing bacterial supplements prefer to use more traditional techniques for improving their strains (Zachopoulos and Hung, 1990).

Bioaugmentation has two main purposes: to minimize and if possible eliminate the lag period (acclimation period) and to improve decomposition of the waste (Golueke and Diaz, 1989). Bioaugmentation can increase the biological

diversity and activity of a population by adding bacteria with enzymatic systems which allow degradation of previously non-biodegradable organics or by adding bacteria which have higher metabolic rates (Lange *et al.*, 1987). An increase in bacterial diversity increases the gene pool available to the population in times of stress. The ability of the organisms to transfer DNA to the indigenous population may be of equal or greater importance than the survival of the organism itself.

The benefits obtained by the use of bioaugmentation can be summarized as follows:

- improved floc formation and settling
- increased rates of waste assimilation and versatility in substrate uptake
- decreased sludge solid yields due to a more efficient breakdown of the colloidal material
- rapid establishment or restoration of the biological activity in the waste treatment process that has experienced a start-up, an upset or chlorination of the return sludge
- increased stability and tolerance to vacillating growth conditions
- odour control
- lower operating and energy costs (Chambers, 1982).

1:10:1 REASONS FOR FAILURE OF BIOAUGMENTATION

Although the use of bioaugmentation in wastewater treatment has potential it is not always successful. The success of a bioaugmentation product is based on the ability of the introduced bacteria to actively compete for the nutrients

available and eventually to gain population dominance (Chambers, 1982). However, abiotic stress in the natural environment is often different from that in the laboratory and because the introduced bacteria may face intense competition, predation or parasitism in nature, the inoculated organisms may fail to bring about the desired levels of biodegradation after their addition to the activated sludge system.

There are several possible reasons for the failure of inoculated organisms to do in nature what they can do in axenic (pure) culture.

1) The concentration of the compound in nature may be too low to support the growth of the inoculated species. Although, species growing on organic substrates other than the compound of interest or non-growing cells may mineralize the test compound, an organism in small numbers whose sole selective advantage in an environment is its ability to multiply at the expense of a particular substrate, may not proliferate if the substrate is below some threshold level.

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 organisms on the low ambient concentration of the chemical of interest may be slower than the rate of predation for example by protozoa, so that the activity of the predators reduces the cell density of the inoculated species.

4) The added organisms may use other organic substrates in the natural environment rather than the pollutant whose mineralization is desired.

5) The organisms may fail to flocculate and can be washed out of the system resulting in increased turbidity of the effluent. Non-flocculated organisms are also more susceptible to grazing by protozoa.
(Goldstein *et al.*, 1985).

1:10:2 CHOOSING A BIOAUGMENTATION PRODUCT

Firstly, it is important to remember that the use of bioaugmentation does not replace the need for sound engineering and process design or for proper plant management. It should be considered as another cost-effective option open to plant operators when effluent problems occur (Stevens, 1989). As detailed previously selection of the correct bacteria is vital to the success of bioaugmentation, therefore considerable thought must be given to the choosing of a bioaugmentation product to ensure that the right product is chosen for a given treatment system. The following are some of the questions that should be asked when evaluating a product :

1) Have the types of organisms present in the product blend been demonstrated as being the same as those that are active in the degradative process?

2) How were the organisms isolated and identified? Can you be assured that the organisms in the starter are the active ones or subcultures of

the active organisms?

3) How has the continuity of the culture been assured? Continuity is an insurance that the organisms in the present product are the same as those originally isolated.

4) What procedures were followed for maintaining the cultures? Maintenance procedures determine the "virility" of the organisms, their ability to function as expected and to compete with indigenous populations.

   It is necessary to be aware of the possibility of mutations, of loss of organisms and of contamination.

5) If enzymes are used in the product, how were they identified as being important to the process?
   (Golueke and Diaz, 1989).

Once a product has been chosen the dosage rate and possible need for maintenance doses must be established. These, of course, are dependent on a given treatment system and the desired effluent quality.

1:10:3 BACTERIA USED IN BIOAUGMENTATION PRODUCTS

There are a number of criteria which are important for the success of a bacterial supplement:-

1) The viability of the bacteria should remain high when added to the indigenous population (Lange, *et al.*, 1987).

2) The supplement should supply an essential type of microorganism not already present in the sludge, should augment a less than adequate microbial population or introduce a type or types of microorganism more effective than the indigenous population (Golueke and Diaz, 1989).

3) Once added, supplements should initiate biodegradation of the target compounds rapidly or degrade compounds not degraded by the indigenous population (Lange *et al.*, 1987).

4) Added supplements should have the necessary characteristics to maintain their population in the reactor (Lange, *et al.*, 1987).

The correct selection of bacteria for a given waste stream is vital to the success of bioaugmentation. Added organisms must compete effectively with the indigenous population in order to establish themselves in the reactor. Factors which provide added bacteria with a competitive edge include : an increased tolerance to toxic materials and a lower limiting substrate requirement needed to drive essential metabolic reactions, a shorter generation time and an ability to respond rapidly to a favourable growth environment (Chambers, 1982).

Activated sludge populations can exist in either a dispersed unicellular state or in a multicellular flocculated state. Flocculent cells are subject to longer residence times in a system where biomass is recycled, but they exhibit lower specific rates of substrate assimilation due to intra-floc diffusion limitations. Unicellular bacteria settle very slowly and consequently are readily lost from the system via the clarified effluent resulting in

increased turbidity of the final effluent. However, the dispersed biomass does exhibit higher specific rates of substrate mineralization since mass transfer resistances are negligible. Some vendors of bioaugmentation products have contended that dispersed cells are the principal agents of biodegradation of potentially hazardous organics in suspended-growth treatment systems such as activated sludge and therefore maintenance doses of the products are essential to replenish dispersed cells lost in the effluent. This, of course, greatly increases operating costs (Hull and Kapuscinski, 1987). A model, developed by Hull and Kapuscinski (1987) predicted that bioaugmentation will only be successful in enhancing the steady-state removal of hazardous organics if the influx of viable biomass is sufficient to effect the accumulation of flocculent, capable biomass and concomitantly, the actual mean cell residence time of the capable population. This model also predicts that bioaugmentation is more likely to be effective when the influent concentration of the hazardous organic is low, as in such cases there would be a lower concentration of capable biomass. As a result there is a greater potential impact of a given bioaugmenting dose on the mean cell residence time and hence, upon the biological removal of the organic compound.

In testing bacteria for their suitability for inclusion in supplements it is vital to remember that conditions in the laboratory and to a considerable extent in pilot-scale operations are maintained at optimum levels. The reality is that in conventional waste treatment practice, conditions are rarely uniformly optimum. Consequently, the potential abilities of the

promising microorganisms fail to come to fruition (Golueke and Diaz, 1989).

1:11 SURVIVAL OF ADDED ORGANISMS

Although there have been a number of studies on the efficacy of bioaugmentation, relatively little work has been done on the survival of the added organisms. Much of the work that has been done is in relation to the release of genetically-engineered microorganisms. Much emphasis has also been placed on the possible detrimental effects of genetic transfer from engineered organisms to indigenous populations and thus, the necessity of monitoring survival of the genes and their transfer in natural ecosystems. In the waste treatment situation the transfer of genetic information regarding pollutant catabolism is obviously beneficial, particularly where that information is encoded on naturally occurring plasmids. Therefore, the successful use of bioaugmentation is dependent on survival of the added organisms or on the survival of the genetic information they contain.

There are a number of possible fates for inoculated organisms following introduction to the environment:-

1) Organism and gene persist
2) Organism and gene proliferate
3) Organism persists and gene dies out
4) Organism proliferates and gene dies out
5) Organism dies out and gene persists
6) Organism dies out and gene proliferates
7) Organism and gene die out

(Jain *et al.*, 1988).

In an activated sludge system one of the major factors affecting the survival of added organisms is their ability to flocculate and thereby become incorporated into sludge flocs. This ensures their maintenance in settled sludge and also protects against predation. Having become established as part of the sludge floc, the ability of the organisms to compete effectively with the indigenous population governs their persistence and proliferation within the system. Other factors influencing the survival of added organisms include resistance to starvation, motility, nutrient (substrate and co-factor) concentration and availability, presence of growth inhibitors and physical factors such as oxygen concentration, temperature and pH (McClure *et al.*, 1991).

Many strains developed in the laboratory are grown at temperatures of 30°C and pH near neutrality. These strains may compete less effectively at the lower temperatures and more variable conditions experienced in natural ecosystems. Some species, for example pseudomonads, are more resistant to starvation than others and are therefore more likely to survive periods of nutrient deficiency (Jain *et al.*, 1987).

As the catabolic pathways for many organic compounds are plasmid-encoded the stability of those plasmids is vital. The maintenance of plasmids has been found to be host, plasmid and environment dependent (Trevors *et al.*, 1989). The non-conjugative plasmid pBR322 was stably maintained in *Klebsiella aerogenes* in sterile agricultural drainage water under both aerobic and anaerobic conditions. A non-conjugative plasmid, pRK2501 was readily lost from *Pseudomonas putida*

under the same conditions. The maintenance of conjugative plasmid, RP4, by *Pseudomonas fluorescens* was markedly affected by the addition of nutrients which caused a loss of the plasmid from cells. The *Klebsiella aerogenes* was more susceptible to starvation conditions than the two *Pseudomonas* spp. The survival of all three strains was negatively affected in non-sterile agricultural drainage water, this may be attributed to competition, antagonism or predation. The results of this study demonstrated the influence of nutrients, oxygen and native microorganisms on the survival of introduced bacterial strains and plasmid stability (Trevors *et al.*, 1989). The presence of a selective pressure for the maintenance of a plasmid increases the potential for its persistence and transfer within an ecosystem.

The stable maintenance of plasmids in bacteria provides a means by which gene pools are maintained and disseminated throughout microbial populations. The potential for genetic interaction in the environment is high if the organisms can survive the dynamic biological, chemical and physical factors that occur. Obviously both the survival and genetic stability of added organisms are important parameters (Trevors *et al.*, 1989).

Although bacteria introduced to a complex ecosystem, such as activated sludge, may rapidly decline in number, enhanced degradation rates can be achieved by transfer of degradative genes to the natural microflora (McClure *et al.*, 1989). The natural microflora resident in such ecosystems are likely to be well adapted to growth and survival. Several studies have found that indigenous

activated sludge bacteria which were recipients of catabolic genes or plasmids could degrade the target pollutant more efficiently than the laboratory strains. If the acquisition of novel degradative abilities by indigenous bacteria is advantageous then growth and proliferation of these strains may result in the establishment of a microbial community better adapted to degrade potential environmental pollutants rapidly (McClure *et al.*, 1990).

Activated sludge mixed liquors contain high concentrations of cells (total numbers > 10^{10} cells/ml, viable count > 10^{8} cells/ml) most in intimate contact within sludge flocs, this provides favourable conditions for plasmid transfer (McClure *et al.*, 1990).

1:12 GENE TRANSFER IN ACTIVATED SLUDGE

There are three possible mechanisms for the transfer of plasmids in activated sludge or other natural environments. Conjugation by plasmids capable of mediating their own transfer and of mobilizing other replicons is probably the most important mechanism for gene transfer in nature. For conjugation to occur cells must come into close contact, cellular interactions are established by the means of conjugative pili. DNA transfer is initiated at the origin of transfer. A complementary DNA strand is synthesized on the transferred strand and on the strand retained in the donor cell. Thus, conjugative DNA transfer is a replicative process that increases the population size of the plasmid (Wilkins, 1990). The other mechanisms of gene transfer are transduction which is bacteriophage mediated and transformation which

is the uptake of naked DNA released from cells which lyse after death.

Factors which influence DNA transfer include:

- repression of transfer systems on conjugative plasmids.
- cell surface properties. Lipopolysaccharide structure is important in stabilizing contacts with pili. Bacteriophage require specific sites for recognition and attachment.
- temperature, spatial distribution and the physiological state of cells can influence the efficiency of transfer.
- surface exclusion inhibits pilus-directed aggregation and reduces DNA transfer. (Wilkins, 1990).

Once the DNA has been successfully transferred other factors affect the maintenance and expression of plasmids. Incompatibility expressed by a resident plasmid and the activity of restriction endonucleases will affect the maintenance stability of an immigrant plasmid. The recipient cell must contain the correct biochemical infrastructure for expression to occur (Saunders *et al.*, 1990).

The evolution of novel catabolic pathways in response to major perturbations in the biosphere caused by the use of xenobiotic compounds provides good evidence that gene transfer does occur. An example of this "natural genetic exchange" is the plasmid-mediated transfer of the chromosomally-encoded dehalogenase I gene from *Pseudomonas putida* PP3 to other bacteria which resulted in transconjugants with a new ability to degrade halogenated compounds (Dwyer *et al.*, 1988).

Evidence for naturally-occurring intergeneric gene transfer comes from the discovery of antibiotic resistance genes, transposons, insertion elements and plasmids with similar DNA sequences in unrelated bacterial isolates (Hirsch, 1990).

Given the variety of barriers to genetic exchange and the varying nature of DNA molecules as they present themselves in potential recipient cells, it can be concluded that in natural microbial communities the net rate of transfer of any given gene will be low. Many of the potential gene transfer events that occur, particularly simple plasmid transfer between related bacteria, will be unproductive in the evolutionary sense of generating new genetic combinations. One effect of barriers to genetic exchange is to produce damage to incoming DNA, which, whilst primarily destructive, may actually be creative since recombination events may be stimulated and novel rearrangements of DNA produced (Saunders *et al.*, 1990). The maintenance of "new" genetic information by the cell is positively affected by the presence of a selective pressure in the environment. A positive selective pressure also provides a strong impetus for plasmid transfer if that transfer is beneficial to the cell. The transfer of degradative genes from introduced bacteria is beneficial to the community as a whole as it increases the ability of the ecosystem to cope with the presence of toxic pollutants (Dwyer *et al.*, 1988). Genes acquired can result in the ability to degrade a wide range of structurally related compounds.

Many degradative plasmids have a very broad host range as they can transfer and replicate in a range

of unrelated Gram negative bacteria. The TOL plasmid pWWO in its original host *Pseudomonas putida* mt-2 is naturally derepressed for transfer. Transfer of TOL occurs at high frequency to *pseudomonad* rRNA group 1 (*P. fluorescens, P. aeruginosa, P. stutzeri* etc.) and also to *E. coli* and *Erwinia chrysanthem* spp. The TOL regulatory circuits are rather complex and some strains may lack some of the required accessory elements. In studies, no transfer of the TOL plasmid was observed in *Acinetobacter, Rhizobium* and *Alcaligenes* spp. or in pseudomonads belonging to rRNA groups II, III, IV (Ramos-Gonzalez *et al.*, 1991).

Numerous studies have shown genetic exchange from added organisms to indigenous populations in natural environments. Much work has been done by McClure *et al* (1989, 1990, 1991) on the survival and transfer of cloned and naturally-occurring catabolic genes in model activated sludge microcosms. The test organism was *Pseudomonas putida* UWCI which carried the recombinant plasmid pD10 coding for 3-chlorobenzoate degradation and Kanamycin resistance. pD10 is non-self transmissible but can be mobilized by certain conjugal plasmids present in the same cell. *P. putida* UWCI persisted in the activated sludge unit for more then 8 weeks, although the population size gradually declined. The plasmid pD10 was stably maintained in the introduced organism but 3-chlorobenzoate degradation was not enhanced. Derivatives of *P. putida* UWCI were isolated from the activated sludge which were able to transfer pD10 to a recipient strain, indicating the *in situ* transfer of mobilizing plasmids from the indigenous sludge microflora to the introduced strain (McClure

et al., 1989).

In further studies, a large increase in the numbers of natural 3-chlorobenzoate degrading bacteria was shown to be due to the acquisition of pD10. These transconjugants may have deleteriously affected the survival of *P. putida* UWCI as they would be better adapted to the natural conditions in the activated sludge system (McClure *et al.*, 1991). Considerable work has also been done with *Pseudomonas* sp. strain B13 which has the ability to degrade chlorocatechols via a modified *ortho* cleavage pathway. The addition of this strain to mixed microbial cultures led to the emergence of a new genotype capable of complete haloaromatic degradation when neither parental strain carried the complete degradative pathway (Reineke and Knackmuss, 1979). Further study demonstrated the transfer of the TOL plasmid to strain B13, which allowed for the degradation of chloroaromatics to chlorocatechol which was then metabolized via the modified *ortho* pathway of strain B13 (Knackmuss, 1981).

The retention of inoculated organisms or transfer of their genetic information is important if bioaugmentation is to enhance degradation of pollutants in wastewater. It is also very important for reasons of process efficiency and cost; hence the recent interest in the use of biofilms and immobilized enzyme systems for wastewater treatment (McClure, 1991).

1:13 THE MONITORING OF ADDED ORGANISMS

Numerous methods have been developed to follow the fate of organisms added to ecosystems containing

mixed microbial populations (Jain *et al.*, 1988).

Conventional methods for the detection of a specific organism include:-

- selective plating and enrichment techniques
- enumeration by most-probable-number (MPN) method
- epifluorescence count technique

Developing methods include:-

- use of plasmid epidemiology and restriction profiles (DNA fingerprinting)
- use of selectable genotypic markers
- use of nucleic acid sequence analysis
- nucleic acid hybridization techniques
- protein and enzyme analysis
- genetically engineered markers

In choosing a technique for monitoring an organism or organisms a number of important criteria must first be considered.

- it should be applicable under a wide variety of environmental conditions
- it should be suitable for technical application in terms of its simplicity
- it should be able to detect, identify and enumerate the added organism
- it should be sensitive and specific to detect a small population
- it should be capable of differentiating the specific organism from other organisms in the environment
- it should be able to discriminate the added organism from other strains of the same species

- it should be efficient, cost effective and time-economic (Jain *et al.*, 1988).

One of the most powerful and reliable methods for monitoring specific organisms is the use of DNA probes either species-specific probes which detect the presence of a complementary sequence unique to the organism to be added or function-specific probes, such as those that identify genes for pollutant degradation. Function-specific probes are not specific to one taxonomic group, but rather measure the potential of the community as a whole to perform a function, though not the actual activity of the function (Ogram and Sayler, 1988).

However, no single technology is perfect for all monitoring needs rather a multiple methodological approach should be used to achieve accurate and sensitive monitoring of specific bacteria in environmental samples. The selection of a technique must be based on a clear definition of the goal of the detection and monitoring strategy (Jain *et al.*, 1988).

2 MATERIALS & METHODS

2:1 MATERIALS

2:1:1 BACTERIAL CULTURES

Pseudomonas species

The *Pseudomonas putida* spp. IGA 7.16, IGA 7.24 IGA 0.92 and the *Pseudomonas fluorescens* sp. IGA 13.42 were obtained from InterBio Laboratories Ltd., Sandyford Ind. Est., Dublin 18, Ireland.

Pseudomonas putida NCIB 10432 carrying a TOL plasmid and *Pseudomonas putida* NCIB 12199 carrying a NAH Plasmid were obtained from the National Collection of Industrial Bacteria, Aberdeen, U.K.

All the *Pseudomonas* cultures were supplied in freeze dried form.

Biolyte CX 80

The bioaugmentation product, Biolyte CX 80 was obtained from InterBio Laboratories Ltd., Sandyford Ind. Est., Dublin 18, Ireland.
Biolyte CX 80 is a specially formulated microbial product designed to degrade a wide range of aliphatic and aromatic compounds in aerobic biological wastewater treatment systems. The CX 80 formulation contains a range of 13 microorganisms, including members of the genera *Aeromonas*, *Bacillus*, *Pseudomonas* and *Rhodococcus*, together with surfactants carried on a cereal base.

Recombinant *E.Coli* strains

E.coli BHB 2600 (pDTG113) carrying the genes for the degradation of naphthalene to salicylate and *E.coli* JM109 (pDTG602) carrying toluene dioxygenase and *cis*-toluene dihydrodiol dehydrogenase genes were kindly donated by Dr. D.T. Gibson, Dept. of Microbiology, University of Iowa, U.S.A.

E.coli K12 (pGSH2836) carrying toluene monooxygenase and benzyl alcohol oxidase genes was kindly donated by Dr. S. Harayama, Departement de Biochimie Medicale, University of Geneva, Switzerland.

The recombinant *E. coli* strains were supplied growing on agar.

2:1:2 MEDIA

All media unless otherwise stated were obtained from Oxoid.
All media were sterilized by autoclaving at 121°C for 15 minutes.

Hugh and Leifsons' Medium

The medium was prepared as outlined in Harrigan and M^{c} Cance (1976).
All the ingredients except Bromothymol blue were dissolved in distilled water and the pH was adjusted to 7.1. The indicator dye was then added. The medium was dispensed into test tubes in 10ml aliquots. Following sterilization, the tubes were cooled rapidly in an iced water bath and inoculated immediately.

	g/l
Peptone	2.0
NaCl	5.0
K_2HPO_4	0.3
Glucose	10.0
Agar	3.0
Bromothymol blue (1% (w/v) aqueous solution)	3.0 ml
pH 7.1	

Gelatin Agar

Gelatin was added to nutrient agar before sterilization to a final concentration of 0.4% (w/v) as outlined in Harrigan and M^c Cance (1976).

Luria-Bertani Medium & Luria Medium

Luria-Bertani (LB) Broth and Luria Broth were 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
Sodium Chloride	10.0
pH 7.0	

Luria Broth	g/L
Tryptone	10.0
Yeast Extract	5.0
Sodium Chloride	5.0
Glucose	1.0
pH 7.0	

Milk Agar

Skim milk powder (Oxoid) was added to nutrient agar before sterilization to a final concentration of 10% (w/v) as outlined in Harrigan and M^c Cance (1976).

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, without sterilizing, for 6-8 weeks.

	g/l
K_2HPO_4	4.36
NaH_2PO_4	3.45
NH_4CL	1.0
$MgSO_46H_2O$	0.91
Trace Salts Solution	1.0 ml
pH 7.0	

Trace salts solution

	g/100ml
$CaCl_2.2H_2O$	4.77
$FeSO_4.7H_2O$	0.37
$CoCl_2.6H_2O$	0.37
$MnCl_2$	0.10
$NaMoO_4$	0.02

Tween 80 Agar

The agar was prepared as outlined in Harrigan and M^c Cance (1976).The ingredients were dissolved in distilled water and the pH adjusted to within 7.0 - 7.4 if required.

	g/l
Peptone	10.0
$CaCl_2$ H_20	0.1
NaCl	5.0
Tween 80	10.0

Agar	15.0
pH 7.0 - 7.4	

2:1:3 BUFFERS

20 x SSC

A stock solution of 20XSSC 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°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 (for phenol assay)

Potassium phosphate buffer was prepared as outlined in Standard Methods for the Examination of Water and Wastewater (1985) by dissolving K_2HPO_4 (104.5g) and KH_2PO_4 (72.3g) in distilled water (1l). The pH of the resulting solution should be 6.8.

Sodium Phosphate Buffer

Sodium phosphate buffer (0.01M) was prepared by dissolving Na_2HPO_4 (0.01M) and NaH_2PO_4 (0.01M) in distilled water. The pH of the resulting solution should be 7.0.

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 with 2M HCl.

2:1:4 SOURCE OF CHEMICALS

The chemicals used were obtained from a number of sources including Riedel-de-Haen, BDH, Sigma, Aldrich and NBS Biologicals.

2:1:5 SOURCE OF GENETIC MATERIAL

Restriction enzymes were supplied by Bethesda Research Laboratories, Life Sciences Inc.,Maryland, USA.

λ DNA (0.25μg/μl) was supplied by Boehringer Mannheim, Germany.

α-^{32}P ATP was supplied by Du Pont, France.

Prime-a-Gene labelling system was supplied by Promega Corp., Madison, USA.

DIG DNA labelling and detection system was supplied by Boehringer Mannheim, Germany.

2:2 METHODS

2:2:1 RESUSCITATION OF FREEZE-DRIED *PSEUDOMONAS* CULTURES

All the *Pseudomonas* strains were supplied freeze-dried.

Sterile nutrient broth (0.2ml) was added to the freeze-dried cultures and the organisms were resuspended with a sterile inoculating loop. A loopful of culture was plated onto nutrient agar using the streak plate technique. Alternatively, a loopful of culture was used to inoculate nutrient broth flasks.

The cultures were incubated at 30°C for 24 hours, with agitation (200 rpm) for liquid cultures.

2:2:2 MAINTENANCE OF BACTERIAL CULTURES

Maintenance of *Pseudomonas* cultures on nutrient agar

Cultures were maintained on nutrient agar slopes at 4°C and were subcultured every 2-3 months.

Maintenance of *Pseudomonas* cultures on aromatic compounds

Cultures were maintained on aromatic substrates by plating on *Pseudomonas* minimal medium (section 2:1:2) incorporating Oxoid bacteriological agar number 1 (1%, w/v) together with the appropriate aromatic compound.

Phenol (5mM) and m-Toluate (5mM) were added to the

medium prior to sterilization. Benzoate (5mM) was dissolved in ethanol (1g/ml) to ensure even distribution in the medium and was added after sterilization. p-Chlorophenol was also dissolved in ethanol (200 mg/ml) and added after sterilization.

Naphthalene crystals (0.15-0.25g) were scattered on the lid of the inverted petri dishes and the organisms were grown in the vapour. Toluene plate cultures were grown in a desiccator (260mm diameter) and a glass beaker with 15-20ml Toluene was placed in the bottom i.e under the grid, to provide a toluene-saturated atmosphere.

All the plates were incubated at 30°C for 48-72 hours.

Maintenance of recombinant *E.coli* cultures

Recombinant strains were grown in accordance with the instructions obtained from Dr. D. T. Gibson and Dr. S. Harayama.

E.coli BHB 2600 (pDTG113) was grown overnight in LB broth incorporating kanamycin (50 μg/ml).

E.coli JM109 (pDTG 602) was grown overnight in Luria broth incorporating ampicillin (100 μg/ml).

E.coli K12 (pGSH 2836) was grown overnight in LB broth incorporating ampicillin (50 μg/ml).

The strains were stored using the method outlined by Maniatis *et al* (1982).

The culture (0.85 ml) was added to glycerol (0.15ml) in sterile eppendorf tubes. The glycerol was sterilized by autoclaving twice at 121°C for 15 minutes. The culture and glycerol were mixed well by vortexing and the tubes were sealed with parafilm. The cultures were stored at -20°C. Cultures stored in this manner are expected to remain viable, without loss of plasmids, for a number of years.

2:3:2 GROWTH OF *PSEUDOMONAS* CULTURES ON AROMATIC SUBTRATES

Cells from nutrient agar or aromatic plates were used to inoculate nutrient broths (10ml). Cultures were incubated overnight at 30°C and agitated at 200rpm.

The cells were harvested in a Labofuge 6000 bench-top centrifuge (5,000 rpm for 10 minutes).

The cultures were washed in sterile 0.1M Sodium phosphate buffer (pH 7.0) or 0.033M Tris-HCl buffer (pH 7.6) (section 2:1:3) and the final pellet resuspended in the same buffer.

This cell suspension was then used to inoculate *Pseudomonas* minimal medium, at a concentration of 2% (v/v).

Phenol and m-toluate were added to the medium, at a concentration of 5mM, before sterilization. Benzoate and naphthalene were added after sterilization to give a concentration of 5mM. p-Chlorophenol (200ppm) was dissolved in a minimum

volume of ethanol and added after sterilization. Toluene (0.5%, v/v) was supplied in the vapour phase by suspending a small tube, with the appropriate volume of toluene, inside the flask. A very tight cotton wool bung was used to minimize loss of toluene vapours to the atmosphere and a large headspace was provided to ensure adequate aeration.

All cultures were incubated a 30°C and agitated at 150rpm for 48 hours.

Specific growth rates were calculated using the computer software package Sigma Plot (Version 4.0), Jandel Corporation. A mathematical transform was used to determine

$$\text{Ln } (X/X_o)$$

where X = absorbance at time t
X_o = initial absorbance

A plot of $\text{Ln}(X/X_o)$ vs t was constructed. Regression analysis was performed on the exponential portion of the curve. The resulting slope being equal to the specific growth rate (μ).

2:2:4 TESTS USED IN THE CHARACTERIZATION OF *PSEUDOMONAS* SPECIES

Antibiotic Sensitivity

An overnight nutrient broth culture (0.1ml) was spread plated onto Oxoid Mueller-Hinton agar. Oxoid Antibiotic Discs were placed on the surface of the agar plates which were incubated at 30°C for 24 hours. The following antibiotics were tested :

chloramphenicol (50μg), novobiocin (30μg), penicillin G (10iu), polymyxin B (300iu), streptomycin (25μg) and tetracycline (30μg). The presence of clear zones around the discs indicated sensitivity to the antibiotic.

API tests

A number of API identification systems were used including API 20B, for heterotrophic aerobes; API 20NE, for non-enteric Gram negative rods and API 50CH for determining carbohydrate assimilation. The identification systems were used according to the manufacturers' instructions (Bio Merieux, 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 0.1M sodium phosphate buffer (pH 7.0) (section 2:1:3). The pellet was resuspended in the medium supplied by the manufacturer for API 20B and API 50CH tests. In the case of API 20NE, the pellet was resuspended in 0.85%(w/v) NaCl (10ml) and used to inoculate a portion of the tests. For the API 20NE assimilation tests, 200μl of this suspension was used to inoculate auxiliary medium supplied by the manufacturer.

The following tests were performed as outlined by Harrigan and McCance (1976) unless otherwise stated.

Casein Hydrolysis

Milk agar plates were inoculated by streaking once across the surface. Plates were incubated at 30°C for 2-14 days. Clear zones visible after

incubation were presumptive evidence of casein hydrolysis. This was confirmed by flooding the plates with 1% (v/v) hydrochloric acid which precipitated unhydrolyzed protein.

DNase Activity (Collins and Lyne, 1985)

The organisms were streaked onto Difco DNase agar and incubated at 30°C overnight. The plates were flooded with 1M hydrochloric acid, which precipitated unchanged nucleic acids. Clear zones around the inoculum indicated a positive reaction.

Gelatin Hydrolysis

Gelatin agar was inoculated by streaking the test organism once across the surface of the medium. Plates were incubated at 30°C for 2-14 days. Plates were flooded with 8-10ml of mercuric chloride solution (15%(w/v) in 20%(v/v) hydrochloric acid). Unhydrolyzed gelatin formed a white opaque precipitate thus a clear zone was recorded as a positive result.

Gram Reaction

The Gram stain was carried out on 18-24 hour cultures according to the Hucker method (Collins and Lyne, 1985).

Lipase Activity

Tween 80 agar was inoculated by streaking once across the surface. Plates were incubated at 30°C for 1-7 days.

Opaque zones surrounding the inoculum consisted of

calcium salts of free fatty acids and were indicative of lipolytic activity.

Morphology

The colony morphology of the *Pseudomonas* spp. was examined following growth on nutrient agar at 30°C for 24 hours. The cell morphology was examined following growth in nutrient broth for 24 hours at 30°C with agitation at 200rpm.

Oxidase Activity

a) Filter paper was impregnated with a 1%(w/v) aqueous solution of tetramethyl-p-phenylene-diamine (with 0.1% ascorbic acid to prevent auto-oxidation). Bacterial culture was gently smeared across the filter paper with a glass rod. The formation of a purple colour within 5-10 seconds indicated oxidase positive cultures.

b) Oxoid oxidase identification sticks were used to take up some bacterial culture. A positive reaction was recorded when purple coloration formed within 30 seconds.

Oxidation - Fermentation Test

Two tubes of Hugh and Leifson's medium were stab inoculated with the test culture. One tube was covered with sterile mineral oil and the tubes were incubated at 30°C for up to 14 days.

Acid production was shown by a change in the colour of the medium from blue-green to yellow. Fermentative organisms produced acid in both tubes but oxidative organisms produced acid only in the

open tube and usually only at the surface.

Pigment Production

Cultures were streaked onto Difco *Pseudomonas* agar F to detect fluorescein production and Difco *Pseudomonas* agar P to detect pyocyanin production. Plates were incubated at 30°C for 1-2 days.

Spore Stain

A smear of the *Pseudomonas* spp. was prepared from a 48 hour nutrient broth culture and heat fixed. The smear was stained with 5% (w/v) aqueous malachite green and kept steaming for 10 minutes (renewing the stain as it evaporated). It was then washed for 30 seconds in tap water and counterstained with 0.25% (w/v) saffarin for 1 minute.

Temperature Profiles

The organisms were grown on nutrient agar and incubated as follows:

4°C	for 7-14 days
20 and 25°C	for 2- 3 days
30 and 37°C	for 1- 2 days

2:5 ASSAY FOR PHENOL COMPOUNDS

Colorimetric assay

Phenol and mono-chlorophenol concentrations were determined by a modification of the method detailed in Standard Methods for the Examination of Water

and Wastewater (1985).

Cells were removed from samples by centrifugation (5,000 rpm for 10 minutes) and the resulting supernatant was diluted if necessary. A range of standards were prepared containing 0-0.05mg of phenol or chlorophenol in 10ml of distilled water. The sample or standard (10ml) was placed in a test tube and 0.5N NH_4OH (0.25ml) was added. The pH was then adjusted to 7.9 ± 0.1 with potassium phosphate buffer (pH 6.8) prepared as outlined in section 2:1:3. 2% (w/v) 4-aminoantipyrene solution (100μl) was added and the tubes were mixed well. Finally, 8% (w/v) potassium ferricyanide (100μl) was added and the tubes mixed. The tubes were allowed to stand at room temperature for 15 minutes and the absorbance was read at 500nm.

Sample standard curves for phenol and p-chlorophenol are presented in Figure 17.

HPLC assay

Samples were centrifuged (5,000 rpm for 10 minutes) to remove cells and the supernatant (5ml) was extracted with ethyl acetate (2ml) in three successive extractions. The resulting organic phase was assayed immediately or stored at 4°C for 2-3 days. Prior to analysis the organic phase was filtered through a Millipore PTFE filter (0.2 μm).

A sample (100μl) was loaded onto a readyne injection loop. A Shimadzu LC-9A Solvent Delivery Unit automatically injected 20μl of sample onto the Novapak C_{18} column (Waters; 4μ particle size, 3.9mm x 150mm stainless steel column). The mobile

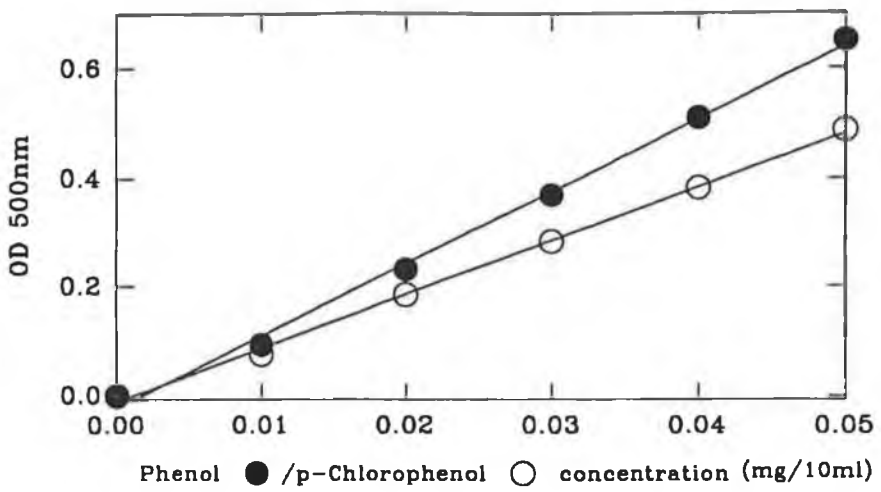


Figure 17. Standard curve for phenol and p-chlorophenol using a colourimetric assay

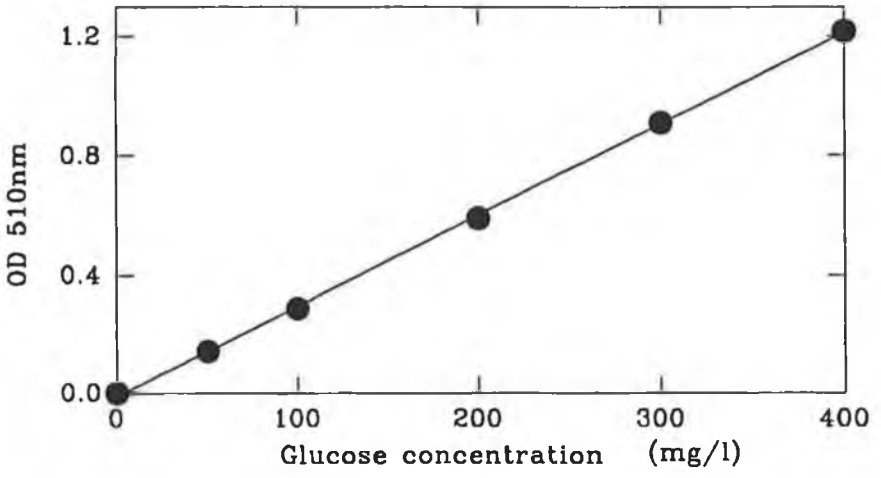


Figure 18. Standard curve for glucose using the GOD-PAP assay

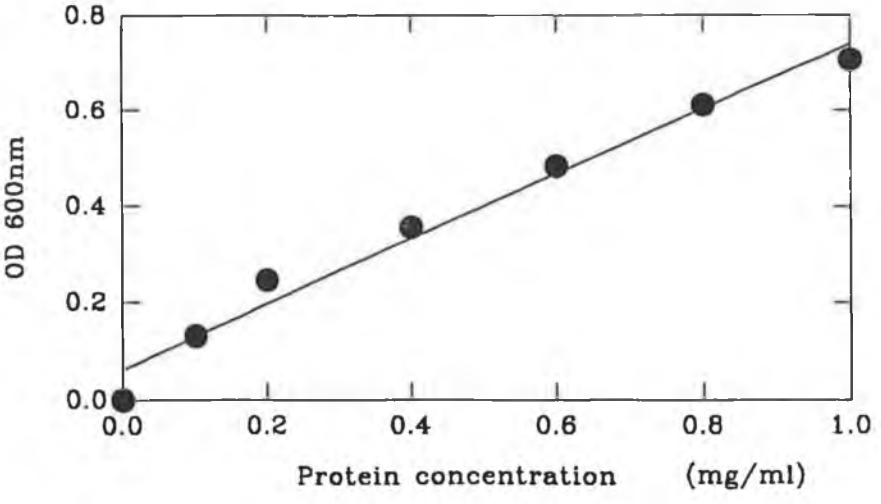


Figure 19. Standard curve for protein using the Lowry assay

phase used was acetic acid (0.075M)/acetonitrile (67:33) at a flowrate of 0.8ml/min. The Shimadzu SPD-6AV detector was set to 270nm with an absorbance sensitivity of 0.01. An Axiomm chromatography computer package was used to monitor and analyze the data. The retention time for p-chlorophenol was 6.0-6.1 minutes.

Following analysis, the column was washed with ultrapure water (20ml), to remove the acetic acid, and was then stored in acetonitrile and water (50:50). The system was washed with methanol. All solutions used were HPLC grade and were filtered (Millipore, 0.45μm PTFE filters) and degassed before use.

2:2:6 GLUCOSE ASSAY

Cells were removed from samples by centrifugation (5,000 rpm for 10 minutes). The glucose concentration in the culture supernatant was determined using a Boehringer Mannheim GOD-PAP kit in accordance with the manufacturers' instructions. The method was sensitive in the range of 0-5mM glucose.

A sample standard curve for glucose is presented in Figure 18 (page 79).

2:2:7 CATECHOL DIOXYGENASE ASSAYS

Preparation of cell-free extracts

Cells were grown on aromatic substrates (200ml) as described previously (section 2:2:3) and harvested in a Sorvall RC-5B high speed centrifuge (5,000 rpm

for 10 minutes). The resulting pellet was washed in 0.033M Tris HCl buffer (pH 7.6) and the final pellet was resuspended in the same buffer (3ml).

The cells were disrupted by sonication for 3 minutes (30 seconds on;30 seconds off) at 16 amplitude microns in a MSE Soniprep 150. The cell suspension was kept on ice throughout sonication.

Whole cells and cell debris were removed by centrifugation at 20,000 rpm and 0-4°C for 20 minutes in a Sorvall RC-5B centrifuge.

The cell-free extract was kept on ice and assayed as soon as possible for catechol dioxygenase activity (Feist and Hegeman, 1969).

Catechol 1,2-dioxygenase activity

The *ortho*-cleavage of catechol by catechol 1,2-dioxygenase was measured by following the formation of *cis, cis*-muconic acid, the cleavage product.

The following reagents were added to a quartz cuvette:

2ml	50mM tris-HCl buffer (pH 8.0)
0.7ml	distilled water
0.1ml	100mM 2-mercaptoethanol
0.1ml	cell-free extract

The contents of the cuvette were mixed by inversion and 0.1ml of catechol (1mM) was then 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 minutes.

Catechol 2,3-dioxygenase activity

Catechol 2,3-dioxygenase activity was determined by following the formation of the *meta*-cleavage product, 2-hydroxymuconic semialdehyde.

The following reagents were added to a plastic cuvette:

2 ml	50mM tris HCl buffer (pH 7.5)
0.6ml	distilled water
0.2ml	cell-free extract

The contents were mixed by inversion and 0.2ml of catechol (100mM) was added and mixed with the contents.

2-Hydroxymuconic semialdehyde production was followed by an increase in absorbance at 375nm over a period of 5 minutes.

Calculation of enzyme activity

The enzyme activity was calculated using the following equation:

$$\text{Activity } (\mu\text{moles product formed/min}) = \frac{ExCxL}{V} \times \frac{\Delta OD}{min}$$

where

E = Molar extinction co-efficient (1/mol/cm)
C = substrate concentration (moles)
L = path length (cm)
V = reaction volume (L)

Catechol 1,2-dioxygenase : E_{260} = 16,800 l/mol/cm

$$\text{Activity} = \frac{16{,}800 \times 10^{-7} \times 1}{3 \times 10^{-3}} \times \frac{\Delta OD}{min}$$

$$= 0.56 \times \frac{\Delta OD}{min}$$

= μmoles product formed/min

Catechol 2,3-dioxygenase : E_{375} = 14,700 mol/l/cm

$$\text{Activity} = \frac{14{,}700 \times 2 \times 10^{-7} \times 1}{3 \times 10^{-3}} \times \frac{\Delta OD}{min}$$

$$= 0.98 \times \frac{\Delta OD}{min}$$

= μmoles product formed/min

The specific enzyme activity is expressed as μmoles of product formed per minute per mg of protein and was calculated from :

$$\text{Specific activity}(\mu\text{moles/min/mg}) = \frac{\text{Activity}}{\text{Total Protein}}$$

Protein determination

The protein concentration in the cell-free extracts was determined by the method of Lowry *et al.*(1951).

Reagent A 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).

A sample standard curve is presented in Figure 19 (page 79).

Reagent A : 50ml 0.2% Na_2CO_3 in 0.1M NaOH
0.5ml 1% $CuSO_4$
0.5ml 2% Na K tartarate

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

2:2:8 ISOLATION OF PLASMID DNA FROM *PSEUDOMONAS* SPECIES

A modified method of Hansen and Olsen (1978) was used for the isolation of large plasmids from *Pseudomonas* spp. (Almond *et al.*, 1985).

An overnight culture in Luria broth (250ml) was harvested at 7,000 rpm for 10 minutes in a Sorvall RC-5B high speed centrifuge. The resulting cell pellet was resuspended in 6ml of 25% (w/v) sucrose in 0.05M Tris (pH 8.0). From this point on, all further additions were mixed by gentle inversion of the tube. First, 1ml of lysozyme (5mg/ml in 0.25M Tris, pH 8.0) was added and the tubes were left on ice for 5 minutes. Then, 2.5ml of EDTA (0.25M, pH 8.0) was added and the tubes were left on ice for a further 5 minutes. Cell lysis was completed by the addition of 2.5ml of SDS (20% w/v in TE), followed by a brief (1-2 minute) incubation at 55°C.

A freshly prepared 3M solution of NaOH (0.75ml) was added and the tubes were mixed by inversion for 3 minutes at room temperature. Then, 6ml of Tris (2M, pH 7.0) was added and mixed thoroughly but

gently. The addition of 3ml of SDS (20% w/v in TE) was immediately followed by the addition of 6ml of ice cold NaCl (5M). The tubes were then mixed gently and left on ice for 5-6 hours or overnight at 4°C.

The precipitated chromosome - membrane complex was removed by centrifugation at 20,000rpm for 30 minutes (4°C) in the Sorvall RC-5B centrifuge. The supernatant was removed to a fresh centrifuge tube and 1/3 volume (approximately 9.25ml) of PEG 6000 (42% w/v in 0.01M Sodium phosphate buffer, pH 7.0) was added. The tubes were mixed by inversion and chilled overnight at 4°C.

The DNA was harvested by centrifugation at 7,000rpm for 6 minutes (4°C) in the Sorvall RC-5B and the resulting pellet was resuspended in 6ml of TE buffer (section 2:1:3). The tubes were kept on ice and gently shaken from time to time to resuspend the DNA. The DNA was visualized using agarose gel electrophoresis or was purified for restriction analysis using a caesium chloride gradient.

2:2:9 SEPARATION OF PLASMID DNA BY AGAROSE GEL ELECTROPHORESIS

Plasmids were separated by horizontal electrophoresis through a 0.7% agarose gel using a Gallenkamp Maxicell submarine gel system. Tris acetate running buffer was used (section 2:1:3). Bromophenol blue solution (0.07% (w/v) in 40% sucrose) was added to the DNA solution (1/5) and 25μl of sample was loaded per well. To separate large plasmids, the gel was run at 35mV overnight using a Gallenkamp Biomed E500 power pack.

Following electrophoresis, the gel was stained in ethidium bromide (0.4μg/ml in distilled water) for 30-40 minutes, destained in distilled water for 15 minutes and visualized on a UV transilluminator. Successful gels were photographed using Kodak Tri-X-pan, 35mm film. The negatives were developed with Kodak Universal Developer (1/8 (v/v) in distilled water) and fixed with Kodafix (1/4 (v/v) in distilled water). The negatives were printed on Kodak F4 photographic paper using the above developing and fixing solutions.

2:2:10 CURING OF THE TOL PLASMID FROM *PSEUDOMONAS PUTIDA* NC1B 10432

Curing was performed using the method of Dunn and Gunsalus (1973). The *Pseudomonas putida* NC1B 10432 was grown in L-broth with 5-20μg/ml of Mitomycin C (added after sterilization of the medium) and incubated at 30°C with agitation at 200rpm for 2 days. Appropriate dilutions were spread - plated onto nutrient agar and grown overnight at 30°C. Colonies were replica plated onto *Pseudomonas* mineral medium incorporating m-toluate (5mM) and incubated at 30°C for 2-4 days. Colonies which failed to grow on m-toluate were streaked from the nutrient agar plates onto fresh medium with m-toluate to verify the result.

The DNA was then isolated from presumptive TOL^- cells using the modified Hansen and Olsen procedure (section 2:2:8) and visualized by agarose gel electrophoresis (section 2:2:9). One of the cultures which had lost the TOL plasmid was selected and maintained on nutrient agar for use as a chromosomal marker on agarose gels.

2:2:11 ISOLATION OF PLASMID DNA FROM *E.COLI*

Plasmids were isolated using the method of Cannon *et al.* (1974).

An overnight culture in Luria broth (250ml) incorporating the appropriate antibiotic was harvested at 10,000rpm for 10 minutes in a Sorvall RC-5B centrifuge. The cells were resuspended in 2ml of 25% (w/v) sucrose in 0.05M Tris (pH 8.0) and transferred to thick-walled Beckman Ultracentrifuge tubes. Firstly, 0.4ml of lysozyme (20mg/ml in 0.25M Tris, pH 8.0) was added and the tubes were incubated at room temperature for 5 minutes.

Secondly, 0.8ml of EDTA (0.25M, pH 8.0) was added and the tubes were incubated at room temperature for a further 10 minutes. Finally, 3.2ml of Triton Lytic mix was added. The Triton Lytic mix comprised 1% (v/v) triton-X-100, 5% (v/v) Tris (1M, pH 8.0) and 25% EDTA (0.25M, pH 8.0) in distilled water. The tubes were incubated at room temperature for 15 minutes.

Cell debris was removed by centrifugation at 40,000rpm for 40 minutes (4°C) in a Beckman L8-80M ultracentrifuge. The plasmid-containing supernatant was removed to a sterile universal and further purified using a caesium chloride gradient.

2:2:12 PURIFICATION OF PLASMID DNA

A modification of the method outlined by Maniatis *et al.* (1982) was used to purify plasmid DNA for restriction analysis and probe preparation.

Caesium chloride (6.9g) was added to 6.5ml of DNA

solution from *E.coli* or *Pseudomonas* sp. and dissolved by mixing gently. This solution was transferred to Beckman Quickseal tubes using a pasteur pipette and 0.18ml of ethidium bromide (10mg/ml in distilled water) was then added. The total weight of solution (excluding tube) was brought to 14.1g with Na_2EDTA (10mM). The tubes were filled with mineral oil and balanced in pairs to within 0.01g. They were then heat-sealed and centrifuged at 50,000rpm and 18°C for 22 hours in a Beckman L8-70M ultracentrifuge.

The lower plasmid band was extracted with a syringe in the presence of UV light and transferred to a sterile plastic tube. This was extracted 4 times with equal volumes of isopropanol saturated with 20XSSC (section 2:1:3) to remove the ethidium bromide. Dialysis tubing was boiled in Na_2EDTA (10mM) for 10 minutes and then boiled in distilled water for a further 10 minutes. The plasmid solution was dialysed against TE buffer (section 2:1:3) twice (including one overnight) to remove the caesium chloride. The dialysed DNA solution was then stored at -20°C.

2:2:13 RESTRICTION OF PLASMID DNA

Restriction enzymes (*Eco* RI, *Hind* III, *Sal* I, *Sst* I and *Xho* I) were supplied, together with the corresponding buffer by Bethesda Research Laboratories, Life Technologies Inc., Maryland, USA. The enzymes were stored at -20°C. The buffers were stored at 4°C.

The DNA solutions, purified by caesium chloride gradients, were cut with restriction endonucleases

according to the suppliers' instructions. λ DNA was cut separately with *Hind* III and *Xho* I and the digests were combined to provide appropriate size markers in the range of 1-33.5kb (Gibco-BRL catalogue).

Restriction digests were set up as follows:

	Plasmid DNA	Probe DNA	λ DNA
DNA solution (μl)	20.5	5.0	4.0
Buffer (μl)	2.5	1.0	4.0
Sterile distilled water (μl)	-	3.0	30.0
Enzyme - 10 iu/ml (μl)	2.0	1.0	2.0
	25.0	10.0	40.0

All samples were incubated at 37°C for 2 hours.

2:2:14 PREPARATION OF PROBE DNA

The recombinant plasmid pDTG113 was isolated and purified for use as the NAH probe. This plasmid was cut with *Eco* RI which resulted in two fragments, the vector and the insert. Due to its large size, the insert alone was used as the probe. A restriction map of pDTG113 is shown in Figure 20.

The recombinant plasmids pDTG602 and pGSH2836 were isolated and purified for use as the toluene-dioxygenase and toluene-monooxygenase probes, respectively. Both of these plasmids were linearized with *Eco* R1, which occupied a single

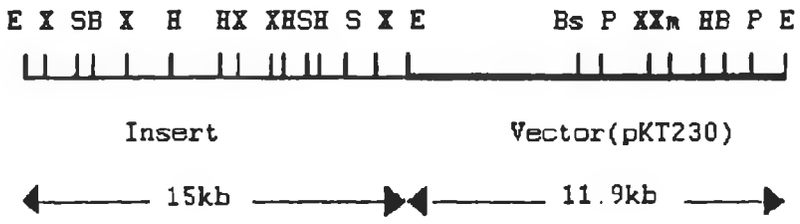


Figure 20. Restriction map of pDTG113
(Serdar and Gibson, 1989)

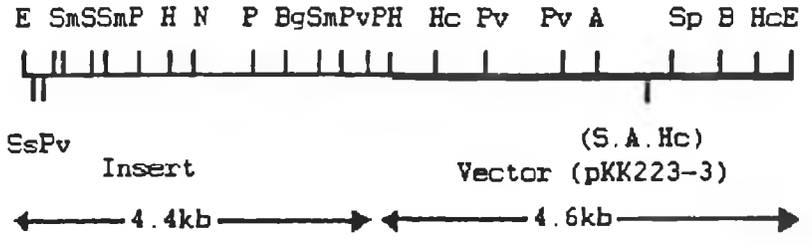


Figure 21. Restriction map of pDTG602
(Zylstra and Gibson, 1989)

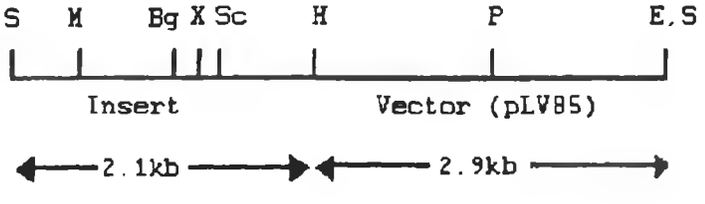


Figure 22. Restriction map of pGSH2836
(Harayama *et al.*, 1986)

restriction site on the plasmid. A restriction map of pDTG602 is shown in Figure 21 and that of pGSH2836 is shown in Figure 22. The linearized DNA was labelled radioactively with ^{32}P or non-radioactively with digoxigenin.

Preparation of the NAH probe

Four 20.5μl aliquots of pDTG113 were cut with *Eco* R1 and the resulting two fragments were separated by electrophoresis through a 1% (w/v) agarose gel (section 2:2:9). Being the largest fragment, the insert was resolved closest to the wells. The insert was cut from the gel with a scalpel and weighed in a preweighed eppendorf. The insert DNA was separated from the agarose and purified using the "Gene-Clean" procedure of Bio 101 Inc., California, U.S.

Twice the volume (by weight) of sodium iodide was added and the tube was incubated at 45-55°C for 5 minutes to dissolve the agarose. The tube was mixed occasionally. Glass milk was supplied by Dr. Thecla Ryan, D.C.U. The glass milk was vortexed well and 5μl was added to the tube. The contents of the tube were mixed by inversion and left on ice for 5 minutes. The tube was then centrifuged in a microfuge at 12,000rpm for 5 seconds and the supernatant was discarded. The pellet was washed three times with New Wash. The tubes were centrifuged at 12,000rpm for 5 seconds after each wash and the pellet was resuspended using a micropipette. The insert DNA was eluted into 10μl of TE buffer(section 2:1:3) by incubating at 45-55°C for 3 minutes. The tube was centrifuged at 12,000rpm for 30 seconds and the plasmid-containing supernatant was removed to a clean sterile

eppendorf. The procedure was then repeated giving a final volume of 20μl of DNA solution. The DNA was stored at -20°C until needed.

Sodium Iodide

A solution of 90.8% (w/v) of sodium iodide in water was stirred until as much as possible had dissolved and then was filtered through Whatman No. 1 filter paper. Sodium sulphite was added and the solution was stored at 4°C.

New Wash

50% Ethanol
10mM Tris-HCl, pH 7.5
1mM EDTA

The reagent was stored at -20°C.

Determination of DNA concentration

Serial dilutions of linearized probe DNA were prepared on parafilm. First, 5μl of TE was placed on the parafilm and 5μl of DNA was added. The dilution was mixed in a micropipette. Then 5μl of this solution was added to a second 5μl of TE and so on. Each sample was mixed with 1μl of bromophenol blue solution and 5μl was loaded onto a 1% (w/v) agarose mini-gel. λ standards of known concentration were also loaded onto the gel. The gel was run at 100V for 30 minutes and stained in ethidium bromide solution. The concentration of probe DNA was estimated by comparing the intensity of fluorescence of the samples with that of λ DNA of known concentration.

Labelling of NAH and TOL probe DNA with ^{32}P

Linearized probe DNA was labelled by random primed incorporation of α-^{32}P ATP (DuPont, France) using the Prime-a-Gene labelling System (Promega Corp., Madison, USA) based on the method developed by Feinburg and Vogelstein (1983). The kit was stored at -20°C. Before use the components were allowed to thaw on ice except Klenow enzyme, which was kept at -20°C and returned to the freezer immediately after use. First 25ng of linearized DNA was added to a sterile eppendorf and the volume was brought to 30μl with sterile distilled water. The DNA was denatured by heating to 95-100°C for 2 minutes and was then cooled rapidly on ice. Then, 1μl of each of the non-labelled dNTP's (dCTP, dGTP, dTTP) was mixed to yield a 3μl solution. The labelling reaction was set up by adding the following reagents to the denatured DNA solution, in their stated order:

10μl	5X labelling buffer
2μl	mixture of non-labelled dNTP's
2μl	nuclease-free BSA
5μl	$\alpha^{32}P$-ATP (50μCi, 3000Ci/mmole)
1μl	Klenow enzyme

The tube was centrifuged briefly to mix contents and was incubated at room temperature for at least 1 hour. The reaction was stopped by heating at 95-100°C for 2 minutes and was subsequently chilled in an ice bath. EDTA was added to a concentration of 20mM and the solution was then used directly for hybridization.

Non-radioactive labelling of NAH and TOL probe DNA

Random primer labelling of linearized probe DNA with digoxigenin was carried out using the DIG DNA labelling kit in accordance with the manufacturers' instructions (Boehringer Mannheim, Germany).

First, 5μl of linearized DNA was added to a sterile eppendorf along with 10μl of sterile distilled water and denatured by boiling for 10 minutes followed by chilling on ice. The kit reagents were then added to the denatured DNA solution in the following order:

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

The tube was centrifuged briefly to mix contents and was incubated in a 37°C waterbath for at least 1 hour. The reaction was stopped by adding 2μl of EDTA (0.2M, pH 8.0) and the labelled DNA was precipitated with 2.5μl of lithium chloride (4M) and 75μl of ethanol (at -20°C). The tube was incubated at -20°C for 2 hours and then centrifuged at 12,000rpm for 10 minutes in a microfuge. The pellet was washed twice with cold ethanol (70% v/v) and then dried at 55°C for 15 minutes. The pellet was resuspended in 50μl of TE buffer. The labelled DNA was then used directly for hybridization or stored at -20°C.

2:2:15 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 gels to nitrocellulose paper

Following electrophoresis one corner of the gel was cut away in order to mark its orientation on the nitrocellulose. The DNA was partially hydrolyzed by acid depurination. The gel was soaked twice for 15 minutes in 0.25M HCl at room temperature and then washed very well with water (4-5 times). The DNA was denatured by soaking the gel in several volumes of 1.5M NaCl and 0.5M NaOH for 1 hour at room temperature with constant shaking. The gel was neutralized by soaking in several volumes of 1M Tris.HCl (pH 7.6) and 1.5M NaCl for 1 hour at room temperature with constant shaking.

Two pieces of nitrocellulose paper (BA85; Schleicher and Schuell, Germany) were cut about 1-2mm larger then the gel in both dimensions. Gloves were always used when handling nitrocellulose. The nitrocellulose filters were floated on the surface of 2XSSC until completely wet from beneath and then submerged for 2-3 minutes. Six pieces of Whatman 3MM paper, cut to exactly the same size as the gel, were also soaked in 2XSSC. The wet nitrocellulose was placed on top of the gel and all air bubbles were removed by gently rolling a glass rod over the surface of the filter. Three sheets of Whatman 3MM paper were then placed on top of the filter and again all air bubbles were removed. A stack of paper towels (approx. 10cm) cut just smaller than the 3MM paper were then placed on the 3MM paper. Finally, a glass plate was placed on top and the whole thing inverted. The process was then repeated with the second nitrocellulose filter. A

500g weight was placed on the top glass plate and the bidirectional transfer of DNA was allowed to proceed overnight. During the first few hours of transfer the gel stack was inverted every 20 minutes to ensure even transfer.

Transfer was allowed to proceed overnight. The filters were soaked in 6XSSC for 5 minutes at room temperature and then dried at room temperature. The dried filter was placed between two sheets of 3MM paper and baked at 80°C for 2 hours.

Hybridization of Southern Filters

The baked filter was floated on the surface of 6XSSC until wet from beneath and then immersed for 2 minutes. The wet filter was placed in a heat-sealable bag. Prehybridization fluid (0.2ml/cm^2 of filter) was warmed to 68°C and then added to the bag.

Prehybridization fluid

6XSSC
0.5% SDS
5X Denhardt's solution
100μg/ml denatured calf thymus DNA

As much air as possible was squeezed from the bag before it was sealed. The bag was incubated for 2-4 hours at 68°C with occasional agitation. Following prehybridization the corner of the bag was cut with a scissors and the prehybridization solution removed. The hybridization solution (50μl/cm^2 of filter) was added with the appropriate precautions for the handling of ^{32}P.

Hybridization solution

6XSSC
0.01M EDTA
^{32}P - labelled denatured probe DNA
5X Denhardt's solution
0.5% SDS
100μg/ml denatured calf thymus DNA

Air was removed from the bag and it was resealed. The filter was incubated at 68°C overnight with occasional agitation.

The following day the filter was removed from the bag and immediately submerged in a solution of 2XSSC and 0.5% (w/v) SDS at room temperature. After 5 minutes the filter was transferred to a solution of 2XSSC and 0.1% (w/v) SDS and incubated at room temperature for 15 minutes. The filter was then transferred to prewarmed solution of 0.1XSSC and 0.5% (w/v) SDS and incubated at 68°C for 2 hours with gentle agitation. The buffer was then changed and incubated for a further 30 minutes. The filter was then dried at room temperature on a sheet of 3MM paper. The dry filter was placed in a heat-sealable bag and applied to x-ray film to obtain an autoradiographic image.

50X Denhardt's Solution

1% (w/v) Ficoll
1% (w/v) Polyvinylpyrrolidone
1% (w/v) Bovine serum albumin

The above reagents were dissolved in distilled water and the solution was stored at -20°C.

Denatured Calf Thymus DNA

Calf thymus DNA was dissolved in water (10mg/ml). The DNA was sheared by passing it through a hypodermic needle several times and was then boiled for 10 minutes. The solution was stored at -20°C in 1ml aliquots. Just prior to use the DNA was heated to 95-100°C for 5 minutes and cooled immediately on ice.

Autoradiography

In the dark, a sheet of Kodak x-ray film was taped to an x-ray film holder (shiny-side down). The filter sealed in a bag was then placed on top of the film and taped securely in place. A second sheet of x-ray film was then taped on top of the film (shiny-side up). The x-ray holder was closed and placed in a black plastic bag. The film was exposed for several days at room temperature. When the desired exposure time had elapsed the films were removed, in the dark, and developed as follows:

> The film was placed in Kodak DX-80 developer for 4 minutes, washed in water for 30 seconds and then fixed in Kodak FX-40 fixer for about 5 minutes and finally washed well under running water.

If necessary another film can be applied to the filter to obtain another autoradiograph.

2:2:16 DOT BLOT HYBRIDIZATION

Total DNA was prepared and hybridized using the method of Maniatis *et al.* (1982). The sample and the control DNA were denatured by boiling for 10 minutes and chilling quickly on ice. The DNA was incubated with an equal volume of 1M NaOH for 20 minutes at room temperature. The DNA was then neutralized with half the volume of a solution of 1M NaCl, 0.3M sodium citrate, 1M HCL and 0.5M Tris.HCL (pH 8.0). The samples were mixed well and immediately chilled on ice.

A sheet of nitrocellulose was cut to the desired size and placed on a sheet of clingfilm. First, 5μl of the DNA solution was spotted onto the filter and allowed to absorb, then another spot of 5μl was applied. The process was repeated until the desired volume of sample had been applied (30μl for sample DNA, 10μl for control or probe DNA). The filter was allowed to dry at room temperature for 1 hour and baked at 80°C for 2 hours. The filter was then used directly for hybridization or stored between two sheets of 3MM paper and wrapped in clingfilm. The dot blots were hybridized using non-radioactively labelled probe (section 2:2:17).

2:2:17 COLONY HYBRIDIZATION

The filters were prepared for hybridization according to the method of Maniatis *et al.* (1982) and were hybridized using the Boehringer Mannheim DIG DNA labelling and detection kit according to the manufacturers' instructions.

Preparation of filters for colony hybridization

Nitrocellulose was cut to size to fit in a 90mm petri dish. The filters were marked with a ball-point pen, placed between sheets of Whatman 3MM paper and then wrapped in aluminium foil. The filters were autoclaved at 121°C for 15 minutes. Using a sterile forceps the filters were overlaid on plate count agar. Colonies were transferred to the nitrocellulose using sterile cocktail sticks and the plates were incubated at 30°C for 24-48 hours.

Lysis of colonies and binding of DNA to nitrocellulose filters

Four pieces of Whatman 3MM paper were cut to the appropriate size to fit into four glass trays. Each piece was saturated with one of the following solutions: 10% (w/v) SDS, denaturing solution (0.5M NaOH and 1.5M NaCl), neutralizing solution (1.5M NaCl and 0.5M Tris.HCl, pH 7.4) and 2XSSC. Any excess liquid was poured off. Using a blunt-ended forceps the nitrocellulose filters were peeled from their plates and placed colony side up on the SDS-impregnated 3MM paper. After 3 minutes exposure of the first filter it was transferred to the second sheet of 3MM paper soaked in denaturing solution. The remaining filters were transferred in the same order that they were removed from their agar plates. Each filter was exposed to the denaturing solution for 5 minutes. When transferring the filters excess liquid was removed by blotting briefly on dry 3MM paper. The filters were transferred to the neutralizing solution for a further 5 minutes. Finally, the filters were transferred to the 2XSSC-soaked paper for 5

minutes. The filters were dried, colony side up, on a sheet of 3MM paper for 30 minutes at room temperature. The filters were then placed between two sheets of 3MM paper and baked at 80°C for 1-2 hours. Filters not used immediately for hybridization were wrapped in cling film and stored at room temperature.

Hybridization to digoxigenin-labelled probe

Baked filters were floated on the surface of a solution of 2XSSC until wet from beneath and were then submerged for 5 minutes. The filters were then washed in several volumes of prewashing solutions at 50°C for 30 minutes with occasional agitation.

Prewashing solution

5XSSC
0.5% (w/v) SDS
1mM EDTA (pH 8.0)

Bacterial debris was gently scraped from the filters using soft tissue soaked in prewashing solution. This reduced the background hybridization without affecting the intensity or sharpness of positive signals.

The filters were prehybridized in heat-sealable plastic bags (2 filters/bag) with at least 20ml of hybridization solution per 100cm^2 of filter.

Hybridization solution

5XSSC
2% (w/v) Blocking reagent
0.1% (w/v) N-laurylsarcosine, sodium salt
0.02% (w/v) SDS

50% (v/v) Formamide

The filters were incubated at 42°C for at least 1 hour. The solution was distributed from time to time. The solution was then replaced with 2.5ml of hybridization solution, per 100cm^2 of filter, containing freshly denatured labelled DNA (section 2:2:14). The filters were incubated at 42°C overnight with the solution being redistributed occasionally.

Following incubation, the filters were removed from the plastic bags and washed twice in 2XSSC and 0.1% (w/v) SDS for 5 minutes at room temperature (50l/100cm^2 filter). The filters were washed twice more for 15 minutes at 68°C in 0.1XSSC and 0.1% (w/v) SDS (50ml/100cm^2 filter). The filters were then used directly for detection or stored air-dried for later detection.

Hybridization solutions could be stored at -20°C and reused several times. Immediately prior to use, the probe DNA was redenatured by heating the hybridization solution to 95°C for 10 minutes.

For dot blot hybridization the above protocol was followed except no prewashing of filters was required, the hybridization solution did not contain formamide and therefore only 1% blocking reagent was added and also prehybridization and hybridization were carried out at 68°C.

Immunological detection of hybridized DNA

All the following incubations were performed at room temperature. All the reactions were carried

out with agitation except for the colour development. The volumes were calculated for 100cm^2 filter size.

The filters were washed briefly (1 minute) in several volumes of buffer 1 (100mM maleic acid, 150mM NaCl; pH 7.5) and then incubated for 30 minutes in 100ml of buffer 2 (1% (w/v) blocking reagent in buffer 1). The filters were then incubated for 30 minutes in 20ml of diluted antibody-conjugate solution (150mU/ml, 1/5000 in buffer 2). Unbound antibody was removed by washing twice for 15 minutes in 100ml of buffer 1. The filters were equilibrated for 2 minutes with buffer 3 (100mM Tris.HCl, 100mM NaCl, 50mM Mg Cl_2; pH 9.5) and then incubated in 10ml of colour solution in the dark. The colour solution was prepared fresh with NBT-solution (4.5μl/ml Buffer 3) and X-phosphate solution (3.5μl/ml Buffer 3). The colour was allowed to develop overnight without shaking or mixing. The reaction was stopped by washing the filters for 5 minutes with 50ml of TE buffer. The filters were stored in sealed plastic bags in TE buffer. The results were documented by photography.

2:2:18 THE ACTIVATED SLUDGE SYSTEM

Operation of the activated sludge system

Activated sludge was obtained from a plant treating chemical wastewater. Duplicate systems (Figure 23) were set up in fermenter pots with a 1 litre working volume.

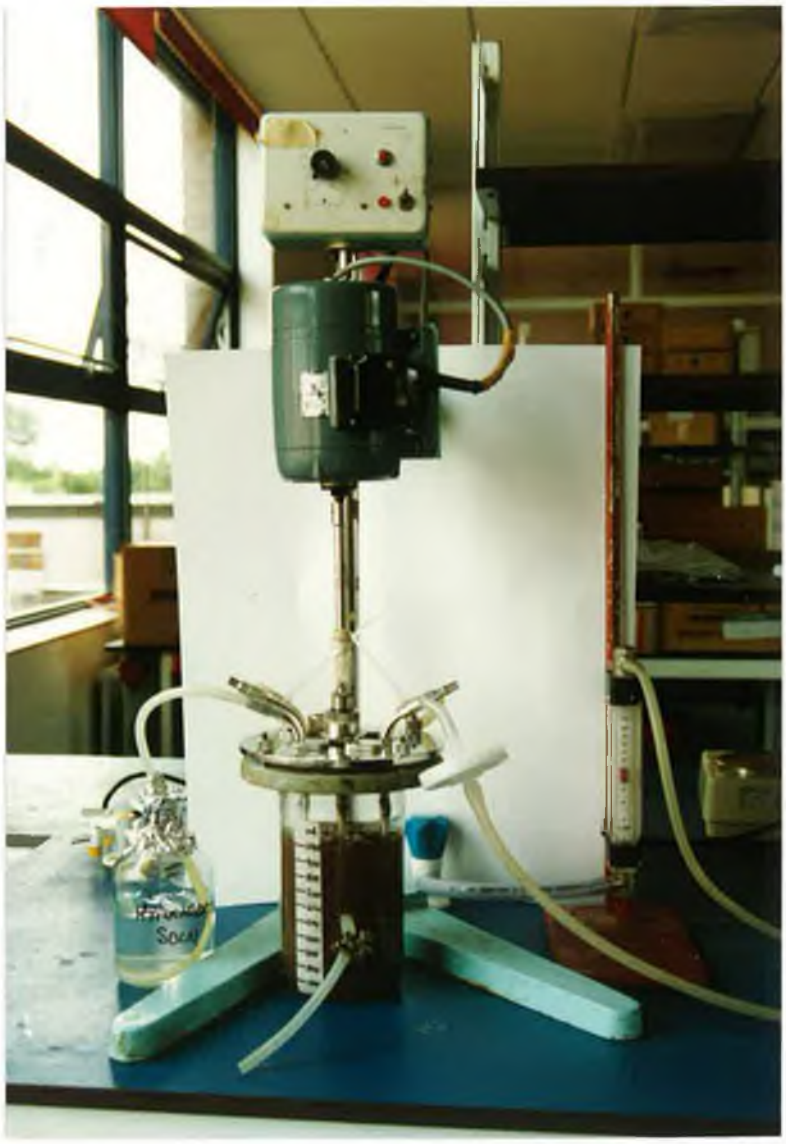


Figure 23. The model activated sludge system.

The systems were operated using the fill and draw technique at room temperature with agitation and aeration (1-2 v/v/min). The activated sludge was fed every 3-4 days. The solids were allowed to settle and the effluent was removed to the 500ml level mark. The volume was brought back to 1 litre with a synthetic effluent comprising *Pseudomonas* minimal medium and naphthalene. Naphthalene (20mM) was supplied as the sole carbon and energy source.

Analysis of the activated sludge system

Temperature, pH, dissolved oxygen

The temperature and dissolved oxygen of the systems were measured using a WTW Oxi 196 dissolved oxygen meter (WTW, Germany) in accordance with the manufacturers' instructions.

The pH of the systems was measured with a Corning pH meter.

Settled Sludge Volume (SSV)

Mixed liquor (100ml) was removed and allowed to settle in a graduated cylinder for 30 minutes. The settled sludge volume was recorded as ml/l after 30 minutes.

Mixed Liquor Suspended Solids (MLSS)

Whatman GF/C, glass fibre filter paper was predried at 103°C for 2-3 hours and then weighed. A sample of mixed liquor (10ml) was filtered through the dried paper which was then dried at 103°C for 24 hours. The weight of the dried solids was then determined and the result recorded as g total

solids/l.

Sludge Volume Index (SVI)

The sludge volume index was calculated as follows:

$$\text{SVI (ml/g)} = \frac{\text{Settled sludge volume (ml/l)}}{\text{Suspended solids (g/l)}}$$

Chemical Oxygen Demand (COD)

The chemical oxygen demand of the effluent was determined using a modification of the method outlined in Standard Methods for the Examination of Water and Wastewater (1985). The effluent was filtered through Whatman GF/C glass fibre filter paper and diluted if necessary.

COD tubes and caps were washed with 20% (v/v) H_2SO_4. Suitably diluted sample or standard (2.5ml) was added to the tube. Digestion solution (1.5ml) was carefully added. Sulphuric acid reagent (3.5ml) was carefully added down the side of the tube. The caps were screwed on tightly and the tubes inverted with care to mix the contents.

The tubes were then placed in a pre-heated digestion block at 150°C for 2 hours. After the elapsed time, the tubes were removed and cooled. The entire contents of the tube were then transferred to a 100ml flask and Ferroin indicator (50μl) was added. The contents were titrated against the FAS reagent. The end-point was a sharp colour change from blue-green to a reddish brown.

The sensitivity of the assay was in the range of 0-500mg O_2/l.

The COD was calculated as follows:-

$$\text{COD (mg } O_2/l) = \frac{(A-B) \times M \times 8.000}{\text{Sample volume}}$$

Where A = volume of FAS used for blank (ml)
B = volume of FAS used for sample (ml)
M = molarity of FAS

$$\text{Molarity of FAS (M)} = \frac{\text{Volume in digestion tube}}{\text{Volume of FAS used for blank}} \times 0.02$$

Reagents

Digestion Solution (Potassium Dichromate Solution)

$K_2Cr_2O_7$ (4.913g), previously dried at 103°C for 2 hours was dissolved in distilled water. Concentrated H_2SO_4 (167ml) and $HgSO_4$ (33.3g) were added, dissolved and the solution allowed to cool. The reagent was then diluted to 1 litre.

Sulphuric acid reagent

$AgSO_4$ (5.5g) was added to H_2SO_4 (545ml) and allowed to stand for 2-3 hours.

FAS reagent (Ferrous Ammonium Sulphate reagent)

Fe $(NH_4)_2(SO_4)_2.6H_2O$ (0.02M) was dissolved in distilled water. Concentrated H_2SO_4 (20ml) was added and the solution allowed to cool. The reagent was then diluted to 0.02M.

Potassium Hydrogen Phthalate Standard

Potassium hydrogen phthalate (425mg), previously dried at 103°C for 2 hours, was dissolved in 1 litre of distilled water.

This solution should give a COD of 500mgO_2/l.

Isolation of Bacteria from Activated Sludge

A modification of the method of Banks and Walker (1979) was used to isolate bacteria from activated sludge.

A well-mixed sample of sludge was diluted (1:10) with sterile tripolyphosphate solution (5mg/L) and sonicated for 1 minute at an amplitude of 4μ in a MSE Soniprep 150. Appropriate dilutions were then made with tripolyphosphate solution and spread-plated (0.1ml) on plate count agar and *Pseudomonas* minimal medium incorporating naphthalene. All plates were incubated at room temperature for 6 days.

The colonies formed were counted and colonies from plate count agar were used for the hybridization experiment (section 2:2:17). Colonies from the naphthalene plates were isolated as pure cultures and were identified using the API 20NE system.

2:2:19 BIOAUGMENTATION OF ACTIVATED SLUDGE.

Bioaugmenation of activated sludge with *Pseudomonas putida* IGA 7.24

A 1 litre-scale bioaugmentation study was carried out on activated sludge treating naphthalene. The

activated sludge system as described in section 2:2:18 was inoculated with the naphthalene-degrading *Pseudomonas putida sp.* IGA 7.24.

The inoculum was prepared from an overnight nutrient broth culture (20ml) of *P. putida* IGA 7.24. The culture was harvested by centrifugation at 5,000rpm for 10 minutes and washed with 0.01M sodium phosphate buffer (pH 7.0). The final pellet was resuspended in the above buffer to give an OD at 660nm of 0.992, this corresponded to approximately 7 x 10^8 cells/ml.

The inoculum was added to the system to give a concentration of 1% (v/v). After a period, the inoculum concentration was reduced to 0.1% (v/v). Analysis was carried out on the system as outlined in section 2:2:18.

Bioaugmentation of activated sludge with Biolyte CX 80

A flask-scale bioaugmentation study was carried out on activated sludge treating p-chlorophenol.

The Biolyte CX-80 was prepared as follows:
The freeze-dried bacteria (10g) were resuscitated by shaking in dilution buffer (90ml) at 30°C for 1 hour. The dilution buffer was prepared by adding 1.25ml of stock buffer solution (0.1M K_2HPO_4, pH 7.2) and 1ml of 10% (v/v) triton-x-100 solution to 1 litre of distilled water. After 1 hour agitation the cereal-based carrier was allowed to settle.

Activated sludge was obtained from a plant treating chemical wastewater. The sludge was allowed to settle, the clarified effluent was removed and then

replaced with *Pseudomonas* minimal medium. The sludge was then dispensed into flasks in 100ml aliquots. p-Chlorophenol, dissolved in ethanol (200mg/ml), was added to each flask to give a final concentration of 200ppm. Flasks were inoculated with Biolyte CX-80 (10% v/v) and incubated at 30°C with agitation at 150rpm.

The bacteria containing supernatant was used directly for the bioaugmentation experiment. Secondary sugars supplied by the cereal carrier were removed by harvesting the bacteria at 5,000rpm for 10 minutes and washing with 0.01M sodium phosphate buffer (pH 7.0). The final pellet was resuspended in the above buffer and this suspension was also used in the bioaugmentation experiment.

The systems inoculated with washed or unwashed CX-80 and a control system were monitored for p-chlorophenol degradation (section 2:2:5).

3 RESULTS

3:1 CHARACTERIZATION OF THE *PSEUDOMONAS* SPECIES

Four *Pseudomonas* spp. from a commercial bioaugmentation product (CX80) were characterized. These organisms comprised three *Pseudomonas putida* spp., designated IGA 7.16, IGA 7.24 and IGA 0.92 and one *Pseudomonas fluorescens*, designated IGA 13.42. The colony and cell characteristics and the Gram reaction of each organism were noted together with their response to a wide range of biochemical tests. The results are outlined in Tables 2-7. The response of the strains in all cases was quite similar. As can be seen in Table 2, the cell characteristics of the four organisms were identical. Apart from the colour of the *P. putida* IGA 7.16 colonies, the colony characteristics of the four *Pseudomonas* spp. were also identical (Table 3).

Table 2. Cell characteristics of the *Pseudomonas* species

	P. putida spp.			*P. fluorescens*
	7.16	**7.24**	**0.92**	**13.42**
Shape	rod	rod	rod	rod
Gram Reaction	-	-	-	-
Spore formation	-	-	-	-
Motility	+	+	+	+

Table 3. Colony characteristics of the *Pseudomonas* species

	P. putida spp.			*P. fluorescens*
	7.16	**7.24**	**0.92**	**13.42**
Colour	white	cream	cream	cream
Shape	round	round	round	round
Edge	entire	entire	entire	entire
Elevation	convex	convex	convex	convex
Diffusible Pigments	+	+	+	+
Fluorescein	+	+	+	+
Pyocyanin	-	-	-	-

The four organisms had similar biochemical properties (Table 4). All were oxidase and catalase positive. *P. putida* IGA 0.92 had a positive response to the nitrate reductase test. *P. putida* IGA 7.16 and *P. fluorescens* IGA 13.42 produced lipase and *P. putida* IGA 13.42 also produced gelatinase. The response of the organisms to the other biochemical tests was negative. The four *Pseudomonas* spp. were capable of using a wide range of carbohydrates as sole carbon source (Table 5). Of the fifty six carbohydrates tested, *P. putida* IGA 7.16 could use thirty, *P. putida* IGA 7.24 could use twenty six, *P. putida* IGA 0.92 could use twenty seven while *P. fluorescens* IGA 13.42 used forty two substrates.

Table 4. Biochemical properties of the *Pseudomonas* species

	P. putida spp.			*P. fluorescens*
	7.16	7.24	0.92	13.42
Hugh and Leifson test	Ox	Ox	Ox	Ox
Oxidase	+	+	+	+
Catalase	+	+	+	+
β-Galacto -sidase	-	-	-	-
Nitrate Reductase	-	+	-	-
Lipase	+	-	-	+
DNase	-	-	-	-
Gelatinase	-	-	-	+
Casein Hydrolysis	-	-	-	-
Indole Form'n	-	-	-	-
H_2S Form'n	-	-	-	-
Urease	-	-	-	-
VP	-	-	-	-

+ positive reaction Ox - oxidative
- negative reaction VP - Voges Proskauer

Table 5. Carbohydrate Utilization at 30°C by the *Pseudomonas* species

	P. putida spp.			*P. fluorescens*
	7.16	7.24	0.92	13.42
N-Acetylglu -cosamine	-	-	-	-
Adipate	-	-	-	-
Adonitol	+	+	+	+
Amygdalin	+	-	-	+
D-Arabinose	-	-	-	+
L-Arabinose	+	+	+	+
D-Arabitol	+	+	+	+
L-Arabitol	+	+	+	+
Arbutin	+	-	-	+
Arginine	+	+	+	+
Caprate	+	+	+	+
Cellobiose	+	-	-	+
Citrate	+	+	+	+
Dulcitol	-	-	-	+
Erythritol	-	+	+	+
Esculin	-	-	-	-
Fructose	+	+	+	+
D-Fucose	+	+	+	+
L-Fucose	-	-	-	-
Galactose	+	+	+	+

cont'd p.115

Table 5. cont'd.

	7.16	7.24	0.92	13.42
B-Gentibiose	-	-	-	+
Gluconate	+	+	+	+
Glucose	+	+	+	+
Glycerol	+	+	+	+
Glycogen	-	-	-	-
Inositol	+	+	+	+
Inulin	-	-	-	-
2-Ketoglucon-ate	-	-	-	-
5-Ketoglucon-ate	-	-	-	-
Lactose	-	-	-	+
D-Lyxose	-	-	-	+
Malate	+	+	+	+
Maltose	-	-	-	-
Mannitol	+	-	+	+
Mannose	+	+	+	+
Melezitose	-	-	-	+
Meliobiose	+	+	+	+
a-Methyl-D-Glucoside	-	-	-	-
a-Methyl-D-Mannoside	-	-	-	-

cont'd p.116

Table 5. cont'd.

	7.16	7.24	0.92	13.42
B-Methyl-D-Xyloside	+	-	-	+
Phenylacet-ate	+	+	+	+
Raffinose	-	+	-	-
Rhamnose	-	-	-	+
Ribose	+	+	+	+
Saccharose	+	+	+	+
Salicin	+	-	-	+
Starch	-	-	-	-
Sorbitol	+	+	+	+
L-Sorbose	-	-	+	+
D-Tagatose	-	-	-	-
Trehalose	+	+	+	+
D-Turanose	-	-	-	+
Xylitol	+	+	+	+
D-Xylose	+	+	+	+
L-Xylose	+	-	-	+

+ positive reaction
- negative reaction

The ability of the organisms to grow on nutrient agar at a range of temperatures (4°C-37°C) was investigated (Table 6). All the organisms were capable of growth at 20°C-30°C. *P. putida* IGA 7.16 was unable to grow at 4°C. *P. putida* IGA 7.24 and *P. fluorescens* IGA 13.42 were unable to grow at 37°C.

Table 6. Growth of the *Pseudomonas* species at Various Temperatures

	P. putida spp.			*P. fluorescens*
°C	7.16	7.24	0.92	13.42
4	-	+	+	+
20	+	+	+	+
25	+	+	+	+
30	+	+	+	+
37	+	-	+	-

+ growth
- no growth

The four *Pseudomonas* spp. were examined for their sensitivity to a number of antibiotics at 30°C (Table 7). The four organisms were capable of growth in the presence of both novobiocin (30μg) and penicillin G (10iu). They were sensitive to polymyxin B (300iu), streptomycin (25μg) and tetracycline (30μg) failing to grow in the presence of these antibiotics. *P. putida* IGA 7.16 was capable of growth in the presence of

chloramphenicol (50μg) while the other three organisms were sensitive to this antibiotic.

Table 7. Growth of the *Pseudomonas* species at 30°C in the presence of antibiotics

	P. putida spp.			*P. fluorescens*
	7.16	7.24	0.92	13.42
Chloramphenicol - 50μg	+	-	-	-
Novobiocin - 30μg	+	+	+	+
Penicillin G - 10iu	+	+	+	+
Polymyxin B - 300iu	-	-	-	-
Streptomycin - 25μg	-	-	-	-
Tetracycline - 30μg	-	-	-	-

+ growth
- no growth

3:2 DEGRADATION OF AROMATIC COMPOUNDS BY THE *PSEUDOMONAS* SPECIES

3:2:1 THE ABILITY OF THE *PSEUDOMONAS* SPECIES TO GROW ON BENZOATE, NAPHTHALENE, M-TOLUATE, PHENOL, CHLOROPHENOL AND TOLUENE.

The ability of the four *Pseudomonas* spp. to grow at the expense of a range of aromatic substrates was investigated. The organisms were grown in liquid culture on different concentrations of benzoate, naphthalene, m-toluate, phenol, chlorophenol and toluene in *Pseudomonas* minimal medium at 30°C and agitated at 200rpm (section 2:2:3). The results are outlined in Tables 8 and 9.

Table 8. The growth of the *Pseudomonas* species on aromatic compounds at 30°C

	Conc'n	*P. putida* spp.			*P.fluor -escens*
Substrate	**(mM)**	**7.16**	**7.24**	**0.92**	**13.42**
Benzoate	5	+	+	+	+
	10	+	+	+	+
	20	+	-	+	+
Naphthalene	5	+	+	+	+
	10	+	+	+	-
	20	+	+	+	-
m-Toluate	5	+	+	+	+
	10	+	+	+	+
	20	+	+	+	+
Phenol	5	+	+	+	+
	10	-	-	+	-
	20	-	-	-	-

+ growth - no growth

Table 9. The growth of the *Pseudomonas* species on chlorophenol and toluene at 30°C

	Conc'n	*P. putida* spp.			*P.fluor -escens*
Substrate	**(ppm)**	**7.16**	**7.24**	**0.92**	**13.42**
o-Chlorophenol	200	-	-	-	-
m-Chlorophenol	200	-	-	-	-
p-Chlorophenol	200	+	-	-	-
	300	-	-	-	-
	400	-	-	-	-
	500	-	-	-	-
	600	-	-	-	-
Toluene	**(µl/ 50ml)**				
	100	+	-	-	-
	250	+	-	-	-
	500	+	-	-	-

+ growth - no growth

The four organisms grew well on benzoate and m-toluate. The three *P. putida* spp. also grew well on naphthalene while the *P. fluorescens* sp. grew only on 5mM naphthalene. All four organisms failed to grow at the higher concentrations of phenol. *P. putida* IGA 7.16 was capable of growth on toluene and on 200ppm p-chlorophenol but not on higher concentrations of p-chlorophenol or on o- or m-chlorophenol. The other three organisms, *P. putida* IGA 7.24, IGA 0.92 and *P. fluorescens* IGA 13.42, failed to grow on any of the chlorophenols or on toluene. The growth of *P. putida* IGA 7.16 on p-

chlorophenol and toluene was the only feature which distinguished this strain from the others.

3:2:2 THE GROWTH OF THE *PSEUDOMONAS* SPECIES ON BENZOATE, NAPHTHALENE AND M-TOLUATE

The four *Pseudomonas* spp. grew well on benzoate, naphthalene and m-toluate. The extent of growth on these aromatic substrates was investigated by following the OD_{660nm} of the cultures until growth at 30°C reached stationary phase or decline. The pH during growth was also noted. The duration of the lag period and the maximum OD_{660nm} attained were recorded and the specific growth rate was calculated (section 2:2:3) for each organism and substrate (Tables 10-13).

In all cases the pH decreased during growth from an initial pH of 7.0. This decrease was most marked in the naphthalene cultures. Where growth occured the biomass produced increased with increasing concentration of substrate as indicated by the maximum OD attained in each case. In most cases where growth occured there was a lag period. This generally extended for a minimum period of 6 hours. Where the lag period increased it did so with increasing substrate concentration. In general, the growth rate also increased with increasing substrate concentrations. *P. putida* IGA 7.16 and IGA 0.92 achieved the fastest growth rates following growth on benzoate. *P. putida* IGA 7.24 and *P. fluorescens* IGA 13.42 achieved the fastest growth rates on m-toluate.

Table 10. Growth rate, maximum OD_{660nm} and lag period of *P. putida* IGA 7.16 during growth on benzoate, naphthalene and m-toluate

Substrate	Conc'n (mM)	Duration of Lag (h)	Maximum OD_{660nm}	pH at max. OD	μ (h^{-1})
Benzoate	5	6	0.750	6.94	0.0692
	10	6	1.150	6.83	0.1049
	20	6	1.582	6.70	0.1644
Naphthalene	5	24	1.601	6.76	0.0518
	10	24	1.967	6.55	0.0757
	20	24	2.071	6.42	0.0763
m-Toluate	5	6	0.677	6.90	0.0356
	10	6	0.734	6.81	0.0787
	20	24	1.146	6.60	0.1581

Table 11. Growth rate, maximum OD_{660nm} and lag period of *P. putida* IGA 7.24 during growth on benzoate, naphthalene and m-toluate

Substrate	Conc'n (mM)	Duration of Lag (h)	Maximum OD_{660nm}	pH at max. OD	μ (h^{-1})
Benzoate	5	6	0.766	6.78	0.1026
	10	24	1.331	6.69	0.1948
	20	-	0	-	0
Naphthalene	5	6	1.307	6.70	0.0882
	10	6	2.075	6.56	0.0968
	20	6	2.737	6.41	0.0999
m-Toluate	5	6	0.460	7.00	0.1650
	10	6	0.888	6.96	0.1835
	20	24	1.378	6.86	0.1781

Table 12. Growth rate, maximum OD_{660nm} and lag period of *P. putida* IGA 0.92 during growth on benzoate, naphthalene and m-toluate

Substrate	Conc'n (mM)	Duration of Lag (h)	Maximum OD_{660nm}	pH at max. OD	μ (h^{-1})
Benzoate	5	0	0.600	6.79	0.3310
	10	0	0.983	6.74	0.3008
	20	6	1.559	6.57	0.1716
Naphthalene	5	6	1.225	6.81	0.1474
	10	24	1.786	6.61	0.2072
	20	24	1.975	6.50	0.2873
m-Toluate	5	24	0.627	6.83	0.0792
	10	24	1.067	6.68	0.1156
	20	24	1.640	6.61	0.1226

Table 13. Growth rate, maximum OD_{660nm} and lag period of *P. putida* IGA 13.42 during growth on benzoate , naphthalene and m-toluate

Substrate	Conc'n (mM)	Duration of Lag (h)	Maximum OD_{660nm}	pH at max. OD	μ (h^{-1})
Benzoate	5	6	0.440	7.00	0.1323
	10	24	0.810	6.99	0.1217
	20	54	1.196	6.92	0.1919
Naphthalene	5	30	0.991	6.87	0.0606
	10	-	0	-	0
	20	-	0	-	0
m-Toluate	5	6	0.559	6.98	0.1494
	10	6	1.010	6.88	0.2511
	20	24	1.455	6.70	0.2884

3:2:3 THE GROWTH OF THE *PSEUDOMONAS* SPECIES ON PHENOL.

The four *Pseudomonas* spp. grew well on 5mM phenol. Cultures were set up in *Pseudomonas* minimal medium incorporating phenol (5mM) as the sole carbon source. The cultures were incubated at 30°C with agitation at 200rpm until the phenol was degraded and the cultures reached stationary phase. The growth rate and the rate of phenol removal of the four organisms were compared. Cell growth was determined by monitoring the OD_{660nm} and the cell number on plate count agar. This is represented in Figures 24-27 along with the phenol removal.

In the case of all the organisms except *P. putida* IGA 0.92 there was an initial period during which phenol was removed very slowly followed by a period of rapid phenol removal. Phenol removal occurred most rapidly in *P. putida* IGA 0.92 cultures, with all the phenol degraded in 24 hours. This was reflected in a rapid growth rate. *P. putida* IGA 0.92 was also the only one of the four organisms capable of growth on 10mM. *P. putida* IGA 7.24 demonstrated a high rate of phenol removal, with all the phenol degraded in 30 hours. This organism demonstrated a relatively slow growth rate, however, there was no lag phase during growth. *P. putida* IGA 7.16 and *P. fluorescens* IGA 13.42 had low rates of phenol removal, with the phenol removed after 54 hours and 48 hours, respectively. Both of these organisms demonstrated lag periods of 30 hours during growth. In all cases the maximum OD_{660nm} and the maximum cell number were reached when the phenol was exhausted.

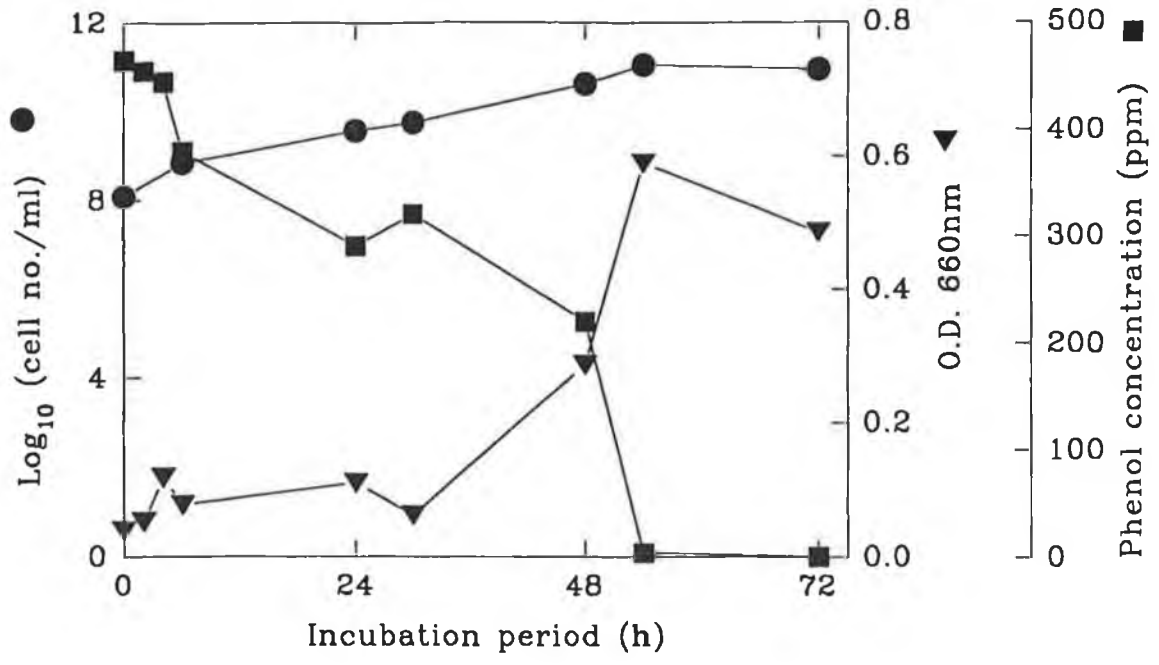


Figure 24. Growth of *P. putida* IGA 7.16 on phenol (μ=0.0920 h^{-1} ± 0.0028)

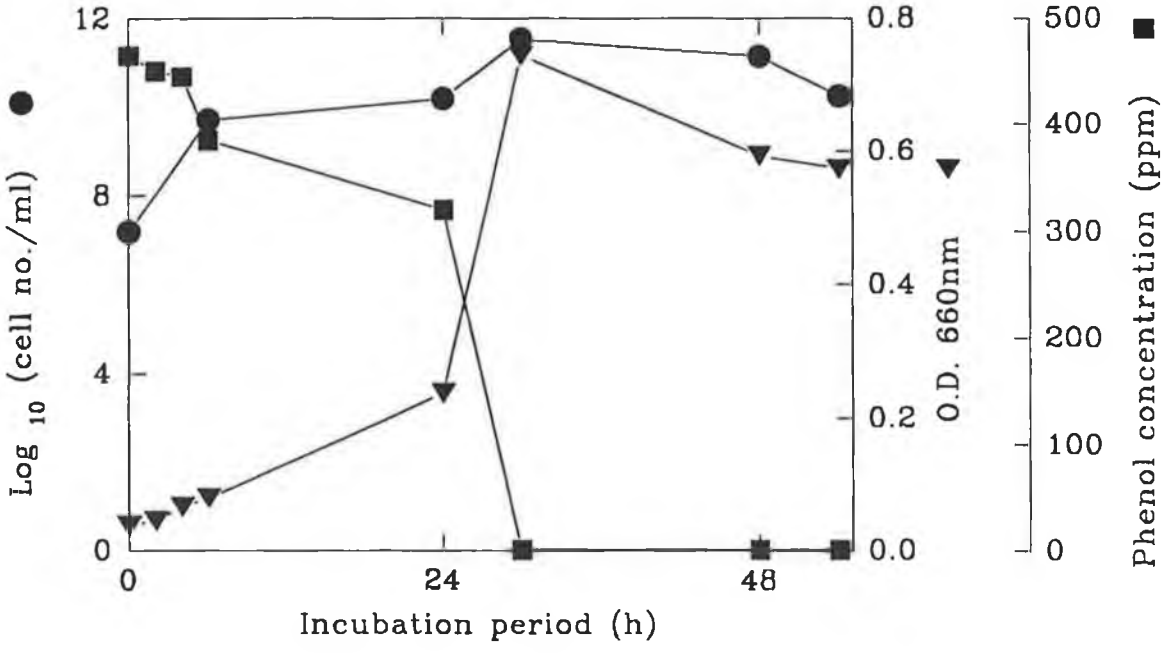


Figure 25. Growth of *P. putida* IGA 7.24 on phenol (μ=0.0884 h^{-1} ± 0.0009)

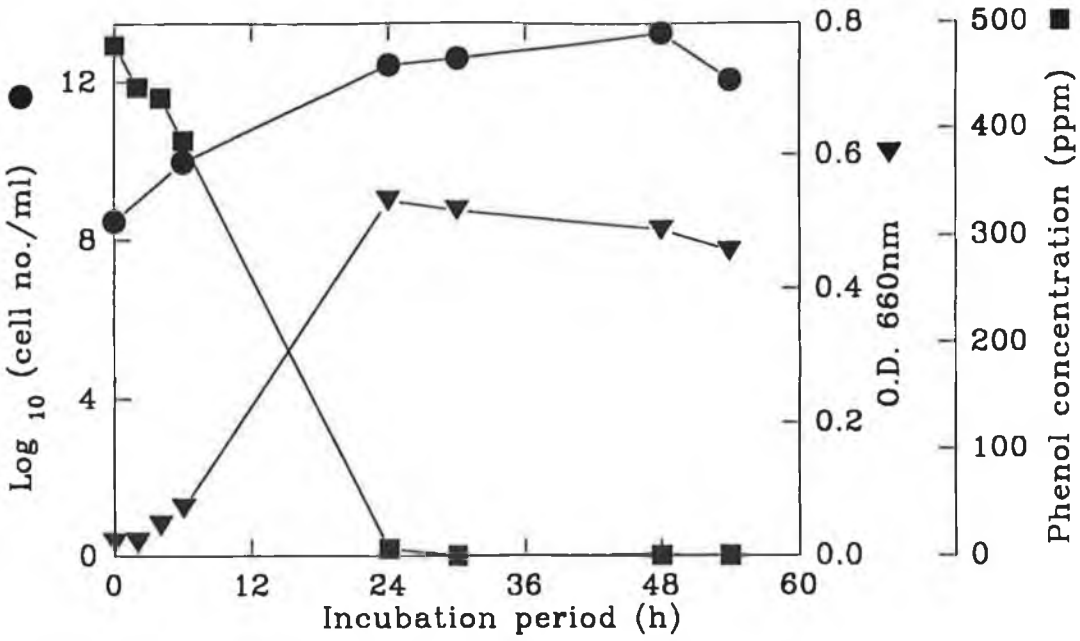


Figure 26. Growth of *P. putida* IGA 0.92 on phenol
(μ=0.1331 h^{-1} ± 0.0018)

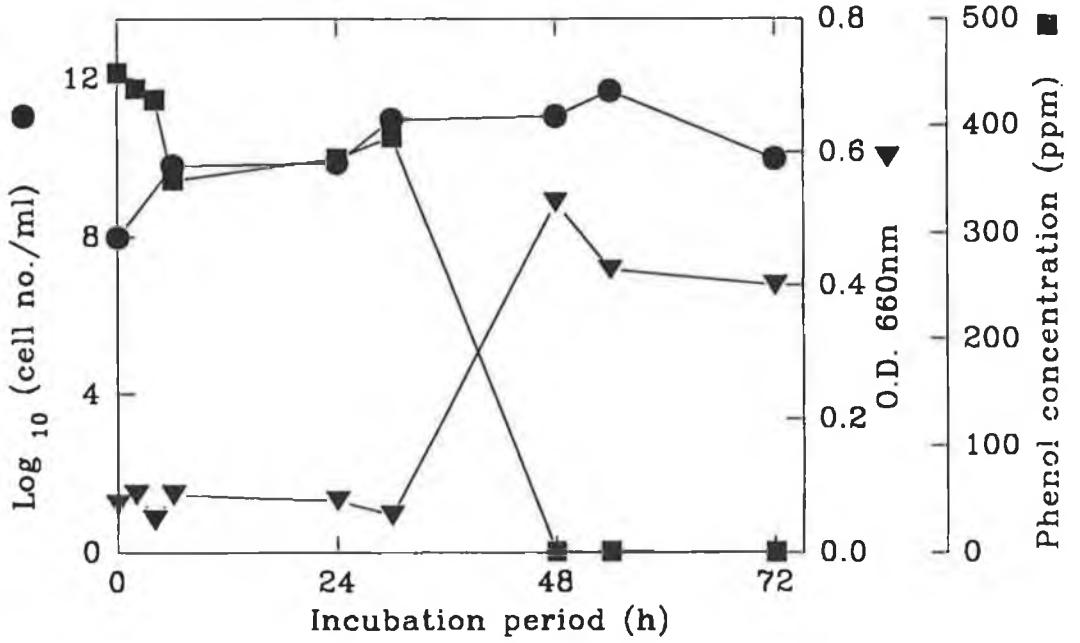


Figure 27. Growth of *P. putida* IGA 13.42 on phenol
(μ=0.1255 h^{-1} ± 0.0015)

3:2:4 GROWTH OF THE *PSEUDOMONAS* SPECIES ON CHLOROPHENOL

The four *Pseudomonas* spp. from CX 80 were examined for their ability to degrade o-, m- and p-chlorophenol. Chlorophenol (200ppm in 100μl ethanol) was added to *Pseudomonas* minimal medium. The cultures were incubated at 30°C and agitated at 200rpm. The pH, OD_{660nm} and chlorophenol concentration were monitored until the cultures reached stationary phase. The pH of the cultures varied little and was always in the range of 6.85-7.00.

P. putida IGA 7.16 was the only organism capable of complete degradation of any of the substrates. It degraded p-chlorophenol in 48 hours (Figure 28) attaining a maximum OD_{660nm} of approximately 0.3. A yellow colour developed in the culture medium in the first 12 to 18 hours but then disappeared. Higher OD was attained on o- and m-chlorophenol but these substrates were not degraded. The increase in OD did not reflect growth and was attributed to the formation of black compounds in the medium. These black compounds formed in the medium following initial degradation of these chlorophenols.

These black compounds were also formed by *P. putida* IGA 7.24, IGA 0.92 and *P. fluorescens* IGA 13.42 on o- and m-chlorophenol (Figures 29-31). The growth of these three organisms on p-chlorophenol resulted in the formation of intensely yellow coloured compounds after 18-24 hours incubation and a very slight increase in OD. There was an initial degradation of p-chlorophenol but once the yellow product formed further degradation was inhibited.

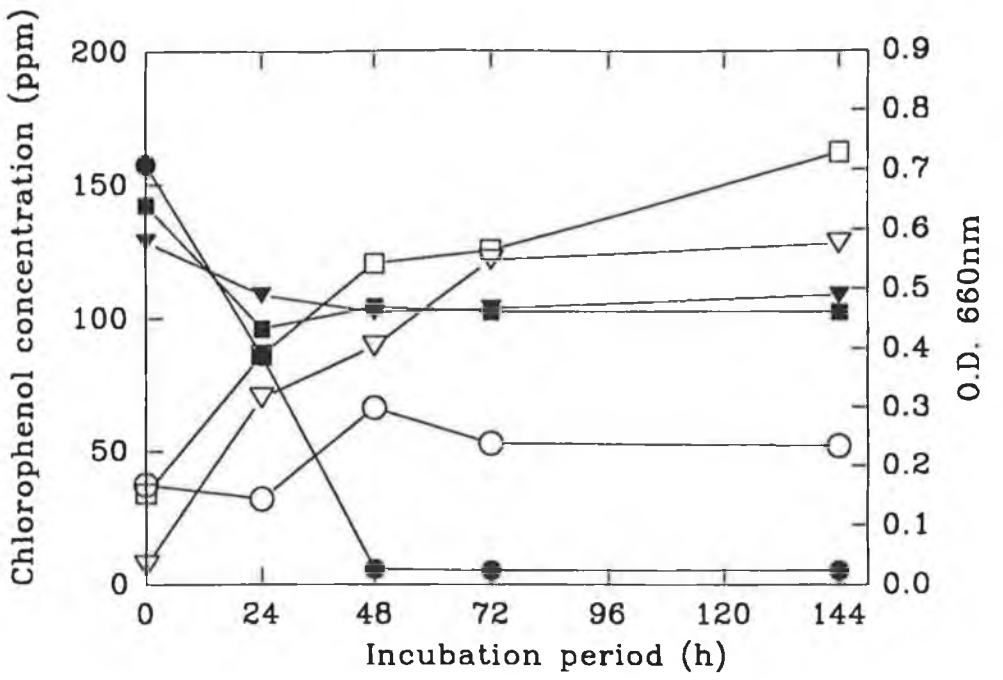
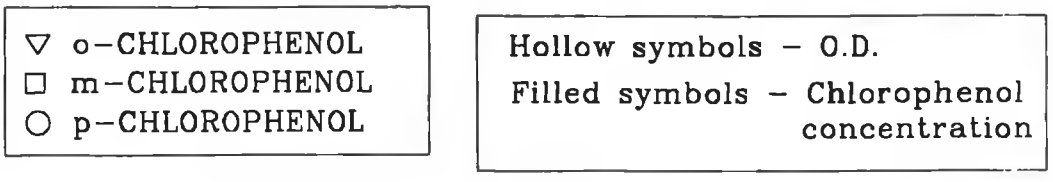


Figure 28. Growth of *P. putida* IGA 7.16 on chlorophenol



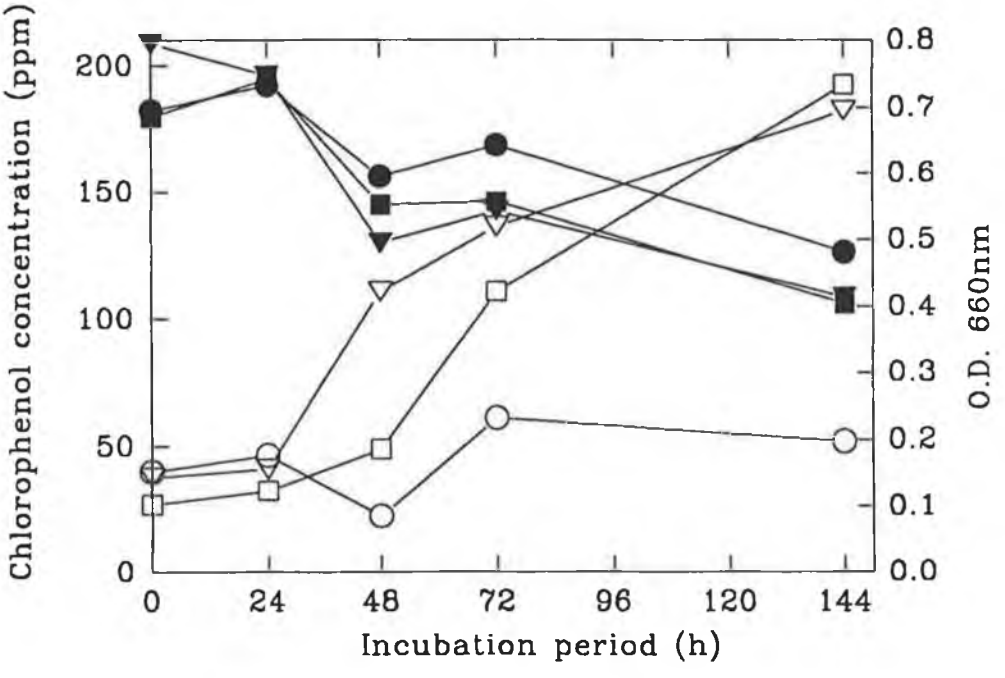


Figure 29. Growth of *P. putida* IGA 7.24 on chlorophenol

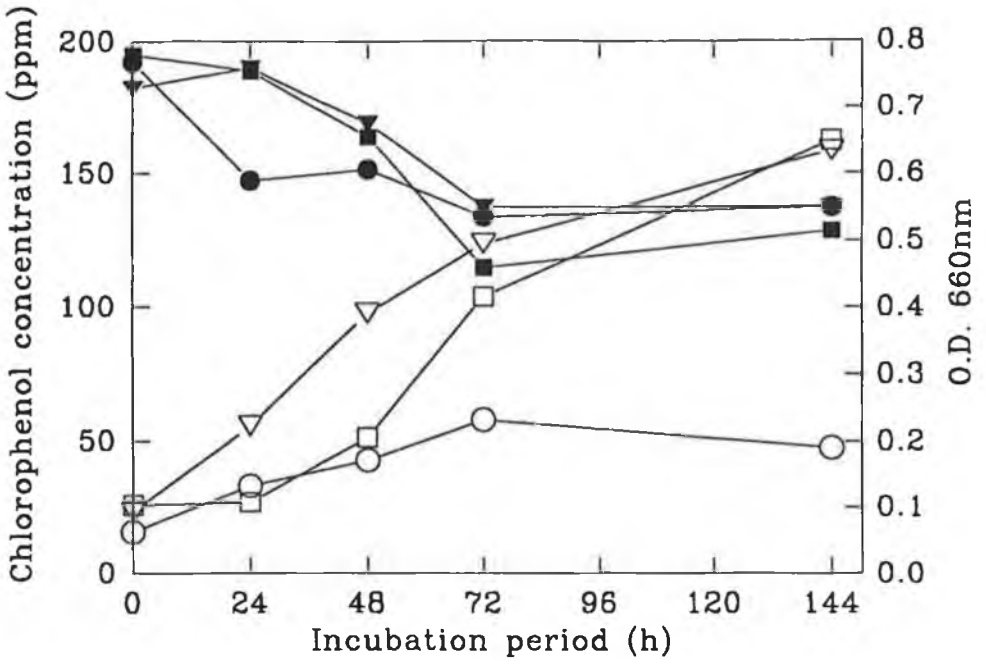
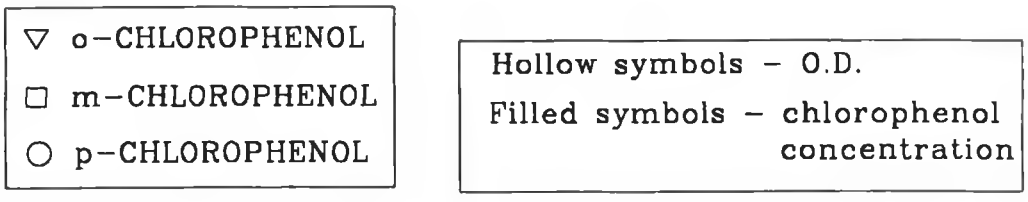


Figure 30. Growth of *P. putida* IGA 0.92 on chlorophenol



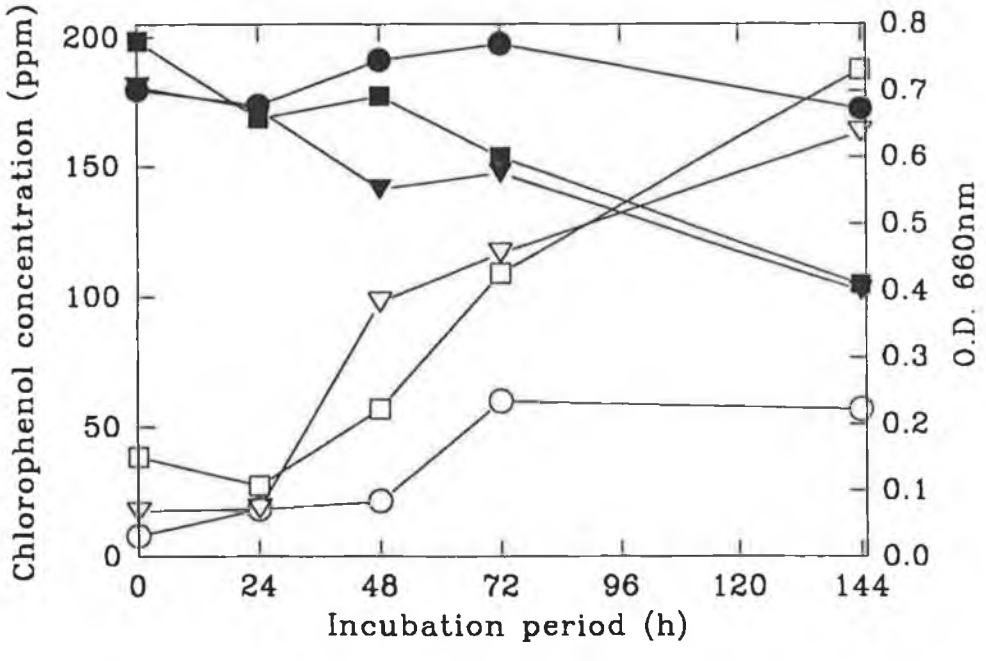


Figure 31. Growth of *P. putida* IGA 13.42 on chlorophenol

3:2:5 THE DEGRADATION OF p-CHLOROPHENOL BY MIXED MICROBIAL CULTURES

CX 80

The commercial bioaugmentation product, CX 80 was found to be unable to degrade o- and m-chlorophenol. Flask cultures were established with *Pseudomonas* minimal medium incorporating p-chlorophenol (200ppm in 200μl ethanol). The freeze dried bacteria of CX 80 were resuscitated and separated from the cereal carrier (section 2:2:19). Both washed and unwashed product were added to the medium and incubated at 30°C with agitation at 200rpm. p-Chlorophenol degradation was monitored (Figure 32).

The product rapidly degraded the p-chlorophenol, removing the substrate in 48 hours (Figure 32). Washing the bacteria, which removed secondary carbon sources carried over from the cereal carrier, adversely affected the performance of the product with the washed product failing to degrade the p-chlorophenol when monitored for 144 hours.

CX 80 and activated sludge

A sample of activated sludge was obtained from a plant treating chemical wastewater and examined for its ability to degrade p-chlorophenol (200ppm). A flask-scale bioaugmentation study was set up with CX 80 as described in section 2:2:19. The ability of the washed and unwashed product to enhance p-chlorophenol was examined (Figure 33).

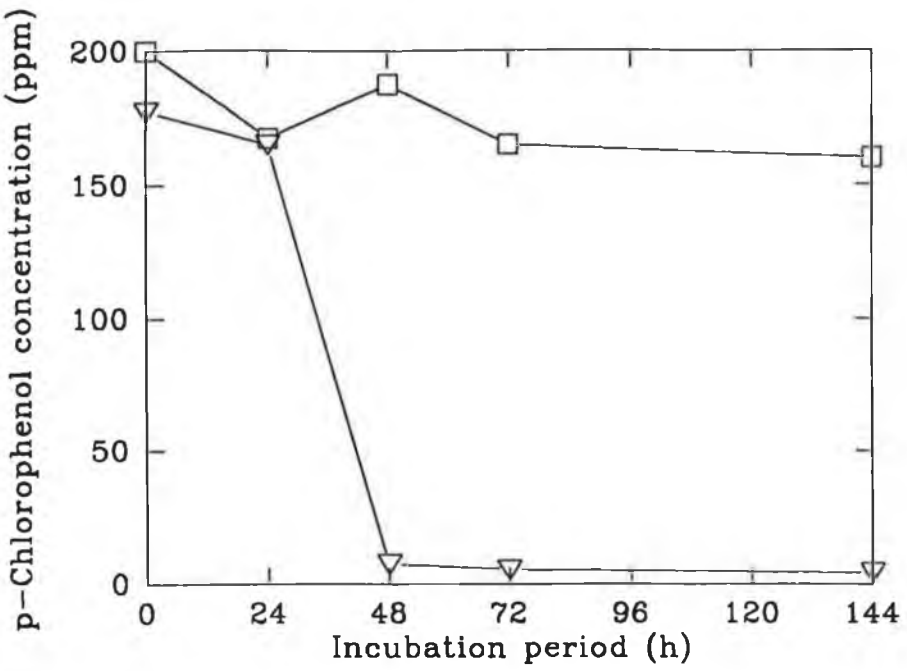
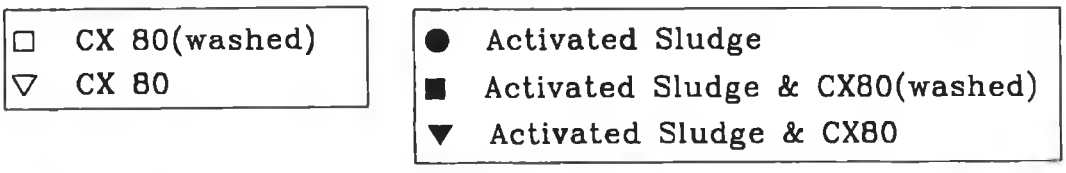


Figure 32. p-Chlorophenol degradation by CX 80



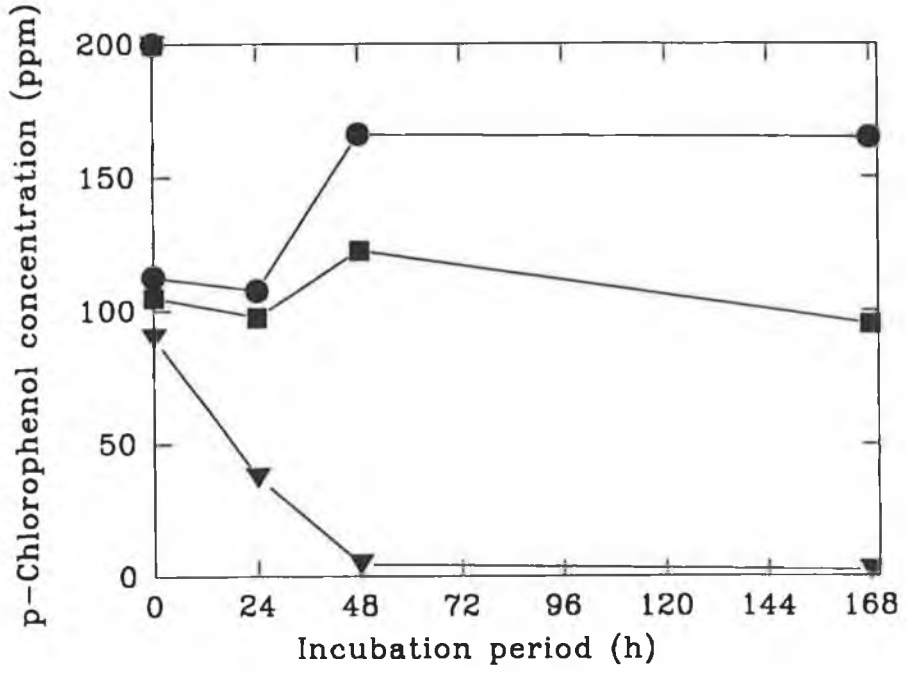


Figure 33. p-Chlorophenol degradation by activated sludge and CX 80

Although 200ppm p-chlorophenol was added to all the flasks, the initial concentration detected in the medium was only approximately 100ppm. This drop in the initial concentration of p-chlorophenol detected was attributed to adsorption on to the sludge flocs. Bioaugmentation of the sludge with CX 80 resulted in rapid degradation of the p-chlorophenol, with all the substrate removed in 48 hours. The activated sludge alone failed to degrade p-chlorophenol after 168 hours. The addition of the washed product resulted in no improvement of sludge performance. Following a period of 48 hours there was an apparent increase in the p-chlorophenol concentration in the flasks containing activated sludge only and activated sludge with washed product. The increase was greater in the case of the activated sludge alone. This increase was attributed to desorption or elution of the substrate from the sludge flocs.

3:2:6 p-CHLOROPHENOL DEGRADATION BY *PSEUDOMONAS PUTIDA* IGA 7.16

Of the *Pseudomonas* spp. examined only *P. putida* IGA 7.16 was capable of p-chlorophenol degradation. This organism was therefore selected for further investigation. The effect of inoculum size on the degradation of chlorophenol was examined. The removal of secondary carbon sources by washing the product CX 80 adversely affected its ability to degrade p-chlorophenol. Therefore the effect of glucose on p-chlorophenol degradation by *P. putida* IGA 7.16 was also investigated.

Table 14. The effect of inoculum concentration on the rate of p-chlorophenol degradation by *P. putida* IGA 7.16

Concentration (ppm)	Inoculum Size (%)	Rate of Removal (ppm/h)
200	2	4.354 ± 0.198
200	5	9.323 ± 0.469

An increased inoculum size from 2% to 5% resulted in the faster removal of p-chlorophenol (Table 14). The increase in the rate of p-chlorophenol removal was approximately proportional to the increase in inoculum size. The lag period before p-chlorophenol degradation was also reduced.

The presence of glucose resulted in a faster rate of p-chlorophenol removal, with the substrate removed after 24 hours compared with 30 hours in the culture without glucose (Figures 34-37). The presence of glucose also resulted in a slightly increased growth rate. In all cases glucose consumption preceded p-chlorophenol degradation. The glucose was consumed within 24 hours with the greatest proportion consumed in the first 6 hours. The presence of glucose resulted in a faster production of biomass. The maximum OD_{660nm} was attained after 24 hours when glucose was present compared with 48 hours in cultures without glucose. However, the total biomass produced was approximately equal in all cultures with or without glucose. The faster production of biomass resulted in a faster utilization of p-chlorophenol. The concentration of glucose supplied did not appear to be critical as the rate of p-chlorophenol removal, the growth rate and the rate of biomass

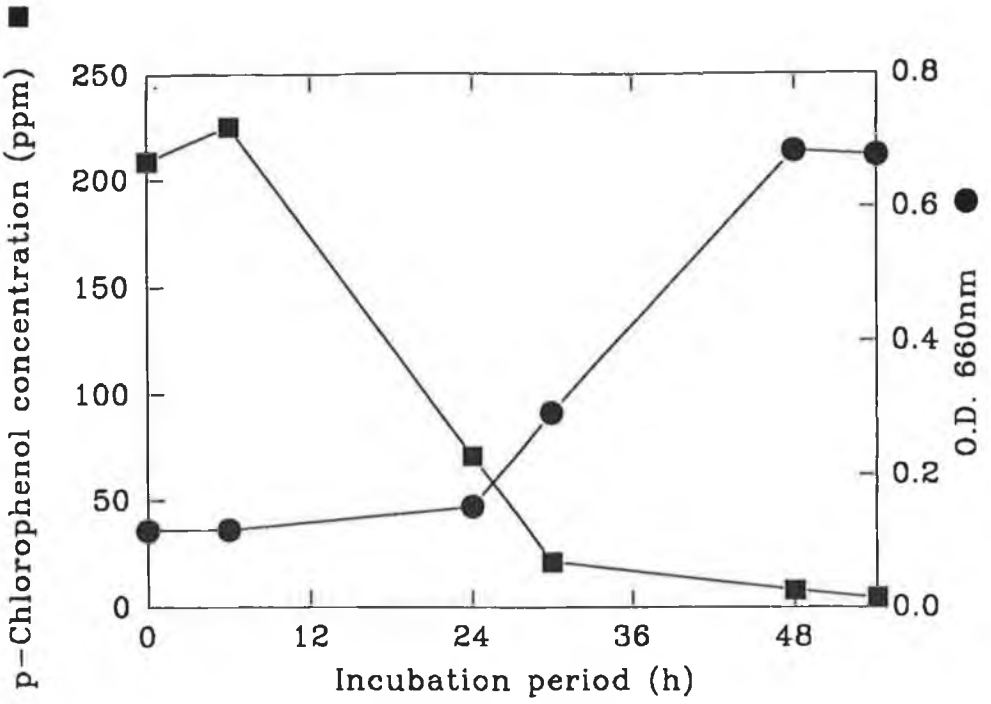


Figure 34. Growth of *P. putida* IGA 7.16 on p-chlorophenol (μ=0.0601 h^{-1} ± 0.0126)

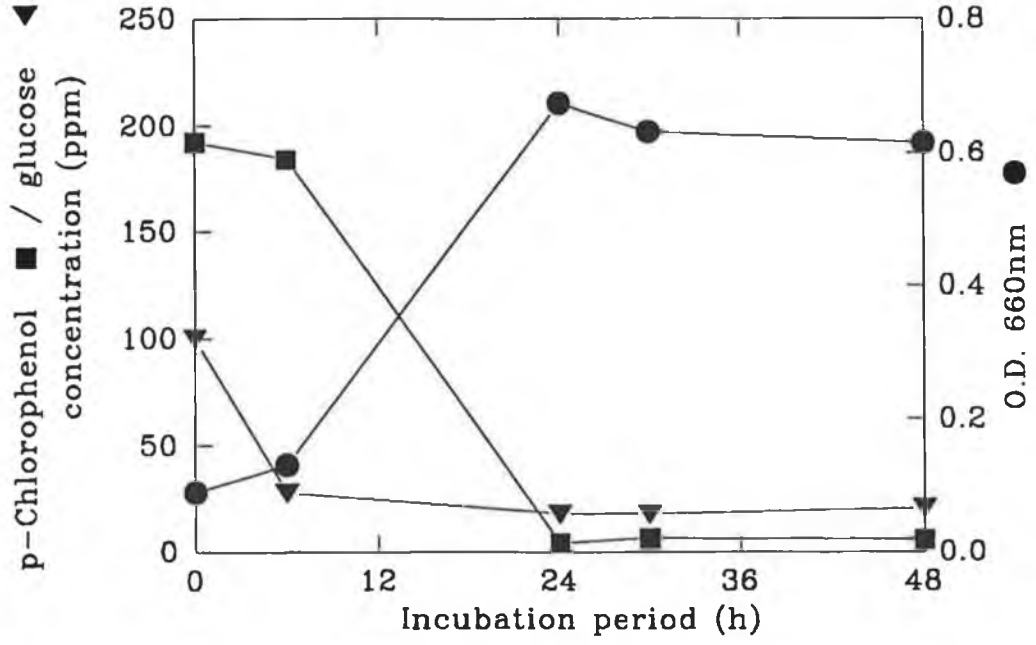


Figure 35. Growth of *P. putida* IGA 7.16 on p-chlorophenol with 100ppm glucose (μ=0.0912 h^{-1} ± 0.0016)

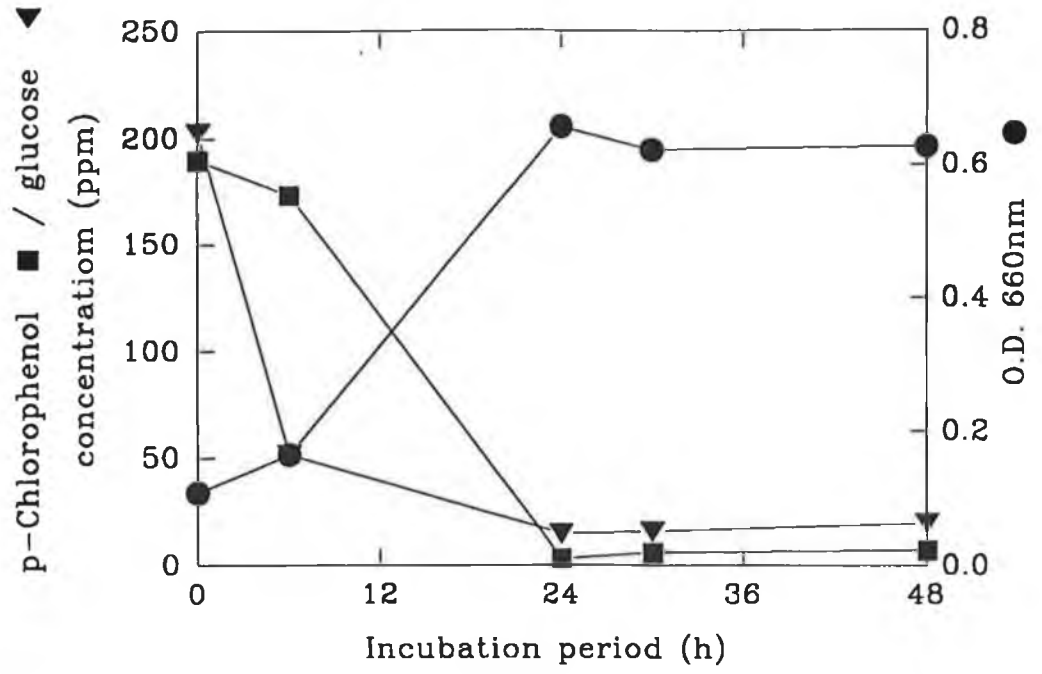


Figure 36 Growth of *P. putida* IGA 7.16 on p-chlorophenol with 200ppm glucose (μ=0.0769 h^{-1} ± 0.0029)

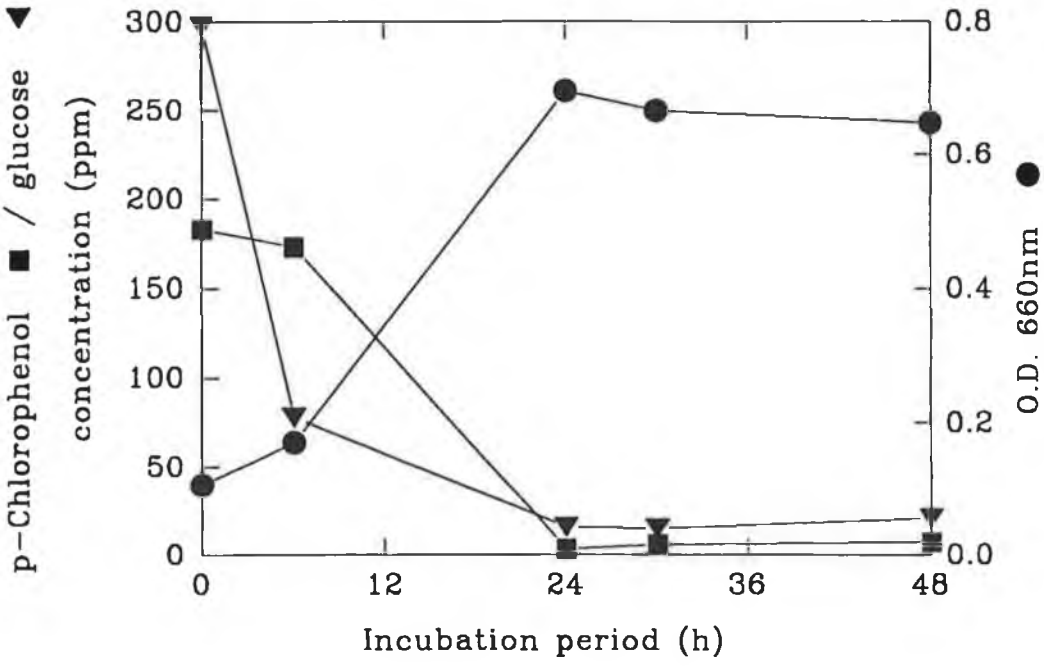


Figure 37. Growth of *P. putida* IGA 7.16 on p-chlorophenol with 300ppm glucose (μ=0.0783 h^{-1} ± 0.0012)

production were similar at all concentrations of glucose examined.

3:2:7 THE GROWTH OF *PSEUDOMONAS PUTIDA* IGA 7.16 ON TOLUENE

P. putida IGA 7.16 was the only organism tested that was capable of growth in the presence of toluene. Toluene was supplied in the vapour phase by suspending a small tube containing toluene within the flask. The volumes of toluene tested were 100, 250 and 500μl per 50ml of *Pseudomonas* minimal medium. The pH and OD_{660nm} of the cultures were monitored.

In all cases the lag phase lasted 24 hours (Figure 38). The pH remained at neutrality except in the culture grown in the presence of 500μl toluene where the pH decreased from 7.0 to 6.5. The biomass produced increased with increasing concentrations of toluene. However, in the presence of 100μl of toluene this increase was small. The growth rate of the organism in the presence of 250μl and 500μl toluene increased with increasing concentration of substrate.

3:3 CATECHOL DIOXYGENASE ACTIVITY

Catechol, the common intermediate in aromatic degradation can be metabolized via either the *ortho* or the *meta* cleavage pathways. To determine the preferred cleavage route for each aromatic substrate in each organism, the activities of catechol 1,2-dioxygenase (*ortho*-cleaving enzyme) and catechol 2,3-dioxygenase (*meta*-cleaving enzyme) were determined.

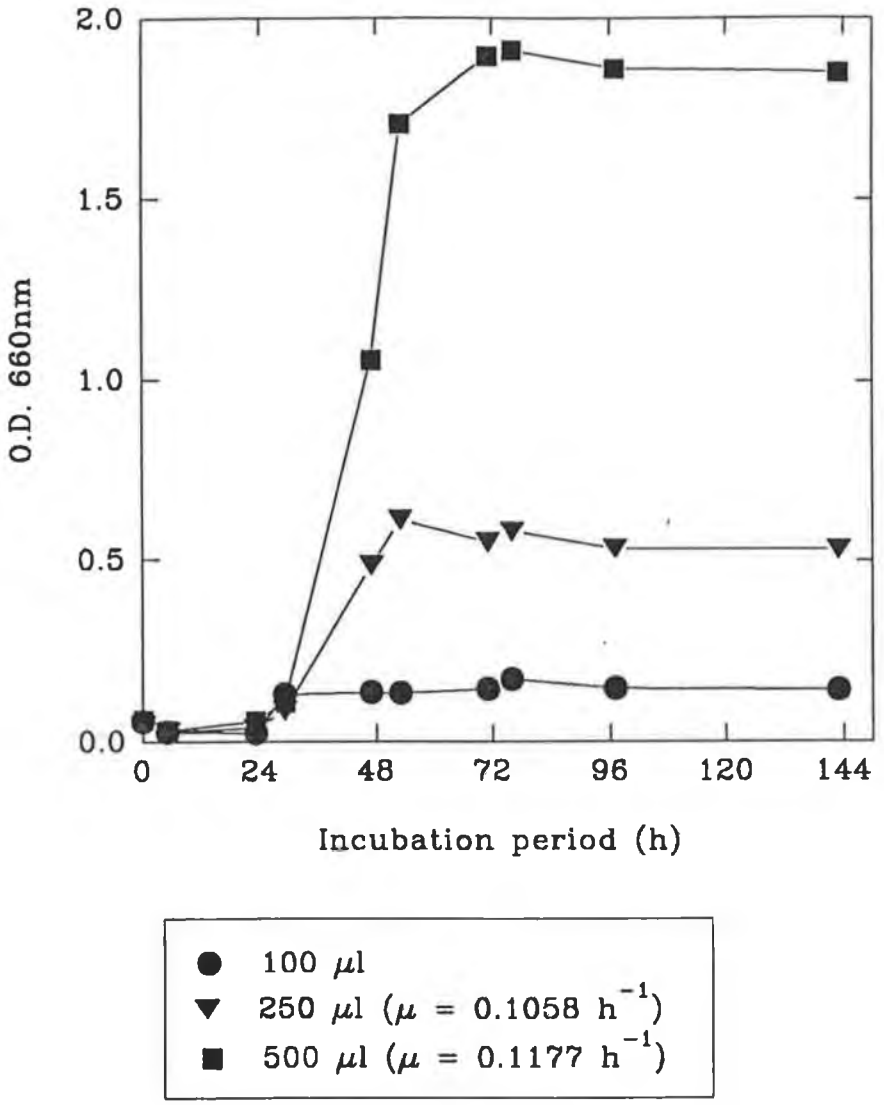


Figure 38. Growth of *P. putida* IGA 7.16 in the presence of toluene

The enzyme activities were determined after 48 hours growth on the aromatic compound (5mM). The compounds examined included benzoate, naphthalene, phenol, m-toluate, toluene and p-chlorophenol.

The activities of acclimated (maintained on aromatic compounds for 6 months) and non-acclimated (maintained on nutrient agar for a similar period) cultures were determined. Duplicate readings were obtained from triplicate flask cultures. The enzyme activity following growth on glucose was examined to establish constitutive levels.

The specific enzyme activities (Figures 39-42) clearly demonstrated the inducible nature of the catechol dioxygenase enzymes. *P. putida* IGA 7.24 demonstrated high constitutive levels of catechol 2,3-dioxygenase. The pattern of the results obtained was similar for both the acclimated and non-acclimated cultures. Growth on benzoate produced predominantly *ortho* activity i.e. catechol 1,2-dioxygenase. While growth on naphthalene, phenol and m-toluate induced predominantly *meta* activity i.e.catechol 2,3-dioxygenase. *P. putida* IGA 7.16 was the only organism capable of growth on toluene and p-chlorophenol. On both these substrates catechol 2, 3-dioxygenase was the predominant activity. Enzyme activity in cultures grown on p-chlorophenol was quite low compared with other substrates.

The strongest activity in *P. putida* IGA 7.16 was determined following growth on m-toluate and toluene. In *P. putida* IGA 0.92 and *P. fluorescens* IGA 13.42 the highest specific activity was also determined following growth on m-toluate. The specific activity of *P. putida* IGA 7.24 was similar

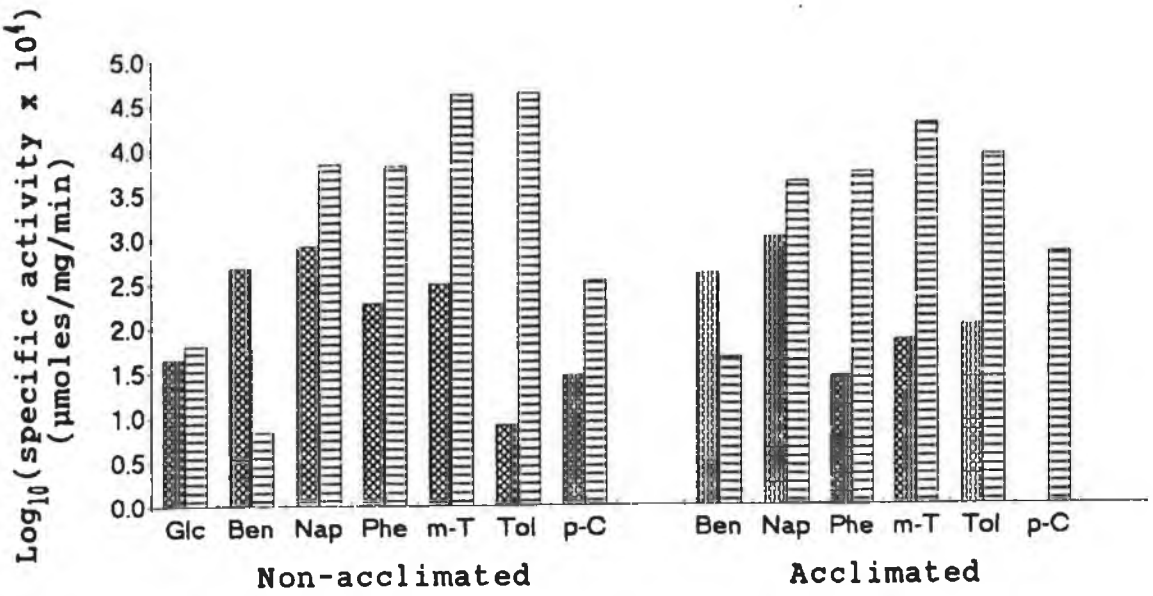


Figure 39. Specific enzyme activity of the catechol dioxygenase enzymes in *P. putida* IGA 7.16

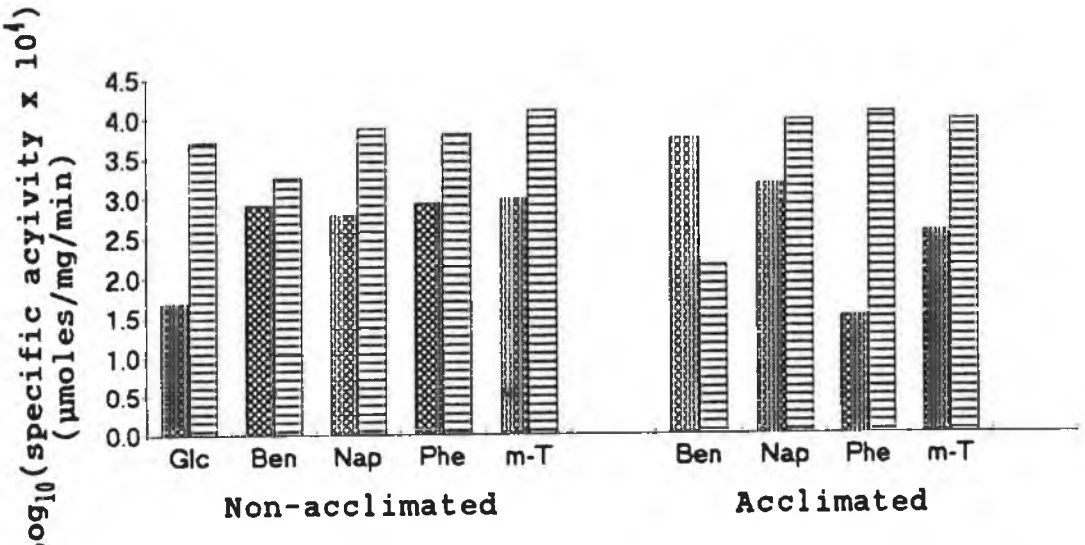


Figure 40. Specific enzyme activity of the catechol dioxygenase enzymes in *P. putida* IGA 7.24

Glc - glucose
Ben - benzoate
Nap - naphthalene
Phe - phenol
m-T - m-toluate
Tol - toluene
p-C - p-chlorophenol

▩ catechol 1,2-dioxygenase

▤ catechol 2,3-dioxygenase

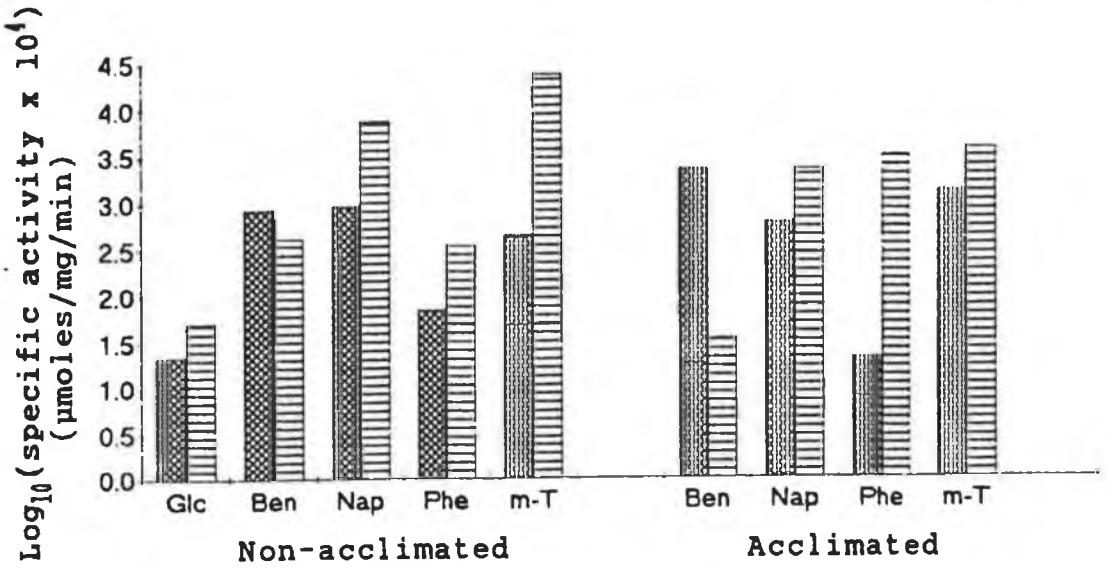


Figure 41. Specific enzyme activity of the catechol dioxygenase enzymes in *P. putida* IGA 0.92

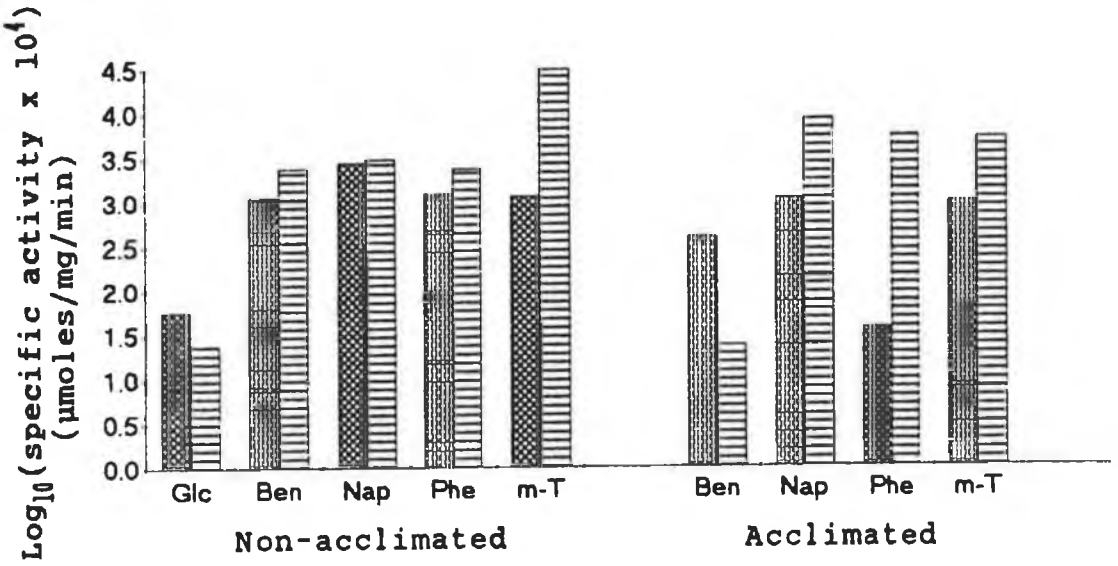


Figure 42. Specific enzyme activity of the catechol dioxygenase enzymes of *P. putida* IGA 13.42

Glc - glucose
Ben - benzoate
Nap - naphthalene
Phe - phenol
m-T - m-toluate

▩ catechol 1,2-dioxygenase

▤ catechol 2,3-dioxygenase

following growth on naphthalene, phenol and m-toluate.

3:4 PLASMID PROFILES OF THE *PSEUDOMONAS* SPECIES

3:4:1 PLASMID PROFILES OF THE *PSEUDOMONAS* SPECIES GROWN ON LURIA BROTH

The four *Pseudomonas* spp. expressed *meta* cleavage activity which is indicative of the presence of degradative plasmids. It was therefore of interest to examine the plasmid profiles of the organisms. Marker strains were used to establish the approximate size of the plasmids (Figure 43). *P. putida* NCIB 10432 (Lane 3) contained a transmissible TOL plasmid (117kb) and *P. putida* NCIB 12199 (Lane 4) contained the NAH plasmid pWW60-22 (85-86 kb). A cured strain *P. putida* C-104 (Lane 1) was used as a chromosomal marker (section 2:2:10).

Plasmid DNA was isolated (section 2:2:8) and was separated by agarose gel electrophoresis (section 2:2:9). The plasmid profiles of *P. putida* IGA 7.16 (Lane 5), IGA 7.24 (Lane 6), IGA 0.92 (Lane 7) and *P. fluorescens* IGA 13.42 (Lane 8) were examined (Figure 43).

Although the TOL and NAH plasmids were not resolved due to their large size the result indicated that the large plasmids isolated from the *Pseudomonas putida* spp. were in the range of 85 to 120 kb in size. A large plasmid band (85-120kb) was visualized in the two *P. putida* spp. IGA 7.16 (Lane 5) and IGA 0.92 (Lane 7). Two large plasmid bands (85-120kb and 35-80kb) and at least three smaller plasmid bands (27, 23 and 8kb) were visualized in

P. putida IGA 7.24 (Lane 6). No plasmids were visualized in *P. fluorescens* IGA 13.42 (Lane 8). No further investigation or genetic profiling of this strain was carried out.

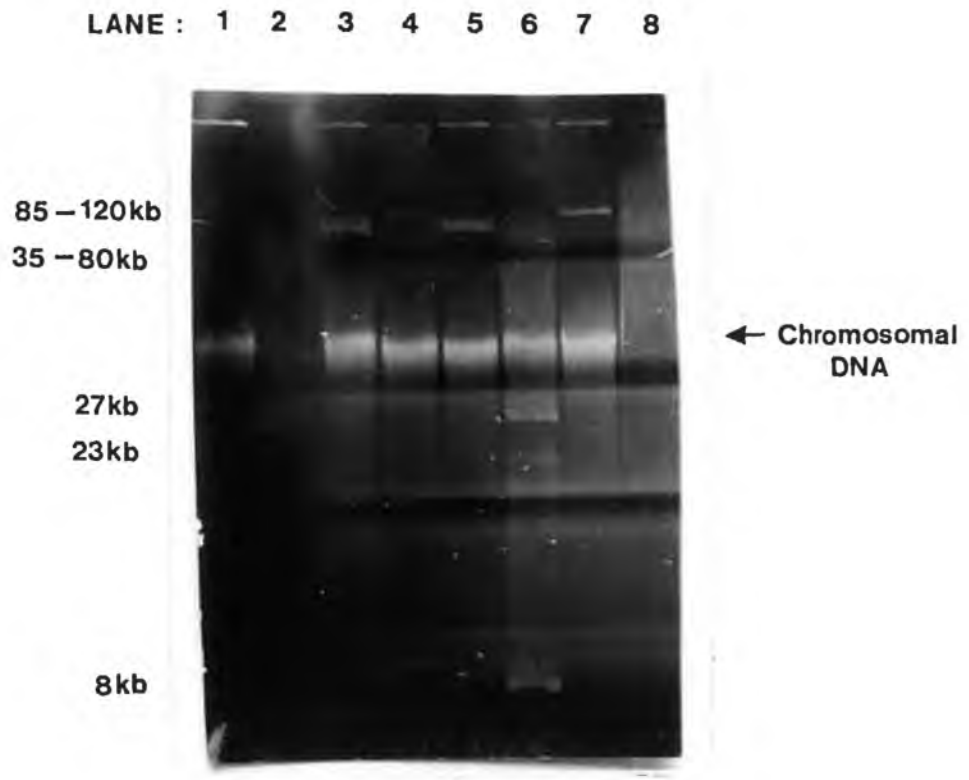


Figure 43. The plasmid profiles of the *Pseudomonas* species grown on luria broth

Lane 1 : *P. putida* C-104
Lane 3 : *P. putida* NCIB 10432 (TOL:117kb)
Lane 4 : *P. putida* NCIB 12199 (NAH:85-86kb)
Lane 5 : *P. putida* IGA 7.16
Lane 6 : *P. putida* IGA 7.24
Lane 7 : *P. putida* IGA 0.92
Lane 8 : *P. fluorescens* IGA 13.42

3:4:2 PLASMID PROFILES OF THE *PSEUDOMONAS PUTIDA* SPECIES FOLLOWING GROWTH ON AROMATIC COMPOUNDS

The *Pseudomonas putida* species had been shown to carry large plasmids following growth on luria broth. It was of interest to determine if growth on aromatic compounds resulted in any alteration of the plasmid profile. The plasmid profiles of the three organisms were examined following growth on 5mM concentrations of benzoate, phenol, naphthalene and m-toluate for 48 hours. The plasmid profile of *P. putida* IGA 7.16 was also determined following growth on toluene (500μl) and p-chlorophenol (200ppm). The resulting plasmid profiles are shown in Figures 44-46.

The large plasmid band, previously sized to approximately 85-120kb, visualized in luria broth cultures of *P. putida* IGA 7.16 (lane 1, Figure 44) and *P. putida* IGA 7.24 (lane 1, Figure 45) was stably maintained following growth on all the aromatic compounds tested (Lanes 2-13, Figure 44 and Lanes 2-9, Figure 45). The large plasmid visualized in *P. putida* IGA 0.92 (85-120kb) luria broth cultures (lane 1, Figure 46) was maintained only in acclimated benzoate (lane 3) and m-toluate (lane 9) cultures.

Two small plasmid bands not previously seen were visualized in *P. putida* IGA 7.16 following growth on naphthalene (lane 4, Figure 44). These plasmids bands were sized to 8.5kb and 22kb using a number of marker plasmids of known size.

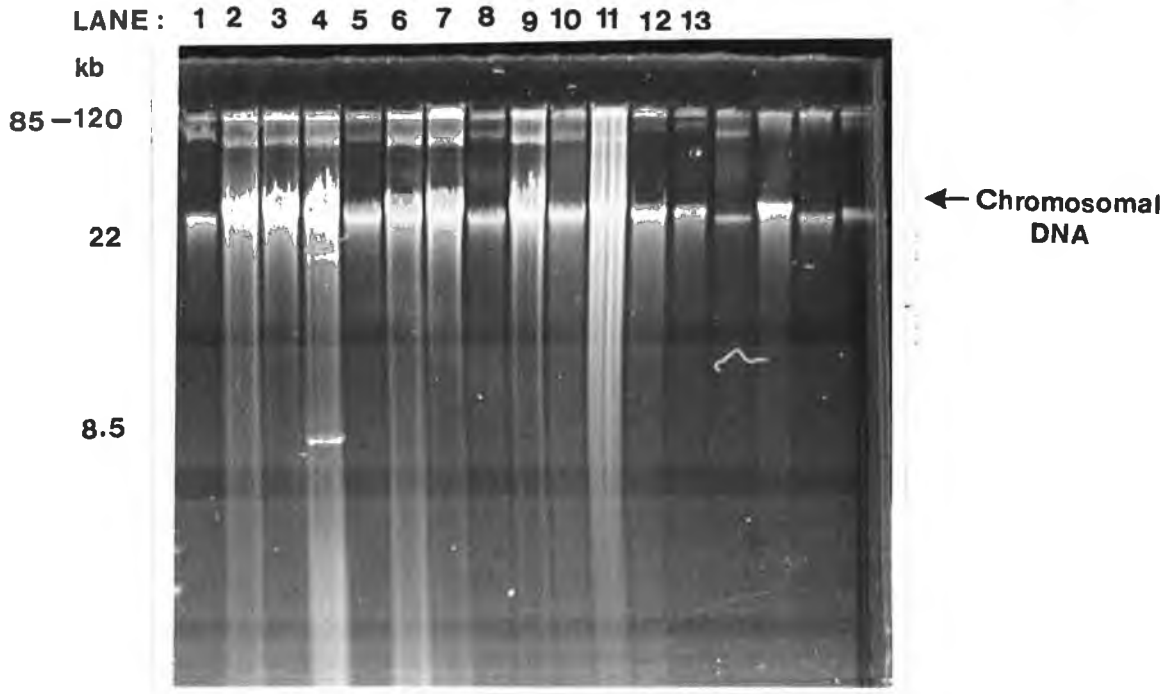


Figure 44. The plasmid profile of *P. putida* IGA 7.16 following growth on aromatic compounds

Lane 1 : L. broth
Lane 2 : Benzoate
Lane 3 : Benzoate (acclimated)
Lane 4 : Naphthalene
Lane 5 : Naphthalene (acclimated)
Lane 6 : Phenol
Lane 7 : Phenol (acclimated)
Lane 8 : m-Toluate
Lane 9 : m-Toluate (acclimated)
Lane 10 : Toluene
Lane 11 : Toluene (acclimated)
Lane 12 : p-Chlorophenol
Lane 13 : p-Chlorophenol (acclimated)

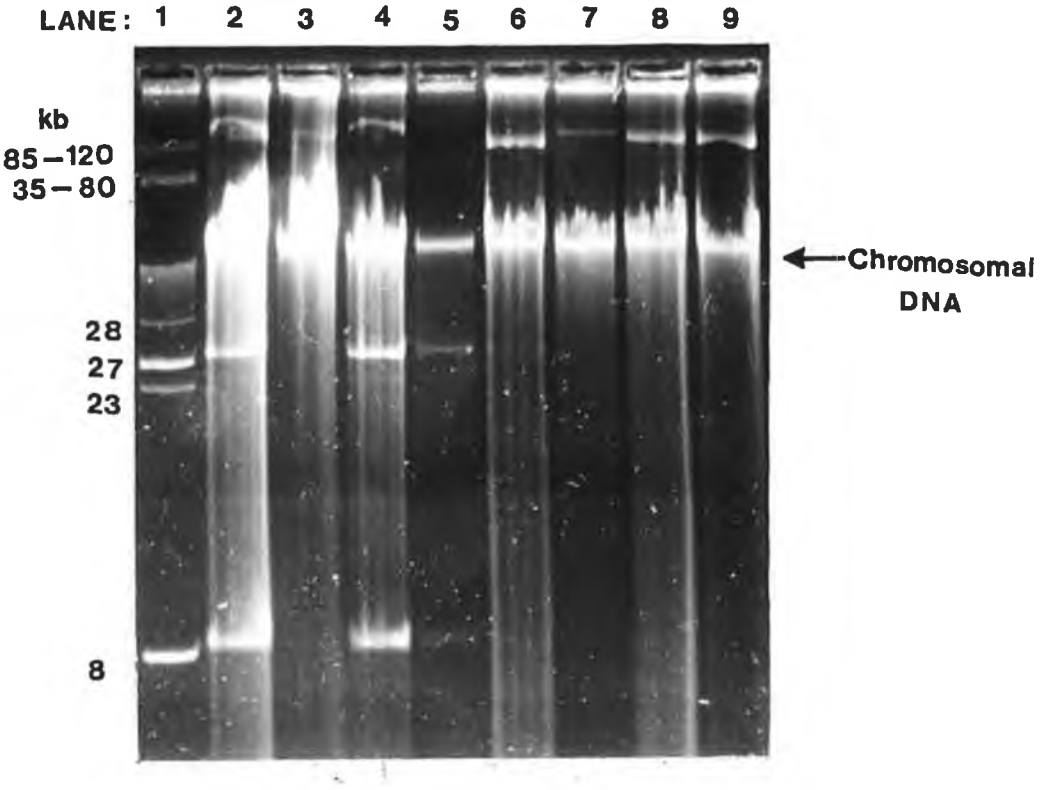


Figure 45. The plasmid profile of *P. putida* IGA 7.24 following growth on aromatic compounds

Lane 1 : L. broth
Lane 2 : Benzoate
Lane 3 : Benzoate (acclimated)
Lane 4 : Naphthalene
Lane 5 : Naphthalene (acclimated)
Lane 6 : Phenol
Lane 7 : Phenol (acclimated)
Lane 8 : m-Toluate
Lane 9 : m-Toluate (acclimated)

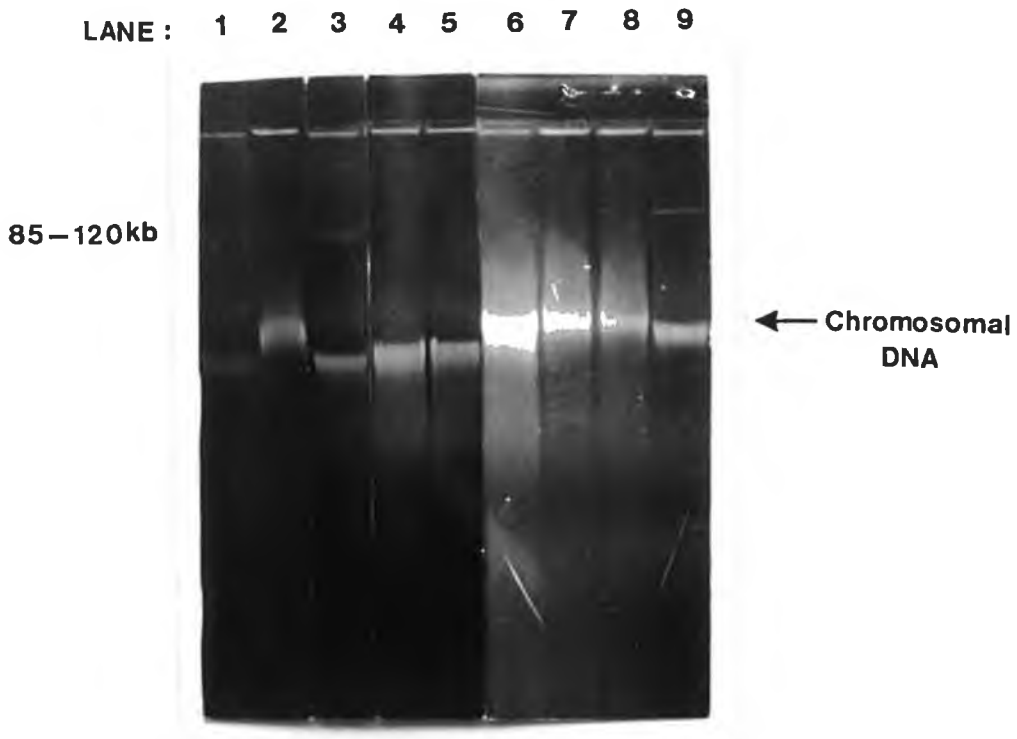


Figure 46. The plasmid profile of *P. putida* IGA 0.92 following growth on aromatic compounds

Lane 1 : L. broth
Lane 2 : Benzoate
Lane 3 : Benzoate (acclimated)
Lane 4 : Naphthalene
Lane 5 : Naphthalene (acclimated)
Lane 6 : Phenol
Lane 7 : Phenol (acclimated)
Lane 8 : m-Toluate
Lane 9 : m-Toluate (acclimated)

The DNA isolated from *P. putida* IGA 7.24 luria broth cultures was purified on a caesium chloride gradient. This resulted in less chromosomal debris and revealed an additional small plasmid of 28kb (lane 1, Figure 45) which was not previously visualized. The growth of *P. putida* IGA 7.24 on the aromatics resulted in the loss of some of its smaller plasmids (Figure 45). A greater degree of loss was evident in the cultures grown on aromatics for an extended period of time. The other plasmid band, which was not accurately sized but which would appear to be in the range of 35-80kb, was maintained only in the non-acclimated benzoate (lane 2) and naphthalene (lane 4) cultures. Three of the small plasmid bands visualized in the luria broth cultures, previously sized to 27kb, 23kb and 8kb, were also maintained in these cultures. The 27kb plasmid band was maintained in acclimated naphthalene cultures (lane 5). These small plasmid bands were lost from all the other aromatic cultures. The 28kb plasmid band was lost from all the cultures.

3:5 IDENTIFICATION OF PLASMIDS BY HYBRIDIZATION

The presence in *P. putida* IGA 7.16 and IGA 7.24 of a large plasmid of similar size to the TOL and NAH plasmids together with the ability of these organisms to grow on naphthalene and of *P. putida* IGA 7.16 to grow on toluene prompted the investigation of these plasmids using TOL and NAH DNA probes. A NAH probe comprising the genes for the degradation of naphthalene to salicylate (upper pathway operon) was prepared from pDTG113 (section 2:2:13). Two TOL probes were prepared - pDTG602 comprising the toluene dioxygenase gene and pGSH2836 comprising the toluene monooxygenase gene.

Due to the difficulty in transferring large DNA fragments to nitrocellulose the plasmid DNA from the two organisms was first cut with restriction endonucleases.

3:5:1 RESTRICTION PROFILE OF PLASMID DNA

Plasmid DNA was isolated from *P. putida* IGA 7.16 and IGA 7.24 and purified by caesium chloride gradient (section 2:2:12) and cut with a range of restriction endonucleases. The enzymes used included *Eco* RI, *Hind* 111, *Sal* 1 and *Xho* 1, *Sst* 1 was also used for plasmid DNA from IGA 7.16. Restricted DNA was visualized on a 1% agarose gel along with λ size markers in the range of 2kb to 33.5kb. NCIB 10432 (TOL) and NCIB 12199 (NAH) were also included as markers.

No restriction fragments were visualized for the plasmid DNA from *P. putida* IGA 7.16 with the 85-120kb plasmid remaining in tact (Lanes 5-9, Figure 47). This DNA failed to cut despite repeated attempts with freshly isolated and purified DNA samples and with new enzyme stocks.
The restriction profile of *P. putida* IGA 7.24 DNA is shown in Figure 47 (Lanes 11-14). The large plasmids from this organism were cut successfully with all the enzymes tested. A number of DNA restriction fragments ranging in size from less than 2kb to approximately 33kb were visualized following incubation with the various endonucleases.

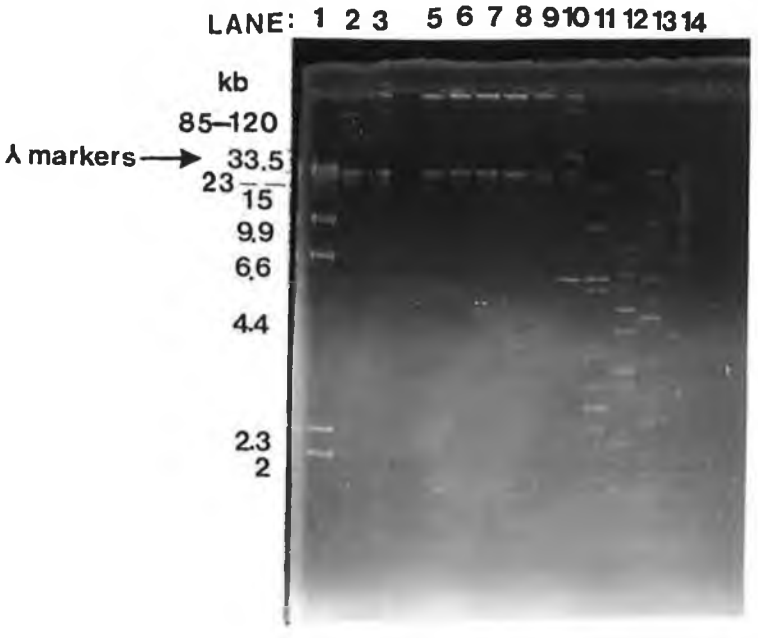


Figure 47. Restriction profiles of *P. putida* IGA 7.24 and IGA 7.16

Lane 1 : λ markers
Lane 2 : *P. putida* NCIB 10432 (TOL:117kb)
Lane 3 : *P. putida* NCIB 12199 (NAH:85-86kb)
Lane 5 : *P. putida* IGA 7.16 (uncut)
Lane 6 : " " *Eco* RI
Lane 7 : " " *Hind* III
Lane 8 : " " *Sal* I
Lane 9 : " " *Xho* I
Lane 10 : *P. putida* IGA 7.24 (uncut)
Lane 11 : " " *Eco* RI
Lane 12 : " " *Hind* III
Lane 13 : " " *Sal* I
Lane 14 : " " *Xho* I

3:5:2 IDENTIFICATION OF A NAH PLASMID IN *PSEUDOMONAS PUTIDA* IGA 7.16 AND IGA 7.24

Southern hybridization

Whole DNA from *P. putida* IGA 7.16 and IGA 7.24 and restricted DNA from *P. putida* IGA 7.24 was fractionated on a 0.7% agarose gel. The DNA was transferred to nitrocellulose filters so that Southern hybridization could be performed (section 2:2:15). The NAH probe was labelled with ^{32}P (section 2:2:14).

The resulting gel is presented in Figure 48a). Lanes 1 to 14 were as described previously in Figure 47. Probe DNA (pDTG113) was visualized in lanes 17 to 21. The uncut probe DNA (lane 17) comprised covalently closed circular, open circular and linear forms of the plasmid DNA. Restriction of the probe DNA with *Eco*R I (lane 18) resulted in the formation of two fragments, the insert and the vector. A number of restriction fragments were formed following restriction with *Hind* III (lane 19) and *Xho* I (lane 21). *Sal* I (lane 20) failed to cut the probe DNA.

The NAH probe hybridized to the large plasmid band of *P. putida* IGA 7.24 (lane 10, Figure 48b) and to a number of restriction fragments (lanes 11-14). The probe hybridized very strongly to the 15 kb *Eco* RI fragment of *P. putida* IGA 7.24 DNA (lane 11). This indicated the strong degree of homology between this fragment and the probe DNA. The NAH probe failed to hybridize to *P. putida* IGA 7.16 DNA. pDTG113 DNA was included as a positive control. The probe DNA hybridized to this DNA quite weakly. There was also no hybridization to

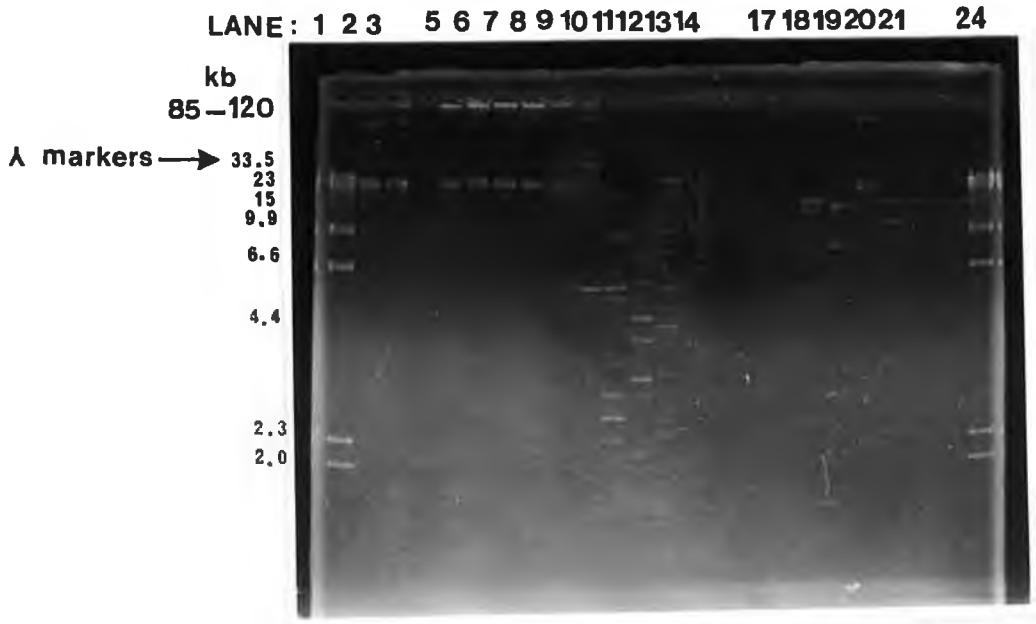


Figure 48. a) Restricted DNA from *P. putida* IGA 7.16 and IGA 7.24 for hybridization with the NAH probe

Lane 1;24	:	λ markers
Lane 2	:	*P. putida* NCIB 10432 (TOL:117kb)
Lane 3	:	*P. putida* NCIB 12199 (NAH:85-86kb)
Lanes 5-9	:	*P. putida* IGA 7.16
Lane 10	:	*P. putida* IGA 7.24 (uncut)
Lane 11	:	" " *Eco* RI
Lane 12	:	" " *Hind* III
Lane 13	:	" " *Sal* I
Lane 14	:	" " *Xho* I
Lane 17	:	pDTG113 (+ve control)
Lane 18	:	" *Eco* RI
Lane 19	:	" *Hind* III
Lane 20	:	" *Sal* I
Lane 21	:	" *Xho* I

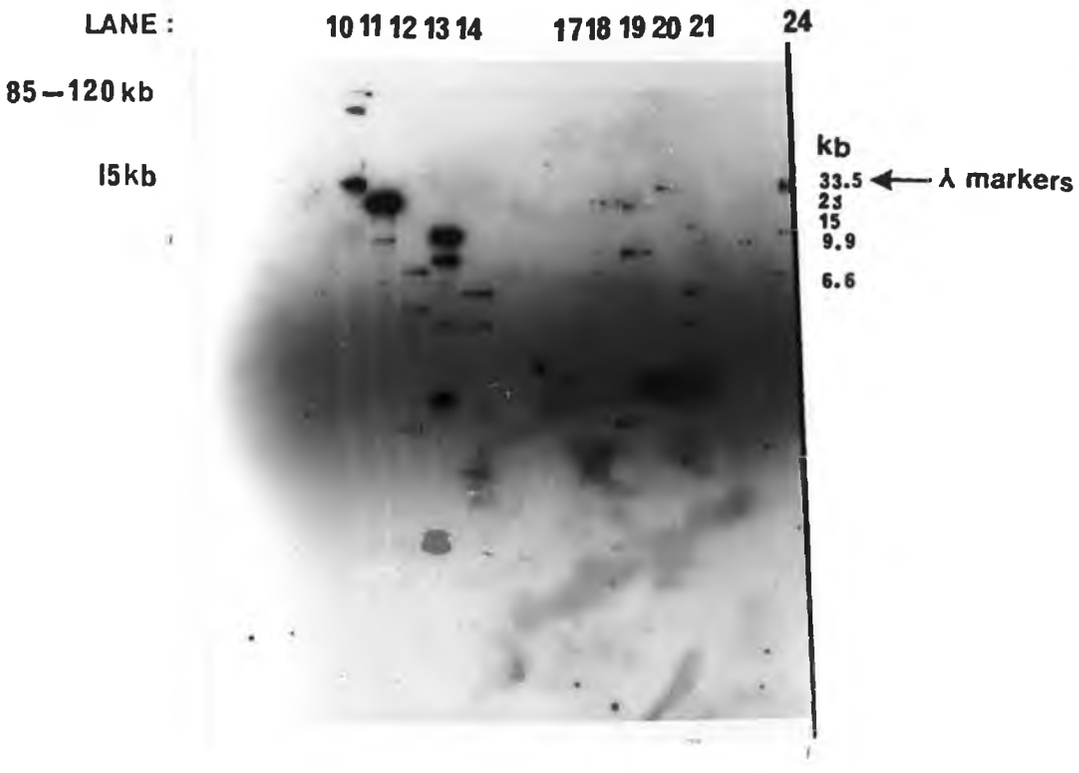


Figure 48. b) Autoradiograph of DNA from *P. putida* IGA 7.16 and IGA 7.24 hybridized with the NAH probe

Lanes : as in Figure 48 a)

the λ DNA in Lane 1 and only weak hybridization to λ DNA in Lane 24. The weak hybridization on both sides of the gel may have been due to inefficient transfer of DNA from such a large gel.

From these results it could be concluded that the large plasmid band (85-120kb) of *P. putida* IGA 7.24 was a NAH plasmid.

Dot blot hybridization

To confirm the presence of a NAH plasmid in *P. putida* IGA 7.24 and the absence of one in *P. putida* IGA 7.16 dot blots of total DNA were prepared (section 2:2:15). DNA from pDTG113 (positive control), *P. putida* NCIB 10432 (TOL), IGA 7.24 and IGA 7.16 was blotted onto nitrocellulose, DNA from *P. putida* IGA 0.92 was also added and a digoxigenin-labelled NAH probe was used.

The NAH probe hybridized strongly to *P. putida* IGA 7.24 DNA (No. 3, Figure 49) and to the positive control (No. 1) but failed to hybridize to the other DNA samples, proving the presence of the NAH plasmid in *P. putida* IGA 7.24 and the absence of a NAH plasmid in *P. putida* IGA 7.16 and IGA 0.92.

3:5:3 IDENTIFICATION OF A TOL PLASMID IN *PSEUDOMONAS PUTIDA* IGA 7.16

Southern hybridization

The DNA from *P. putida* IGA 7.16 did not cut successfully so whole DNA from this organism was run on an agarose gel along with *P. putida* IGA 7.24 and positive controls (pDTG602 and pGSH2836) and

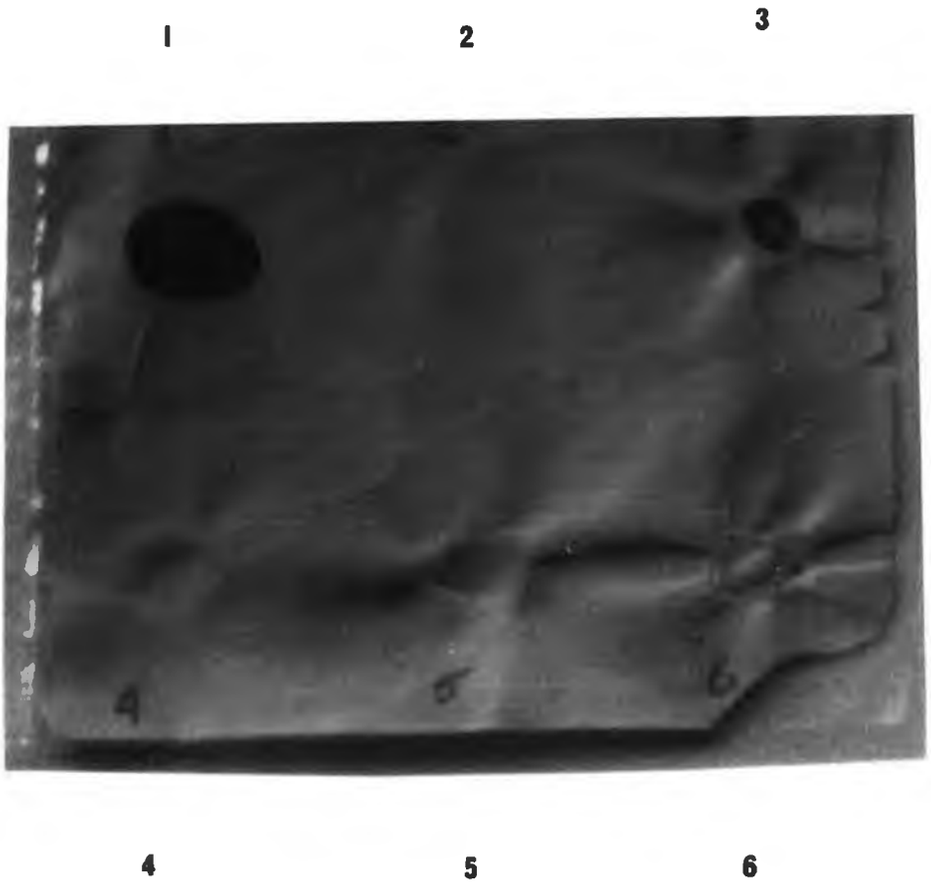


Figure 49. Dot blot hybridization to the NAH probe

1 : pDTG113 (positive control)
2 : *P. putida* NCIB 10432
3 : *P. putida* IGA 7.24
4 : *P. putida* IGA 7.16
5 : *P. putida* IGA 7.16 on toluene
6 : *P. putida* IGA 0.92

nitrocellulose filters were prepared. The two TOL probes were labelled with ^{32}P. The DNA from both *P. putida* IGA 7.16 and IGA 7.24 failed to hybridize to either probe. Both probes hybridized to their respective positive controls.

Dot blot hybridization

The large size of the plasmid DNA may have hindered its transfer to nitrocellulose, therefore these results were confirmed with dot blot hybridization using digoxigenin-labelled TOL probes. Positive control DNA (pDTG602 or pGSH2836), DNA from *P. putida* NCIB 10432, IGA 7.24, IGA 7.16 and IGA 0.92 was blotted onto nitrocellulose. Hybridization of the DNA to both the monooxygenase (pGSH2836) and dioxygenase (pDTG602) probes were examined.

The toluene monooxygenase probe hybridized strongly to positive control DNA (No. 1, Figure 50) and to *P. putida* IGA 7.16 DNA (No. 4). This probe did not hybridize to the other DNA samples.

The toluene dioxygenase probe failed to hybridize to the positive control DNA and hybridized only weakly to *P. putida* IGA 7.16 DNA (Figure 51). This probe did not hybridize to other DNA samples.

The toluene monooxygenase gene is encoded on the TOL plasmid. Therefore, its hybridization to DNA from *P. putida* IGA 7.16 indicated the presence of a TOL plasmid in this organism.

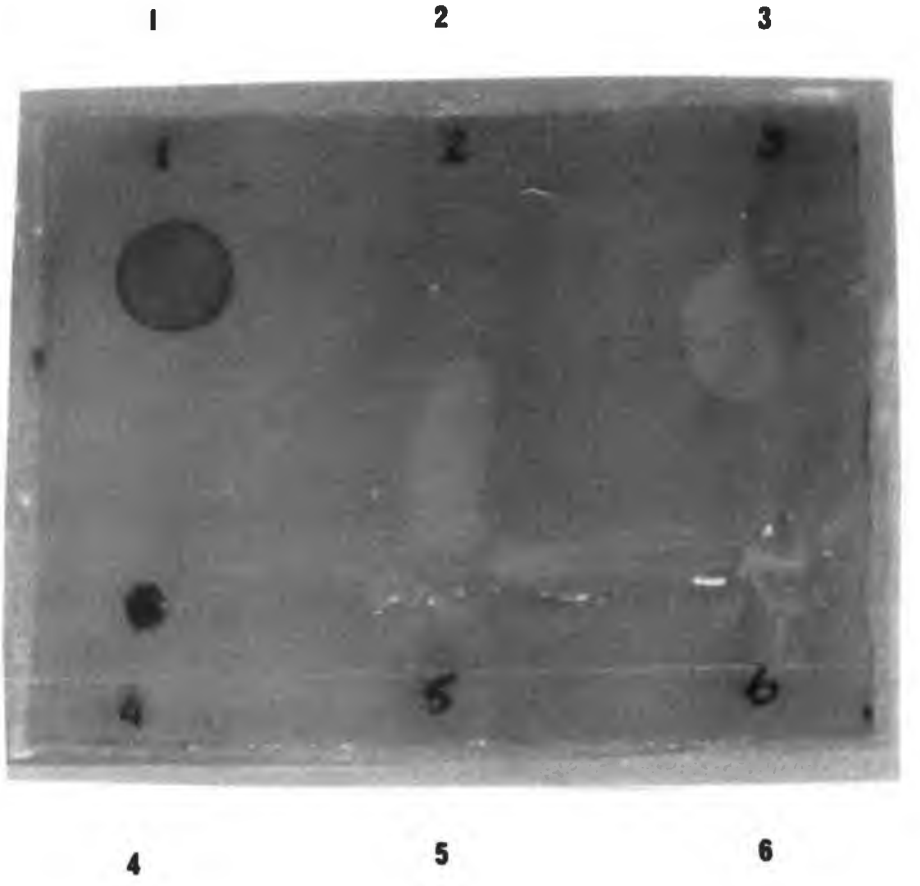


Figure 50. Dot blot hybridization to the toluene monooxygenase probe (pGSH2836)

1 : pGSH2836 (positive control)
2 : *P. putida* NCIB 10432 (TOL)
3 : *P. putida* IGA 7.24
4 : *P. putida* IGA 7.16
5 : *P. putida* IGA 7.16 on toluene
6 : *P. putida* IGA 0.92

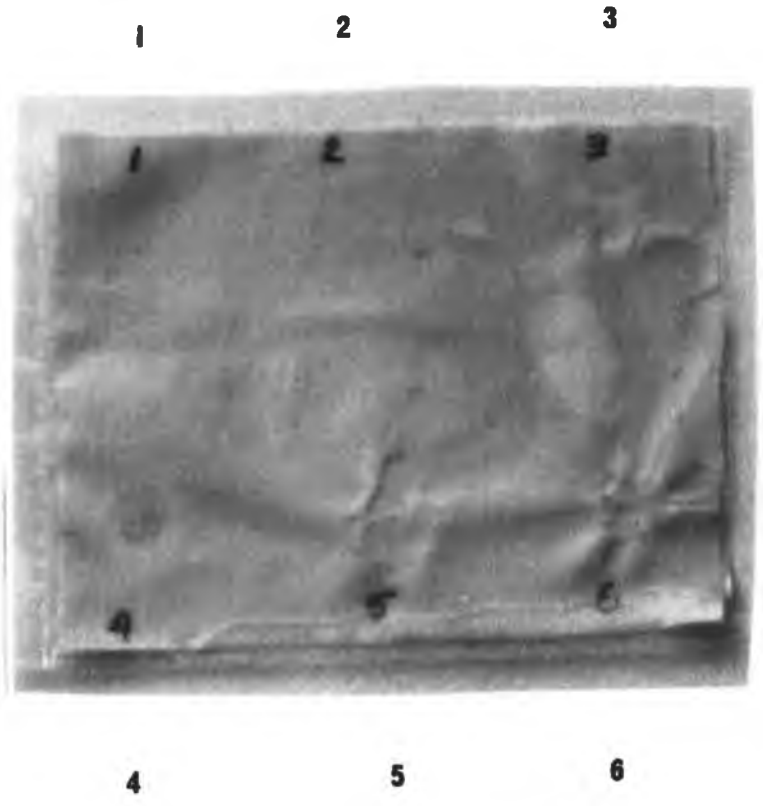


Figure 51. Dot blot hybridization to the toluene dioxygenase probe (pDTG113)

1 : pDTG602 (positive control)
2 : *P. putida* NCIB 10432 (TOL)
3 : *P. putida* IGA 7.24
4 : *P. putida* IGA 7.16
5 : *P. putida* IGA 7.16 on toluene
6 : *P. putida* IGA 0.92

3:5:4 HYBRIDIZATION OF DNA FROM *PSEUDOMONAS PUTIDA* IGA 7.24 FOLLOWING GROWTH ON AROMATIC SUBSTRATES

The stable maintenance of the large plasmid in *P. putida* IGA 7.24 following growth on various aromatic compounds was examined using Southern hybridization. DNA was isolated from acclimated and non acclimated cultures of *P. putida* IGA 7.24 grown on 5mM concentrations of benzoate, phenol, naphthalene and m-toluate. From this nitrocellulose filters were prepared. Hybridization of the large plasmid band with the ^{32}P-labelled NAH probe was investigated. λ markers (2-33.5kb) and positive control DNA (pDTG113) were also included on the gel.

Lanes 8-16 of the gel (Figure 52a) are as described previously for Figure 45. The NAH probe hybridized to the large plasmid band from luria broth cultures (lane 7, Figure 52b) and to the 15kb *Eco*R I fragment from *P. putida* IGA 7.24 DNA (lane 8) confirming the earlier result. The probe also hybridized to the positive control DNA (lane 3).

The NAH probe hybridized to the large plasmid visualized in non-acclimated benzoate (lane 9) and phenol (lane 13) cultures and also in both acclimated and non-acclimated naphthalene cultures (lanes 11 and 12). This indicated the stable maintenance of a NAH plasmid in these cultures. However, the plasmid visualized in acclimated benzoate (lane 10) and phenol (lane 14) cultures and in both m-toluate cultures (lanes 15 and 16) failed to hybridize to the NAH probe. It appeared therefore that the plasmid in these cultures had lost the genes of the naphthalene upper pathway which the probe encoded.

The NAH probe again failed to hybridize to DNA from *P. putida* IGA 7.16 (lane 18) confirming the earlier result.

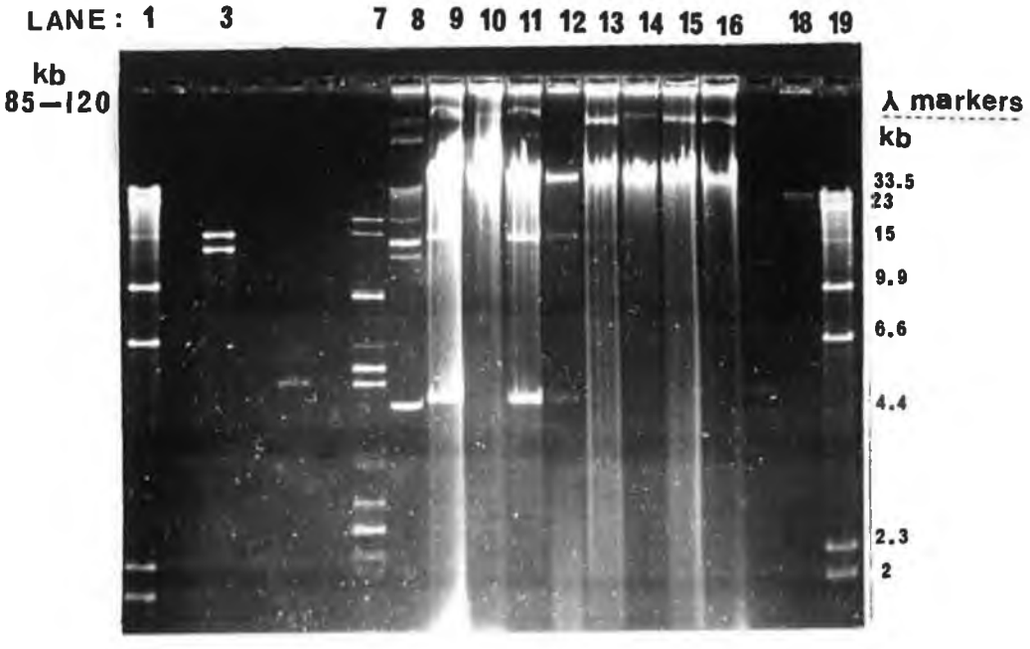


Figure 52. a) Plasmid profile of *P. putida* IGA 7.24 DNA from cultures following growth on aromatic substrates

Lane 1, 19	:	λ markers
Lane 3	:	pDTG113 (*Eco* RI)
Lane 7	:	*P. putida* IGA 7.24 (*Eco* RI)
Lane 8	:	" " L. broth
Lane 9	:	" " Benzoate
Lane 10	:	" " " (accl'd)
Lane 11	:	" " Naphthalene
Lane 12	:	" " " (accl'd)
Lane 13	:	" " Phenol
Lane 14	:	" " " (accl'd)
Lane 15	:	" " m-Toluate
Lane 16	:	" " " (accl'd)
Lane 18	:	*P. putida* IGA 7.16

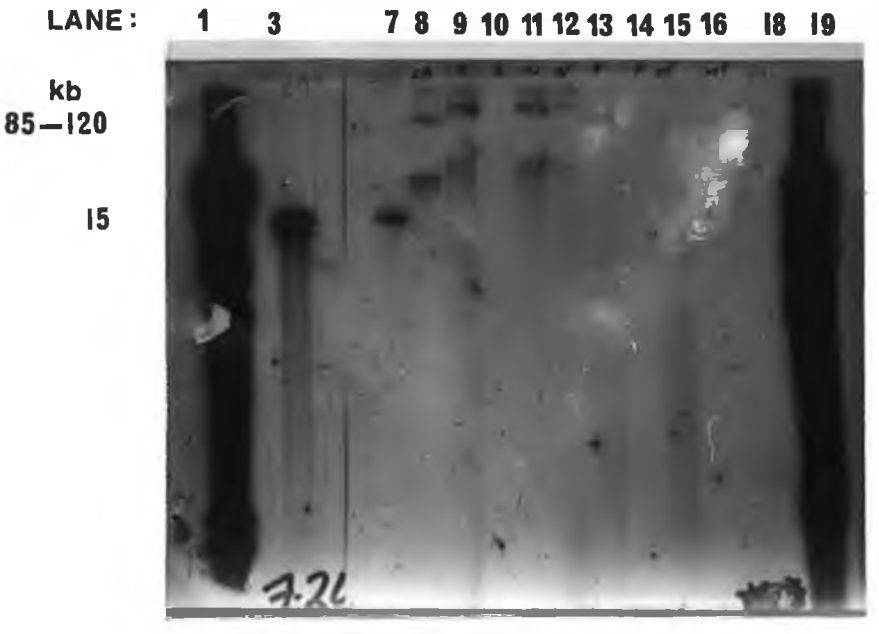


Figure 52 b) Hybridization of DNA from *P. putida* IGA 7.24 aromatic cultures with the NAH probe

Lanes : as in Figure 52 a)

3:6 MONITORING *PSEUDOMONAS PUTIDA* IGA 7.24 IN ACTIVATED SLUDGE

P. putida IGA 7.24 was capable of growth on naphthalene and retained its NAH plasmid following prolonged exposure to this substrate. It was of interest to monitor this organism in a mixed microbial system and therefore it was decided to use the organism for the bioaugmentation of an activated sludge system.

A control and a test system were set up as described in section 2:2:18. The two systems were identical in all respects except for the addition of *P. putida* IGA 7.24 to the test system. Both systems were fed with a synthetic effluent comprising *Pseudomonas* minimal medium incorporating naphthalene (20mM). The two systems were monitored daily for 30 days.

3:6:1 THE MICROBIOLOGY OF THE SYSTEM

Total bacterial numbers and the naphthalene degrading bacteria in the mixed liquor

The total number of viable bacteria and the naphthalene degrading bacteria were determined in the mixed liquor of both systems prior to feeding. The numbers in the test system were also monitored after feeding and the addition of *P. putida* IGA 7.24.

The initial total population in both systems was approximately 10^8 cells/ml (Figures 53 and 54). The naphthalene degrading population comprised approximately 10^6 cells/ml. This rose to 10^7

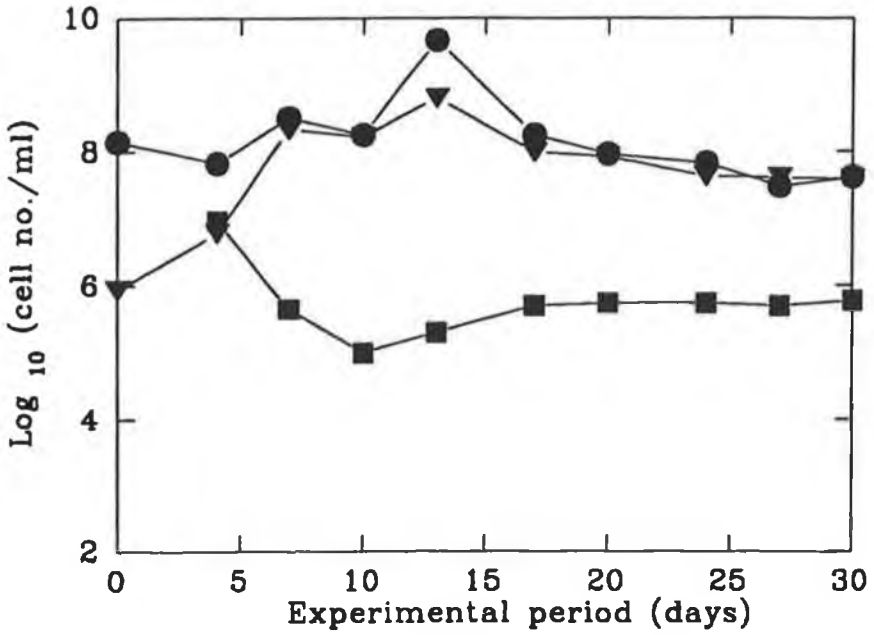


Figure 53. Total & naphthalene degrading cells in the control activated sludge system

● Total cell count in the mixed liqour (plate count)
▼ Naphthalene cell count in the mixed liquor (plate count)
■ Cell count in the supernatant (haemocytometer)

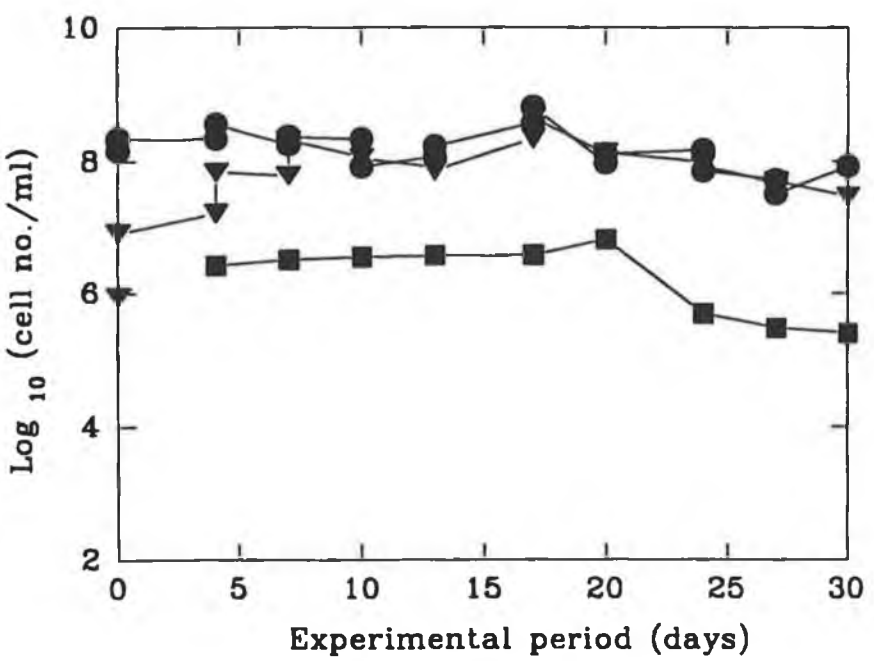


Figure 54. Total & naphthalene degrading cells in the test activated sludge system

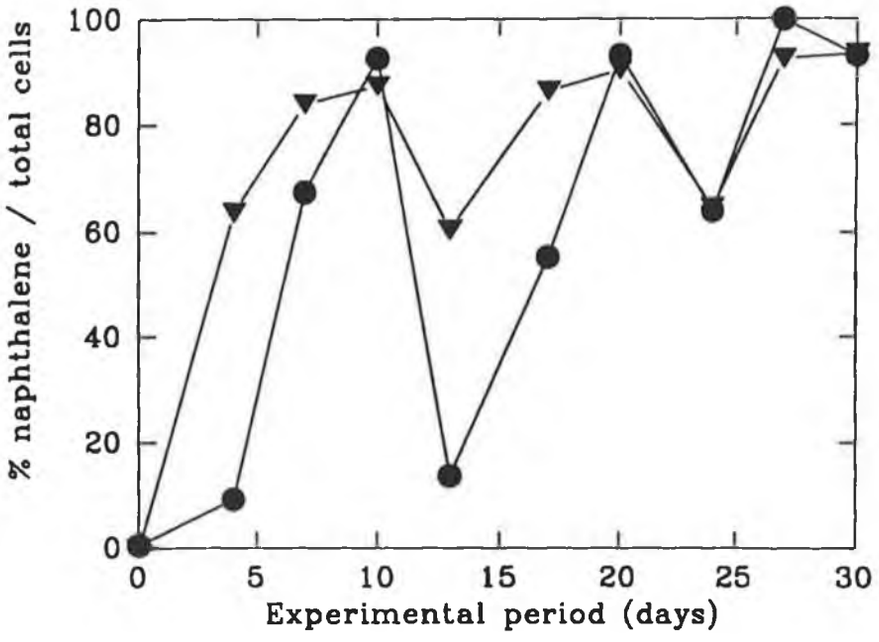


Figure 55. Percentage of naphthalene degrading to total cells in the control activated sludge system

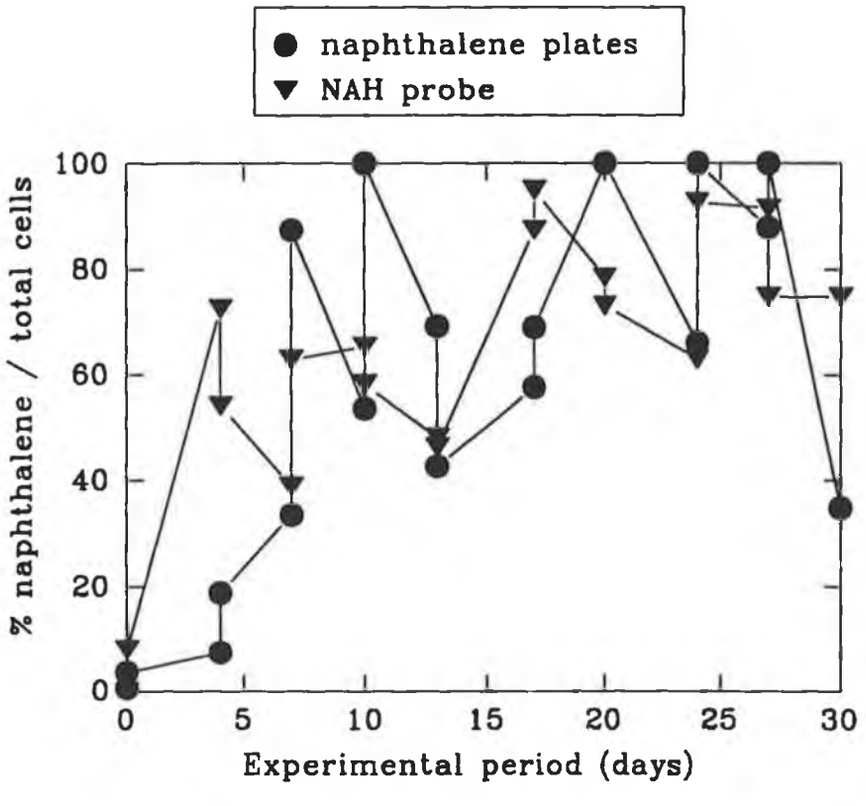


Figure 56. Percentage of naphthalene degrading to total cell in the test activated sludge system

cells/ml in the test system following addition of *P. putida* IGA 7.24. During the first week of operation the naphthalene population in the mixed liquor increased rapidly in both systems and then remained approximately constant at 10^8 cells/ml. The percentage of naphthalene degrading organisms in the total population was determined from the selective counts on naphthalene vapour plates. It was also determined using colony hybridization of the non-radioactively labelled NAH probe (section 2:2:17) to PCA colonies. The results of these methods are compared in Figures 55 and 56. The naphthalene population increased rapidly over the first week and reached 90 to 100% of the total population. Colony hybridization was more sensitive than the naphthalene vapour plates for the detection of low numbers of naphthalene degrading cells. Colony hybridization indicated a greater initial increase in the naphthalene degrading population of the test system during the first four days when compared with the control system. The percentage of naphthalene degrading cells in the total population of the test system was very erratic. The addition of *P. putida* IGA 7.24 may have disturbed the system.

Free-swimming bacteria in the systems

The sludge in both systems was allowed to settle prior to feeding and a sample of supernatant was removed. A total count of free-swimming bacteria was determined using a haemocytometer. The number of free-swimming bacteria in the control system was initially high at around 10^7 cells/ml. This number decreased during the first ten days to approximately 10^5 cells/ml, rising only slightly thereafter. In the test system the number of free-

swimming bacteria was initially slightly lower than the control system at approximately 5 x 10^6 cells/ml. However the numbers remained at this value throughout the experiment, decreasing in the final week only when the maintenance dose of *P. putida* IGA 7.24 was reduced from 1% to 0.1%. This suggested that a proportion of the free-swimming bacteria comprised the added organism, *P. putida* IGA 7.24.

Other microbial populations in the systems

Filamentous growth and protozoan populations were monitored by microscopic examination of the mixed liquor in both the test and control systems (Table 16 and 17). Very little filamentous growth was observed in either system. The protozoan population increased in both systems over the first week of the experiment and then remained approximately constant. However, in the control system there was a decrease in protozoan numbers on day 13. This corresponded to a sharp increase in the total bacterial numbers in that system. The increase in the bacterial population seems to have been due to a proliferation of organisms other than the naphthalene degraders as indicated by a sharp decline in the percentage of naphthalene degrading bacteria in the total population (Figure 53). The protozoan numbers increased again on day 14 and the bacterial numbers decreased over the following three days until they reached the level recorded prior to day 13. It was also noted that the numbers of attached and free-swimming protozoa were approximately equal.

Table 15. Filamentous growth and protozoan populations in the control activated sludge system

Day	Filamentous growth	Attached protozoa	Free-swimming protozoa
3	-	++	-
4	-	++	++
5	+	+	++
6	+	+	+++
7	+	-	+++
10	+	+++	+++
11	+	+++	+
12	+	+++	+
13	+	++	+
14	+	++++	+++
17	+	++++	+
18	+	+++	++
20	+	+++	+++
24	+	+++	+++
25	+	++++	++++
27	+	++++	++++
28	+	++++	++++

- none
+ very few
++ few
+++ many
++++ very many

Table 16. Filamentous growth and protozoan populations in the test activated sludge system

Day	Filamentous growth	Attached protozoa	Free-swimming protozoa
3	-	++	-
4	+	++	-
5	+	++	-
6	+	++	+++
7	+	++	+++
10	+	+++	+++
11	+	+++	+++
12	+	+++	+++
13	-	+++	++
14	+	++++	+++
17	-	+++	+++
18	+	++++	++++
20	-	+++	+++
24	-	+++	+++
25	+	+++	+++
27	+	+++	+++
28	+	+++	+++

- none
+ very few
++ few
+++ many
++++ very many

Identification of naphthalene degrading bacteria isolated from the activated sludge systems

The population in both the control and test systems quickly adapted to naphthalene, with a proliferation of naphthalene degrading bacteria in both systems.

It was therefore decided to identify the naphthalene degrading bacteria isolated from both systems. Colonies showing different colony characteristics were removed from naphthalene vapour plates carrying bacteria isolated on day 10.

All the bacteria identified were Gram negative, non-spore forming, oxidase positive rods exhibiting oxidative metabolism of glucose and were found to belong to the genus *Pseudomonas*. The majority of the isolates were identified as *Pseudomonas putida* spp. However, the species identified could be distinguished from *P. putida* IGA 7.24. A non-motile *P. putida* sp. with similar biochemical properties to those of *P. putida* IGA 7.24 was isolated from both systems. *P. putida* IGA 7.24 was isolated only from the test system.

3:6:2 OPERATING PARAMETERS OF THE SYSTEMS

Chemical oxygen demand

The Chemical Oxygen Demand (COD) of both systems was determined in order to monitor their performance (Figures 57). When the two systems were initially set up the COD was determined at a value of 300-350 mg/l. The COD of each system was

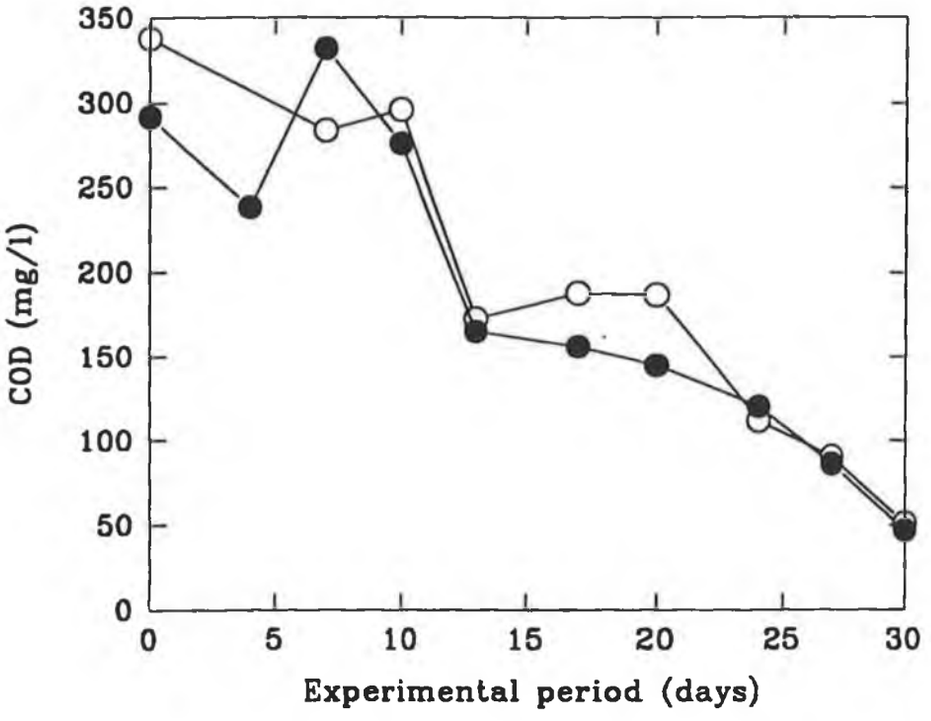


Figure 57. Chemical oxygen demand of the test and control activated sludge systems

subsequently determined before fresh feed was added to the system. The COD remained approximately constant for the first seven to ten days of operation. Decreasing COD values were then observed as the population adapted to the naphthalene. The performance of both systems in terms of COD removal was very similar.

Temperature, pH, dissolved oxygen and solids

The temperature, pH and dissolved oxygen of the two systems were monitored (Figures 58 and 59). The systems were maintained at room temperature. The mean temperature in the control system was 20.6°C and in the test system was 19°C. The pH of both systems was initially at neutrality. The pH decreased slightly as the naphthalene was used up but was restored on addition of fresh medium. The pH range in the control system was 7.33 - 5.68 and in the test system was 7.25 - 5.89. The dissolved oxygen of the two systems was initially quite erratic and there was a sharp drop in dissolved oxygen in the control system on day 5 due to technical difficulties. The mean dissolved oxygen concentration in the control system was 7.6 mg/l and that in the test system was 8.0 mg/l.

The mixed liquor suspended solids (Figure 60) remained approximately constant with a mean concentration of 3.8 g/l in both systems. The sludge volume index (Figure 61) was initially poor with a value of 220 ml/g in both systems. The SVI decreased to below 100 ml/g in both systems. This decrease occured more rapidly in the test system, showing considerable improvement after two weeks of operation compared with three weeks in the control system.

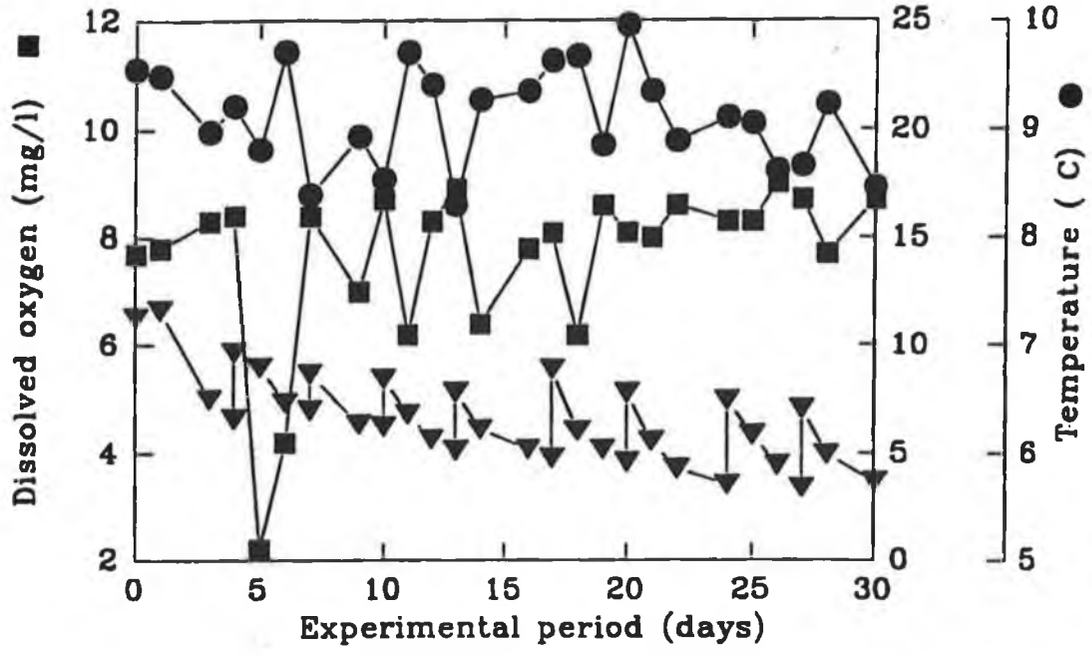


Figure 58. Temperature, pH & dissolved oxygen in the control activated sludge system

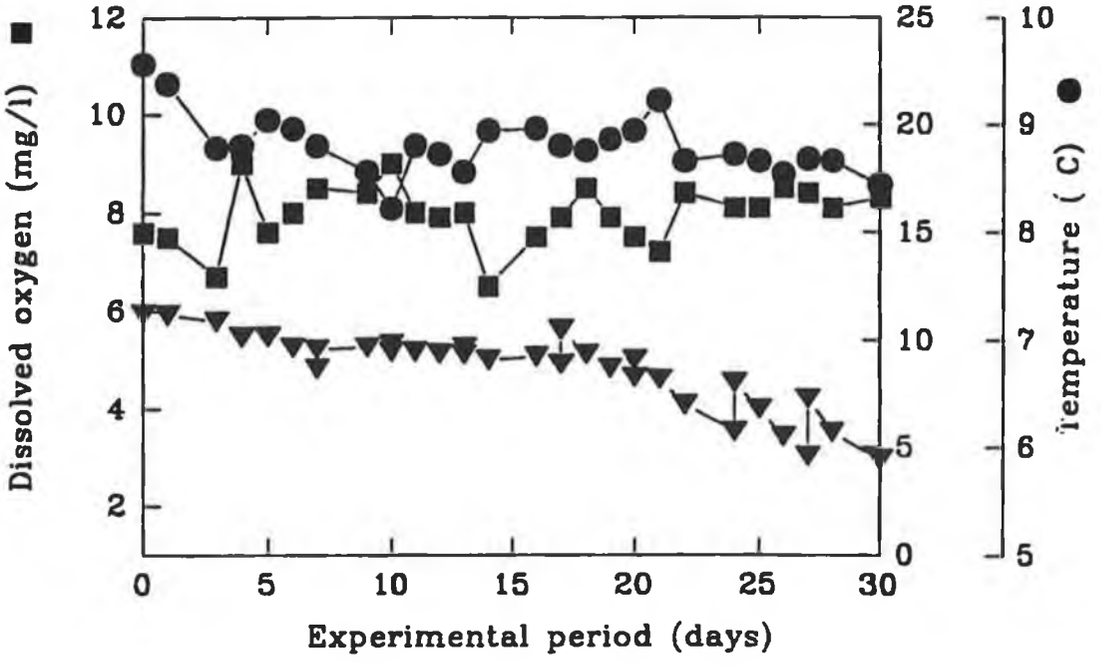


Figure 59. Temperature, pH & dissolved oxygen in the test activated sludge system

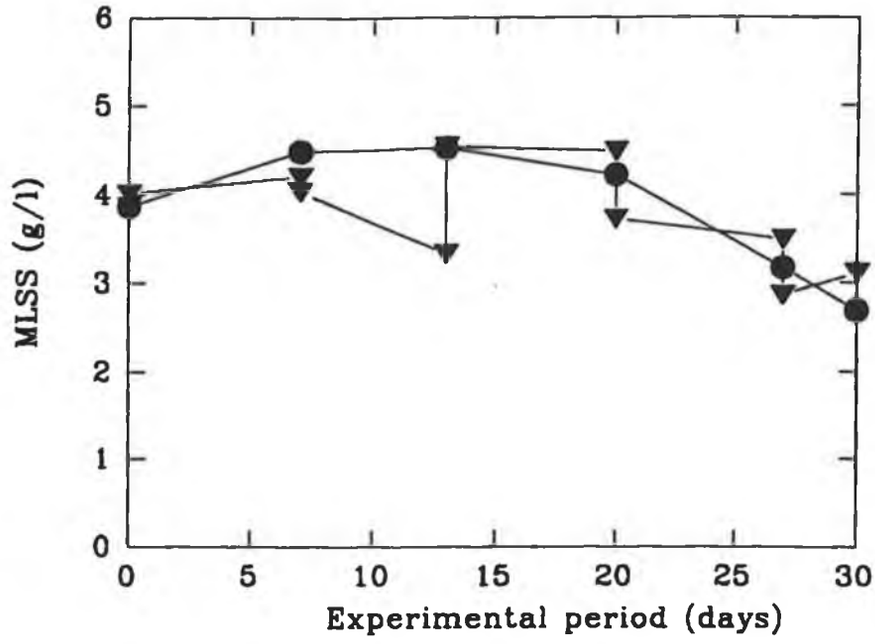


Figure 60. Mixed liquor suspended solids of the control and test activated sludge systems

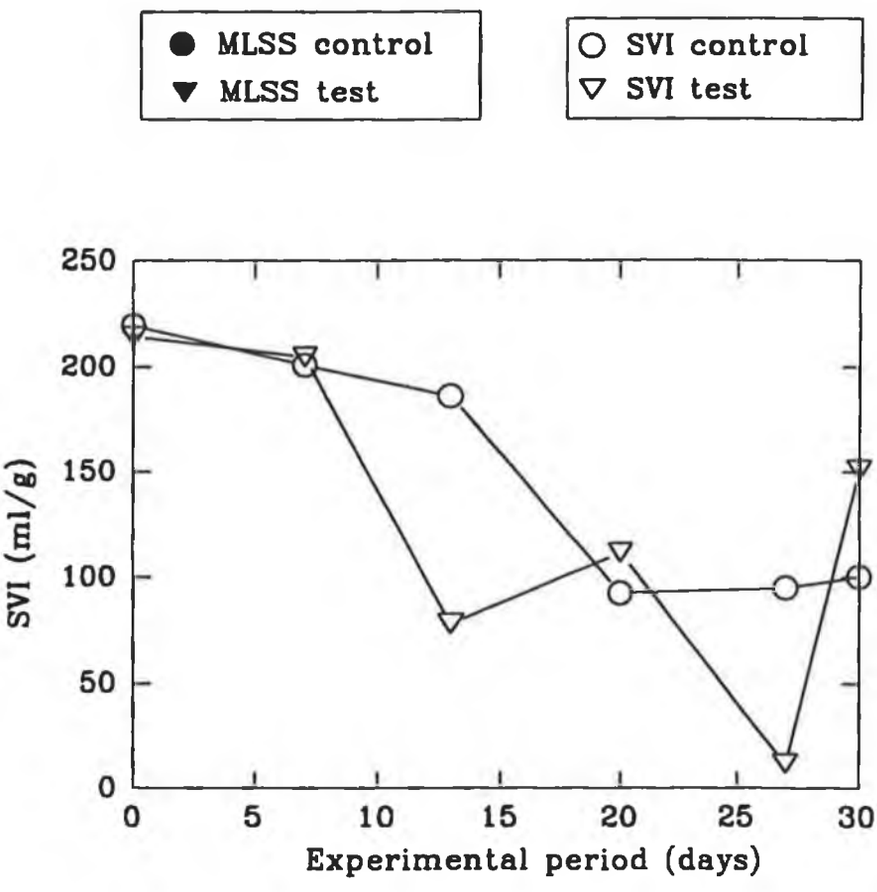


Figure 61. Sludge volume index of the control and test activated sludge systems

4 DISCUSSION

The commercial bioaugmentation product, Biolyte CX 80, produced by InterBio Laboratories Ltd., Ireland, was designed to provide a selection of organisms able to degrade a wide range of complex aromatic and aliphatic compounds in aerobic biological wastewater treatment systems. The formulation contained a range of thirteen microorganisms together with surfactants carried on a cereal base. The bacterial strains in the product included members of the genera *Bacillus*, *Pseudomonas*, *Aeromonas* and *Rhodococcus*. These bacteria had been selected and adapted to give optimum performance in degrading a wide variety of organic chemicals including benzenes, phenols and naphthalenes. They were grown in pure culture, harvested and preserved by freeze-drying before being blended in the final formulation (manufacturer's literature).

Four *Pseudomonas* spp., three *P. putida* spp. (IGA 7.16, IGA 7.24 and IGA 0.92) and one *P. fluorescens* sp. (IGA 13.42), from CX 80 were selected for investigation. The ultimate goal was to monitor the survival of one or more of these organisms once added to an activated sludge system. To this end the four *Pseudomonas* spp. were characterized in order to determine whether some unique feature or features would allow for simple differentiation between them and between these organisms and other *Pseudomonas* spp.

The organisms were characterized based on the scheme outlined by Hendrie and Shewan (1979) for the identification of pseudomonads. The four organisms conformed to the traditional definition of aerobic

pseudomonads as outlined by Stanier *et al.* (1966), being unicellular, straight rods, motile, Gram negative, non-spore forming, oxidase positive with oxidative metabolism of glucose. The four *Pseudomonas* spp. were almost identical in both their cell and colony morphology.

The four organisms were fluorescent pseudomonads characterized primarily by their ability to produce water-soluble, yellow-green , fluorescent pigments. They all produced diffusible fluorescein pigments when grown on a suitable medium. As with other fluorescent pseudomonads, these organisms were members of the RNA group I (Stanier *et al.*, 1966).

The many biochemical properties of the four *Pseudomonas* spp. examined were typical for their species as outlined in Bergey's Manual for Systematic Bacteriology (1984). An important feature which distinguished the *P. fluorescens* sp., IGA 13.42, from the *P. putida* spp. was its ability to liquefy gelatin. This is one of the main differences between these two species (Stanier *et al.*, 1966). *Pseudomonas* spp. are well known for their metabolic versatility (Palleroni, 1986) and this was borne out by the ability of the four organisms to use a large number of the carbohydrates tested for growth. The *P. fluorescens* sp., IGA 13.42, was by far the most nutritionally versatile using seventy-five percent of the carbohydrates tested. This species has been called the epitome of nutritional versatility among bacteria (Stanier *et al.*, 1966).

The optimum growth temperature for most *Pseudomonas* spp. is 28°C but many are capable of growth in the range of 4°C to 43°C. *P. putida* IGA 0.92 grew at all temperatures tested but *P.putida* IGA 7.16 failed to

grow at 4°C and *P. putida* IGA 7.24 and *P. fluorescens* IGA 13.42 failed to grow at 37°C. The antibiotic sensitivity of the four organisms was similar. All were resistant to penicillin which is a typical feature among *Pseudomonas* spp. (Bergey, 1984). Antibiotic resistance is frequently encoded on plasmids termed R-factors which are transmissible among organisms (Bergey, 1984), as such antibiotic resistance alone does not make a suitable choice as a selectable marker.

The ability of the four *Pseudomonas* spp. to grow at the expense of a range of aromatic compounds was examined in the basal salts medium of Goulding *et al.* (1988), which provided a source of nitrogen, phosphate and trace salts, incorporating the aromatic compound as the sole source of carbon for energy and growth. The solubility of the aromatic compounds in the aqueous medium can be important for their metabolism. Phenol, benzoate and m-toluate dissolved readily in the medium. Naphthalene and p-chlorophenol did not dissolve completely. It was necessary to dissolve the p-chlorophenol in a minimum volume of ethanol. Toluene which has a solubility in water of 0.067% (w/w) at 23.5°C (Merck Index) was supplied as a vapour. A number of bacteria have been found to be capable of metabolizing aromatic compounds with low water solubilities. Various mechanisms have been suggested for this. Some bacteria may facilitate the uptake of poorly soluble compounds by producing emulsifiers or by the possession of a hydrophobic cell surface. It is also possible that the organisms grow only at the expense of the compound in solution and that the rate of dissolution of such compounds might govern the rate of biodegradation (Stucki and Alexander, 1987).

Benzoate has been shown to support the growth of a variety microorganisms (Reiner, 1971). The four *Pseudomonas* spp. grew readily on benzoate. The enzymes for benzoate degradation are chromosome encoded and are ubiquitous in *Pseudomonas* spp. (Harayama and Rekik, 1990). The pathway for benzoate metabolism is induced by benzoate itself (Cuskey and Sprenkle, 1988). The degree of growth of the four organisms was greater on higher concentrations of benzoate. In many cases the time taken to acclimate to the benzoate was also greater on the increased benzoate concentrations.

Catechol, an intermediate in benzoate metabolism, is usually degraded via the *ortho* cleavage pathway (Williams and Murray, 1974). Both the *ortho* and *meta* cleavage enzymes (catechol 1,2-dioxygenase and catechol 2,3-dioxygenase, respectively) were clearly induced by benzoate when compared with the activity levels determined following growth on glucose. However, *ortho* cleavage activity was predominant following both initial and prolonged growth on benzoate.

As is typical of several *Pseudomonas* spp. (Davies and Evans, 1964) the four organisms were capable of growth on naphthalene. Many of the compounds formed during naphthalene degradation are acidic (Yen and Serdar, 1988). A drop in the culture pH was observed as the naphthalene was metabolized. This drop was greater at the higher growth rates achieved on the increasing naphthalene concentrations. The lag period on naphthalene tended to be longer than for the other substrates. The naphthalene degradative pathway is induced by the intermediate, salicylate (Yen and Serdar, 1988) and therefore some initial degradation is required by the low levels of

constitutive enzymes resulting in the formation of salicylate before the pathway enzymes are fully induced.

Growth on naphthalene, including prolonged growth induced predominantly *meta* cleavage activity. This was as expected as the majority of aromatics including naphthalene are cleaved via the *meta* pathway (Williams and Murray, 1974).

The four *Pseudomonas* spp. grew on phenol at the lower concentration of 5mM. However, the higher concentrations were inhibitory for all the organisms except *P. putida* IGA 0.92 which was capable of growth on 10mM but not 20mM. The enzymes for phenol degradation are induced by phenol (Feist and Hegeman, 1969). A short lag period was demonstrated in *P. putida* IGA 0.92 and IGA 7.24, however, a longer period of acclimation was necessary for *P. putida* IGA 7.16 and *P. fluorescens* IGA 13.42 before phenol degradation proceeded.

Phenol also induces catechol 2,3-dioxygenase and the other enzymes of the *meta* cleavage pathway (Feist and Hegeman, 1969). Catechol 2,3-dioxygenase activity was predominant in all the organisms following initial and prolonged growth on phenol.

m-Toluate is an intermediate in the degradation of m-xylene by the TOL plasmid encoded pathway (Davey and Gibson, 1974). All the organisms were capable of growth on m-toluate and as with previous substrates, the degree of growth as indicated by the specific growth rate and maximum OD attained increased on the higher m-toluate concentrations. It was thought that the ability to grow on m-toluate would also give some indication as to the ability to grow on toluene.

However, this was found not to be the case as only *P. putida* IGA 7.16 was capable of growth on toluene and was subsequently shown to carry a TOL plasmid. This discrepancy may have been due to the fact that the genes for toluate dioxygenase form part of the lower pathway operon on the TOL plasmid and this operon is similar in many different catabolic plasmids (Harayama and Rekik, 1990).

Methyl-substituted aromatic compounds induce the enzymes of the *meta* cleavage pathway. Growth on m-toluate by the four *Pseudomonas* spp. for both short and prolonged periods induced predominantly catechol 2,3-dioxygenase activity.

P. putida IGA 7.16 was the only organism capable of growth in the presence of toluene. Toluene is a highly toxic solvent and can kill most microorganisms at very low concentrations (Inuoe *et al.*, 1991). Toluene was provided as a vapour by suspending a small tube containing liquid toluene inside the flask. The degree of growth increased greatly at the higher concentrations of toluene suggesting a greater availability of substrate for metabolism.

There are two main pathways for the degradation of toluene in *Pseudomonas* spp. , that encoded by the TOL plasmid (Burlage *et al.*, 1989) and a chromosome encoded pathway found in *P. putida* F1 (Zylstra *et al.*, 1988). In both of these pathways catechol is an intermediate and is degraded via the *meta* cleavage pathway. Growth of *P. putida* IGA 7.16 on toluene induced chiefly catechol 2,3-dioxygenase activity in both acclimated and non-acclimated cultures.

P. putida IGA 7.16 was also the only organism capable

of degrading p-chlorophenol. This ability may be linked to its ability to metabolize toluene as a number of studies have demonstrated the broad substrate specificity of the enzymes involved in toluene metabolism. The enzymes of the TOL plasmid encoded pathway, toluene monooxygenase, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase, have all been shown to act on chlorinated substrates (Abril *et al.*, 1989). The chromosome encoded toluene dioxygenase of the *P. putida* F1 toluene degradative pathway has also been shown to catalyze the conversion of several chlorophenols (Spain *et al.*, 1989).

The growth of *P. putida* IGA 7.16 on p-chlorophenol induced catechol 2,3-dioxygenase activity and this activity was greater following prolonged exposure to the substrate. The *meta* cleavage pathway usually leads to the formation of dead-end products so ortho cleavage is the preferred route following growth on chlorinated substrates (Williams and Murray, 1974). Oxidation of p-chlorophenol results in the formation of 4-chlorocatechol which when cleaved by catechol 2,3-dioxygenase yields 5-chloro-2-hydroxymuconic semialdehyde, a dead-end product which accumulates in the medium resulting in an intense yellow colour (Knackmuss, 1981). A strong yellow colour, indicating the accumulation of the dead-end product, formed in the culture medium of *P. putida* IGA 7.24 and IGA 0.92 and *P. fluorescens* IGA 13.42 which failed to metabolize p-chlorophenol. A faint yellow colour formed in *P. putida* IGA 7.16 cultures but disappeared as the p-chlorophenol was degraded indicating that an alternative route was used.

The four organisms were unable to degrade o- and m-chlorophenol. The presence of a small volume of

ethanol in the medium may have contributed to the initial increase in OD. Control cultures set up with ethanol only increased slightly in OD after 24 hours and then declined rapidly. Controls with chlorophenol but no organism showed no loss of chlorophenol due to volatilization over a period of 144 hours. Black compounds formed in the medium following initial degradation of both o- and m-chlorophenol. These may also have contributed to the rise in OD observed. Chlorophenols can be fortuitously metabolized by the catabolic enzymes of various pathways which have a broad substrate specificity (Knackmuss and Hellwig, 1978; Spain *et al.*, 1989). The oxidation of both o- and m-chlorophenol results in the formation of 3-chlorocatechol which is a suicide substrate for catechol 2,3-dioxygenase resulting in the formation of an acylchloride. This in an acylating agent which irreversibly inactivates catechol 2,3-dioxygenase resulting in the accumulation of chlorocatechol and its black autooxidation products (Bartels *et al.*, 1984). Therefore, the formation of the black compounds in the medium of the four organisms indicated that their catechol 2,3-dioxygenase enzyme had been inactivated.

When bacteria are cultivated in a medium containing glucose and a less easily metabolized carbon source, the glucose is preferentially consumed (Bailey and Ollis, 1986). When *P. putida* IGA 7.16 was grown on p-chlorophenol in the presence of glucose, the glucose was indeed consumed preferentially. The presence of glucose can also lead to the 'glucose effect'. This gives rise to catabolite repression blocking the induction of genes for the degradation of the second carbon source due to the low levels of cyclic AMP in cells growing rapidly on glucose

(Bailey and Ollis, 1986).

This, however, was not found to be the case with p-chlorophenol and glucose where the presence of glucose resulted in the faster removal of p-chlorophenol. This seemed to have been due to a faster formation of biomass in the presence of glucose. The presence of increased concentrations of glucose had no additional effect on the biomass and the total biomass produced was approximately the same in all cases with and without glucose. Papanastasiou and Maier (1982) carried out a study on the degradation of 2,4-dichlorophenoxyacetate (2,4-D) in the presence of glucose. p-Chlorophenol is an intermediate breakdown product in 2,4-D metabolism. Glucose was found to improve 2,4-D metabolism due to a faster production of biomass. When the biomass concentration was increased by doubling the inoculum size a proportionate increase in p-chlorophenol removal by *P. putida* IGA 7.16 was observed.

The bioaugmentation product CX 80 which included the four *Pseudomonas* spp. failed to degrade o- or m-chlorophenol but rapidly degraded p-chlorophenol. The beneficial effect of a secondary, easily assimilated carbon source was again observed. When the product was washed to remove alternative carbon sources provided by the cereal carrier, the cells failed to degrade p-chlorophenol. The secondary carbon sources may have boosted the numbers of degraders in the product mix thus enhancing p-chlorophenol degradation.

These results were borne out when the product was used to bioaugment a sample of activated sludge. Again only the addition of unwashed product resulted

in complete p-chlorophenol degradation. The p-chlorophenol was added at a concentration of 200ppm, however, the initial concentration detected in the culture supernatant was only in the range of 90-110ppm. This may have been due to adsorption of some of the substrate by the activated sludge flocs. The phenomenon of adsorption by activated sludge flocs is widely recognised (Hamer, 1985). After 48 hours there was an increase in the concentration of p-chlorophenol detected in the supernatants of the culture containing activated sludge only and that containing activated sludge and washed product. This may have been due to some desorption of the substrate.

Meta cleavage activity is plasmid encoded and is known to be carried on a number of different catabolic plasmids (Harayama and Rekik, 1990). The catechol 2,3-dioxygenase activity of the four *Pseudomonas* spp. was therefore indicative of the presence of catabolic plasmids. A number of different DNA isolation techniques were examined to release plasmids from the organisms.

The rapid isolation procedures of Birnboim and Doly (1979) and Eckhardt (1978) for the extraction of large plasmids were examined but no plasmids were visualized. The triton lytic procedure of Crosa and Falkow (1981) for the isolation of plasmids from Gram negative organisms including *Pseudomonas* spp. was also investigated without success. A rapid method for the isolation of large, stable and unstable plasmids involved in the degradation of aromatic compounds in *Pseudomonas* spp. was also investigated (Wheatcroft and Williams, 1981). The main problem encountered with this technique was the handling of the highly viscous solution which resulted. No

plasmids were visualized using this technique.

Degradative plasmids tend to be large in size and are closely associated with the chromosome, these factors make them difficult to isolate (Hansen and Olsen, 1978). The procedure of Hansen and Olsen (1978) was designed for the isolation of large plasmids from *Pseudomonas* spp. It involved cell lysis with lysozyme and SDS followed by alkaline denaturation. This procedure was not successful initially with the main problem being a failure to reduce the pH following alkaline denaturation. Alkaline denaturation is an important step in the isolation of plasmid DNA as it irreversibly denatures chromosomal DNA thus allowing plasmid DNA to be isolated. A number of modifications as outlined by Almond *et al.* (1982) were incorporated. The chief modification was in the proportions of NaOH and Tris added during the alkaline denaturation step. The volume of Tris used in the modified method was eight times the volume of NaOH used compared to twice the volume as used in the original method. This successfully reduced the pH of the DNA solution. The isolation procedure was also simplified by eliminating the washing step which removed residual medium at the beginning of the procedure. The heating step was also changed to 55°C for 2 minutes compared to eight cycles of heat pulse at 55°C and mixing. A number of other slight modifications to the volumes of reagents added were also incorporated. This modified method proved to be very reliable and reproducible for the isolation of large plasmids from *Pseudomonas* spp.

The plasmid profiles of the four organisms grown on a non-selective medium, luria broth, revealed the presence of large plasmids in the three *P. putida* spp., IGA 7.16, IGA 7.24 and IGA 0.92. A number of

smaller plasmids were also visualized in *P. putida* IGA 7.24. No plasmids were visualized in *P. fluorescens* IGA 13.42 using any of the methods examined, therefore further investigation of this organism was not undertaken. Due to the growth characteristics of the organisms it was thought they might carry a TOL or NAH type plasmid. To investigate this further strains carrying a TOL and NAH plasmid were obtained from the National Collection of Industrial Bacteria (NCIB), Aberdeen, UK, for comparison. NCIB 10432 was *Pseudomonas putida* AL (mt-2, PaM1, PaW1), originally isolated from soil, carrying a transmissible TOL plasmid of approximately 117kb in size. NCIB 12199 was *Pseudomonas putida* PaW736 containing a NAH plasmid, pWW60-22, 85-87kb in size. A chromosomal marker was obtained by curing *P. putida* NCIB 10432 of its TOL plasmid using mitomycin C (5-20μg/ml) in a method developed for the curing of large degradative plasmids (Dunn and Gunsalus, 1973).

Due to their large size the TOL and NAH plasmids were resolved close to the wells of the gel and their similarity in size meant there was little resolution between them. Therefore the only conclusion that could be drawn at this stage with regard to the large plasmids visualized in the *P. putida* spp. was that they were of similar size range, approximately 85-120kb. In order to identify the large plasmids DNA probes to the NAH and TOL plasmids were obtained.

Most naturally occurring plasmids are stably maintained by the host, even during growth on non-selective media, that is in the absence of any selective pressure (Stephens and Dalton, 1988). However reports in the literature have indicated that some large degradative plasmids undergo modification,

such as excision of segments of DNA or even complete loss of the plasmid, when grown in the presence of certain aromatic compounds. Alterations in the plasmid profiles of organisms were encountered following growth on a range of compounds including benzoate, methylbenzoate, toluene and xylene (Stephens and Dalton, 1988; Carney and Leary, 1989). The studies by Carney and Leary (1989) also found that prolonged growth on these substrates resulted in the maintenance of the altered plasmid profile or a greater degree of alteration.

The stability of degradative plasmids is of particular importance for organisms that form part of a bioaugmentation product for use in the treatment of wastes from the chemical industry where they may be exposed to a large number of compounds. The stable maintenance of catabolic plasmids provides a means by which gene pools are maintained and disseminated throughout the microbial population (Trevors *et al.*, 1989). The large plasmid visualized in *P. putida* IGA 7.16 and IGA 7.24 cultures appeared to be stably maintained following both initial exposure and prolonged growth on the range of aromatics tested. However, subsequent investigation of the plasmid profiles of *P. putida* IGA 7.24 using Southern hybridization to the NAH probe showed that this was not actually the case. In many cases the genes for the naphthalene upper degradative pathway, of which the probe comprised, had been lost from the plasmids visualized. This was found in m-toluate cultures and in the phenol and benzoate cultures following prolonged growth only. As expected Southern hybridization confirmed the presence of the naphthalene pathway genes in both naphthalene cultures. These genes were also retained in the cultures following initial exposure to benzoate and

phenol. However, the expression in all the cultures of catechol 2,3-dioxygenase activity, which is plasmid encoded, indicated that the genes for the *meta* pathway were retained. The large plasmid visualized in *P. putida* IGA 0.92 was completely lost following growth on most of the aromatic substrates.

The identity of the large plasmid of *P. putida* IGA 7.16 and IGA 7.24 was investigated using Southern hybridization to the TOL and NAH probes. Difficulties can be encountered in transferring large DNA fragments to nitrocellulose (Maniatis et al., 1982). Therefore, the plasmid DNA was first cut with restriction endonucleases. Due to the high salt concentration and the presence of PEG in the DNA preparations from the Hansen and Olsen method (1978), samples were purified by caesium chloride gradient and dialysis. Difficulties were encountered cutting DNA from *P. putida* IGA 7.16 cultures. Particulate matter remained associated with the plasmid band visualized in the caesium chloride gradient which may have interfered with the action of the restriction enzymes. The DNA from *P. putida* IGA 7.24 was cut successfully with a number of the restriction enzymes.

Southern hybridization was used for the identification of the large plasmid carried by *P. putida* IGA 7.24. The NAH probe used comprised the genes of the naphthalene degradative upper pathway encoding the degradation of naphthalene through salicylate. Due to the large size of the recombinant plasmid obtained, the insert carrying the NAH genes was separated from the vector plasmid and used as the probe. This also prevented possible cross-reactivity between the vector and similar material that may be

found in the sample for probing (Ogram and Sayler, 1988).

The large (85-120kb) plasmid carried by *P. putida* IGA 7.24 was identified as a NAH plasmid due to its hybridization to the NAH probe. The probe also hybridized to a number of restriction fragments from *P. putida* IGA 7.24 DNA. It hybridized particularly strongly to the 15kb *Eco* RI fragment. The naphthalene upper pathway, of which the probe comprised, was 15kb in length and was flanked by *Eco* RI restriction sites (Serdar and Gibson, 1989). This indicated therefore that the 15kb fragment identified in *P. putida* IGA 7.24 was the naphthalene upper pathway operon.

The presence of a NAH plasmid was confirmed using a dot blot hybridization procedure with non-radioactively labelled probe DNA. The probe DNA was labelled by random primed incorporation of digoxigenin-labelled deoxyuridine-triphosphate. After hybridization to the target DNA the hybrids were detected by enzyme-linked immunoassay using an antibody-conjugate and subsequent enzyme-catalyzed colour reaction. The formation of a purple/blue precipitate was indicative of a positive hybridization. The labelling and detection systems were obtained in kit form (Boehinger Mannheim) allowing ease of operation. Compared with the radioactive system it gave faster results with safer handling. It was performed without the special precautions necessary when handling ^{32}P. According to its manufacturer, the sensitivity and specificity of the digoxigenin system are comparable to those obtained with radioactive systems (manufacturer's literature). Non-radioactively labelled probes have been used successfully to detect bacterial cells in

contaminated foodstuffs (Dovey and Towner, 1989) and to monitor the survival of bacteria in an aquatic environment (Amy and Hiatt, 1989).

Probes were obtained which were homologous to the genes of the initial enzymes of the two major toluene degradative pathways in *P. putida* spp. One comprised the toluene monooxygenase gene which encodes the enzyme responsible for the initial step in toluene degradation by cells carrying the TOL plasmid. The other probe comprised the toluene dioxygenase and *cis*-toluene dihydrodiol dehydrogenase genes which encode the initial steps in the degradation of toluene in *Pseudomonas putida* F1 which carries a chromosome-encoded pathway. Southern hybridization with ^{32}P-labelled TOL probes failed to identify the presence of a toluene monoxygenase or a toluene dioxygenase gene in *P.putida* IGA 7.16. This may have been due to the failure of DNA from *P. putida* IGA 7.16 to cut with the restriction endonucleases and the difficulties encountered in transferring large DNA fragments to nitrocellulose filters.

Therefore, the dot blot hybridization procedure, which does not necessitate restriction of the sample DNA was employed. Using this procedure the presence of a TOL plasmid was identified in *P. putida* IGA 7.16 as the toluene monooxygenase probe hybridized strongly to DNA from this organism. The chromosome-encoded toluene dioxygenase probe hybridized only weakly to the DNA from *P. putida* IGA 7.16. This was perhaps due to some cross-reactivity between the two toluene probes owing to similarities between the two degradative enzymes.

DNA from luria broth cultures of *P. putida* IGA 0.92 was included on the filters for dot blot

hybridization but failed to hybridize to either of the TOL probes or to the NAH probe. The large plasmid visualized in *P. putida* IGA 0.92 therefore remained unidentified.

Of the four organisms, *P. putida* IGA 7.24 was the most extensively characterized on a genetic basis, positively identified as carrying a NAH plasmid. For this reason this organism was chosen for study in a bioaugmentation experiment. The *Pseudomonas putida* IGA 7.24 was added to an activated sludge system, to monitor its survival and also to investigate its effect on the system. Due to the stability of the NAH plasmid in *P. putida* IGA 7.24 when grown on naphthalene, the presence of a NAH plasmid in the sludge population was monitored as a means of monitoring this organism. The transmissible nature of the NAH plasmid meant that indigenous bacteria which either already had the plasmid or had acquired the plasmid were also detected. Monitoring the NAH plasmid or naphthalene degradative capability reflected the effect of *P. putida* IGA 7.24 on the system.

Two identical activated sludge systems were set up in separate locations in order to prevent cross-contamination between the control and test systems. That this was achieved was confirmed by the detection of *P. putida* IGA 7.24 in the test system only. An interesting feature of the systems was the isolation of a non-motile *P. putida* spp. from both systems. Although very rare, non-motile *Pseudomonas* spp. have previously been isolated from environmental samples (Bergey, 1984). The systems were operated on a fill and draw basis. This was convenient to operate and has become increasingly widespread in industrial-scale wastewater treatment plants as it can provide

a good solution to denitrifying problems and to some bulking problems encountered in single stage completely mixed plants (O'Flaherty, 1991). The fill and draw system has also been used in previous studies on the effectiveness of bioaugmentation (Kennedy *et al.*, 1990).

The test system was augmented with 10^6 cells/ml of *P. putida* IGA 7.24, a concentration of 1% of the total population. A variety of inocula sizes are cited in the literature concerning bioaugmentation. A rate of 1% based on cell numbers was used by McClure *et al.* (1991) in their study of the survival of 3-chlorobenzoate degrading organisms in an activated sludge unit. Zachopoulos and Hung (1990) also chose a 1% ratio of cells when studying the effect of bioaugmentation on activated sludge kinetics.

There are a number of mechanisms by which specific pollutants are removed in the activated sludge process, these include air stripping, sorption onto flocs and biological oxidation (Eckenfelder, 1989). The naphthalene was not completely soluble in the aqueous medium used and microscopic examination revealed what appeared to be the naphthalene crystals entrapped within the flocs. However, studies on naphthalene have shown that greater than 95% is biodegraded by activated sludge while less than 5% is air stripped (Eckenfelder, 1989). The COD of both systems showed an overall downward trend after a period of acclimation. The performance of both systems with regard to COD removal was similar.

The activated sludge was obtained from a plant treating chemical waste of a complex nature and was operated with a solids content of 3 to 4 g/l at ambient temperature and neutral pH. A value for the

sludge volume index (SVI) greater then 150 ml/g is considered to represent bulking sludge. The SVI of both systems was initially poor at greater than 200 ml/g. Both systems fed with naphthalene showed an improvement in the settleability in time, with the SVI dropping to below 100 ml/g. The suspended solids of both systems was unchanged during the experiment remaining at 3 to 4 g/l. This indicated that there was no net growth of the biomass. The systems were operated at ambient temperature during the summer months and temperatures in both systems were approximately 20°C throughout.

The pH in the two systems decreased during the period between feeds indicating naphthalene metabolism. A similar pH effect had been observed when pure cultures of *P. putida* IGA 7.24 and the other *Pseudomonas* spp. were grown on naphthalene.

The organisms in an activated sludge system can exist in either a flocculated or dispersed state. Flocculated organisms tend to have a longer residence time in the system. Dispersed organisms are more susceptible to grazing by protozoa and are readily lost from the system as they settle poorly. As the organisms in many bioaugmentation products exist in dispersed form, maintenance doses are required to maintain the population (Hull and Kapuscinski, 1987). The dispersed bacteria in the effluent of the control system decreased during the first week, most likely due to grazing by protozoa as the protozoan population increased during this period. Thereafter, the dispersed bacteria were maintained at approximately 10^5 cells/ml. Protozoa can rarely reduce bacterial numbers below this level (Wiggins and Alexander, 1988). The numbers of dispersed bacteria in the test system remained high throughout

the experiment, decreasing only when the maintenance dose of *P. putida* IGA 7.24 was reduced. This suggested that *P. putida* IGA 7.24 failed to flocculate and constituted a good proportion of the dispersed bacteria detected in the test system. Due to this failure to flocculate, *P. putida* IGA 7.24 would have been lost from the system had maintenance doses not been applied.

The initial low numbers of protozoa increased rapidly during the first week. The presence of protozoa has been shown to lengthen the acclimation period necessary for the mineralization of organic compounds due to their grazing on potential degrading populations (Wiggins and Alexander, 1988). The low initial levels of protozoa in both systems may have allowed the rapid increase in the naphthalene degrading population. Protozoa are regarded as an indicator of sludge quality. Their role involves the ability to feed on dispersed bacteria, which if not removed represent an unacceptable biochemical oxygen demand and suspended solids concentration in the treated effluent. Plants for the exclusive treatment of industrial wastewaters frequently experience problems with respect to maintaining appropriate populations of protozoa due to the presence of toxic or inhibitory compounds (Hamer, 1985). The healthy protozoan populations rapidly established in both systems was therefore an indication of the success with which the bacterial population could metabolize the naphthalene.

The ingesting of bacteria by protozoa exemplifies the interactions of prey and predators (Pirt, 1975). The protozoan population in the control system decreased sharply on day 13 allowing a proliferation of the bacterial population. Bacterial numbers returned to

their previous level once the protozoan population had reestablished itself.

The effect of bioaugmentation on the naphthalene degrading capability of the systems was investigated using selective plate counts on naphthalene vapour plates. Total viable counts were performed on plate count agar. A number of important factors affect the reliability of plate counts. Firstly, the presence of dormant, non-culturable bacteria in populations is likely to distort results (Saunders, 1990). Other factors include the temperature of incubation, the pH of the medium, possible toxic constituents in the medium and most importantly the release and dispersion of the cells from sludge flocs. Sonication was used to disperse the flocs and approximately 10^8 cells/ml were recovered on plate count agar. This is a typical recovery level on a non-selective medium following sonication (Banks and Walker, 1977).

Selective plate counts as a means of determining the naphthalene degrading capability take advantage of cellular growth to amplify low density populations. However, a number of factors besides those mentioned previously can affect the reliability of selective plate counts. The assumption that microorganisms capable of growth on a compound as a sole carbon source must be capable of catabolism of that substrate is flawed. Numbers may be under-estimated due to a number of problems including poor or slow growth of a population, requirements for auxotrophic nutrients, cross-feeding, lack of sensitivity for populations representing a small percentage of the total, no growth due to cometabolism and colony-colony inhibition (Jain *et al.*, 1988). An additional problem was encountered using naphthalene as the

carbon source due to insolubility in the medium. Naphthalene crystals were scattered on the lid of the petri dish and the organisms were grown in the vapour. A similar procedure was employed by Blackburn *et al.*, (1987) in their study of naphthalene degrading populations in activated sludge. However, despite its drawbacks selective plating techniques are likely to remain a major preliminary tool for environmental monitoring.

A number of procedures which would allow the direct detection of *P. putida* IGA 7.24 were considered. The use of antibodies either monoclonal or polyclonal, could offer a sensitive and specific means for monitoring the introduced organism. The chief drawbacks encountered were the complicated and expensive production of antibodies. There can also be the problem of low sensitivity for the enumeration of small populations and the possibility of interference with antibody binding by other constituents in the sample (Jain *et al.*, 1988). The development of hybridization techniques allows for more specific and direct monitoring of specific DNA sequences in microbial populations.

Colony hybridization eliminates the need for selective cultivation. Media which result in higher recovery can be used. However, this procedure does still require cultivation of the cells and therefore does not yield a fully representative sample (Jain *et al.*, 1988). A developing method does away with the need for cultivation. It involves the direct extraction of DNA from the environmental sample, the DNA sequence of interest must be amplified by PCR prior to hybridization to a specific probe (Ogram and Sayler, 1988). This technique is likely to become increasingly important in the study of specific

populations in the environment.

A gene probe which is specific for complementary DNA sequences provides a reliable, powerful and specific detection and monitoring tool. The two types of gene probes used are species-specific probes, which require the presence of a complementary sequence unique to the test organism, and function-specific probes which identify genes for a given function such as pollutant degradation that are not specific to one taxonomic group. Function-specific probes such as the one used in this experiment measure the potential of a community to perform a function but does not measure the actual function (Ogram and Sayler, 1988). The use of the NAH probe in this experiment therefore identified those cells with a naphthalene degrading genotype, that is the catabolic potential of the system, but was not necessarily an indication of expression of those genes. However, as naphthalene was provided as the sole source of carbon, the cells would be under considerable selective pressure to express the naphthalene degrading genes.

Colonies grown on plate count agar were transferred to nitrocellulose filters and were lysed to release the DNA which was heat-fixed to the filters. A number of lysis procedures were tried (Maniatis *et al.*, 1982) and the one which resulted in the sharpest signal following hybridization was used. As the non-radioactively labelled probe had been used successfully previously this procedure was followed again. Colony hybridization has been used to monitor the presence of both TOL and NAH genotypes in environmental samples. Using colony hybridization, the number of naphthalene degrading organisms was up to ten times higher than when determined by traditional plate analysis using naphthalene as a

sole carbon source (Blackburn *et al.*, 1987). The rapid increase in the numbers of naphthalene degrading bacteria in both the test and control systems was more sensitively determined using colony hybridization to the NAH probe compared with the use of naphthalene vapour plates.

The naphthalene degrading bacteria that were identified in both systems were all *Pseudomonas* spp. which is typical for activated sludge treating chemical wastewater (Hamer, 1985). The majority of isolates were identified as *P. putida* spp. but could be distinguished from *P. putida* IGA 7.24 on the basis of biochemical characteristics. The goal of this experiment had been to monitor *P. putida* IGA 7.24 in an activated sludge system. This organism was detected only in the system to which it was added. The presence of large numbers of *Pseudomonas* spp. obviously contributed to the capability of the sludge to metabolize naphthalene. The addition of *P. putida* IGA 7.24 therefore had little impact on the performance of the sludge. However, this experiment demonstrated the usefulness of colony hybridization for the detection of naphthalene degrading genotypes in activated sludge.

5. CONCLUSIONS

The goal of this research was to monitor one or more of the *Pseudomonas* spp. from CX 80 in an activated sludge system. Therefore, the four organisms were extensively characterized. The response of the three *P. putida* spp. IGA 7.16, IGA 7.24 and IGA 0.92 and the *P. fluorescens* IGA 13.42 to a number of biochemical tests was typical for their species (Bergey, 1984). Further investigation of the ability of these organisms to grow on a range of aromatic compounds as sole carbon source revealed *P. putida* IGA 7.16 to be the only organism capable of growth on toluene and p-chlorophenol. All four organisms could grow on benzoate, naphthalene, m-toluate and phenol.

An examination of catechol-dioxygenase activity demonstrated the presence of both *ortho* and *meta* cleavage pathways. The *meta* cleavage activity was indicative of the presence of degradative plasmids (Harayama and Rekik, 1990). Large plasmids were detected in the three *P. putida* spp. only. The large plasmid in *P. putida* IGA 7.24 was identified as a NAH plasmid and that in *P. putida* IGA 7.16 was identified as a TOL plasmid. The plasmid in *P. putida* IGA 0.92 was not identified.

The presence of a NAH plasmid in *P. putida* IGA 7.24 was selected as a means of monitoring this organism in an activated sludge system. The transmissible nature of this plasmid (Yen and Serdar, 1988) meant that indigenous organisms which acquired this plasmid were also detected. Plasmid transfer is a positive feature of bioaugmentation as it increases the catabolic potential of the system. However, the impact of *P. putida* IGA 7.24's addition was negligible due to the presence of cells carrying a NAH plasmid among the indigenous population of the activated sludge.

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